High-throughput genome-wide expression analysis of a non-model organism: The chickpea root and nodule transcriptome under salt and drought stress

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Este trabajo está especialmente dedicado a mis padres, Carlos y Clara, y a mi esposa Stefanie por el enorme apoyo que me han brindado en estos agnos. Gracias por muchas ensenazas de vida, gracias por su incondicional ayuda

Contents

Abbreviations

ABA		Abscisic acid
-	ABRE	ABA-responsive element
-	AREB	ABRE-binding factor
-	ABF	ABA-binding factor
ACS		Acetyl-CoA synthetase
ADP		Adenine di-phosphate
-	ARF	ADP-ribosylation factor
AOX		Alternative oxidase
APKs		Ankyrin protein kinases
APX		Ascorbate peroxidase
AR		Ascorbate reductase
bZIP		Basic leucine zipper protein
CAT		Catalase
CBL		Calcineurin B-like protein
-	CIPK	CBL-interacting protein kinase
CDPK		Ca^{2+}-dependent protein kinase
CHI		Chalcone isomarase
CHS		Chalcone synthase
CS		Cysteine synthase
CY3		Cyannine-3
CY5		Cyannine-5
CYP		Cytocrhome P450
ΔCT		Differential of cycle threshold
DHAR		Dehydroascorbate reductase
DNA		Deoxyribonucleic acid
-	cDNA	Complementary DNA
dNTP		deoxyribonucleotide tri-phosphate
-	dATP	deoxiadenine tri-phosphate
-	dCTP	deoxicitosine tri-phosphate
-	dGTP	deoxiguanine tri-phosphate
-	dTTP	deoxitimine tri-phosphate
DRE		Dehydration-responsive element
-	DREB	DRE-binding protein
Enod40		Early nodulin class 40
ERBF		Ethylene-responsive binding factor
EST		Expressed sequenced tag
GI		Gene indices
GO		Gene Ontology
GOAT		Glutamine synthetase
GOGAT		Glutamate synthase
GPX		Glutathione peroxidase
GR		Glutathione reductase
GS		Glutamine synthetase
GSR		Gene score re-sampling
GST		Glutathione S-transferase

HDZ		Homeodomain leucine zipper protein
HMG		High mobility group protein
HRPG		Hydroxyproline-rich glycoproteins
HsF		Heat shock transcription factor
HsP		Heat shock protein
ICDH		Isocitrate dehydrogenase
LB		Leghemoglobin
MAPK		Mitogen activated protein kinase
-	MAPK	MAP-kinase
-	MAPKK	MAPK-kinase
-	MAPKKK	MAPKK-Kinase
MDAR		Monodehydroascorbate reductase
MDH		Malate dehydrogenase
MIP		Major intrinsic protein
MOR		Malate oxidoreductase
MPC		Magnetic particle capturer
MT1		Metallothionein-like protein 1
NCED		Nine-cis-epoxycarotenoid dioxygenase
nr		Non-redundant nucleotide database (NCBI)
NTP		Nucleotide tri-phosphate
-	ATP	Adenine tri-phosphate
-	CTP	Citosine tri-phosphate
-	GTP	Guanine tri-phosphate
-	UTP	Uracil tri-phosphate
P		Statistical probability
PBM		Peribacteroid membrane
PCR		Polymerase chain reaction
-	qRT-PCR	Quantitative real-time PCR
PDH		Proline dehydrogenase
PEPC		Phosphoenolpyruvate carboxylase
PIP		Plasma-membrane intrinsic protein
PK		Protein-kinase
$Poly(A)^+$		Polyadenylated mRNA
PP		Protein phosphatase
PPi		Pyrophosphate
ProT		Proline transporter
$R_{(ln)}$		Natural logarithm of expression ratio
RACE		Rapid amplification of cDNA ends
RLK		Receptor-like protien kinase
RNA		Ribonucleic acid
-	mRNA	Messenger RNA
-	miRNA	Micro RNA
-	rRNA	Ribosomal RNA
-	siRNA	Small interfering RNA
-	snoRNA	Small nucleolar RNA

ROS		Reactive oxygen species
RT		Reverse transcriptase
SAGE		Serial analysis of gene expression
SNARE		Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNF		Symbiotic nitrogen fixation
SNP		Single nucleotide polymorphism
-	SAAT	SNP-associate alternative tag
SOD		Superoxide dismutase
SOS		Salt ovelry sensitive
SPDS		Spermidine synthase
SuperTag		SuperSAGE Tag
SUS		Sucrose synthase
SUT		Sucrose transporter
TC		Tentative consensus sequence
TF		Transcription factor
TIGR		The institute of genomic research
TIP		Tionoplast intrinsic protein
TPS		Trehalose 6-phosphate
U		Unit (enzymatic activity)
UGPase		UDP-glucose pyrophosphorylase
UniTag		Unique Tag
UPS		Ubiquitin/proteasome system
VAMP		Vesicle-associated membrane proteins

1 Introduction

1.1 Chickpea: a world-wide important non-model crop

Chickpea (*Cicer arietinum* L.), a member of the *Fabaceae,* is the third most important food legume world-wide with over 10 million hectares under cultivation (Millan et al., 2006). It is mostly grown in arid and semi-arid regions, predominantly in undeveloped countries (90% of its cultivated area). At present, the most important chickpea-producing countries are India (64%), Turkey (8%), Pakistan (7%), Iran (3%), Mexico (3%), Myanmar (3%), Ethiopia (2%), Australia (2%), and Canada (1%) (**Figure 1-1**).

Chickpea has one of the most balanced nutritional compositions, and its protein digestibility is the best among the dry season food legumes. Apart from human consumption, this crop has economical importance as animal feed as well as in herbal medicine. Chickpea seeds contain 20-30% protein, and approximately 40% carbohydrates. Additionally, they are a good source of calcium, magnesium, potassium, phosphorus, iron, zinc, and manganese. This crop provides more beneficial carotenoids than the genetically engineered "golden rice", and, in comparison to other grain legumes, has almost no anti-nutritive components (Hayriye Ibrikci, 2003). Ecologically, chickpea is known as an efficient N_2-fixing system due to its capability of symbiotic nitrogen fixation (SNF), a process taking place at the root nodules, specialized structures formed upon *Rhizobium* infection, a N_2-fixing bacteria (ICRISAT, www.icrisat.org).

Like every extensively cultivated crop, this legume is facing the consequences of the continuously deteriorating environmental conditions on this planet , which are leading to always more rigorous temperature regimes and dry soils (abiotic stress; **Figure 1-2**). To counteract this global phenomenon, extensive artificial irrigation is required to achieve acceptable harvest yields in many of the chickpea cultivating regions (Bakht et al., 2006). However, on the long term this practice results in increased soil salinization and therefore in a depression in productivity. One of the most affected processes influenced by abiotic stresses in chickpea, and in legumes generally, concerns the SNF function. Although many of the effects of abiotic stresses on nodulation, growth, and N_2-fixation have been well studied in this crop, little is known about the physiological, biochemical, and transcriptional stress-responses as e.g. compatible osmolyte accumulation, ammonium assimilation, photosynthesis, and active ion transport (Soussi et al., 1998). Apart from drought and salt stress, many of the chickpea cultivated areas are subject to cold temperatures during

wintertime, constraining the productivity even more drastically (Singh et al., 1992; Bakht et al., 2006).

The above mentioned aspects emphasize the need of transferring chickpea from the group of "under-researched crops" into a transition group, in which the molecular and biochemical characterization of stress responses already started. In some of these fronts, some advances have already been achieved. For example, Boominathan and collaborators (2004) isolated around hundred drought-inducible transcripts from dehydrated chickpea roots via SSH subtracted libraries. In a later study, Mantri and co-authors (2007) reported on a deeper transcriptome analysis, for which 768 pre-selected genes were spotted onto microarrays to track their behavior under salt, drought, and cold stresses. In that study, the authors observed more than 2-fold transcriptional changes for 109, 210 and 386 genes after drought, cold and high-salinity treatments, respectively. Despite these preliminary insights, the amount of information is still at least 20-fold lower than in other legumes, as e.g. *Medicago truncatula*, a legume model crop that profits from the massive EST sequencing of more than 184,599 cDNAs (Cheung et al., 2006), and standardized microarrays originally containing more than 16,000, now about 21,000 genes (Buitink et al., 2006).

In an attempt to fill the big gap of missing information, and to profit from the massive knowledge from other legumes (i.e. *M. truncatula, Lotus japonicus, Glycine max, Phaseolus vulgaris*), the work in this thesis presents the expression profiles of more than 30,000 unique transcripts under salt and drought stress in chickpea roots and nodules. For this purpose, more than 270,000 cDNA fragments (in the form of 26bp cDNA tags) were massively sequenced and statistically analyzed for stress differential expression. Additionally, the results were confirmed by independent techniques such as qRT-PCR and microarray hybridizations, proving that the extracted information can be transferred to other platforms.

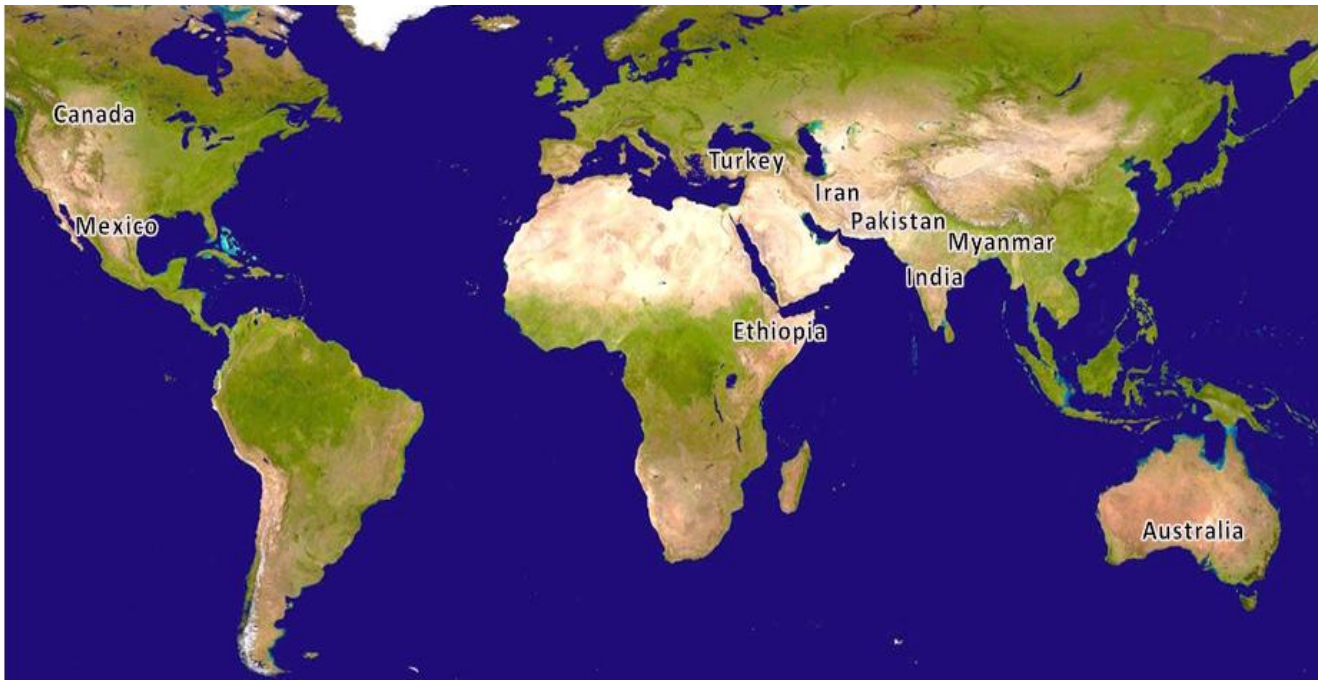


Figure 1-1 **Main chickpea-growing regions in the world**

Figure 1-2 **Chickpea cultivation in desiccated areas on the Mediterranean basin**

1.2 Abiotic stress in plants

Plants, as sessile organisms, are exposed to changes in their environment, which they cannot escape. Therefore, any new condition implicating a decrease in their performance, or in the probability of survival of an individual, will be perceived as stress. As a consequence, plants are obliged to deploy different physiological strategies to overcome any adversity encountered on their surrounding (Albrecht et al., 2003).

In crop plants in particular, abiotic stresses account for the major part of the difference between potential and real harvest yields in agricultural areas world-wide. Year after year, high salinity, drought, heavy metal exposure, excess of radiation, heat, and cold temperatures are responsible for uncountable losses with major economical and social consequences, most of them in undeveloped countries (Yamaguchi-Shinozaki and Shinozaki, 2006).

1.2.1 Drought and salt stress

Among the abiotic stresses, the decrease on water availability (commonly known as drought stress) is considered a major limiting factor for plant development and growth. Severe changes of water potential in the environment and consequently in the plant may lead to osmotic stress, disturbing the normal cellular functions, and eventually leading to cell death. To counteract these effects, various molecular, cellular, and whole-plant responses are triggered, as e.g. changes in life cycle, or morphological alterations in root and shoot development, ionic re-adjustments, and modifications in the metabolism of carbohydrates or synthesis of compatible osmolytes (Hasegawa et al., 2000).

In turn, high salt concentrations (commonly known as salt stress) cause in plants ionic disequilibrium and hyperosmosis. The adverse effects of exposition to high salinity conditions are manifested in the inhibition of germination, growth reduction, or even arrest, and stop of development (Zhu, 2002). Due to the toxicity of high Na^+ concentrations, the control of the cytosolic levels of this cation is of vital importance for the plant cell. The principal mechanisms involved in this control involve prevention of uptake as well as an increase of Na^+ export (Zhu, 2003).

In chickpea, and legumes in general, drought causes a 40-50% reduction in yield globally (Ahmad et al., 2005). Additionally, most legumes are known to be salt-sensitive, a fact of future concern, since the increasing use of artificial irrigation world-wide suggests that, by the year 2050, 50% of all arable land will be salinized (Wang et al., 2003). Common for

drought and high salinity, two major highways exist through which stress responses in plants are processed (highways here referred to as "attributes": i) an ionic-, and ii) an osmotic-attribute (Xiong et al., 2002) (**Figure 1-3)**. For both stresses, the specific combination of these two attributes is directing the responses of the plant towards activation of physiological processes aiming at alleviating the environmental pressure. Additionally, the alterations on the plant metabolism caused by the osmotic- and ionic-disequilibrium confront the plant with the overproduction of reactive oxygen species (ROS), leading to oxidative stress, a disturbance that may also reach lethal levels (**Figure 1-3**) (Apel and Hirt, 2004).

1.2.1.1 The osmotic attribute

Higher plants are exposed to different degrees of water stress at some stages in their developmental process. The type and strength of water stress can vary from atmospheric humidity changes and net radiation up to soil water deficits (drought) in arid environments. In plants of more arid regions, tolerance to water stress usually involves low osmotic potentials (high solute levels), which are a combination product of the differences in the basal osmotic potential and the solute accumulation in response to water deficit. The aim of this strategy is then to maintain the turgor and hence a steady plant growth rate (Morgan, 1984). In plants under salt stress, alterations in the osmotic equilibrium are caused by the high concentrations of Na^+ ions. Although there may be no water deficit in the environment, the differential osmotic pressure will nevertheless lead to loss of water and thus, dehydration of the plant tissues.

As general consequences in high salinity and dehydration, the plant's altered water status leads to initial growth reduction through inhibition of cell division and expansion, membrane disorganization, reactive oxygen species production, metabolic toxicity, photosynthesis inhibition, and attenuated nutrient acquisition (Hasegawa et al., 2000).

1.2.1.2 The ionic attribute

The excess of Na^+ ions in the vicinity of a plant cell under salt stress causes a major disequilibrium in K^+ and H^+ transporter activities. Since many transport systems of the cell membranes do not completely discriminate between K^+ and Na^+, the plant cells are "forced" to import Na^+ from the apoplastic space to satisfy the need of K^+ for several physiological purposes. The increasing accumulation rate of Na^+ ions reaches then toxic intracellular levels, that disrupt several cellular processes including active transport, protein biosynthesis,

and various other metabolic pathways (Hasegawa et al., 2000). As a consequence of the metabolic disequilibrium, plants experience growth rate reduction, oxidative stress, and cell death in the end.

The Na^+ export process involves mainly two strategies, which are both dependent on energy. The first general strategy consists of extruding Na^+ ions out of the cell via Na^+-plasma membrane antiporters, normally ATP-driven. In this case, the extruded Na^+ immediately becomes a potential intake ion as soon as it enters the apoplastic space. The second strategy consists of exporting Na^+ ions transiently into special compartments (e.g. vacuoles) for later extrusion via exocytosis. This second strategy rests upon vacuolar proteins such as vacuolar H^+-ATPases, membrane proteins that catalyze the exclusion of the major part of the active Na^+ out of the cell (Low et al., 1996; Gaxiola et al., 2002).

1.2.2 Reactive oxygen species and oxidative stress in plants

Superoxide- (O_2^-), hydrogen peroxide- (H_2O_2), and hydroxyl-radicals (OH^-), collectively known as reactive oxygen species (ROS), are by-products of the plant cell metabolism. Under normal conditions ROS are produced in only low quantities, in compartments like mitochondria, chloroplasts, and/or peroxisomes, as derivates of processes like chloroplast and mitochondrial respiration (Moller, 2001; Del Rio et al., 2003).

ROS interact with a broad array of bio-molecules inducing alterations in their functions, therefore, they are considered as toxic at high concentrations (Apel and Hirt, 2004). In parallel, ROS can function as signaling molecules by triggering several signal transduction cascades. For these reasons, the ROS-generation and -scavenging machinery in plants is tightly controlled by a redundant and complex network involving dismutase enzymes, and cellular buffers, aside of the ROS generators (Gechev et al., 2006).

Salt and drought stresses induce a strong metabolic disequilibrium in the afflicted plant cell, leading to ROS overproduction, which follows different routes in different plant organs. In leaves, the major ROS production occurs in chloroplasts and peroxisomes, whereas in non-photosynthetic tissues, ROS are mainly generated in mitochondria (Gadjev et al., 2006). ROS overproduction under drought and salt stress conditions can rapidly reach toxic levels on the cell, which, if not controlled, can lead to increased mitochondrial electron transport, resulting in turn in ATP depletion, and even apoptosis (Tiwari et al., 2002).

As mentioned above, the various reactive oxygen species may also act as signaling radicals/molecules. However, much about the ROS-triggered signaling cascade(s) in plants

remains obscure, inspite of the reported interaction of these radicals with several components of diverse pathways, as e.g. several RLKs, MAPKs, and proteins involved in Ca^{2+} signaling (Dat et al., 2000; Samuel et al., 2005; Del Rio et al., 2006).

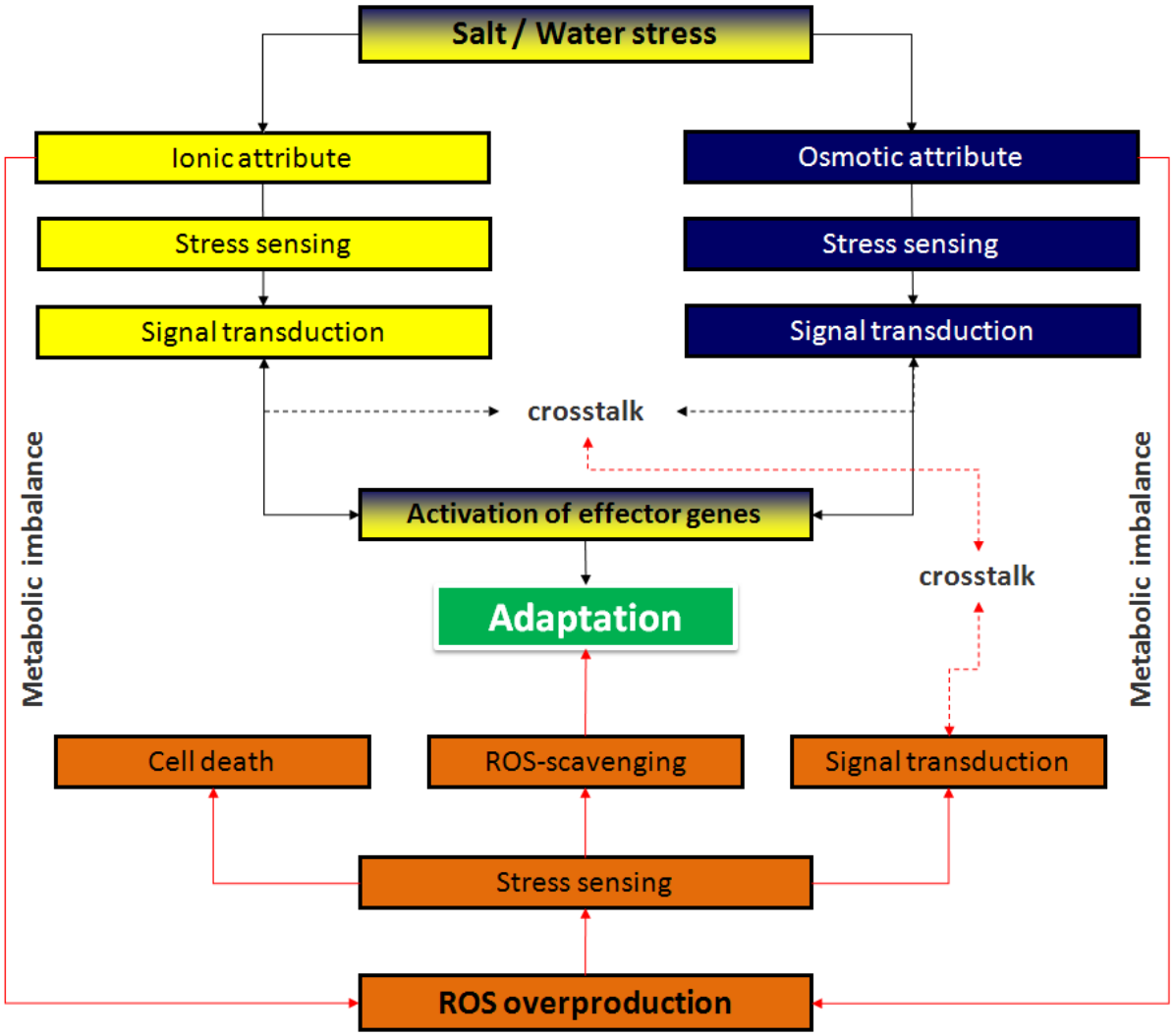


Figure 1-3 **General scheme of drought and salt stress attributes in plants**

In the above general scheme, water and salt stresses in plants are perceived through two main highways or "attributes": i) the **ionic,** and ii) **osmotic** stress components. Both components are activating signaling cascades which have specific as well as shared events. The last component of such cascades induces whole batteries of different effector genes acting on different physiological sceneries, and thereby overcoming the environmental adversity. In parallel and as a consequence of metabolic disturbances by both osmotic and ionic attributes, **reactive oxygen species (ROS)** production increases. ROS themselves are highly toxic for the cell and impose additional stress onto the plant. Therefore, ROS-scavenging machineries play a crucial role for the stressed plant. Apart from being highly toxic, ROS are also known to function as signaling molecules, triggering diverse cascades with several components shared with other stress-related pathways.

1.3 Legumes and symbiotic nitrogen fixation

As previously mentioned, nutritionally and ecologically, SNF makes of chickpea (and legumes in general) an important object of study. In these plants, all the processes downstream the conversion of atmospheric nitrogen (N_2, di-nitrogen) into its organic form (ammonia) turn around a single structure: the root nodules.

1.3.1 Legume nodules as the nitrogen-fixing organs in roots

Nitrogen (N_2) is one of the rate-limiting elements in plant growth processes. Therefore, it is the mineral nutrient needed in greatest abundance by higher plants (Crawford, 1995). Normally, the N_2 available in the biosphere is continuously depleted by de-nitrification processes. Only some prokaryotes reduce di-nitrogen to an organic form (ammonia) and fix it in the biosphere through a quite complex and oxygen-sensitive process. Among these prokaryotes, *Rhizobia*, a class of nitrogen-fixing bacteria, establishes symbiotic partnerships with higher plants, which supply them with energy and protect the N_2 fixation machinery from deleterious oxygen. In the framework of SNF, leguminous plants evolved the capability to form new organs, the root nodules, in response to *Rhizobia* invasion (Mylona et al., 1995).

The symbiotic interaction between legumes and *Rhizobia* begins with signals recognition by both partners, integrating the bacterial invasion at the root-hairs epidermis and the initiation of cell division in the root cortex cells, several cell layers away from the bacteria primary attachment sites (Oldroyd and Downie, 2008). Subsequently, the root hairs start curling, and the bacteria invade the plant by a newly formed infection thread growing through it. In parallel, a nodule primordium is shaped by cortical cells. When the infection thread reaches the primordium, the bacteria are released into the cytoplasm of the host cells and surrounded by a plant-derived peribacteroid membrane (PBM). At this stage, the bacteria are already differentiated into their symbiotic form, known as bacteroids (Mylona et al., 1995).

The PBM biogenesis is regulated through differential expression of genes of both the host legume and *Rhizobia*, inducing the synthesis of nodulins, bacteroidines, fatty acids, polysaccharides, and other components. At N_2-fixing stage, the PBM provides selectivity for metabolite and ion transport, and facilitates the signaling between both the prokaryotic (bacteria) and eukaryotic (host plant) cell (Krylova et al., 2007). In a general scheme, legume nodules consist of five distinct regions as shown in **Figure 1-4**: i) nodule meristem, ii) prefixation zone, iii) interzone, iv) N_2-fixation zone, and v) senescence zone. The

conformation of the mature nodules offers physical barriers (nodule parenchyma) as well as enzymatic mechanisms (leghemoglobin activity), that keep the nitrogen fixation zone in an O_2-free state. In this way the plant secures the protection the extremely O_2-sensitive bacterial nitrogenase.

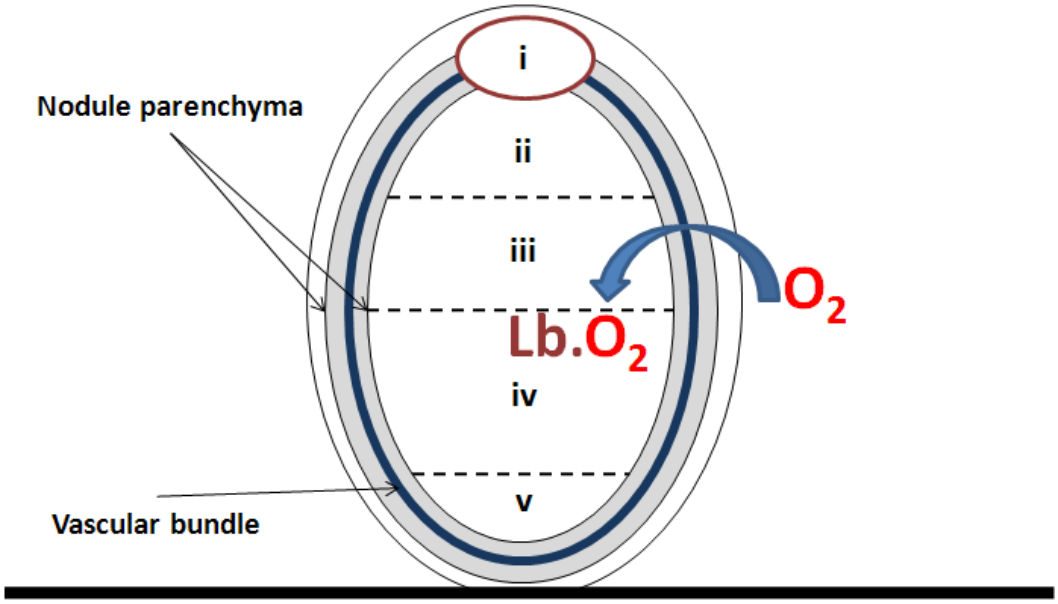


Figure 1-4 A schematic view on a legume's nodule

Five different regions can be distinguished in a functional nodule:
i) nodule meristem, ii) prefixation zone, iii) interzone, iv) N_2-fixation zone, and v) senescence zone. The nodular parenchyma (represented in light blue colors) builds up an oxygen barrier which efficiently isolates the N_2-fixation region. However, since this barrier is interrupted at the nodule meristem (i), the activity of leghemoglobin (Lb), an oxygen quenching enzyme, is needed to constantly protect the extremely oxygen-sensitive bacteroid nitrogenases

1.3.2 Legume nodules and abiotic stresses

One of the major bottlenecks in SNF in plants is the sensitivity of the interaction between both partners to abiotic stresses. In many legume species, particularly under high salt conditions, the ability of the plants to keep functional nodules has been directly related to stress tolerance. Studies in other legume genera (e.g. *Vicia sp.*) proved, that the activity of enzymes directly involved in SNF, such as glutamine synthetase (GS) and glutamate synthase (GOGAT), is drastically decreased under high Na^+ concentrations (Cordovilla et al., 1994). In general, most of the explanations for the negative effect of salt and drought on SNF are

turning around the diminished photosynthate production and its supply into the nodules, a reduced flux of respiratory substrates into the bacteroid, and alterations of the oxygen diffusion barrier protecting the nitrogenases (Soussi et al., 1998). In studies evaluating physiological parameters in the salt- tolerant chickpea variety INRAT-93 (the same variety used for the present work), O_2-conductance values were lower than in varieties known to be salt-sensitive, such as Amdoun (L'Taief et al., 2007). This relatively low O_2 conductance may well directly govern the ability to keep functional nodules and therefore could be related to salt tolerance.

A further aspect playing a very important role in the physiology of nodules concerns the generation of ROS, which represent a ubiquitous danger for aerobic organisms. This risk is especially elevated in legume root nodules due to the strongly reducing conditions, the high rates of respiration, the tendency of leghemoglobin to autoxidize, the abundance of non-protein Fe ions and the presence of several redox proteins that leak electrons to O_2 (Becana et al., 2000). Consequently, nodules are particularly rich in both quantity and diversity of antioxidant defenses. These include enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase , glutathione reductase (GR), and metabolites such as ascorbate, glutathione, and other thiol tripeptides (Iturbe-Ormaetxe et al., 2001).

Abiotic stresses, especially salt stress, enhance the generation of ROS in root nodules, thereby introducing even more destabilizing pressure on the N_2-fixation machinery. In essence, the knowledge of how plants manage this situation on the molecular level may increase our understanding of general responses to abiotic stresses.

1.4 Expression profiling as an important tool in molecular biology

Genome-wide expression profiling techniques became some of the most frequently used analytical tools for the understanding of many biological systems over the past 20 years. Starting in the eighties and early nineties with nylon membranes as ancestors of today´s micro-arrays, the term “expression profiling” has been inflated exponentially, as has the number of publications and related methods (Stoughton, 2005). In a very general view, expression profiling techniques can be divided into two categories: i) closed- and ii) open-architecture methods.

1.4.1 Hybridization-based "closed architecture" gene expression profiling

Closed architecture methods are based on spotting a determinate number of cDNAs or oligonucleotide probes onto a solid surface (e. g. micro- and macro-arrays) and their hybridization to target nucleic acids. Microarrays constitute the most efficient and advanced example for this kind of approach (Schena et al., 1995). Since the early days of this technique, considerable efforts have been made to reduce costs, to avoid bias, to simplify the procedures, and to establish standard rules for experimental design and evaluation (e.g. MIAME). Additionally, thanks to major efforts dedicated to the continuous sequencing of transcriptomes of model and non-model organisms (e. g. the *Arabidopsis* CATMA project, Aubourg et al., 2007 ; and the cancer genome anatomy project, Krizman et al., 1999), the supply of sequence information for microarray platforms has continuously increased at high rates. Consequently, especially during the last decade, the growing number of publications and research groups involved in expression profiling have allowed large projects, that contributed to create large gene-expression databases publicly available, e. g. ArrayExpress (Brazma et al., 2006), gene expression omnibus GEO (Edgar et al., 2002), Arabidopsis gene expression database AtGenExpress (Kilian et al., 2007), and GeneBins (Goffard and Weiller, 2007), among others.

1.4.2 Sequence-based "open architecture" gene expression profiling

In parallel to microarray-based techniques, quantitative expression profiling procedures emerged, based on the sequencing of a representative sample of an mRNA population. These "open-architecture" techniques do not require previous cDNA or oligonucleotide spotting, and therefore the number of analyzed probes and genes is variable. A specific type of open-architecture technology requires sequencing of small discrete fragments (so called "tags") derived from mRNA populations by the use of special restriction endonucleases. The most representative example for such a method is the procedure known as serial analysis of gene expression (SAGE) (Velculescu et al., 2000).

Despite the good performance of SAGE, especially as far as the number of analyzed transcripts is concerned, this technique has drawbacks for the annotation of the small tags, restrictions in multiplexing and the amount of starting material, and difficulties in reproducibility (Anisimov and Sharov, 2004; Maillard et al., 2005). More recently, a broad palette of SAGE-derived sub-techniques emerged, and a few of them have partially overcome some of the above mentioned drawbacks, whereas others only added minor improvements.

As examples, procedures like SADE, a SAGE adaptation for downsized extracts (Virlon et al., 1999); SAGE-lite, a SAGE variant starting from small mRNA amounts (Peters et al., 1999); gene expression fingerprinting (GIF), a SAGE variant using a different set of restriction enzymes (Zajchowski et al., 2000); gene identification signature (GIS), a SAGE variant which also samples 5'-cDNA ends (Ng et al., 2005); LongSAGE, a SAGE variant producing a bigger tag size (Wahl et al., 2005); TOGA total analysis of gene expression (Lo et al., 2001); MiniSAGE, another SAGE variant developed for small samples (Ye et al., 2000); PCR-SAGE (Neilson et al., 2000); rapid analysis of gene expression (Margulies et al.), a SAGE variant using a still different set of restriction enzymes (Wang et al., 1999); and massively parallel signature sequencing (MPSS; Reinartz et al., 2002), can be highlighted.

1.4.3 SuperSAGE and its application in a non-model organism

SuperSAGE, one of the many variants of the SAGE technique, is a procedure originally described by Matsumura and co-authors (2003). This technique substantially improves the tag size to 26 bp, in comparison to the original SAGE (14 bp) and LongSAGE tag length (20 bp). Therefore, one of the main advantages of this procedure is the more accurate tag annotation in public EST databases, thanks to the longer sequence information. Apart from the original work in which this technique was tested in rice leaves infected with the rice pathogen *Magnaporthe grisea*, this procedure has also been proved successfully in banana (*Musa acuminata*), where the expression of more than 10,000 tags representing more than 5,000 transcripts was monitored (Coemans et al., 2005).

Methodologically, the SuperSAGE method relies on the class III restriction endonuclease *Eco*P15I. This enzyme cleaves a DNA molecule 26 bp away from its recognition site, which consists of two 5'-GACGAC-3' repeats in head-to-head orientation (Mucke et al., 2001). The use of this endonuclease in combination with the frequent cutter *Nla*III allows retrieving a 26 bp cDNA fragment from about 98.0% of the cDNAs represented in a poly(A)$^+$ mRNA population (proportion theoretically calculated in *Arabidopsis;* Robinson et al., 2004). A detailed step-by-step procedure of the SuperSAGE technique is portrayed in section **2.3** under Materials and Methods. After massive amplification and sequencing, the obtained 26 bp tags are grouped *in silico* in unique tags categories (UniTags) and annotated in public EST databases. Quantitatively, the number of copies (counts) of each retrieved UniTag is used to estimate its expression ratio. A general scheme of the SuperSAGE data-generation is shown in **Figure 1-5**.

For the present work, SuperSAGE has been chosen to analyze the whole transcriptome changes in chickpea roots and nodules upon salt and drought stress. By further improvements in the methodology, such as the introduction of massive parallel pyrosequencing via the 454-technology (Margulies et al., 2005), the amount of sequenced information has been up-scaled at least 20-fold.

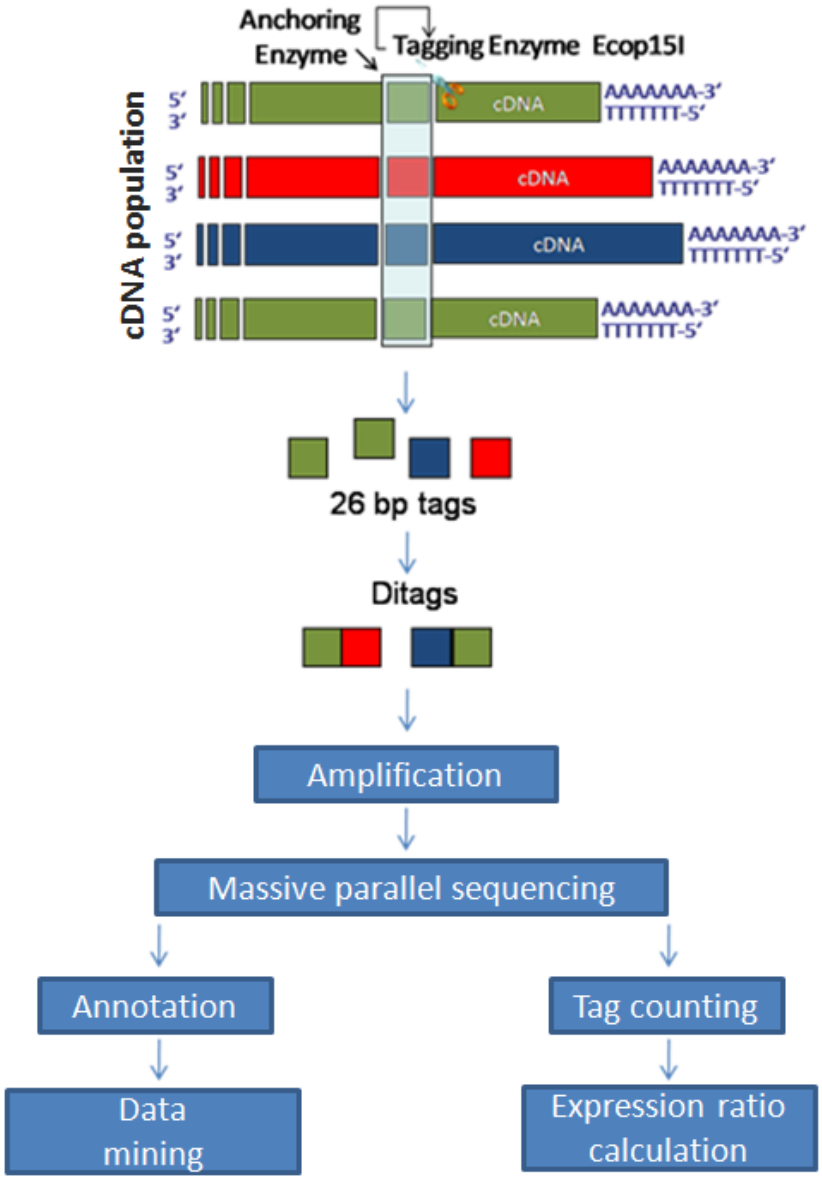


Figure 1-5 **General scheme of the SuperSAGE data generation process**

SuperSAGE libraries consist of 26bp tags generated from a discreet position within each cDNA in a population. To be massively amplified and sequenced, tags are randomly coupled into "ditags". After sequencing, the resulting tags are grouped into UniTags, counted, and annotated. Subsequently, normalized counts are used to calculate the expression ratio of each UniTag. A detailed step-by-step description of the SuperSAGE methodology is deposited on section **2.3 (Materials and Methods)**.

1.5 Large-scale transcriptome profiling studies of drought- and salt-stressed plants

With the advent of massively parallel sequencing of model organisms and the use of expression profiling techniques like microarrays (Schena et al., 1995), the number of studies on the plant transcriptome responses upon abiotic stresses has substantially increased. In the last decade, at least 37 publications on large-scale expression profiling in plants under salt or water stress appeared, not including sub-transcriptome analyses. Logically, most experiments have been carried out for model organisms such as *Arabidopsis* and rice. A compilation of the organisms, number of analyzed elements and references is deposited in **Table 1-1**.

For example, during the last decade, the amount of analyzed information in *Arabidopsis* has gradually increased more than 10-fold in comparison to the first published salt and drought transcriptome analyses. By using microarrays, Seki and co-workers (2001) analyzed the expression of 1,300 full- length cDNAs to identify drought- and cold-inducible genes, and targets of the DREB1A/CBF3 transcription factors, known to control stress-inducible gene expression. One year later, the same authors reported on a higher density microarray, in which 7,000 full- length cDNAs were characterized under drought, cold, and salinity stress (Seki et al., 2002). In parallel, characterization works carried out by Kreps and co-authors (2002) increased the numbers of analyzed full-length cDNAs up to 8,300. In the last four years, the number of analyzed full-length unique cDNAs (or genes) increased at least 3-fold. Using microarrays, Jiang and Deyholos (2006) and Kilian and co-authors (2007), respectively, reported on the characterization of 23,686 and 24,000 *Arabidopsis* genes under diverse abiotic stresses.

In rice, the same tendency has been observed. For example, the characterization upon salt stress of 1,728 full-length cDNAs derived from root EST libraries was carried out by Kawasaki and co-authors (2001) by microarray analysis. This initial amount of information has been more than 20-fold expanded in the past few years. Zhou and co-authors (2007) reported on the expression profile of 37,000 unique rice genes in response to drought and high salinity, also by the use of microarrays.

In legumes, the tendency towards an increased amount of analyzed sequence information is less notorious. The maximum number of analyzed elements has been reported by Buitink and co-authors (2006) in a microarray-based expression profile of 16,086 cDNAs in response to drought stress in the model legume *Medicago truncatula*. In *Lotus japonicus*, also a widely characterized and sequenced legume, Sanchez and co-authors

(2008) described an integrative approach, in which more than 10,000 full-length cDNAs were analyzed via the construction of an Affymetrix™ gene chip. For chickpea, a non-model legume, the panorama looks much less promising up to now. Only two "large-scale" transcription profiling papers on responses to water or salt stress have been published (Boominathan et al., 2004; Mantri et al., 2007), together covering less than 1,000 unique transcripts.

Table 1-1 Large-scale transcription profiling in water- or salt-stressed plants during the last decade

Main features of each work such as organism, authors and number of analyzed genes are shown. Publications on salt or drought stress in legumes are denoted at the bottom of the table in a separate section.

Organism	Authors	Year	Technique	Analyzed unique transcripts
Arabidopsis	Seki et al.	2001	microarray	1'300
Arabidopsis	Kreps et al.	2002	microarray	8'300
Arabidopsis	Seki et al..	2002	microarray	7'000
Arabidopsis	Rizhsky et al.	2004	microarray	nd*
Arabidopsis	Kawaguchi et al.	2004	EST sequencing	2'000
Arabidopsis	Sunkar and Zhu	2004	miRNA sequencing	nd*
Arabidopsis	Jiang et al.	2006	microarray	23'700
Arabidopsis	Kilian	2007	microarray	24'000
Rice	Kawasaki et al.	2001	microarray	1'700
Rice	Reddy et al.	2002	SSHs	1'000
Rice	Rabbani et al.	2003	microarray	1'700
Rice	Sahi et al.	2003	SSHs	1'260
Rice	Shiozaki et al.	2005	PCR-SSHs	384
Rice	Zhou et al.	2007	microarray	37'000
Rice	Gorantla et al.	2007	EST sequencing	5'800
Maize	Yu and Setter	2003	microarray	2'500
Maize	Poroyko et al.	2007	EST sequencing	15'700
Barley	Talame et al.	2006	microarray	1'600
Barley	Oztur et al.	2002	microarray	1'400
Populus	Street et al.	2006	microarray	13'500
Populus euphratica	Brosché et al.	2005	EST sequencing	14'000
Thellungiella halophila	Wong et al.	2005	EST sequencing	6'600
Thellungiella halophila	Wong et al.	2006	microarray	3'600
Sorghum	Buchanan et al.	2005	microarray	12'900
Sorghum	Pratt et al.	2005	EST sequencing	55'800
Tobacco	Rizhsky et al.	2002	macroarrays	nd*
Tomato	Ouyang et al.	2007	SSHs / microarray	2'500
Potato	Rensink et al.	2005	EST sequencing	20'700
Citrus	Terol et al.	2007	EST sequencing	54'000
Sunflower	Fernandez et al	2008	microarray	317
Glycine soja	Ji et al.	2006	EST sequencing	2'000
Lotus japonicus	Sanchez et al.	2008	microarray	> 10'000
Medicago truncatula	Buitink et al.	2006	microarray	16'000
Medicago	Merchán et al.	2007	SSHs / macroarray	384
Glycine max	Irsigler et al.	2007	microarray	5'700
Chickpea	Boominathan et al.	2004	SSHs / macroarray	100
Chickpea	Mantri et al.	2007	microarray	768
Chickpea	**Present work**	**2008**	**SuperSAGE**	**30'000**

*number of analyzed unique transcripts (or genes) not defined in the cited publication

1.6 Contribution of genome-wide expression profiling and transgenic approaches to the understanding of water and salt stress in plants

Large-scale transcript profiling shows, that water- and salt-stress responses in plants involve up- and down-regulation of a large number of genes. In general, apart from the transcripts involved in physiological adaptation (encoding e.g. enzymes for the synthesis of osmolytes, ion transporters, and ROS-scavengers), transcripts encoding proteins regulating transcriptional and translational machineries revealed to play major roles in water and salt stress responses in plants (Sahi et al., 2006). In the following sub-sections, relevant achievements in the search for genes involved in water- and salt-stress responses made through transgenic approaches and large-scale expression profiling will be shortly highlighted. However, these sections will be restricted to some relevant functional categories of genes, such as those encoding proteins working in ABA-related pathways, signal transduction, transcriptional regulation, and the SOS pathway for ionic detoxification in plants (Qiu et al., 2002). Additionally a more general summary of functional categories, genes, and the probable role of these genes in the salt and drought stress context is deposited in **Table 1-2**, following the review work of Sahi and co-authors (2006).

Up to now, information about the expression dynamics of most of the genes and gene-categories mentioned above is totally missing in chickpea. The use of the knowledge accumulated in other plant species and its transfer to this crop would, therefore, represent a great advance in the basic understanding of the stress responses of this important legume.

1.6.1 ABA: the most important drought and salt stress signaling hormone in plants

The important role of abscisic acid (ABA) as a signaling compound in plants under drought and salt stress is an obligatory topic. This hormone, which is involved in processes like germination, seed dormancy, plant development, cell division, and control of stomata closure, is also a key regulator for the integration of the various signals triggered by ionic- and osmotic-disequilibrium in plants (Knight and Knight, 2001). In drought- and salt-stressed plants, ABA levels rise significantly within hours after onset of stress, and decline after rehydration, suggesting the direct involvement of this plant hormone very early after stress induction (Xiong and Zhu, 2003).

ABA is a natural sesquiterpenoid compound of plants, which is partly synthesized in chloroplasts and other plastids, but also indirectly through the production and breakdown of carotenoids (40-carbon compounds) via the violaxanthin pathway (Milborrow, 2001).

Table 1-2 Examples for genes involved in drought and salt stress responses, identified by transcription profiling and transgenic studies in plants

Function	Genes	Possible role in stress
Signaling	RLKs, MAPKs, histidine kinases, PP2C, G-PCRP,AAA-type ATPase, calmodulin, serine-threonine protein kinases, ADPRFs, calcineurin, EF-hand containing proteins	stress signal transduction and gene expression
Transcriptional and post-transcriptional regulation	DREB, EREBP, MYB, MYC and Zn-finger transcription factors, RING finger proteins, MADS box proteins, HDZ, CBF, TATA-binding protein, GCN-like proteins, glycine-rich and zinc finger RNA-binding proteins, RNA polymerase, splicing factors, micro RNAs	transcriptional regulation of stress gene expression, transcript stability, turnover, processing
Translation	ribosomal proteins, translation initiation and elongation factors, t-RNA synthetases	stress-regulated protein translation, selective translation, transport, localization
Protein folding	F-box, WW, WD40, postsynaptic density protein, Disc-large, Zo1 (PDZ), tetratricopeptide repeat (TPR)-domain-containing proteins, HSPs, PPIases, PDIase, DnaJ, DnaK like proteins, calrecticulin	maintenance of protein structures, protein folding, prevention of protein denaturation, protein sorting, targeting
Protein turnover	polyubiquitins, ubiquitin conjugating enzymes and ligases, components of the proteasome pathway, proteases, protease inhibitors	regulation of protein metabolism, targeted protein degradation in response to stress
Osmoprotectants	pyrroline carboxylate reductase, proline oxidase, cholineoxidase, trehalose phosphate synthase; LEA, cor,dehydrins, water stress proteins	osmotic adjustment, protection of cellular structures and macromolecules
Transport	water, amino acid, sugar and metal transporters, aquaporins, membrane proteins, antiporters, ion channels, sulphate transporters, ABC-type transporters, amino acid permeases, Na+ and K+ transporters, plasma membrane and vacuolar ATPases	ion homeostasis during stress, compartmentalization of solutes and amino acids
ROS scavengers, cell death, senescence and ageing	SOD, peroxidases, oxido-reductases, PAL, CAT, GST, cytochrome c-oxidase, glyoxalase, cyclin H1, histones, tumor suppressors	detoxification of free oxygen radicals, cell death, hypersensitive response
Metal-binding proteins	metallothionin, ferritin, Cu- and Zn-binding proteins, calmodulin	cellular metabolism, metal ion homeostasis, cofactors of critical reactions, signaling, metal toxicity, secondary stress responses, oxidative stress

Table 1-2 continuation

Function	Genes	Possible role in stress
Photosynthesis	Chlorophyll *a/b*-binding protein, photosystem I subunit PSI-like protein, ATP sulphurylase, rubisco activase	regulation of photosynthesis
Defense	WRKY family of transcription factors, chitinase, glucanases, protease inhibitors, myrosinase-binding protein, thaumatin	protection against biotic stress including viral, bacterial and fungal infestation
Hormone-related proteins	zeaxanthin epoxidase, GDA-1 (GA-induced gene), ASR-1 (abscisic acid responsive), ACC Synthase, ABI-3 interacting protein, allene oxide synthases, NCED	hormonal homeostasis and gene expression
General metabolism	NDPK, arginine decarboxylase, glucosyltransferases, mannosyltransferases, methyl and acetyl transferases, choline kinase, lipoxygenase, fatty acid desaturase, GAPDH, lipase, ferredoxin nitrite reductase, aldolase, enolase, alanine transaminases, methionine synthase, asparagine synthetase, tryptophan synthase, acetohydroxyacid synthase, NADP-ME, fructose bis-phosphatase, malate dehydrogenase, enzymes of the photorespiratory and pyruvate cycle pathways, acetyl Co-A synthetase, phenylpropanoid pathway	housekeeping, metabolic pathways, carbohydrate-, fatty acid- and protein-synthesis and modifications, membrane fluidity, nitrogen metabolism, carbon and nitrogen fixation
Unclassified	genes encoding proteins with uncharacterized domains and tissue specific genes	unknown

Because of the broad palette of physiological processes positively correlated with endogenous ABA levels, ABA biosynthesis is considered a rate-limiting step in many plant stress-response events (e.g the closure of stomata under plant dehydration) (Leung and Giraudat, 1998). The identification of genes coding for enzymes involved in ABA biosynthesis has up to now revealed details on their respective organ-specific expression, which is indicative for complex regulation of these genes in response to environmental conditions (Seo and Koshiba, 2002). From the many proteins involved in ABA-biosynthesis, 9-epoxicarotenoid dioxygenase (NCED) has been postulated to catalyze one of the key steps (i.e. the cleavage of neoxanthin to form xanthoxin). Therefore, the stress-triggered induction of transcripts coding for this protein has become an indicator of ABA-activity on the plant (Iuchi et al., 2000). Additionally, apart from starting the *de novo* ABA biosynthesis machinery, ABA concentration in the cell has been proven to increase by other mechanisms, which also react upon stress, like the release of ABA conjugates by the action of ß-glucosidases (Dietz et al., 2000).

As far as the direct interaction of this hormone with signaling and transcriptional pathways is concerned, at least three classes of transcription factors (TFs) are ABA- and stress-induced. First, ABA-responsive element binding transcription factors (AREBs/ABRE-

binding factors/ABFs) bind to the abscisic acid-responsive elements (ABRE) in the promoter region of certain stress-responsive genes (Choi et al., 2000; Kim et al., 2004). This TF type is a sub-class of the basic leucine zipper (bZIP) family, a type of TF widely related to abiotic stresses (Kim et al., 2004; Baena-Gonzalez et al., 2007). Additionally, TFs from the MYB, MYC and WRKY classes mediate responses common to ABA and abiotic stresses, and frequently play signaling integrator roles (Abe et al., 2003; Zou et al., 2004).

1.6.2 Stress sensing and signal transduction

Understanding the early events in plant stress sensing as well as the subsequent signal transduction cascades is prerequisite for strategies to engineer stress tolerance in plants. The most important achievements in this field have been reviewed by several authors (Hasegawa et al., 2000; Knight and Knight, 2001; Xiong et al., 2002; Zhu, 2002; Boudsocq and Lauriere, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006).

Single abiotic stresses are composed of diverse components, most of them shared by several stresses. Therefore, an integrated and specific response from the plant to each stimulus needs to be processed and delivered to guarantee its survival (McCarty and Chory, 2000). In this sense, signal transduction elements are the regulators of a very complex and redundant network of events, involving stress recognition and early signaling, protein phosphorylation and de-phosphorylation cascades, and transcriptional and translational control.

1.6.2.1 Calcium: the most important signal in early events upon water and salt stress in plants

Changes in Ca^{2+} concentration (Ca^{2+}-transients) represent the major signaling source for the plant cell under ionic and osmotic stress. In the early salt stress stages, Ca^{2+}-transients are the key steps linking the excess of Na^{+} with subsequent signaling events, leading to the perception of ionic and osmotic disequilibrium (Sanders et al., 1999; Hirschi, 2004; Lecourieux et al., 2006). Due to the availability of Ca^{2+} in the apoplastic fluid, its non-toxicity, and the predominating high differential ratio between apoplastic and cytoplasmic Ca^{2+} concentrations [$Ca^{2+}_{cyt/apo}$], this ion offers a broad palette of spatial and temporal scenarios in which the concentration changes could be perceived as signal triggers. Ca^{2+} signals can be subdivided into two categories: i) primary general signals, and ii) amplified secondary specific signals. In primary events, an exogenous [Ca^{2+}] change is perceived,

normally by sensors which receive feedback from the apoplastic fluid (e.g. G-proteins). Following, an amplified and more specific signal is then generated by a subsequent Ca^{2+} release from internal storages in a pathway, in which inositol-triphosphate (IP3) acts as mediator, and in which, genes coding for proteins such as phospholipase-C are supposed to control rate-limiting steps (Xiong et al., 2001; Meijer and Munnik, 2003).

Time and localization of this secondary Ca^{2+}-release are controlling the specificity of particular signals (Lecourieux et al., 2006; Ma et al., 2006). Due to the importance of Ca^{2+} in plant stress signaling, genes coding for proteins that directly or indirectly interact with this ion, such as calcineurin-B like proteins (CBLs), calmodulin, calmodulin-binding proteins, calcium-dependent protein kinases (CDPKs), and various calcium channels, have been pinpointed as major players in salt and drought stress signaling (Ikeda, 2001; Romeis et al., 2001; Albrecht et al., 2003). For example, the Ca^{2+}-dependent protein kinase, OsCDPK7 of rice is suggested to be a positive regulator of the tolerance to cold, salt, and drought stress (Saijo et al., 2001). Moreover, in tobacco, two CDPK-coding genes were induced upon defense elicitation and osmotic stress treatments, and therefore were considered to play major roles in stress tolerance (Romeis et al., 2001).

1.6.2.2 Protein-kinase and protein-phosphatase cascades

Since water deficit is sensed by different receptors on the plant's membrane, it is probable that different signals (ionic, osmotic, or mechanical) are simultaneously activated by different receptors. Subsequently, events involving second messengers, hormones and reactive oxygen species (ROS) activate different signaling cascades. Many of the components of these cascades are common for several pathways, which promotes their cross-talk forming a very complex network. For instance, the MAP-kinase (MAPK) family of proteins own integrative roles between ROS-, salt-, and drought stress-derived signaling (Teige et al., 2004; Samuel et al., 2005).

As an example, reported by Teige and co-workers (2004), the *Arabidopsis* MAPK-kinase MKK2 was specifically activated by cold and salt stress, and also by the stress-induced MAP-kinase MKK1. MKK2 in turn was found to directly target the MAPKs MK4 and MK6. Additionally, a genome-wide transcriptome analysis of MKK2-overexpressing plants demonstrated altered expression of 152 genes involved in transcriptional regulation, signal transduction, cellular defense, and stress metabolism.

Along with MAPKs, other kinds of protein kinases (PKs) like the CBL-interacting protein kinases (CIPKs) (Liu et al., 2000), receptor-like protein kinases (RLKs;(He et al., 2004), shaggy and GSK-proteins (Bianchi et al., 1994), ankyrin protein kinases (APKs;(Chinchilla et al., 2003), and several other PK-types are involved in salt and drought stress responses. In many cases, the transcription levels of the respective genes were altered by the environmental pressure.

Protein phosphatases (PPs) are also main components of the stress signaling networks, playing positive and negative roles in the regulation of, and cross-talk between different signaling pathways (Smith and Walker, 1996). For example, ABI1, a member of the PP2C family, has been reported to negatively regulate ABA-mediated signal transduction in plants under osmotic disequilibrium. At the same time, this protein interacts with a specific domain of SOS2, a CIPK involved in the control of plant ion homeostasis (Ohta et al., 2003) See also section **1.4.1.4**).

1.6.2.3 Transcriptional regulators and protein cycle-related proteins

At the transcriptional regulation level, several expression profiling studies and several transgenic approaches have pinpointed TF families to be highly involved in drought and salt stress responses of plants (Zhu, 2002). Due to the involvement of abscisic acid in stress signaling in plants, AREB, a sub-class of bZIP TFs, has been detected as major transcription regulator under high salinity or water deficit (Choi et al., 2000). TFs belonging to the WRKY, MYB and MYC families are also induced by high salinity, drought, and ABA, and are therefore considered to play integrator roles between different signaling pathways in plants (Abe et al., 2003; Zou et al., 2004; Xie et al., 2005).

Dehydration-responsive-element binding (DREB) proteins additionally represent a class of TFs, reportedly major regulators in salt, drought, and cold stress responses in plants. More specific for salt and drought stress responses, DREB2A and DREB2B are highly induced upon water deficit and increase in NaCl concentration (Nakashima et al., 2000). However, as reported by Liu and co-authors (1998) in *Arabidopsis*, plants over-expressing DREB2 showed only a weakly induced expression of the DREB2 target genes under control conditions, which suggests a need for post-transcriptional modifications of the these TFs for their activation. DREB2 transcription factors have been reported to act in an ABA-independent manner (Shinozaki and Yamaguchi-Shinozaki, 2000), representing an alternate way to the ABA-dependent signaling pathways in plants under abiotic stresses.

Further on, heat shock TFs (HsFs) are thought to function as a highly redundant and flexible gene regulatory network that controls the response of plants to different environmental stresses. This type of proteins may also directly sense ROS, which makes them mediators in stress-related signal transduction (Miller and Mittler, 2006).

Transcript synthesis and stability are also considered as other essential parameters in plant stress responses. For example, RNA and DNA helicases enhance stress tolerance in transgenic plants (Gong et al., 2002; Sanan-Mishra et al., 2005). Further on, the regulation of translation also seems to be an important component of the cellular stress responses (Bailey-Serres, 1999). As a confirmation, a broad array of ribosomal proteins has been reported to be cold-, drought-, and salt stress-responsive in different plants (Bartels and Salamini, 2001).

Last but not least, the control of the protein turn-over machinery is also of major importance in plant stress responses. Genes coding for different classes of proteases, protease inhibitors, and ubiquiltin-cycle-related proteins are induced during drought and salt stress, suggesting that the degradation of non-functional proteins is also a crucial event in plants against stress, as for example observed in the transcriptome-wide analysis conducted by Kawasaki and co-authors (2001) and Buchanan and co-authors (2005) in rice and sorghum, respectively.

1.6.3 The salt overly sensitive pathway for ionic stress in plants

For several of the signaling cascades in abiotic stress responses of plants, many of the components and many of the functions of the involved proteins are still not known, which interferes with the full characterization of the series of events leading to stress tolerance. One of the few exceptions, for which almost a straight line of events has been characterized, is represented by the salt overly sensitive (SOS) pathway of *Arabidopsis* under ionic stress (Qiu et al., 2002). In this pathway, a calcium sensor known as SOS3 (a CBL protein;(Sanchez-Barrena et al., 2004)) forms a complex with a CIPK known as SOS2 (Liu et al., 2000). The SOS3-SOS2 complex is then directly activating a Na^+/H^+ antiporter known as SOS1, which exports Na^+ out of the cell. In parallel, several vacuolar ATPases involved in the Na^+ translocation into vacuoles are indirectly regulated by this CIPK-CBL complex (Batelli et al., 2007). Additionally, several CBL and CIPK family members apart from SOS3 and SOS2 have been characterized, demonstrating that there is certain flexibility to form an active complex,

which allows these proteins to interact with several other signaling pathways (Albrecht et al., 2003).

Recently, components of this Arabidopsis pathway have been characterized in other plant species like rice (Martinez-Atienza et al., 2007), demonstrating its degree of conservation. At present, there is no such information available for chickpea. Therefore, the detection of transcripts coding for SOS-related proteins in this crop can be of great value.

1.7 Aims of the present work

Presently, chickpea is a nutritionally and commercially important legume crop, but unfortunately under-researched in many (especially molecular) aspects. The present study therefore aimed at changing this situation and focused on the following objectives:

- To gain a first overview of the transcriptome in chickpea roots and to characterize its re-adjustment to salt and drought stress, employing an advanced version of SuperSAGE technology.
- To exploit the accumulated sequence information of the related model plants *M. truncatula* and *L. japonicus* to reduce the information gap between them and chickpea via annotation of the sequenced cDNA fragments (SuperSAGE tags) derived from the genome-wide transcription profiling.
- To observe differences of the transcriptome between different organs (i.e. nodules and roots) of the same chickpea plant under non-stress conditions, and to monitor the shared and non-shared stress responses.
- To identify a set of candidate transcripts (genes), whose expression dynamics indicate their involvement in drought and salt stress management.
- To test the transferability of information obtained from genome-wide expression profiling via SuperSAGE to other technology platforms such as e.g. microarrays and qRT-PCR.
- To convert the massive transcriptome data into Gene Ontology (GO) categories to facilitate a better knowledge of the stress-responsive metabolic pathways in roots and nodules.

1.8 Structure of the present thesis

The following sections of the present thesis are organized in three major parts

- Materials and Methods — Chapter 2
- Results
  - Technical results — Chapter 3
  - Chickpea transcriptome data mining — Chapters 4 to 7
- General discussion — Chapter 8

Concerning the results of the present work, Chapter 3 describes the most relevant methodological achievements along with the results of the SuperSAGE profiles-confirmation and additional experiments. Subsequently, the transcriptome of chickpea roots or nodules under salt and drought stress is detailed in Chapters 4 to 6. Since data-mining results require a considerable input of background information, along these chapters, a good portion of the presented data is accompanied by brief discussion and remarks. Chapter 7 conserves the same structure, but is oriented to highlight common drought and salt stress transcriptome responses in chickpea roots.

Closing, the discussion comprised in Chapter 8 is directed in the first sections to the methodological achievements and problems of SuperSAGE, followed by general aspects of the transcriptome responses of chickpea under salt and drought stress (emphasizing on roots). General conclusions are summarized at the end of this chapter.

2 Materials and methods

2.1 Plant material

For the present experiments, the following cultivars/accessions of chickpea (Cicer arietinum L.) were used:

2.1.1 ICC588: A drought-tolerant chickpea variety

Accession ICC588 is the product of a breeding process to improve drought-tolerance in chickpea, in which eight varieties were pre-selected and phenotypically evaluated in the field at ICARDA, Syria. From these test crosses, four lines were classified as drought-susceptible: Amit, ILC3279, ILC10606 and ILC9955, whereas line ICC588 proved to be drought-tolerant, as are lines ILC3182, ICCV2 and CDC Chico. (Rehman et al. ICARDA, http://www.pulse.usask.ca/6cprw/poster/reh.pdf)

Based on its field performance and the accumulated information background, ICC588 was selected for e.g. the generation of segregant populations ICC588 X ILC3279, and therefore was appropriately selected for genome expression profiling via SuperSAGE.

2.1.2 INRAT-93: A salt-tolerant chickpea variety

Accession INRAT-93 (Beja 1) is a chickpea variety bred at the National Institute for Agricultural Research of Tunisia (INRAT) and released in 2003. (http://www.icarda.org /seed_unit /SeedUnit/catalogue/tunisia.htm). The salt tolerance of this variety has been tested by L'taief and co-workers (2007). These authors suggested that the good performance under saline conditions of INRAT-93 may be correlated to its very low nodule conductance for O_2, one of the major factors for the inhibition of N_2-fixation in saline media. Due to the good background information, and its nodulation performance under salt stress conditions, INRAT-93 was selected for SuperSAGE expression profiling. It should be noted, that the growing conditions of INRAT-93 seedlings, harvested for SuperSAGE libraries, were identical in time and location to the conditions reported by L'taief and co-authors (detailed information in **section 2.2.1**)

2.1.3 ILC8262: A cold-tolerant chickpea variety

The selection of line ILC8262 results from massive screening of more than 7,000 chickpea lines under cold conditions (Singh et al., 1995). This variety was among the best 25 accessions with excellent performance in a five-winters test period, and still nowadays is used as standard for cold-tolerance screenings (Toker, 2005). Further tests have confirmed the performance of this variety in low temperatures under controlled conditions, again a fact recommending this line for the establishment of SuperSAGE libraries.

2.2 Plant stress treatments

For the development of SuperSAGE libraries aiming at the evaluation of drought-, salt-, and cold-responsive transcriptomes in chickpea (*Cicer arietinum*), seedling germination, plant growth, and treatments were carried out in two locations selected on the basis of international cooperation: i) International Center for Agricultural Research in the Dry Areas (ICARDA, Aleppo, Syria), and ii) National Institute of Agricultural Research (INRA, Montpellier, France). For each location and type of assay (i.e. drought-, salt-, and cold-stress), unstressed control, and stressed chickpea plants were processed in parallel. Principal aspects of the SuperSAGE libraries and stress treatments are detailed in **Table 2-1**.

Table 2-1 Main aspects of SuperSAGE libraries developed in the present work

Investigated cultivars, types of stress treatment, selected organ, and experimental details for each of the eight SuperSAGE-libraries constructed in the present work. For subsequent pair-wise comparisons, control and stress libraries were developed from each tissue and for each type of stress.

Variety	Phenotype	Treatment	Experimental Details	Location	Organ	Library code
Ca-ICC588	drought- tolerant	drought	well watered	Syria	complete roots	ICC588-D-Ct
Ca-ICC588	drought- tolerant	drought	desiccation, 6h	Syria	complete roots	ICC588-D-Str
Ca-INRAT-93	salt- tolerant	salt	0.0 mM NaCl	France	Roots	I93-S-Rt-Ct
Ca-INRAT-93	salt- tolerant	salt	0.0 mM NaCl	France	Nodules	I93-S-Nd-Ct
Ca-INRAT-93	salt- tolerant	salt	25 mM NaCl, 2h	France	Roots	I93-S-Rt-Str
Ca-INRAT-93	salt- tolerant	salt	25 mM NaCl, 2h	France	Nodules	I93-S-Nd-Str
Ca-ILC8262	cold- tolerant	cold	12°C, hardened	Syria	Leaves	ILC8262-Ct
Ca-ILC8262	cold- tolerant	cold	-5°C, 5h	Syria	Leaves	ILC8262-Str

2.2.1 Salt stress treatment

Surface-sterilized INRAT-93 seeds were germinated for 5 days on 0.9% agar at INRA-EMSAM (Montpellier, France). Seedlings with a minimum root length of 5 cm were inoculated with *Mesorhizobium ciceri* (strain UPMCa7), and transferred to 40 L hydroaeroponics buckets, each one with capacity for 15 individuals. INRAT-93 seedlings were further grown for 15 days in temperature-controlled glasshouse conditions with a day/night temperature regime of circa 28/20°C and a 16h photoperiod with additional light of 400 µmol PAR $m^{-2}s^{-1}$. Micro- and macro-nutrients concentrations in the growth medium were adjusted to the following levels: 0.7mM K_2SO_4, 1mM $MgSO_4$·7H2O, 1.65mM $CaCl_2$, 22.5 mM H_2PO_4 (macronutrients), and 6.6 mM Mn^{2+}, 4 mM Bo^{3+}, 1.5 mM Cu^{2+}, 1.5 mM Zn^{2+}(micronutrients)**.** Additionally, 2.0 g L^{-1} $CaCO_3$ were used as pH regulator. After one round of compression and filtering, a constant air flow of 3 x 400 mL $[L_{(sln)}$ 1min$]^{-1}$ was induced in each bucket.

For salt-stress treatment, the grown chickpea plants were transferred into freshly prepared buckets with the above mentioned conditions plus additional 25mM NaCl. In parallel, control plants were placed into new nutritive solution without NaCl. Control and 25 mM NaCl-treated roots and nodules from three plant replicas were harvested separately, and frozen in liquid nitrogen 1 and 2 hours, respectively, after onset of the stress. The arrangement of the buckets is exemplified in **Figure 2-1**. Additional buckets were prepared to monitor the fresh and dry weight of the INRAT-93 plants (roots, shoots, and nodules) 4 days and 5 weeks, respectively, after stress induction. Buckets containing plants of salt-sensitive ICC4958 served to check the survival rate of both phenotypes.

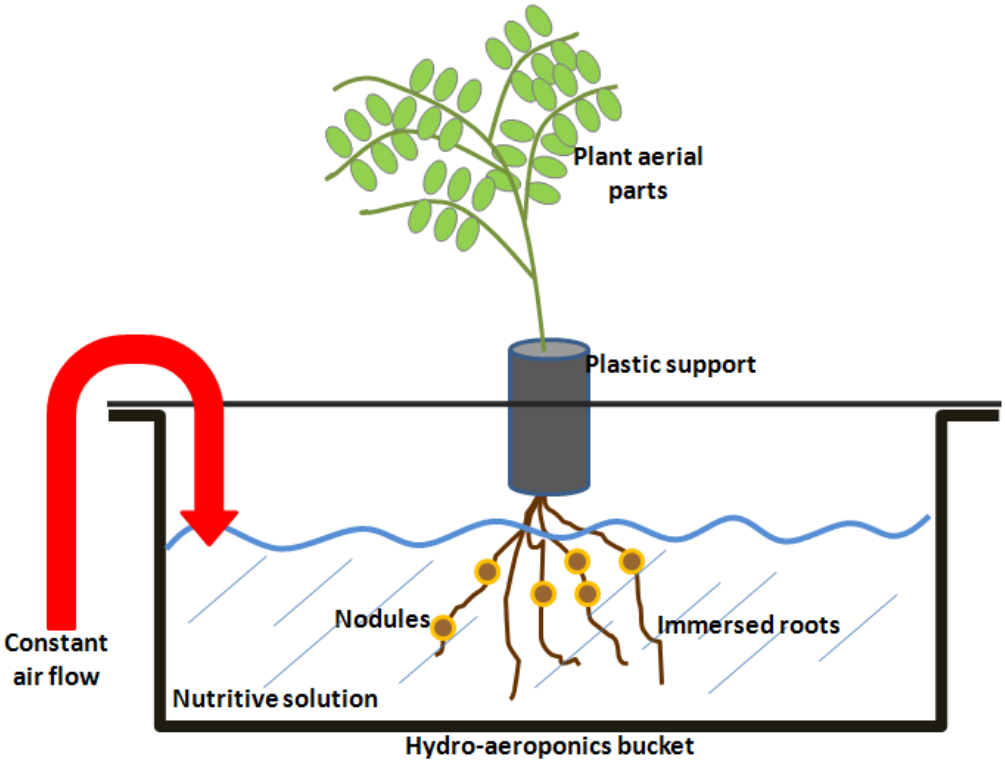


Figure 2-1 **Salt stress-assay with chickpea plants grown in hydroaeroponics**

Main design schema of the hydro-aroponics buckets used for the growth and subsequent salt stress treatment of the chickpea plants from the salt tolerant variety INRAT-93

2.2.2 Drought-stress treatment

Surface-sterilized seeds of drought-tolerant chickpea variety ICC588 were germinated in filter paper boxes at ICARDA (Aleppo, Syria). Seedlings were grown in growth-chambers at a constant temperature of 22^{0}C, a photoperiod of 12 h light/12 h dark and normal watering. After eight days, the seedlings were transferred onto composite soil for a hardening period of 20 days at 20 - 25°C during day/ 15 - 20°C during night with a photoperiod of 16 hours light and 8 hours dark. Control plants were removed from their pots and their roots immediately frozen in liquid nitrogen at the same time that the stressed plants were harvested. Desiccated plants were carefully removed from the composite soil, thereby preventing mechanical damage, and subjected to dehydration for 6h at room temperature (**Figure 2-2**). After this period, the plants showed wilting symptoms (leaves drooped). Subsequently, the roots were separated from the shoots and shock-frozen in liquid nitrogen. For each condition (control and 6 hours desiccation), three replica plants were harvested.

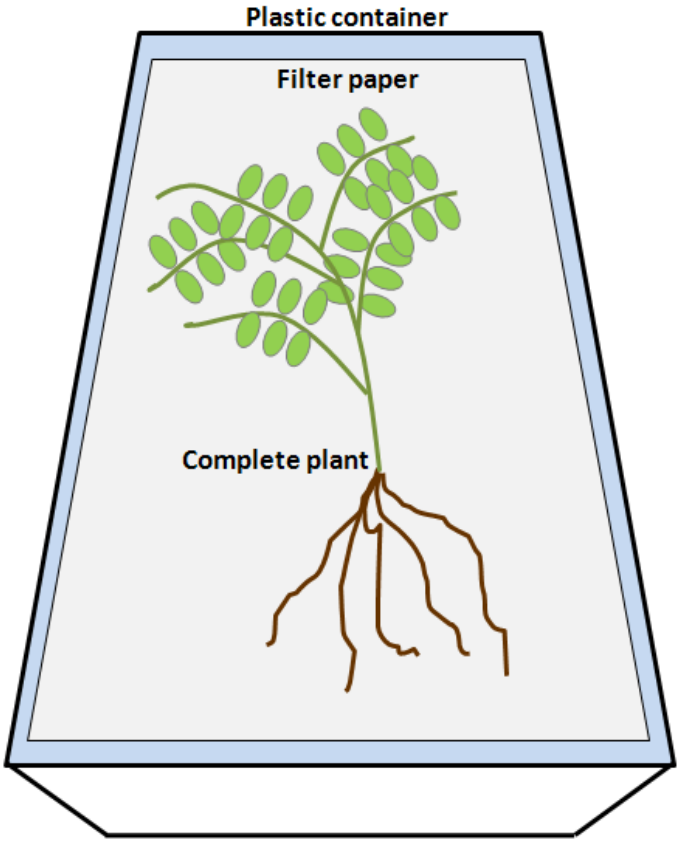


Figure 2-2 **Drought stress-assay with chickpea plants grown in normal soil**

2.2.3 Cold stress treatment

At ICARDA (Aleppo, Syria), surface-sterilized seeds of ILC8262 were germinated on 1% agar-agar in Petri dishes in the dark at 30°C for a period of 6 to 10 days. After germination, plants were transferred to pots containing a sand-soil mixture, and grown for a period of 15 days in glass-house conditions under a natural day-night light cycle. After a 15 days hardening period, the plants were transferred to climatic chambers with an initial pre-stress temperature decrease over 24 hours (down to 12°C). Subsequently, a subset of plants was incubated in a cold chamber at -5°C. The set of control plants that remained at 12°C was harvested after five hours simultaneously with the cold-stressed material. For each treatment, four plant replicas were separately collected, and their shoots shock-frozen in liquid nitrogen for subsequent RNA extraction.

2.3 Construction of SuperSAGE libraries

Initially, establishment of chickpea SuperSAGE libraries mainly followed the protocol of Matsumura et al. (2003), a very elegant but also time-, skill-, and cost-demanding procedure. After the introduction of several optimization steps in the present work, time and costs were reduced by up to 50% in comparison to the previous methodology, based on the resulting amount of information. The major change, namely the adaptation of direct sequencing via 454-Life Science technology (454-Life Sciences, Branford, CT, USA) shortened the SuperSAGE protocol appreciably.

The principle of SuperSAGE relies on the use of the type III restriction endonuclease *Eco*P15I, an enzyme which cleaves a DNA molecule 26 base pairs (bp) away from its recognition site (Mucke et al., 2001), enabling the recovery of a defined cDNA fragment (tag) from each transcript in a poly(A)$^+$-RNA population. The sequence of steps in SuperSAGE libraries construction is detailed in the following sections and depicted on **Figures 2-3A and 2-3B**.

2.3.1 Total RNA isolation and cDNA synthesis

Total RNA was isolated from control and stressed roots using a modified CTAB procedure (Weising, 2005) followed by precipitation of the RNA in 3M LiCl at 4°C overnight. From approximately 1 mg of total RNA, 1 to 3 µg of poly(A)$^+$-RNA were purified using the Oligotex^{TM} mRNA Mini-Kit^{TM} (QIAGEN, Hilden, Germany) according to the manufacturer's batch protocol. Poly(A)$^+$-RNA was reverse-transcribed with the Superscript^{TM} double-stranded cDNA synthesis kit (INVITROGEN, Karlsruhe, Germany) using an oligo-dT including one of the recognition sites for *Eco*P15I (see **Table 2-2**). Briefly, approximately 3 µg poly(A)$^+$-RNA were incubated one hour at 37°C in the presence of 250 pmol oligo-dT primer, 1x SuperScript^{TM} first strand reaction buffer, 300 µM each of dATP, dCTP, dGTP, and dTTP, 10 mM DTT, and 1 U of SuperScript III^{TM} reverse transcriptase. After first strand synthesis, second strand reaction continued for two hours at 16°C in the presence of 200 µM each of dATP, dCTP, dGTP, and dTTP, 10 U *E. coli* DNA polymerase I, 1 U *E. coli*. DNA ligase, and 1 U of RNAse H.

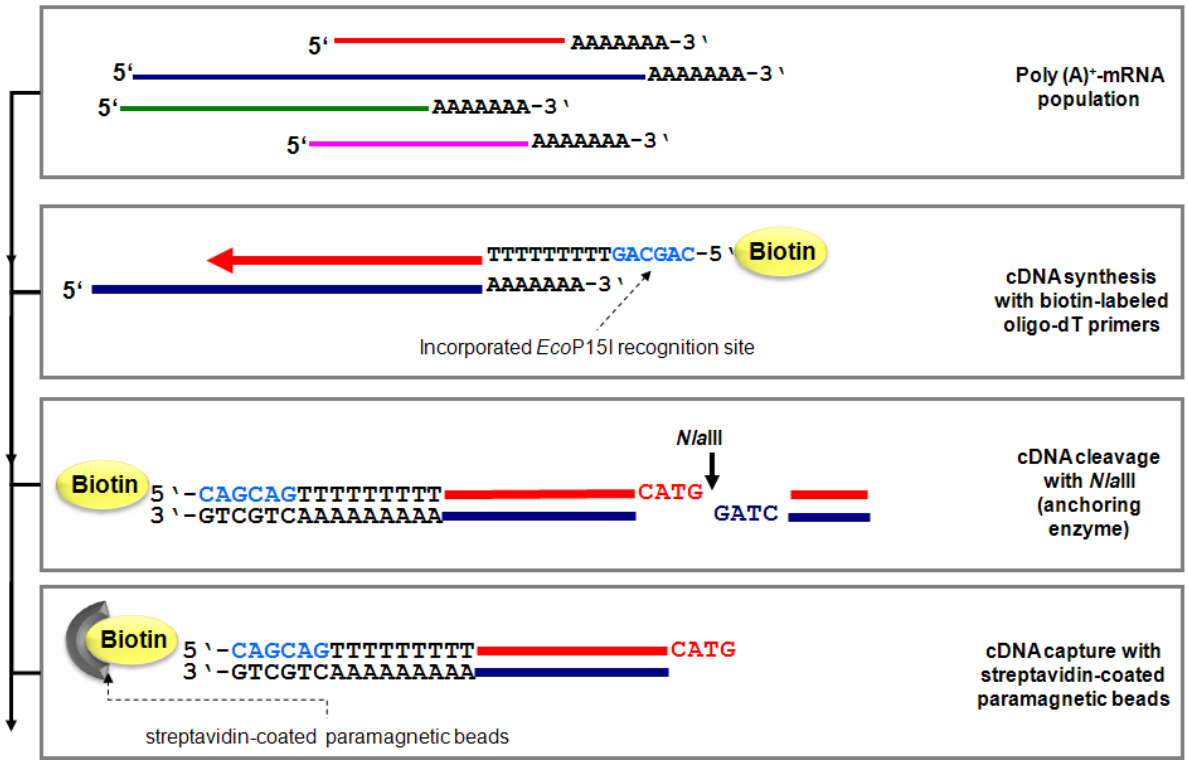


Figure 2-3A First steps in SuperSAGE library construction

Poly(A)$^+$-RNA is reverse transcribed to cDNA using a 5' biotin- labeled oligo-dT containing a small 5'-CAGCAG-3' stretch which provides one of the two necessary recognition sites for the type III restriction endonuclease *EcoP15I*. The resulting cDNA is subsequently cleaved with the restriction endonuclease *Nla*III (anchoring enzyme), leaving a 5'-CATG cohesive overhang.

2.3.2 *Nla*III digestion

Reverse transcribed cDNAs were quantified by conventional OD 260/280 measurement (Sambrook and Russell, 2001), and re-confirmed with Caliper™-chip quantification (CALIPER, Hopkinton MA, USA). Subsequently, *NlaIII* digestion reactions were prepared in 150 µl final volumes containing 2.0 to 4.0 µg cDNA, 150 U *NlaIII* (NEW ENGLAND BIOLABS, Frankfurt, Germany), 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, and 1 µg ml^{-1} Bovine Serum Albumin (BSA), and incubated at 37°C for 1.5 hours. After a first round of digestion, cDNA probes were **re-digested** a second time under identical conditions.

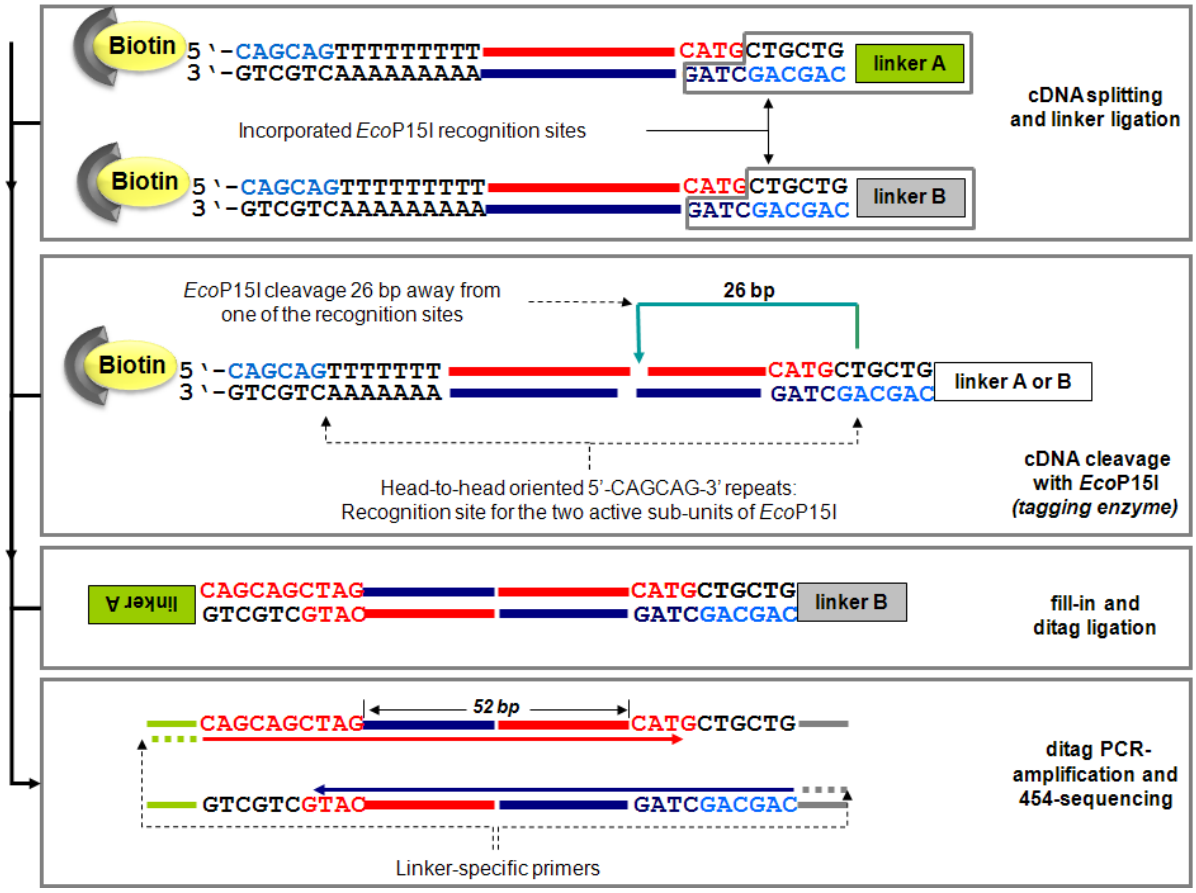


Figure 2-3B Subsequent steps in the SuperSAGE procedure

After cDNA-binding to paramagnetic beads, each probe is split, and the two resulting fractions ligated to two different DNA-linkers (linker A and linker B, respectively). Linker-cDNA fragments are then restricted with *Eco*P15I, releasing a DNA fragment consisting of the previously ligated linker plus a 26bp cDNA tag**.** Linker-tag fragments were recovered and ligated to linkerA-tag-tag-linkerB ditags. Resulting fragments were amplified with Linker-specific primers, and directly sequenced via the 454-pyrosequencing technology.

2.3.3 cDNA capture with paramagnetic beads and linker ligation

Each *Nla*III-restricted cDNA was bound to 1.0 mg of M-280™ streptavidin-coated paramagnetic Dynabeads™ (DYNAL BIOTECH, Hamburg, Germany) through incubation at room temperature in 1x binding-buffer (5 mM Tris-HCl, pH 7.5, 0.5mM EDTA, 1 M NaCl). After incubation, the cDNAs bound to the paramagnetic particles- were separated from non-bound material in a magnetic particle capturer (MPC), washed twice with 1x binding-buffer, and re-suspended in Low-TE Buffer (3 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, pH 7.5).

As depicted in **Figure 2B**, subsequent steps aim at releasing linker-tag cDNA fragments from the immobilized beads by *Eco*P15I. For efficient cleaving, *Eco*P15I requires two -5'-CAGCAG-3' recognition sites in a head-to-head polarity (Mucke et al., 2001). Two pre-formed recognition sites for this enzyme were added in the SuperSAGE process as follows: i) a 5'-

CAGCAG-3'-stretch was included in the oligo-dT primer used for cDNA synthesis, and ii) a 5'-CAGCAG-3' stretch was part of the SuperSAGE DNA-linkers (Matsumura et al. 2003). SuperSAGE DNA-linkers were prepared in equimolar proportions of both linker-oligos (see **Table 2-2**) at 100 µM final concentration. Linker annealing was carried out by heating the oligo solution at 95°C for 10 min, followed by a gradual temperature decrease to 22°C. Linkers were ligated to the Dynabead-immobilized cDNAs in 50µl reactions, each containing 100 pmol linker, 5 U T4 DNA ligase (INVITROGEN, Karlsruhe, Germany), 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, and 0.5 mM ATP, by incubation at 16°C for two hours. After ligation, the paramagnetic beads were captured and washed with 1x binding-buffer to eliminate non-ligated DNA fragments.

2.3.4 Release of linker-tag fragments

Prior to *Eco*P15I digestion, the Dynabeads containing the captured cDNAs were washed and re-suspended twice in 1x *Eco*P15I reaction buffer (50 mM Tris-HCl pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 1 µg ml^{-1} BSA). *Eco*P15I restriction reactions were carried out with 10 units of *Eco*P15I (NEW ENGLAND BIOLABS, Frankfurt, Germany) in the presence of 1 mM ATP and a final volume of 100µl at 37°C for 1 hour. After a first round of restriction, the paramagnetic beads were captured, the remaining solution was recovered (Linker-Tag)**,** and a new round of digestion was carried out. Both digestion mixtures were purified with phenol:chloroform:isoamyl alcohol (vol/vol 25:24:1) and precipitated with ice-cold 70% ethanol and 0.75 M NH_4OAc, in the presence of 200µg of glycogen (ROCHE, Mannheim, Germany).

Table 2-2 SuperSAGE required oligo-nucleotides

Oligonucleotides used in the SuperSAGE procedure, their sequences and modifications, and the purpose for modifications. Customized recognition sites for *EcoP151* are highlighted in red

		Modifications		
Oligo name	Sequence 5'- 3'	5'-end	3'-end	Purpose
SuperSAGE-dT	CTGATCTAGAGGTACCGGATCCCAGCAGTTTTTTTTTTTTTTTT	biotin	" - "	cDNA synth.
Linker A-1	TTTGGATTTGCTGGTGCAGTACAACTAGGCTTAATACAGCAGCATG	" - "	" - "	Linker A
Linker A-2	CTGCTGTATTAAGCCTAGTTGTACTGCACCAGCAAATCCAAA	phosphate group	amino-C7	Linker A
Linker B-1	TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGCAGCAGCATG	" - "	" - "	Linker B
Linker B-2	CTGCTGCGTACATCGTTAGAAGCTTGAATTCGAGCAGAAA	phosphate group	amino-C7	Linker B
Linker A primer	ACAACTAGGCTTAATACAGCAG	biotin	" - "	Ditag PCR
Linker B Primer	CTTCTAACGATGTACGCAGCAG	biotin	" - "	Ditag PCR

2.3.5 Purification of linker-tag fragments

Precipitated linker-tag fragments were washed, re-suspended, and purified via electrophoresis in 2.0% low melting agarose. Fragments of the expected size (SuperSAGE linker + 26bp tag) were visualized under UV light after staining with ethidium-bromide, and excised from the gel. Agarose plugs containing the excised bands were digested with Agarase (AgarACE™, PROMEGA, Mannheim, Germany) according to the manufacturer's instructions.

2.3.6 Filling-in and ditag ligation

Since *Eco*P15I creates a 5'-overhang of two bases at its cleaving site (Mücke et al. 2001), a filling-in step of high efficiency is a major requirement preceding the ditag ligation. Filling-in and ditag ligations were carried out with the Blunting-high™ kit (TOYOBO, Japan). Initial filling-in reactions were started by incubating 3.5 µl of cDNA template (linker-tag fragments) in the presence of 2.5 U of KOD1 (*Pyrococcus sp.)* DNA polymerase, 120 mM Tris-HCl, pH8.0, 10mM KCl, 1.5 mM $MgCl_2$, 6 mM $(NH_4)2SO_4$, 200 µM dNTPs, 0.1% TritonX-100, and 0.001% BSA at 72°C for 2.5 minutes. Then 5.0 µl of LoTE were added along with 10 µl of Ligation-high™ mix, and linker-tag fragments incubated at 16°C overnight. Resulting ditags (linkerA+tag-tag+linkerB fragments) were 10-fold diluted with nuclease-free water for PCR amplification.

2.3.7 PCR amplification of ditags

For PCR amplification, different dilutions in nuclease-free water of the ligated ditags were first prepared. Test PCRs with decreasing amounts of template were carried out with 0.5 µl and 1.0 µl of [1/1], [1/10], [1/100] ditag dilutions, respectively. PCR amplifications proceeded in 25 µl reactions containing 0.625 U Platinum *Taq*™ DNA polymerase (INVITROGEN, Karlsruhe, Germany), 600 µM of each dATP, dCTP, dGTP, and dTTP, 1.5 mM $MgCl_2$; 20 mM Tris-HCl (pH 8.4), 50 mM KCl; and 15 pmol of each linker-A- and linker-B-specific primers. Temperature cycling was started with an initial denaturation at 94°C for 2 min, followed by 28 cycles of 30 sec at 94°C, 45 sec at 55°C, and 60 sec at 70°C and a final extension at 70°C for 2 min. Additionally, no-sample control amplifications were carried out for each library and linker combination. Amplicon sizes were checked via 8% non-denaturing PAGE.

2.3.8 Massive ditag amplification and purification for direct sequencing via 454-technology

After checking the ditag test PCRs, 30 to 50 parallel amplification reactions using the optimal amount of sample were carried out for each processed SuperSAGE library under the conditions described in the previous step. Amplicons were purified via Qiaquick™ PCR purification columns (QIAGEN, Hilden, Germany) according to the provider's instructions. Purified amplicons were re-purified by 8.0% PAGE, and the bands of the expected ditag size excised, and electro-eluted in 400 µl electroelution tubes (ROTH, Karksruhe, Germany) by applying 9 volt cm^{-1} for 60 minutes in an electrophoresis chamber in the presence of 1xTBE. Electro-eluted fragments were recovered and purified with Qiaquick™ Mini-elute™ PCR-purification columns (QIAGEN, Hilden, Germany). Purified fragments were directly sequenced with 454-technology (454-Life Sciences, Branford, CT, USA).

2.4 Data analysis

2.4.1 SuperSAGE tags counting and libraries normalization

For each library, 26 bp long SuperSAGE tags (here called SuperTags) were extracted from the raw sequences using the GXP-SuperTagsorter™ (GENXPRO GmbH, Frankfurt am Main, Germany). After counting, all SuperTags were sorted into unique tags groups (here called UniTags). SuperSAGE libraries normalization, comparisons and primary statistical treatments were carried out with DiscoverySpace 4.01 software (Canada's Michael Simith Genome Sciences Centre, available at http://www.bcgsc.ca/discoveryspace). Scatter plots of the distribution of the expression ratios ($R_{(ln)}$) based on direct comparisons of libraries, and significance levels were calculated according to the algorithms for expression data from Audic and Claverie (1997).

Due to the diversity of chickpea varieties, organs, and locations involved in this work, direct comparisons between inter-stress and inter-tissue libraries were not possible in all cases. To analyze similar responses between the different stress treatments and varieties, secondary indirect comparisons were carried out based on the expression ratios control-library/stress-library from each treatment (**Figure 2-4**).

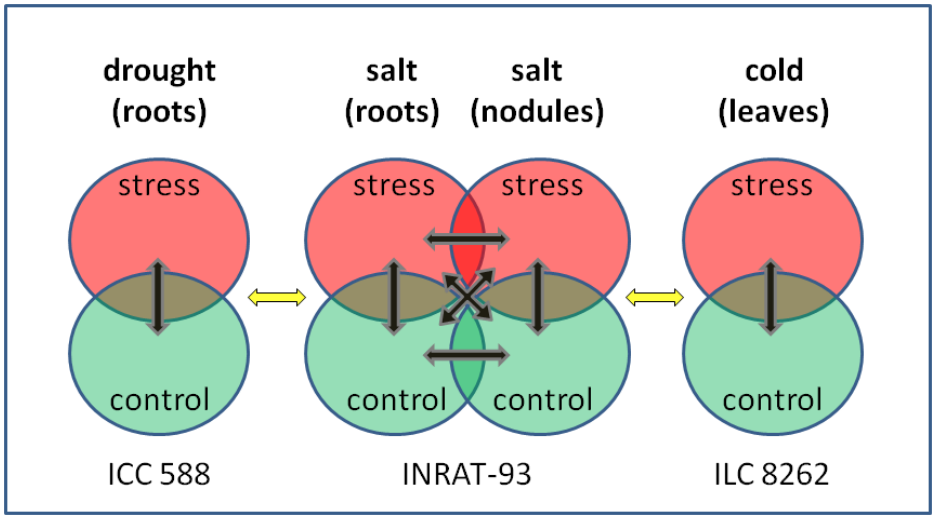


Figure 2-4 SuperSAGE libraries from drought-, salt-, and cold-stressed chickpea plants

The transcriptional responses of chickpea plants to drought, salt, and cold stresses, respectively, are covered by 8 SuperSAGE libraries (stress: red; control: green). The diversity of locations and plant varieties precludes direct comparisons for differential expression which are only possible in the indicated direction (**black arrows**). Extrapolation of results between different stress situations was possible at a secondary level **(yellow arrows)**.

2.4.2 Sequence homology alignment of 26-bp SuperTags

UniTags sequences were BLASTed (Altschul et al., 1990) against different public databases discriminating the hits in a hierarchical-taxonomical manner using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). All obtained sequences were first BLASTed against the NCBI non-redundant DNA databases, limiting the output hits with the highest priority level to *Cicer arietinum* and members of the *Fabaceae* family (legumes) by using the routine BLASTcl3 (NCBI, www.ncbi.org). Subsequently, individual local BLAST searches were carried out in the TIGR gene indices for members of the *Fabaceae* family. After legume-restricted database search, TIGR gene indices from *Arabidopsis*, rice and maize completed the annotation (http://compbio.dfci.harvard.edu/tgi/plant.html). Detailed information about the screened databases is shown in **Table 2-3**.

Table 2-3 Screened EST- and genomic-databases for annotation of SuperSAGE tags

Overflow of anonymous hits (i.e. cDNA clones and complete chromosomes) was avoided by annotating in hierarchical manner. After each BLAST round, non-informative entries were eliminated before continuing with the next step.

Database	BLASTing routine	Hierarchical level
NCBI (nr) *Fabaceae* (Priority: *Cicer arietinum*)	Netblast - Blastcl3	1
TIGR gene index - *Medicago truncatula*	Localblast - Blastn	2
TIGR gene index - *Phaseolus vulgaris*	Localblast - Blastn	3
TIGR gene index - *Glycine max*	Localblast - Blastn	4
TIGR gene index - *Lotus japonicus*	Localblast - Blastn	4
TIGR gene index - *Arabidopsis thaliana*	Localblast - Blastn	5
TIGR gene index - *Oryza sativa*	Localblast - Blastn	6
TIGR gene index - *Zea mays*	Localblast - Blastn	7

2.4.3 Cluster analysis and functional category distribution analysis of SuperSAGE tags

Cluster analysis of the SuperSAGE expression ratios [$R_{(ln)}$] used the software package Cluster 3.0 (Stanford University, 1989, http://bonsai.ims.utokyo.ac.jp /~mdehoon/ software/cluster). A distance matrix for the $R_{(ln)}$ was calculated with Pearson's correlation distance method (Eisen et al., 1998), and UniTags were grouped using the average linkage clustering routine under hierarchical clustering.

Shared tendencies analysis of expression ratios from different stress treatments was conducted by analyzing the output of the Cluster analysis with the software Venn maper 1.0 available at http://www.gatcplatform.nl/vennmapper/index.php.

Over-representation P values for Gene Ontology (GO) categories (biological processes) observed in the different stress situations were calculated and correlated with the UniTag expression ratios ($R_{(ln)}$) by applying the Gene Score Re-sampling (GSR) analysis of the ErmineJ 2.0 software package (University of British Columbia, 2006, http://www.bioinformatics .ubc.ca/ermineJ), according to Breslin and co-authors (2004), as indicated by the software developers.

2.5 Confirmatory experiments

2.5.1 Rapid amplification of cDNA ends (3'- and 5'-RACE) using UniTags as PCR primers

A subset of 26bp SuperSAGE tags was selected for direct use as 3'- and 5'-RACE PCR primers. For 3'-RACE, cDNA amplifications were carried out with an initial denaturation step of 94°C for 2 min, followed by 30 cycles each of 94°C for 40 sec, 55°C for 1 min, and 72°C for

1 min, with a final extension step at 72°C for 4 min. Reactions contained 15–20 ng cDNA template, 10 pmol 26bp Tag-based primer, 10 pmol oligo-dT 14-NV primer, 200 µM dNTPs, 0.4 U *Taq* DNA polymerase (Genecraft, Germany) in buffer containing 1.5 mM $MgCl_2$ supplied by the provider. Amplification of the region flanked by the SuperSAGE 26bp Tag and the 5'-end of each selected cDNA was carried out with the SMART™ 5'-RACE kit (CLONTECH, CA, USA) according to the providers instructions. Detailed information about the multiple steps of the 5'-RACE procedure is available at (http://www.clontech.com/images/pt/PT3269-1.pdf).

RACE (5' and 3') products were separated in 1.5% preparative agarose gels, and bands corresponding to unequivocal amplicons were excised and purified with Qiaquick™ cleanup columns (QIAGEN, Hilden, Germany). Cloning of PCR products as well as colony PCR screening followed standard blue-white screening procedures (Sambrook and Russell, 2001). Positive clones were sequenced via ABIprism™ multi-colour fluorescence-based DNA analysis system (APPLIED BIOSYSTEMS, Foster City CA, USA).

2.5.2 Confirmation of SuperSAGE expression profiles via qRT-PCR

Parallel RNA extractions of tissues, from which the SuperSAGE libraries were derived, were carried out as described in **section 2.3.1**. Approximately 500 ng of total RNA were further processed to poly(A)$^+$-RNA with Oligotex™ matrix (QIAGEN, Hilden, Germany). cDNA was synthesized using the Superscript III™ double-stranded cDNA synthesis kit (INVITROGEN, Karlsruhe, Germany). Resulting cDNA was quantified with two parallel methods: i) Nanodrop™ spectrometer measurement (NANODROP, Willmington DE, USA), and ii) Caliper™ chip quantification (CALIPER, Hopkinton MA, USA).

*Taq*Man™ probe and SYBRgreen oligonucleotide design was carried out with software package Primer Express, version 2.0, provided by Applied Biosystems (Foster City, CA, USA), with 3'- or 5'-RACE products from selected SuperTags as starting points. The real-time PCR reactions for SYBRgreen™ and *Taq*Man™ assays used the Power-SYBRgreen™ PCR master mix and the *Taq*Man™-Universal PCR Master mixes, respectively (Applied Biosystems). RT-PCR amplifications were carried out in a StepOne™ RT-PCR System machine with the following temperature profile for SYBRgreen™ assays: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec. and 60°C for 20 sec. (annealing and elongation). *Taq*Man™ assay profiles consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec. and 65°C for 30 sec. Amplicon quality was checked

by an additional melting curve gradient with fluorescence measures after each temperature step. The amplification of the target genes at each cycle was monitored by SYBRgreen- or *Taq*Man probe-released fluorescence. The Ct, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, was used as a measure for the copy number of the target gene at the start. Relative quantitation of the targets amplified via SYBRgreen™ assays was performed by the comparative ΔΔCt method. Genes amplified by *Taq*Man™ assays were quantified via the Relative Standard Curve Method (Applied Biosystems). The efficiency of each primer pair was checked with cDNAs from control and 6h-desiccated plants as standard templates. The RT-PCR data were normalized with the relative efficiency of each primer pair.

2.5.3 Confirmation of expression profiles via UniTags microarray

2.5.3.1 Micro-array design and spotting

SuperSAGE expression profiles were confirmed by direct spotting of a selection of 26 bp Tags onto microarray supports from two different platforms. The first platform was selected according to the standard guidelines of Array-on GmbH (Gatersleben, Germany), and contained a total of three hundred oligonucleotides representing Tags with different expression levels in chickpea roots and nodules under drought and salt stress regimes. Additionally, for a small subset of Tags, oligos with mismatches in positions 7, 13, and 20 were included. Each oligo was spotted in ten different positions along a microarray section ("quadrant"). Each quadrant was reproduced four times in four microarray sections to generate a total of 40 replicas per oligo.

For a second and deeper analysis, an Agilent ™ 16K oligo microarray was designed (AGILENT TECHNOLOGIES, Santa Clara CA, USA). Three thousand UniTags with different expression levels under cold, drought, and salt stresses were selected. From these, a subset of 2,796 oligos was spotted in duplicate onto different sections of the microarray. Additionally, for each of the 3,000 selected tags, oligos with mismatches were spotted onto the microarray in three sets as follows: i) mismatch at position 7; ii) mismatches at positions 7 and 13; respectively, and iii) mismatches at positions 7, 13, and 20, respectively. Background correction was achieved by the Feature Extraction software™ (Agilent Technologies), subtracting the mismatch intensities for each spotted UniTag.

Microarray design, spotting and hybridizations were carried out at the Array-on facilities (ARRAY-ON, Gatersleben, Germany) according to the Agilent™ directions (AGILENT TECHNOLOGIES, Santa Clara CA, USA).

2.5.3.2 cDNA synthesis and cRNA labeling

For the first platform, total RNA extractions, poly(A)$^+$-mRNA isolation and cDNA synthesis (1.0 µg) from tissues also used for SuperSAGE were carried out as described in **section 2.3.1**. The obtained cDNAs were transcribed to cRNA by using the MEGAscript™ T7-RNA amplification Kit (AMBION, Austin TX, USA), following the provider's protocol. cRNAs were eluted in 15µl nuclease-free water and incubated for 35 minutes at 95°C in 1x fragmentation buffer (40 mM Tris-ac, 100 mM KOAc, and 30 mM MgOAc). Prior to hybridization, fragmented cRNAs were checked by ethidium-bromide staining in 1.5% 1xTBE agarose gels.

For the 16K Agilent microarray, total RNA from each probe (200 ng) as well as internal "spike-in" RNA controls were labeled with Cy3- or Cy5-CTP using the Two-Color Microarray-based Gene Expression Analysis kit (Agilent Technologies). Initially, total RNA was reverse transcribed at 40°C for two hours in the presence of 1x first strand buffer (Agilent), 10 mM DTT, 100 pmol T7-promoter oligo-dT, 300 µM each of dATP, dCTP, dGTP, and dTTP, and 1U MMLV reverse transcriptase. The obtained first-strand cDNA was then transcribed to cRNA in 1x transcription buffer (Agilent technologies), 10 mM DTT, 1x Agilent NTP mix, 5% PEG, 1U inorganic pyrophosphatase, 1U T7-RNA polymerase, and 1.0 mM Cy3-CTP or Cy5-CTP, respectively, by incubation at 40°C for two further hours. Obtained cRNAs were purified via RNAeasy™ Mini-elute™ columns (QIAGEN, Hilden, Germany). Fragmentation and hybridization of cRNAs were carried out under the Agilent guidelines at Array-On (Gatersleben, Germany). Additional to the standard labeling, in which control and treated probes for each type of stress were labeled with cy3 and cy5, respectively, dye-swap sets of cRNAs were included with the opposite labels.

3 A snapshot of the chickpea transcriptome using SuperSAGE

After sequencing a total of eight chickpea SuperSAGE libraries for the first time using the high-throughput potential of the 454-technology, the amount of generated data per experiment was at least 8-fold higher than in previous studies (Matsumura et al., 2003; Coemans et al., 2005). With 278,387 effective 26-bp SuperTags (excluding singletons and twin-ditags), representing 30,144 unique transcripts (UniTags), the present study reveals the largest tag-based transcription profiles available for chickpea up to date.

3.1 General aspects of the chickpea SuperSAGE-based transcriptome: Transcripts in very high copy numbers are not frequent

3.1.1 Frequencies of tag copy numbers in INRAT-93 SuperSAGE libraries (roots)

Upon salt-treatment of the salt-tolerant variety INRAT-93 (control and stressed conditions), a total of 86,919 tags from roots represented 17,918 UniTags. In both libraries, less than 1% percent of the tags were present in very high copy numbers (>5'000 copies million^{-1}), whereas 9% and 90% of the transcripts were present between 100 to 1'000 and less than 100 copies million^{-1}, respectively (**Table 3-1**).

3.1.2 Frequencies of tag copy numbers in INRAT-93 SuperSAGE libraries (nodules)

In nodules of the same INRAT-93 plants from which the root libraries were developed, 13,115 UniTags were extracted out of 57,281 sequenced tags. As already observed in roots, less than 1% of the transcripts were present in very high copy numbers (> 5,000 copies million^{-1}). However, the number of transcripts present between 100 to 1,000, and less than 100 copies million^{-1} varied to some extent. Fifteen percent of the transcripts fell in between 100 and 1,000 copies million^{-1}, contrasting the 10% found in roots. Transcripts detected in less than 100 copies million^{-1} made up ~ 85% of the total UniTags (**Table 3-1**).

3.1.3 Frequencies of tag copy numbers in ICC588 SuperSAGE libraries (roots)

For the two libraries of drought-treated chickpea plants (drought-tolerant variety ICC588, either subjected to 6h desiccation or from well-watered controls), a total of 82,012 tags from roots were sequenced and represented 17,498 unique transcripts (UniTags). Similar to the results in the INRAT-93 root experiment, less than 1% of the tags were detected at very high copy numbers (> 5,000 copies million^{-1}), whereas 10% and 89% of the

transcripts were present between 100 to 1,000 and less than 100 copies million^{-1}, respectively (**Table 3-1**).

3.1.4 Frequencies of tag copy numbers in ILC8262 SuperSAGE libraries (leaves)

The transcriptome of chickpea leaves harvested from the cold-tolerant variety ILC8262 (control and cold treated) varied from that of roots and nodules from the varieties ICC588 and INRAT-93. Still, transcripts present in more than 5,000 copies million^{-1} represent only a very low portion (< 1%). On the other hand, the proportion of tags between 100 and 1,000 copies million^{-1} accounted for almost 20% of the UniTags, 10 and 5% more than observed for roots and nodules, respectively. A total of 52,175 sequenced tags represented 10,115 UniTags (**Table 3-1)**.

Table 3-1 Primary results after 454-sequencing of chickpea SuperSAGE libraries

After statistical filtering, a total of 278,387 SuperTags, representing more than 30'000 unique transcripts (UniTags), were used for further library comparisons. Differential expression, abundancy classes, and annotation aspects are denoted for each assay.

		Drought (roots)		Salt (roots)		Salt (nodules)		Cold (leaves)	
		number of Tags	%	number of Tags	%	number of Tags	%	number of Tags	%
Sequencing	Sequenced Tags	82,012		86,919		57,281		52,175	
	Number of Unique Transcripts (UniTags)	17,498		17,918		13,115		10,115	
Differential expression	Up-regulated (> 8.0-fold)	388	*2.22*	346	*1.93*	96	*0.73*	595	*5.88*
	Up-regulated (> 2.7-fold)	2,556	*14.61*	2,279	*12.72*	2,190	*16.70*	3,093	*30.58*
	Down-regulated (> 2.7-fold)	4,975	*28.43*	3,962	*22.11*	1,439	*10.97*	1,224	*12.10*
	Down-regulated (> 8.0-fold)	589	*3.37*	2,055	*11.47*	72	*0.55*	323	*3.19*
Transcript abundancy classes	> 5,000 copies million^{-1}	2	*0.01*	3	*0.02*	5	*0.04*	7	*0.07*
	3,000 to 5,000 copies million^{-1}	10	*0.06*	9	*0.05*	10	*0.08*	11	*0.11*
	1,000 to 3,000 copies million^{-1}	48	*0.27*	72	*0.40*	61	*0.47*	63	*0.62*
	100 to 1,000 copies million^{-1}	1,718	*9.82*	1,688	*9.42*	1,956	*14.91*	1,948	*19.26*
	2 to 100 copies million^{-1}	15,720	*89.84*	16,146	*90.11*	11,083	*84.51*	8,086	*79.94*
Annotation	ESTs Linked to UniProt entries	3,858	*22.05*	3,998	*22.31*	3,006	*22.92*	1,994	*19.71*

3.2 UniTag annotation to ESTs deposited in public databases

From the 30,144 UniTags of the complete chickpea SuperSAGE dataset (drought, salt, and cold treatments), 5,915 significantly matched with ESTs linked to characterized Uniprot entries. Individual values for each treatment and tissue are depicted in **Table 3-1**. Uncharacterized entries such as anonymous genomic DNA, whole chromosomes, anonymous ESTs, and shotgun sequencing clones were excluded for further analysis due to incompatibility with functional databases such as gene ontology (**GO**; http://www.geneontology.org). From Uniprot-linked annotations, 49.6, 17.4, and 15.8%, respectively, corresponded to *Medicago truncatula*-TIGR, *Lotus japonicus*-TIGR, and *Cicer arietinum-NCBI*(nr) *databases* (**Figure 3-1**). Surprisingly, chickpea entries could not be called maximally. Considering the sizes of the screened databases (i.e. the number of deposited ESTs), chickpea, with only 1,542 core nucleotide- and 2,486 EST-entries at NCBI, is clearly inferior to the massively sequenced species like *M. truncatula* (226,923 deposited ESTs) or *L. japonicus* (109,618 deposited ESTs), fact reflected by the observed results.

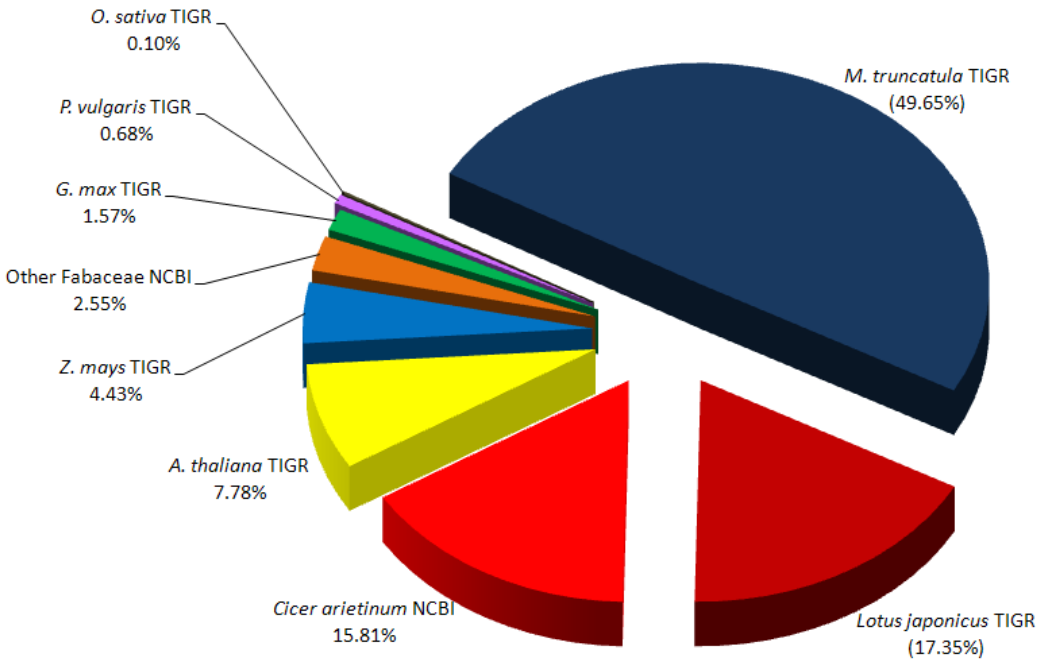


Figure 3-1 **Distribution of UniTags linked to Uniprot-entries according to the screened public databases**

The fact that chickpea as an "orphan crop" cannot profit from the massively accumulated knowledge on related model species, justifies i) the use of open-architecture techniques like SuperSAGE, and ii) the attempt to efficiently transfer this massive knowledge from the model legumes. With the advent of the large-scale transcription profiling techniques, the focus of the analyses will continue to shift from examining the expression and action of single genes to whole transcriptomes (Brady et al., 2006). Therefore, techniques with high annotation versatility are required.

3.3 The resolution of the SuperSAGE technology: Unique transcripts vs transcript-isoforms

Some of the most frequent questions arising during SAGE-based profile analyses concern the diversity of the tags of a sequenced library, the representation of a tag in the population, the handling of tags with very similar sequences, and the correlation between tag size and similarity of tags (Stern et al., 2003). To clarify these questions, *in silico* analyses were carried out to compare the results observed in chickpea with previous SuperSAGE-derived datasets, as well as with data derived from other tagging techniques.

3.3.1 SuperSAGE and other tagging techniques

To define the degree of tag sequence similarity within chickpea SuperSAGE libraries, the UniTags dataset obtained from libraries Ca-ICC588-D-Ct and Ca-ICC588-D-Str (drought-control and drought-stressed chickpea, respectively) were selected for self-BLASTing via local BLAST (Altschul et al., 1990). For a comparison to previous SuperSAGE experiments and to SAGE datasets with shorter tags, the SuperSAGE dataset developed from *Musa acuminata (GPL2542)* (Coemans et al., 2005), the maize Long-SAGE dataset GSM30936 (Gowda et al., 2004), and the rice SAGE dataset GPL5365 (unpublished), were additionally retrieved from the gene expression omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and self-BLASTed. The lowest threshold for considering two different SuperSAGE tags as similar was set by the BLAST software starting from sequence homologies larger than 20 bp. For Long-SAGE (21 bp) and SAGE (14 bp) tags, the lowest threshold limits were set at 16 and 10 bp, respectively.

In the three evaluated SuperSAGE datasets (chickpea and banana), 70% of the UniTags did not find high homologies (>20 bp) to any other UniTag within the own dataset (**Figure 3-2**). In much lower proportions, 15, 4, and 2% of the UniTags, respectively, found one, two, and three similar hits within the same libraries. For all similarity levels, the banana (Coemans et al., 2005) and chickpea SuperSAGE libraries showed very similar characteristics.

If the techniques with shorter tags are compared with the SuperSAGE technology, then only 45% of the Long-SAGE, and only 19% of the SAGE UniTags were distinguishable from others within the same database by more than 5 and 6 bp, respectively. In Long-SAGE, 21, 9, and 4%, respectively, of the UniTags found one, two, and three similar hits within the own dataset. In the normal SAGE, 23, 20, and 15% of the UniTags, respectively, were categorized as having 1, 2, and 3 similar hits within the own database (**Figure 3-2**).

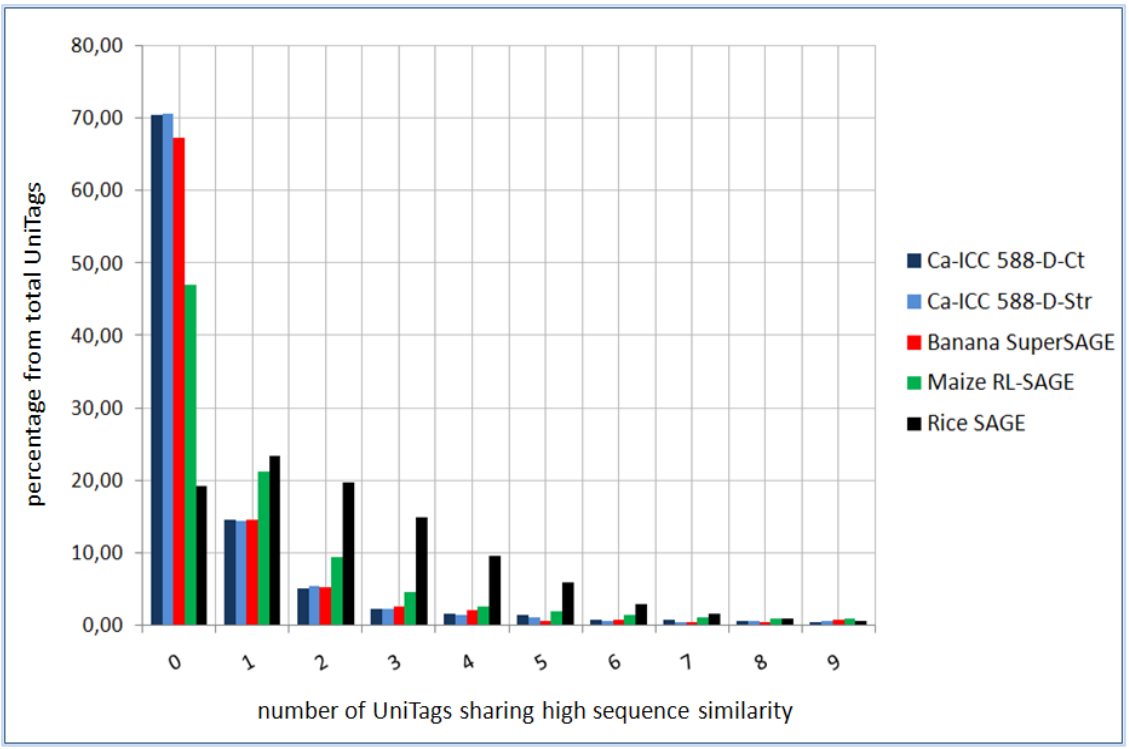


Figure 3-2 Resolution of SuperSAGE and other tagging techniques

Proportion of total UniTags (ordinate) versus number of similar hits for a given tag (abscissa) after self-BLASting of the chickpea ICC588 datasets (blue columns). Results from ICC588 were compared to other self-BLASTed SuperSAGE as well as Long-SAGE and SAGE libraries retrieved from the NCBI gene expression omnibus. Close to 70% of the SuperSAGE tags did not find high similarity hits, whereas 15 and 4% found high similarity hits (> 20 bp) with one and two other UniTags, respectively, within the own datasets.

0 : Percentage of UniTags with low similarity hits within the own dataset

1 - 9 : Number of similar hits for a given UniTag (in percentage of total tags)

3.3.2 SNP-associated alternative tags in chickpea datasets

In the tested chickpea ICC588 root libraries, a survey of SNP-associated alternative tags (here called SAATs) was carried out. After self-BLASTing the chickpea ICC588 dataset, 430 out of the total 17,498 analyzed UniTags (2.45%) revealed to have one SNP-associated alternative hit. Further on, 0.3% (66 out of 17'498) and 0.1% (18 out of 17'498) of the analyzed UniTags revealed to have two and three SAATs, respectively. The occurrence of SNPs within the region contiguous to the anchoring enzyme recognition site in SAGE-related procedures (*Nla*III for SuperSAGE) has been previously analyzed in the human transcriptome (Silva et al., 2004). According these authors, although sequencing errors cannot be discarded, there is enough evidence to consider the SAATs as separate entities. In the present study, each SAAT was treated also as an individual transcript.

3.4 Gene-expression changes upon abiotic stresses in chickpea: A large portion of the transcriptome is stress-responsive

In the present section, apart from the results showing the drought- and salt-responsive chickpea transcriptome, the "transcriptome-remodeling" of cold-stressed chickpea leaves is additionally shown. Although the cold stress responses are not deeply analyzed in the present thesis, in a near future, the already generated data will enable subsequent studies to deeply dive into the chickpea leaves gene-expression.

According to the results obtained after the pair-wise comparisons between control and stress libraries, and in agreement with several previous plant abiotic-stress expression profile studies with different types of platforms (Kawasaki et al., 2001; Seki et al., 2002; Seki et al., 2002; Rabbani et al., 2003), a large portion of the chickpea transcriptome reacts upon stress with changes in expression. However, the proportion of differentially expressed genes may vary, partly because of diverse resolution levels of the different techniques. In the chickpea SuperSAGE profiles, a large portion of the transcriptome is present at very low copy numbers (< 100 copies million^{-1}). This detection level is certainly better than the levels achieved by many hybridization-based techniques.

After twin-ditag and singleton filtering, control vs stress SuperSAGE libraries for each treatment were compared by using the software package Discoveryspace 4.0 (http://www.bcgsc.ca/platform/bioinfo/software/ds). Differentially expressed transcripts (UniTags) were selected with two thresholds according to the absolute value of the natural

logarithm of their expression ratios [here denoted as $R_{(ln)}$], as follows: i) more than 2.7-fold differential expression [$R_{(ln)}$>1.0], and ii) more than 8.0-fold differential expression [$R_{(ln)}$>2.0].

3.4.1 Salt stress-induced "transcriptome remodelling" in chickpea roots and nodules

In roots of the salt-tolerant chickpea variety INRAT-93, 35% of the UniTags were at least 2.7-fold up- or down-regulated, respectively, after only 2 hours of 25 mM NaCl-treatment (**Figure 3-3, Table 3-1**). More than 2,000 UniTags (11%) were at least 8-fold down-regulated, a much higher proportion than the mere 1.93% (346 UniTags) showing more than 8-fold up-regulation, and also, far more than the 0.55 and 0.73% (72 and 96 UniTags, respectively) showing at least 8-fold down- and up-regulation in nodules of the same plants.

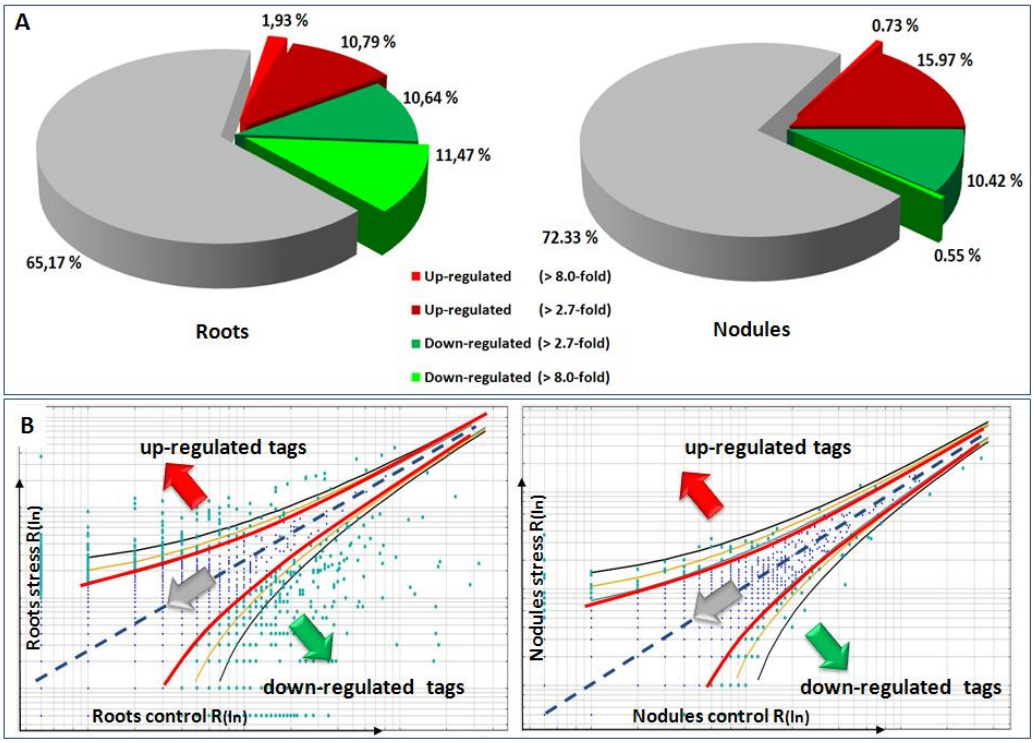


Figure 3-3 Chickpea roots and nodules salt-responsive transcriptome

A) Schematic representation of UniTags expression changes in chickpea roots (left) and nodules (right) from the salt-tolerant variety INRAT-93 2 hours after 25 mM NaCl-treatment.
Differentially expressed transcripts: red and green
Constitutively expressed transcripts: grey

B) Zero-axed ln-scale scattered plot of [$R_{(ln)}$] from chickpea UniTags in SuperSAGE libraries from control (abcissa) and salt stressed (ordinate) roots and nodules.
Black line: 99.9% significance threshold
Yellow line: 99.0% significance threshold
Red line: 95.0% significance threshold
Interrupted blue line: Regression line

3.4.2 Drought stress-induced "transcriptome remodelling" in chickpea roots

In roots of the drought-tolerant chickpea variety ICC588, almost 45% of the transcriptome is showing at least 2.7-fold up- or down-regulation, already after 6 hours of dehydration (**Table 3-1, Figure 3-4**). A lower portion of the transcriptome is more than 8-fold either up- (2.22%) or down-regulated (3.37%). UniTags with expression changes between 2.7- and 8-fold up- or down-regulation represent more than 37% of the total unique transcripts.

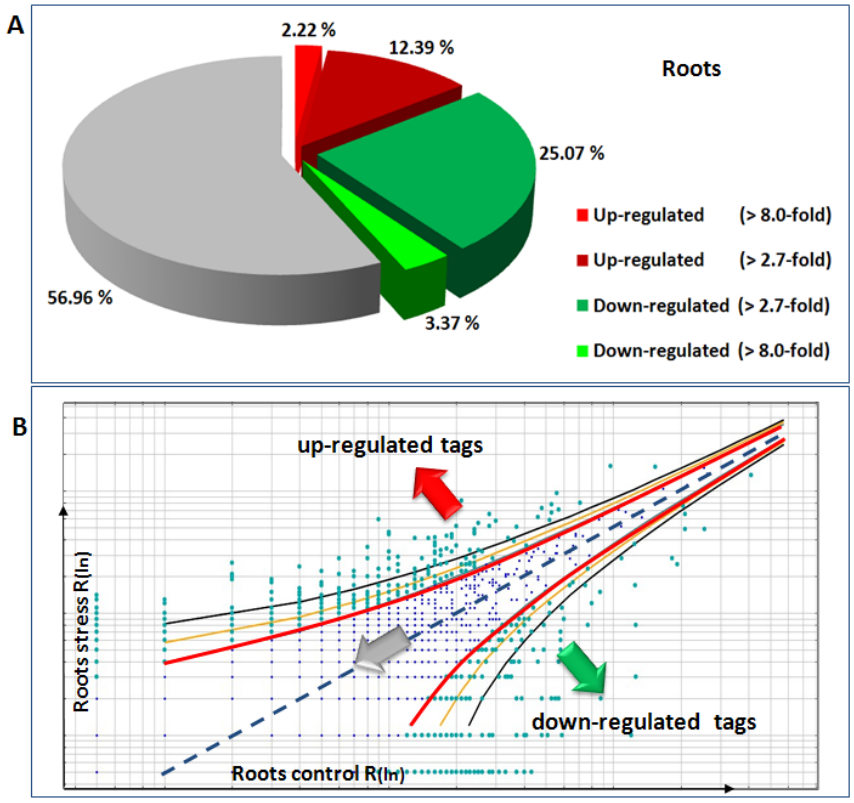


Figure 3-4 The drought-responsive transcriptome of chickpea roots

A) Schematic representation of UniTags expression changes in chickpea roots of the drought-tolerant variety ICC588 6 hours after start of desiccation.
Differentially expressed transcripts: red and green
Constitutively expressed transcripts: grey

B) Zero-axed ln-scale scattered plot of [$R_{(ln)}$] from chickpea UniTags in SuperSAGE libraries from control (abscissa) and drought stressed (ordinate) roots.
Yellow line: 99% significance threshold
Grey line: 99.9% significance threshold
Red line: 95.0% significance threshold
Interrupted blue line: Regression line

3.4.3 Cold stress-induced "transcriptome remodelling" in chickpea leaves

In chickpea leaves of the cold-tolerant variety ILC8262, 43% of the transcriptome reacted upon the onset of chilling temperatures with expression changes of at least 2.7-fold up- or down-regulation (**Figure 3-5**). In contrast to the transcriptome reaction of chickpea roots under salt stress, more than 3,000 unique transcripts (30.5%) were up-regulated after 5 hours at -5°C, and of these, 595 (5.8%) showed at least 8-fold expression changes (**Figure 3-5, Table 3-1**).

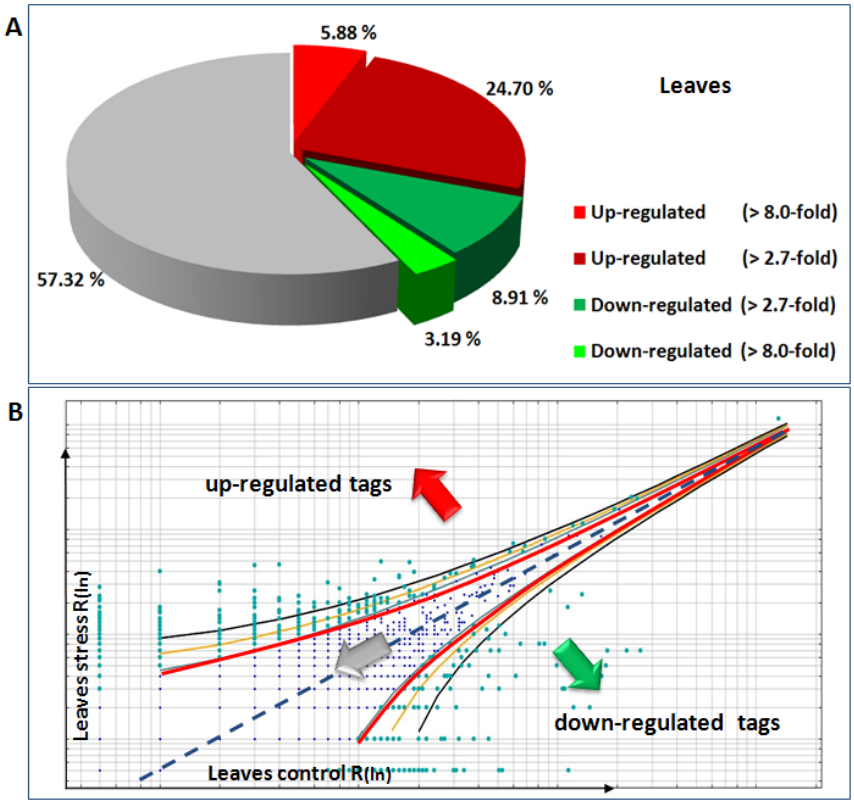


Figure 3-5 **The cold-responsive transcriptome of chickpea leaves**

A) Schematic representation of cold stress-induced transcriptome changes in chickpea leaves. In contrast to roots and nodules under drought and salt stress, respectively, the majority of differentially expressed transcripts is up-regulated.

B) Zero-axed ln-scale scattered plot of [$R_{(ln)}$] from chickpea UniTags in SuperSAGE libraries from control (abscissa) and cold stressed (ordinate) leaves.
Yellow line: 99% significance threshold
Grey line: 99.9% significance threshold
Red line: 95.0% significance threshold
Interrupted blue line: Regression line

3.5 Confirmation of SuperSAGE expression profiles

In order to confirm the expression profiles obtained with SuperSAGE, two parallel transcription profiling techniques were applied, using the sequence information from the 26-bp long tags derived from the different libraries and treatments. The sequence information was used directly or in combination with other techniques like 3'- and 5'-RACE in order to generate: i) oligos to spot on microarrays, and ii) longer cDNA stretches from which qRT-PCR-specific primers and *Taq*Man™ probes could be developed.

3.5.1 Microarray hybridization of spotted SuperSAGE-derived oligos

To characterize the chickpea transcriptome under diverse abiotic stresses (i.e. drought, salt, cold), sequence information from SuperSAGE profiles was used to design an Agilent 16K microarray, onto which 3,000 selected UniTags were spotted for a comparison of both profiling techniques. On the microarray, the majority of the oligos with original tag sequences were spotted twice (twin-replicas). Additionally, oligos with different mismatch numbers from each original tag as well as a small sub-set of longer RACE-derived sequences were also included. A general plan for the design of the 16K Agilent chickpea microarray is shown in **Table 3-2**

Table 3-2 Features of the Agilent 16K chickpea microarray

Sequences from 3,000 different UniTags were selected from the transcription profiles revealed by three main stress treatments (drought, salt, cold). Together with the original UniTag sequences and their twin-replicas, oligos containing 1, 2, and 3 mismatches were spotted for background correction. A subset of 120 probes containing 3'RACE products of selected UniTags was also included.

Type of probe	Number of spots
Original UniTag selection	3,000
1-Mismatch oligos	3,000
2-Mismatch oligos	3,000
3-Mismatch oligos	3,000
UniTag Twin-replicas	2,796
3'RACE 60-mer oligos	120
Internal control spikes	1,084
Total	16,000

*Microarray selected oligos are denoted in the main data matrix deposited in the **Electronic Appendix; File 1**.

For the different hybridization rounds, replicas from salt- and drought-treated roots total RNAs of the chickpea varieties INRAT-93 and ICC588 were extracted and labelled as denoted in **Table 3-3**. Additionally, as detailed under Materials and Methods **(section 2.5.3.2)**, normally labelled probes were co-hybridized with dye-swapped labelled cRNAs to exclude biases introduced by different Cy5 or Cy3 incorporation efficiencies.

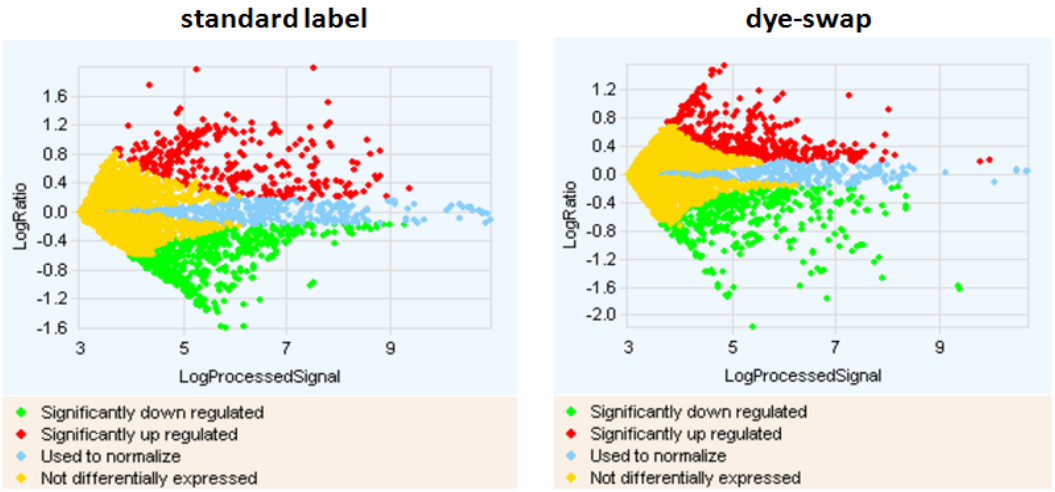
Table 3-3 cRNA probes and main features of total RNAs for the chickpea Agilent 16K microarray hybridizations

Labelled probes were prepared as described under Materials and Methods using the standard labelling strategy along with dye-swapped cRNAs. In each of the hybridizations, internal "spike-in" controls were labelled and mixed with the original probes, following the Agilent guidelines.

Hyb. ID	Plant phenotype	Treatment	Probe / replica	Normal label / dye-swap	Labeled RNA (ng)	Dye
C.a-Array-1	Drought tolerant	control	DRG-CT / R1	Normal	2400	Cy3
		6h desiccation	DRG-6H / R1		2400	Cy5
C.a-Array-2	Drought tolerant	control	DRG-CT / R1	Dye-swap	2400	Cy5
		6h desiccation	DRG-6H / R1		2400	Cy3
C.a-Array-3	Drought tolerant	control	DRG-CT / R2	Normal	800	Cy3
		6h desiccation	DRG-6H / R2		800	Cy5
C.a-Array-4	Drought tolerant	control	DRG-CT / R2	Dye-swap	800	Cy5
		6h desiccation	DRG-6H / R2		800	Cy3
C.a-Array-5	Salt tolerant	2h 0.0 mM NaCl	I93-CT / R1	Normal	2400	Cy3
		2h 25.0 mM NaCl	I93-2H / R1		2400	Cy5
C.a-Array-6	Salt tolerant	2h 0.0 mM NaCl	I93-CT / R1	Dye-swap	2400	Cy5
		2h 25.0 mM NaCl	I93-2H / R1		2400	Cy3

After statistical treatment of the different internal twin-replicas, normalization, and mismatch background correction (**Figure 3-6**), reproducible signals among all hybridization rounds (i.e. RNA replicas, and dye-swapped samples) were selected for cluster analyses together with SuperSAGE expression ratios. Microarray expression ratios for the selected spots are listed in in the main data matrix **(Electronic Appendix; File-1)**. Cluster analysis for IC588 and INRAT-93 is shown in **Figure 3-7**. For the selected INRAT-93 root UniTags, 78.75% of the data points showed punctual shared regulation tendencies between both platforms. Similarly for ICC588 drought-treated roots, a punctual shared tendency of 79.05% was observed between microarrays and SuperSAGE (**Table 3-4**).

A



B

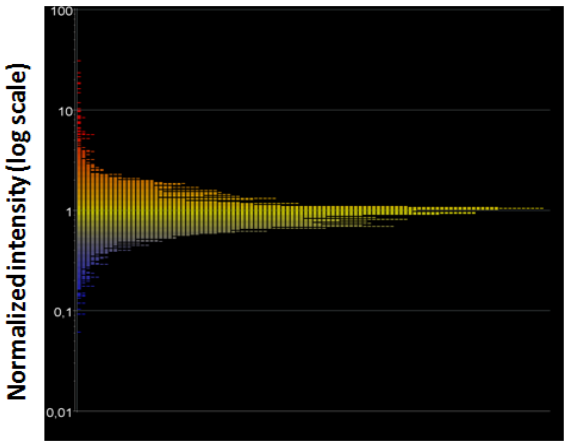


Figure 3-6 **Dye-swap correction and normalization of Agilent microarray signal intensities for spotted SuperSAGE UniTags**

A) Comparative log-scaled diagram showing signal intensities of Cy3- (control) and Cy5-(6h desiccation) labelled ICC588 root cRNAs (left), compared to dye-swapped probes (right)

B) Signal intensities normalization of hybridized ICC588 cRNAs

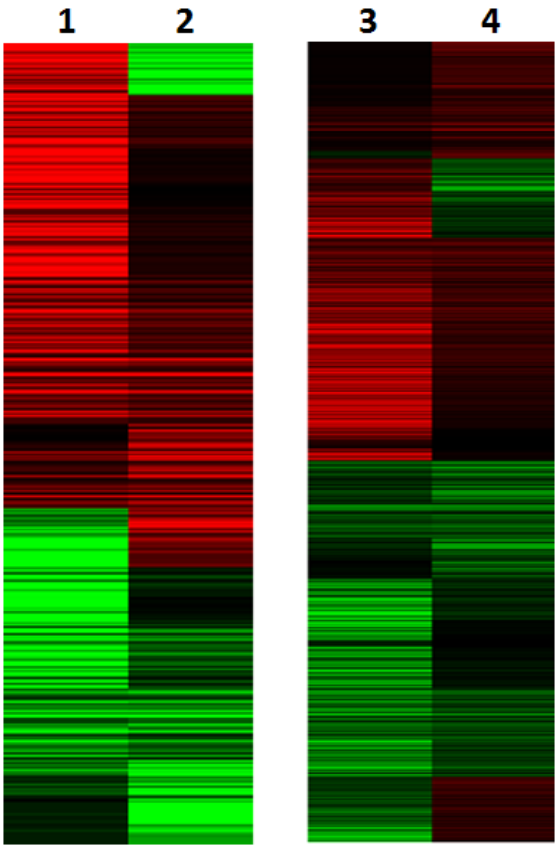


Figure 3-7 **Comparative heat maps of microarray and SuperSAGE profiles**

Cluster 3.0 comparative heat map of SuperSAGE- and Agilent-generated expression ratios in roots of chickpea varieties INRAT-93 and ICC588

1) SuperSAGE expression ratios of UniTags selected for profile confirmation of salt-stressed INRAT-93
2) Microarray expression ratios of averaged and background-corrected hybridized INRAT-93cRNAs. Shared (punctual) up- or down-regulation: 78.75%.
3) SuperSAGE expression ratios of UniTags selected for profile confirmation of drought-stressed ICC588
4) Microarray expression ratios of averaged and background-corrected hybridized ICC588 cRNAs. Shared (punctual) up or down-regulation: 79.05%

Table 3-4 Shared tendencies between SuperSAGE and microarray profiles for transcripts from drought- and salt-stressed chickpea roots

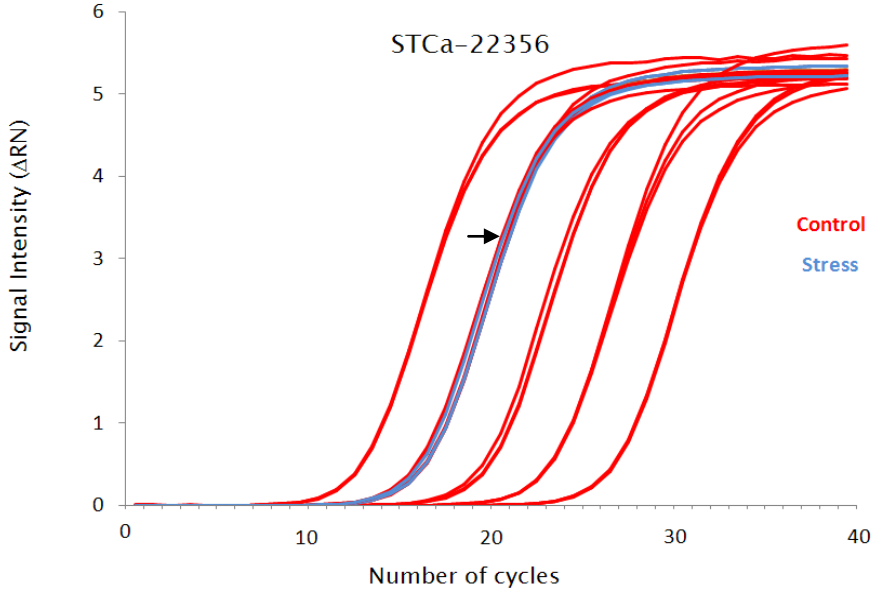
Stress / organ	Drought / roots	Salt / roots
Total selected spots	1,056	739
Up-regulation	425	349
Down-regulation	417	233
Contrasting tendency	214	157

3.5.2 Confirmation of SuperSAGE profiles via qRT-PCR

SuperSAGE ICC588 drought expression profile results were exemplarily confirmed by quantitative real-time (qRT) PCR using *Taq*Man™ probes derived from the following tags: STCa-22356 (O65741_CICAR, mRNA for putative transmembrane channel protein) and STCa-7975 (anonymous drought-induced EST). Additionally, SYBR-Green™ assays were carried out with UniTags STCa-1921 (O65760_CICAR, extensin), STCa-17627 (Q700A7_CICAR, putative universal stress protein), STCa-8434 (anonymous drought-induced EST), STCa-17859 (AJ515033, *C. arietinum* hypothetical protein), STCa-8000 (AJ250836, *C. arietinum* PAL gene), and STCa-22717 (AJ487043, *C. arietinum* CYP450). For all assays, the sequence for either the forward or the reverse PCR primer was derived directly from the SuperSAGE tags, whereas the complementary primers were derived from 3'- or 5'-RACE sequences.

Confirming the SuperSAGE expression levels, amplifications in the SYBR Green™ assay STCa-2271 and with the TaqMan™ probe STCa-22356 revealed constitutive levels of expression ($\Delta\Delta Ct < 0.5$) (**Figure 3-8**). Amplifications in SYBR-Green™ assays with STCa-1921, STCa-17627, STCa-8434 as well as the TaqMan™ probe STCa-7975 revealed an up-regulation of the respective transcripts under stress ($\Delta\Delta Ct > 0.5$) (**Figure 3-8**). These results confirmed our SuperSAGE analysis. Stress-induced down-regulation of UniTags was corroborated by SYBR Green™ assays for STCa-17859 and STCa-8000 ($\Delta\Delta Ct < -0.5$). However, for STCa-8000, amplification profiles as well as post-qRT-PCR amplicon melting curves suggested partially unspecific priming.

A



B

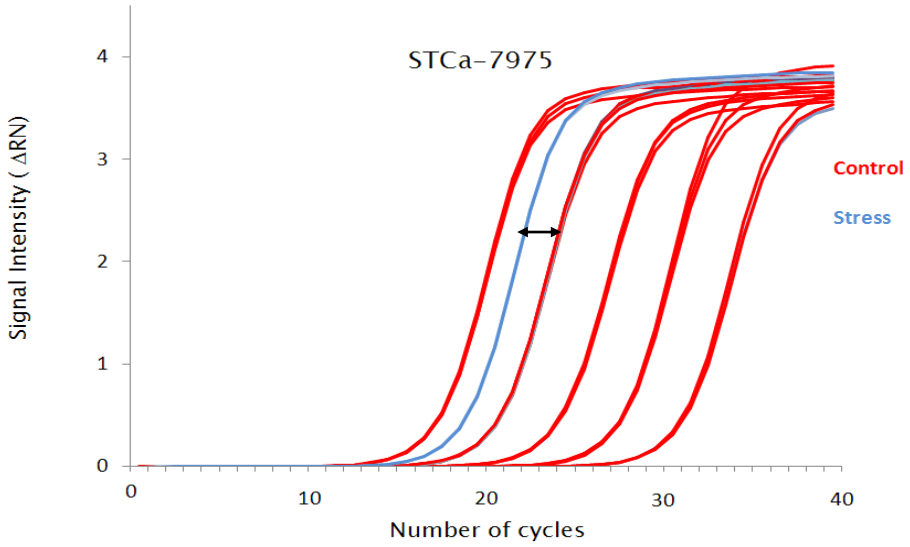


Figure 3-8: **TaqMan™ qRT-PCR confirmation of SuperSAGE data in drought-stressed roots**

A) *Taq*Man™ assay with UniTag-derived primer STCa-22356 (3'-RACE product; O65741_CICAR, putative trans-membrane channel protein). Result: no difference between control and desiccated roots in the same sample concentration range, i.e. constitutive expression (black arrow).
Method for measure of differential expression: relative quantification curve

B) *Taq*Man™ assay with UniTag-derived primer STCa-7975 (3'-RACE product; anonymous drought-induced EST). Result: earlier C_T for the cDNA from desiccated roots (stress up-regulation) in the same concentration range (black double-headed arrow).
Method for measure of differential expression: relative quantification curve

Control roots cDNA: red curve
Drought-stressed roots cDNA: blue curve

3.5.3 Confirmation of UniTags annotation by sequencing of 5'- and 3'-RACE products

In addition to the confirmation of SuperSAGE results by qRT-PCR, 5- and 3'-RACE products of selected UniTags were sequenced to check validity of the annotation of the resulting cDNAs. For this procedure, the 26 bp tags were used directly for cDNA amplification as described under Materials and Methods (**section 2.5.1**).

A total of 25 fragments from the 5'- or 3'-end of cDNAs derived from 13 UniTags were sequenced and the sequences BLASTed against public EST and genomic DNA databases. Four of the sequenced fragments (corresponding to three UniTags) did not confirm the expected annotation. Two cDNA fragments (274 and 124 bp) from UniTag STCa-424261 (receptor-like kinase, Q9SWU7) were sequenced, but no high homology hits with any fully characterized ESTs from public databases was found. However, after re-BLASTing the original 26 bp tag, the initial annotation reassured.

From Unitag STCa-8061 (beta-1,3-glucanase-like protein, Q94G86), five RACE products were sequenced. From these, four products showed the expected annotation, whereas the annotation of the largest fragment (343 bp) did not correlate with the initial homology hit (GB|AAM61695.1AY085142).

One 288 bp cDNA fragment originated from UniTag STCa-15690 (Q9FT05, cationic peroxidase) possessed homologies not corresponding to the original annotation. However, after re-BLASTing the 26-bp tag against the NCBI(nr) database, cationic peroxidase emerged as the hit with the highest homology. A compilation of the sequenced 3'- and 5'-RACE products along with the corresponding tags and annotations is shown in **Table 3-5**. Corresponding cDNA sequences for each amplified fragment are deposited in the **Electronic Appendix; File-2**.

The above results suggest that in the majority of the cases 3'- or 5'-RACE sequencing brought the expected annotation. However, unspecific amplification also produces other undesirable products. As can be seen in **Table 3-5**, more than one product per RACE amplification was obtained in many cases.

Table 3-5 5'- and 3'-RACE fragments amplified from SuperSAGE tags

RACE products, their size and annotation together with the ID of the corresponding tag, e-values, and Uniprot accession codes are depicted. In several cases, more than one fragment per UniTag was amplified.
Grey boxes: RACE fragment annotation not corresponding to original UniTag

Tag ID	e-value	Uniprot acc	Annotation	Sequence length	3' RACE- annotation (TIGR or NCBI Fabaceae)
STCa-42461	0.00938	Q9SWU7	Receptor-like kinase (RLK)	274 bp	TC100394 weakly similar to UP|Q7X9I9 (Q7X9I9) Caffeic acid O-methyltransferase
				124 bp	TC95485 homologue to PIR|S41773|S41773 glycine-rich RNA-binding protein
STCa-2964	4.0E-07	O65847	Protein phosphatase 1	102 bp	TC101732 / UP|O65847 Protein phosphatase 1
				187 bp	TC101732 / UP|O65847 Protein phosphatase 1
STCa-12274	1.0E-04	PP12_ARATH	Protein phosphatase PP1 isozyme 2	156 bp	M93409.1 Phosphoprotein phophatase-type 1
				119 bp	Protein serine/threonine phosphatase (TOPP2)
STCa-1016	4.0E-07	O65844	Protein phosphatase 1 (PP1)	283 bp	Medicago sativa mRNA for protein phosphatase 1
STCa-16453	3.0E-07	O04391	Cinnamyl alcohol dehydrogenase	512 bp	TC100392 UP|O04391 Cinnamyl alcohol dehydrogenase
STCa-4896	2.4E-03	O82469	Protein phosphatase-2C (PP2C)	344 bp	BB920958 Trifolium pratense leaf Trifolium pratense cDNA clone RCE27063, mRNA sequence.
STCa-17682	4.0E-07	Q8L5G0	Tonoplast intrinsic protein	101 bp	AJ489613.1 mRNA for tonoplast intrinsic protein (Tip gene)
STCa-15004	4.0E-07	Q9SMK0	Peroxidase	315 bp	AJ275313.1 Cicer arietinum partial mRNA for peroxidase (3e-142)
				163 bp	AJ275313.1 Cicer arietinum partial mRNA for peroxidase 6e-66
STCa-8061	3.0E-04	Q94G86	Beta-1,3-glucanase-like protein	245 bp	UP|Q94G86 Beta-1,3-glucanase-like protein
				202 bp	UP|Q94G86 Beta-1,3-glucanase-like protein
				221 bp	UP|Q94G86 Beta-1,3-glucanase-like protein
				212 bp	UP|Q94G86 Beta-1,3-glucanase-like protein
				343 bp	GB|AAM61695.1AY085142 hydroxyproline-rich glycoprotein-like protein
STCa-15690	1.0E-04	Q9FT05	Cationic peroxidase	288 bp	UP|Q41026 HMG 1 protein
STCa-23456	4.0E-07	Q5RLY0	Acidic glucanase	132 bp	AJ131047.2 Cicer arietinum mRNA for glucan-endo-1,3-beta-glucosidase (1e-60)
					AY804253.1 Medicago sativa acidic glucanase (Glu) mRNA (6e-52)
STCa-15246	2.0E-05	Q9SSZ9	Peroxidase 1 (peroxidase)	238 bp	EF456705.1 Medicago truncatula peroxidase (PRX3)
STCa-35200	2.0E-05	Q43437	Type I chlorophyll a/b-binding protein	159 bp	AJ131044.1 Cicer arietinum mRNA for chlorophyll a/b binding protein
				171 bp	AJ131044.1 Cicer arietinum mRNA for chlorophyll a/b binding protein
				173 bp	AJ131044.1 Cicer arietinum mRNA for chlorophyll a/b binding protein
				160 bp	AJ131044.1 Cicer arietinum mRNA for chlorophyll a/b binding protein
				179 bp	AJ131044.1 Cicer arietinum mRNA for chlorophyll a/b binding protein

3.6 Additional experiments: UniTags conservation between two non-related legumes: chickpea and lentil

As outlined in the first part of the present chapter, the interpretation of expression profiles relies on the annotation of the detected UniTags to already deposited EST sequences from *Cicer* or other legumes (e.g. *M. truncatula, L. japonicus, G. max, and P. vulgaris*). Up to now, annotation of short 26 bp tags against complete cDNAs or mRNAs has shown that the transfer of information from massively-sequenced legumes to crops like chickpea is acceptable (**section 3.2**).

To test the probability that UniTags derived from one species could also be found in libraries from other genera, a SuperSAGE library developed from non-stressed lentil (*Lens culinaris cv. AKM-302*) leaves was directly compared to the SuperSAGE library denoted as ILC8262-Ct (non-stressed leaves of chickpea cultivar ILC8262; section **2.2**). Plants from both species were grown in parallel under identical conditions, as described in section **2.2.3** for experiments initially planned to evaluate responses of chickpea plants to cold stress.

3.6.1 Description of libraries

After sequencing, filtering of twin ditags and tag counting, a total of 31,941 transcripts from non-stressed chickpea leaves were detected, representing 7,660 UniTags (unique transcripts). For lentil leaves, a total of 9,840 UniTags were represented by a total of 44,507 transcripts.

3.6.2 Common UniTags-proportion related to copy numbers in chickpea and lentil leaves

Figure 3-9 shows that the proportion of common UniTags is relatively stable throughout the different copy number abundancy categories. However, the probability to find the same UniTag in both libraries will be slightly higher for transcripts that are present in high copy numbers. Copy numbers of common and total UniTags are depicted in **Table 3-6**.

Although there is a considerable degree of overlap between both libraries (approximately 50% of the total UniTags found at > 50 copies million^{-1}), this proportion is not satisfactory for expression profiling by directly comparing libraries of two species. In many cases, the basis of up- or down-regulation of transcripts would not be distinguishable between: i) genuine expression changes ii) changes related to inter-specific taq sequence differences.

Table 3-6 Numbers of total and common UniTags detected in non-stressed lentil and chickpea leaves

Copies million^{-1}	Lentil UniTags	Chickpea UniTags	Common UniTags
> 1,000	84	92	58
> 500	227	285	158
> 100	2,263	1,889	1,164
> 50	3,762	3,980	2,164
> 1	9,840	7,660	4,153

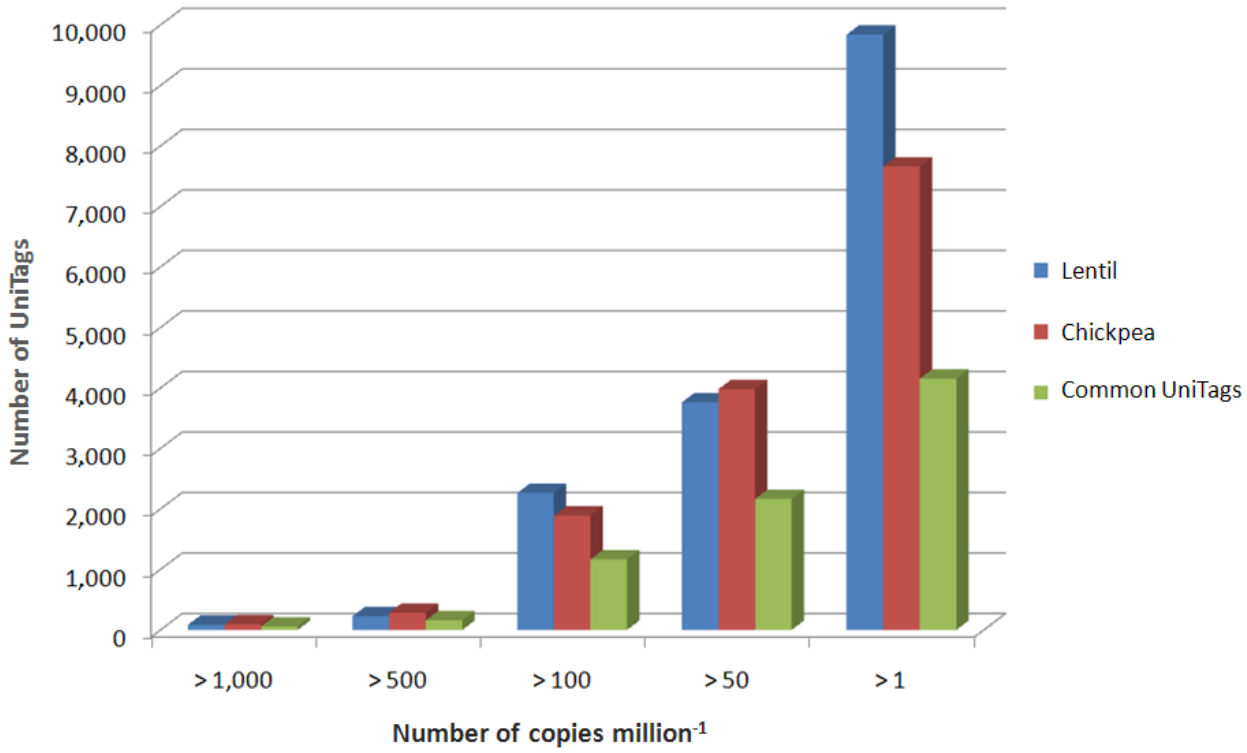


Figure 3-9 Relative abundancies of common and total UniTags of lentil and chickpea based on their copy numbers (copies million^{-1})

4 The salt stress-responsive transcriptome of chickpea roots

4.1 Confirmatory physiological measurements in INRAT-93

The effects of two hours of 25 mM NaCl treatment on chickpea were observed as the plants gradually wilted. To monitor the consequences of saline stress for the biomass of roots and shoots, salt-tolerant INRAT-93 plants were grown in parallel with plants from the salt-sensitive variety ICC4958 for a period of five weeks. For each variety, 45 plants were grown, and groups of fifteen plants harvested two hours, four days, and 5 weeks after an initial 25 mM NaCl-treatment.

During the first week of salt stress, the plants from both varieties experienced loss of fresh weight in roots and aerial parts (shoots). From an averaged fresh weight of 14.3 and 7.01 g, respectively, INRAT-93 plants reduced their biomass down to 9.49 and 7.25g for shoots and roots, respectively (per plant), after 4 days. A similar decrease of biomass was also observed in ICC4958 plants (**Figure 4-1**). Ever five weeks after salt stress, INRAT-93 plants continued to live and re-gained root growth, showing final average weights of 6.25 and 13.46g for shoots and roots, respectively. At this time, ICC4958 plants suffered from a high degree of wilting and starvation, which was reflected by loss of fresh weight to final average values of 4.76 and 1.20 g, respectively, for shoots and roots (**Figure 4-2**).

In parallel to plants inoculated with *Mesorhizobium ciceri*, INRAT-93 seedlings were grown in urea (8.0 mM) as main nitrogen source, and harvested at identical times to measure biomass. After four days, the effects of stress were more pronounced in *Rhizoboium*-inoculated plants than in plants fertilized with urea. The fresh weight of shoots from urea-fertilized plants showed small variation (11.1 to 11.5g), whereas the fresh weight of roots decreased from 9.8 to 6.8 g. After five weeks of stress, roots from urea-fertilized plants regained growth at a relatively low rate when compared to inoculated plants (5.85g average weight), whereas shoot biomass decreased similarly.

As already observed by L'Taief and co-authors (2007), the present results confirm the general tendency in inoculated INRAT-93 plants under salt stress to promote root growth. This tendency is less pronounced in non-nodulated (non-inoculated) plants.

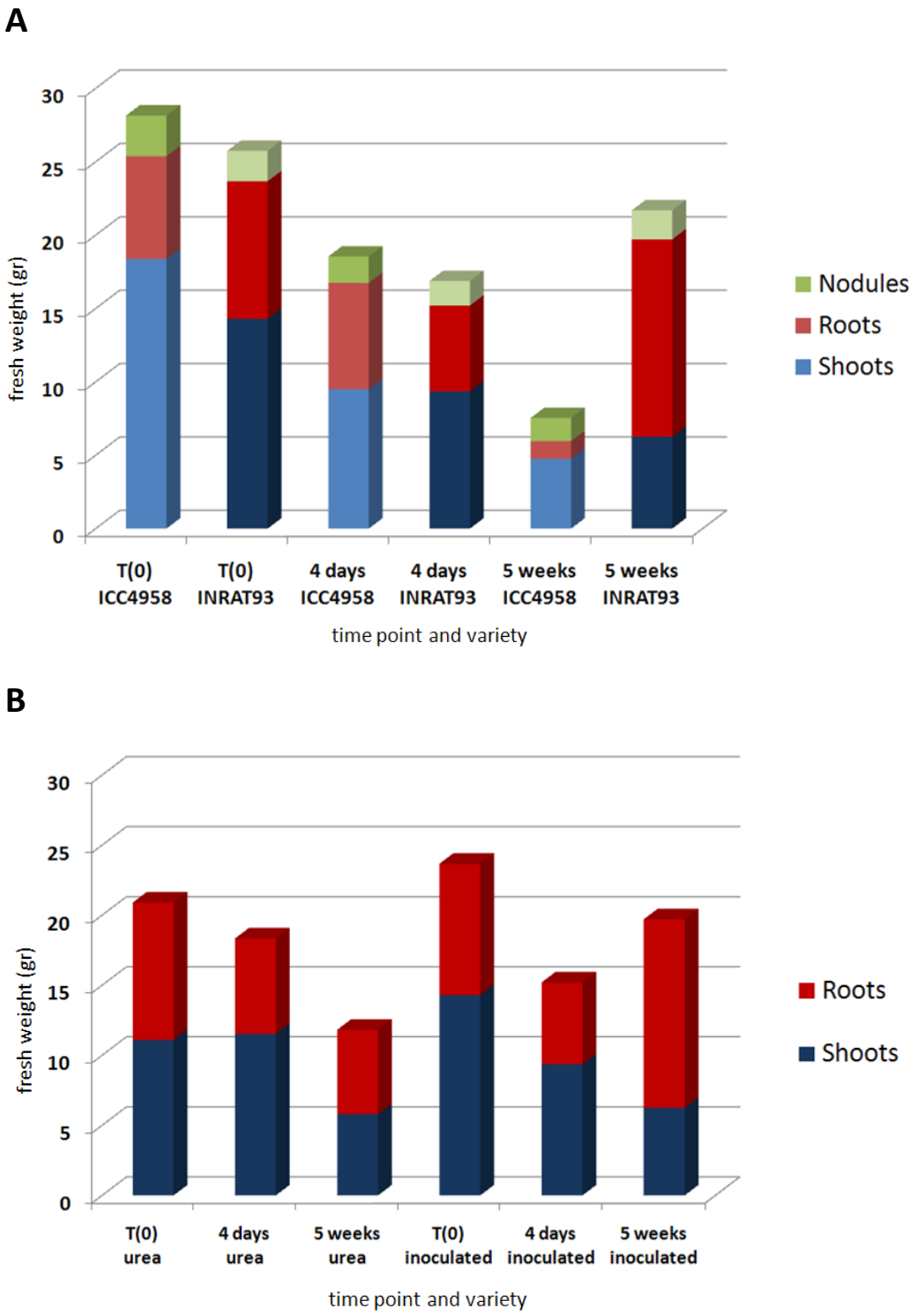


Figure 4-1 Fresh weight of chickpea plants at different time points after treatment with 25mM NaCl in hydro-aeroponics

A) Fresh weight of nodules, roots, and shoots of chickpea plants from varieties INRAT-93 (dark colors) and ICC4958 (light colors) at the day of stress onset [T(0)], 4 days, and 5 weeks of salt treatment.

B) Fresh weight of roots and shoots of INRAT-93 plants fertilized with urea (first three columns) and inoculated with *Rhizobium* (columns 4 to 6), respectively, at different time points.

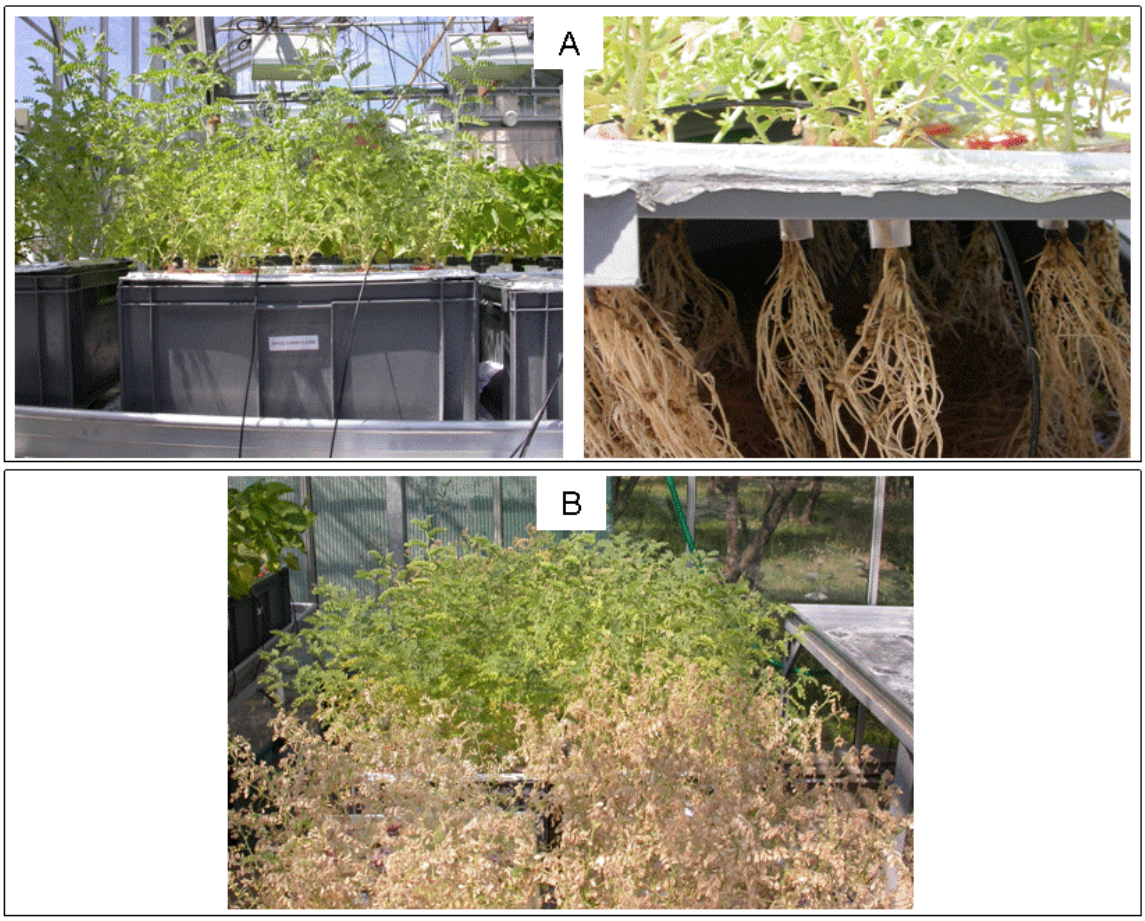


Figure 4-2 **Salt-treated chickpea plants grown in hydro-aeroponics**

A) Left: Growth buckets in INRA greenhouse facilities
Right: Nodulated roots of chickpea (INRAT-93)
B) Chickpea lines with different salt sensitivity 5 weeks of stress induction
Foreground : ICC4958 (salt-sensitive)
Background: INRAT-93 (salt-tolerant)

4.2 Salt stress-induced differential gene expression in chickpea roots

4.2.1 Top salt stress-up-regulated UniTags in INRAT-93 roots

After 2 hours of 25 mM NaCl treatment, 34% of the sampled chickpea transcriptome was responsive, showing at least 2.7-fold ($R_{(ln)}$>1) differential expression. From this salt-responsive proportion, 1.93 and 11.47%, respectively, of the UniTags were more than 8-fold up- or down-regulated (see section **3.4.1**). The 40 most significantly salt stress-up- or down-regulated transcripts matching well-characterized genes in public databases are listed in **Tables 4-1A** and **4-1B,** respectively. The following subsections will be restricted to approach the most up-regulated genes in order to highlight the **salt stress-induced** transcriptome responses in chickpea.

Table 4-1A: Top 40 salt stress up-regulated annotatable UniTags from INRAT-93 roots

Tag ID	Protein	$R_{(ln)}$	Associated process	Uniprot ID
STCa-18884	Early nodulin 40*	5.69	Nodulation	NO40_SESRO
STCa-7896	Superoxide dismutase	3.70	ROS-scavenging	Q9ZNQ4_CICAR
STCa-318	Trypsin protein inhibitor 3	3.59	Endopeptidase inhibitor	Q5WM51_CICAR
STCa-19021	Extensin	3.40	Cell wall organization	O65760_CICAR
STCa-17087	Dormancy-associated protein	3.38	No associated process	O22611_PEA
STCa-7166	NADP-dependent isocitrate dehydrogenase I	3.25	Metabolism	Q6R6M7_PEA
STCa-1381	Acetyl-CoA synthetase	3.19	Metabolism	Q9ZR69_SOLTU
STCa-2982	Cysteine synthase	3.15	Protein metabolism	O65747_CICAR
STCa-15648	Mitochondrial 24S mt-RNL ribosomal gene*	3.10	No associated process	No Uniprot link
STCa-20215	Putative extracellular dermal glycoprotein	3.08	Proteolysis	Q9FSZ9_CICAR
STCa-20066	14-3-3-like protein A	3.03	Protein domain-specific binding	1433A_VICFA
STCa-15159	Disease resistance protein DRRG49-C	2.98	Response to stress	DRR4_PEA
STCa-17434	Gb|AAD20160.1	2.92	No associated process	Q9FYR1_ARATH
STCa-22427	Fiber protein Fb19	2.88	Response to stress	Q6T7D1_GOSBA
STCa-4531	Isoflavone 3'-hydroxylase	2.88	No associated process	Q2ENF7_ASTME
STCa-14437	60S acidic ribosomal protein P1	2.83	Protein biosynthesis	RLA1_MAIZE
STCa-1385	1-aminocylopropane-1-carboxylate oxidase	2.83	Metabolism	Q41681_9FABA
STCa-12309	Ankyrin-like protein	2.83	No associated process	Q9FMJ2_ARATH
STCa-23197	Hypothetical protein	2.78	Response to stress	Q9LEN3_CICAR
STCa-8459	UDP-glucose pyrophosphorylase	2.78	Metabolism	Q8W557_9FABA
STCa-12035	Cytochrome P450 monooxygenase	2.73	Electron transport/metal ion binding	Q9XFX0_CICAR
STCa-11051	Retinoblastoma-related protein	2.68	No associated process	Q8H0J6_MAIZE
STCa-7975	T5A14.10 protein	2.68	No associated process	Q9ZVU7_ARATH
STCa-14984	40S ribosomal protein S4	2.68	Protein biosynthesis	RS4_PRUAR
STCa-21666	Low temp. salt-responsive protein LTI6B	2.68	Integral to membrane	RCI2B_ARATH
STCa-1958	Gibberellin-stimulated protein	2.68	Hormone response	Q53AN3_ORYSA
STCa-17272	10 kDa photosystem II polypeptide	2.68	Oxygen evolving complex	Q6V7X5_TRIPR
STCa-24178	Phosphoglycerate mutase	2.62	Metabolism/metal ion binding	PMGI_MESCR
STCa-13313	Chalcone isomerise	2.62	Flavonoid biosynthesis	CFI_VITVI
STCa-23978	Inorganic pyrophosphatase-like protein	2.62	Phosphate metabolism	Q9LFF9_ARATH
STCa-10123	Synaptobrevin-like protein	2.62	Transport/integral to membrane	Q69WS1_ORYSJ
STCa-11172	Caffeic acid 3-O-methyltransferase	2.56	Lignin biosynthesis	COMT1_MEDSA
STCa-181	Myo-inositol-1-phosphate synthase	2.56	Inositol 3P biosynthesis/Ca^{2+} release	O22611_PEA
STCa-15340	Alfin-1	2.56	Regulation of transcription	Q40359_MEDSA
STCa-24453	Tonoplast intrinsic protein	2.56	Transport	Q8L5G0_CICAR
STCa-4528	Cytochrome P450 monooxygenase	2.56	Electron transport/metal ion binding	Q9SML2_CICAR
STCa-5543	Epsilon subunit of mitochondrial F1-ATPase	2.56	ATP-coupled proton transport	Q8L5Q1_CICAR
STCa-11309	60S ribosomal protein L18a	2.49	Protein biosynthesis	RL18A_CASSA
STCa-16808	Histone H2B	2.49	Response to DNA damage stimulus	Q9M3H6_CICAR
STCa-22470	Glutathione S-transferase	2.49	ROS-scavenging	Q948X4_MEDSA

*****The annotation of UniTags STCa-18884 and STCa-15648 is still ambiguous. However, early nodulin 40 and mitochondrial 24S mt-RNL ribosomal gene are the most probable homologies in all screened databases

Table 4-1B: Top 40 salt stress down-regulated annotatable UniTags from INRAT-93 roots

Tag code	Protein	$R_{(ln)}$	Associated process	Uniprot ID
STCa-6887	Type II chlorophyll a/b binding protein	-4.65	Photosynthesis	Q41038_PEA
STCa-18085	Serine/threonine-protein phosphatase BSL2	-4.42	Protein dephosphorylation/signaling	BSL2_ARATH
STCa-3563	Putative GDP-L-fucose synthase 2	-4.33	Metabolism	FCL2_ARATH
STCa-18432	Auxin-independent growth promoter	-4.30	No associated process	Q9LIN9_ARATH
STCa-3207	Drought-induced protein	-4.30	Response to stress	Q941N0_9FABA
STCa-14228	Glycine-rich RNA-binding protein GRP2A	-4.16	Nucleotide binding	GRP2_SINAL
STCa-14232	Glycine-rich RNA-binding protein GRP2A	-4.08	Nucleotide binding	GRP2_SINAL
STCa-6878	Type II chlorophyll a/b binding protein	-4.08	Photosynthesis	Q41038_PEA
STCa-18340	Auxin-independent growth promoter	-3.99	No associated process	Q9LIN9_ARATH
STCa-12693	Type II chlorophyll a/b binding protein	-3.99	Photosynthesis	Q41038_PEA
STCa-18321	Auxin-independent growth promoter	-3.91	No associated process	Q9LIN9_ARATH
STCa-14659	MtN1 protein precursor	-3.89	Response to stress	P93331_MEDTR
STCa-13900	Aminotransferase-like protein	-3.89	Transaminase activity	Q6K3L3_ORYSJ
STCa-14223	Glycine-rich RNA-binding protein GRP2A	-3.89	Nucleotide binding	GRP2_SINAL
STCa-2558	Serine/threonine protein kinase	-3.82	Protein phosphorylation/signaling	Q53VE2_LOTJA
STCa-18372	Auxin-independent growth promoter	-3.79	No associated process	Q9LIN9_ARATH
STCa-7806	Pollen-specific protein-like At4g18593	-3.79	No associated process	Q570P7_ARATH
STCa-21045	Cytochrome P450 monooxygenase CYP78A	-3.79	Electron transport/metal ion binding	Q2MJ07_MEDTR
STCa-6160	Putative extensin	-3.79	Cell wall organization	Q9FSY9_CICAR
STCa-6786	Tonoplast intrinsic protein	-3.79	Transport	Q8L5G0_CICAR
STCa-14898	Glucose-6-phosphate isomerise	-3.67	Metabolism	Q76E42_ORYSA
STCa-6884	Type II chlorophyll a/b binding protein	-3.67	Photosynthesis	Q41038_PEA
STCa-14630	Aminotransferase-like protein	-3.67	Transaminase activity	Q6K3L3_ORYSJ
STCa-18318	Auxin-independent growth promoter	-3.67	No associated process	Q9LIN9_ARATH
STCa-13974	Beta-glucosidase	-3.54	Metabolism/ABA-release	BGLS_TRIRP
STCa-14782	Aminotransferase-like protein	-3.54	Transaminase activity	Q6K3L3_ORYSJ
STCa-17899	Cytochrome P450	-3.54	Electron transport/metal ion binding	Q9XGL7_CICAR
STCa-18316	Auxin-independent growth promoter	-3.54	No associated process	Q9LIN9_ARATH
STCa-4801	ADP-glucose pyrophosphorylase precursor	-3.54	Starch/glycogen biosynthesis	Q43819_PEA
STCa-4842	NAP1Ps	-3.54	Nucleosome assembly	P93488_PEA
STCa-4930	BZIP transcription factor (BZIP)	-3.54	Regulation of transcription	Q93XM6_ARATH
STCa-18327	Auxin-independent growth promoter	-3.54	No associated process	Q9LIN9_ARATH
STCa-12919	14-3-3-like protein	-3.42	Protein-protein interactions	O24533_VICFA
STCa-12140	At1g20110/T20H2_10	-3.38	Metal ion binding	Q9ASS2_ARATH
STCa-13246	NAM-related protein 1	-3.38	Regulation of transcription	Q7XJ90_MAIZE
STCa-14368	UVI1	-3.38	No associated process	Q9AUH7_PEA
STCa-13700	S-adenosylmethionine synthase	-3.38	Metabolism	Q6J9X6_MEDSA
STCa-18297	Auxin-independent growth promoter	-3.38	No associated process	Q9LIN9_ARATH
STCa-18317	Auxin-independent growth promoter	-3.38	No associated process	Q9LIN9_ARATH
STCa-18320	Auxin-independent growth promoter	-3.38	No associated process	Q9LIN9_ARATH

UniTags annotated to the Enod40 protein (early nodulin 40, NO40_SESRO), superoxide dismutase (SOD, Q9ZNQ4_CICAR), trypsin protein inhibitor-3 (Q5WM51_CICAR), extensin (O65760_CICAR), dormancy-associated protein (O22611_PEA), NADP-dependent isocitrate dehydrogenase I (Q6R6M7_PEA), acetyl-CoA synthetase (Q9ZR69_SOLTU), cysteine synthase (O65747_CICAR), an ortholog of a mitochondrial 24S mt-RNL ribosomal protein gene, putative extracellular dermal glycoprotein (Q9FSZ9_CICAR), 14-3-3-like protein (1433A_VICFA), disease resistance response protein DRRG49-C (DRR4_PEA), the *Arabidopsis* gene AAD20160.1 (Q9FYR1_ARATH), and a fiber protein Fb19 (Q6T7D1_GOSBA) were most up-regulated in INRAT-93 roots after 2h of 25 mM NaCl-treatment.

4.2.1.1 Early nodulin 40

In INRAT-93 roots after 2h NaCl-treatment, UniTaq STCa-18884 was more than 250-fold induced. In the context of root transcriptome responses, this is the first report of such an induction of an enod40 gene in legumes under salt-stress. Apart from its function in the early stages of nodule formation (Takeda et al., 2005), it is suggested that enod40 modulates the action of auxin, and may function as plant growth regulator that alters phytohormone responses (http://www.uniprot.org/uniprot/O24369). Thus, this UniTag represents a transcript that merits with no doubt further characterization.

4.2.1.2 Superoxide dismutase (SOD)

Apart from affecting the ionic and osmotic disequilibrium, salt stress induces major alterations in metabolism (e.g. high respiration rates), that in turn lead to production of ROS. Superoxide ions (O_2^-), the major by-products of chloroplast and mitochondrial respiration, are rapidly dismutated to H_2O_2 by SODs in plant cells (Gechev et al., 2006). In the context of salt stress, SODs and other ROS-scavenging proteins (e.g. glutathione peroxidase GPX, catalase CAT, ascorbate peroxidase APX) have been reported to be very active (Del Rio et al., 2003; Gadjev et al., 2006), fact that could explain the up-regulation of the SOD-annotated UniTag STCa-7896 (Q9ZNQ4_CICAR) in chickpea roots. A more detailed description of the dynamics of transcription of genes encoding several ROS-scavenging proteins in chickpea roots under salt stress is provided **section 4.4.1** of the present chapter.

4.2.1.3 Trypsin protein inhibitor 3

Trypsins are serine proteases, which in several cases are secreted by attacked plants to prevent successful insect and bovine herbivory. However, rapid accumulation of

transcripts coding for trypsin inhibitors have also been reported in plants under salt, drought, high aluminium stress, wounding, fungal infection, and ABA and jasmonate applications (Sanchez-Hernandez et al., 2004). Particularly in the first stages of salt stress responses in rice, various isoforms of trypsin inhibitors are very active (Kawasaki et al., 2001). The induction of genes encoding trypsin inhibitors in salt-stressed plants is probably part of a general non-specific stress response.

4.2.1.4 Extensin

Extensins are a family of hydroxyproline-rich proteins that are generally involved in counteracting mechanical pressures by strengthening cell walls. Pressures derived from mechanical stress, and differences in water potentials (Tire et al., 1994). In plants, Increased accumulation rates of transcripts coding for extensin and other cell wall proteins like cellulose synthase have already been observed in root systems under salt stress (Ueda et al., 2007), most markedly towards the apical region. Up-regulation of the UniTag STCa-19021 (O65760_CICAR) in INRAT-93 roots suggests that, already 2 hours after initial NaCl-treatment, the afflicted plant reacts to overcome the strong mechanical pressure caused by the osmotic disequilibrium.

4.2.1.5 Dormancy-associated proteins

Although dormancy-associated proteins in legumes have already been reported to be salt stress-induced in a *M. truncatula* microarray screening (de Lorenzo et al., 2007), very little is yet known about their exact functions. The Uniprot accession O22611_PEA is particularly assigned to the family of auxin-repressed proteins (www.ebi.ac.uk/interpro/IPR008406). As growth-promoting phyto-hormones, auxins function in the regulation of root development in salt-stressed plants (He et al., 2005). Therefore, the over-expression of the UniTag STCa-17087 (dormancy-associated protein, O22611_PEA) in salt-treated INRAT-93 roots may be linked to auxin activity and root growth regulation. In fact, one of the most notorious reactions observed in plants of this variety, that were subjected for 5 weeks to 25 mM NaCl-treatment.

4.2.1.6 NADP-dependent isocitrate dehydrogenase

Popova and co-authors (2002) reported on an active NADP-dependent isocitrate dehydrogenase (ICDH) isoform in the facultative halophyte *Mesembryanthemum crystallinum* L. and in pea leaves (*P. sativum*) under salt stress. According to these authors,

glutamate and glutamine, the main forms of symbiotically-fixed N_2 in legumes, can act as activators of for this enzyme. If this information is transferable to INRAT-93 roots, the high NADP-dependent ICDH transcript accumulation under salt stress can be indicative of the functioning of the SNF-machinery. Additionally, ICDH enzymes play an important role in generating NADPH to keep antioxidants like glutathione in a reduced state in mitochondria, acting thereby in ROS-scavenging processes (Moller, 2001).

4.2.1.7 Acetyl-CoA synthetase

Acetyl-CoA synthetase (ACS), a key enzyme in acetate production in *Saccharomyces cerevisiae*, was extensively characterized by Akamatsu and collaborators (2000), but there are no major reports of its induction under osmotic or ionic stresses. In legumes, acetyl-CoA serves as intermediate in the biosynthesis of malate (Prell and Poole, 2006), the major export product of the symbiotic plant host cell into the bacteroid. Thereby, the expression levels of UniTag STCa-1381 (ACS, Q9ZR69_SOLTU) in INRAT-93 roots under salt stress can be linked to SNF-related processes.

4.2.1.8 Cysteine synthase

Up to now, information about the role of cysteine synthase in plants under salt stress is rather limited. However, from the point of view of managing oxidative stress, the activity of cysteine synthases (and the amino acid cysteine) gains importance. Apart from its general roles in protein biosynthesis and as a sulfur donor, cysteine is one of the main components of the anti-oxidant glutathione (along with glutamate; Noji et al., 2001). Glutamate and ascorbate are the major redox buffers in plants, constituting the ascorbate/glutathione cycle (Apel and Hirt, 2004). A logic explanation for the high up-regulation of cysteine synthase transcripts (STCa-2982, O65747_CICAR) in salt-treated INRAT-93 roots could be then the supply of glutathione to the ROS scavenging machinery.

4.2.1.9 Extra cellular dermal glycoprotein D14550

Extra cellular dermal glycoproteins (EDGPs) are proteases of the family of aspartic peptidases (www.ebi.ac.uk/interpro/IPR009007). In *Arabidopsis,* Gong and co-authors (2001) reported strong accumulation of transcripts coding for an extracellular dermal glycoprotein (EDGP, gblD14550) in wild-type (Col-0 gl1) seedlings in contrast to SOS mutants. Therefore, a co-regulation of EDGPs and genes encoding components of the SOS pathway was proposed.

Despite this result, up to date there is not enough background information about the specific functions or possible targets (substrates) of this type of EDGP. The up-regulation of UniTag STCa-20215 (Q9FSZ9_CICAR) in salt stressed chickpea roots could be related to proteolytic processes. Further characterization of this transcript and the exact function of the coded protein may be a source of valuable information.

4.2.1.10 14-3-3-like proteins

14-3-3 proteins form a multi-member family, which is involved in protein-protein interactions and signal cascades cross-talk (Roberts et al., 2002). Plants particularly have large 14-3-3 gene families with high specificity of target proteins (Ferl, 1996). In tomato, the transcription profiles of all 14-3-3 family members were evaluated under salt stress, showing a broad range of regulation levels (Xu and Shi, 2006). In a similar work in rice, at least four 14-3-3 transcript isoforms were induced by drought and salt-stress (Chen et al., 2006). In chickpea, the up-regulation of the UniTag STCa-20066 (1433A_VICFA) can lead to identify specific stress-induced 14-3-3 family members in legumes, making this transcript a good candidate for deeper analyses (e.g. siRNA gene silencing).

4.2.1.11 Further relevant annotated UniTags

Associated with hormone and stress response processes, at least four UniTags were observed among the most up-regulated transcripts in salt stressed chickpea roots (DRRG49-C protein, fiber protein Fb19, hypothetical protein Q9LEN3, and one gibberellin-stimulated protein). However, the specific function of their coded proteins is up to now unknown. A low temperature- and salt-responsive protein LTI6B, associated with membrane processes, was also found. LTI6B is also designated as RCI2B (rare cold induced protein 2B), a protein first characterized in *Arabidopsis*, where it is highly induced under cold, drought and salt stresses, but no information about its possible function(s) is known (Medina et al., 2001)

Among the most salt-up-regulated UniTags, a transcript annotated to an ankyrin protein (STCa-12309, Q9FMJ2_ARATH), which belongs to a relatively newly described class of protein kinases (APKs), has also been reported to be induced by osmotic stress in alfalfa (*Medicago sativa*), and was connected to nodulation, although induction was also detected in other plant organs (Chinchilla et al., 2003).

Related to protein biosynthesis, transcripts annotated to three types of ribosomal proteins were among the most salt-responsive in chickpea roots (60S acidic ribosomal protein P1, 60S ribosomal protein L18a, and 40S ribosomal protein S4). In previous studies,

several ribosomal proteins, including the 60S L18, were found to be salt-responsive in rice ESTs (Sahi et al., 2003; Wu et al., 2005).

Not directly associated to signal transduction, but linked to inositol 3-phosphate biosynthesis, one UniTag annotated to myo-inositol-1-phosphate synthase was 13-fold up-regulated (STCa-181, O22611_PEA). Inositol 3-phosphate is triggering the release of Ca^{2+} out of internal reservoirs such as vacuoles. Variations in cytoplasmic Ca^{2+} concentration in plant cells are considered one of the major early signaling events upon salt stress (Lecourieux et al., 2006).

4.2.1.12 Non-annotable highly up-regulated salt-induced UniTags

Although the majority of the salt stress up-regulated UniTags from INRAT-93 roots were annotated, the remainingtranscripts remain to be linked to characterized ESTs. The un-annotated UniTags may represent potentially new genes, or at least potentially new isoforms of already characterized ESTs, which may play specific roles in salt stress responses of legume roots. Sequences and fold-changes of the most up-regulated anonymous UniTags are compiled in **Table4-2**.

Table 4-2 Top 30 up-regulated non-annotable UniTags in salt-stressed INRAT-93 roots

Tag ID	Sequence	$R_{(ln)}$	Differential expression (fold)
STCa-16261	CATGGTGGTTTTTATGATAATTAAAG	4.35	77.09
STCa-19168	CATGTATGTTTGTTTAATTATGTTTT	3.90	49.50
STCa-5894	CATGATTTACAAATCCTTAGAAATAG	3.53	34.09
STCa-5877	CATGATTGTTTACTGTGAAATTGAAT	3.43	30.85
STCa-3844	CATGAGTATTAGTTTCTAAGTTAAGG	3.38	29.22
STCa-283	CATGAAACAGATGCTTAGAGAGGTTT	3.32	27.58
STCa-10582	CATGGAAAATCTATTGCACAAATCTC	3.26	25.97
STCa-6410	CATGCAACTTTAATATTAAACCTATG	3.24	25.56
STCa-8669	CATGCCGCGTGCAGGAAGAAGGCGCT	3.19	24.34
STCa-15189	CATGGGTTACTCGATCCTAAGATATA	3.16	23.52
STCa-24330	CATGTTTTGAAGTCTAATATAGAGTT	3.09	21.91
STCa-13750	CATGGGAATTTATTATTTTCTAGCTA	3.05	21.09
STCa-22299	CATGTTAATTTTAAATCCATTATTTG	3.03	20.70
STCa-21916	CATGTGTGTTTTAGTGAAGAAGAATC	3.03	20.70
STCa-18427	CATGTAGGATTTATGTTTATCTTAGA	3.01	20.29
STCa-24398	CATGTTTTGTTACAAATTTTATGTTA	3.01	20.29
STCa-23821	CATGTTTAGTTTGATTATCAGTTGAA	3.01	20.29
STCa-1885	CATGAATGAATTGATTAAGATATATA	2.97	19.47
STCa-387	CATGAAAGAAAATCAATTATGTGGGC	2.97	19.47
STCa-22950	CATGTTCTATAACAATATACTTTGAT	2.97	19.47
STCa-21993	CATGTGTTCGTATTAATGATTTATGA	2.97	19.47
STCa-7445	CATGCAGGGGGAACCCGGGGAACTGA	2.88	17.85
STCa-20130	CATGTCTGGAAAATAAATTTGTCTTA	2.88	17.85
STCa-23784	CATGTTTACTTTGTTACTATCATTAT	2.88	17.85
STCa-22619	CATGTTATGTCTACTGTAATAATAAA	2.88	17.85
STCa-4616	CATGATCATTATGTATTTTCTTCCTG	2.84	17.05
STCa-10115	CATGCTGTTAATGGAACAAATGGATA	2.84	17.05
STCa-15886	CATGGTGAACACTTGTTTTTCTCTGT	2.79	16.23
STCa-24351	CATGTTTTGCAAGAAGTAAAAGCTAT	2.79	16.23
STCa-16461	CATGGTTACTTGAATAATTTATTATC	2.76	15.83

4.3 Correlation of SuperSAGE profiles with GO categories in salt-stressed INRAT-93 roots

A more holistic approach evaluating the entire UniTag dataset generated from both control and salt-treated root libraries was carried out by correlating the differential expression ratios [$R_{(ln)}$] to defined standard functional gene categories (i.e. biological processes and cellular components) from the Gene Ontology (GO) database (www.geneontology.org). Statistics for GO categories over-representation were calculated with the help of the Gene Score Re-sampling (GSR) analysis from the ErmineJ 2.0 software (www.bioinformatics .ubc.ca/ermineJ/).

4.3.1 Most over-represented GO biological processes in INRAT-93 salt-stressed roots

After data analysis, GO biological processes such as RNA biosynthesis (GO:0032774), Post-translational protein modifications (GO:0043687), Cellular component organization (GO:0016043), Protein folding (GO:0006457), and Phosphorus metabolic process (GO:0006793), could be identified with the highest ranks for over-representation in early stages of salt stress in chickpea roots (all with P values < 0.00001) (**Table 4-3A**).

Further, still at very high significance, several metabolic processes like Cellular carbohydrate metabolism (GO:0044262), Catabolic process (GO:0009056), Macromolecule catabolic process (GO:0009057), RNA metabolic process (GO:0016070), and Lipid metabolic process (GO:0006629), accompanied external stimulus-related categories such as Response to stress (GO:0006950), Response to stimulus (GO:0050896), and Defense response (GO:0006952). Also, other expected categories like Signal transduction (Knight and Knight, 2001; Apel and Hirt, 2004; Boudsocq and Lauriere, 2005), Regulation of transcription (Chen and Zhu, 2004), and the self-grouped category “ROS-detoxification” (Gechev et al., 2006), belonged to the over-represented processes.

The over-representation of processes related to RNA-metabolism and -biosynthesis can give a hint of the degree of “re-modelling” of the transcriptome of chickpea roots already 2 hours after 25 mM NaCl-treatment. As reported in several other studies, among them a SAGE transcriptome analysis of *Arabidopsis* (Kreps et al., 2002), more than 30% of the transcriptome underlies expression changes under abiotic stress. General metabolic changes in the stressed plant are also reflected by over-expression of diverse metabolism-related GO categories. However, these metabolism-involving GO terms (e.g. Cellular carbohydrate metabolic process**,** Catabolic process, Macromolecule catabolic process, and Lipid metabolic process) are too broad to allow concrete conclusions.

Table 4-3A Overrepresented GO: biological processes as deduced from transcript abundancies (annotated to UniProt entries) in salt-stressed chickpea roots, calculated by the program ErmineJ 2.0

GO ID	GO Biological process	Rank	P
GO:0032774	RNA biosynthetic process	1	1.00E-12
GO:0043687	Post-translational protein modification	2	1.00E-12
GO:0016043	Cellular component organization and biogenesis	3	1.00E-12
GO:0006457	Protein folding	4	1.00E-12
GO:0006793	Phosphorus metabolic process	5	1.00E-12
GO:0044262	Cellular carbohydrate metabolic process	6	1.00E-12
GO:0007165	Signal transduction	7	1.00E-12
GO:0009056	Catabolic process	8	1.00E-12
GO:0016310	Phosphorylation	9	1.00E-12
GO:0009057	Macromolecule catabolic process	10	1.00E-12
GO:0016070	RNA metabolic process	11	1.00E-12
GO:0046907	Intracellular transport	12	1.00E-12
GO:0050896	Response to stimulus	13	1.00E-12
GO:0006950	Response to stress	14	1.00E-12
GO:0044249	Cellular biosynthetic process	15	1.00E-12
GO:0006355	Regulation of transcription, DNA-dependent	16	1.00E-12
GO:0006091	Generation of precursor metabolites and energy	17	1.00E-12
GO:0009607	Response to biotic stimulus	18	1.00E-12
GO:0006629	Lipid metabolic process	19	1.00E-12
GO:0006468	Protein amino acid phosphorylation	20	1.00E-12
GO:0044248	Cellular catabolic process	21	1.00E-12
GO:0042221	Response to chemical stimulus	22	1.00E-12
CMC-1	ROS scavenging enzymes*	23	1.00E-12
GO:0006952	Defense response	24	1.00E-12
GO:0051641	Cellular localization	25	1.00E-12
GO:0006807	Nitrogen compound metabolic process	26	1.00E-12
GO:0015031	Protein transport	27	1.00E-12
GO:0019219	Regulation of nucleic acid metabolic process	28	1.00E-12
GO:0045449	Regulation of transcription	29	1.00E-12
GO:0019438	Aromatic compound biosynthetic process	30	1.00E-12

*Custom-made category

Table 4-3B Underrepresented GO biological processes as deduced from transcript abundancies (annotated to UniProt entries) in salt-stressed chickpea roots, calculated by the program ErmineJ 2.0

GO ID	GO Biological process	Rank	P
GO:0006350	Transcription	1	1.00E-12
GO:0032774	RNA biosynthetic process	2	1.00E-12
GO:0044262	Cellular carbohydrate metabolic process	3	1.00E-12
GO:0050794	Regulation of cellular process	4	1.00E-12
GO:0050789	Regulation of biological process	5	1.00E-12
GO:0016070	RNA metabolic process	6	1.00E-12
GO:0030154	Cell differentiation	7	1.00E-12
GO:0006091	Generation of precursor metabolites and energy	8	1.00E-12
GO:0045449	Regulation of transcription	9	1.00E-12
GO:0019222	Regulation of metabolic process	10	1.00E-12
GO:0006118	Electron transport	11	1.00E-12
GO:0031323	Regulation of cellular metabolic process	12	1.00E-12
GO:0015979	Photosynthesis	13	0.0001
GO:0006952	Defense response	14	0.0004
GO:0044264	Cellular polysaccharide metabolic process	15	0.0008
GO:0008219	Cell death	16	0.0010
GO:0006073	Glucan metabolic process	17	0.0012
GO:0032502	Developmental process	18	0.0023
GO:0006259	DNA metabolic process	19	0.0032
GO:0006793	Phosphorus metabolic process	20	0.0033
GO:0043687	Post-translational protein modification	21	0.0035
GO:0065003	Macromolecular complex assembly	22	0.0035
GO:0009059	Macromolecule biosynthetic process	23	0.0036
GO:0016310	Phosphorylation	24	0.0049
GO:0016051	Carbohydrate biosynthetic process	25	0.0061
GO:0019684	Photosynthesis, light reaction	26	0.0067
GO:0044265	Cellular macromolecule catabolic process	27	0.0070
GO:0007001	Chromosome organization and biogenesis	28	0.0086
GO:0006468	Protein amino acid phosphorylation	29	0.0090
GO:0005975	Carbohydrate metabolic process	30	0.0094

4.3.2 Simultaneously over- and under-represented GO biological processes

Curiously, some of the processes appearing in the top list of overrepresented GO biological processes are also observed in the list of most underrepresented ones (**Tables 4-3A, and 4-3B**). This apparent contradiction is a consequence of the very general coverage in assignment of GO categories to characterized genes. Here, instead of focusing on specific pathways, diverse processes are associated to one, or a group of related GO terms (http://www.geneontology.org). This aspect is very well exemplified by UniTags belonging to the GO category Regulation of transcription in the present work. In chickpea roots from the salt-tolerant variety INRAT-93, more than 123 UniTags, belonging to more than 24 TF classes (see Section **4.4.3**), differentially reacted upon salt treatment, which is displayed as a wide range of regulation levels.

GO biological processes like RNA biosynthesis, Cellular carbohydrate metabolism, RNA metabolism, Generation of precursor metabolites and energy, Defense response, Phosphorus metabolic process, and Post-transcriptional protein modifications contain further examples.

Not only transcripts (genes) assigned to similar processes, but even transcripts coding for basically the same protein, reveal very contrasting regulation levels. A very strong case in salt stressed chickpea roots is given by extensin, whereas UniTag STCa-19021 (O65760_CICAR) is 30-fold upregulated [$R_{(ln)}$ = 3.4], UniTag STCa-6160 (Q9FSY9_CICAR), annotated to a different extensin accession, is more than 40-fold down-regulated [$R_{(ln)}$ = 3.79] (**Tables 4-3A and 4-3B**). A further case is exemplified by the very large family of cytochrome P450-containing proteins (CYPs). From 107 CYP-annotated UniTags, four are up-regulated at least 11-fold [$R_{(ln)}$ = 2.4], and two are down-regulated at least 34-fold [$R_{(ln)}$ = 3.5]. Another clear example for varying reactions of UniTags annotated to similar proteins is given by SODs**.** In salt-stressed roots of chickpea, six UniTags annotated to ESTs coding for SODs were found, from these, two were at least 6-fold up-regulated (STCa-7896, and STCa-3770), two were only 1.5-fold up-regulated (STCa-7895, and STCa-7897), and two were constitutively expressed (STCa-18941, and STCa-19660).

The results described in the previous section prove, that the “re-programming” of cellular processes in roots under salt-stress indeed involves repression and induction of components belonging to closely related pathways at the same time.

4.3.3 Most over-represented GO cellular components in salt-stressed INRAT-93 roots

GO cellular components such as ribonucleoprotein complex (GO:0030529), Protein complex (GO:0043234), Organelle part (GO:0044422), Plastid (GO:0009536), and Mitochondrion (GO:0005739), rank within the highest overrepresented categories ($P<0.005$). Further down on the list, and still with high overrepresentation levels ($P<0.1$), GO cellular components related to protein biosynthesis such as the different ribosomal sub-units, and components associated with chromosomes and chromosome-organization, were also found (**Table 4-3C**).

GO cellular components associated with protein biosynthesis appear to play distinct roles in chickpea roots under salt stress. However, the terminology of gene ontology is too broad to pinpoint groups of proteins, whose biosynthesis is favoured. In general, an abrupt "proteome re-modelling" involving the biosynthesis of new proteins along with a changed protein turn-over is observed in the first stages of salt stress responses. This aspect is considered in several reports on proteome dynamics in plants (Amme et al., 2006; Larrainzar et al., 2007).

Further on, over-representation of the term "Mitochondria" as one of the cellular components that suffers most under salt stress (Gechev et al., 2006), and as one of the most abundant organelles in SNF-engaged plant tissues (Iturbe-Ormaetxe et al., 2001), agrees well with the high expression levels of UniTags annotated to genes involved in oxidative stress management in INRAT-93 roots (e.g encoding SODs, GSTs, ICDHs).

Table 4-3C Over-represented GO: cell components as deduced from transcript abundances (annotated to UniProt entries) in salt-stressed chickpea roots, calculated by the program ErmineJ 2.0

GO ID	GO Cellular component	Rank	P
GO:0030529	Ribonucleoprotein complex	1	1.00E-12
GO:0043234	Protein complex	2	1.00E-12
GO:0044422	Organelle part	3	1.00E-12
GO:0009536	Plastid	4	5.42E-08
GO:0005739	Mitochondrion	5	2.80E-03
GO:0005856	Cytoskeleton	6	0.01
GO:0033279	Ribosomal subunit	7	0.01
GO:0005694	Chromosome	8	0.02
GO:0005783	Endoplasmic reticulum	9	0.02
GO:0000785	Chromatin	10	0.03
GO:0044427	Chromosomal part	11	0.04
GO:0000786	Nucleosome	12	0.04
GO:0015935	Small ribosomal subunit	13	0.04
SOS pathway	CBLs - CIPKs - Proton pumps	14	0.05
GO:0005576	Extracellular region	15	0.05
GO:0000502	Proteasome complex (sensu Eukaryota)	16	0.06
GO:0015630	Microtubule cytoskeleton	17	0.06
GO:0015934	Large ribosomal subunit	18	0.06
GO:0031090	Organelle membrane	19	0.06
GO:0005618	Cell wall	20	0.07
GO:0044428	Nuclear part	21	0.07
GO:0044430	Cytoskeletal part	22	0.09
GO:0005874	Microtubule	23	0.11
GO:0012505	Endomembrane system	24	0.11
GO:0005839	Proteasome core complex	25	0.12
GO:0005875	Microtubule associated complex	26	0.13
GO:0005794	Golgi apparatus	27	0.21
GO:0031966	Mitochondrial membrane	28	0.22
GO:0048475	Coated membrane	29	0.82

4.4 Diverse salt stress-related processes and expression profiles of involved UniTags in roots from salt-treated INRAT-93 plants

In the following sub-sections, expression levels in chickpea roots and background information about genes and gene families involved in several salt stress-associated processes will be detailed.

4.4.1 ROS production and scavenging in salt-stressed chickpea roots

As one of the first responses of plant cells under salt stress, ROS (singlet oxygen- ($^1O_2$), superoxide- (O_2^-), hydroperoxide- (H_2O_2), and hydroxyl- ($OH^·$) radicals) are generated. These ROS, produced by the challenged cell, trigger a wide range of physiological reactions including programmed cell death (PCD) and general oxidative stress (Apel and Hirt, 2004). Even at low but more so at higher concentrations, ROS are toxic for the cell. Therefore, an elaborate and highly redundant network composed of ROS-producers and ROS-detoxifiers is maintaining this radicals under control in plants (Gechev et al., 2006).

The strong expression changes of many of the UniTags involved in the above processes, some of which are detailed in the following sub-sections, provide enough evidence to suggest that, apart from the osmotic stress and toxic Na^+ concentration, salt-stressed roots of chickpea plants are vigorously involved at a fierce “third front”: coping with the oxidative stress.

4.4.1.1 Mitochondiral respiration and ROS generation

Mitochondrial respiration generates considerable amounts of ROS (esspecially O_2^-), although 20-fold lower than the amount chloroplasts would produce (Moller, 2001). As described in section **4.2.1.6**, high expression (26-fold up-regulation) of a UniTag annotated to an NADP-dependent isocitrate dehydrogenase (ICDH;STCa-7166, Q6R6M7_PEA) was detected in salt-stressed chickpea roots. ICDH generates NADPH to keep antioxidants like glutathione in a reduced state in mitochondria (Moller, 2001). Since root tissue of whatever kind contains only few chloroplasts, the mitochondria can be considered a major ROS generator in the salt-stressed root system. Additionally, UniTags annotated to another ROS-related mitochondrial enzyme, the alternative oxidase (AOX), whose action minimizes the production of (O_2^-) under over-energization conditions (Umbach et al., 2005), showed salt stress down-regulation. From a total of six UniTags annotated to AOX, four (STCa-20476, STCa-2667, STCa-14421, STCa -14429) were at least 6-fold down regulated whereas the other two transcript variants were expressed at constitutive levels (**Table 4-5**). Similar results

have been observed in a *Vigna unguiculata* salt-tolerant variety, showing AOX transcripts repression under salt stress conditions (Costa et al., 2007).

4.4.1.2 Superoxide (O_2^-) dismutation

Dismutation of (O_2^-) ions derived from mitochondrial or chloroplast respiration occurs very quickly by the action of SOD (Dat et al., 2000). According to SuperSAGE profiles from chickpea salt-stressed roots (section **4.2.1.2**), transcripts coding for SODs were among the most up-regulated ones (e.g. STCa-7896; Q9ZNQ4_CICAR, 40-fold). A total of six SOD-annotated UniTags were detected in the INRAT-93 root dataset (**Figure 4-4**). From these, four were constitutive, whereas two others showed more than 2.7-fold up-regulation ($R_{(ln)}>1$).

4.4.1.3 Glutathione/ascorbate cycles and catalase activity

In the initial ROS-scavenging process, (O_2^-) radicals are dismutated to hydrogen peroxide (H_2O_2) by SODs. Hydrogen peroxide, which is more stable than (O_2^-), is scavenged via the ascorbate- and glutathione-cycles, or by the action of catalases (CAT), a class of ROS scavenging enzymes (Gechev et al., 2006). Expression profiles of UniTags annotated to the main ROS detoxification enzymes, along with a detailed scheme of their pathways, are depicted in **Figure 4-4**. According to the expression levels of APX (STCa-11617, Q9SXT2_CICAR), DHAR (STCa- 1532, Q84UH4_TOBAC), some transcript variants of GPX (STCa-3305, GPX4_CITSI), and CAT (STCa-668, Q9ZRU4_CICAR); the initial battery of defense against oxidative stress is deployed in chickpea roots already 2 hours after onset of salt stress. Additionally, glutathione S-transferases (Street et al., 2006), a class of enzymes with DHAR activity as well, and active in the detoxification of lipid hydroxyperoxides (Dixon et al., 2002); are represented by 15 different UniTags. From these, seven transcript variants showed constitutive levels, four and two were at least 2.5- and 8-fold up-regulated (STCa-11623, Q9AYN3_9ASTR; STCa-22470, Q948X4_MEDSA), respectively, whereas two transcript variants were 4.5-fold down-regulated (**Table 4-4**).

Also, transcripts coding for peroxyredoxin and thioredoxin proteins, enzymes also involved in ROS-detoxification but less well characterized (Dietz, 2003; Meyer et al., 2005), show slightly differential expression (**Table 4-4**). After 2 hours of salt stress, a single UniTag annotated to peroxiredoxin is 2.2-fold down-regulated (STCa-23663, Q6UBI3_9CARY), whereas from five UniTags annotated to thioredoxin, three were constitutively expressed, and one UniTag was at least 2.5-fold up-regulated (STCa-16323, Q8H6X3_TOBAC).

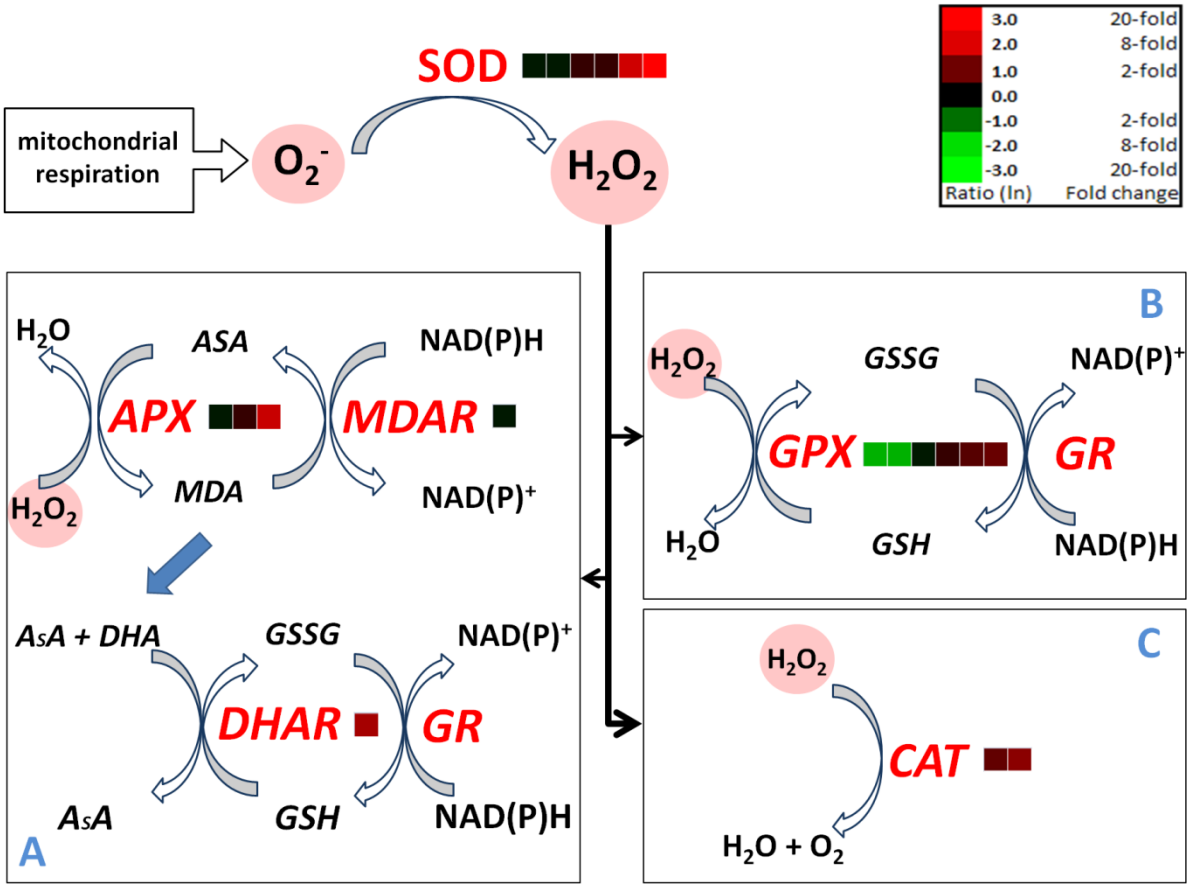


Figure 4-4 Major ROS scavenging processes in plant cells along with transcription profiles of related UniTags in salt-stressed chickpea roots

Superoxide radicals (O_2^-) are primarily dismutated to H_2O_2 by superoxide dismutases (SOD). Following, hydrogen peroxide (H_2O_2) is scavenged via:

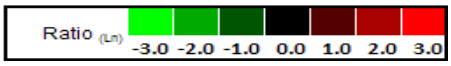
A) Ascorbate cycle: ascorbate peroxidase (APX), monodehydroascorbade reductase (MDAR), dehydroascorbate reductase (Sreedhar et al.), and glutathione reductase (GR), are acting together with ascorbate (AsA) as electron donor.

B) Glutathione cycle: glutathione peroxidase (GPX) and glutathione reductase (GR) are involved to balance oxidized- (GSSH) against reduced-glutathione (GSH).

C) Catalase activity: Catalase (CAT) is detoxifying H_2O_2 without associated reductors

Table 4-4 Additional UniTags annotated to ROS metabolism-related genes

Metabolic process	Protein	Number of representative Supertags (1 5 10 15 20)
Mitochondrial metabolism	Alternative oxidase	
Mitochondrial metabolism	NADP-dependent ICDH	
ROS scavenging	Glutathione S-transferase	
ROS scavenging	Peroxiredoxin	
ROS scavenging	Thioredoxin	
Metal ion binding	Metallothionein-like protein 2	
Metal ion binding	Metallothionein-like protein 1	



4.4.1.4 Counteraction of toxic metal ions

Chemically, over production of (O_2^-) leads to inactivation or alteration of the catalytic activities of enzymes containing Fe-S clusters, whereas H_2O_2 inactivates a broad range of proteins by oxidizing their thiol groups. However, the major and frequently lethal danger of these two ROS does not rely on themselves, but on their protonation to hydroxyl radicals ($OH^·$). This mechanism generally involves metal ions, that catalyse the Fenton reaction ($Fe^{2+}+H^2O^2 \rightarrow Fe^{3+}OH^·+OH^-$). Hydroxyl radicals have no antagonistic scavenging enzyme, and can practically react with any cellular compound coming across their ways. In chickpea roots under salt stress, very high transcriptional induction and also repression of a broad range of metal ion-sequestering enzymes has been detected. A strong case is exemplified by metallothionein-like proteins MT1 and MT2. For this class of proteins, 29 UniTags were detected with regulation levels between 20-fold down-, and 10-fold up-regulation. In this context, UniTag STCa-3424 (MT2_CICAR) was the transcript variant showing the highest induction level (**Table 4-4**).

4.4.2 ROS-triggered and general stress-related signal transduction

On the other hand, plants under salt stress can also use ROS radicals as signals (Xiong et al., 2002), where both chemical identity and intracellular location determine their specific activity (Laloi et al., 2004). Three main, very general categories of genes are involved in ROS-triggered signal transduction: i) H_2O_2 sensors, ii) protein-kinases and -phosphatases, and iii) transcription factors (**Figure 4-5**). The ROS-signaling network seems to be redundant and complex, and rather than being exclusive, its components are common to several other signaling pathways (Foyer and Noctor, 2003). Therefore, the following sub-sections will

detail the expression profiles of genes involved in ROS-triggered- as well as general stress-signal transduction.

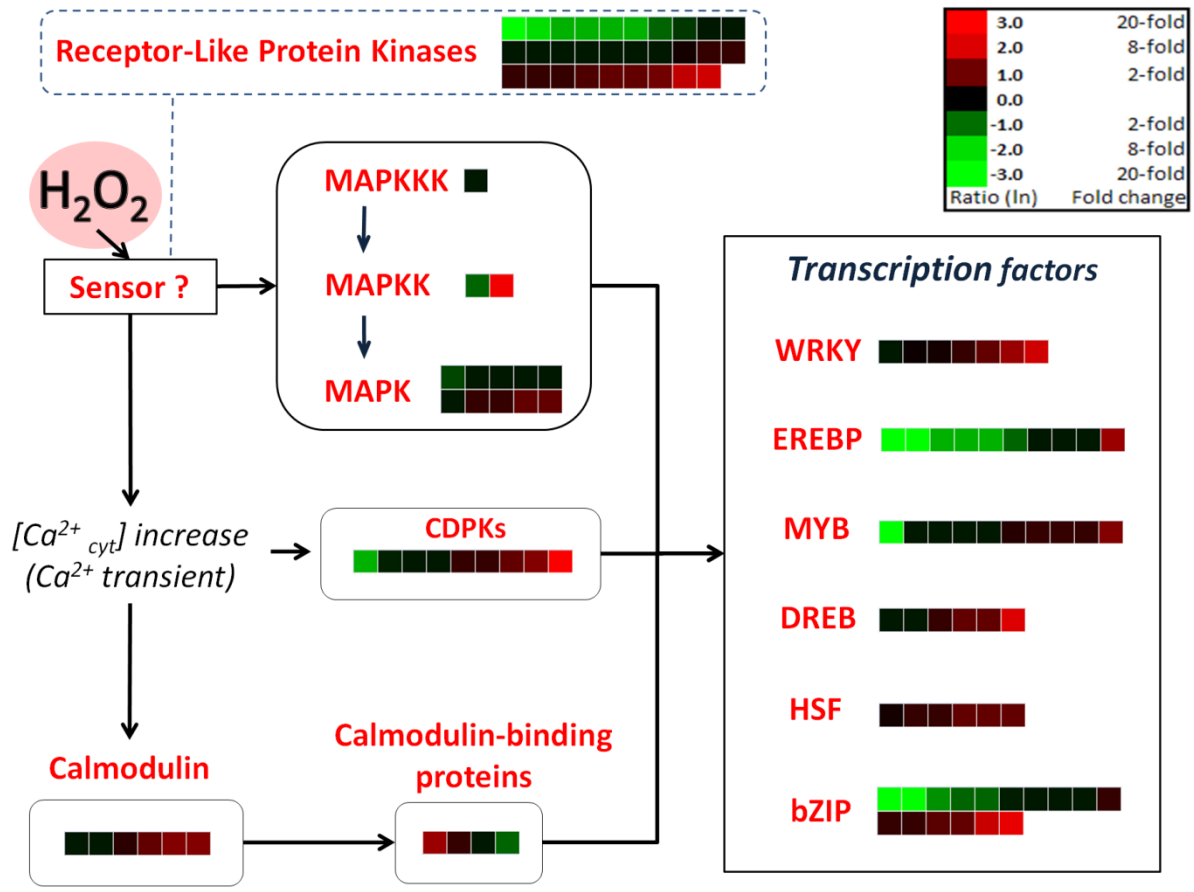


Figure 4-5 Pathway heat-maps showing ROS involvement in plant stress signaling, including UniTag profiles from salt-stressed chickpea roots

Receptor-like protein kinases (RLKs) act as H_2O_2 sensors, where positive sensing can activate MAP-kinase cascades. In turn, Ca^{2+} transients (signals caused by abrupt changes in Ca^{2+} concentration) activated by H_2O_2 can be sensed by Ca^{2+}-binding proteins like calmodulin and calcium-dependent protein kinases (CDPKs). Subsequently, a broad array of transcription factors is activating the transcription of several effector (response) genes.

4.4.2.1 Signaling sensors

Their properties in signal perception predispose receptor like protein kinases (RLKs) as major players in ROS-triggered signaling (Ouelhadj et al., 2007). Further on, transcription of RLK-encoding genes was induced in plants under salt stress (He et al., 2004). In the entire chickpea INRAT-93 dataset, 29 UniTags annotated to diverse RLKs were detected showing expression levels between 6-fold down-regulation and up to 10-fold up-regulation (**Figure 4-5**). SuperSAGE data from salt-stressed roots alone, however, do not allow deciding, whether the up-regulation of some of the RLKs transcript variants is an exclusive response to salt-stress itself, or a response triggered by oxidative stress. Nevertheless, candidates for deeper characterization can already be selected on the basis of the observed salt-stress induction. For example, UniTags STCa-12500, STCa -24316, and STCa-7800; each with >3-fold up-regulation, would belong to such candidates.

4.4.2.2 MAP-kinases-related signal transduction

Signaling cascades controlled by mitogen-activated protein kinases (MAPKs), MAPK-kinases and MAPKK-kinases, play an important role in ROS-related signal transduction (Samuel et al., 2005), and have also been widely associated to salt stress signaling in plants (Mizoguchi et al., 1996). As an example in *Arabidopsis*; Teige and co authors (2004) described the salt stress-specific activation of the MAPK-kinase MKK2, which in turn activates two downstream MAPKs, namely MPK4 and MPK6.

In chickpea, and in contrast to the large number of family members observed in other plants (Hardie, 1999), only a single UniTag annotated to a MAPKK-kinase was detected (STCa-8893, Q75PK5_LOTJA), showing no major expression changes upon salt stress (**Figure 4-5**). From two UniTags annotated to MAPK-kinases, UniTag STCa-5798 (Q93WR7_MEDVA) showed high induction levels (9.0-fold up-regulation), whereas UniTag STCa-13107 (Q9AYN9_TOBAC) was 2.5-fold down-regulated. Further on, a total of 10 UniTags was annotated to MAP-kinases, many of them showing constitutive expression levels. UniTags STCa-10057 (Q43466_SOYBN) and STCa-2409 (Q1PCG0_MEDSA) revealed the highest induction (2.5-fold), whereas STCa-4402 (MMK2_MEDSA) was most down-regulated (2.0-fold) 2h after onset of NaCl-treatment (**Figure 4-5**).

Components of the MAPKKK-MAPKK-MAPK signaling cascades are generally considered as cross-talk nodes between specific signal transduction pathways (Knight and Knight, 2001;

Boudsocq and Lauriere, 2005). Therefore, mixed patterns of up- and down-regulation of the various components of the cascade, as found in chickpea roots, may be logically expected.

4.4.2.3 Calcium-dependent signaling proteins

Calcium signals, also known as calcium transients, are considered the starting points in signal transduction processes triggered by abiotic stresses (Hirschi, 2004; Lecourieux et al., 2006). As a consequence, almost all salt stress-involved signaling pathways involve Ca^{2+}-sensing proteins in their initial steps (Leung and Giraudat, 1998; Saijo et al., 2001). Different classes of Ca^{2+}-binding proteins such as Ca^{2+}-dependent protein kinases (CDPKs), calmodulin, and calmodulin binding proteins are involved in ROS- and salt-stress signaling (Romeis et al., 2001; Rentel and Knight, 2004). In chickpea roots, the transcription profiles of UniTags annotated to CDPKs, calmodulins, and calmodulin-binding proteins reveal salt-stress-induced responses. Three out of the nine transcripts annotated to CDPKs showed 2.5-, 3.0- and 9.0-fold up-regulation, respectively (STCa-16072, Q7XZK5_CICAR; STCa-19016, Q8W4I7_ARATH; and STCa-17567, Q5D875_MEDTR), whereas STCa-17568 (Q5D875_MEDTR) was at least 6-fold down-regulated (**Figure 4-5**). On the other hand, three out of the six UniTags annotated to calmodulin were at least 2.5-fold up-regulated (STCa-17530, STCa-14865, and STCa-21464), and two out of four UniTags annotated to calmodulin-binding proteins reacted either positively (STCa-20534, 4.0-fold up-regulation) or negatively (STCa-6206, 2.5-fold down-regulation) 2 hours after the onset of salt stress (**Figure 4-5**).

4.4.2.4 ABA-dependent and -independent signaling in chickpea roots under salt stress

In the previous section, some of the components of the ABA-dependent and -independent signaling pathways have been introduced (MAP-kinases, calcium sensors, and CDPKs; see also (Leung and Giraudat, 1998). In the present section, some of the ABA signaling-related genes will be briefly highlighted to better understand their transcriptional dynamics in chickpea roots upon salt stress. The most common components of ABA-dependent and -independent signaling pathways in plants are depicted in **Figure 4-6**.

Briefly, salt, drought and heat stress all activate Ca^{2+} signals that are sensed, either by activators of the ABA-dependent- or ABA-independent-cascades (Zhu, 2002; Shinozaki et al., 2003). In an ABA-independent pathway, dehydration-responsive element binding TFs (DREB-TFs) are activated by CBLs (calcineurin B-like proteins). Therefore, genes encoding DREBs and CBLs are expected to be induced by salt stress (Nakashima et al., 2000). Indeed,

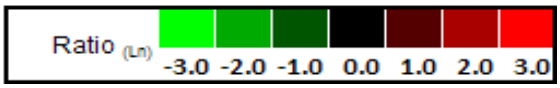
three out of seven DREB-annotated UniTags were at least 2.5-fold up-regulated, among them UniTag STCa-10794 (Q75UJ6_CUCME) as the most differentially expressed one (7.0-fold). From five CBL-annotated UniTags, STCa-2642 (Q8L7F6_PEA) was highest up-regulated (2.7-fold; see also Section **4.4.4**) **(Figure 4-6**).

In an ABA-dependent pathway, the enzyme nine-*cis*-epoxycarotenoid dioxygenase (NCED) is probably the rate-limiting step in the ABA biosynthesis machinery (Milborrow, 2001). Curiously, in chickpea roots under salt stress, both UniTags annotated to NCED show constitutive (STCa-18782, Q1T3T3_MEDTR) and down-regulation levels, respectively (STCa-21012, Q8LP17_PEA; 3.5-fold) (**Table 4-5**). These results suggest, that *de-novo* synthesis of ABA is probably not favoured in the early stages of salt stress in chickpea roots.

On the other hand, the liberation of abscisic acid from ABA-glucose conjugates by beta-glucosidase is an alternative way to increase ABA levels in plants (Dietz et al., 2000). In INRAT-93 roots, 19 UniTags annotated to transcripts coding for beta-glucosidase were detected. At least 6 transcript variants showed more than 2.5-fold up-regulation (STCa-15729, -22461, -16215, -228, -9452, -16531) upon salt stress (**Table 4-5**).

Table 4-5 UniTags from two genes involved in ABA biosynthesis/release in plants and their transcription profiles in salt-stressed chickpea roots

		Number of representative SuperTags				
Metabolic process	***Protein***	***1***	***5***	***10***	***15***	***20***
ABA biosynthesis	9-cis-Epoxycarotenoid dioxygenase					
ABA conjugates release	Beta-glucosidase					



At the transcription factor (TF) level, ABA-responsive element-binding factors (AREB or ABFs) are a sub-class of bZIP-TFs, that directly interact with ABA-dependent signaling (Choi et al., 2000). In chickpea, no UniTag was detected with high similarity to ABFs. However, bZIP transcription factors were among the most represented TFs in the dataset. Thus, with larger sequence information, probably some of the bZIPs-annotated transcripts would be re-assigned to AREBs. MYBs represent a second class of TFs involved in ABA-dependent signaling (Yanhui et al., 2006). In INRAT-93 roots, ten UniTags were annotated to MYB TFs, and are differentially regulated. Among them, STCa-11693 (Q94AX9_ARATH) was most up-regulated (3.0-fold), and STCa-4609 (Q84UB0_MALXI) the strongest down-regulated (20-fold) transcript (**Figure 4-6**).

The observed transcription profiles in salt stressed roots suggest, that both ABA-dependent and -independent signaling pathways may control early responses to salt stress in chickpea roots (i.e. 2h after onset of NaCl-treatment). Nevertheless, the *de novo* synthesis of ABA may not be favoured. The up-regulation of two UniTags (STCa-16967:2.6-fold; STCa-16257:9.0-fold), annotated to uncharacterized ABA-responsive proteins (Q9FMW4_ARATH), confirms the importance of ABA-signaling (**Figure 4-6**).

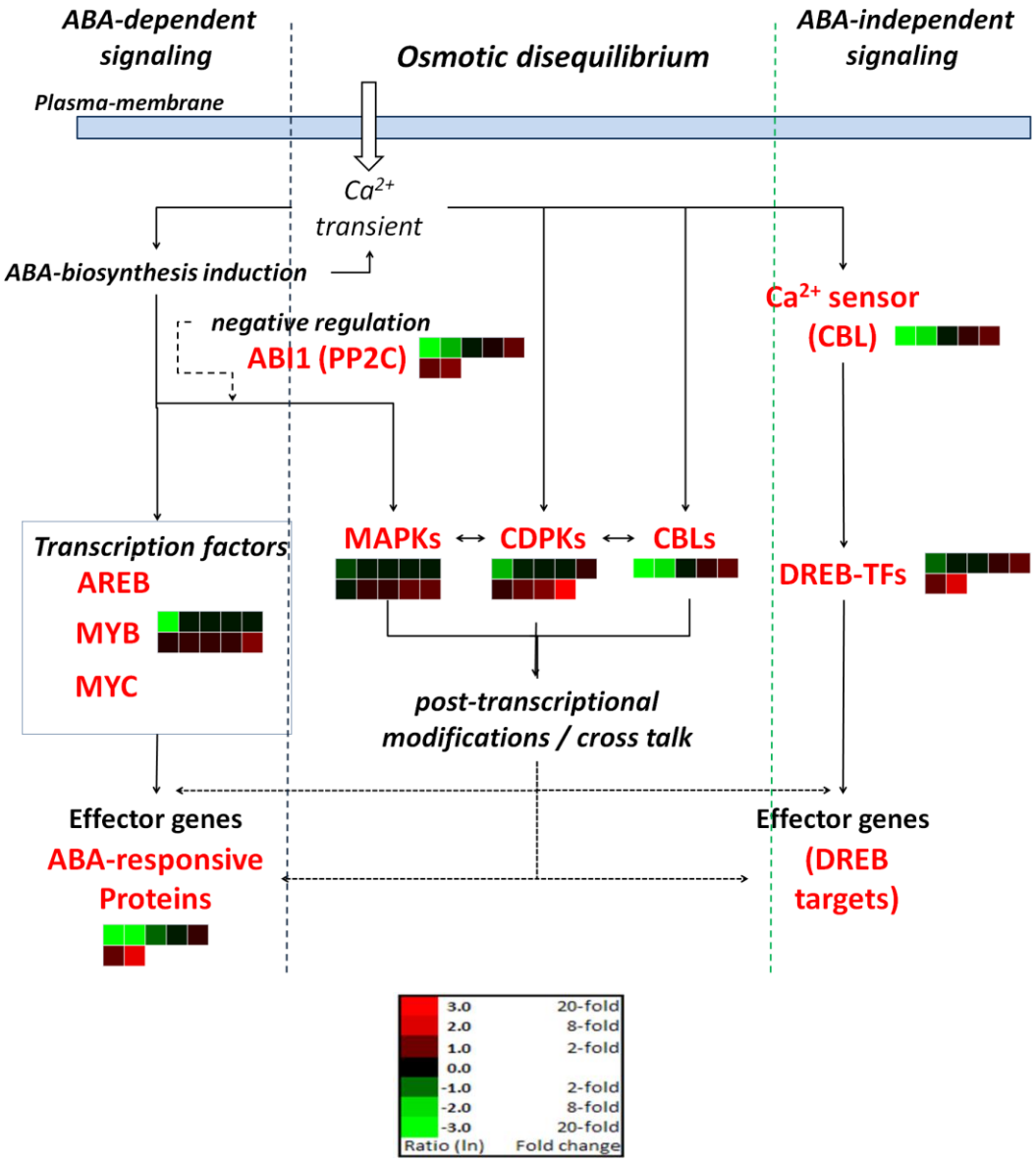


Figure 4-6 Pathway representation of ABA-dependent and -independent signaling cascades along with transcription profiles of chickpea UniTags in salt stressed roots

Abscisic acid, the most important hormone in plants under abiotic stress, triggers a complex signaling cascade involving ABA-responsive transcription factors, and the action of several protein kinases. In parallel, an ABA-independent signaling pathway is activating dehydration-responsive (DREB) TFs through calcium sensors like CBL proteins.

4.4.3 Expression of genes encoding transcription factors in salt-stressed INRAT-93 roots

In control and salt-stressed INRAT-93 libraries, a total of 123 UniTags, annotated to at least 24 different transcription factor (TF) classes were detected (**Figure 4-7**). The most represented TFs were: basic leucine zipper [bZIP], and homeo-domain leucine zipper [HDZ] (each with 16 annotated UniTags), followed by high mobility group [HMG] (15), ethylene-responsive binding factor [ERBF] (10), and MYB-type TFs (10 UniTags). As detailed in **Table 4-6,** expression profiles of UniTags annotated to TFs display a wide range of differential expression in salt-stressed chickpea roots.

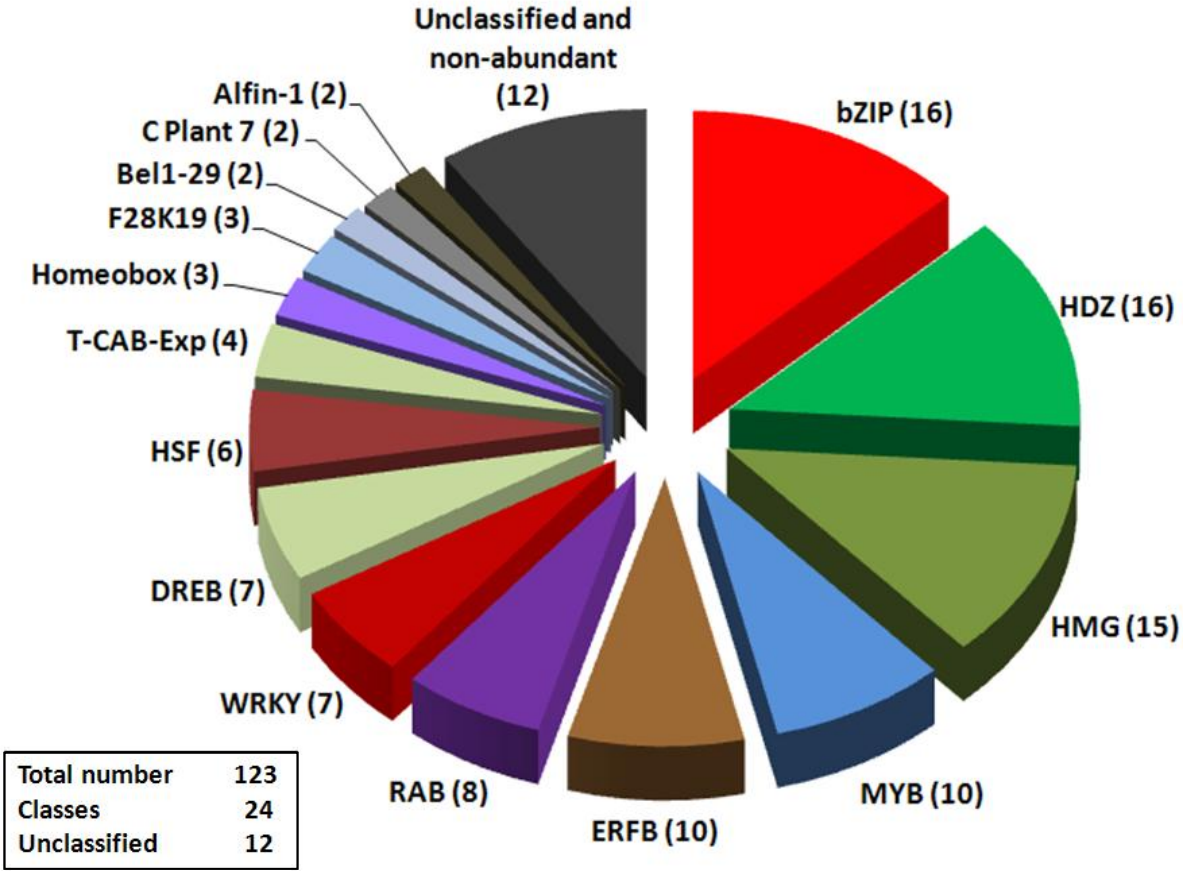
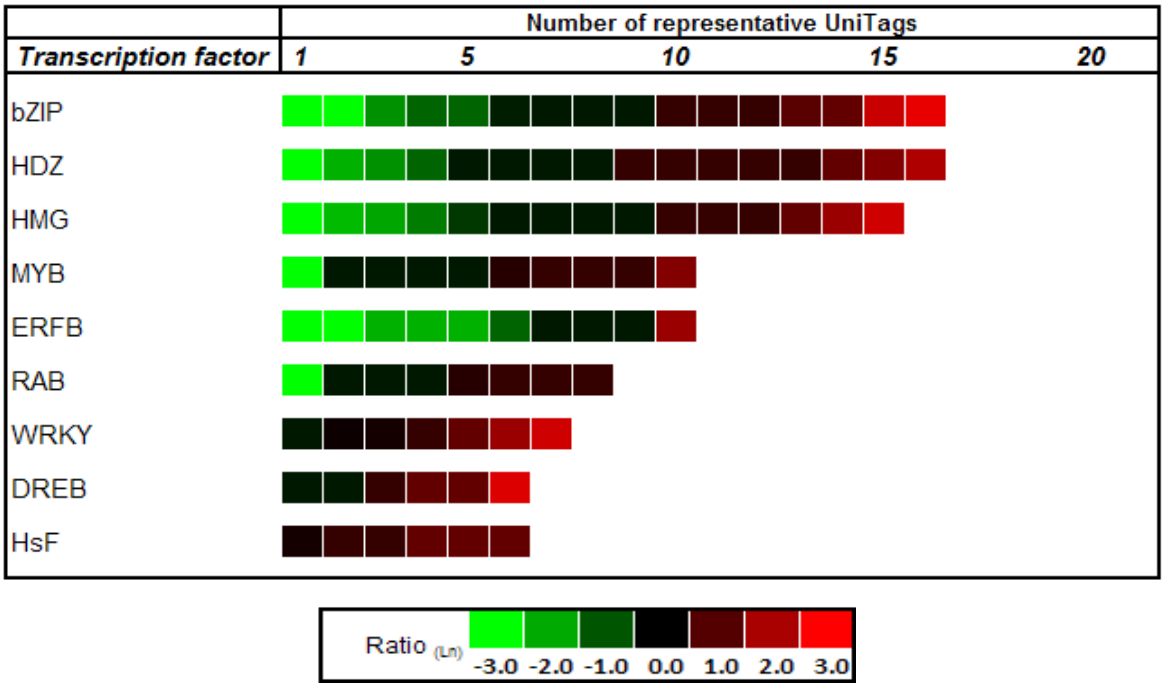


Figure 4-7 **Transcription factor classes in SuperSAGE libraries from control and salt-stressed chickpea INRAT-93 roots**

Table 4-6 Most abundant transcription factor classes in the INRAT-93 root UniTag dataset



4.4.3.1 bZIP transcription factors are most abundant in INRAT-93 roots

Transcription factors of the bZIP-type have been associated with salt stress responses of plants by several authors (Choi et al., 2000; Lopez-Molina and Chua, 2000; Kim et al., 2004; Liu et al., 2007). For example, in *Arabidopsis*, Liu and co-workers (2007) report on a plant stress signaling pathway sharing similarities with endoplasmic reticulum (ER) stress-responses. In this pathway, a membrane-localized bZIP-TF (AtbZIP17) plays a crucial role. Also in *Arabidopsis*, mutations in GIA1, a predicted bZIP-TF from the ABF subfamily, confer ABA-insensitivity (Lopez-Molina and Chua, 2000).

The broad range of differential expression of the 16 UniTags annotated to bZIPs in chickpea roots under salt stress (35-fold down-regulation versus 10-fold up-regulation) are proof of the diversity of processes in which this class of TFs are involved (**Table 4-6, Figure 4-7**). UniTags STCa-13520 (Q8L5W2_SOYBN, 6.0-fold) and STCa-22623 (Q9FUD3_ARATH, 10-fold) revealed the highest up-regulation levels in chickpea roots 2h after onset of NaCl-treatment.

The present results indicate a very wide range of expression levels for bZIPs in salt stressed chickpea roots. Although the sequence information is still not sufficient to identify sub-classes of this TF family (e.g. ABFs), salt stress-responsive candidates can be yet selected to be further characterized.

4.4.3.2 HDZ transcription factors

As observed for the bZIP-type TFs, the broad range of transcript variants and expression levels of HDZs reflects a complex pattern (16 different UniTags with different regulation levels). UniTags STCa-10666 (Q39123_ARATH) and STCa-247 (Q93XA4_PHAVU) revealed the highest HDZ induction levels after 2 hours of salt stress in chickpea roots (3.0- and 6.0-fold up-regulation, respectively; **table 4-6**). The involvement of HDZs (a class of plant-specific TFs) in osmotic stress-, cold-, ABA- and H_2O_2-induced responses has been proven in detail in *Arabidopsis* (Lee and Chun, 1998), *Craterostigma plantagineum (Deng et al., 2006), Brassica napus* (Yu et al., 2005), and sunflower (Palena et al., 1999). However, much is unknown about the possible targets of this TF type.

4.4.3.3 HMG-Box transcription factors

The HMG-box domain was originally identified as the domain that mediates the DNA-binding of chromatin-associated high-mobility group (HMG) proteins of the HMGB type. HMG-box domains have been found in various DNA-binding proteins including transcription factors (Stros et al., 2007). In plants, still is much to be understood about the specific functions of HMG-box proteins in the context of stress responses.; Nevertheless, there are already reports on the differential expression of diverse HMG-box family members upon various abiotic stresses in *Arabidopsis* (Kwak et al., 2007). The observed results in salt-stressed chickpea roots corroborate the findings in *Arabidopsis*. Fifteen UniTags were annotated to HMG-box proteins showing a broad range of expression levels (10-fold down-regulated versus 6-fold up-regulated; **Table 4-6**). However, information about possible targets or regulatory pathways, in which this class of TFs may be involved, is still missing.

4.4.3.4 MYB transcription factors

As briefly detailed in Section **4.4.2.4,** MYB TFs are involved in several regulatory processes induced by osmotic stress in plants, especially in connection with ABA-mediated stress responses (Abe et al., 2003). Expression levels of MYB-coding mRNAs were up-regulated in a broad range plant species under osmotic stress (Seki et al., 2002; Buitink et al., 2006). Curiously for INRAT-93 roots under salt stress, from ten transcript variants annotated to MYB-TFs, only one UniTag was more than 3.0-fold up-regulated (STCa-11693). In contrast, one of the transcript variants (STCa-4609) was 20-fold down-regulated. The remaining 9 UniTags were constitutively expressed. As demonstrated by Poroyko and co-authors (2007),

transcript profiles for several genes change within very small distances from the root tip in maize roots. The authors argue that in roots the action of ABA may be locally different, and therefore the regulation of ABA-responsive genes may also vary. The observed differential transcription of MYBs (and some bZIPs) in chickpea roots may also rest on this phenomenon.

4.4.3.5 Ethylene-responsive transcription factors

The role of ethylene signaling in salt stress responses of plants is still obscure to date. Nevertheless, previous reports like the work of Cao and co-authors (2007) in *Arabidopsis*, indicated cross-talk of ethylene and salt-stress responses. From 10 UniTags annotated to ERBFs, six were at least 2.5-fold down-regulated, three were showing constitutive levels, and only one UniTag was 4.0-fold up-regulated (STCa-1631, Q75UJ4_CUCME; **Table 4-6**). The transcription profiles of ERBFs indicate, that in spite of the up-regulation of one transcript variant, ethylene-related transcription regulation is not highly involved in salt stress-induced responses in chickpea roots.

4.4.3.6 WRKY transcription factors

In plants, TFs of the WRKY family probably play important roles in responses to biotic and abiotic stresses, and during development (Wu et al., 2005). Also, this TF class own integrator roles in signaling between ABA-mediated pathways and ABA-independent pathways triggered by abiotic stresses (Zou et al., 2004). The expression levels of UniTags annotated to WRKY transcription factors in salt-stressed chickpea roots agree well with their expected dynamics. Out of seven UniTags, three were at least 2.5-fold up-regulated, whereas three presented constitutive levels. UniTag STCa-11618 (Q2PJR6_SOYBN) revealed the highest up-regulation (6.5-fold; **Table 4-6**).

4.4.3.7 DREB transcription factors

Dehydration-responsive element binding (DREB) TFs are switchboards for ABA-independent signal transduction responses in plants under drought, heat, and salt stress (Liu et al., 1998; Nakashima et al., 2000). As reported elsewhere (Kasuga et al., 1999; Seki et al., 2002), DREB-encoding genes may react with increased transcription upon salt stress. In salt-stressed chickpea roots, out of a total of 7 UniTags annotated to DREBs, UniTags STCa-10794 (Q75UJ6_CUCME, 7.0-fold), STCa-4170 (Q7Y0Y9_SOYBN, 2.5-fold), and -13360 (Q0H2C5_CICAR, 2.5-fold) were at least slightly up-regulated after salt treatment. However,

in contrast to the expected induction, one of the transcript variants was also slightly down-regulated (STCa-16074, 2.5-fold; **Table 4-6**). In spite of its high resolution, SuperSAGE can unfortunately not differentiate between the subclasses DREB1 and DREB2. From earlier reports it is known, that DREB2 is dehydration-responsive (salt and water stress), whereas DREB1 is principally induced by low temperatures (Liu et al., 1998), which may explain the different expression levels observed in salt-stressed chickpea roots. However, this hypothesis needs direct sequencing confirmation.

4.4.3.8 Heat shock factors

Heat shock factors (HsFs) belong to a TF class, that is highly involved in stress responses (Schulz-Raffelt et al., 2007). As an example, transcription profiles in early salt-tress stages of tomato revealed, that members of the HsF family were salt-induced (Ouyang et al., 2007). Also, in non-plant systems like the fungus *Candida tropicalis*, optimal halotolerance can be achieved by transforming yeast strains with a fragment highly homologous to an HsF (Ali et al., 2001). In chickpea, from six UniTags annotated to HsFs, three were slightly up-regulated (STCa-7994, HSF2_ARATH; STCa-1571, Q9M597_MEDSA; STCa-9159, Q43457_SOYBN; 2.5-fold) whereas the remaining three retained their constitutive levels.

4.4.4 Ion homeostasis and the salt overly sensitive pathway in chickpea

The cascade of events, known as salt overly sensitive (SOS), has been discovered and characterized in detail through knock-out mutant analysis in *Arabidopsis* (Gong et al., 2001; Zhu, 2002). Further on, this pathway has also been found in other plants, like rice (Martinez-Atienza et al., 2007). The starting component of this pathway is known as SOS3, a CBL protein, that acts as Ca^{+2}-sensor (Sanchez-Barrena et al., 2004). In turn, SOS2, a CBL-interacting protein kinase (CIPK) , forms an SOS2-SOS3 complex (Liu et al., 2000), which either directly activates SOS1 (an Na+/H+ antiporter), or indirectly a series of other ion channel proteins. SOS2 also interacts with several other CBL-family members, thereby exerting integrator roles between different signaling cascades.

In the present results, the accumulated evidence suggests, that the SOS pathway is at least partly conserved in chickpea roots (**Figure 4-8**). However, the available information does not allow differentiating between members of each gene class.

4.4.4.1 SOS3/CBLs

In 25 mM NaCl-treated INRAT-93 roots, five CBL-annotated UniTags were detected with different regulation levels (**Figure 4-8**). Interestingly, one CBL UniTag was 25-fold down-regulated (STCa-18807, Q5ZAG5_ORYSA), whereas another one (STCa-2642, Q8L7F6_PEA) was maximally 2.4-fold up-regulated. Although this result seems to contradict logical expectations, there is evidence that the regulation of SOS3 takes place at post-translational (phosphorylation and de-phosphorylation) rather than transcriptional level (Gong et al., 2004). The present results indicate that, although CBLs play a major role in stress signaling, they are not highly stress-induced at transcriptional level in salt-stressed chickpea roots.

4.4.4.2 SOS2/CIPKs (CBL-interacting protein kinases)

As already observed with CBLs, several UniTags annotated to CIPKs were detected in the INRAT-93 dataset. From six UniTags, two were 2.5-fold and 4.0-fold up-regulated, respectively (STCa-19111 and STCa-19209; Q6X0M7_SOYBN), whereas the remaining four kept constitutive levels (**Figure 4-8**). The same principle valid for SOS3-like proteins is also valid for CIPKs within this pathway: the activity of these kinases is rather controlled by the inactivation of its auto-inhibition domain than by its rate of expression (Gong et al., 2004).

4.4.4.3 SOS1 (Na+/H+ exchangers) and diverse proton pump ATPases

In the screened chickpea root dataset, no UniTags with homologies to ESTs encoding plasma-membrane Na+/H+ exchangers (SOS1) were detected. However, as reported by Batelli and co-authors (2007), SOS2 can also interact with the regulatory sub-units of vacuolar H^+-ATPases (V-ATPases), and this interaction is enhanced by salt stress. In chickpea roots, 2 hours after treatment with 25 mM NaCl, four of the nine UniTags annotated to V-ATPases were at least 2.5-fold up-regulated (STCa-19649, STCa-13394, STCa-8702, and STCa-9760) (**Figure 4-8**).

4.4.4.4 AAA-type ATPases, SOS-independent ion channels

In parallel to proteins controlled by the SOS pathway, AAA-type ATPases also play a role in salt-stress tolerance through their participation in the ER-Golgi mediated compartmentalization of excess of Na^+ ions under high salinity (Jou et al., 2006). In chickpea, ten UniTags were annotated to AAA-type ATPases, but only one of these is 2.7-fold up-regulated (STCa-3185, Q9SEA8_MESCR), which is taken as evidence, that no enhanced

transcript accumulation encoding this type of protein is activated in salt-stressed chickpea roots (**Figure 4-8**).

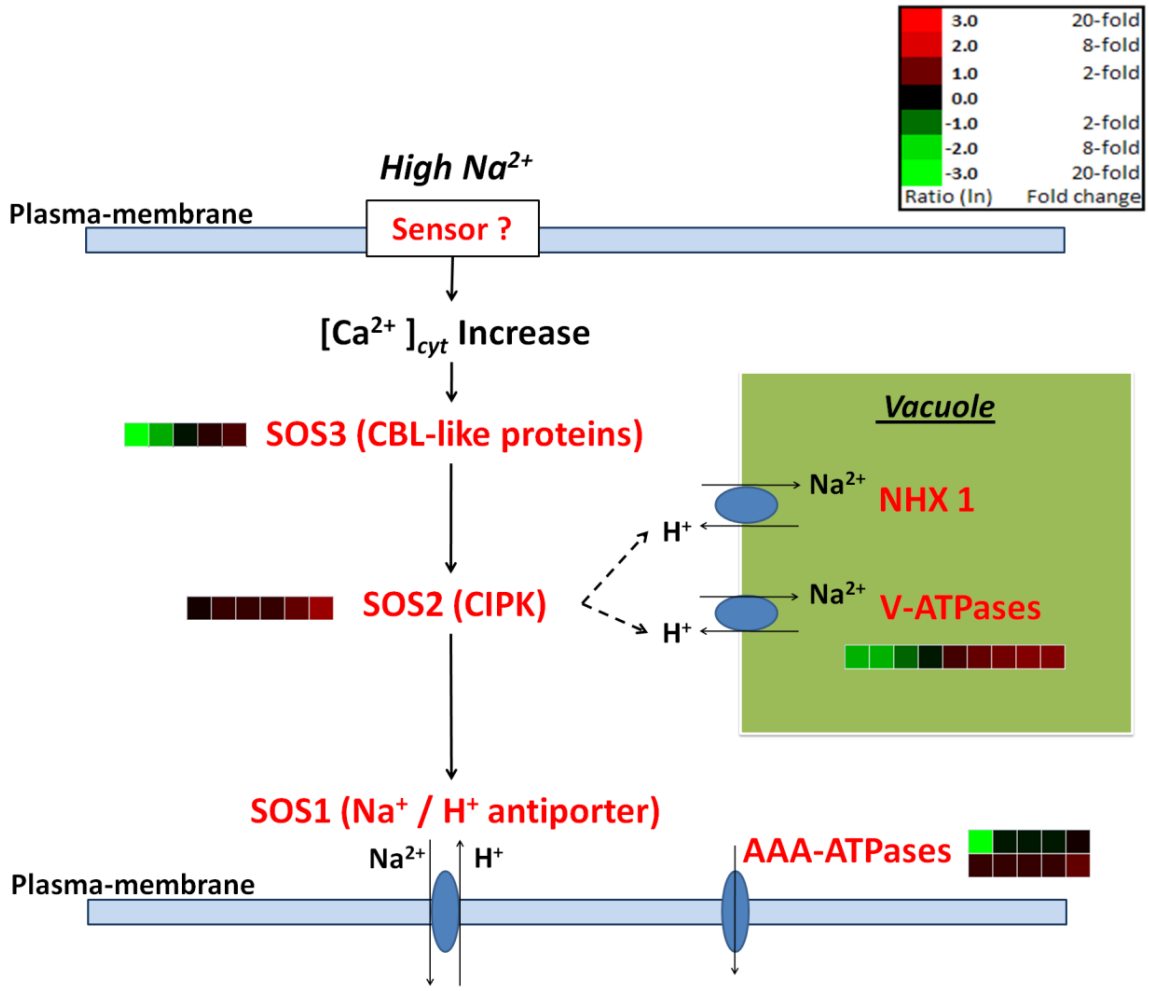


Figure 4-8 **Representation of the SOS pathway and transcription profiles of related UniTags from salt-stressed chickpea roots**

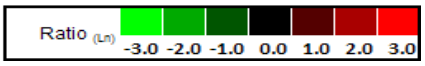
An increase in intracellular Na^+ concentration causes Ca^{2+} transient signals sensed by SOS3, a calcineurin-binding protein (CBL). In turn, SOS3 binds to SOS2, a CBL-interacting protein kinase (CIPK), interrupting its auto-inhibition. The complex SOS2-SOS3 directly activates a plasma-membrane Na^+/H^+ exchanger (SOS1), and indirectly some vacuolar ATPases, which function as proton pumps.

4.4.5 UniTags annotated to proteins active in compatible osmolyte accumulation

Beyond the broad repertoire of signaling events triggered by salt stress, the accumulation of compatible osmolytes to cope with the osmotic disequilibrium is one of the most widespread strategies of plants to enhance their tolerance to high salt concentrations (Seki et al., 2007). Several genes involved in biosynthesis, transport as well as intermediary and catabolic pathways related to this strategy have genetically and functionally been characterized over the past years (Garg et al., 2002; Capell et al., 2004; Urano et al., 2004). In INRAT-93 roots, at least 13 genes (transcripts), related to osmolyte accumulation altered their expression after two hours of 25 mM NaCl-treatment (**Table 4-7**).

Table 4-7 UniTags related to compatiple osmolyte accumulation in salt-stressed chickpea roots

		Number of UniTags
Osmolyte class	*Protein*	*1 5 10*
Sugars	Sugar transporter	
Sugars	Trehalose 6-phosphate synthase	
Sugars	Trehalose-6-phosphate phosphatase	
Sugars	Galactinol synthase	
Sugars	Sucrose transport proteins	
Sugars	Sucrose synthase	
Sugars	Sucrose phosphate synthase	
Amino acids	Betaine/proline transporter	
Amino acids (repressor)	Proline dehydrogenase	
Amino acids	Betaine aldehyde dehydrogenase	
Polyamines	S-adenosylmethionine decarboxylase	
Polyamines	Arginine decarboxylase	
Polyamines	Spermidine synthase 1	



4.4.5.1 The accumulation of sugars as osmotic equilibrators

Among the sugars serving as compatible osmolytes, trehalose plays an eminent role in salt-stressed plants (Garg et al., 2002; Avonce et al., 2004). In crops like rice, increasing the levels of trehalose by genetic engineering improves salt tolerance (Penna, 2003). In salt-stressed chickpea roots, three UniTags annotated to trehalose 6-phosphate synthase were detected. Two of these were about 2.7-fold up-regulated (STCa-18759, Q9LRA7_ARATH; STCa-8231, O23617_ARATH), and one was markedly down-regulated (STCa-18758, Q9LRA7_ARATH; 10-fold down-regulation; **Table 4-7**).

Galactinol synthase plays a positive role in osmotic stress tolerance, because it catalyzes the accumulation of raffinose oligosaccharides (Taji et al., 2002). However, no significant changes in the expression of two UniTags annotated to this protein were detected in salt-stressed chickpea roots.

In addition to its role as compatible osmolyte, sucrose has also signaling properties in plants. Therefore, it can be expected, that salt stress will increase its levels (Price et al., 2004). From four UniTags annotated to sucrose synthase, two showed constitutive expression levels, whereas two others were at least 4.5-fold up-regulated (STCa-20450, Q9XG55_LOTJA; STCa-1110, SUSY_PHAAU). Sucrose transport proteins were represented by a single transcript, that was 3.0-fold up-regulated (STCa-854, Q9SXU7_CICAR; **Table 4-7**).

Taken together, evidence is emerging that sugar metabolism and transport positively react upon the onset of salt stress in chickpea INRAT-93 roots.

4.4.5.2 Accumulation of amino acids

Apart from sugars, amino acids like proline also serve as compatible osmolytes and accumulate in plants under salt/water stress (Verslues and Bray, 2006; Wang et al., 2007). In chickpea roots, neither UniTags coding for proteins involved in proline accumulation (Betaine/proline transporter, Q8LP44_AVIMR, STCa-24308) nor the repressor proline dehydrogenase (Q6JA03_MEDSA, STCa-8454, STCa-19711) significantly changed their expression levels 2 hours after salt stress. Additionally, and opposite to previous reports on the accumulation of other osmolyte amino-acids under osmotic stress (McCue and Hanson, 1992), the expression level of the single UniTag annotated to betaine aldehyde dehydrogenase, a key biosynthetic enzyme for glycine betaine (Vojtechova et al., 1997), indicated transcription repression (STCa-14752, Q6S9W9_GOSHI; 6-fold down-regulation).

In summary, there is no evidence for strong transcriptional alterations in amino acid accumulation-related genes as a positive response to salt stress in chickpea roots.

4.4.5.3 Accumulation of poly-amides

The accumulation of the poly-amides spermidine and putrescine has been suggested as mechanism of defense against osmotic stress (Trung-Nghia et al., 2003; Capell et al., 2004). In the case of chickpea roots, the transcription profiles of genes encoding key enzymes for polyamide accumulation show differential expression upon salt stress. A single transcript variant annotated to arginine decarboxylase revealed 6.0-fold up-regulation (STCa-8875, SPE1_PEA). Further on, one UniTag out of four transcripts annotated to S-

adenosylmethionine decarboxylase, a key enzyme in spermidine biosynthesis (Thu-Hang et al., 2002), was 11-fold up-regulated (STCa-23965; Q8LKJ7_9ROSI). Additionally, one single UniTag annotated to spermidine-synthase (STCa-612; SPD1_PEA) kept constitutive levels (**Tables 4-7**).

The present SuperSAGE profiles support the view, that the accumulation of some transcripts encoding proteins involved in poly-amides accumulation is a positive response of chickpea roots in the early stages of salt stress.

5 The transcriptome of chickpea nodules and its response to salt stress

5.1 Differential gene expression in nodules as compared to non-stressed roots

In order to identify UniTags with positive differential expression in non-stressed nodules as compared to roots, INRAT-93 libraries from both unstressed organs (harvested from the same plants) were directly compared. A total of 51,545 tags from both libraries represented 11,525 different UniTags. From these, 7,941 showed less than 3.0-fold differential expression between both organs. Being more prevalent in nodules, 2,098 UniTags showed more than 3.0-fold differential expression. With a higher threshold, 140 transcripts were more than 8.0-fold prevalent in the symbiotic organs (**Figure 5-1**).

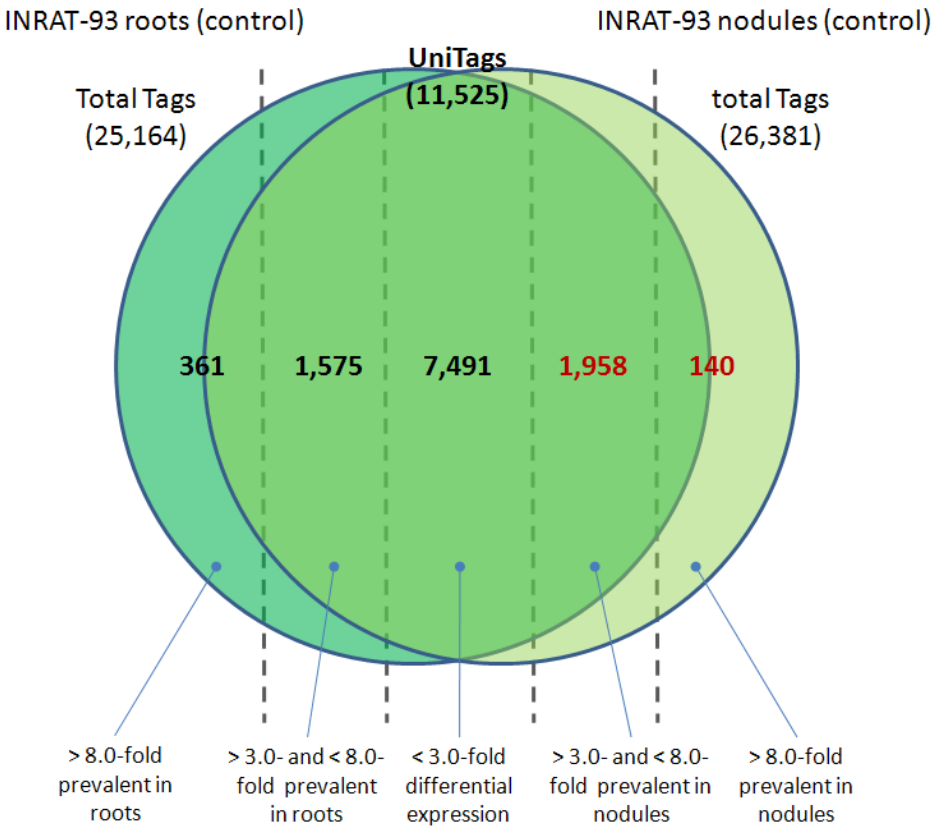


Figure 5-1 Venn diagram representing some features of libraries developed from non-stressed nodules and non-stressed chickpea roots, respectively

In order to extract the nodule-specific component (right part) out of the INRAT-93 UniTag population, both root and nodule SuperSAGE libraries were directly compared using the Discoveryspace 4.0 software. A total of 2,098 UniTags wereat least 3.0-times more prevalent in nodules. From these, 140 transcripts were more than 8.0-times more prevalent in nodules.

Top differentially expressed UniTags in unstressed INRAT-93 nodules

The 40 most differentially expressed (annotated) transcripts in nodules as a component of the chickpea root system are shown in **Table 5-1**. In the following subsections, UniTags from the top of the list will be approached providing literature background information.

5.1.1.1 Class I chitinase

The most differentially expressed UniTag (25-fold) in INRAT-93 non-stressed nodules was annotated to a class I chitinase (STCa-23843, Q76KW5_PEA; **Table 5-1**). In a survey of nodulation-induced gene expression from different members of the chitinase family in *M. truncatula*, different chitinase classes with specific regulation patterns were detected. Compared with pathogen-induced expression, several chitinase isoforms appeared to be induced by either pathogens or symbiosis (Salzer et al., 2004). In non-stressed nodules of chickpea, the induction of class I chitinase may therefore be related to functions affecting the symbiotic program, e.g. the cleavage of exceeding Nod factors (Perret et al., 2000), modification of extra-cellular proteins (N-acetylglucosamine-containing arabinogalactan proteins; (van Hengel et al., 2001), and even signaling events (Kim et al., 2000; Kim et al., 2003)

5.1.1.2 Superoxide dismutase, glutathione S-transferase, and metallothionein-2

Three of the four most differentially expressed UniTags in nodules as compared to roots are involved in ROS detoxification processes, represented by transcripts coding for SOD (STCa-7896, Q9ZNQ4_CICAR), GST (STCa-22470, Q948X4_MEDSA), and one metal metallothionein-like MT2 protein (STCa-3424, MT2_CICAR; **Table 5-1**). In legume nodules, mitochondria, one of the main ROS producers in plants, are organelles abundant in the *Rhizobium*-infected region (Becana et al., 2000). In this context, the observed results are in complete congruence with previous works, like the report of Iturbe-Ormaetxe and co-authors (2001), in which very high activities of SOD and other enzymes involved in the GSH-ascorbate cycle were observed in mitochondria from legume nodules.

Another challenge for an active antioxidant machinery in nodules is the high concentration of iron ions (heme and non-heme), which in turn catalyze the formation of hydroxyl radicals from H_2O_2 (Gechev et al., 2006). The relatively high expression of one MT2-annotated UniTag in chickpea nodules could therefore be interpreted as a stress response

supporting the sequestration of metal ions for prevention of the Fenton reaction (protonation of H_2O_2 to $HO^{\cdot}$).

5.1.1.3 Protein phosphatase 2A and other signaling proteins

Protein phosphatases (PPs), represented by UniTag (STCa-21852, Q8L5L1_MUSAC), are involved in a broad array of events during plant signal transduction (Smith and Walker, 1996). As an example in legume nodules, the activity of nodulin-26 proteins, the most abundant PMB water channel, relies on phosphorylation and de-phosphorylation events (Guenther et al., 2003). Aditionally, other differentially expressed UniTags annotated to signaling proteins like TGF-beta receptor-interacting protein-1 (STCa-21330, Q94KS2_PHAVU), signal recognition particle receptor-like proteins (STCa-24180, Q9M0A0_ARATH), Cytokinin-regulated kinase 1 (STCa-23601, Q9FUK3_TOBAC), and GTP-binding protein (STCa-3643, Q9FSZ5_CICAR) (**Table 5-1**), agree well with their active participation in signaling events in the legumes symbiosome (Van de Velde et al., 2006; Oldroyd and Downie, 2008).

5.1.1.4 Protein-transport protein Sec61

Transport proteins of the type Sec61, represented by UniTag STCa-21953 (SC61G_ORYSJ; **Table 5-1**), are membrane-bound proteins which are present in high copy numbers in *M. truncatula* nodules (Gyorgyey et al., 2000). This type of proteins is linked to peptide translocations across the peri-bacteroid membrane, one of the most important SNF-related processes. Therefore, this result can be interpreted as a confirmation of the transcriptional induction of SNF-related genes in chickpea nodules.

5.1.1.5 ADP-ribosylation factor

Also belonging to the protein transporter category, UniTag STCa-21691, annotated to an ADP-ribosylation factor, was more prevalent in INRAT-93 nodules than in roots (**Table 5-1**). Massive EST-sequencing in *M. truncatula* detected transcripts coding for ADP-ribosylation factors with high expression levels in young nodules (Journet et al., 2002). However, a considerable gap still exists about the putative transport targets for these classes of proteins. The SuperSAGE results observed in INRAT-93 confirm the observations of Journet and co-authors (2002).

Table 5-1 Top 40 annotable UniTags differentially expressed between non-stressed nodules and non-stressed roots of chickpea variety INRAT-93

Tag code	Protein	$R_{(ln)}$	Associated process	Uniprot ID
STCa-23843	Class I chitinase	3.21	Cell wall catabolism/defense	Q76KW5_PEA
STCa-7896	Superoxide dismutase	2.94	ROS scavenging	Q9ZNQ4_CICAR
STCa-22470	Glutathione S-transferase	2.94	ROS scavenging	Q948X4_MEDSA
STCa-21852	Protein phosphatase 2A	2.94	Signal transduction	Q8L5L1_MUSAC
STCa-3424	Metallothionein-like protein 2	2.94	Metal ion binding	MT2_CICAR
STCa-21953	Protein-transport protein SEC61	2.84	Protein transport	SC61G_ORYSJ
STCa-21691	ADP-ribosylation factor	2.72	Protein transport and sorting	ARFG3_HUMAN
STCa-19859	Translationally controlled tumor-like protein	2.72	No associated process	Q8LPE3_CICAR
STCa-16288	F6A14.17 protein	2.72	No associated process	Q9M9U3_ARATH
STCa-22330	Beta-amylase	2.59	Metabolism (carbohydrates)	Q9LIR6_ARATH
STCa-21330	TGF-beta receptor-interacting protein 1	2.59	Signal transduction	Q94KS2_PHAVU
STCa-24180	Signal recognition particle receptor-L protein	2.51	Signal transduction	Q9M0A0_ARATH
STCa-8706	Histone H2B	2.43	Chromosome organization	H2B_GOSHI
STCa-15890	Formin binding protein 3-like	2.43	No associated process	Q5JM35_ORYSJ
STCa-3014	F17O7.4 protein	2.43	No associated process	O64594_ARATH
STCa-11675	F22C12.5 protein	2.43	Redox activity	Q9SH69_ARATH
STCa-10302	Poly(A)-binding protein	2.43	Regulation of translation	Q9M6E6_TOBAC
STCa-23601	Cytokinin-regulated kinase 1	2.43	Signal transduction	Q9FUK3_TOBAC
STCa-3289	WRKY DNA-binding protein 11	2.35	Regulation of transcription	WRK11_ARATH
STCa-23197	Hypothetical protein	2.25	Response to biotic stimulus	Q9LEN3_CICAR
STCa-14984	40S ribosomal protein S4	2.25	Protein biosynthesis	RS4_PRUAR
STCa-10877	Histone H2A variant 1	2.25	Chromosome organization	H2AV1_ARATH
STCa-22803	26S proteasome p55-like protein	2.25	Protein turnover	Q9FIB6_ARATH
STCa-19290	Tonoplast intrinsic protein	2.25	Transport (trans-membrane)	Q8L5G0_CICAR
STCa-23637	Dof zinc finger protein	2.25	Regulation of transcription	Q9SXG8_ORYSA
STCa-11694	ADR226Cp protein	2.25	No associated process	Q759P7_ASHGO
STCa-3643	GTP-binding protein	2.25	Signal transduction	Q9FSZ5_CICAR
STCa-1179	AAF34232.1 protein	2.25	No associated process	Q9FH30_ARATH
STCa-16556	Serine carboxypeptidase-like protein	2.25	Protein turnover	CBPX_PEA
STCa-1381	Acetyl-CoA synthetase	2.25	Metabolism	Q9ZR69_SOLTU
STCa-23164	At2g21045 protein	2.25	No associated process	Q7Y234_ARATH
STCa-2426	Pleiotropic drug resistance protein 3	2.255	Transport (trans-membrane)	PDR3_TOBAC
STCa-22897	Ribosomal protein L37	2.25	Protein biosynthesis	Q6SPR2_SOYBN
STCa-858	Histone H2B	2.25	Chromosome organization	Q9M3H6_CICAR
STCa-4858	F7G19.18 protein	2.25	No associated process	O04035_ARATH
STCa-13085	Mono-lipoyl E2	2.25	No associated process	Q94IP5_ARATH
STCa-21949	Thiolprotease	2.25	Protein turnover	Q41064_PEA
STCa-5681	Hydroxyproline-rich glycoprotein	2.25	Cell wall organization	Q39865_SOYBN
STCa-12663	Orcinol O-methyltransferase	2.15	Lignin biosynthesis	Q8L5K8_ROSHC
STCa-20215	Putative extracellular dermal glycoprotein	2.03	Protein turnover	Q9FSZ9_CICAR
STCa-4531	Isoflavone 3'-hydroxylase	2.03	No associated process	Q2ENF7_ASTME

5.1.1.6 Translationally controlled tumor-like protein

In chickpea, UniTag STCa-19859, annotated to a translationally controlled tumor like protein (Q8LPE3_CICAR), was at least 15-fold more observed in nodules than in roots (**Table 5-1**). In other legumes such as *Glycine max* (soybean), Unigene clusters for translationally controlled tumor-like proteins are also reported among ESTs frequently encountered in nodules (http://www.ncbi.nlm.nih.gov/UniGene/ library.cgi). However, up to now no physiological function for this protein is known.

5.1.1.7 Beta-amylase

High expression levels of transcripts encoding enzymes involved in starch metabolism like beta-amylases were already reported for root systems of legumes. In alfalfa (*Medicago sativa*), transcripts coding for this multigene family are linked to storage processes (Gana et al., 1998). Beta-amylase catalyzes the hydrolysis of starch molecules releasing maltose and producing beta-limit dextrin. Nodules in general contain a considerable content of sucrose, maltose, and alpha-trehalose (Streeter, 1982), which may explain the high expression levels of UniTag STCa-22330 (Q9LIR6_ARATH;**Table 5-1**) in chickpea.

5.1.1.8 Uncharacterized proteins or genes not directly linked with symbiotic N_2-fixation

In general, a considerable part of most differentially expressed UniTags in non-stressed chickpea nodules versus roots can be linked to information derived from previous studies. Nonetheless, a substantial part still remains fully uncharacterized (i.e. F6A14.17-F17O7.4-, F22C12.5-, ADR226Cp-, AAF34232.1, At2g21045, F7G19-proteins).

Also, expression profiles of transcripts annotated to fully characterized proteins, whose function is still not totally clear in the SNF context, represent a gap to be filled. As examples in chickpea nodules, UniTags annotated to histones H2B (STCa-8706, H2B_GOSHI), H2A (STCa-10877, H2AV1_ARATH), and to several proteins linked to general metabolic processes, showed high nodule- versus root differential expression.

5.1.1.9 Non annotable UniTags

Although many of the differentially expressed UniTags were annotated, a large portion of transcripts remains to be linked to any characterized EST. Observed from another point of view, the possibilities of finding new genes (or concretely, new transcript isoforms)

playing important roles in SNF are still open. Sequences and fold-changes of the most nodules-differentially-expressed anonymous UniTags are listed in **Table 5-2**.

Table 5-2 Top 30 differentially expressed non-annotable UniTags in non-stressed nodules of INRAT-93 in relation to non-stressed roots

Tag code	Sequence	$R_{(ln)}$	Differential expression (fold)
STCa-21738	CATGTGTGAGGCATTGTCATTTTATG	3.13	22.90
STCa-21993	CATGTGTTCGTATTAATGATTTATGA	3.00	20.03
STCa-3959	CATGAGTGTTTGGTCTTTAGGCTTCG	2.95	19.09
STCa-21798	CATGTGTGCGATGGCTTAATTATTGT	2.84	17.17
STCa-20692	CATGTGATGTTGGAAGTGATGAAAAT	2.84	17.17
STCa-20954	CATGTGCTTGGATCATAATGTTCTAC	2.84	17.17
STCa-21947	CATGTGTTACCTCGTTACCTGTAGGT	2.84	17.17
STCa-22456	CATGTTAGGTTGTGTGTGATGAAGAG	2.73	15.26
STCa-7875	CATGCATCGATGGTGAGCTCACTGTT	2.73	15.26
STCa-21727	CATGTGTGACCTGTATATTTGTGTTG	2.73	15.26
STCa-21389	CATGTGGTGTTGTATGAAATTGAGGT	2.73	15.26
STCa-22483	CATGTTAGTTGTTCTCCTTGTATGGT	2.73	15.26
STCa-16588	CATGGTTCTGCTTAGTGTGAACCAGC	2.73	15.26
STCa-23655	CATGTTGTGTGGTGTTTAGGGATGGG	2.73	15.26
STCa-13408	CATGGCTCAGTCATCCGCAGAAGATG	2.73	15.26
STCa-24108	CATGTTTGGAGAGTGACTTACCAATT	2.73	15.26
STCa-23420	CATGTTGGCAATTATGGCACCATTAT	2.73	15.26
STCa-7844	CATGCATCACCATCAACAGCCTGAAC	2.73	15.26
STCa-22932	CATGTTCTAAGCATACACACTACCTA	2.59	13.36
STCa-3458	CATGAGCTTTGGAGTGCTGAGTTTTG	2.59	13.36
STCa-4258	CATGATACTCAACACGTTCTCGTCGA	2.59	13.36
STCa-7858	CATGCATCATATAGCGGATGATCATA	2.59	13.36
STCa-22977	CATGTTCTCTCAACATAATGTGTACT	2.59	13.36
STCa-1516	CATGAAGGTTATGTGAATTGTGGTTA	2.59	13.36
STCa-5989	CATGATTTGGTCTGGTGATCGTGTGC	2.59	13.36
STCa-8401	CATGCCAGTTCTGTTGCTGTGGTATT	2.59	13.36
STCa-23413	CATGTTGGATTAGTGGAAAGGTGTCT	2.59	13.36
STCa-24026	CATGTTTGAGCACATATTGCACCGAT	2.59	13.36
STCa-23142	CATGTTGACAGGTTGATAAGAGTAAT	2.59	13.36
STCa-19499	CATGTCAATGTCAGTTTCACTGTGTT	2.59	13.36

5.1.2 Correlation of SuperSAGE profiles with GO categories in non-stressed chickpea nodules versus roots

Gene score re-sampling analysis (GSR) for over-representation of GO functional categories in correlation with the differential expression of UniTags between non-stressed roots and nodules (organ-wise comparison) was carried out with the Ermine 2.0 software.

As expected from an active symbiotic interchange between host-cell and bacteroid, the present analysis indicates over-representation of several membrane-related GO biological processes and cellular components, corroborating previous proteomic findings in nodules (Wienkoop and Saalbach, 2003). Also, processes involving metabolism and active transport were over-represented as reported by Udvardi and Day (1997).

5.1.2.1 Most over-represented GO biological processes in non-stressed nodules

The thirty most over-represented GO biological processes in INRAT-93 nodules are listed in **Table 5-3A**. Biological processes like Macromolecule biosynthetic process (GO:0009059), Cellular component organization and biogenesis (GO:0016043), Intracellular protein transport (GO:0006886), Catabolic process (GO:0009056), Translation (GO:0006412), Response to stimulus (GO:0050896), Generation of precursor metabolites and energy (GO:0006091), Cellular carbohydrate catabolic process (GO:0044275), and Electron transport (GO:0006118) were over-represented with P values lower than 1.0e-12. Going further down on the list, at least eleven processes were linked either to metabolism or catabolism, five biological processes either to stimulus responses (oxidative stress, chemical stimulus, stress), and four further processes to transport.

5.1.2.2 Most over-represented GO cellular components in non-stressed nodules

The same analysis was carried out for over-representation of GO cell component categories. As a result, terms like Intrinsic to membrane (GO:0031224), Nucleus (GO:0031224), Membrane part (GO:0044425), Ribonucleoprotein complex (GO:0030529), Integral to membrane (GO:0016021), Intracellular non-membrane-bound organelle (GO:0043232), Protein complex (GO:0043234), Outer membrane (GO:0019867), and Ribosome (GO:0005840); exhibited highest over representation ($P<1.0E-12$). In general, from the twenty listed categories, at least eight GO cellular components were linked to envelopes or membranes, three categories to mitochondria, and three categories to protein and ribosome complex (**Table 5-3B**).

Table 5-3A Over-represented GO biological processes in INRAT-93 nodules versus roots from the same plants

GO term	GO Biological process	Rank	P
GO:0009059	Macromolecule biosynthetic process	1	1.00E-12
GO:0016043	Cellular component organization and biogenesis	2	1.00E-12
GO:0006886	Intracellular protein transport	3	1.00E-12
GO:0009056	Catabolic process	4	1.00E-12
GO:0006412	Translation	5	1.00E-12
GO:0050896	Response to stimulus	6	1.00E-12
GO:0006091	Generation of precursor metabolites and energy	7	1.00E-12
GO:0044275	Cellular carbohydrate catabolic process	8	1.00E-12
GO:0006118	Electron transport	9	1.00E-12
GO:0044248	Cellular catabolic process	10	0.0001
GO:0005996	Monosaccharide metabolic process	11	0.0002
GO:0006605	Protein targeting	12	0.0002
GO:0015031	Protein transport	13	0.0002
GO:0009057	Macromolecule catabolic process	14	0.0003
GO:0046365	Monosaccharide catabolic process	15	0.0003
GO:0006979	Response to oxidative stress	16	0.0007
GO:0042221	Response to chemical stimulus	17	0.0007
GO:0051641	Cellular localization	18	0.0007
GO:0044262	Cellular carbohydrate metabolic process	19	0.0008
CMC-1	ROS scavenging	20	0.0010
GO:0046907	Intracellular transport	21	0.0012
GO:0006950	Response to stress	22	0.0012
GO:0044265	Cellular macromolecule catabolic process	23	0.0012
GO:0006508	Proteolysis	24	0.0018
GO:0006519	Amino acid and derivative metabolic process	25	0.0024
GO:0006096	Glycolysis	26	0.0028
GO:0006066	Alcohol metabolic process	27	0.0037
GO:0006457	Protein folding	28	0.0045
GO:0065003	Macromolecular complex assembly	29	0.0051
GO:0045045	Secretory pathway	30	0.0058

Table 5-3B Over-represented GO cellular components in INRAT-93 nodules versus roots from the same plants

GO term	GO Cellular component	Rank	P
GO:0031224	Intrinsic to membrane	1	1.00E-12
GO:0005634	Nucleus	2	1.00E-12
GO:0044425	Membrane part	3	1.00E-12
GO:0030529	Ribonucleoprotein complex	4	1.00E-12
GO:0016021	Integral to membrane	5	1.00E-12
GO:0043232	Intracellular non-membrane-bound organelle	6	1.00E-12
GO:0043234	Protein complex	7	1.00E-12
GO:0019867	Outer membrane	8	1.00E-04
GO:0005840	Ribosome	9	1.00E-04
GO:0044422	Organelle part	10	0.0003
GO:0005783	Endoplasmic reticulum	11	0.0006
GO:0005739	Mitochondrion	12	0.0019
GO:0031090	Organelle membrane	13	0.0052
GO:0005829	Cytosol	14	0.0055
GO:0044429	Mitochondrial part	15	0.0193
GO:0005856	Cytoskeleton	16	0.0248
GO:0031975	Envelope	17	0.0299
GO:0031966	Mitochondrial membrane	18	0.0391
GO:0000786	Nucleosome	19	0.0476
GO:0012505	Endomembrane system	20	0.0522

5.2 Salt stress-induced differential gene expression of chickpea nodules

5.2.1 Top up-regulated UniTags in salt-stressed chickpea nodules

As mentioned in the previous section, in parallel to the screening of whole-transcriptome responses to salt stress in roots, nodules of the same plants were separately harvested for development of SuperSAGE libraries (control and 2h 25 mM NaCl-treatment). In contrast to chickpea roots under salt stress (346 UniTags up-, 2055 down-regulated), only 95 and 72 UniTags, respectively, revealed at least 8.0-fold up- or down-regulation. The top 40 most up-regulated transcripts in chickpea nodules after 2 hours of salt stress are listed in **Table 5-4**, and the most differentially expressed will be detailed in the following subsections.

Table 5-4 Top 40 up-regulated annotatable UniTags in salt stressed nodules.

Tag code	Protein	$R_{(ln)}$	Associated process	Uniprot ID
STCa-18884	Probable* early nodulin 40	4.11	Nodulation	NO40_SESRO
STCa-15648	24S Mitochondrial ribosomal mt-RNL gene	3.17	Translation	X55832
STCa-11090	40S ribosomal protein SA	2.73	Protein biosynthesis	RSSA_CICAR
STCa-17434	AAD20160.1 protein	2.61	No associated term	Q9FYR1_ARATH
STCa-1958	Gibberellin-stimulated protein	2.61	No associated term	Q53AN3_ORYSA
STCa-3760	Cysteine proteinase inhibitor	2.48	Inhibition of proteolysis	O04720_SOYBN
STCa-89	Drought-induced protein	2.48	Response to stress	Q941N0_9FABA
STCa-16482	40S ribosomal protein S9-2	2.48	Protein biosynthesis	RS92_ARATH
STCa-10316	NtEIG-E80 protein	2.33	No associated term	Q9FXS6_TOBAC
STCa-3321	Leghemoglobin	2.33	Oxygen transport	Q42928_MEDSA
STCa-1263	Benzoyltransferase-like protein	2.33	No associated term	Q9FLM5_ARATH
STCa-13055	Nonspecific lipid-transfer protein precursor	2.33	Transport (lipids)	NLTP_CICAR
STCa-22149	Acyl carrier protein	2.33	Lipid biosynthesis	ACPM_ARATH
STCa-10862	F6N18.8 protein	2.33	No associated term	Q9LPJ4_ARATH
STCa-21007	Two-component response regulator PRR37	2.33	Regulation of transcription	PRR37_ORYSA
STCa-4833	T13M11_21 protien	2.14	Regulation of transcription	Q94JL3_ARATH
STCa-8434	Fiber protein Fb2	2.14	No associated term	Q8GT87_GOSBA
STCa-23572	F7K24_140 protein	2.14	Signal transduction	Q8RWQ4_ARATH
STCa-7572	Protein phosphatase 2A	2.14	Signal transduction	Q9FVD5_MEDVA
STCa-1895	GDP-mannose 3,5-epimerase	2.14	Ascorbic acid biosynthesis	GME_ARATH
STCa-16007	Aquaporin PIP-type 7a	1.92	Transport (trans-membrane)	PIP2_PEA
STCa-2175	Glutathione S-transferase	1.92	ROS scavenging	GSTX6_SOYBN
STCa-12406	Coatomer subunit beta'-2	1.92	Protein transport	COB22_ARATH
STCa-12523	T23K23_9 protein	1.92	No associated term	Q8L7S4_ARATH
STCa-269	Phytochrome B	1.92	Signal transduction	PHYB_ARATH
STCa-1589	Beta-galactosidase	1.92	Metabolism (carbohydrates)	O65736_CICAR
STCa-19649	Vacoular ATPase subunit A	1.92	Ion transport	VATA_CITUN
STCa-22041	Root nodule extensin	1.92	Cell wall organization	Q94ES4_PEA
STCa-199	Nodulin-like protein	1.92	Transport (trans-membrane)	Q6NMB7_ARATH
STCa-542	Prolyl 4-hydroxylase	1.92	ROS scavenging	Q9FKX6_ARATH
STCa-13688	O-methyltransferase	1.92	Lignin biosynthesis	Q96424_GLYEC
STCa-15530	NADH ubiquinone oxidoreductase	1.92	Electron transport	Q9SP38_LUPLU
STCa-16514	NADH dehydrogenase	1.92	Electron transport	Q9FNN5_ARATH
STCa-22816	F17F16.27 protein	1.92	No associated term	Q8W589_ARATH
STCa-4167	Syringolide-induced protein	1.92	Metabolism (carbohydrates)	Q8S902_SOYBN
STCa-2241	Putative extensin	1.92	Cell wall organization	Q9FSY9_CICAR
STCa-319	Trypsin protein inhibitor 3	1.92	Inhibition of proteolysis	Q5WM51_CICAR
STCa-9781	Eukaryotic translation initiation factor 3	1.92	Protein biosynthesis	IF38_SCHPO
STCa-1461	HMG 1 protein	1.92	Regulation of transcription	Q41026_PEA
STCa-13993	F8K7.2 protein	1.92	No associated term	Q9XI18_ARATH
STCa-16201	Homeodomain protein	1.92	Regulation of transcription	Q9ZTA8_MALDO
STCa-10496	F2J10.14 protein	1.92	No associated term	Q9XJ35_ARATH
STCa-10360	Chalcone synthase	1.92	Metabolism	Q9ZRV7_CICAR

*****The annotation of UniTags STCa-18884, and STCa-15648 is presently ambiguous. However, the best homologies in all screened databases identify both UniTags as early nodulin 40 and mitochondrial 24S mt-RNL ribosomal genes, respectively.

5.2.1.1 Early nodulin 40

As detailed in the previous chapter (Section **4.2.1.1**), Unitag STCa-18884 (NO40_SESRO) also represented the most expressed annotatable transcript in chickpea roots under salt-stress. Early nodulins 40 (enod40) are proteins involved in the earliest stages of legume nodulation (Dey et al., 2004). The extremely high expression levels of enod40 transcripts observed in INRAT-93 salt-stressed nodules therefore present new clues for alternative functions of this protein. Probably, an alternative strategy of the nodules against salt stress consists of triggering further nodulation. However, the relative low E-value for the annotation of UniTag STCa-18884 leaves the possibility, that this transcript may be considered only an Enod40-similar variant, implying differences in function.

5.2.1.2 mt-RNL gene encoding the large sub-unit of mitochondrial rRNA (24S) exon1

On the annotation process, UniTag STCa-1564 was highly homologous to the cDNA TC229517 from a root hair subtracted soybean library, deposited at the TIGR *Glycine max* gene indices. Up to now, there is no annotation to any characterized legume protein for this tentative consensus (TC) sequence. However, this entry is linked to the *Neurospora crassa* mt-RNL gene by sequence homology via the TIGR-orthologs database. Due to its rare nature, the presence of UniTags annotated to the mitochondrial 24S ribosomal mt-RNL gene among the most salt-stress up-regulated transcripts could be a surprising result. Up to now, there is no information about the function of this cDNA in legumes.

5.2.1.3 40S ribosomal proteins

In chickpea nodules, UniTags STCa-11090 and STCa-16482, annotated to the 40S ribosomal proteins RSSA_CICAR and RS92_ARATH, respectively, were found among the most differentially expressed transcripts upon salt stress (**Table 5-4**). Ribosomal proteins in general, and proteins of class 40S in particular, have already been reported to be induced under water stress in roots from *Citrus limonia* (Boscariol-Camargo et al., 2007). Several previous studies indicate that the re-adjustment of the protein biosynthesis machinery is one of the initial responses after onset of salt stress in plants. Previous reports also suggest, that in nodules certain responses may also be translationally controlled, including modifications of ribosomal proteins, elongation factors, and other translation controllers (Van de Velde et al., 2006), which would be a possible logical explanation for the observed result.

5.2.1.4 Gibberellin-stimulated protein

Gibberellins are plant hormones controlling cell division and elongation, and delay of senescence in plants. Also, under environmentally adverse conditions, gibberellin-like compounds are thought to alleviate stress-triggered induction of senescence (Hubick et al., 1986). In legume nodules, various studies have spotted high levels of this plant hormone, indicating the promotion of maintenance of nodule growth as one of its possible functions (Evensen and Blevins, 1981; Dobert et al., 1992). In the context of salt-stressed chickpea nodules, up-regulation of UniTag (STCa-1958, Q53AN3_ORYSA; **Table 5-4**), annotated to a gibberellins-stimulated protein, may be taken as an indication of enhanced gibberellin activity.

5.2.1.5 Cysteine proteinase inhibitors

Induction of cysteine proteinase inhibitors is an early defense strategy in legumes, mostly in response to wounding and mechanical damage (Botella et al., 1996; Zhao et al., 1996). In soybean root nodules, the expression of cysteine proteinase inhibitors in several developmental stages (i.e. recently formed nodules and mature functional nodules) has been linked to counteraction of senescence processes (Alesandrini et al., 2003). In salt-stressed chickpea nodules, the induction of the cystein proteinase inhibitor-annotated UniTag STCa-3760 (O04720_SOYBN) may be linked to the delay or prevention of early senescence processes, which could be switched on by salt stress-induced ROS overflow.

5.2.1.6 Tobacco protein NtEIG-E80

The tobacco gene coding for protein photoassimilate-responsive protein-1 Q9FXS6_TOBAC, in chickpea represented by UniTag STCa-10316, has previously been reported to be induced under biotic stress (Takemoto et al., 2003), however, its possible function remains unknown. Up to now, the present work is the first report on the transcription of genes encoding this protein in nodules, information that can be valuable for the future assignment of its functional role.

5.2.1.7 Drought-induced proteins

The increased transcript accumulation of drought-induced proteins is known as a general response upon drought and salt stress in plants. However, much information about their role in stress management is still missing. Search for further background information deposited in public databases about putative function(s) of the legume protein

Q941N0_9FABA revealed the dehydrin domain as the most conserved feature. In general, dehydrins are proteins induced by water stress that probably have a protective effect on membranes (http://pfam.sanger.ac.uk/PF00257), which is most likely also the role they may play in salt-stressed chickpea nodules.

5.2.1.8 Leghemoglobin

Leghemoglobin proteins play one of the most important roles in nodules. As an O_2 buffer, these proteins are actively reducing the concentration of free oxygen in the cytoplasm of host cells, protecting the structural integrity and activity of the bacterial nitrogenases, the enzymes in charge of fixing N_2 into ammonium (NH_4), its organic form (Santana et al., 1998). Nodule conductance to O_2 diffusion is suggested to inhibit N_2-fixation by soil salinity in grain legumes (Aydi et al., 2004). In a study parallel to the present work, L'Taief and co-authors (2007) measured conductance levels of nodules from the salt-tolerant variety INRAT-93 in comparison to the salt-sensitive Amdoum1. Apart from fully preventing nodule formation, salinity notoriously induced a decrease in shoot and root biomass in Amdoun1 as compared to INRAT-93. Further on, O_2 uptake and nodule conductance measurements also indicated a drastic difference between both varieties (i.e. INRAT-93 had a much lower level of conductance). The high expression levels of transcripts annotated to leghemoglobin detected in the same nodules (UniTag STCa-3321, Q42928_MEDSA), support the hypothesis formulated by L'taief and co-workers. According to this hypothesis, salt tolerance of INRAT-93 may be associated with stability of O_2 uptake and nodule conductance.

5.2.1.9 Lipid-transfer proteins

The expression of genes coding for lipid transfer proteins was induced in symbiotic systems such as the *Rhizobium*-nodulated legume *Astragalus sinicus* (Chou et al., 2006). Although their function is not totally clear, these proteins are involved in membrane biogenesis, secretory pathways, and signaling in plants (Kader, 1996). In the salt-stressed nodules of chickpea, the high expression of UniTag STCa-13055 (non-specific lipid-transfer protein precursor, NLTP_CICAR), could therefore be linked to membrane re-arrangements.

5.2.1.10 UniTags annotated to uncharacterized proteins

Among the most salt up-regulated UniTags from INRAT-93 nodules, homologies to several ESTs linked to Uniprot uncharacterized proteins were found. This is exemplified by UniTags with homologies to *Arabidopsis* proteins like F6N18.8, T23K23, F17F16, F8K7, and F2J10, which were distributed in the list of the top 40 most up-regulated transcripts depicted in **Table 5-4**. Up to now, there are no reports on their functions, or involvement in abiotic stress responses.

5.2.1.11 Non-annotable UniTags

UniTags with high induction levels, but with no high homology to any ESTs in the screened databases, are listed in **Table 5-5**. Still, UniTags with very high differential expression levels remain un-annotated, like, for example, the UniTags STCa-15981 and STCa-19965, that are both more than 20-fold up-regulated. As observed in the previous section, the detection of non-annotable transcripts leaves open the possibility to find new genes, which may play relevant roles in salt-stress responses in legume nodules.

Table 5-5 Non-annotatable most salt-tress up-regulated nodule UniTags

Tag code	Sequence	$R_{(ln)}$	Differential expression (fold)
STCa-15981	CATGGTGATAGCATCCCGGCCCAGAA	3.53	34.12
STCa-19965	CATGTCGGGTGATGTCGCCAGGAATT	3.10	22.18
STCa-9450	CATGCTAAGGCTGCGGCTGTTATTCA	2.61	13.65
STCa-22299	CATGTTAATTTTAAATCCATTATTTG	2.61	13.65
STCa-2116	CATGAATTCGTTTCGACAATATAAGA	2.61	13.65
STCa-13463	CATGGCTGGAGGTTGGAACTGTCATC	2.61	13.65
STCa-5362	CATGATGTAATATGCGAAATGTTGCT	2.61	13.65
STCa-11740	CATGGAGAGTTGAGAAATTGAGAGGG	2.48	11.94
STCa-15605	CATGGTATGTACCATATAACTATAAT	2.48	11.94
STCa-5357	CATGATGTAACTCTACCCACTGTTTT	2.48	11.94
STCa-8350	CATGCCACTTAGGGTTGATATTTTCT	2.48	11.94
STCa-5037	CATGATGATGTGTTTGCTTTGTACAT	2.48	11.94
STCa-175	CATGAAAATAATTGTCTATTTAGGTG	2.33	10.24
STCa-705	CATGAAATTGTAACATTGAAATTGAG	2.33	10.24
STCa-6694	CATGCAATGGATGAAATTATAAAGTA	2.33	10.24
STCa-4478	CATGATATTAGATTTGCTTGTAATAT	2.33	10.24
STCa-6099	CATGCAAAACATCAATTAGATGCTTT	2.33	10.24
STCa-19240	CATGTATTCTAACTGGTATTTGCTAT	2.33	10.24
STCa-621	CATGAAATGCGAAGGACAATAGAGTA	2.33	10.24
STCa-7445	CATGCAGGGGGAACCCGGGGAACTGA	2.33	10.24
STCa-7855	CATGCATCAGGGATGAAGTATGGAGT	2.33	10.24
STCa-6059	CATGATTTTTGGAGAGAACTAGTTGG	2.33	10.24
STCa-18545	CATGTAGTCTCAAGCAGGGGTTGTGT	2.33	10.24
STCa-15235	CATGGGTTATTTTTGCAGTACTTTTT	2.33	10.24
STCa-20520	CATGTGAGAACACTTCTATTTTGTAA	2.33	10.24
STCa-933	CATGAACCTGCTAGGAGGCCTAGCTT	2.33	10.24
STCa-305	CATGAAACCATTACGTTTTGCAAGGC	2.33	10.24
STCa-2196	CATGAATTTGTATTTAGCTTATGTTA	2.33	10.24
STCa-7136	CATGCACTTATAGACACACAGTTTGT	2.33	10.24
STCa-11119	CATGGAATGTATTAGTGATTAGCTTT	2.33	10.24

5.3 Correlation of SuperSAGE profiles of salt-stressed nodules with GO categories

5.3.1 Most over-represented GO biological processes in salt-stressed nodules

GO biological processes like Signal transduction (GO:0007165), Ion transport (GO:0006811), Cell communication (GO:0007154), Oxidative phosphorylation (GO:0006119), Purine nucleotide metabolic process (GO:0006163), Monocarboxylic acid metabolic process (GO:0032787), Purine nucleoside triphosphate metabolic process (GO:0009144), and Regulation of metabolic process (GO:0019222) were the most over-represented biological processes ($P<0.008$) in salt-stressed chickpea nodules. Since these processes are involved in nodule functioning (Atkins, 1987), it can be assumed that symbiotic nitrogen fixation may still be active in INRAT-93 nodules 2h after 25 mM NaCl-treatment.

From the 40 most over-represented GO categories, only two processes were linked to ion transport to and to response processes, respectively. Three GO categories were linked to signal transduction, five to either transcription, translation, or post-translational modifications, and eight categories to metabolic processes (**Table 5-6A**).

Five over-represented processes were also listed under the 40 most under-represented ones (i.e. Regulation of transcription, RNA biosynthesis, Regulation of nucleic acids metabolism, Transcription, and Regulation of cellular metabolic processes, **Table 5-6B**). These findings are in agreement with results from salt stressed roots (Section **4.3.2**). This general tendency indicates that, rather than "ON-OFF" switching of whole systems upon salt stress, the chickpea's responses may rely on fine-tuning of specific metabolic pathways included in these. Adversely, the resolution of the transcriptome-wide analysis of GO categories over-representation is still poor in comparison to model organisms in which specific pathway analysis is possible (e.g. Arabidopsis; Mueller et al., 2003).

Table 5-6A Over-represented GO biological processes (P< 0.1) as deduced from transcript abundances (annotated to UniProt entries) in salt-stressed chickpea nodules.

GO ID	GO Biological process	Rank	P
GO:0007165	Signal transduction	1	1.00E-12
GO:0006811	Ion transport	2	0.0002
GO:0007154	Cell communication	3	0.0002
GO:0006119	Oxidative phosphorylation	4	0.0043
GO:0006163	Purine nucleotide metabolic process	5	0.0065
GO:0032787	Monocarboxylic acid metabolic process	6	0.0071
GO:0009144	Purine nucleoside triphosphate metabolic process	7	0.0082
GO:0019222	Regulation of metabolic process	8	0.0084
GO:0006350	Transcription	9	0.0086
GO:0045449	Regulation of transcription	10	0.0086
GO:0050789	Regulation of biological process	11	0.0086
GO:0019219	Regulation of nucleic acid metabolic process	12	0.0090
GO:0031323	Regulation of cellular metabolic process	13	0.0090
GO:0006793	Phosphorus metabolic process	14	0.0095
GO:0016310	Phosphorylation	15	0.0095
GO:0050794	Regulation of cellular process	16	0.0095
GO:0006412	Translation	17	0.0095
GO:0006091	Generation of precursor metabolites and energy	18	0.0095
GO:0009628	Response to abiotic stimulus	19	0.0127
GO:0009059	Macromolecule biosynthetic process	20	0.0129
GO:0065007	Biological regulation	21	0.0129
GO:0044249	Cellular biosynthetic process	22	0.0152
GO:0006164	Purine nucleotide biosynthetic process	23	0.0192
GO:0050896	Response to stimulus	24	0.0193
GO:0043687	Post-translational protein modification	25	0.0195
GO:0005984	Disaccharide metabolic process	26	0.0210
GO:0000160	Two-component signal transduction system (phosphorelay)	27	0.0210
GO:0032774	RNA biosynthetic process	28	0.0215
GO:0006633	Fatty acid biosynthetic process	29	0.0255
GO:0015674	Di-and tri-valent inorganic cation transport	30	0.0266

Table 5-6B **Most under-represented GO biological processes (P< 0.1) as deduced from transcript abundances (annotated to UniProt entries) in salt-stressed chickpea nodules.**

GO ID	GO Biological process	Rank	P
GO:0009100	Post-translational protein modification	1	1.00E-12
GO:0006396	Macromolecule biosynthetic process	2	1.00E-12
GO:0009057	Cellular component organization and biogenesis	3	1.00E-12
GO:0016311	Phosphorus metabolic process	4	1.00E-12
GO:0046365	Protein modification process	5	1.00E-12
GO:0044275	Catabolic process	6	1.00E-12
GO:0006730	RNA metabolic process	7	1.00E-12
GO:0008652	Translation	8	1.00E-12
GO:0006790	Biopolymer modification	9	1.00E-12
GO:0043413	Generation of precursor metabolites and energy	10	1.00E-12
GO:0006006	Protein amino acid phosphorylation	11	1.00E-12
GO:0006399	Electron transport	12	1.00E-12
GO:0009056	Regulation of biological process	13	1.00E-12
GO:0044265	Regulation of cellular process	14	1.00E-12
GO:0045449	Regulation of metabolic process	15	1.00E-12
GO:0016070	Carbohydrate metabolic process	16	1.00E-12
GO:0043285	Biological regulation	17	1.00E-12
GO:0006519	Phosphorylation	18	1.00E-12
GO:0015031	Transcription	19	0.0012
GO:0009309	Regulation of nucleic acid metabolic process	20	0.0012
GO:0006096	Regulation of transcription	21	0.0012
GO:0032774	Macromolecule catabolic process	22	0.0022
GO:0019219	Regulation of cellular metabolic process	23	0.0027
GO:0006350	Response to stimulus	24	0.0030
GO:0030163	Cellular catabolic process	25	0.0032
GO:0006767	Cellular carbohydrate metabolic process	26	0.0040
GO:0031323	RNA biosynthetic process	27	0.0046
GO:0009308	Protein transport	28	0.0046
GO:0007049	tRNA metabolic process	29	0.0050
GO:0006520	Glycoprotein metabolic process	30	0.0055

5.3.2 Most over-represented GO cellular components in salt-stressed nodules

From the thirteen GO cellular component categories (P<0.1) over-represented under salt stress in INRAT-93 nodules and listed in **Table 5-6C,** five were linked to the protein biosynthesis complex (GO:0043234, GO:0033279, GO:0030529, GO:0015935, GO:0005840), three to membrane (GO:0016021, GO:0044425, GO:0031224), two to ATPase proton pump complex (GO:0016469, GO:0045259), and one to mitochondrion (GO:0005739).

These results reflect the importance of the maintenance of membrane-related processes (e.g. metabolites exchange) in chickpea nodules, and their enhanced activity under salt stress (Day et al., 2001; Krylova et al., 2007). As also observed in roots, the protein machinery of the nodules experiments a strong re-modelling as a response to the salt treatment. The present results also agree with the abundance of mitochondria in legume symbiotic organs (Iturbe-Ormaetxe et al., 2001), and with the active involvement of ATPases as proton pumps in plants under ionic disequilibrium (Gaxiola et al., 2002).

Table 5-6C Most over-represented GO cell components (P< 0.1) as deduced from transcript abundances (annotated to UniProt entries) in salt-stressed chickpea nodules

GO ID	GO Cellular component	Rank	P
GO:0043234	Protein complex	1	1.00E-12
GO:0016021	Integral to membrane	2	1.00E-12
GO:0044425	Membrane part	3	0.0018
GO:0031224	Intrinsic to membrane	4	0.0058
GO:0033279	Ribosomal subunit	5	0.0060
GO:0044422	Organelle part	6	0.0116
GO:0030529	Ribonucleoprotein complex	7	0.0138
GO:0043232	Intracellular non-membrane-bound organelle	8	0.0143
GO:0016469	Proton-transporting two-sector ATPase complex	9	0.0169
GO:0015935	Small ribosomal subunit	10	0.0199
GO:0005840	Ribosome	11	0.0211
GO:0045259	Proton-transporting ATP synthase complex	12	0.0545
GO:0005739	Mitochondrion	13	0.0620

5.4 Two-dimensional expression analysis of chickpea roots and nodules in organ- and stress-directed manner

SuperSAGE analyses are very versatile, if one considers them in the context of *in silico* data handling. Provided there is robust support in the sampling method, more than two different SuperSAGE libraries can be compared to each other to identify differentially expressed genes, making the direct identification of gene responses to more than one variable (e.g. stress-treatment and sampled-organ) possible. In the present work, the developed SuperSAGE libraries from the salt tolerant variety INRAT-93 were compared in two dimensions, as shown in **Figure 5-2(A).**

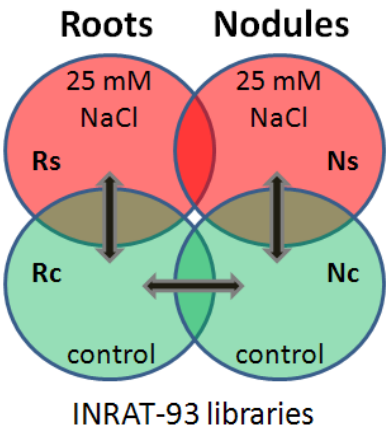
A first dimension contemplates the expression changes observed between organs of the same plant (i.e. nodules and roots). The second dimension covers the changes in both organs upon salt stress. A two dimensional analysis could be compared per analogy with a Cartesian graphic, in which each point (UniTag) has coordinates in two axes: abscissa (stress-wise) and ordinate (organ-wise; **Figure 5-2(B)**).

5.4.1 Two-dimensional cluster analysis

Cluster analysis of stress- and organ-specific expression profiles of INRAT-93 nodules and roots, respectively, was carried out via Pearson correlation distance calculation by using the Cluster 3.0 software. Two relevant clusters could be extracted from the two-dimensional analysis. Of special interest, one of them highlights UniTags that are prevalent in non-stressed nodules, and become highly expressed in roots upon salt stress only (**Figure 5-3)**.

The observed results indicate that, for a determinate set of transcripts, over-expression can be triggered by salt stress and by nodulation in chickpea. In particular, UniTags prevalent in nodules, which are only highly expressed in roots after salt treatment, may suggest that some SNF-related processes can induce a certain degree of stress. In the following section, with the help of further analyses for filtering of expression data, some of the most relevant UniTags sharing this expression pattern will be highlighted.

A



B

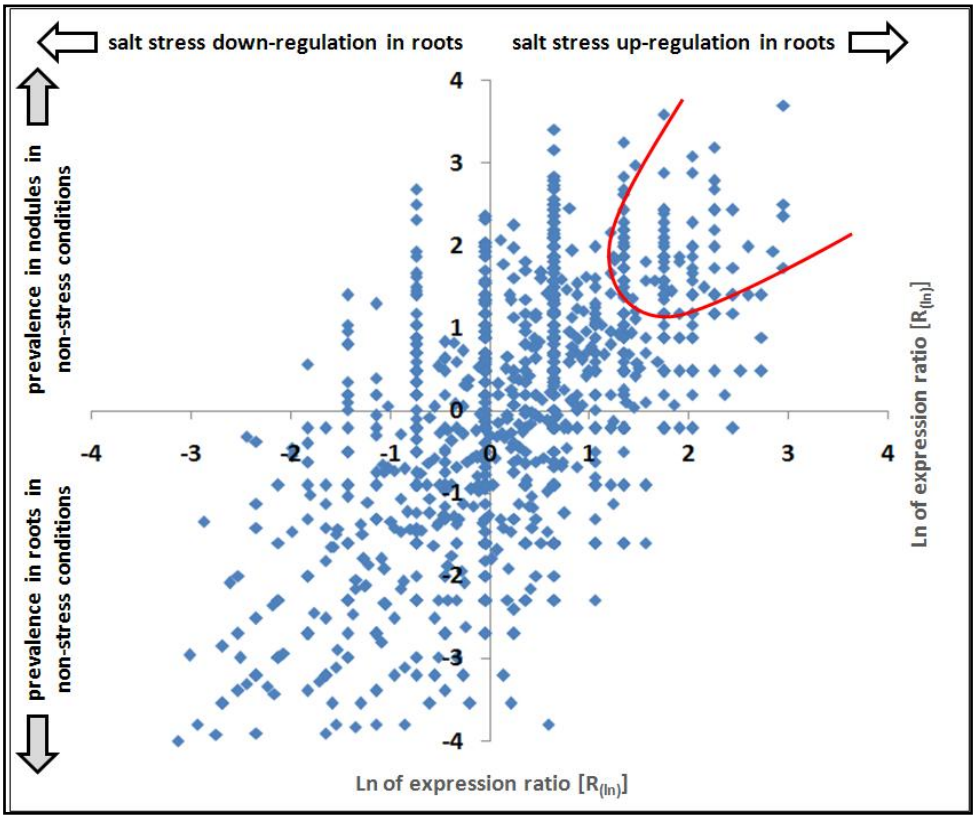
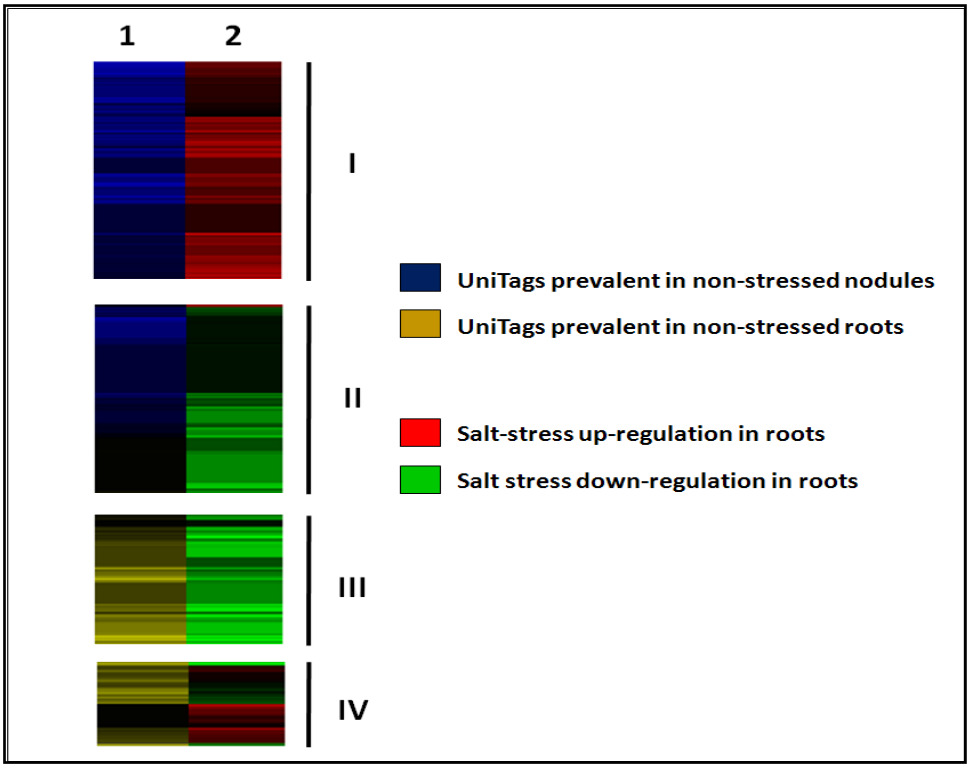


Figure 5-2 Schematic view of a direct two-dimensional comparison between SuperSAGE libraries from nodules and roots, respectively, from the salt tolerant variety INRAT-93

A) UniTag counts from nodule control libraries (**Nc**) were directly compared to UniTags from both nodules under salt stress (**Ns**), and control roots (**Rc**).

B) Scatter plot of organ-wise(ordinate) and stress-wise (abscissa) differential expression Red curve: UniTags prevalent in nodules, that are highly up-regulated in roots only upon salt stress

A


1
2
I
II
III
IV
UniTags prevalent in non-stressed nodules
UniTags prevalent in non-stressed roots
Salt-stress up-regulation in roots
Salt stress down-regulation in roots

B

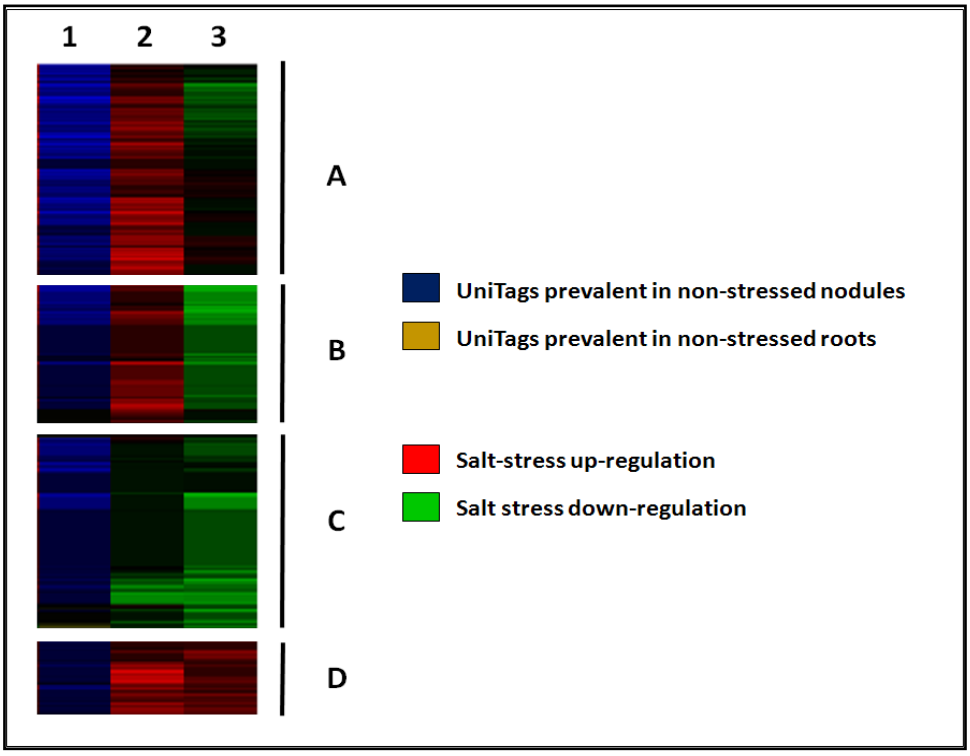

1
2
3
A
B
C
D
UniTags prevalent in non-stressed nodules
UniTags prevalent in non-stressed roots
Salt-stress up-regulation
Salt stress down-regulation

Figure 5-3 Two-dimensional expression analysis in INRAT-93 roots and nodules

A) Two-dimensional cluster analysis in INRAT-93:
1) Organ-wise comparison (yellow-blue scale), non-stressed nodules versus roots
2) Stress-wise comparison (green-red scale) for salt-treated roots of INRAT-93
   - Cluster I) UniTags prevalent in non-stressed nodules that are highly expressed in roots only after salt stress
   - Cluster II) UniTags prevalent in nodules that are down-regulated in roots after salt stress
   - Cluster III) UniTags prevalent in roots that are down-regulated after salt stress in the same organ*
   - Cluster IV) Mixed pattern containing UniTags prevalent in roots that are up-regulated after salt stress in the same organ*

   *The depicted heat map has been filtered for UniTags that are counted in both organs. Exclusive UniTags have been sorted out.

B) Two-dimensional cluster analysis in INRAT-93 (magnified section):
1) Organ-wise comparison, only UniTags prevalent in nodules (blue colour)
2) Stress-wise comparison (green-red scale) for salt-treated roots of INRAT-93
3) Stress-wise comparison (green-red scale) for salt-treated nodules of INRAT-93
   - Cluster A) UniTags prevalent in nodules, only up-regulated in roots after salt stress, and maintaining their expression levels in stressed-nodules
   - Cluster B) UniTags prevalent in nodules that show contrasting stress reactions in nodules and roots, respectively
   - Cluster C) UniTags prevalent in nodules that are down-regulated in both organs upon salt stress
   - Cluster D) UniTags prevalent in nodules showing up-regulation in roots and nodules after salt stress

5.4.2 Shared transcriptome responses during nodulation and salt-stress at different threshold levels

In combination with the output from the cluster analysis, expression profiles from **non-stressed nodules versus non-stressed roots**, and **salt-treated roots versus nodules**, were filtered using the software Venn Mapper 1.01. Three different thresholds for data selection were set by the software as follows: i) 3-fold, ii) 8-fold, and iii) 20-fold differential expression. Numbers of common up- and down-regulated UniTags for each threshold are depicted in (**Table 5-7**).

With a minimum threshold of 3-fold differential expression, from 2,098 UniTags prevalent in non-stressed nodules, 515 (24.5%) were 3-fold up-regulated in roots under salt stress. These 515 UniTags represented 23.3% of the >3-fold salt up-regulated root transcripts. On the other hand, only 10 out of 2,098 UniTags >3-fold prevalent in control nodules were more than 3-fold up-regulated in salt-stressed nodules. Between salt-stressed

roots and salt-stressed nodules, 363 UniTags were commonly more than 3-fold up-regulated (16.7% from nodules, and 16.4% from roots; **Table 5-7,** upper panel**)**. Concerning down-regulation, 1,729 out of 1,936 UniTags considered prevalent in non-stressed roots, were more than 3-fold down-regulated in roots after 2h of salt treatment. A total of 275 UniTags were commonly more than 3-fold down-regulated in both roots and nodules under salt-stress.

Rising the threshold up to 8-fold differential expression, 37 out of 140 UniTags prevalent in non-stressed nodules were more than 8-fold up-regulated in salt-stressed roots. Upon salt stress in both organs, 22 UniTags were commonly more than 8-fold up-regulated. On the other hand, no UniTags prevalent in non-stressed nodules were more than 8-fold up-regulated upon salt stress in the same organ (**Table 5-7,** mid panel). Annotatable UniTags with high prevalence in non-stressed nodules (>8-fold) and high stress-induction in roots (>8-fold) are listed in **Table 5-8**.

Four UniTags were more than 20-fold differentially expressed in non-stressed nodules versus roots. From these, no response overlap with any >20-fold salt-induced root or nodule transcript was observed. Two UniTags were at least 20-fold induced by salt stress in both organs (**Table 5-7,** lower panel). From these, UniTag STCa-18884 was the most up-regulated transcript in both organs upon salt stress (>250-fold up-regulated in roots; 60-fold up-regulated in nodules). Annotatable shared UniTags from salt stressed nodules and roots with a minimum threshold of 20-fold up-regulation in response to salt stress are listed in **Table 5-9**.

Table 5-7 **Venn Mapper output detailing shared responses (number of UniTags) between salt-stressed roots and nodules, respectively, along with non-stressed nodules in relation to roots**

3.0-fold

			25 mM NaCl-treated roots (2h)		Non-stressed nodules vs roots		25 mM NaCl-treated nodules (2h)	
			Up-regulated	Down-regulated	Nodule-prevalent	Root-prevalent	Up-regulated	Down-regulated
		Total	2'207	3'938	2'098	1'936	2'162	1'384
25 mM NaCl-treated roots (2h)	Up-regulated	2'207			**515**	6	**363**	106
	Down-regulated	3'938			24	1'729	204	275
Non-stressed nodules vs roots	Nodule-prevalent	2'098	**515**	24			**10**	901
	Root-prevalent	1'936	6	1'729			174	17
25 mM NaCl-treated nodules (2h)	Up-regulated	2'162	**363**	204	**10**	174		
	Down-regulated	1'384	106	275	901	17		

8.0-fold

			25 mM NaCl-treated roots (2h)		Non-stressed nodules vs roots		25 mM NaCl-treated nodules (2h)	
			Up-regulated	Down-regulated	Nodule-prevalent	Root-prevalent	Up-regulated	Down-regulated
		Total	337	1'962	140	361	95	72
25 mM NaCl-treated roots (2h)	Up-regulated	337			**37**		**22**	1
	Down-regulated	1'962				325	1	6
Non-stressed nodules vs roots	Nodule-prevalent	140	**37**					14
	Root-prevalent	361		325			3	
25 mM NaCl-treated nodules (2h)	Up-regulated	95	**22**	1		3		
	Down-regulated	72	1	6	14			

20.0-fold

			25 mM NaCl-treated roots (2h)		Non-stressed nodules vs roots		25 mM NaCl-treated nodules (2h)	
			Up-regulated	Down-regulated	Nodule-prevalent	Root-prevalent	Up-regulated	Down-regulated
		Total	31	281	4	36	4	1
25 mM NaCl-treated roots (2h)	Up-regulated	31					**2**	
	Down-regulated	281				32		1
Non-stressed nodules vs roots	Nodule-prevalent	4						
	Root-prevalent	36		32				
25 mM NaCl-treated nodules (2h)	Up-regulated	4	**2**					
	Down-regulated	1		1				

Table 5-8 Overlapping transcriptome responses between UniTags prevalent in non-stressed nodules and salt-treated roots in INRAT-93

Threshold in stress-treatment differential expression: $R_{(ln)}$ 2.0 (8-fold)
Threshold in organ-wise differential expression: $R_{(ln)}$ 2.0 (8-fold)
A) $R_{(ln)}$ of UniTags differential expression in control nodules vs control roots
B) $R_{(ln)}$ of UniTags expression in salt-stressed roots

		A	B		
Tag ID	**Protein**	**$R_{(ln)}$**	**$R_{(ln)}$**	**Associated process**	**Uniprot ID**
STCa-13424	Chickpea cDNA similar to Posphate-induced protein	2.3	3.3	Uncharacterized mRNA	no match
STCa-22470	Glutathione S-transferase	2.5	2.9	ROS scavenging	Q948X4_MEDSA
STCa-7896	Superoxide dismutase	3.7	2.9	ROS scavenging	Q9ZNQ4_CICAR
STCa-3424	Metallothionein-like protein 2	2.4	2.9	ROS scavenging	MT2_CICAR
STCa-9670	*Arachis stenosperma* uncharacterized cDNA	2.7	2.7	Uncharacterized mRNA	no match
STCa-8706	Histone H2B	2.4	2.4	Chromosome organization	H2B_GOSHI
STCa-23197	Hypothetical protein	2.8	2.3	No associated process	Q9LEN3_CICAR
STCa-22897	Ribosomal protein L37	2.4	2.3	Protein biosynthesis	Q6SPR2_SOYBN
STCa-14984	40S ribosomal protein S4	2.7	2.3	Protein biosynthesis	RS4_PRUAR
STCa-858	Histone H2B	2.2	2.3	Chromosome organization	Q9M3H6_CICAR
STCa-2426	Pleiotropic drug resistance protein 3	2.4	2.3	Membrane proteins	PDR3_TOBAC
STCa-1381	Acetyl-CoA synthetase	3.2	2.3	Metabolism	Q9ZR69_SOLTU

Table 5-9 Overlapping transcriptome responses of UniTags that are highly up-regulated in roots and nodules upon salt stress

Threshold in organ-wise differential expression: $R_{(ln)}$ 2.0 (8-fold)
Threshold in stress-treatment differential expression: $R_{(ln)}$ 2.0 (8-fold)
B) $R_{(ln)}$ of UniTags expression in salt-stressed roots
C) $R_{(ln)}$ of UniTags expression in salt-stressed nodules

		B	C		
Tag ID	**Protein**	**$R_{(ln)}$**	**$R_{(ln)}$**	**Associated process**	**Uniprot ID**
STCa-18884	Early nodulin 40	5.69	4.12	Nodulation	NO40_SESRO
STCa-24417	Lipoxygenase	3.19	2.50	Lipid biosynthesis	Q9M3Z5_CICAR
STCa-15648	Mitochondrial 26S ribosomal gene	3.11	3.17	Translation	no match
STCa-17434	AAD20160.1	2.93	2.61	No associated process	Q9FYR1_ARATH
STCa-1958	Gibberellin-stimulated protein	2.68	2.61	No associated process	Q53AN3_ORYSA
STCa-8434	Fiber protein Fb2	2.36	2.14	Stress response	Q8GT87_GOSBA
STCa-7166	NADP-dependent isocitrate dehydrogenase I	3.26	1.92	Metabolism	Q6R6M7_PEA
STCa-199	Nodulin-like protein	2.36	1.92	Membrane processes	Q6NMB7_ARATH
STCa-10656	Putative UDP-glycose	2.32	1.92	Metabolism	Q9M3H8_CICAR
STCa-542	Prolyl 4-hydroxylase	2.19	1.92	Protein metabolism	Q9FKX6_ARATH
STCa-15530	NADH ubiquinone oxidoreductase	2.09	1.92	Mitochondrial electron transport	Q9SP38_LUPLU
STCa-13688	O-methyltransferase	2.09	1.92	Lignin biosynthesis	Q96424_GLYEC
STCa-10316	NtEIG-E80 protein	1.99	2.33	No associated process	Q9FXS6_TOBAC

5.5 Organ- and stress-wise expression profiles of UniTags annotated to genes involved in nodule functions

In general, the main processes involving symbiotic nitrogen fixation (SNF) in legumes are turning around three principal aspects: i) delivery of carbon sources from the plant cell to the bacteroids, ii) delivery of fixed N_2 from the bacteroids to the plant cells, and iii) communication between the plant and the bacterioid part (Schultze and Kondorosi, 1998). In the following subsections, the general regulation tendencies of several chickpea SNF-related genes will be detailed regarding control-nodules vs control-roots differential expression and salt stress-induced transcription changes. Due to the complexity of the obtained patterns, the identification of single UniTags by ID will be avoided wherever possible.

Due to methodological barriers presented by the restrictive sampling of poly(A)$^+$-type mRNA in SuperSAGE; bacteroid transcripts were not detectable, which constrains the results to an unilateral point of view (that of the plant). Nevertheless, the amount of accumulated information is already sufficient to take a deeper look into several relevant processes.

5.5.1 Carboxylate flow from the host cell to the bacteroid

5.5.1.1 Malate metabolism

Malate is the major carbon export metabolite from the host cell into the bacteroid during nodulation (Tesfaye et al., 2006). Expression levels of genes involved in malate metabolism, like the gene encoding malate dehydrogenase (MDH) and nodulin 56 show down-regulation in nodules upon salt stress. In parallel, the same UniTags appear to be at least slightly induced in roots. In contrast, a single transcript annotated to malate oxidoreductase (MOR; STCa-19145, Q9LEN2_CICAR) kept its transcription level in nodules, but was down-regulated in roots after NaCl-treatment (**Figure 5-4**). In concordance with the SuperSAGE profiles in nodules from INRAT-93, malate accumulation and MDH activity were reduced during salt stress in chickpea variety ILC1919 (Soussi et al., 1998). In roots under toxic ionic stress, malate is proposed to also be a chelating factor (Tesfaye et al., 2001).

Also in agreement with Soussi and co-authors (1998), a single UniTag annotated to phosphoenolpyruvate carboxylase (PEPC; STCa-3390, CAPP_PHAVU), an enzyme considered crucial for nodule metabolism (Nomura et al., 2006), was about 2-fold over-expressed in non-stressed nodules versus roots, and was 2.4-fold down-regulated in salt-stressed nodules (**Figure 5-4)**. PEPC enzymes catalyze the conversion of phosphoenolpyruvate to oxaloacetate, which in turn can be rapidly converted to aspartate or malate.

5.5.1.2 Sucrose metabolism and transport

As an intermediate, sucrose is synthesized in aerial parts of the plant and then transported into the nodules, before it is catabolised to malic acid (Udvardi and Day, 1997). Sucrose synthase (SUS), a rate-limiting enzyme in the sucrose biosynthesis, has been suggested to be essential for salt tolerance in *P. sativum*, where N_2-fixation rates were directly influenced by the activity of this enzyme (Gordon et al., 1999). In chickpea nodules under control conditions, two UniTags annotated to SUS were detected with no major differential expression between control roots and nodules (SCCa-1110 ad STCa7149; SUSY_PHAAU). Under salt stress, both transcripts maintained their expression levels, and additionally a stress-specific UniTag (STCa-20450, Q9XG55_LOTJA) was detected.

Further on, no UniTags annotated to sucrose transporters (SUT) were detected either in nodules or roots under control conditions. In contrast, one single transcript was induced by salt in both organs (STCa-854, Q9SXU7_CICAR; **Figure 5-4)**.

The function of sucrose in plants under osmotic disequilibrium is not limited to its role as carbon sink. Sucrose also plays a role as compatible osmolyte and as signaling molecule (Zhu, 2002). These facts may explain the stress-induction of some of the UniTags related to sucrose metabolism in chickpea roots and nodules. However, no confirmatory information about sucrose levels in salt stressed chickpea roots and nodules is available, a fact that impairs withdrawing a concrete interpretation of these results.

5.5.2 Nitrogen assimilation

5.5.2.1 Glutamine synthetase and glutamate synthase

Nitrogenase in the bacteroid catalyzes the fixation of N_2 into ammonium (NH_4), which is then incorporated into glutamine and glutamate, and translocated out of the root nodules (Mylona et al., 1995). UniTags annotated to glutamine synthetase (GS), a key enzyme in N_2-assymilation, show a quite complex expression pattern in INRAT-93 roots and nodules. After 2h of salt-treatment, three out of four UniTags detected in stressed nodules show only very slight expression changes (close to constitutive levels); whereas one transcript is at least 3.5-fold up-regulated. In roots under salt stress, four out of seven detected GS UniTags were very slightly up-regulated (**Figure 5-4**). Interestingly, UniTag STCa-5779 (GLNA1_LOTJA), which was slightly prevalent in non-stressed nodules, is down-regulated upon salt stress in both organs, whereas UniTag STCa-19339 (O04998_MEDTR)

exhibits the opposite behaviour. No UniTags annotated to glutamate synthase were detected in the dataset from INRAT-93 roots and nodules.

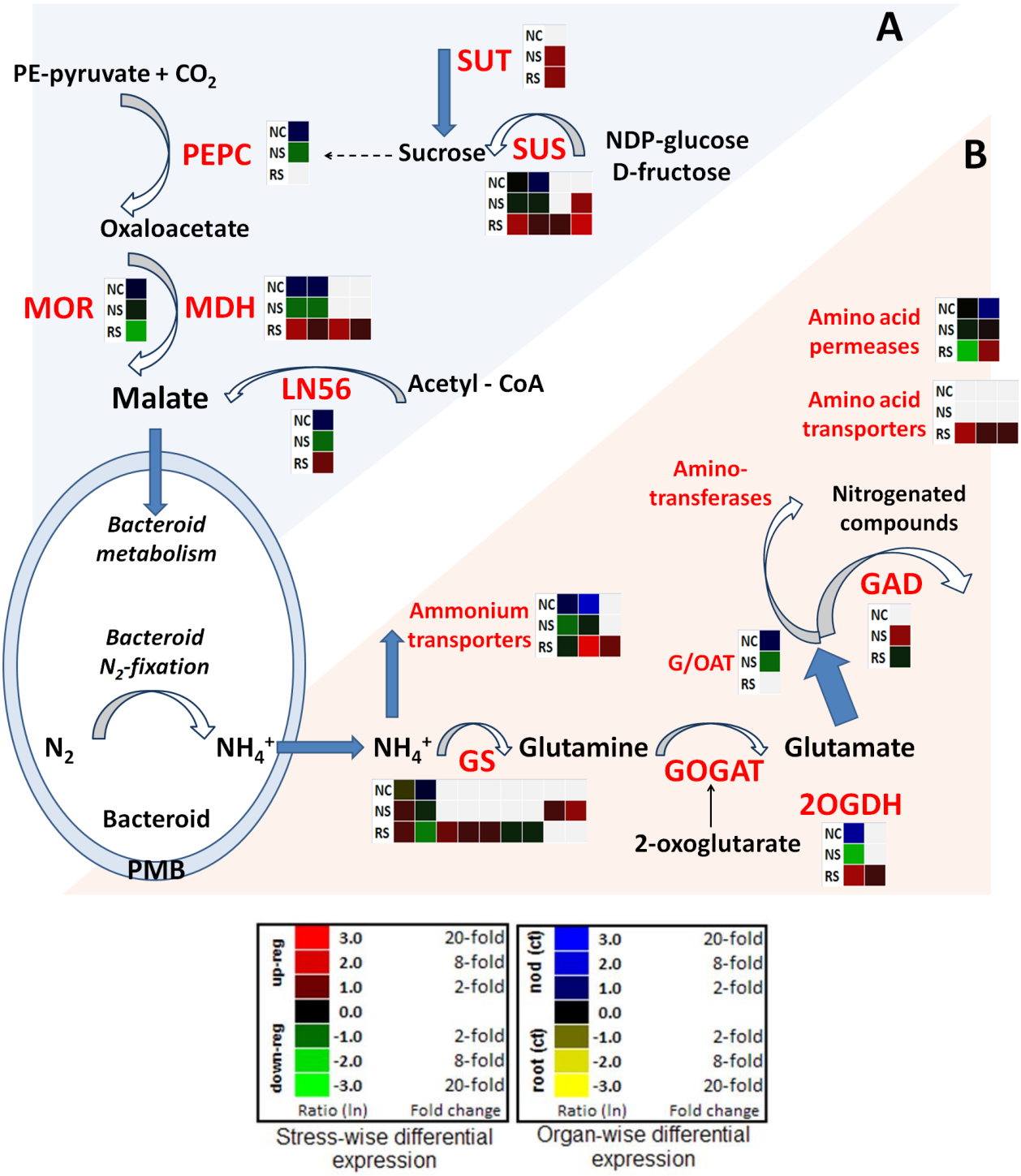


Figure 5-4 Main pathways of malate metabolism and nitrogen assimilation in legume nodules

A) Blue background: Main malate metabolic pathways and involved enzymes and their transcripts (or transcript isoforms)
Phosphoenol-pyruvate carboxylase (PEPC) catalyzes the production of oxaloacetate, which is converted to malate by malate oxidoreductase (MOR) and malate dehydrogenase (MDH). Late nodulin 56 (LN56) in turn catalyzes the conversion of acetyl-CoA to malate. Sucrose, and consequently sucrose transporters influence the activity of PEPC, an enzyme considered to be rate-limiting for malate production in nodules.

B) Light red background: Main nitrogen assimilation pathways in legumes
Fixed nitrogen (NH_3/NH_4) is exported from the nodule to the host cell to be incorporated into glutamine by glutamine synthetases (GS). Glutamine can be converted to glutamate by glutamate synthase (GOGAT) in the presence of 2-oxoglutarate. Ammonium can also be translocated from the symbiotic organs to other locations on the plant by ammonium transporters. Glutamate in turn can be incorporated into nitrogenated compounds involving glutamate amino-transferase (G/OAT), amino acid transporters, and amino-transferases.

NC) Organ-wise UniTag expression profiles indicating -prevalence in nodules with various intensities of blue

NS and RS) Stress-wise Unitag expression profiles of nodules and roots indicating up- and down-regulation in salt stress with intensities of red and green, respectively.

5.5.2.2 UniTags annotated to other enzymes of glutamate metabolism, amino-transferases, and synthethases of nitrogenated compounds

Transcripts annotated to proteins relevant for N_2-assimilation-, such as aminotransferase proteins, asparagine and spermidin synthases, glutamate decarboxylases, 2-oxoglutarate dehydrogenases, glutamine/ornithine acetyltransferases, amino acid permeases, and amino acid transporters (Day et al., 2001; Tesfaye et al., 2006), follow very diverse regulation patterns in chickpea roots and nodules (**Figure 5-4**). Due to the diversity of transcripts and their expression levels, UniTags assigned to the GO biological process Nitrogen compound metabolic process (GO:0006807), including the genes listed in **Table 5-10**, were loaded onto the ErmineJ 2.0 software for a closer look into their over-representation. After GSR analysis, transcripts from salt stressed nodules assigned to N_2-metabolism revealed a P value of 0.25 (not shown in **Table 5-6A**). This value is neither low enough to consider this category as over-represented, nor high enough to consider it as "not-represented". With the restrictions belonging to an analysis solely based in transcription profiles, this result suggest that N_2-fixation and nitrogenated compounds

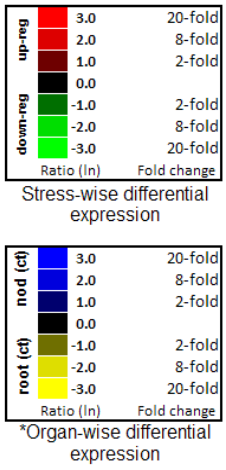
metabolism are keeping at least a steady rate of their activity (even with slight increase) in chickpea nodules after 2 hours of salt stress. Even despite of a possible prevention of the delivery of carbon compounds into the bacteroid (Section **5.4.1**).

Previous reports of an increase in amino acid concentration and activity of N_2-metabolic enzymes upon salt stress are in concordance with the present results from INRAT-93 nodules and roots (Fougere et al., 1991; Marquez et al., 2005). Upon salt stress, several processes involve the use of nitrogenated compounds in the plant. Among them, accumulation of amino acids (e. g. proline) as compatible osmolytes (Wang et al., 2007), and protein biosynthesis.

Table 5-10 **Differential expression levels of transcripts annotated to amino-transferases, and other N_2-metabolism proteins in INRAT-93 roots and nodules**

Nc vs Rc: control nodules versus control roots
Nc vs Ns: control nodules versus stressed nodules
Rc vs Rs: control roots versus stressed roots

			Number of representative UniTags				
Metabolic process	***Protein***	***Comparison***	***1***	***5***	***10***	***15***	***20***
Nitrogen assimilation	Asparagine synthetase	Nc vs Rc* Nc vs Ns Rc vs Rs					
Nitrogen assimilation	Spermidine synthase 1	Nc vs Rc* Nc vs Ns Rc vs Rs					
Nitrogen assimilation	Glu-1-semialdehyde NH_3-transferase	Nc vs Rc* Nc vs Ns Rc vs Rs					
Nitrogen assimilation	Aspartate aminotransferase	Nc vs Rc* Nc vs Ns Rc vs Rs					
Nitrogen assimilation	Aminotransferase-like protein	Nc vs Rc* Nc vs Ns Rc vs Rs					
Nitrogen assimilation	BC-amino-acid NH_3-transferase	Nc vs Rc* Nc vs Ns Rc vs Rs					
Nitrogen assimilation	Acetylornithine aminotransferase	Nc vs Rc* Nc vs Ns Rc vs Rs					
Nitrogen assimilation	Alanine-glyoxylate aminotransferase	Nc vs Rc* Nc vs Ns Rc vs Rs					



5.5.3 ROS generation and ROS scavenge

5.5.3.1 Leghemoglobin (Lb) and ferric-Lb reductase

In nodules, only the ferrous form of Lb (Lb^{2+}) is able to bind O_2, forming oxyleghemoglobin ($Lb^{2+}O_2$), which can spontaneously autoxidize to form ferric Lb (Lb^{3+}) and superoxide (O_2^-) (Gunther et al., 2007). The released O_2^- radicals can be quenched by other $Lb^{2+}O_2$ molecules, which in turn can be oxidized to Lb^3 (**Figure 5-5**). A single UniTag annotated to Lb was detected in INRAT-93 showing 4.5-fold and 10-fold up-regulation in stressed roots and nodules, respectively (STCa-3321, Q42928_MEDSA). UniTags annotated to leghemoglobin reductase, the enzyme catalyzing the conversion of Lb^{3+} to Lb^{2+}(Becana and Klucas, 1990), were highly prevalent in non-stressed roots, but not in nodules. Under salt stress, the four observed transcript variants were strongly down-regulated in roots, whereas two UniTags were slightly stress-induced in nodules (**Figure 5-5**).

The dynamics of expression of the UniTags annotated to these two enzymes under control and stress conditions in INRAT-93 roots and nodules, respectively, allow the following assumptions: i) Under control conditions, the conversion of Lb^{2+} to Lb^{3+} may be prevented by high SOD activity, reflected by high SOD transcription levels (**Figure 5-5**), ii) under salt stress in nodules, the mechanism converting Lb^{3+} to Lb^{2+} may be activated by an overflow of (O_2^-) radicals.

5.5.3.2 SODs and enzymes related to glutathione/ascorbate cycles

Symbiotic N_2-fixation (SNF) induces intensive mitochondrial respiration and therefore high ROS production rates (Becana et al., 2000). In chickpea nodules, various UniTags annotated to ROS-scavengers (i.e. SOD, CAT, GST, GPX, and DHAR) are more expressed in nodules than in roots, even under control conditions (**Figure 5-5**). These results are in concordance with several previous studies reviewed by Gechev and co-authors (2006).

Apart from the ROS derived from the N_2-fixation process, reactive oxygen species are also produced by the metabolic changes induced by abiotic stresses such as drought and high salinity (Apel and Hirt, 2004). Therefore, salt stress- and SNF-induced ROS overflow may trigger similar responses in some degree in chickpea plants. This aspect is well exemplified by the expression levels of UniTag STCa-7896, annotated to a SOD protein (Q9ZNQ4_CICAR). This particular tag was already over-expressed in non-stressed nodules (20-fold differential expression). After two hours of salt treatment, its expression level remained constant in this organ, whereas in roots, a 40-fold induction was observed (**Figure 5-5**). The same pattern

holds for UniTag STCa-22470 (GST, Q948X4_MEDSA): 20-fold over-expression already in non-stressed nodules in contrast to roots. After 2h of salt stress, STCa-22470 was at least 12-fold up-regulated in roots, but kept its transcription level in stressed nodules. On a minor scale, UniTag STCa-1532 (DHAR, Q84UH4_TOBAC) showed a similar behaviour. In other cases, transcripts such as UniTags STCa-11623 (GST, Q9AYN3_9ASTR), and STCa-11617 (APX, Q9SXT2_CICAR), not showing major differences between both organs under non-stressed conditions, were up-regulated upon salt stress in roots and nodules.

The observed expression patterns from UniTags annotated to ROS-scavengers in INRAT-93 nodules and roots allow considering a shared tendency between SNF- and stress-responses in chickpea. Nodules from the salt-tolerant variety INRAT-93 may already be under severe oxidative stress, before any additional ROS as stress by-products are generated after salt treatment. In chickpea, nodulation capacity has been already correlated with salt tolerance (Singh et al., 2005). In connection with this correlation, the results from INRAT-93 roots and nodule profiles suggest that the early over-expression of ROS-scavengers in chickpea nodules may be advantageous for the plant when facing an additional stress. However, confirmation of this hypothesis requires further monitoring of both organs (nodules and roots) under salt treatment, additional to transcriptome-based analyses.

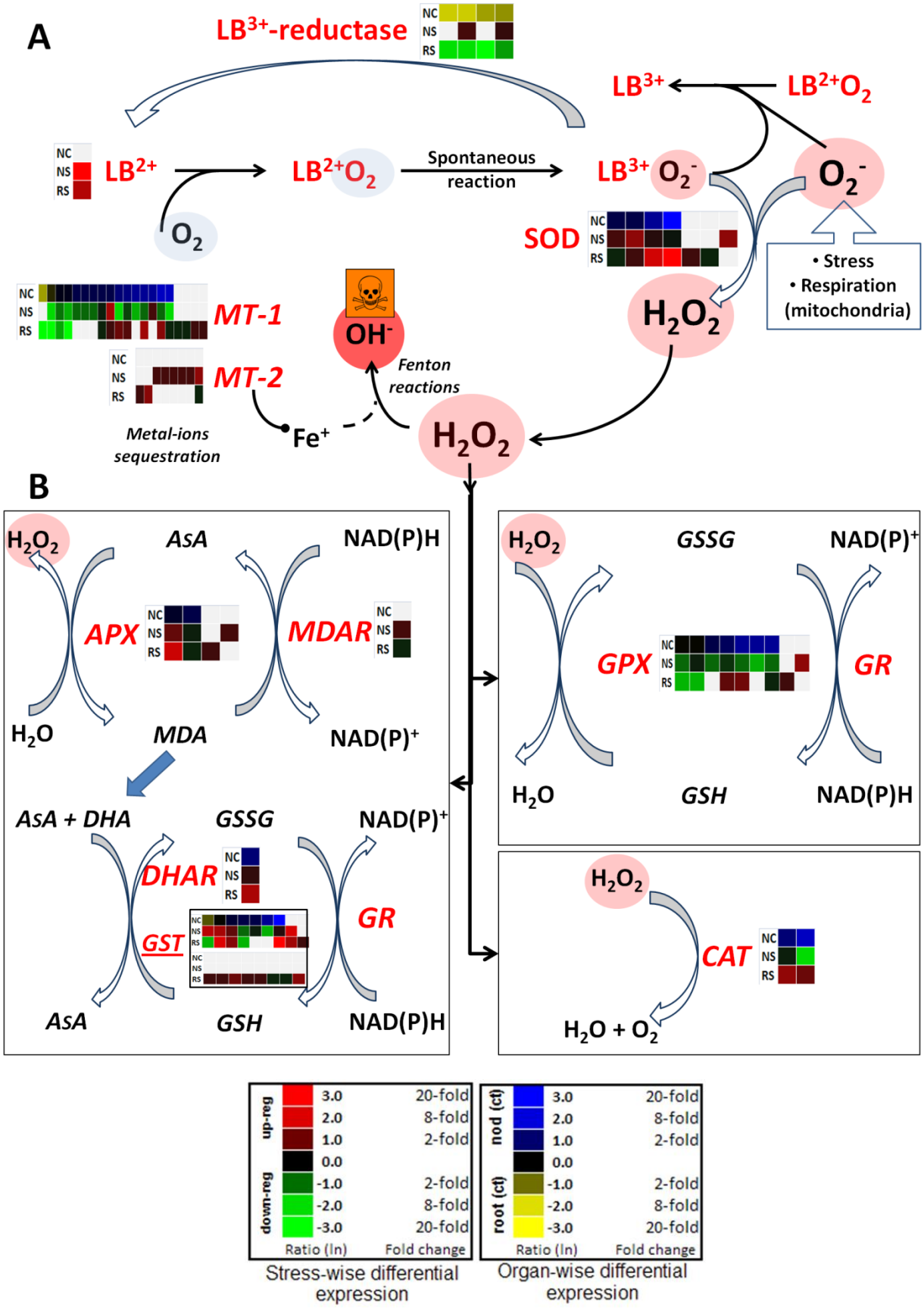

A
LB3+-reductase
NC
NS
RS
LB3+
LB2+O2
LB2+
O2
LB2+O2
Spontaneous reaction
LB3+ O2-
O2-
SOD
• Stress
• Respiration (mitochondria)
H2O2
MT-1
MT-2
OH-
Fenton reactions
Metal-ions sequestration
Fe+
H2O2
B
H2O2
AsA
NAD(P)H
APX
MDAR
H2O
MDA
NAD(P)+
AsA + DHA
GSSG
NAD(P)+
DHAR
GST
GR
AsA
GSH
NAD(P)H
H2O2
GSSG
NAD(P)+
GPX
GR
H2O
GSH
NAD(P)H
H2O2
CAT
H2O + O2
up-reg
down-reg
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
20-fold
8-fold
2-fold
2-fold
8-fold
20-fold
Ratio (ln)
Fold change
Stress-wise differential expression
nod (ct)
root (ct)
Organ-wise differential expression

Figure 5-5 Expression profiles of genes encoding proteins producing or destroying reactive oxygen species (ROS) in legume nodules

A) In the very intricate and redundant ROS pathway in legume nodules, superoxide radicals (O_2^-) are derived from elevated mitochondrial respiration rates. In turn, leghemoglobin (LB^{2+}), the enzyme keeping the nodules free of molecular oxygen (O_2), can spontaneously be converted to ferric LB (LB^{3+}), generating new O_2^-. These radicals can further induce more conversions of LB^{2+} to LB^{3+}. The generated superoxide can be directly dismutated by SOD to H_2O_2, which must be immediately counteracted, as depicted in (B). On the other hand, H_2O_2 can generate hydroxyl radicals (OH^-) in the presence of abundant free Fe^+ ions, which are sequestered by metallothionein-like proteins (Chelysheva et al.).

B) Hydrogen peroxide can be scavenged via the glutathione/ascorbate cycles or the action of catalases (CAT), as already described in chapter 4 (Section **4.4.1.2**).

NC) Organ-wise UniTag expression profiles indicating -prevalence in nodules with various intensities of blue

NS and RS) Stress-wise Unitag expression profiles of nodules and roots indicating up- and down-regulation in salt stress with intensities of red and green, respectively.

5.5.4 Trans-membrane channels (Aquaporins)

The SNF process in root nodules of legumes requires continuous exchange of compounds, and communication between host cell and bacteroid through the peribacteroid membrane (PBM). Therefore, trans-membrane (TM) channels, commonly known as aquaporins or major intrinsic proteins (MIPs), have been reported to play crucial roles in nodulated legumes (Udvardi and Day, 1997).

Already under normal conditions, chickpea nodules and roots of the salt-tolerant variety INRAT-93 exhibit organ-specific transcription profiles for several classes of trans-membrane channel proteins (**Table 5-11**). In total, 69 UniTags were annotated to MIP transcripts. From them, 47 remained unclassified among the MIP family, 2 were annotated to plasma-membrane intrinsic proteins (PIP), 19 were annotated to tonoplast intrinsic proteins (TIP), and one UniTag was annotated to the NOD26-like intrinsic protein (Knipp and Honermeier) subclass.

The single UniTag annotated to nodulin 26 (NOD26; STCa-11512, Q39883_SOYBN), a nodule-abundant PMB- water channel (Weaver et al., 1994), was detected in chickpea being 2.0-fold more abundant in nodules than in roots. Transcripts annotated to TIP aquaporins, a class of trans-membrane (TM) channels also known to be abundant in legume nodules (Fleurat-Lessard et al., 2005), displayed mixed expression patterns. From nineteen detected

UniTags, two were at least 4.5- and 2.0-fold, respectively, prevalent in non-stressed roots, whereas three and one UniTags, respectively, were more than 2.0- and 9.0-fold abundant in non-stressed nodules. From the remaining 47 unclassified MIPs, 27 UniTags appeared prevalent in nodules under control conditions, whereas 18 transcript variants resulted to appear only under stress conditions either in roots or in nodules, however, with low induction levels.

Additionally to the above detailed expression profiles, the intense involvement of membrane-related processes in chickpea nodules is confirmed by the gene score re-sampling (GSR) analysis for over-representation of GO cellular components. Six membrane-related GO cell component categories were found among the most over-represented ones in non-stressed nodules in respect to roots (**Table 5-2B)**.

After salt stress, the single UniTag annotated to nodulin 26 was 3.5-fold and 5.0-fold up-regulated in nodules and roots, respectively. With exception of UniTags STCa-24453 and STCa-8037, both annotated to the TIP-aquaporin Q8L5G0_CICAR (>8.0-fold and >5.0-fold up-regulated in roots and nodules, respectively), and the UniTag STCa-21968 annotated to a PIP2 protein (Q8W4T8_MEDTR), no marked up-regulation was observed. For nodules in particularly, the down-regulation tendency of aquaporins was more evident (**Table 5-11**).

In agreement with these results, barley did not react upon salt-stress with changes in the transcription rate for a series of aquaporins, at least not within the first hours after treatment (Katsuhara and Shibasaka, 2007). Also *Nicotiana glauca* did not change the expression levels of several MIPs, TIPs and PIPs after the onset of stress (Smart et al., 2001). Some proteins of this family are also serving as channels for malate and other carboxy acids (Weaver et al., 1994). Therefore, a possible decrease in malate concentration in salt-stressed nodules may reduce the transcription level of specific aquaporins.

5.5.5 Proteins functioning in signal transduction

SNF in legume nodules activates various signaling cascades, which control communication between bacteroids and host-cells at all nodulation stages (Kistner and Parniske, 2002). In chickpea roots and nodules, the expression levels of signaling-related transcripts (e.g. RLKs, CDPKs, Calmodulin, MAPKs, and PPs; **Table 5-11**), revealed different organ- and stress-wise regulation patterns.

Multigenic families, like the RLK family, were represented by at least 35 annotated UniTags with organ-wise expression levels ranging between 4.5-fold prevalence in **roots** and

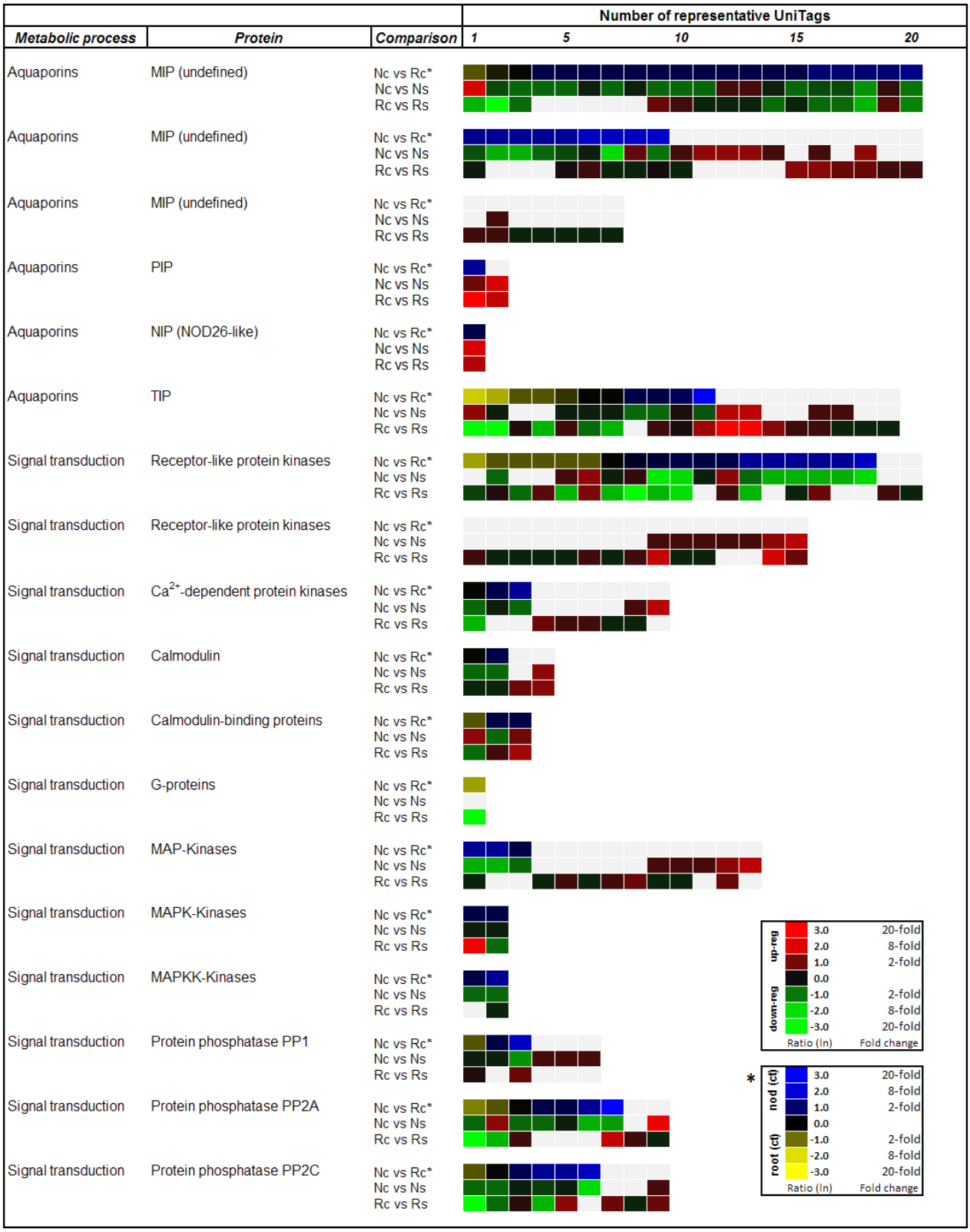
4.5-fold prevalence in **nodules**. After salt stress, expression changes in both organs (roots and nodules) ranged between 8- and 6-fold down- and up-regulation, respectively (**Table 5-11**). Although the resolution of SuperSAGE may lack of power to discriminate between members of the RLK family, the combination of organ- and stress-wise transcription profiles allows selecting transcripts that can be considered interesting targets for further analyses. For example, UniTags STCa-7800 (Q70I30_LOTJA) and STCa-24316 (O82432_MALDO) are only up-regulated in roots after salt stress (6.0- and 5.0-fold, respectively). In nodules in particular, UniTag STCa-492 (Q70I28_LOTJA) appears to represent a stress-induced transcript.

As examples in other gene classes, among Ca^{2+}-dependent signaling proteins (i.e.CDPKs, calmodulin and calmodulin-binding proteins) the UniTag STCa-14946 (CDPK, Q7XZK4_CICAR) was only observed in nodules after salt stress (at least 5-fold up-regulation). From UniTags annotated to MAPKs, MAPKKs, and MAPKKKs, also transcripts with relevant expression patterns could be selected: UniTag STCa-5798 (MAPKK, Q93WR7_MEDVA) was slightly prevalent in non-stressed nodules (1.8-fold differential expression), and became highly induced in roots only after salt stress (8-fold up-regulated). From transcripts annotated to PP2A proteins, UniTags STCa-7572 (Q9FVD5_MEDVA; 5-fold up-regulation) and STCa-21852 (Q8L5L1_MUSAC; 8.0-fold up-regulation) were only detected after salt stress in nodules and roots, respectively.

Due to the diversity of signaling cascades in which proteins from the above detailed classes are involved (Smith and Walker, 1996; Leung and Giraudat, 1998; Ikeda, 2001; Zhu, 2002; Shinozaki et al., 2003; Samuel et al., 2005), the information derived from SuperSAGE is not sufficient to identify the specific pathways to which the proteins represented by the observed UniTags are related. However, the sequence information contained in the UniTags with relevant expression patterns can be used as starting point for further characterization.

Table 5-11 Differential expression levels of transcripts annotated to trans- membrane (TM) channels and to signal transduction-related proteins in INRAT-93 roots and nodules

Nc versus Rc: control nodules versus control roots
Nc versus Ns: control nodules versus stressed nodules
Rc versus Rs: control roots versus stressed roots



6 The drought-responsive transcriptome of chickpea roots

6.1 Drought-induced differential gene expression in chickpea roots

A total of 82,012 tags from roots of the drought-tolerant variety ICC588 were sequenced, and represented 17,498 unique transcripts (UniTags). After 6 hours of dehydration, 7,531 UniTags (43%) were differentially expressed in stressed as compared to control roots ($R_{(ln)}$>1.0; 2.7-fold). Of these, 388 and 589 were more than 8.0-fold up- and down-regulated, respectively (as detailed in Section **3.1**). In the present chapter, the principal results of the process of filtering and extracting relevant information out of this large dataset will be presented.

6.1.1 The most up-regulated UniTags in drought-stressed ICC588 roots

The 40 most up- and down-regulated transcripts in droughted roots matching well-characterized genes in public databases are presented in **Tables 6-1A** and **6-1B**, respectively. The annotatable transcripts coding for a 14-3-3 protein (1433A_VICFA), extensin (O65760_CICAR), NADP-dependent isocitrate dehydrogenase (Q6R6M7_PEA), S-receptor kinase-like protein 1 (Q70I30_LOTJA), chalcone isomerase (Q9SXS9_CICAR), myo-inositol-1-phosphate synthase (Q94C02_SOYBN), UDP-glucose pyrophosphorylase (Q8W557_9FABA), ATP synthase (ATP4_PEA), aquaporin PIP-2 (Q8W4T8_MEDTR), and polygalacturonase (A2Q3E3_MEDTR) were the most up-regulated interpretable transcripts under drought stress, though not the most up-regulated in the complete ICC588 dataset (see section **6.1.1.11**). In the following sub-sections, background information about the genes with the highest differential expression levels will be detailed.

Table 6-1A Top 40 annotatable UniTags up-regulated after 6h of dehydration stress in ICC588 roots

Tag code	Protein	$R_{(ln)}$	Associated process	Uniprot ID
STCa-20066	14-3-3-like protein A	3.84	Protein domain specific binding	1433A_VICFA
STCa-19021	Extensin	3.69	Cell wall organization and biogenesis	O65760_CICAR
STCa-7166	NADP-dependent isocitrate dehydrogenase I	3.57	Carbohydrate metabolism	Q6R6M7_PEA
STCa-7800	S-receptor kinase-like protein 1	3.57	Protein amino acid phosphorylation	Q70I30_LOTJA
STCa-10145	Chalcone isomerase	3.51	Flavonoid biosynthesis	Q9SXS9_CICAR
STCa-181	Myo-inositol-1-phosphate synthase	3.41	Inositol 3P biosynthesis/Ca^{2+} release	Q94C02_SOYBN
STCa-8459	UDP-glucose pyrophosphorylase	3.34	Metabolism	Q8W557_9FABA
STCa-6190	ATP synthase (mitochondrial)	3.34	Proton pump	ATP4_PEA
STCa-21968	Aquaporin PIP-2	3.32	Transport	Q8W4T8_MEDTR
STCa-7762	Polygalacturonase	3.30	Cell wall protein	A2Q3E3_MEDTR
STCa-228	Beta-glucosidase	3.26	Carbohydrate metabolism	Q9FSY8_CICAR
STCa-20422	Specific tissue protein 1	3.21	No associated process	Q39449_CICAR
STCa-21666	Low temp. and salt-responsive protein LTI6B	3.17	Integral to membrane	RCI2B_ARATH
STCa-23486	S-adenosyl-L-methionine synthetase	3.12	One-carbon compound metabolism	Q9AT56_ELAUM
STCa-14806	Cysteine proteinase	3.12	Proteolysis	CYSEP_VIGMU
STCa-2982	Cysteine synthase	3.07	Protein biosynthesis	O65747_CICAR
STCa-22698	Putative adenosine kinase	2.91	Purine ribonucleoside salvage	Q8L5Q4_CICAR
STCa-12550	60S ribosomal protein L13	2.86	Protein biosynthesis	RL131_ARATH
STCa-12406	Coatomer subunit beta'-2	2.85	Protein transport	COB22_ARATH
STCa-17627	Putative universal stress protein	2.79	Response to stress	Q700A7_CICAR
STCa-542	Prolyl 4-hydroxylase	2.72	Protein metabolism	Q9FKX6_ARATH
STCa-1589	Beta-galactosidase	2.72	Carbohydrate metabolism	O65736_CICAR
STCa-8720	S-adenosylmethionine synthetase	2.65	Metabolism	Q9AT56_ELAUM
STCa-10123	Synaptobrevin-like protein	2.65	Transport / integral to membrane	Q69WS1_ORYSJ
STCa-2044	Fiber protein Fb11	2.64	No associated process	Q8GT82_GOSBA
STCa-227	Beta-glucosidase	2.56	Carbohydrate metabolism	Q9FSY8_CICAR
STCa-866	Protein kinase Pti1	2.56	Protein amino acid phosphorylation	Q84P43_SOYBN
STCa-15340	Alfin-1	2.56	Regulation of transcription	Q40359_MEDSA
STCa-16114	Cytosolic acetoacetyl-coenzyme A Thiolase	2.56	No associated process	Q5XMB8_TOBAC
STCa-16514	NADH dehydrogenase	2.56	Mitochondrial electron transport	Q9FNN5_ARATH
STCa-5543	Epsilon subunit of mitochondrial F1-ATPase	2.52	Transport	Q8L5Q1_CICAR
STCa-8853	Ribosomal protein L10 homolog	2.48	Protein biosynthesis	Q42149_ARATH
STCa-23978	Inorganic pyrophosphatase-like protein	2.48	Phosphate metabolism	Q9LFF9_ARATH
STCa-857	Histone H2B	2.38	Response to DNA damage stimulus	Q9M3H6_CICAR
STCa-21625	Serine protease inhibitor-like protein	2.38	No associated process	Q8RV99_ORYSA
STCa-24140	Putative 14-kDa proline-rich protein	2.38	Lipid transport	Q9LEN8_CICAR
STCa-16415	NADPH-cytochrome P450 oxidoreductase	2.38	Electron transport	Q7M275_TOBAC
STCa-923	Ribosomal protein S26	2.28	Protein biosynthesis	Q9SWS9_PEA
STCa-1343	Apyrase-like protein	2.28	No associated process	Q84UE1_MEDTR
STCa-2122	Histone H2A	2.28	Chromosome organization	H2A_CICAR
STCa-6603	Polygalacturonase PG11 precursor	2.28	Carbohydrate metabolism	Q84TM8_MEDSA
STCa-7388	Aldolase	2.28	No associated process	Q945F2_CICAR
STCa-8045	CaM protein	2.28	Calcium related signal transduction	Q7DLT8_CICAR
STCa-14940	TGA-type basic leucine zipper protein	2.28	Regulation of transcription	Q93XA1_PHAVU
STCa-15506	Delta-COP	2.28	Intracellular protein transport	Q9M640_MAIZE
STCa-16257	ABA-responsive protein	2.28	Stress response / ABA dependent	Q9FMW4_ARATH
STCa-16760	Elongation factor 1-alpha	2.28	Protein biosynthesis	O81921_CICAR

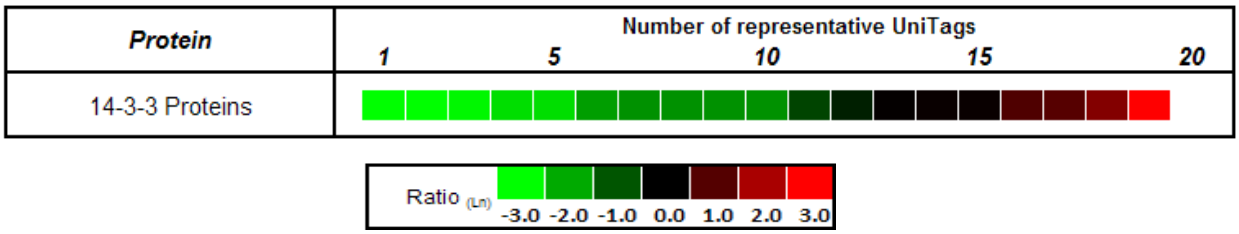
Table 6-1B Top 40 annotatable UniTags down-regulated under drought stress

Tag code	Protein	$R_{(ln)}$	Associated process	Uniprot ID
STCa-1804	Expansin-like protein (fragment)	-3.09	Sexual reproduction	Q7XHJ2_QUERO
STCa-13652	40S ribosomal protein S23	-3.09	Protein biosynthesis	RS23_EUPES
STCa-4802	ADP-glucose pyrophosphorylase precursor	-2.91	Glycogen biosynthesis	Q43819_PEA
STCa-5076	Ribosomal protein L32	-2.91	Protein biosynthesis	Q45NI6_MEDSA
STCa-7347	3-hydroxybutyryl-CoA dehydrogenase	-2.91	Fatty acid metabolism	Q9LDF5_ARATH
STCa-8227	Histone H3	-2.91	Chromosome organization	H3_ONOVI
STCa-13267	Allene oxide synthase precursor	-2.91	Lipid biosynthesis	Q7X9B4_MEDTR
STCa-17859	Hypothetical protein 275	-2.91	No associated process	Q8GTD8_CICAR
STCa-21081	Vestitone reductase	-2.86	Cellular metabolism	Q40316_MEDSA
STCa-3331	60S ribosomal protein L18	-2.69	Protein biosynthesis	RL18_CICAR
STCa-10792	CIPK protein	-2.69	Signal transduction	Q84XC0_PEA
STCa-12317	Heat shock protein 70-3	-2.69	Response to unfolded protein	Q67BD0_TOBAC
STCa-18274	NADPH-ferrihemoprotein reductase	-2.69	Electron transport	Q43235_VICSA
STCa-19040	DNA-directed RNA polymerase subunit B	-2.69	Transcription	Q70Q06_VICSA
STCa-19432	KI domain interacting kinase 1-like protein	-2.69	Protein amino acid phosphorylation	Q9T058_ARATH
STCa-19785	Reduced vernalization response 1	-2.69	Regulation of transcription	Q8L3W1_ARATH
STCa-19870	Transaldolase	-2.69	Carbohydrate metabolism	O04894_SOLTU
STCa-18410	Cytochrome P450	-2.55	Electron transport	Q9ZRW6_CICAR
STCa-18321	Auxin-independent growth promoter	-2.49	No associated process	Q9LIN9_ARATH
STCa-1286	Eukaryotic translation initiation factor	-2.40	Translational initiation	Q7XJB0_LACSA
STCa-3390	Phosphoenolpyruvate carboxylase	-2.40	Carbon utilization	CAPP_PHAVU
STCa-3855	ThiF family protein-like	-2.40	No associated process	Q653N8_ORYSA
STCa-3897	20S proteasome alpha subunit C	-2.40	catabolism	PSA4_SPIOL
STCa-5074	Pectin methyl-esterase PER precursor	-2.402	Cell wall modification	Q9SC90_MEDTR
STCa-5237	F-box family protein-like	-2.402	No associated process	Q5VR67_ORYSA
STCa-5681	Hydroxyproline-rich glycoprotein	-2.402	Cell wall organization	Q39865_SOYBN
STCa-6267	Transcription factor MYBS3	-2.402	Regulation of transcription	Q8H1D0_ORYSA
STCa-6374	Putative extensin	-2.402	Cell wall organization	Q9FSY9_CICAR
STCa-6426	Protein kinase	-2.402	Protein amino acid phosphorylation	Q9ZRU3_CICAR
STCa-6928	40S ribosomal protein S19	-2.402	Protein biosynthesis	Q9ZRW2_CICAR
STCa-6991	Cytochrome P450	-2.402	Electron transport	Q9XGL7_CICAR
STCa-7688	Narf-like protein	-2.402	Electron transport	Q5VR67_ORYSA
STCa-8832	chalcone synthase	-2.402	Biosynthesis	Q39865_SOYBN
STCa-9049	Translocon-associated subunit A-precursor	-2.402	No associated process	Q8H1D0_ORYSA
STCa-9308	MIP Aquaporin	-2.402	Transport	Q9FSY9_CICAR
STCa-11376	60S ribosomal protein L10	-2.402	Protein biosynthesis	Q9ZRU3_CICAR
STCa-11527	Bet v I family protein (bet gene)	-2.402	No associated process	Q93YF9_MEDTR
STCa-12919	14-3-3-like protein	-2.402	Protein domain specific binding	Q9ZRV7_CICAR
STCa-13826	Coatomer alpha subunit-like protein	-2.402	Protein targeting	SSRA_ARATH
STCa-14803	ATP synthase alpha chain. mitochondrial	-2.402	Transport	Q8GTE0_CICAR

6.1.1.1 14-3-3 proteins

UniTag STCa-20066 (1433A_VICFA) represented the most up-regulated transcript (45-fold) 6 hours after dehydration in ICC588 roots. In drought-stressed chickpea, a total of nineteen UniTags annotated to 14-3-3-proteins were detected. From these, four were more than 2.0-fold up regulated, whereas ten showed more than 4.0-fold down-regulation (**Table 6-2**). In rice, Chen and co authors (2006) reported on very diverse expression patterns for the 14-3-3 family under biotic as well as under abiotic (drought, salt, ABA applications) stresses, a result that agrees with the profiles observed here. As already observed in Section **4.2.1.10**, UniTag STCa-20066 was also among the most up-regulated transcripts (20-fold) in roots of salt-treated INRAT-93 plants. The members of this multi-protein family own a very high target specificity (Ferl, 1996). Thus, the up-regulation of this UniTag should lead to the characterization of a chickpea protein playing a major role in common salt and drought responses.

Table 6-2 UniTags annotated to 14-3-3 proteins and their expression levels in drought-stressed chickpea roots



6.1.1.2 Extensin

UniTag STCa-19021, annotated to the extensin accession O65760_CICAR was the second most up-regulated transcript in drought-stressed ICC588 roots. As also observed in salt-stressed INRAT-93 roots, this particular UniTag is shared between responses to drought and salt stresses in chickpea. As previously discussed (chapter 4, Section **4.2.1.4**), extensin is involved in cell wall enforcement, probably one of the principal defense mechanisms of chickpea root cells against water and salt stress.

6.1.1.3 NADP-dependent isocitrate dehydrogenase I

UniTag STCa-7166 (Q6R6M7_PEA), a component of the common response of chickpea against drought and salt stresses, was 35-fold up-regulated in drought-stressed roots. As detailed in Section **4.2.1.6**, the high expression of NADP-dependent ICDHs in chickpea roots could be related to the glutathione recycling activity during counteraction of

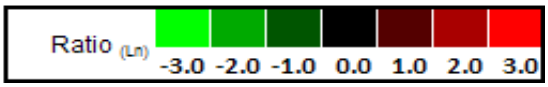
strong oxidative stress (Moller, 2001), or could also be linked to the SNF process. However, ICC588 plants did not nodulate, so that the latter aspect may only be of minor importance, if at all.

6.1.1.4 S-receptor kinase-like protein 1

The important role of early stress sensing and signaling events in drought stress responses of chickpea plants is reflected by the expression levels of UniTag STCa-7800, annotated to the receptor-like protein kinase (RLK) 1 (Q70I30_LOTJA). In the entire chickpea ICC588 dataset, 36 RLK transcript variants were discovered. Two of these increased in abundance more than 20-fold, fourteen were 2.0 to 8.0-fold up-regulated, and twelve UniTags were more than 2.7-fold down-regulated under drought stress (**Table 6-3**). In the drought-response model arising from research in *Arabidopsis* and rice, the first step of signaling is the perception of the stress through G-protein coupled receptors (GPCRs), inositol polyphosphates, or receptor-like kinases (Xiong et al., 2002). In these both organisms, as well as in chickpea, a broad range of isoforms of the transcripts encoding these proteins and various expression levels can be detected.

Table 6-3 UniTags annotated to RLKs and expression levels in drought-stressed chickpea roots

Protein	Number of representative UniTags *1* *5* *10* *15* *20*
Receptor-like protein kinases	



6.1.1.5 Chalcone Isomerase

Chalcone isomerase (CHI) along with chalcone synthase (CHS) are two key enzymes in the biosynthesis of flavonoids in plants (Saslowsky and Winkel-Shirley, 2001). In the context of drought stress, flavonoids protect plants against oxidative stress (Pourcel et al., 2007). In chickpea drought-stressed roots, high up-regulation of UniTag STCa-10145 (Q9SXS9_CICAR) suggests that, as an alternative to the common ROS-scavenging mechanism, flavonoid production in these organs can counteract stress-induced oxidative stress damage. In salt-stressed INRAT-93 roots, one chalcone isomerase UniTag also belonged to the most up-regulated ones. However, different in sequence (UniTag STCa-13313, **Section 4.2.1**).

6.1.1.6 Myo-inositol-1-phosphate synthase

UniTag STCa-181 is also a shared component in the responses of chickpea roots towards salt and drought stresses. In the ICC588 root drought stress dataset, this UniTag was 30-fold up-regulated. The production of inositol-3-phosphate in plants, an event in which myo-inositol-1-phosphate synthases are involved, is a key event for the release of Ca^{2+} from internal storages (Meijer and Munnik, 2003). In the drought and salt stress context, Ca^{2+}-release and Ca^{2+}-dependent early signaling are ruled by the same mechanisms, only differing from each other by the timing and localization of the specific signals (Ca^{2+}-transients; Ikeda, 2001). Thus, the present result can be interpreted as a common stress response in chickpea.

6.1.1.7 UDP-glucose pyrophosphorylase

UniTag STCa-8459 (Q8W557_9FABA), annotated to an UDP-glucose pyrophosphorylase (UGPase) was 28-fold up-regulated in drought-stressed ICC588 roots. As it has been observed in many of the UniTags depicted in **Table 6-1**, the same UniTag was 16-fold up-regulated under salt stress (**Section 4.2.1**). UGPase is a key enzyme producing UDP-glucose from glucose-1-phosphate and UTP, which is involved in several metabolic pathways, among them, the synthesis of sucrose and cellulose (Ciereszko et al., 2001). Sucrose and cellulose themselves are compounds in the management of drought/salt stresses, especially in main metabolism, signaling, and structural organization of the cell (Smeekens and Rook, 1997).

6.1.1.8 Mitochondrial ATP synthase

Mitochondrial membrane-associated ATP synthase produces ATP from ADP and inorganic phosphate in the presence of a proton gradient across the inner membrane, which is generated by electron transport complexes of the respiratory chain (http://www.uniprot.org/uniprot/Q41000). In chickpea roots under drought stress, UniTag STCa-6190 (ATP4_PEA) annotated to an ATP synthase was 28-fold up-regulated. The role of this enzyme in drought stress is only poorly understood presently. The up-regulation of transcripts encoding this protein may possibly be a consequence of the enhanced mitochondrial respiration rate induced by drought, as observed by Rizhsky and co-authors (2002) in tobacco. Additionally, ATP synthases are also known act as proton pumps at the expenses of ATP, therefore, over-expression of this protein may be also related with maintenance of ionic equilibrium in plants under stress (Taylor et al., 2003).

6.1.1.9 Aquaporin PIP-2

PIP aquaporins are water-channel proteins that belong to the major intrinsic-protein family. Most aquaporins are highly selective for water, though some also facilitate the movement of small uncharged molecules such as glycerol (www.ebi.ac.uk/interpro/IPR012269). In legumes under drought stress, several factors regulate aquaporin expression: among others, leaf transpiration rate, leaf water status, abscisic acid (ABA), and soil water content (Aroca et al., 2006). In drought-stressed roots of chickpea, UniTag STCa-21968 (Q8W4T8_MEDTR), annotated to a plasma-membrane intrinsic protein (PIP2), was 28-fold up-regulated after 6 hours of dehydration, which corroborates the results of Aroca and co-workers (2006) in common bean. However, these authors found discrepancies between transcript accumulation rates and protein levels in root and leaf extracts. In Section **6.3.5**, the behavior of all aquaporin transcript variants found in the ICC588 roots dataset will be described in detail.

6.1.1.10 Polygalacturonase

Polygalacturonase proteins form a widely distributed class of plant enzymes, which are generally linked to ripening processes, cell wall degradation, and cell wall separation (Cassab and Varner, 1988; Roberts et al., 2002). Up to date, there are no concise reports on the differential expression of polygalacturonase-encoding genes in legumes under drought stress. In chickpea, the up-regulation of UniTags coding for this enzyme (STCa-7762, A2Q3E3_MEDTR) in drought-stressed roots may be related to structural re-arrangements of cell walls and counteraction of mechanical pressures.

6.1.1.11 Non-annotable up-regulated UniTags in chickpea roots under drought

As already observed in the salt-stress dataset, many of the UniTags were homologous to ESTs annotated to characterized Uniprot entries. However, some of the up-regulated tags still remain to be assigned to the gene they are originating from. The most up-regulated, but non-annotatable UniTags in the ICC588 drought-stress dataset are listed in **Table 6-4**.

Table 6-4 Most up-regulated non-annotable UniTags in drought-stressed chickpea roots

Tag code	Sequence	$R_{(ln)}$	Differential expression (fold)
STCa-4092	CATGATAAAGTTTGTTTCTTATATCT	4.18	65.17
STCa-23915	CATGTTTCAGCTTATGAAGAACAAGT	3.77	43.47
STCa-3818	CATGAGTAGTGTGAACTTTTTCTCTT	3.42	30.42
STCa-18846	CATGTATATTTGCTTTATGGGATCCT	3.34	28.25
STCa-9961	CATGCTGCAGAACTACTATTCTTTCC	3.34	28.25
STCa-6550	CATGCAAGTGCATCAAAAGGAAGGGG	3.30	27.17
STCa-21621	CATGTGTATTTCTTTATGCTATATAG	3.30	27.17
STCa-4590	CATGATCAGTAGATCACTAAATAAAT	3.30	27.17
STCa-5391	CATGATGTATCAGCTCGTAGTAAGAG	3.26	26.08
STCa-105	CATGAAAACATTGATGCTATGTGTAT	3.26	26.08
STCa-16605	CATGGTTGAAGCAAAATAAATTGTTA	3.26	26.08
STCa-19256	CATGTATTGAATAAAAGTTATGATGA	3.22	24.98
STCa-18178	CATGTAGAAGTTTTAATTCATCTATG	3.17	23.90
STCa-387	CATGAAAGAAAATCAATTATGTGGGC	3.17	23.90
STCa-24344	CATGTTTTGATGAAGTTTTAAGGATT	3.17	23.90
STCa-12193	CATGGATATTGAATTCGAGCAGAAAA	3.13	22.81
STCa-5638	CATGATTATTATTATTGTTGTAATGG	3.10	22.26
STCa-22062	CATGTGTTTACCATTTTCTAATATTG	3.08	21.74
STCa-23884	CATGTTTATTTGTTAACGTTCCTTTT	3.08	21.74
STCa-10367	CATGCTTGGTTAGATATGTTGTTTTT	3.03	20.64
STCa-170	CATGAAAATAAGACATCATAAGAACT	3.00	20.11
STCa-3839	CATGAGTATGTTTGAAAATAAATTGT	2.97	19.55
STCa-5928	CATGATTTATTATACCTTGCCAAGAT	2.92	18.47
STCa-175	CATGAAAATAATTGTCTATTTAGGTG	2.86	17.39
STCa-5308	CATGATGGTATTAGTGAATAAAAAGA	2.86	17.39
STCa-23006	CATGTTCTGGGAATCAAAAAAAAAAA	2.86	17.39
STCa-219	CATGAAAATGAGGTGGTGCTGAAGGA	2.86	17.39
STCa-10095	CATGCTGTCTCACAAATGAGATTGAC	2.86	17.39
STCa-16058	CATGGTGCGATTGAGTCTAAAAGGAG	2.79	16.30
STCa-19365	CATGTATTTTGAGTCTAGAATGAATG	2.79	16.30

6.2 Correlation of SuperSAGE profiles with GO categories in roots of drought-stressed chickpea plants

6.2.1 Most over-represented GO biological processes in drought-stressed roots

As shown in **Table 6-5A**, GO biological processes such as Translation (GO:0006412), Response to stimulus (GO:0050896), Generation of precursor metabolites and energy (GO:0006091), Response to biotic stimulus (GO:0009607), Proteolysis (GO:0006508), Protein amino acid phosphorylation (GO:0006468), Defense response (GO:0006952), and Protein

transport (GO:0015031) scored highest in over-representation in ICC588 roots after 6h of dehydration (P<1.0E-12). In general, 3 of the 30 listed categories are associated with defense and response mechanisms, 7 categories with active transport, 2 categories with cell wall, 5 categories with nucleotide metabolism and biosynthetic processes, 5 categories with general metabolism, and one category with ROS-scavenging.

As already described for salt stress-related processes (Section **4.3.2**), some GO categories may involve transcripts associated with the same GO term, but possessing contrasting degrees of regulation. For example, in drought-stressed roots, biological processes like translation, response to stimulus, generation of precursor metabolites and energy, protein amino acid phosphorylation, defense response, carbohydrate metabolic process, and electron transport are also found among the drought under-represented GO biological processes. (**Table 6-5B**).

The present results suggest, that chickpea roots respond to drought stress with the expected stress response, but additionally undergo a strong global re-arrangement of their metabolism and protein machinery.

6.2.2 Most over-represented GO cellular components in roots of drought-stressed chickpea plants

After a GSR analysis selecting for the representation of GO cellular component categories in chickpea roots dehydrated for 6h, Ribonucleoprotein complex (GO:0030529), Mitochondrion (GO:0005739), Endoplasmic reticulum, Cytosol (GO:0005829), and Organelle part (GO:0044422), were over-represented with P<6.2 E-3. Still with high significance, three components related to proton and small molecule transport as well as other components associated with membranes and cytoskeleton, were detected (**Table 6-5C**). These observations are in concordance with the GO biological processes and suggest, that the protein biosynthesis machinery is significantly reacting upon drought stress. Also, metabolic disorders are reflected in components like mitochondrion, where processes like respiration and increased ROS production take place.

Table 6-5A Over-represented GO biological processes as deduced from transcript abundances (annotated to UniProt entries) in drought-stressed chickpea roots

GO ID	GO Biological process	Rank	P
GO:0006412	Translation	1	1.00E-12
GO:0050896	Response to stimulus	2	1.00E-12
GO:0006091	Generation of precursor metabolites and energy	3	1.00E-12
GO:0009607	Response to biotic stimulus	4	1.00E-12
GO:0006508	Proteolysis	5	1.00E-12
GO:0006468	Protein amino acid phosphorylation	6	1.00E-12
GO:0006952	Defense response	7	1.00E-12
GO:0015031	Protein transport	8	1.00E-12
GO:0005975	Carbohydrate metabolic process	9	1.00E-12
GO:0006118	Electron transport	10	1.00E-12
GO:0006812	Cation transport	11	1.00E-12
GO:0007047	Cell wall organization and biogenesis	12	1.00E-04
GO:0046907	Intracellular transport	13	1.00E-04
GO:0044248	Cellular catabolic process	14	1.00E-04
GO:0051641	Cellular localization	15	1.00E-04
GO:0006807	Nitrogen compound metabolic process	16	1.00E-04
GO:0015672	Monovalent inorganic cation transport	17	1.00E-04
GO:0009117	Nucleotide metabolic process	18	1.00E-04
GO:0006164	Purine nucleotide biosynthetic process	19	2.00E-04
GO:0006753	Nucleoside phosphate metabolic process	20	2.00E-04
GO:0006730	One-carbon compound metabolic process	21	2.00E-04
GO:0006119	Oxidative phosphorylation	22	2.00E-04
CMC-1	General ROS scavenging enzymes	23	2.00E-04
GO:0055086	Nucleotide metabolic process	24	3.00E-04
GO:0009108	Coenzyme biosynthetic process	25	3.00E-04
GO:0006811	Ion transport	26	5.00E-04
GO:0015992	Proton transport	27	5.00E-04
GO:0009057	Macromolecule catabolic process	28	6.00E-04
GO:0009664	Cellulose and pectin-containing cell wall organization	29	6.00E-04
GO:0009165	Nucleotide biosynthetic process	30	6.00E-04

Table 6-5B Under-represented GO biological processes as deduced from transcript abundances (annotated to UniProt entries) in drought-stressed chickpea roots

GO ID	GO Biological process	Rank	P
GO:0006464	Protein modification process	1	1.00E-12
GO:0043412	Biopolymer modification	2	1.00E-12
GO:0006457	Protein folding	4	1.00E-04
GO:0043687	Post-translational protein modification	3	1.00E-04
GO:0006468	Protein amino acid phosphorylation	5	1.00E-03
GO:0032502	Developmental process	6	1.80E-03
GO:0030154	Cell differentiation	7	0.00
GO:0006952	Defense response	8	0.00
GO:0006512	Ubiquitin cycle	9	0.00
GO:0016310	Phosphorylation	10	0.00
GO:0006793	Phosphorus metabolic process	11	0.00
GO:0019941	Modification-dependent protein catabolic process	12	0.00
GO:0009059	Macromolecule biosynthetic process	13	0.00
GO:0008219	Cell death	14	0.01
GO:0006091	Generation of precursor metabolites and energy	15	0.01
GO:0006412	Translation	16	0.01
GO:0006118	Electron transport	17	0.02
GO:0006605	Protein targeting	18	0.02
GO:0050789	Regulation of biological process	20	0.02
GO:0050794	Regulation of cellular process	19	0.02
GO:0065007	Biological regulation	21	0.02
GO:0006915	Apoptosis	22	0.02
GO:0048519	Negative regulation of biological process	23	0.02
GO:0016043	Cellular component organization and biogenesis	24	0.03
GO:0006350	Transcription	25	0.04
GO:0009056	Catabolic process	26	0.04
GO:0031323	Regulation of cellular metabolic process	27	0.04
GO:0005975	Carbohydrate metabolic process	28	0.04
GO:0019222	Regulation of metabolic process	29	0.04
GO:0050896	Response to stimulus	30	0.04

Table 6-5C Over-represented GO cell components as deduced from transcript abundances (annotated to UniProt entries) in drought-stressed chickpea roots

GO ID	GO cellular component	Rank	P
GO:0030529	Ribonucleoprotein complex	1	1.00E-12
GO:0005739	Mitochondrion	2	5.00E-04
GO:0005783	Endoplasmic reticulum	3	7.00E-04
GO:0005829	Cytosol	4	1.80E-03
GO:0044422	Organelle part	5	6.20E-03
GO:0033178	Proton-transporting two-sector ATPase complex	6	0.01
GO:0012505	Endomembrane system	7	0.01
GO:0044445	Cytosolic part	8	0.01
GO:0045259	Proton-transporting ATP synthase complex	9	0.02
CMC-2	Aquaporins and transmembrane channels	10	0.03
GO:0005576	Extracellular region	11	0.04
GO:0009536	Plastid	12	0.06
GO:0005839	Proteasome core complex	13	0.08
GO:0005856	Cytoskeleton	14	0.09
GO:0031090	Organelle membrane	15	0.09
GO:0000785	Chromatin	16	0.11
GO:0048046	Apoplast	17	0.12
GO:0031975	Envelope	18	0.14
GO:0005694	Chromosome	19	0.15

6.3 Diverse drought-related processes and expression profiles of involved genes in drought-stressed chickpea roots

6.3.1 ROS production and detoxification

6.3.1.1 Mitochondrial respiration

Considering that UniTag STCa-7166, annotated to a NADP-dependent isocitrate dehydrogenase, was one of the most up-regulated transcripts ($R_{(ln)}$=3.58; 36.0-fold) after onset of drought in chickpea roots (**Table 6-1A**), and further considering that one of the other three ICDH transcript variants also showed high expression levels (2.7-fold up-regulation, **Table 6-6**), it can be postulated that mitochondrial respiration is increasing in chickpea under dehydration. The expression profiles of these mitochondrial respiration "indicators" suggest that mitochondrial ROS production may also increase upon drought stress in chickpea roots (6h of dehydration), mimicking the response of salt-stressed roots. Further on, and congruent with the situation in salt-stressed INRAT-93, from eight UniTags annotated to alternative oxidase (AOX), five transcript variants (STCa-14424, STCa-14426, STCa-20476, STCa-14428, STCa-14427) were at least 4.0-fold down-regulated (**Table 6-6**).

6.3.1.2 Scavenging of superoxide- and hydrogen peroxide-radicals

Three out of seven SOD-annotated UniTags (STCa-3770, STCa-7894, and STCa-7896) were more than 2.7-fold up-regulated in dehydrated chickpea roots, whereas the remaining four transcript variants remained constitutively expressed. Moreover, one of two CAT-annotated transcripts was more than 2.7-fold up-regulated (STCa-24141). Both DHAR-annotated UniTags remained constitutively expressed, and one of the three APX transcripts was 4.0-fold down-regulated (STCa-11616; **Figure 6-1**). From 14 UniTags annotated to GSTs, four transcript variants (STCa-977, STCa-2175, STCa-20830 and STCa-12384) were at least 2.7-fold down-regulated, whereas three of them revealed 2.7-fold up-regulation (STCa-3042, STCa-12502 and STCa-22470; **Table 6-6**). The transcription of UniTags annotated to thioredoxin and peroxiredoxin, two enzyme with ROS-scavenging activity, did not reveal up-regulation. On the contrary, from three peroxiredoxin transcript variants, two were more than 8-fold down-regulated (STCa-10052 and STCa-23664; **Table 6-6**).

Regarding enzymatic recycling of oxidized gluthatione, this process appears to have a high relevance in the ROS-scavenging machinery of chickpea roots. This assumption is

supported by the strong up-regulation ($R_{(ln)}$=3.08; 22-fold) of a cysteine synthase (Crespi et al.) encoding UniTag (STCa-2982) (**Table 6-1A**). As a consequence of an increased CS activity in rice, both, the total glutathione and the reduced glutathione pools were significantly increased in response to aluminium stress (Yang et al., 2007)

The general tendency, extracted from these results, indicates that the ROS-scavenging machinery in chickpea ICC588 roots reacts upon dehydration. However, probably not to the same magnitude as observed in salt-stressed INRAT-93 plants.

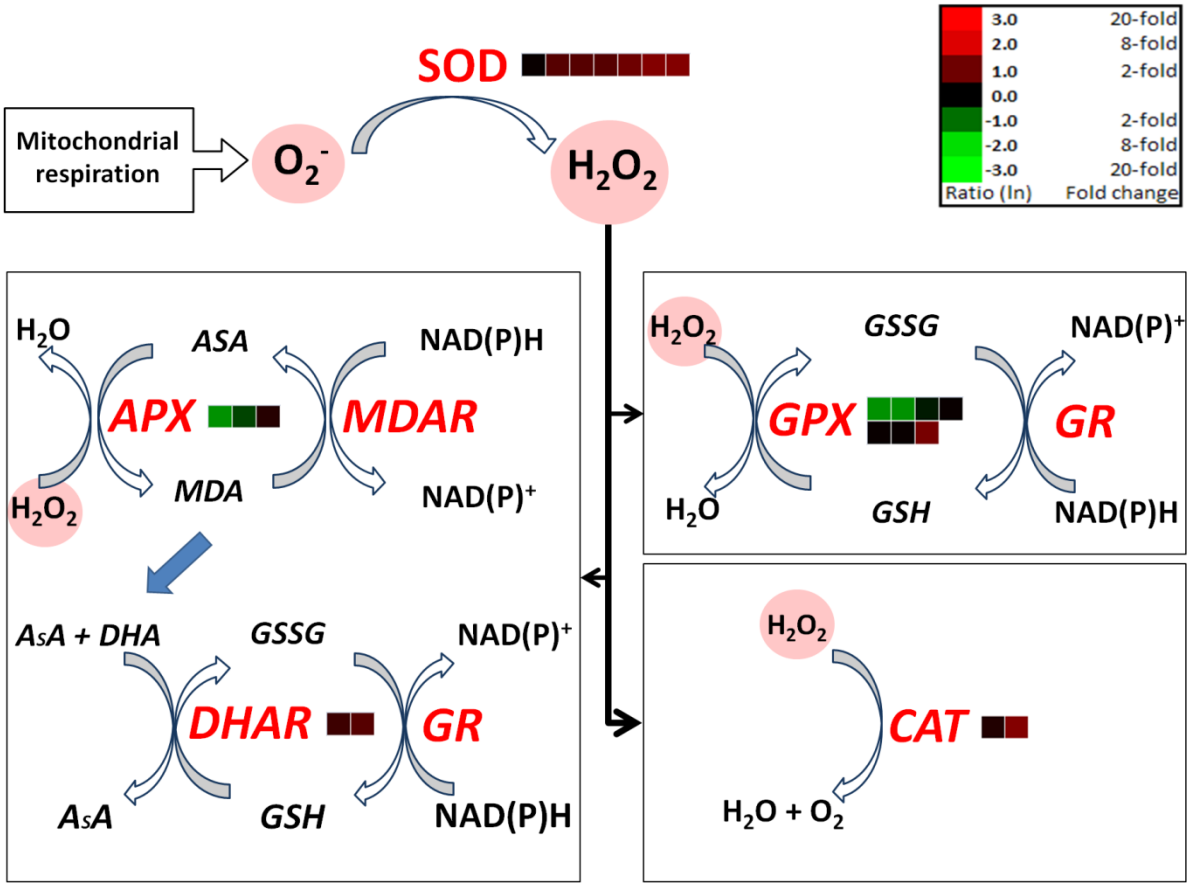
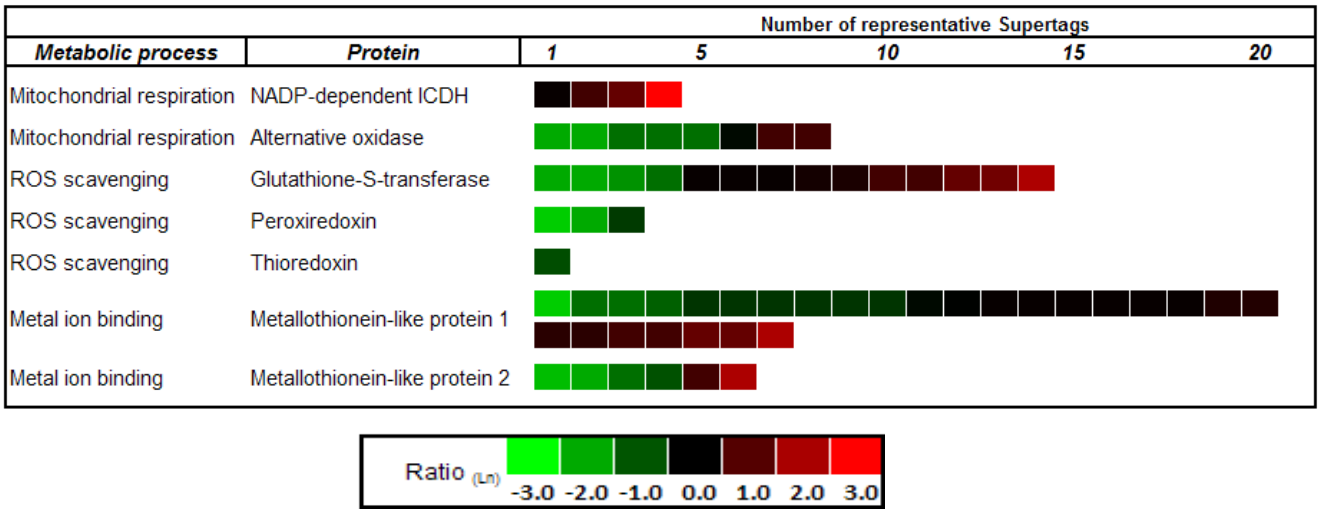


Figure 6-1 Major ROS scavenging processes in plant cells along with transcription profiles of related UniTags from drought stressed chickpea roots

Table 6-6 Additional UniTags annotated to genes encoding proteins of ROS metabolism and their transcription levels in drought- stressed chickpea roots



6.3.2 ROS-triggered and general stress-related signal transduction

6.3.2.1 Signaling sensors

As discussed in Section **6.1.1.4**, RLK genes are transcribed into a broad array of transcript variants and regulated to various levels in drought-stressed chickpea roots. No clues for a differentiation between drought/salt- and H_2O_2-triggered signaling cascades, respectively, can be extracted from the information contained within the 26bp tag sequence. However, by combining the salt and drought chickpea information, potential candidate RLKs with positive responses to both stresses can be identified. Only two UniTags, STCa-7800 and STCa-24316, among the many RLK transcript variants in dehydrated ICC588 roots (36, **Figure 6-2**) are commonly more than 2.7-fold up-regulated under both stresses. However, UniTag STCa-7800 revealed a much higher stress-induced differential expression in desiccated roots (35-fold drought- vs 7-fold salt-up-regulation), a fact that can be relevant for its stress-induction specificity.

6.3.2.2 Calcium-dependent signaling events

Transcript variants encoding proteins involved in Ca^{2+}-driven signaling cascades were up- as well as down-regulated in response to drought stress in chickpea roots. These included transcripts encoding a wide range of kinases such as CDPKs (Romeis et al., 2001), calmodulin, and calmodulin-binding proteins. However, there is clear difference between the transcription levels of CDPKs versus the latter two classes of proteins. In drought-stressed chickpea roots, transcript levels of UniTags annotated to calmodulin and calmodulin-binding proteins show a general down-regulation tendency (6 out of 8 UniTags

were more than 2.7 fold down-regulated). On the other hand, from seven CDPK-annotated UniTags, four were more than 2.7-fold up-regulated (STCa-4552, STCa-16072, STCa-4079, and STCa-17567; **Figure 6-2**). This contrasts with the transcription levels observed under salt stress, where calmodulin and calmodulin-binding proteins showed a slight up-regulation tendency.

6.3.2.3 MAP-kinases-related signal transduction

The interplay between protein kinases and protein phosphatases balances the activation and inactivation of several signaling cascades (Smith and Walker, 1996; Hardie, 1999). However, from a transcriptional point of view, MAP-kinases do not seem to interact much with early drought-stress signaling in chickpea roots. From 11 discovered MAPK-transcripts, 4 were down-regulated more than 2.7-fold (STCa-13432, STCa-10032, STCa-6718, and STCa-4402), 6 were similarly expressed as in control roots, and only one transcript variant was slightly up-regulated (STCa-10942, 2.0-fold). Whereas none of the two detected MAPK-kinases was more than 2.7-fold up-or down-regulated, respectively. From three UniTags annotated to MAPKK-kinases, two were at least 4.0-fold down-regulated (STCa-8893, STCa-10844), and one transcript was 6-fold up-regulated (STCa-2124) (**Figure 6-2**).

Aside of the transcriptional regulation of genes encoding proteins of the MAP-kinase cascades, the activity of the various proteins within the cascade is regulated by post-transcriptional modifications, all embedded in a delicate and redundant network of cross-talk events (Hardie, 1999). For that reason it is difficult to draw conclusions about the behaviour of these genes in drought-stressed chickpea roots on the basis of only transcript levels.

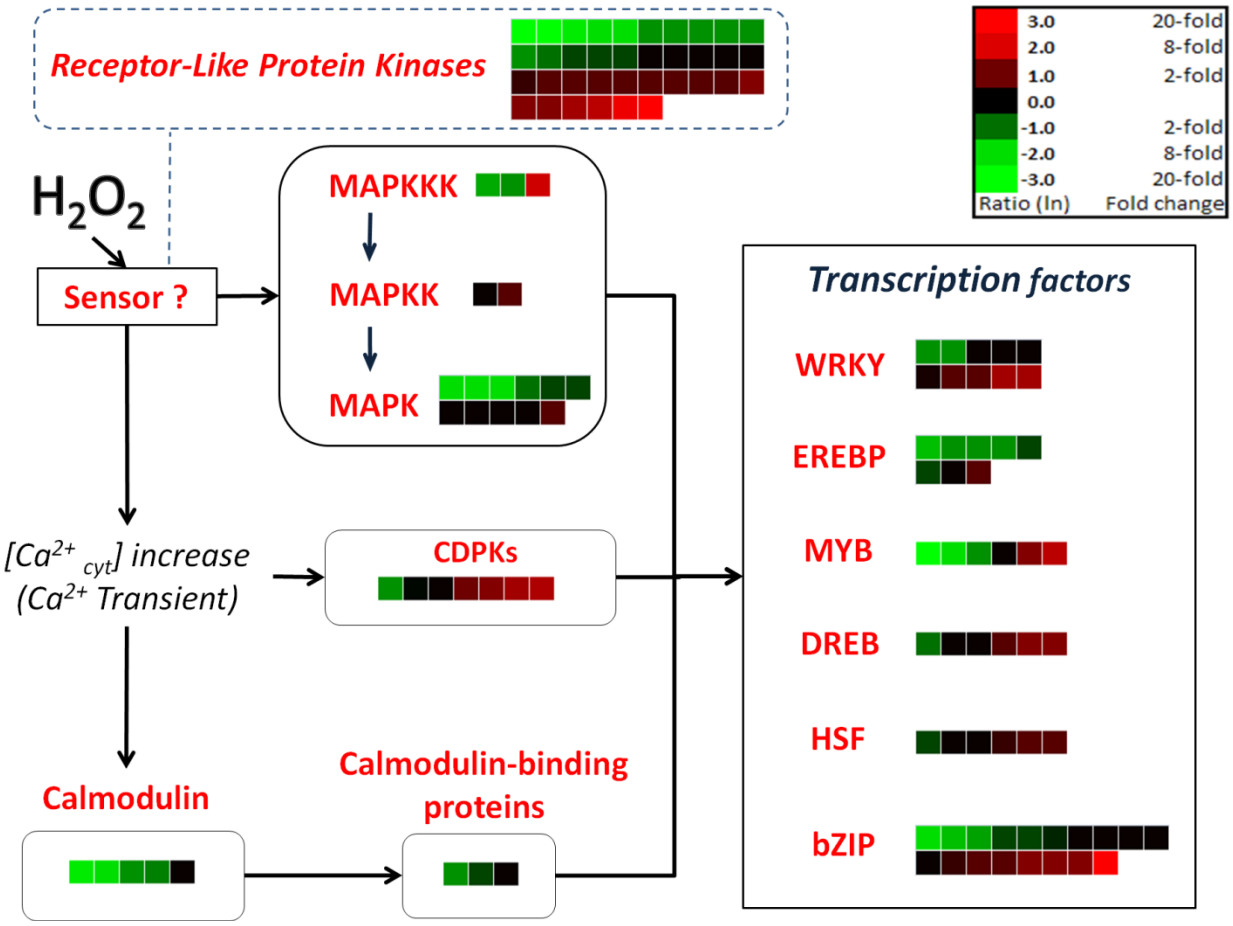


Figure 6-2 Oxydative stress signaling pathways along with transcription profiles of related UniTags in drought-stressed chickpea roots

6.3.3 Transcription factors

A general distribution of the TF classes found in the SuperSAGE dataset of chickpea ICC588 roots, based on the number of representative UniTags, is shown in **figure 6-3**. Additionally, the expression levels of each transcript variant annotated to the most abundant TF classes after drought stress are detailed in **Table 6-7**.

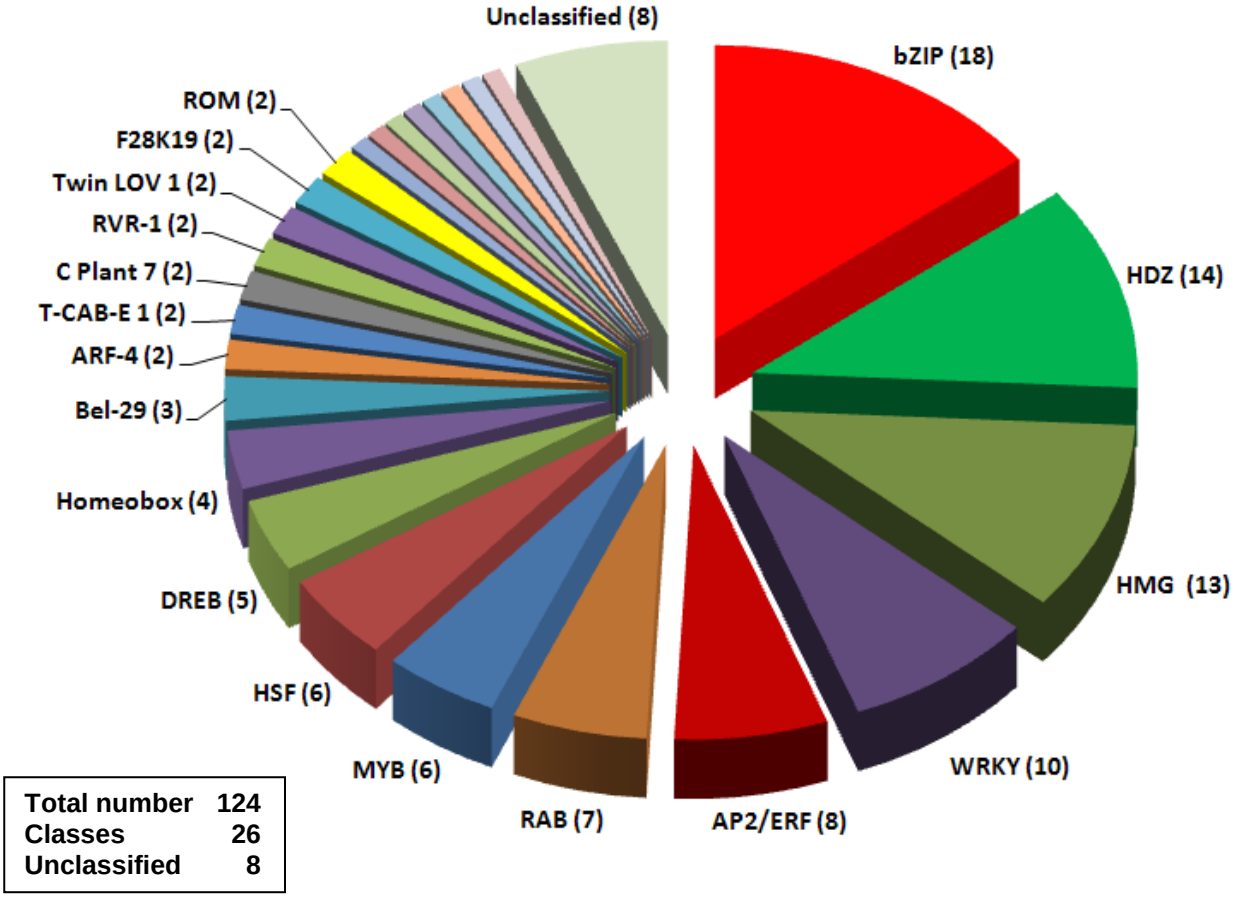
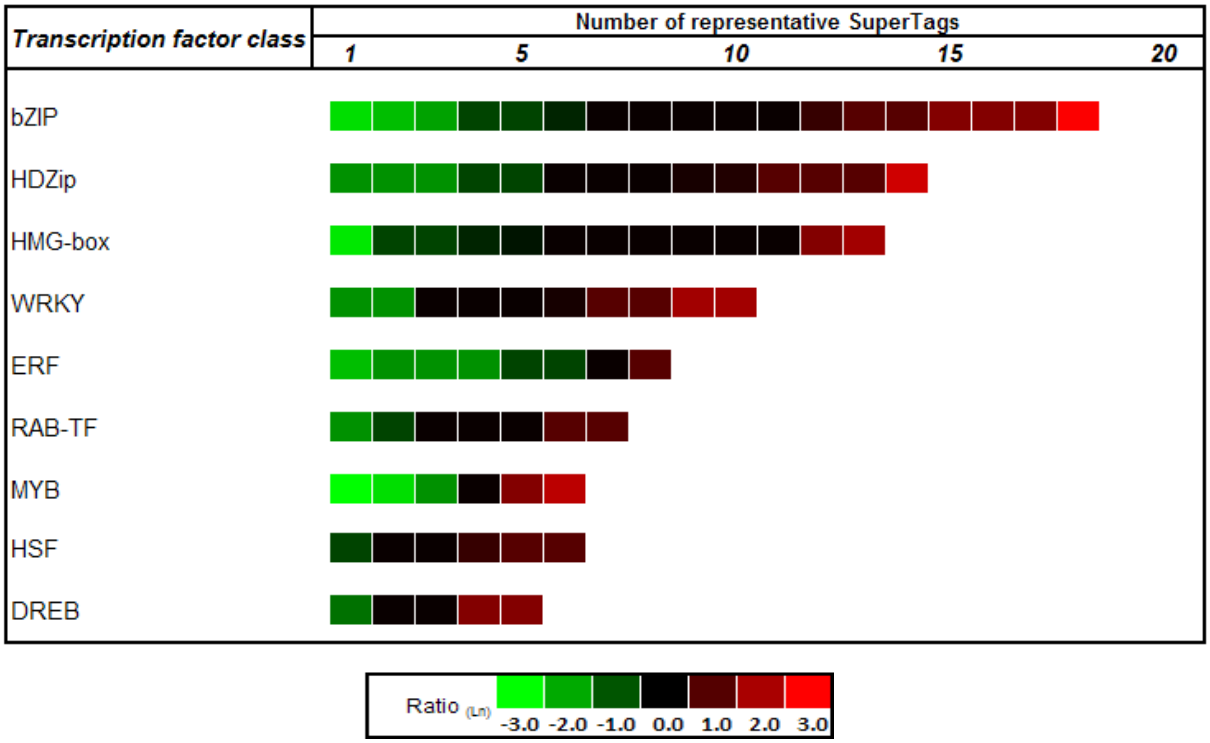


Figure 6-3 **Transcription factor classes in SuperSAGE libraries from drought-stressed chickpea roots.**

Numbers in parentheses represent the number of UniTags annotated to each class

Table 6-7 **Expression levels of UniTags annotated to different TF classes in the root of chickpea ICC588**



6.3.3.1 bZIP transcription factors in roots of ICC588 plants

As previously observed in the salt-responsive transcriptome of INRAT-93 roots, the most represented TF class (regarding number of annotated UniTags) is the bZIP category. A total of 18 bZIP-annotated UniTags were detected in the dataset from ICC588 roots. From these, four UniTags were more than 2.7-fold up-regulated (highest up-regulation: STCa-14940, 9.8-fold), and three UniTags were more than 2.7 fold down-regulated (highest down-regulation: STCa-1765, 7.4-fold; **Table 6-7)**. Neither in this, nor the INRAT-93 dataset any UniTag annotated to AREB/ABF-TFs could be detected, though this bZIP subclass is well represented in drought-stressed tissue (Choi et al., 2000). Therefore, further characterization of drought-responsive bZIP UniTags, like UniTag STCa-14940, should be therefore a priority for future steps.

6.3.3.2 HDZ transcription factors in roots of ICC588 plants

A total of 14 UniTags was detected in ICC588 roots, that could be annotated to HDZ TFs. From these, four and three tags, respectively, were more than 2.7-fold up- or down-regulated. The highest induction level was reached by UniTag STCa-247 (6.5-fold), whereas UniTag STCa-23634 was the most down-regulated (3.7-fold; **Table 6-7**). Generally, HDZ TFs are represented by a considerable number of transcript variants. However, the range of

responses upon drought stress is relatively narrow, when compared to other TF classes (e.g. bZIPs). Previous studies report on the involvement of HDZs in drought responses. As an example, Dezar and co-authors (2005) improved the drought tolerance of *Arabidopsis* plants transformed with the sunflower gene Hahb-4, a HDZ TF that proved to be ABA- and drought-responsive.

6.3.3.3 HMG-box transcription factors

In the ICC588 dataset, a total of 13 UniTags were annotated to HMG-box TFs. Only two and one transcript variant, respectively, were more than 2.7-fold up- or down-regulated. From these, UniTag STCa-8711 was the most strongly differentially expressed after drought stress (8.1-fold down-regulated) (**Table 6-7**). As detailed in Section **4.4.3.3**, not much information is known about the role of HMG-box TFs in abiotic stress responses.

6.3.3.4 WRKY transcription factors

In the context of drought stress, WRKY TFs interact with elements of the ABA-responsive signaling pathway (Zou et al., 2004). In a previous study, which functionally characterized the whole rice WRKY super-family with respect to interactions with ABA-dependent signaling, TFs of this class played positive as well as negative regulator roles (Xie et al., 2005). In drought-stressed chickpea roots, ten UniTags were annotated to TFs of the WRKY class. From these, two transcript variants were up-regulated more than 2.7-fold (STCa-11618 and STCa-11619), whereas two UniTags revealed 2.7-fold down-regulation (STCa-3289 and STCa-19868; **Table 6-7**).

6.3.3.5 Ethylene-responsive transcription factors

In drought-stressed roots of chickpea plants, UniTags annotated to ERFs show a general down-regulation tendency. From eight transcript variants, no UniTag was detected to be more than 2.7-fold up-regulated. On the other hand, four of the detected UniTags were at least 2.7-fold down-regulated (STCa-14442, STCa-14837, STCa-3847, STCa-2398; **Table 6-7**). In previous studies, ethylene has been reported to have negative effects on drought tolerance. For example, in wheat, a physiological effect caused be ethylene known as “drought stress syndrome” was found to be closely related to ROS production (Beltrano et al., 1999), suggesting that this plant hormone accelerates the oxidative stress under drought conditions. Further on, similar effects were reported in holm oak, where airborne ethylene

reduced the oxidative stress protection and the water deficit tolerance young plants (Munne-Bosch et al., 2004). The results observed in chickpea suggest, from the transcriptional point of view, that ethylene-controlled cascades are suppressed in drought-stressed roots. Probably, as a strategy to prevent ROS overproduction.

6.3.3.6 MYB transcription factors

As previously described in the context of salt stress, TFs belonging to the MYB class play important roles in ABA-dependent signaling (Yanhui et al., 2006). In drought stressed chickpea roots, six UniTags were annotated to MYB TFs. Quite contrasting with what would be expected for major regulators of drought responses, only two of the transcript variants were more than 2.7-fold up-regulated (STCa-11693, 5.5-fold; STCa-22351, 3.2-fold). On the other hand, three transcript variants were more than 2.7-fold down-regulated, showing UniTag STCa-6267 as the most repressed (11-fold; **Table 6-7**). In rice, Dai and co-authors (2007) reported on the enhancement of drought tolerance through the over expression of a single MYB-TF (out of thirteen). This observation suggests that although MYBs are mostly down-regulated in drought-stressed chickpea roots, the few up-regulated transcripts may still play important roles. These results evidence, that there must be a considerable component of "fine tuning" in the orchestration of responses against drought stress in ICC588 roots. Such fine tuning of drought responses has previously been emphasised for plants in general by Seki and co-authors in an extensive review (Seki et al., 2007).

6.3.3.7 Heat shock factors

Heat shock transcription factors (Hsfs) are TFs encoded by a large gene family in plants. They are thought to function as a highly redundant and flexible gene network, that controls the response of plants to different environmental stress conditions. In the drought (and salt) stress context, Hsfs have been proposed to actively interact with signaling pathways, functioning as potential H_2O_2 sensors (Miller and Mittler, 2006). In chickpea drought-stressed roots, none of the six UniTags annotated to Hsfs was more than 2.7 fold up- or down-regulated, respectively (**Table 6-7**).

6.3.3.8 DREB transcription factors

DREB transcription factors are thought to regulate plant responses to dehydration in an ABA-independent manner (Liu et al., 1998). In drought-stressed roots from the salt-tolerant chickpea variety ICC588, six transcript variants were annotated to this class of TFs.

From them, two (STCa-4170 and STCa-4212) and one UniTag (STCa-13360), respectively, were more than 2.7-fold up- and down-regulated, whereas the remaining kept constitutive levels (**Table 6-7**). As already approached in the context of salt stressed roots (Section **4.4.3.7**), the sequence information contained within the 26bp tag is not powerful enough to differentiate between DREB1 (mostly cold-responsive) and DREB2 (mostly salt/drought-responsive) sub-classes. This may explain the different regulation levels observed in drought stressed chickpea roots.

6.3.4 ABA-dependent and ABA-independent signaling

Briefly, in the ABA-dependent pathway at least one of the UniTags annotated to each detected component (i.e. bZIP and MYB Tfs, MAPKs, CDPKs, CBLs, **ABA-responsive proteins**, and the negative regulator PP2C) is up-regulated, supporting from the transcriptional point of view an active ABA-dependent pathway in drought-stressed chickpea roots (**Figure 6-4**). However, UniTags annotated to proteins involved in ABA-biosynthesis are not reacting to drought stress. Two UniTags (STCa-18782, and STCa-21012) annotated to 9-*cis*-Epoxycarotenoid dioxygenase, a rate-limiting protein in ABA biosynthesis (Iuchi et al., 2000), were constitutively expressed and slightly down-regulated, respectively (**Table 6-8**).

On the other hand, 19 UniTags annotated to beta-glucosidase, an enzyme catalyzing ABA-release from conjugates (Dietz et al., 2000), exhibited diverse regulation levels (**Table 6-8**). From these, three transcript variants were highly induced (STCa-228, 25-fold; STCa-227, 12-fold; and STCa-16215, 9-fold). This result indicates that *de novo* synthesis of ABA is not a primary reaction of chickpea plants upon drought stress, as also observed in salt-stressed INRAT-93 roots (**section 4.4.2.4**). However, ABA signaling may be active through the use of other ABA-sources.

As far as the ABA-independent pathway is concerned and as detailed in section **6.3.3.8**, at least two UniTags annotated to DREB TFs are transcriptionally active upon drought stress. On the other hand, UniTags annotated to CBL proteins remained constitutive (or only slightly up-regulated). These results indicate, that the ABA-independent signaling pathway is also transcriptionally active in dehydrated chickpea roots.

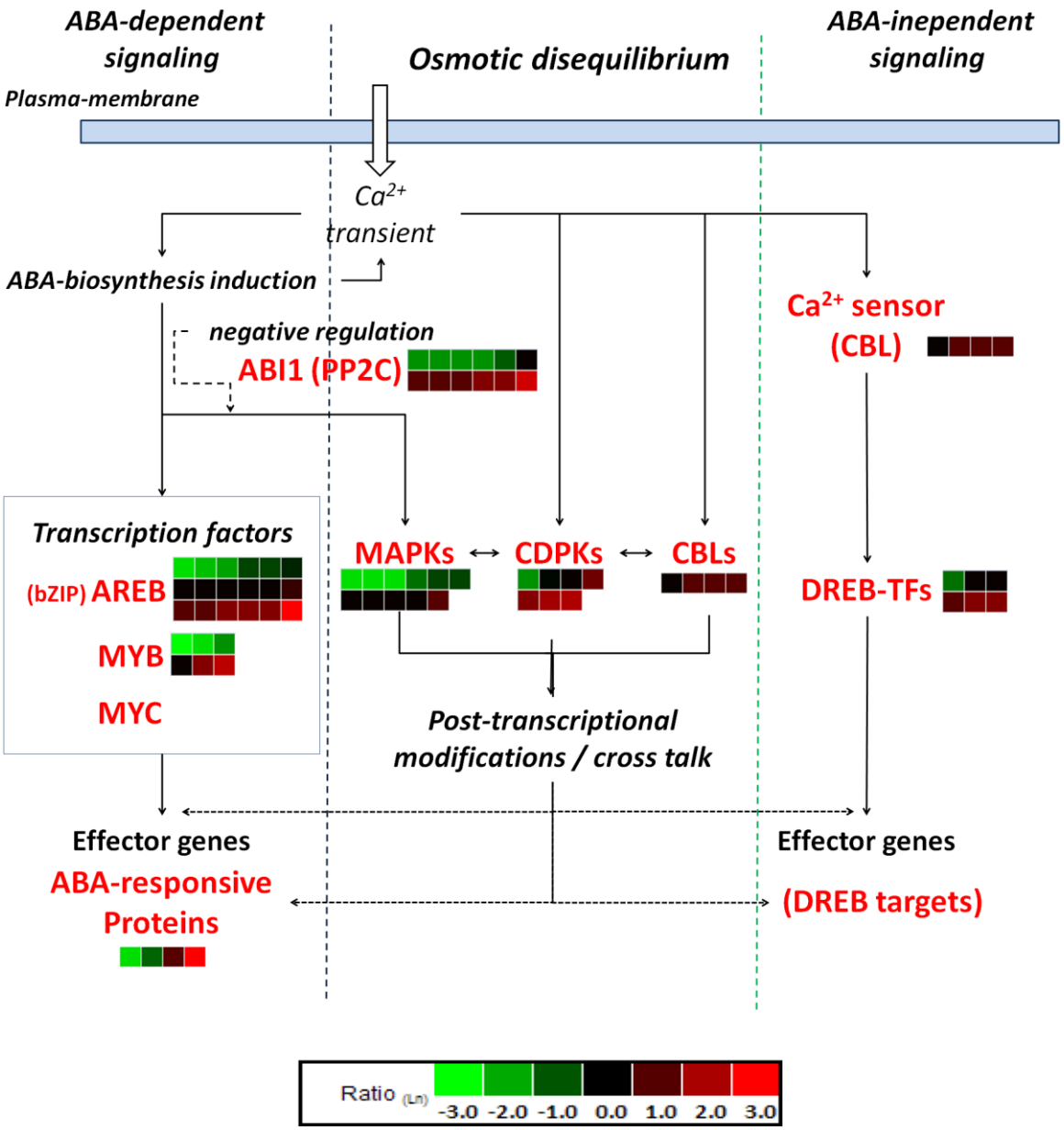
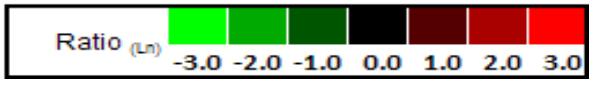


Figure 6-4 **ABA-dependent and -independent signaling cascades along with transcription profiles of related UniTags in drought-stressed chickpea roots**

Table 6-8 **UniTags annotated to transcripts encoding proteins for ABA-biosynthesis and release of ABA-conjugates and their expression levels in chickpea drought-stressed roots**

		Number of representative SuperTags				
Metabolic process	***Protein***	***1***	***5***	***10***	***15***	***20***
ABA biosynthesis	9-cis-Epoxycarotenoid dioxygenase					
ABA conjugates release	Beta-glucosidase					



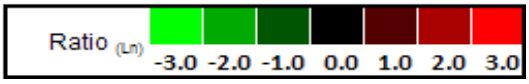
6.3.5 Transcriptional regulation of genes encoding aquaporins under drought stress

In ICC588 roots, 62 UniTags annotated to MIP aquaporins were detected, that were diversely regulated after 6h of dehydration. From them, 39 UniTags remained as unclassified MIPs, 19 were annotated to the TIP, 3 to the PIP, and one UniTag was annotated to the NIP subclass. UniTag STCa-21968, annotated to a PIP2 protein, showed the highest up-regulation among all aquaporins (28-fold). The unclassified MIPs revealed expression levels between 11-fold and 5-fold down- and up-regulation, respectively. Regarding tonoplast intrinsic proteins, from 19 transcript variants, only one (UniTag was more than 8-fold up-regulated (STCa24453), whereas the remaining 18 showed expression levels between 8- and 4-fold down- and up-regulation, respectively (**Table 6-9)**. Similar results showing diverse expression levels of aquaporin members under drought stress have been reported by Alexanderson and co-authors (2005) in *Arabidopsis*. After evaluating the expression levels of more than 35 aquaporin members, up- and down-regulation was correlated in many cases with the sampled organ (roots, flowers, leaves). Since in chickpea only roots were analyzed, some of the up- or down-regulated transcript variants may have contrasting regulation levels in other organs after dehydration.

Interestingly, the over-expression of a certain aquaporin isoform in transgenic *Arabidopsis* plants resulted in altered expression patterns of other MIPs and implied changes in seed germination rates, seedling growth, and responses of the plants under various stress conditions (Jang et al. 2007). These results suggest a concerted transcriptional regulation of at least a subset of aquaporin genes, a fact that cannot be proven with the present chickpea profiles, but not also excluded.

Table 6-9 Expression profiles of UniTags annotated to MIP and TIP proteins in drought stressed chickpea roots

Protein	Number of representative UniTags *1* *5* *10* *15* *20*
MIP (unclassified)	
TIP	
PIP	
NIP	



6.3.6 UniTags annotated to proteins active in compatible osmolyte accumulation in drought-stressed chickpea roots

After screening the ICC588 dataset for transcripts annotated to genes related to osmolyte accumulation, transcription profiles from 12 genes involved in sugar-, amino acid-, and polyamide-accumulation were identified. Detailed information for each osmolyte category is presented in the following sub-sections.

6.3.6.1 Sugar accumulation

As far as sugar accumulation is concerned, one UniTag annotated to trehalose-6-phosphate synthase (STCa-18759, 2-fold down-regulated), and three UniTags annotated to trehalose-6-phosphate phosphatase (STCa-9149 3-fold up-regulated; STCa-11438 3-fold down-regulated; STCa-21065 constitutive) were detected (**Table 6-10**). Trehalose plays an important role as compatible osmolyte and signaling molecule under drought stress (Garg et al., 2002; Avonce et al., 2004). However, the single UniTag annotated to threhalose-6-phosphate synthase, one of the rate-limiting enzymes in trehalose production, was down-regulated. This result indicates there is not a marked transcriptional activation of trehalose biosynthesis as a response to drought stress in chickpea roots.

Additionally, one 4.4-fold up-regulated UniTag annotated to galactinol synthase (STCa-11968), as well as at least three UniTags (STCa-19100, STCa-8449, STCa-16426) representing transcripts related to sucrose metabolism and transport (each showing more than either 2.7-fold up- or down-regulation) suggest, that the dynamics of sugar metabolism, transport, and accumulation is altered in response to drought stress in chickpea roots.

6.3.6.2 Accumulation of amino acids

Several UniTags annotated to amino acid transport- and -accumulation-related genes were detected in the ICC588 root dataset. UniTag STCa-24308 with homology to a proline/betain transporter was 6-fold up-regulated, whereas UniTags STCa-8454 STCa-8455 representing a negative regulator for proline accumulation (proline dehydrogenase; Verdoy et al., 2006) were moderately down- or up-regulated, respectively. Further on, one transcript annotated to betaine aldehyde dehydrogenase (STCa-14752) revealed a slight down-regulation (1.5-fold; **Table 6-10**).

The present results reveal that expression changes of genes involved in amino acids accumulation react positively as well as negatively upon drought stress in chickpea roots,

however no clear up-regulation tendency allows suggesting that the accumulation of this type of osmolytes is markedly promoted (at transcription level).

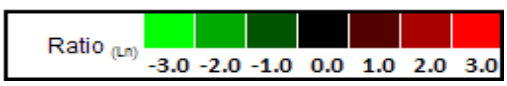
6.3.6.3 Accumulation of polyamines

Arginine decarboxylase and spermidine synthase share important roles in the accumulation of putrescine and spermidine, which act as compatible osmolytes in plants (Capell et al., 2004). One UniTag coding for each arginine decarboxylase and spermidine synthase, respectively, were 2-fold (STCa-8875) and 3-fold (STCa-611) up-regulated (**Table 6-10**). Thus, there is at least a slight response of some components of the polyamine metabolism upon drought stress in chickpea roots.

The results of the present Section indicate that sugars-, aminoacids- and polyamines-accumulation mechanisms in chickpea roots are reacting by inducing the transcription of some of their involved genes upon drought stress. However, as mentioned in the previous chapter, this observations need to be corroborated by direct measures of these compatible osmolytes in stress affected organs.

Table 6-10 **UniTags annotated to genes encoding proteins involved in compatible osmolyte accumulation and their expression profiles in drought-stressed chickpea roots**

		Number of UniTags
Osmolyte class	***Protein***	***1 5 10***
Sugars	Sugar transporter	
Sugars	Trehalose 6-phosphate synthase	
Sugars	Trehalose-6-phosphate phosphatase	
Sugars	Galactinol synthase	
Sugars	Sucrose transport proteins	
Sugars	Sucrose synthase	
Sugars	Sucrose phosphate synthase	
Amino acids	Betaine/proline transporter	
Amino acids (repressor)	Proline dehydrogenase	
Amino acids	Betaine aldehyde dehydrogenase	
Polyamines	Arginine decarboxylase	
Polyamines	Spermidine synthase 1	



7 Similar transcriptome responses of chickpea roots under drought and salt stress

In order to select transcripts with similar or identical responses to salt and drought stress in chickpea roots, the expression ratios ($R_{(ln)}$) of common UniTags from the chickpea varieties ICC588 and INRAT-93 were analyzed with the Venn mapper 1.0 software. Three different thresholds for changes in expression were set by the software (3-fold, 8-fold, and 20-fold) to identify transcripts with similar or identical regulation tendencies under both stresses. Numbers of transcripts for each threshold are comparatively shown in **Table 7-1**.

7.1 UniTags sharing similar or identical responses to different stresses

Over all, a total of 12,117 UniTags representing 143,460 tags were commonly contained in the datasets from INRAT-93 and ICC588 roots. When the minimum threshold was set to 3-fold differential expression, then 673 (30.0%) out of 2,210 INRAT-93 root UniTags were also more than 3-fold up-regulated in drought stressed roots from ICC588 plants, representing 26.2% from the 2,529 drought-induced (>3-fold) transcripts. Substantially more, namely 1,417 UniTags were commonly down-regulated, accounting for 35.0% of the salt-, and 29.0% of the drought-repressed transcripts, respectively (**Table 7-1,** upper panel)**.**

With a threshold of 8-fold, 64 out of 337 (18%) and 282 (22%) salt- and drought-up-regulated UniTags, respectively, shared a similar or identical regulation tendency. Concerning down-regulation, the high numbers of salt-repressed UniTags (threshold: 8-fold) strongly influenced the proportions of shared responses of the transcriptomes. The overlapping proportion represented only 5.0% of the salt-, in contrast to 19% of the drought-repressed UniTags (**Table 7-1**, middle panel).

Only five UniTags were more than 20-fold up-regulated in both varieties and stress treatments (**Table 7-1**, lower panel): STCa-21968 (PIP-aquaporin; Q8W4T8_MEDTR), STCa-7166 (NADP-dependent isocitrate dehydrogenase; Q6R6M7_PEA), STCa-20066 (14-3-3-like protein A; 1433A_VICFA), STCa-19021 (extensin; O65760_CICAR), and STCa-2982 (cysteine synthase, O65747_CICAR). Annotatable UniTags with a minimum up-regulation threshold of 8-fold in both stress treatments are listed in **Table 7-2**. On the other extreme, 8 (out of 433) INRAT-93- and 49 ICC588-UniTags were commonly more than 20-fold down-regulated.

Table 7-1 Venn Mapper output showing the number of UniTags with shared regulation tendency in drought- and salt-stressed chickpea roots

Three different thresholds for changes in expression were selected by the software: 3-fold, upper panel; 8-fold, middle panel; and 20-fold, lower panel.

3.0-fold

			6h dehydration ICC588		2h NaCl-treatment INRAT-93	
			Up-regulated	down-regulated	Up-regulated	down-regulated
		Total	2,529	4,814	2,210	3,951
6h dehydration ICC588	Up-regulated	2,529			**673**	82
	down-regulated	4,814			183	**1,417**
2h NaCl-treatment INRAT-93	Up-regulated	2,210	**673**	183		
	down-regulated	3,951	82	**1,417**		

8.0-fold

			6h dehydration ICC588		2h NaCl-treatment INRAT-93	
			Up-regulated	down-regulated	Up-regulated	down-regulated
		Total	282	584	337	1,962
6h dehydration ICC588	Up-regulated	282			**64**	4
	down-regulated	584			2	**111**
2h NaCl-treatment INRAT-93	Up-regulated	337	**64**	2		
	down-regulated	1,962	4	**111**		

20.0-fold

			6h dehydration ICC588		2h NaCl-treatment INRAT-93	
			Up-regulated	down-regulated	Up-regulated	down-regulated
		Total	39	49	36	433
6h dehydration ICC588	Up-regulated	39			**5**	
	down-regulated	49				**8**
2h NaCl-treatment INRAT-93	Up-regulated	36	**5**			
	down-regulated	433		**8**		

Table 7-2 Annotated UniTags with shared high up-regulation tendency in drought- and salt-stressed chickpea roots

		$R_{(ln)}$			
Tag ID	**Protein**	**Drg**	**Slt**	**Associated process**	**Uniprot ID**
STCa-21968	Aquaporin / Aquoporin PIP-2	3.32	3.53	Transport / transmembrane	Q8W4T8_MEDTR
STCa-24453	Tonoplast intrinsic protein	2.28	2.56	Transport / transmembrane	Q8L5G0_CICAR
STCa-22993	ADP-ribosylation factor 1-like protein	2.16	2.50	Transport	Q70XK1_HORVD
STCa-10123	Synaptobrevin-like protein	2.65	2.62	Transport / integral to membrane	Q69WS1_ORYSJ
STCa-5543	Mitochondrial F1-ATPase	2.53	2.56	Proton pump	Q8L5Q1_CICAR
STCa-7166	NADP-dep. isocitrate dehydrogenase I	3.58	3.26	Metabolism	Q6R6M7_PEA
STCa-8459	UDP-glucose pyrophosphorylase	3.34	2.79	Metabolism	Q8W557_9FABA
STCa-15241	Enolase-phosphatase	2.28	2.09	Metabolism	Q9FN41_ARATH
STCa-1131	4,5-DOPA extradiol dioxygenase	2.16	2.09	Metabolism /O_2 incorporation	Q70FG7_BETVU
STCa-8720	S-adenosylmethionine synthetase	2.65	2.28	Metabolism	Q9AT56_ELAUM
STCa-23978	Inorganic pyrophosphatase-like protein	2.48	2.62	Phosphate metabolism	Q9LFF9_ARATH
STCa-2982	Cysteine synthase	3.08	3.16	Protein metabolism	O65747_CICAR
STCa-542	Prolyl 4-hydroxylase	2.72	2.19	Protein metabolism	Q9FKX6_ARATH
STCa-13511	Ubiquitin-conjugating enzyme	2.31	2.32	Proteolysis	Q6PQ37_9CARY
STCa-12550	60S ribosomal protein L13	2.86	2.28	Protein biosynthesis	RL131_ARATH
STCa-923	Ribosomal protein S26	2.28	2.19	Protein biosynthesis	Q9SWS9_PEA
STCa-15340	Alfin-1	2.57	2.56	Regulation of transcription	Q40359_MEDSA
STCa-20066	14-3-3-like protein A	3.84	3.03	Protein domain binding	1433A_VICFA
STCa-181	Myo-inositol-1-phosphate synthase	3.42	2.56	IP3 biosynthesis / Ca^{2+} release	Q94C02_SOYBN
STCa-19021	Extensin	3.69	3.40	Cell wall organization	O65760_CICAR
STCa-21666	Low temp. and salt-responsive protein 6	3.17	2.68	Integral to membrane	RCI2B_ARATH
STCa-857	Histone H2B	2.39	2.09	Chromosome organization	Q9M3H6_CICAR
STCa-16257	ABA-responsive protein GEML-8	2.28	2.09	Response to stress	Q9FMW4_ARATH
STCa-8434	Fiber protein Fb2	2.16	2.36	Response to stress	Q8GT87_GOSBA
STCa-24349	Gibberellin 2-beta-dioxygenase	2.28	2.28	Metal ion binding	G2OX_PHACN
STCa-17272	10 kDa photosystem II polypeptide	2.28	2.68	Oxygen evolving complex	Q6V7X5_TRIPR
STCa-17434	Gb|AAD20160.1	3.45	2.93	No associated process	Q9FYR1_ARATH
STCa-10999	Predicted proline-rich protein	2.72	2.28	No associated process	Q9M0H8_ARATH
STCa-17087	Dormancy-associated protein	2.67	3.38	No associated process	O22611_PEA

7.2 Annotation of UniTags showing high up-regulation shared tendency and associated biological processes

One of the main objectives of the present work was the identification of stress-responsive genes in chickpea roots. Since drought and salt stresses are environmental pressures having many attributes in common, such as the disturbance of the osmotic and ionic equilibrium in the cell (Xiong et al., 2002; Zhu, 2002), one can expect, that at least part of the transcriptome responses are common as well. The different varieties used, the varying

growth conditions and sampling time points may, however, restrict the interpretation of the enormous data sets generated by SuperSAGE expression analysis. Although salt and drought stresses share many common aspects, they may trigger any transcriptome response at different times after the onset of stress, thereby influencing the dynamics of the response. For example, in *Arabidopsis*, the time points at which the maximum transcription changes occurred, was specific for the different stresses (Seki et al., 2002). After monitoring more than 7,000 genes, the expression changes induced by drought reached their maximum peak after 5 hours of dehydration. On the other hand, in salt stressed plants, an early peak was identified within the first 2 hours, whereas a second less pronounced peak was observed for a few genes after 10 hours of NaCl treatment.

7.2.1 UniTags annotated to aquaporins, proton pumps, and transport proteins

Four UniTags associated with water channels, transport, and proton pump processes, respectively, were more than 8.0-fold (R_{ln}>2.0) up-regulated in chickpea roots after drought **and** salt stress. To this category belong two aquaporin transcripts (STCa-21968, PIP-2 Q8W4T8_MEDTR; and STCa-24453, TIP Q8L5G0_CICAR), one ADP-ribosylation factor-1 protein (STCa-22993, Q70XK1_HORVD), one synaptobrevin-like protein (STCa-10123, Q69WS1_ORYSJ), and one mitochondrial F1-ATPase (STCa-5543, Q8L5Q1_CICAR; **Table 7-2**). Additional to the role of widely stress-reported genes approached already in previous sections (e.g. aquaporins), the important involvement of other types of proteins, some of them previously not associated to stress, is approached here.

ADP-ribolsylation factors (ARFs) are proteins highly involved in protein trafficking roles into the cell by acting in conjunction with small GTPases. In plants, ARFs are suggested to act in the retrograde protein transport process from the Golgi apparatus to the ER (Matheson et al., 2007). Particularly, the ARF1 has been suggested to be crucial in the maintenance of the integrity of the Golgi membranes and the ER export sites. Also, this same protein is proposed to be involved in protein transport to the vacuole, and in secretory pathways (Stefano et al., 2006). In roots, ARFs have been shown to be highly active in the polar growth regulation in tip cells, by supporting the secretion of a large amount of membrane and cell wall materials at the growing region for the sustainment of rapid elongation rates (Song et al., 2006).

Synaptobrevins, known to belong to the VAMP (vesicle-associated membrane proteins) class of trafficking proteins on the plant cell, are highly active components of secretory pathways into vacuoles, being the major components of the SNARE complex (soluble N-

ethylmaleimide-sensitive factor attachment protein receptor; Leshem et al., 2006). The plant vacuoles play an important role in plant salt and drought tolerance, where their physiological and biochemical identity is determined by correct targeting of vesicles and their cargo. For example, in *Arabidopsis*, the role of VAMPs mediating the vesicle complexes docking to the tonoplast has been shown to play an important role in salt tolerance by assisting the sodium-sequestering machinery (Gaxiola et al., 2002; Mazel et al., 2004).

By using the proton gradient caused by oxidative phosphorylation, mitochondrial F1-ATPases are the main ATP producers in the plant cell mitochondrion. In some other cases, ATPases can also work backwards-wise by using the energy derived from ATP to generate a proton gradient (www.ebi.ac.uk/interpro/ac=IPR006721). Probably not with the same function that other ATPases have on the plant cell under salt and drought stress (mainly Na^+ ions exportation), mitochondrial F1-ATPases may be directly involved in the synthesis of ATP as an energy source for many physiological processes. The high expression levels of mitochondrial F1-ATPases-annotated UniTags in chickpea roots under salt and drought stress could be then related to the high demand of energy that this plant experiences during the first stress stages.

7.2.2 UniTags annotated to genes encoding proteins of cell metabolism

At least seven UniTags annotated to genes encoding proteins involved in metabolic processes were up-regulated during both stresses in chickpea roots, among them NADP-dependent isocitrate dehydrogenase (STCa-7166, Q6R6M7_PEA), cysteine synthase (STCa-2982, O65747_CICAR), and UDP-glucose pyrophosphorylase (STCa-8459, Q8W557_9FABA). These transcripts and the corresponding genes have been separately dealt with in chapters 4 and 6 of the present work. Additionally, UniTags annotated to enolase phosphatase (STCa-15241, Q9FN41_ARATH), S-adenosylmethionine synthetase (Q9AT56_ELAUM), inorganic pyrophosphatase-like protein (Q9LFF9_ARATH), 4,5-DOPA extradiol dioxygenase (STCa-1131, Q70FG7_BETVU), and prolyl 4-hydroxylase (STCa-542, Q9FKX6_ARATH), respectively, were detected (**Table 7-2**).

Enolase-phosphatase enzymes are involved in salvage processes by regenerating methionine from methylthioadenosine (http://cmr.tigr.org/tigr-scripts/CMR/ HmmReport. cgi?hmm_acc=TIGR01691). The field of action of this enzyme in the central metabolism in plants is very broad, and up to now there are no major reports of enolase-phosphatase induction under abiotic stresses. The up-regulation of transcripts coding for this protein in

chickpea suggests, as could be logically expected, major metabolism re-arrangements in the stress early stages.

4,5-DOPA extradiol dioxygenase is an enzyme that has been reported to play a rate-limiting step in the betalain biosynthesis in plants (Christinet et al., 2004). In turn, betalains are plant pigments whose function has up to now not been fully characterized. However, it has already been suggested that in some cases betalains could act as antioxidants and could be induced under stress (Gentile et al., 2004; Sepulveda-Jimenez et al., 2005). After the first characterization in sweet beet (*Beta bulgaris*), betalains have been reported to be present in roots of other legumes, including chickpea (Watson and Goldman, 1997).

In general, inorganic pyrophosphatases and phosphatases have not been directly reported to be stress induced in plants. However, the action of this enzymes has been postulated to work as an alternative energy source on the cell when ATP sources are depleted, by using pyrophosphate (Tiainen et al.) as energy donor (Dobrota, 2006). After computing the relative importance of PPi versus ATP In plants, it has been revealed that PPi, as an alternative energy source, can reach high proportions in stress situations (Davies et al., 1992). In chickpea roots under salt and drought stress, ATP consuming processes, such as proton pumping, markedly increase their activities (Low et al., 1996). This feature may induce the PPi usage boosting the expression of PPiase-coding transcripts.

4-Hydroxyproline-rich glycoproteins (HRGPs) are found ubiquitously in the extracellular matrix of plants, accounting for as much as 10–20% of the dry weight of their cell walls. These proteins are implicated in all aspects of plant growth and development including apoptosis and responses to stress (Kieliszewski and Shpak, 2001). In turn, prolyl 4-hydroxylases, enzymes in charge of HRGP-modifications, have not been much characterized in higher plants. Up to now, there are only two studies reporting on two different *Arabidopsis genes* coding for this protein (Tiainen et al., 2004). Consequently, the possible roles for this type of proteins under stress conditions are still a dark matter.

7.2.3 UniTags annotated to genes coding for proteins involved in protein biosynthesis and turn-over

Three UniTags with shared up-regulation tendency were annotated to genes involved in protein biosynthesis and degradation. One transcript (STCa-13511, Q6PQ37_9CARY) was homologous to a gene encoding an ubiquitin-conjugating enzyme that catalyzes a step in the ubiquitin cycle (proteins turn-over). On the other hand, one 60S and one 26S ribosomal protein-annotated UniTags (STCa-12550, RL131_ARATH; and STCa-923, Q9SWS9_PEA, respectively) represented genes, whose products are involved in protein biosynthesis (**Table 7-2**). The ubiquitin/proteasome system (UPS) targets proteins for degradation. In this system, ubiquitin acts as an adapter that makes the target protein recognizable by the proteasome, in a process that involves the activity of ubiquitin-conjugating enzymes (Dreher and Callis, 2007). On the other hand, 60S and 26S ribosomal proteins are part of an extended group of proteins comprising more than 81 classes (Degenhardt and Bonham-Smith, 2008). For both types of proteins, the salt and drought up-regulation provides very valuable information in the search for specific stress-induced isoforms in chickpea.

7.2.4 UniTags annotated to genes encoding proteins involved in signal transduction, protein-protein interaction(s) and regulation of transcription

No UniTags associated with signal transduction *per se* were more than 8-fold up-regulated by both stresses in chickpea roots. However, three transcripts were indirectly associated to signaling cascades. For example, transcripts annotated to a 14-3-3 protein (STCa-20066, 1433A_VICFA), to one Alfin-1 transcription factor (STCa-15340, Q40359_MEDSA), and to myo-inositol-1-phosphate synthase (STCa-181, Q94C02_SOYBN), (**Table 7-2**).

As briefly approached in Section **4.2.1.11**, myo-inositol-1-phosphate synthase activity can be linked to the Ca^{2+} release mechanisms in the cell through the production of Inositol 3-phosphate, one of the most important early signaling events in plants under salt stress (Lecourieux et al., 2006). As mentioned in Sections **4.2.1.10** and **6.1.1.1,** the role of 14-3-3 protieins as adapters in protein-protein interactions is widely known in plants under stress conditions (Chen et al., 2006). Proteins of this family are represented in many plant species by several members displaying diverse expression patterns (Rosenquist et al., 2000; Roberts et al., 2002; Xu and Shi, 2006). Therefore, the up-regulation of UniTag STCa-20066 under both stresses provides a direct link to a stress-induced specific isoform. Further

characterization of this transcript, the encoded protein, and its possible targets become high priority in future studies.

Regarding Alfin-1 proteins, the function of this TF-class in the activation of genes conferring stress tolerance has been reported already in legumes. In alfalfa (*M. sativa*), over-expression of an Alfin-1 transcript regulated the expression of a proline-rich protein (PRP-2) involved in alleviation of high salinity effects (Winicov and Bastola, 1999). Alfin-1 transcripts encode a member of the zinc-finger family of proteins. This TF is expressed predominantly in roots, and appears to be unique or a low-copy gene in the genomes where it has been detected (e.g. rice, and *Arabidopsis*; Bastola et al., 1998). The over-expression of UniTag STCa-15340 in drought- and salt-stressed chickpea roots highlights the role of this TF-class in plant stress responses. Apart from the TFs widely reported to modulate stress responses, e.g. DREBs, ABFs, MYBs, WRKYs, this relatively novel type adds up to the repertoire of transcription regulators activated on legumes upon adverse environmental conditions.

7.2.5 UniTags annotated to genes encoding proteins directly involved in stress response(s)

Among the chickpea root transcripts highly up-regulated under salt and drought stresses, only two UniTags were annotated to genes directly associated to stress responses: UniTag STCa-16257, corresponding to the ABA-responsive protein GEML-8 (Q9FMW4_ARATH), and UniTag STCa-8434, corresponding to fiber protein Fb2 (Q8GT87_GOSBA) (**Table 7-2**). Up to know, the specific functions of these types of genes is not known. Therefore, the over-expression of transcripts annotated to these proteins can be only interpreted as a confirmative result.

7.2.6 UniTags annotated to genes encoding proteins for cell-wall organization

Regarding genes/proteins involved in cell-wall organization, the UniTag STCa-19021, annotated to the extensin accession O65760_CICAR, was more than 8-fold up-regulated under salt and drought stress in chickpea roots (**Table 7-2**). As observed by Tire and co-authors (1994) in *Nicotiana plumbaginifolia*, high extensin transcription levels are involved in cell wall enforcement as a response to biotic and abiotic stresses. This result indicates that mechanical pressures should be also considered major drought and salt stress attributes in chickpea roots.

7.2.7 UniTags annotated to genes encoding proteins involved in ROS-metabolism

No UniTag directly related to ROS-scavenging was more than 8.0-fold up-regulated in both treatments of chickpea roots. However, one UniTag each annotated to a Gibberellin 2-beta-dioxygenase (STCa-24349, G2OX_PHACN), and a 10 kDa photosystem II polypeptide (STCa-17272, Q6V7X5_TRIPR), both indirectly involved in oxidative stress management (Bergantino et al., 1995), shared up-regulation in response to the stresses (**Table 7-2**).

7.2.8 Non-annotatable UniTags with shared up-regulation tendency in salt- and drought-stressed chickpea roots

Although several up-regulated transcripts shared sequence homologies to ESTs in public databases linked to fully characterized proteins, still several UniTags remain un-assigned. Despite being non-informative for the annotation procedure, these 26bp tags nevertheless may represent starting points for the discovery of novel genes, or at least new transcript isoforms playing some roles in drought and salt stress responses in chickpea. Non-annotatable UniTags together with their closest hits after an "EST-linked" annotation procedure are listed in **Table 7-3**.

7.3 UniTags showing contrasting responses in salt and drought stressed chickpea roots

Apart from selecting transcripts (genes) with similar or identical responses to salt- and drought-stresses, the chickpea root dataset from salt- or drought-treated plants was analyzed for the distribution of expression ratios (via Venn mapper) to select transcripts with contrasting responses. The main parameters for selection of UniTags were: i) the candidate UniTag had to be at least 8-fold ($R_{(ln)}$>2.0) up-regulated under one stress, and no more than 1.2-fold up-regulated under the other stress ($R_{(ln)}$<0.2), and ii) different UniTags but linked to the same Uniprot accession were excluded. After data analysis, at least 30 annotated UniTags with salt and drought contrasting responses in chickpea roots were detected (**Tables 7-4A** and **7-4B**).

Table 7-3 Non-annotated UniTags with shared high up-regulation tendency in drought- and salt-stressed chickpea roots

		Ratio (ln)			
Tag code	**Sequence**	**Drg**	**Slt**	**Annonymous EST hit**	**EST annotation***
STCa-1789	CATGAATCAATTCAATAACTTCTGAA	2.57	2.19	FE671780.1	No match
STCa-4590	CATGATCAGTAGATCACTAAATAAAT	3.30	2.19		
STCa-16261	CATGGTGGTTTTTATGATAATTAAAG	2.57	4.35		
STCa-19168	CATGTATGTTTGTTTAATTATGTTTT	2.53	3.90		
STCa-24351	CATGTTTTGCAAGAAGTAAAAGCTAT	2.65	2.79		
STCa-20347	CATGTGAACTTAGGTTTGTTTATGTT	2.10	2.62		
STCa-15259	CATGGGTTGGCCATTATTTTGTTTAG	2.16	2.09		
STCa-5894	CATGATTTACAAATCCTTAGAAATAG	2.53	3.53		
STCa-175	CATGAAAATAATTGTCTATTTAGGTG	2.86	2.62	EY478278.1	Transcription elongation factor 1
STCa-16605	CATGGTTGAAGCAAAATAAATTGTTA	3.26	2.56	CA912439.1	Protein AT4g37830
STCa-170	CATGAAAATAAGACATCATAAGAACT	3.00	2.50		
STCa-11740	CATGGAGAGTTGAGAAATTGAGAGGG	2.28	2.36		
STCa-387	CATGAAAGAAAATCAATTATGTGGGC	3.17	2.97		
STCa-18846	CATGTATATTTGCTTTATGGGATCCT	3.34	2.68		
STCa-6259	CATGCAAATCGAATCGGTTTAAATGC	2.28	2.56		
STCa-6777	CATGCAATTTGGTCTTAAGGAATATA	2.39	2.43		
STCa-18178	CATGTAGAAGTTTTAATTCATCTATG	3.17	2.28		
STCa-22163	CATGTTAAATAAGGGTTCATCTGTAT	2.65	2.28	FE672240.1	2 dihydroflavonol reductase
STCa-9004	CATGCGACTCTTAAATTATATTATGT	2.28	2.09		
STCa-705	CATGAAATTGTAACATTGAAATTGAG	2.28	2.09	FE672182.1	Arabidopsis MAPK-20 protiein
STCa-6410	CATGCAACTTTAATATTAAACCTATG	2.15	3.24	FE669969.1	Auxin response factor 14
STCa-4616	CATGATCATTATGTATTTTCTTCCTG	2.65	2.84		
STCa-13756	CATGGGAATTTGATAATAAAAGAACC	2.33	2.62		
STCa-10367	CATGCTTGGTTAGATATGTTGTTTTT	3.03	2.43		
STCa-22151	CATGTTAAAGAAATTCAATAATATTG	2.39	2.43		
STCa-24251	CATGTTTTAGATTGAATTTTCATACT	2.57	2.28		
STCa-18230	CATGTAGAGATTGAAATGAAAATTAA	2.28	2.09		
STCa-21605	CATGTGTATTATTCATTAATTAATTA	2.74	2.47	FE672139.1	Two-comp. response regulator
STCa-17408	CATGTAAGTTTTGATTGATGGAGAAG	2.72	2.28		
STCa-4092	CATGATAAAGTTTGTTTCTTATATCT	4.18	2.14		

*****An EST-linked annotation procedure was carried out after BLASTing the non-annotated 26bp uniTags against the NCBI anonymous EST database for high homology hits in Fabaceae entries (E< 1.0E-5). When high homology hits were obtained, complete EST sequences were retrieved and re-BLASTed against the NCBI (nr) and TIGR -GI databases. Seven out of 30 UniTags could be annotated this way.

Table 7-4A Chickpea root UniTags with the highest probability to be exclusively induced by drought

		$R_{(ln)}$			
Tag ID	**Protein**	**Drg**	**Slt**	**Associated process**	**Uniprot acc.**
STCa-1224	Receptor protein kinase-like (RLK)	2.16	0.20	Signal transduction	Q6K703
STCa-866	Protein kinase Pti1	2.57	-0.21	Signal transduction	Q84P43
STCa-7584	Avr9/Cf-9 induced kinase 1	2.16	-0.21	Signal transduction	Q84QD9
STCa-1016	Protein phosphatase 1 (PP1)	2.16	0.20	Signal transduction	O65844
STCa-11965	Probable oligopeptide transporter 3	2.48	-0.21	Transport / integral to membrane	O23482
STCa-13877	Putative phospholipid-transporting ATPase 4	2.03	-0.34	Transport / integral to membrane	Q9LNQ4
STCa-16528	Exostosin-like protein	2.16	-1.60	Membrane	Q2HVN7
STCa-14806	Cysteine proteinase (Sulfhydryl-endopeptidase)	3.13	-2.29	Peptidase / proteolysis / PCD	CYSEP
STCa-12638	26S proteasome regulatory subunit-like protein	2.03	-0.50	Protein complex	Q69Q88
STCa-6821	26S proteasome non-ATPase regulatory subunit 3	2.48	-0.75	Protein complex	Q06364
STCa-9974	60S ribosomal protein L5	2.28	-0.21	Protein biosynthesis	Q6UNT2
STCa-8945	Pyruvate dehydrogenase E1 beta subunit	2.22	-0.90	Metabolism / oxidoreductase	O24458
STCa-12590	Glutamate: glyoxylate aminotransferase 1	2.16	-0.90	Biosynthesis / N_2-assimilation	Q9LR30
STCa-16163	1-aminocyclopropane-1-carboxylic acid oxidase	2.16	-0.21	Iron metal binding	Q84L58
STCa-9933	Putative desacetoxyvindoline 4-hydroxylase	2.16	-0.21	No associated term	Q1SAV8

Table 7-4B Chickpea root UniTags with the highest probability to be exclusively induced by salt

		$R_{(ln)}$			
Tag ID	**Protein**	**Drg**	**Slt**	**Associated process**	**Uniprot acc.**
STCa-24417	Lipoxygenase	0.08	3.19	Fatty acids biosynthesis	Q9M3Z5
STCa-9604	Chloroplast 50S ribosomal protein L14	0.08	2.19	Protein biosynthesis	RK14
STCa-19047	Tubulin alpha-3/alpha-5 chain	-0.21	2.09	Protein polymerization	P20363
STCa-21989	Elongation factor 2 (EF-2)	-0.32	2.28	Protein biosynthesis / translation	O23755
STCa-815	SVP-like floral repressor	-1.30	2.43	Regulation of transcription	Q7Y1U9
STCa-20215	Putative extracellular dermal glycoprotein	-1.30	3.09	Proteolysis	Q9FSZ9
STCa-1385	1-aminocylopropane-1-carboxylate oxidase	0.08	2.84	Iron ion binding / oxidoreductase	Q41681
STCa-4531	Isoflavone 3'-hydroxylase	-1.12	2.88	Iron ion binding / oxidoreductase	Q2ENF7
STCa-23782	Zinc finger protein 5	0.08	2.09	Metal ion binding	Q8LCZ7
STCa-1381	Acetyl-CoA synthetase	-1.02	3.19	Metabolism	Q9ZR69
STCa-1477	6-phosphogluconate dehydrogenase	-0.39	2.39	Metabolism / NADP binding	Q40311
STCa-5165	Actin depolymerizing factor	-0.61	2.19	Actin binding	Q9XEN2
STCa-15030	Uridine kinase-like protein	-0.61	2.09	Biosynthesis / cAMP	Q6YV21
STCa-11000	Fiber protein Fb27	0.08	2.09	Stress response	Q6UA10
STCa-5798	MAP kinase kinase	0.08	2.19	Signal transduction	Q93WR7
STCa-12035	Cytochrome P450 monooxygenase	-0.61	2.74	Electron transport / CYP superfamily	Q9XFX0
STCa-2426	Pleiotropic drug resistance protein 3	-0.79	2.43	No associated process	PDR3

7.3.1 Signal transduction-related genes

Filtered as exclusively drought-induced UniTags, transcripts coding for three different protein kinases (STCa-1224, RLS; STCa-866, protein kinase Pti1, and STCa-7584, Cf-9 induced kinase 1) were highly expressed in drought-stressed roots, but either constitutively expressed or down-regulated in the same organ under salt stress (**Table 7-4A**). In contrast**,** only one UniTag (STCa-5798) annotated to a MAPK-kinase was up-regulated under salt-, but not under drought stress (**Table 7-4B**).

7.3.2 Genes encoding transport proteins

In this category, two UniTags were detected as up-regulated in drought-stressed, but only lowly expressed in salt-stressed roots: STCa-11965 (oligopeptide transporter 3), and STCa-16528 (phospholipid-transporting ATPase 4). Additionally, one UniTag annotated to an exotosin-like protein (STCa-16528), linked to membrane processes, was also up-regulated in drought-, and almost constitutively expressed in salt-stressed roots (**Table 7-4A)**.

7.3.3 Genes encoding proteins involved in protein synthesis and turn-over

Belonging to this category, UniTags annotated to two 26S proteasome-related proteins (STCa-12638, and STCa-6821), one cystein proteinase (STCa-14806), and one 60S ribosomal protein (STCa-9974), respectively, were up-regulated under drought, but showed no major expression changes under salt-stress (**Table 7-4A)**. On the contrary, UniTags annotated to the 50S chloroplast ribosomal protein L14 (STCa-9604), tubulin alpha-3/alpha-5 chain (STCa-19047), and elongation factor 2 (EF-2) (STCa-21989) were up-regulated under salt, but constitutively expressed under drought stress (**Table 7-4B**).

7.3.4 General remarks

The results shown in sections 7.2 and 7.3 are solely based on the transcript variant level. Therefore, three main assumptions should be considered when defining responses as “shared” or “contrasting” in salt- and drought-stressed chickpea roots:

i) The probability, that two different transcripts generate two 26bp SuperSAGE tags of identical sequence is very low. Thus, tags shared by tissues responding to different stresses (**section 7.2**) code for the same protein with a high probability.

ii) On the other hand, two different tags (one over-expressed under drought only, and the other one only under salt stress), considered as part of “contrasting” responses

(**section 7.3**), may still be derivatives of similar transcripts. As observed in previous studies, transcripts undergoing processes that cause changes in their sequences like exon skipping (Bournay et al., 1996), or alternative splicing (de la Mata et al., 2003; Wang and Brendel, 2006; Reddy, 2007) may code for similar proteins.

iii) Different tags derived from different transcripts (genes) with contrasting expression levels may code for closely related members of the same protein family (Wahl et al., 2005).

8 General discussion

8.1 454-pyrosequencing of SuperSAGE tags as a transcriptome-survey technique

For the first time, the pyrosequencing technology of 454 Life Sciences has been applied for the sequencing of 26bp SuperSAGE tags at all. Here, more than 270,000 tags representing >30,000 unique transcripts (UniTags) were sequenced and monitored for differential expression in salt- and drought-stressed chickpea roots. Regarding the amount of processed cDNAs, the present report is the largest high-throughput transcriptome survey in *C. arietinum* up to date.

Certainly, the 454 pyrosequencing method has demonstrated to be powerful enough to sequence large amounts of transcripts. In comparison to previous studies (Matsumura et al., 2003; Coemans et al., 2005), the combination of this technology with SuperSAGE has boosted the quantity of analyzed transcripts at least 20-fold. In a previous work on legumes, Cheung and co-authors (2006) already reported of more than 290,000 *M. truncatula* ESTs in a single 454-sequencing machine run. However, no quantitative information about differential gene expression could be delivered.

In the following sections, the technical and biological aspects of the present chickpea transcriptome survey will be discussed.

8.2 Aspects of the chickpea transcriptome

8.2.1 SNPs can be a frequent phenomenon in chickpea transcripts

As exemplarily reported for human cancer cells (Boon et al., 2002), some tags generated by SAGE-related techniques are very often differentiated from each other only by SNPs, forming the so called SNP-associated alternative tags (Silva et al., 2004), here refered to as SAATs. These previous reports are in congruence with the present results. In a test dataset composed by the ICC588 chickpea root SuperSAGE libraries, approximately 2.5% of the 17,498 extracted UniTags revealed SNP-differences with at least one other tag (section **3.3.2)**.

Considering the likeliness of the appearance of very similar transcripts in an organism, the occurrence of SNPs within ESTs in humans is linked to the high flexibility of the transcriptome. According to Cheng et al. (2005) and Kapranov et al. (2005), this high flexibility allows the generation of multiple transcript “isoforms” from a single locus, among them, SNP-containing variants. Further on, Silva and co-authors (2004) suggest a link

between the flexibility of the transcriptome and the occurrence of SAATs. By comparing experimental and *in silico* extracted tags with fully characterized human transcripts, these authors found that 1,136 out of 44,033 cDNAs (2.6%) harboured a SNP contiguous to the *Nl*aIII recognition site.

Exemplifying this phenomenon in chickpea, eleven SAATs were annotated to the aquaporin (MIP) protein Q8GTE0_CICAR (AJ515031). As depicted in **Figure 8-1**, these UniTags showed different regulation levels in roots of the variety ICC588. And further on, nine of them were also found in roots from the variety INRAT-93. After a restricted BLAST of the eleven tags against *C. arietinum* anonymous ESTs (NCBI), four high-homology hits were obtained for all sequences (gi169748172, gi169745411, gi169744679, gi169744392, gi169743991, gi169743949). Each of the resulting ESTs was reBLASTed against the NCBI(nr) database, displaying the entry AJ515031 as the hit with the highest E-value (the same hit obtained by each of the eleven tags). This supports then the validity of the aquaporin SAATs in chickpea. However, information about the genic region (or regions) generating these transcripts is still missing.

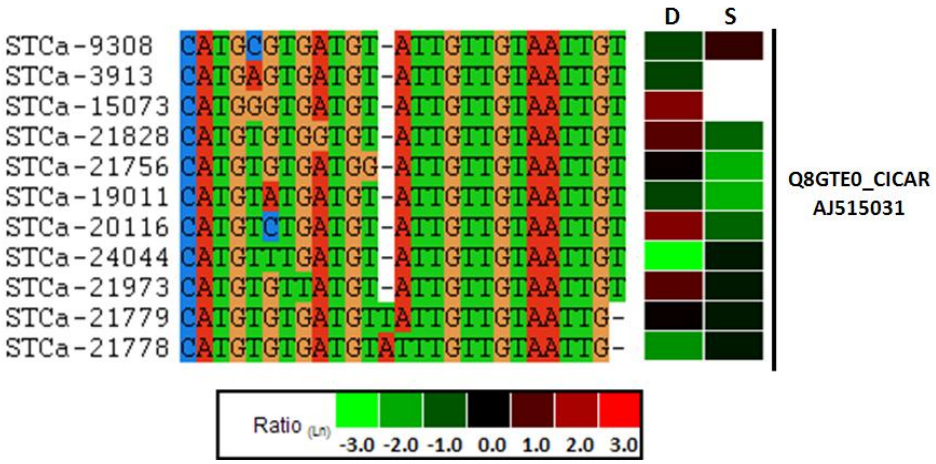


Figure 8-1 **Example of SNP-associated alternative tags (SAATs) in chickpea roots**

Exemplifying the occurrence of SNPs in chickpea root tags, eleven UniTags annotated to a single aquaporin (Q8GTE0_CICAR) are depicted. These transcripts showed different expression levels after 6h of drought stress in the variety ICC588 (D). Also, nine of them were present in salt-stressed roots of the variety INRAT-93 (S).

8.2.2 Transcript isoforms with dissimilar sequence: Evidence of alternative splicing in the chickpea transcriptome?

Additionally to the occurrence of SAATs (previous Section) within the chickpea transcriptome, groups of UniTags with dissimilar sequence were also annotated to the same gene (or protein). As an instructive example, **Table 8-1** shows 34 UniTags annotated to a single chickpea metallothionein-1 accession (MT1; X95708.1). As depicted in **Figure 8-2,** the tags were derived mainly from two cDNA sites, that are both preceded by a CATG sequence, which logically explains the tag origin. According to the SAGE methodology (Velculescu et al., 1995), it is expected that the most probable site for a tag extraction is the CATG site most proximal to the mRNA 3'-end (position 463; **Figure 8-2**). However, UniTags originating from position 245 were observed in all screened chickpea varieties and tissues, which supports their validity. Obviously a group of transcripts lost part of their sequences, where a CATG site was originally present.

This result reflects alternative mRNA-splicing (Robinson et al., 2004; Wang and Brendel, 2006). As reported by Reddy (2007) in an extensive review, alternative splicing in plants plays an important role in post-transcriptional regulation, and may vary under stress conditions. For example, more alternative-spliced isoforms of GSTs were observed in maize after Cadmium stress onset than in control conditions (Marrs and Walbot, 1997), which also

explains many transcript isoforms detected exclusively in stressed chickpea plants, not only from GSTs but from many other proteins (genes).

Additionally, several studies in *Arabidopsis* postulate the 3'-ends of mRNAs (3'UTRs) as hot spots for transcript variation (Alexandrov et al., 2006; Nagasaki et al., 2006; Wang and Brendel, 2006). In SAGE, it is very likely that many tags are derived from 3'UTRs (Velculescu et al., 1995), which explains, that various tags can be annotated to a single protein (gene) in chickpea.

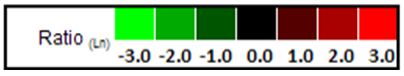
Considering the relationship between similar transcript isoforms and the differences in the encoded proteins, still much remains to be understood in plants. In general, it involves changes in properties like structure-stability, loss of function in specific domains, enzyme activity, and post-translational modifications (Stamm et al., 2005). As demonstrated for mouse by comparing the brain tissue transcriptome and proteome (Irmler et al., 2008), these changes can also reflect the variety of expression levels observed in transcripts variants coding for the same (similar) protein.

The chickpea genome is still under-sequenced, which impairs concrete conclusions concerning the validity of transcripts isoforms. As will be discussed in Sections **8.3.2** and **8.3.3**, also methodological failures may lead to the detection of non-genuine tags. Therefore, unless there is no confirming additional information, up to now it is impossible to discriminate false from genuine transcripts of similar sequence with certainty.

Table 8-1 Example of multiple UniTags annotated to the same mRNA accession

UniTags annotated to the chickpea MT1 accession X95708.1 along with their respective sequences and expression levels under drought (D, roots), salt (S, nodules and roots), and cold stress (C, leaves) are depicted. Due to the comparative nature of this example, UniTags detected in chickpea leaves of the variety ILC8269 (not deeply analyzed in the present work) were included. Diverse regulation tendencies, including the regulation of some of the isoforms exclusively under stress were observed.

Tag ID	Sequence	D(rt)	S(rt)	S(nd)	C(lv)	Origin position	Total counts	Special features
STCa-20474	CATGTGACCCTTGCAACTGCAAGTGA					245	79	
STCa-28902	CATGTGCAGCTTGATCCACACTTGCA					248	2	Anti-sense
STCa-3780	CATGAGTAAGCAAATATAGCTAATAT					463	7	Stress-exclusive
STCa-9248	CATGCGTAAGCAAATATAGCTAATAT					463	25	
STCa-14936	CATGGGTAAGCAAATATAGCTAATAT					463	8	Stress-exclusive
STCa-18649	CATGTATAAGCAAATATAGCTAATAT					463	10	
STCa-20302	CATGTGAAAGCAAATATAGCTAATAT					463	2	
STCa-21431	CATGTGTAAACAAATATAGCTAATAT					463	2	
STCa-21475	CATGTGTAAGCAAATATAGACTAATA					463	2	
STCa-21476	CATGTGTAAGCAAATATAGCCAATAT					463	3	
STCa-21477	CATGTGTAAGCAAATATAGCTAAATA					463	5	
STCa-37487	CATGTGTAAGCAAATATAGCTAAATC					463	1	
STCa-21478	CATGTGTAAGCAAATATAGCTAACAT					463	3	
STCa-21479	CATGTGTAAGCAAATATAGCTAATAA					463	14	
STCa-21480	CATGTGTAAGCAAATATAGCTAATAC					463	16	
STCa-21481	CATGTGTAAGCAAATATAGCTAATAT					463	1,632	
STCa-21482	CATGTGTAAGCAAATATAGCTAATCC					463	28	
STCa-21483	CATGTGTAAGCAAATATAGCTAATGT					463	3	
STCa-21484	CATGTGTAAGCAAATATAGCTAATTA					463	2	
STCa-21485	CATGTGTAAGCAAATATAGCTGACAA					463	4	
STCa-21486	CATGTGTAAGCAAATATAGCTGACGC					463	2	
STCa-21487	CATGTGTAAGCAAATATAGCTTAATA					463	3	
STCa-21488	CATGTGTAAGCAAATATAGTCTAATA					463	2	
STCa-21489	CATGTGTAAGCAAATATATCTAATAT					463	4	
STCa-21494	CATGTGTAAGCAAATTAGCTAATATG					463	38	
STCa-21498	CATGTGTAAGCAATATAGCTAATATG					463	4	
STCa-21735	CATGTGTGAGCAAATATAGCTAATAT					463	4	
STCa-21956	CATGTGTTAGCAAATATAGCTAATAT					463	2	Stress-exclusive
STCa-23731	CATGTTTAAGCAAATATAGCTAATAT					463	6	Stress-exclusive
STCa-16331	CATGGTGTGTAAGCAAATATAGCTAA					465	2	
STCa-16269	CATGGTGTAAGCAAATATAGCTAATA					466	9	
STCa-21325	CATGTGGTAAGCAAATATAGCTAATA					468	3	
STCa-6212	CATGCAAAGCAAATATAGCTAATATG					470	2	Stress-exclusive
STCa-21559	CATGTGTAGCAAATATAGCTAATATG					471	2	



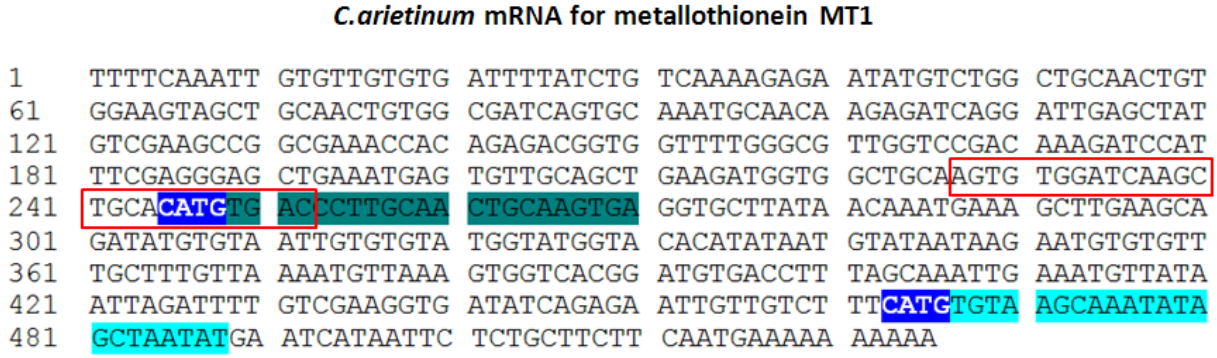


Figure 8-2 Example of multiple UniTags annotated to the same mRNA accession

From all chickpea tissues and varieties (ICC588: roots; INRAT-93: roots and nodules; ILC-8269: leaves) used in the present study, 34 UniTags were annotated to a single chickpea MT1 mRNA accession (X95708.1). The 26bp fragments were derived mainly from site 463 (highlighted in light blue) and 245 (highlighted in mat blue). UniTags derived from position 245 were less frequent than from position 463. However, they were observed in all chickpea tissues and consequently in several SuperSAGE libraries developed and sequenced at different times. Additionally, one of the UniTags was derived from a sequence in anti-sense orientation (red-framed regions).

8.2.3 Complex regulation patterns of UniTags annotated to genes from multi-member families

Additionally to the annotation of several transcripts to a single accession, several chickpea UniTags were linked to proteins represented by more than one gene, displaying contrasting expression levels in many cases. In plants, this has already been observed in genes belonging to different functional categories. Fourteen-three-three (14-3-3) proteins, which are encoded by a multi-member gene family, are a good example of this regulatory flexibility. In tomato, the expression levels of twelve 14-3-3 transcripts revealed notable differences after salt stress (Xu and Shi, 2006). Also in rice, the expression of eight different 14-3-3 genes under diverse abiotic stresses showed similar patterns (Chen et al., 2006). These observations agree well with the present results of chickpea under salt and drought stress, where 30 UniTags were annotated to at least ten 14-3-3 accessions, displaying expression levels between 45-fold up- and 30-fold down-regulation. In general 14-3-3 proteins have a broad array of protein targets and act in diverse signaling pathways (Ferl, 1996), which explains the diversity of their transcript profiles.

At this point, the suitability of the statistical treatment and interpretation of data, especially related to the appearance of SAATs and other types of transcript isoforms, can be questioned. When should expression levels of transcripts coding for similar proteins better

be averaged?, when treated separately? This is absolutely unclear up to now. The mouse transcriptome and proteome analyses reported by Irmler and co-authors (2008) provide useful data to approach this problem. At the protein level, the authors found 16 out of 106 proteins with contrasting expression ratios present in more than one 2D-PAGE spot. From the 106 proteins, the expression of 75 was regulated at the transcript level. Fourteen out of the latter were represented by multiple oppositely regulated transcripts. Five of them underwent alternative splicing in exonic regions, giving rise to different protein variants. Consequently, their expression ratios were treated independently. In contrast, nine transcripts differed at the 3'UTR only, suggesting that they were coding for the same protein. Therefore, their expression ratios could be averaged.

The biological meaning of this contrasting regulation needs to be completely understood. Most probably, it relies on aspects like: i) changes in RNA stability and rates of productive translation (Wollerton et al., 2004), ii) repression or induction of transcripts encoding proteins with new binding properties, iii) changes in intracellular localization of the coded protein (Stamm et al., 2005), iv) changes in enzymatic and signaling activities (Li and Koromilas, 2001), v) changes in protein stability, vi) insertions of domains subjected to post-translational modifications, vii) and changes in very specific functions, like e.g. ion-channel properties (Tian et al., 2001).

Adversely, the wealth of information about the chickpea transcriptome alone is insufficient to generate any sound conclusions on the functional or regulatory importance of the many transcript variants showing contrasting regulation levels (as discussed in previous sections).

8.2.4 Low-copy-number transcripts are a major component of the chickpea root poly(A)$^+$-RNA landscape

The tag copy numbers within the chickpea cDNA populations suggest, that a substantial proportion of the sampled transcripts is present at low abundance (at least 80% are found at 2 to 100 copies million^{-1}; Section 3.1). This observation is not at all new for plants. In SAGE-based transcriptome analyses in *Arabidopsis* and maize (Fizames et al., 2004; Poroyko et al., 2005), at least 70% of the detected transcripts (excluding singletons) were low abundant. In several other organisms outside the plant kingdom (i.e. yeast, mouse, and humans), large differences between abundant and rare transcripts have also been observed (Kim et al., 2006).

Why this proportion of low copy number transcripts is so big in many transcriptomes, it is not clear. It has been suggested that transcripts found in high abundance represent a limited number of house-keeping genes, whereas "rare" transcripts are derived from genes with more specialized functions. For example, in a yeast SAGE analysis, transcripts encoding glycolytic enzymes are present in a few copies per cell, whereas some physiologically important transcription factors are expressed in high abundance (Holland, 2002). However, in other cases, transcripts encoding proteins with the same function can also display very different ranges of copy numbers. For example, in an analysis restricted to TFs of *Arabidopsis*, differences in copy numbers of transcripts by six orders of magnitude were detected (0.001 to 100 copies per cell;(Czechowski et al., 2004).

The fact, that probably not all the transcripts found within a transcriptome are also translated into proteins, should be considered. Several examples demonstrate that there is no perfect correlation between transcriptome and proteome in many species (de Nobel et al., 2001; Hu et al., 2006; Trauger et al., 2008), which can disfavour the rare transcripts. Additionally, there is no doubt that rare UniTags can be artefacts produced by RNA instability, an usual phenomenon in transcription profiling studies (Copois et al., 2007).

The chickpea results, as presented here, can only be taken as a confirmation of the complexity of a transcriptome. Low-copy number UniTags need to be further analysed to prove their validity. Given the case, hopefully in a close future, that the transcripts found in very low copy number are proven to be 100% valid, these results can have very relevant implications on the resolution of many profiling techniques.

8.3 Methodological drawbacks of a SAGE-based transcriptome survey

Despite the great advances in our understanding of the chickpea transcriptome made possible by SuperSAGE, there are still drawbacks in this technique, that have to be overcome to achieve a complete transcriptome survey. In the following subsections, these main obstacles will be approached.

8.3.1 Exclusive sampling of polyadenylated RNAs: A large portion of the transcriptome is not analyzed

In the present work, as well as in many profiling reports, the sampled transcripts are exclusively polyadenylated RNAs (poly(A)$^+$-RNA). Nowadays, the traditional concept of a "gene", defined as a genomic region encoding a poly(A)$^+$ RNA, that in turn is translated into

a protein, is increasingly blurred and controversial, especially since a series of differing definitions for the term “gene” exists (Johnson et al., 2005). The recent emergence of reports on large numbers of unannotated transcripts, many of them non-polyadenylated, and with apparently little protein-coding capacity, is forcing a revaluation of the physical boundaries of what we consider genic regions (Gingeras, 2007).

For example, in a survey by Cheng and co-workers (2005), in which, sites of transcription of polyadenylated and non-polyadenylated RNAs for 10 human chromosomes were mapped at 5bp resolution, 43.7% of all transcribed sequences were non-polyadenylated. Overall, the transcribed portions of the human genome are predominantly represented by interlaced networks of both poly $(A)^+$-and poly $(A)^-$ annotated and unannotated transcripts. If this also holds for other systems, the present survey of a chickpea transcriptome may represent less than 60% of the plant’s potential genes (transcripts).

Therefore, it has to be considered that many transcripts coding for rare peptides, non-polyadenylated transcripts with potential regulatory functions (i.e. miRNAs, snRNAs, siRNAs;(Johnson et al., 2005), and rare transcripts of unknown functions are being excluded from this analysis, in fact, from most pertinent analyses at the present time. The importance of this type of transcripts has already been recognized for plants under abiotic stresses. In *Arabidopsis* seedlings exposed to dehydration, salinity, and ABA, more than 20 miRNAs forming 15 new families were detected (Sunkar and Zhu, 2004). Additionally, more than 100 novel endogenous small RNAs were identified in the same plants, several of them up-or down-regulated, suggesting that they play important roles in stress responses.

8.3.2 Tag generation is restricted to transcripts with *Nla*III recognition sites

Additionally to the exclusive sampling of poly$(A)^+$ transcripts, a further major obstacle is faced by SuperSAGE: Not all polyadenylated transcripts may possess an *Nla*III recognition site (5’-CATG-3’). This aspect has been discussed in detail by Pleasance and co-authors (2003) for *D. melanogaster* and *C. elegans,* using a conceptual transcriptome approach. After analyzing full-length cDNA populations derived from both organisms and comparing conceptual vs. experimental tags, 2.0 and 3.0% of the *D. melanogaster* and *C. elegans* transcripts, respectively, lacked *Nla*III recognition sites. The same kind of analysis was made in humans, showing that, from 54,645 analyzed mRNA sequences corresponding to 20,300 Unigene clusters, 0.1% did not contain an *Nla*III recognition site (Silva et al., 2004). Further on, there is also evidence that the same tendency is conserved in plants. According to an extensive SAGE analysis carried out in *Arabidopsis*, in 2.0% of the transcripts deposited in

public databases the sequence CATG was absent (Robinson et al., 2004). This suggests that, in the most favorable case, at least 600 transcripts can be missed in a population of 30,000 chickpea tags.

Beyond the problem of finding CATG sites in every existing transcript of a given cell or tissue, problems intrinsic to the *Nla*III-recognition site cleaving function can reduce the efficiency of SuperSAGE. This restriction enzyme occasionally decreases its activity, leaving uncut *Nla*III sites in cDNA pools, thereby producing false tags from a transcript (Angelastro et al., 2000). As depicted in **Figure 8-3**, the ideal SuperSAGE tag is derived from the *Nla*III recognition site located most proximal to the polyadenylated 3'-end of a cDNA. If such a site remains uncleaved, a false tag will be derived from the second closest position**.** Although in the present work several *Nla*III-cleaving rounds were applied to the chickpea cDNAs, it cannot be proven, that the *Nla*III-cleaving step was 100% efficient.

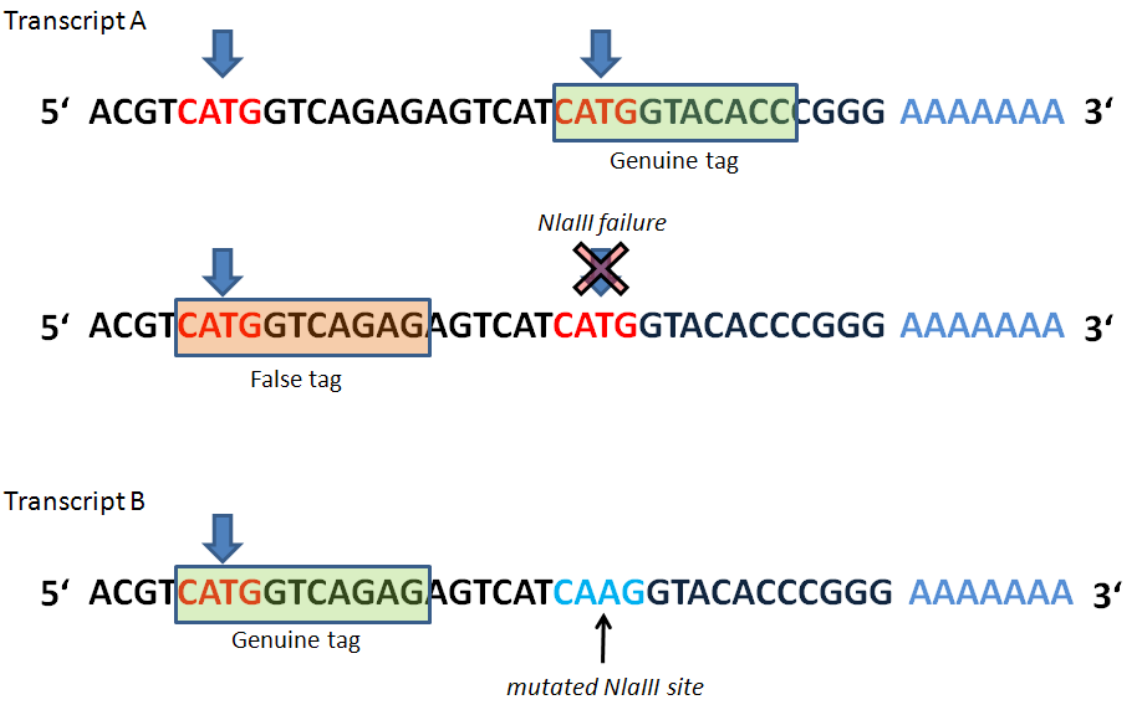


Figure 8-3 Failure of *Nla*III to cleave correctly as a source for bias in SAGE-related techniques

As depicted for transcript A, the ideal SAGE tag is derived from the most proximal *Nla*III site (CATG) to the poly(A)$^+$ 3'-end in a cDNA (green box). Failures in the *Nla*III performance leave uncut CATG sites, which can lead to the generation of a false tag (red box). In the analysis, the false tag will be considered a different transcript. Further on, this false tag cannot be differentiated from genuine tags derived from very similar transcripts (transcript isoforms), in which CATG sites either are naturally mutated, or have been deleted (Transcript B, green box).

Considering the aspects discussed in Sections **8.2.1** and **8.2.2**, it is inevitable to conclude that the chickpea transcriptome is far from being totally surveyed. Nevertheless, the present work is a first step towards describing the transcriptome of this crop, and understanding the changes induced by environmental stresses.

8.3.3 Sequencing- and amplification-induced errors may introduce false tags

DNA amplification- and sequencing-errors may also be a source of biases in SAGE-based techniques, leading to the detection of "false" tags (e.g. non-genuine SAATs). In SuperSAGE, ditags are amplified via PCR before 454-pyrosequencing. If non-robust DNA polymerases are used, the probability of introducing mismatches in the amplicons can considerably increase. In the present work, massive ditag amplifications were exclusively carried out with proof-reading DNA polymerases (i.e. Phusion^{TM} high fidelity DNA polymerase, NEB, Germany) in order to reduce this source of experimental errors.

Also, thanks to a selective step in the SAGE experimental procedure, known as twin-ditag elimination (Velculescu et al., 1995), most of the amplification or sequencing errors can be detected and eliminated (**Figure 8-4**). In SAGE libraries, each registered tag must originate from a different ditag combination. Even for frequent transcripts, e.g. a transcript represented 1,000 times in a total of 50,000, the probability of forming twin ditags is $4.0\ 10^{-4}$ (40 ditags in 1,000,000 possible combinations). Based on this principle, twin ditags are filtered *in silico* after primary data analysis. Nevertheless, if amplification failures introduce SNPs in one of the 26bp fragments of a ditag, a twin ditag will not be recognized, and a false tag would be registered. However, since each single error can be detected only once, it will generate a singleton, which is eliminated *in silico* in a subsequent step (**Figure 8-4 A**). On the other hand, tags carrying real SNPs will couple themselves randomly with other tags before amplification and sequencing, so they are not filtered out in the twin-ditag exclusion (**Figure 8-4 B**). According to Stern and co-authors (2003), who estimated the occurrence of false tags in a bioinformatic approach, the probability of scoring tags containing sequencing errors is lower than 1 false tag $\times 1{,}000.000^{-1}$. In the present study, this would mean that the number of false tags per SuperSAGE library is close to 0.05 tags $\times 50{,}000^{-1}$. This estimation assumes that sequencing errors will only appear in tags found in very low copy numbers, most probably observed once in a dataset (singletons). As highlighted above, singletons were eliminated *in silico* in the present work as a standard procedure.

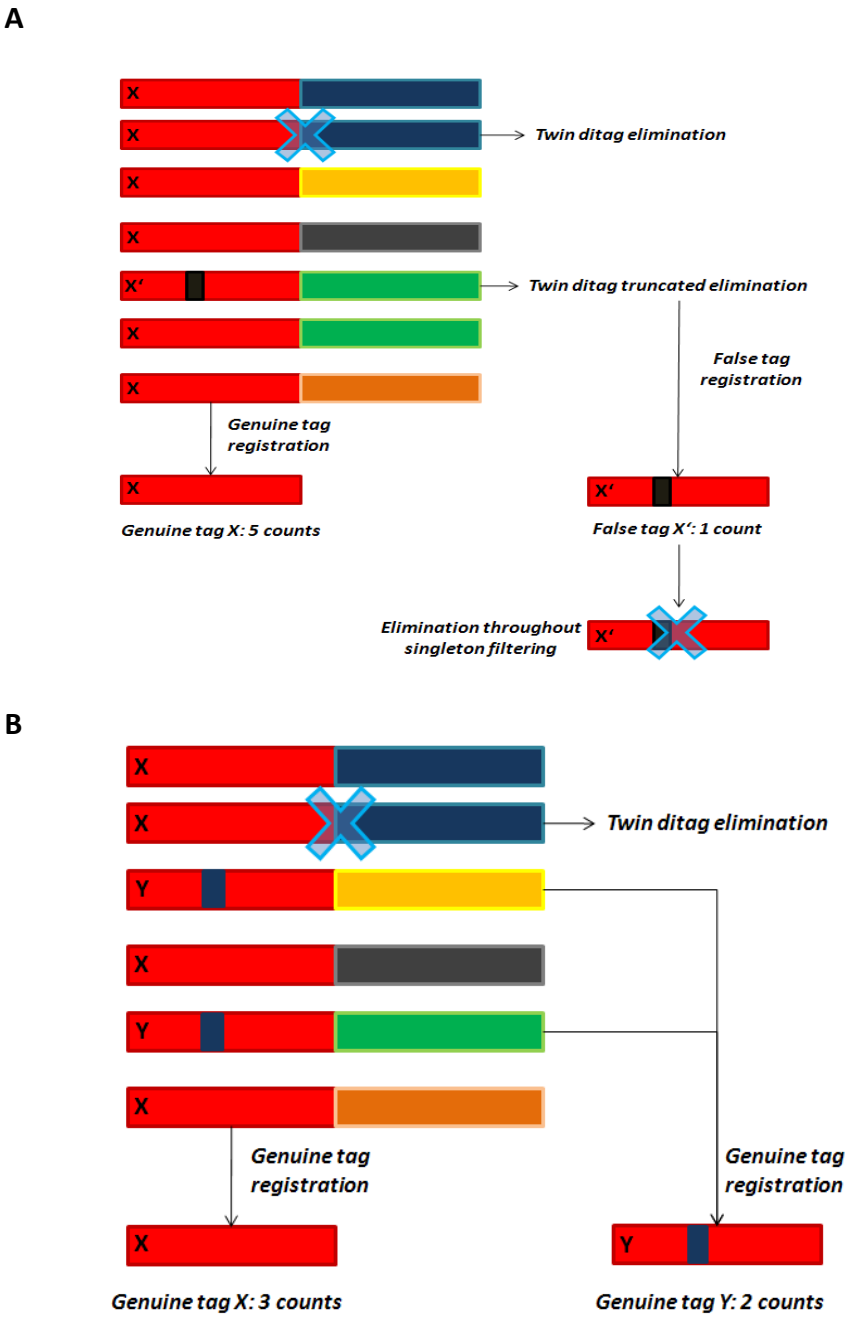


Figure 8-4 Elimination of sequencing errors in SAGE-related procedures

A) In SAGE-related procedures, twin-ditags are filtered *in silico* after a primary data analysis. For a valid registration, every single tag should be derived from a different ditag combination (**tag X**), which guarantees a random sampling of all transcripts. SNPs introduced by amplification may lead to registration of false tags (**tag X'**), albeit at low frequencies, allowing their elimination after singleton filtering.

B) Real SNP-associated alternative tags (**tag Y**) should be randomly coupled with other tags for ditag formation. After amplification and sequencing, only tags from different ditag combinations will be retained. In this way, most of the false SNP-associated tags can be differentiated from the genuine ones.

8.3.4 *In silico* analysis of massive genetic data: A must for standardization of terminology

More than 30,000 UniTags derived from the SuperSAGE experiments in chickpea nodules and roots were sequentially annotated against two out of ca. 18 plant gene databases present in the public repository (Galperin, 2008): Genbank (http://www.ncbi.nlm.nih.gov) and TIGR gene indices (GIs) (Lee et al., 2005). When filtering the massive amount of data, several major problems arose, making the need for better standardized descriptions, nomenclature**,** and cross-links between different databases for the annotated transcripts, obvious.

As far as the low level of cross-information between databases is concerned, from the almost 6,000 annotations of UniTags linked to characterized proteins, approximately 2,000 annotations did not have congruent and consistent descriptions between GenBank and TIGR-GI platforms. Whereas GenBank catalogued thousands of sequences as anonymous clones or chromosomes, the same sequences were bridged to characterized mRNAs by the TIGR-GIs through tentative consensus (TCs) entries (Quackenbush et al., 2001). The strategy followed in the present study, in which individual datasets are filtered for obsolete annotations after each hierarchically ordered BLAST round, at least overcame this problem partially. Nevertheless, data filtering for users with no expertise in managing local genetic databases or without knowledge in programming languages such as BioPerl (Stajich et al., 2002) will be inefficient in terms of amount of time and invested efforts.

At present, data filtering of large genomic datasets can only be done by text-mining of entry descriptions, which involves drawbacks caused by the inconsistencies in terminology that accumulate by the action of hundreds of database curators and entry submitters. For example, DREB TFs, one of the most important transcriptional regulators in salt and drought stress in plants (Liu et al., 1998), are described in at least four different ways: (1) as dehydration-responsive element binding protein (Q7Y0Y9_SOYBN, (Trauger et al.), as DRE-binding transcription factor (Q6IVL3_GOSHI), (3) as drought-responsive element binding protein (Q5RM57_GLYSO), and (4) as DREB-like protein (Q75UJ6_CUCME). This ambiguity corrupts even the use of the term “dehydration” or “drought” as characteristic designators.

Another example, MAP kinases, the most important signaling proteins in stressed plants (Teige et al., 2004), are described in several ways: e.g. as mitogen-activated protein kinase (Q1PCG0_MEDSA), or MAP kinase protein (Q9SMJ7_CICAR), MAP3K epsilon protein kinase (O81809_ARATH), or MAPK PsMAPK2 (Q9M6R8_PEA). This incongruence of terminology enormously hinders the automatic processing and analyses of data. Like the two highlighted

cases, several other examples exist in genomic databases (probably one example per annotated gene family). The consequences of these inconsistencies are generally multiplied, when sets made of thousands of annotations are filtered according to gene families or functional categories, or when the primary data annotation is coupled with other databases (e.g. Uniprot: Apweiler et al., 2004 ; and gene ontology: Crangle and Zbyslaw, 2004).

Additionally to the cases in which genes coding for the same protein or the same family of proteins are not described concordantly, some problems intrinsic to the annotation processes of the reference sequences deposited in public databases also appear. As analysed by Steven Brenner (1999) in a publication aimed at pinpointing the most frequent errors in genome annotation, the annotation of the *Mycoplasma genitalium* genome by three independent groups was evaluated giving sometimes terrifying results. According to this study, an 8% error was found in the annotation process of the 340 analyzed genes. If one extrapolates these numbers to plants, they can reach considerable dimensions, because plant genomic databases contain informations from multiple species, and therefore are annotated and curated by several groups independently.

The consequences of the above mentioned problems for chickpea, as an organism relying on the knowledge generated for related model plants, can be very serious unless errors in the assignment of obtained ESTs or sequence tags to already characterized genes are not systematically eliminated. Unfortunately, the question of how large could be the error (in percentage) in the presently 6,000 annotated chickpea transcripts, remains open.

8.3.5 Perspectives for improvement of SuperSAGE-based transcription profiling

As previously described, the current methodology of SuperSAGE is directed towards sampling poly $(A)^+$-RNAs. In recent years, however, non-coding RNAs emerged as important regulators of transcription and translation (Sunkar and Zhu, 2004; Gingeras, 2007).

Since the characteristics of the different RNA classes have only few overlapping features (e.g. they own very different sizes, different secondary and tertiary structures, and absence/presence of a poly$(A)^+$ tail), it is difficult to catch all transcript classes of a tissue with a single method. For example, a completely different RNA purification method is required to recover all small- and micro-RNAs and separate them from the high-molecular-weight transcripts (Sunkar et al., 2005; Yao et al., 2007), impairing the simultaneous analysis of both fractions. For that purpose, future genome-wide expression analyses should enable the possibility to merge “sub-transcriptomes” (e.g. sRNAs + miRNAs + high molecular weight

mRNAs + non-poly(A^+) RNAs). This implicates, that polyadenylation-independent reverse transcription protocols, size-optimized isolation procedures, and adapted bioinformatics should be coupled with SuperSAGE.

8.4 Is the SuperSAGE-derived sequence information transferable to other systems?

One of the main objectives of choosing SuperSAGE as the main technique to conduct a transcriptome-wide gene expression survey in chickpea was the transfer of the retrieved information to other screening platforms in the most possible direct way. Two methodological approaches were tested in the present work: i) direct spotting of 26bp tags onto microarrays, and ii) use of the 26bp tags as start point for 5' and 3' amplifications, for subsequent design of TaqMan™ and SYBRgreen™ qRT-PCR probes.

8.4.1 Spotting of 26 bp UniTags onto microarrays

Previously, Matsumura and co-authors (2006) reported on the transfer of sequence information from SuperSAGE to microarrays (Matsumura et al., 2006). In the present study, information from chickpea expression profiles was transferred to Agilent- (16K array), and Array-on-platforms (300 oligos array). In general, the background levels were relatively high for both types of systems, leading to loss of information**.** However, after comparing the results from the 16K agilent array with SuperSAGE, a high proportion of data points showed shared regulation tendencies (**section 3.5**). Despite this good proportion of shared tendencies (79%), the signal intensities did not correlate completely with the SuperSAGE expression ratios. The results were congruent indicating either up- or down-regulation, but were not congruent indicating the degree of differential expression.

One of the major drawbacks of the microarray-based techniques in general, and therefore for the transfer of information from SAGE-based techniques to cDNA chips in particular, is the loss of resolution. Whereas very similar transcripts could be differentiated *in silico* by SuperSAGE (e.g. SAATs), this degree of differentiation can present problems for hybridization-based techniques (Stoughton, 2005). At present, diverse technologies have already been applied for the detection of SNPs on microarrays. However, these approaches are more directed towards genotyping than to expression profiling (Erdogan et al., 2001; Fan et al., 2003; Shen et al., 2005).

Another big obstacle in the use of SuperSAGE microarrays is presented by low-abundant transcripts**.** As described by Evans and co-workers (2003) for neuronal tissue, an example of

transcriptomes full of low-abundant mRNAs, the power of microarrays is limited when "rare" messages are evaluated. In the present study, most of the spotted 26bp tags were selected on the basis of their up- or down-regulation, implying that their copy numbers might be relatively high. In this way, the microarray profiles do not support the monitoring of tags found in low abundance.

The above described drawbacks emphasize, that better strategies are needed to transfer information from SuperSAGE expression profiling to hybridization-based platforms.

8.4.2 Use of the 26pb tag sequence information in PCR-based procedures

As detailed in Section 3.5.3 (**Table 3-5**), and parallel to spotting onto microarrays, the sequence information from the 26bp tags was used as starting point for 3'- and 5'-RACE procedures. The amplification of chickpea sequences results in more than one product, that can be amplified from a single UniTag. Nevertheless, in most of the cases the amplified products preserve the same homology with EST accessions shown by the original UniTags. An exception of this tendency was shown by UniTag STCa-8061, which was initially annotated to a ß-1,3 glucanase. From five 3'- and 5'-RACE fragments of different lengths, one was annotated to a different gene. This example raises two main questions: i) how specific are RACE amplification reactions, and ii) what is the probability, that *different* transcripts are amplified when a tag is derived from a sequence region conserved by many transcripts? Along these lines it was demonstrated that the possibility of amplifying different alternative fragments out of one single transcript section could be higher than expected (Johnson et al., 2005; Gingeras, 2007).

These facts can have repercussions on the transfer of information from SuperSAGE to PCR-based techniques requiring larger sequence stretches, for example, qRT-PCR or *in situ* PCR. Provided the RACE amplification is specific enough to detect the "genuine" cDNA fragment from which a given tag is derived, qRT-PCR guarantees high specificity for expression profiling assays. In a previous report on the characterization of more than 1,400 TFs in *Arabidopsis*, the proportion of non-specific qRT-PCR products was about 4% (Czechowski et al., 2004). In the present study, the results of the SYBRgreen^{TM} and TagMan^{TM} assays were in congruence with the SuperSAGE profiles of the selected UniTags (**Section 3.6.2**). However, the number of assays is not large enough to be considered as statistically representative. For that purpose, hundreds of TaqMan^{Tm} or SYBRgreen probes would be needed.

Parallel to the present work, as an additional confirmation of the transferability of the generated information, *in situ* PCR (Bagasra, 2007) assays were tested. In the research group of Jean-Jacques Drevón (INRA, Montpellier, France), successful localization of various chickpea messenger RNAs was achieved in fixed slices of chickpea roots and nodules (**Figure 8-5**).

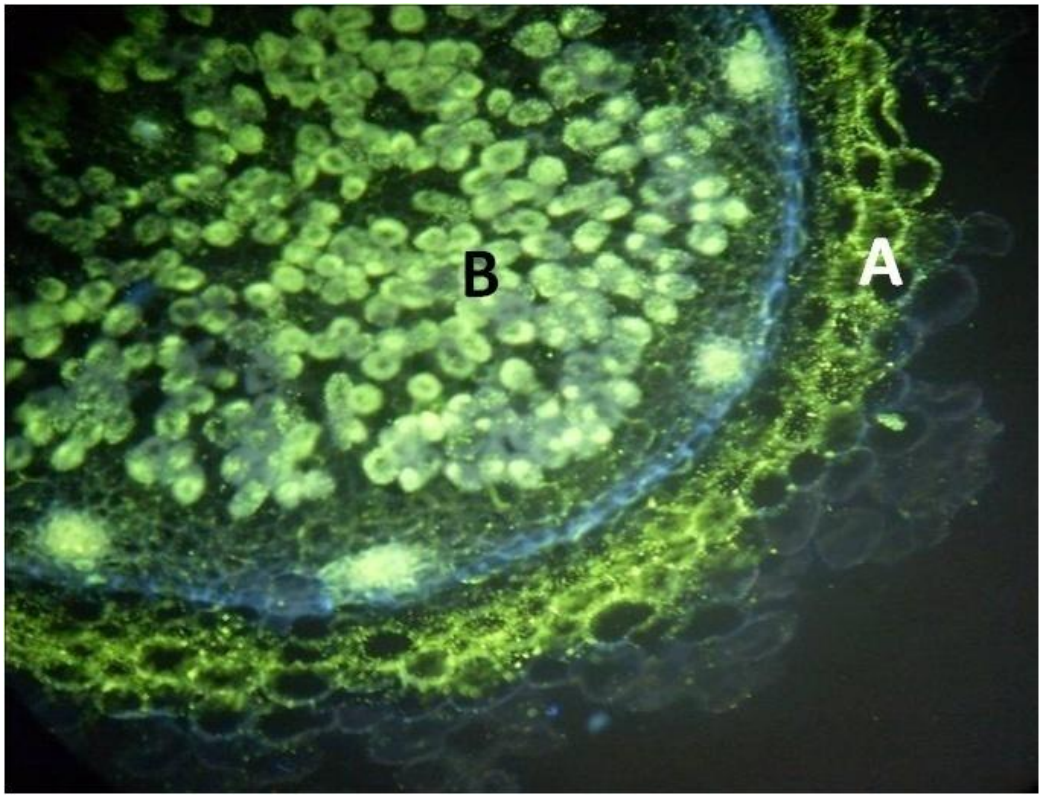


Figure 8-5 ***In situ*** **detection and localization of a PP1 transcript in chickpea nodules**

In situ PCR amplification of the protein phosphatase 1 transcript derived from the 3'RACE amplification of UniTag STCa-1016 and its localization in a cross-section of chickpea INRAT-93 nodules. Microscope image kindly provided by Drs. Mainassara Zaman and Jean Jacques Drevón (INRA, Montpellier, France). Green fluorescence in the nodule cortex (**A**) and in the bacteroids (**B**) indicates high PP1 transcript accumulation.

8.5 Correlation of UniTag expression ratios with GO categories: What can the chickpea transcriptome tell us in a holistic approach?

Up-regulated genes in the present study are expected to deliver valuable information about proteins that are important for processes triggered by new environmental conditions in chickpea. However, it should be stressed, that downregulated or constitutively expressed transcripts also contain important information for the survival of living beings. With the advent of high-throughput profiling techniques, many studies started to consider the part of the transcriptome that is repressed under stress as an important source of information. As an example, by studying the responses of more than 16,000 transcripts responding to desiccation in *Medicago*, Buitink and co-authors (2006) dedicate part of their results section to the down-regulated genes. However, their discussion did not approach these genes individually, and remained mainly descriptive. In a more recent publication, reporting the analysis of 750 chickpea stress-responsive genes, Mantri and co-authors (2007) treated down- and up-regulated transcripts equally by grouping them into biological processes. Similarly, Irsigler and co-authors (2007) followed the same lines in a study on osmotically stressed soybean leaves.

In a similar approach, the present study grouped all the annotated transcripts according to their related gene ontology (GO) biological process and cell component categories. Additionally, the expression ratios of each transcript were added up to each GO category via GSR analysis (Lee et al., 2005). In this way, over- and under-represented GO biological processes and cell components in salt- or drought-stressed chickpea roots were revealed. Subsequently, common tendencies between both types of stress were filtered out with the intention to reveal general osmotic- and ionic-stress responsive processes in chickpea roots **(Table 8-2)**. In the coming sections, the following three main categories will be shortly discussed: i) Exclusively overrepresented GO biological processes common for drought- and salt stressed chickpea roots (induction), ii) Exclusively underrepresented GO biological processes common for salt- and drought-stressed chickpea roots (repression), and iii) Biological processes simultaneously over- and under-represented under both stresses (readjustment).

Table 8-2 GO biological processes with common representation tendencies in drought- and salt-stressed roots of chickpea plants

Upper panel: Exclusively overrepresented biological processes common for salt- and drought-stressed chickpea roots (bright red arrow)
Center panel: Biological processes simultaneously over- and under-represented under both stresses (light green and red arrows)
Lower panel: Exclusively underrepresented biological processes common for salt- and drought-stressed chickpea roots (bright green arrow)

GO ID	GO Biological process
GO:0009607	Response to biotic stimulus
GO:0015031	Protein transport
GO:0046907	Intracellular transport
GO:0044248	Cellular catabolic process
GO:0051641	Cellular localization
GO:0006807	Nitrogen compound metabolic process
CMC-1	General ROS scavenging enzymes
GO:0009057	Macromolecule catabolic process
GO:0006091	Generation of precursor metabolites and energy
GO:0006468	Protein amino acid phosphorylation
GO:0006952	Defense response
GO:0005975	Carbohydrate metabolic process
GO:0006350	Transcription
GO:0050794	Regulation of cellular process
GO:0050789	Regulation of biological process
GO:0030154	Cell differentiation
GO:0019222	Regulation of metabolic process
GO:0031323	Regulation of cellular metabolic process
GO:0008219	Cell death
GO:0032502	Developmental process
GO:0006793	Phosphorus metabolic process
GO:0043687	Post-translational protein modification
GO:0009059	Macromolecule biosynthetic process
GO:0016310	Phosphorylation

8.5.1 Common salt and drought over-represented (induced) biological processes

Surprisingly, no direct stress-related GO biological process (e.g Response to stress) was exclusively over-represented under salt and drought stress in chickpea roots. However, the GO category 0009607 (Response to stimulus) was detected, possibly reflecting the activation of non-specific response mechanisms. Another category, namely the group of ROS-scavenging proteins (CMC-1), indicates transcriptional activation of responses against oxidative stress, a common phenomenon upon salinity stress and desiccation in plants (Gechev et al., 2006). Nitrogen metabolism also represents one process which is normally

boosted under stress, and could be linked to stress responses like proline accumulation, or to general protein cycle re-adjustments (Verdoy et al., 2006). Further on, two transport-related categories indicate that re-localization processes are also very active under both stresses in chickpea roots. Confirming this result, Taylor and co authors (2003) report on the adjustments of several protein import pathways in plant mitochondria under abiotic stresses.

Although no stress categories *per se* are consequently over-represented, processes like intracellular transport, nitrogen compounds metabolism, and ROS scavenging demonstrate to have stress-responsive components in chickpea roots.

8.5.2 Common salt and drought under-represented (repressed) biological processes

Among the common GO categories detected to be exclusively transcriptionally repressed in chickpea roots under salt and drought stress, four processes are related to regulation (i.e. cellular process, biological process, metabolic process, and cellular metabolic process). The broad coverage of these GO terms does not allow concrete conclusions as to what specific biological or metabolic process is concerned. On the other hand, more discrete terms like Cell death (0008219) are easier to approach to some extent. For chickpea, it has already been suggested that the repression of ageing and death-related events can lead to stress tolerance (Mantri et al., 2007), a fact that confirms the present results.

For other GO categories, like cell differentiation and development it cannot be excluded that their underrepresentation may reflect the particular transcriptome of some root sections rather than the one of the whole organ. For example, ABA in stressed roots can act as growth promoter or inhibitor, depending on its place of action. This compartmentalized action can consequently be reflected by the contrasting expression levels of ABA-responsive elements along different root sections (Sharp and Lenoble, 2002).

The present result suggests a transcriptional repression of developmental processes in the stressed root. However, it cannot be excluded that particular root sections may show a contrasting tendency, and may even promote their development. Repression of cell death has to be further researched in chickpea, because it owns great potential for stress-tolerance.

8.5.3 Simultaneously induced and repressed biological processes in salt- and drought-stressed chickpea roots

The discovery of simultaneously over- and under-represented GO biological processes in drought- and salt-stressed chickpea roots is of great value, because it touches two very important aspects:

I) There are processes in chickpea roots, in which some of the component pathways could be repressed, whereas some others could be induced. For instance, the whole palette of defense mechanisms in chickpea may **not** be completely deployed by the roots under ionic/osmotic stress. As an example, oxidative burst is a promoted reaction to biotic stresses in plants as a defense strategy against phytopathogens (Bolwell et al., 2002). On the other hand, the same reaction is repressed as a defense mechanism from plants against abiotic stresses in order to prevent cell death. In this way, GO categories like Defense responses (GO:0006952), which may cover biotic- and abiotic-stress responses, may show **readjustment** rather than exclusively induction or repression.

II) The resolution of the GO categories is too low to discriminate the activation or de-activation of more specific processes. Therefore, one should refrain from misleading interpretations.

The present results emphasize, that although the gene ontology database can be considered an important source of information, this database standardizes descriptive terms related to a gene, rather than replaces a more specific pathway analysis (Rhee et al., 2008). Analyses like GSR do not consider the interactive relations between genes encoding proteins working in the same processes. Therefore, results extracted from this kind of analysis should be considered as supportive information only, and are recommended to be critically evaluated.

8.6 What could be the physiology beyond the transcriptome responses?

In the individual Results sections of the present work (chapters 4, to 6), massive information about the “re-modelling” of the chickpea root and nodule transcriptomes upon salt and drought treatments has been presented. Along with these results, background information was provided about the main protein classes and gene families involved in salt and drought stress responses in plants. Regarding their biological meaning, according to Shinozaki and Yamaguchi-Shinozaki (2007), the proteins involved in general plant stress

responses can be grouped into two big categories: i) regulatory level, and ii) physiological effector level (**Figure 8-6**).

In the previous Results sections, extensive information has already been given about the proteins involved in regulatory processes during stress responses and the expression of their annotated UniTags in chickpea (i.e. signal transduction, and regulation of transcription). Therefore, the following sections will mainly focus on the relationship between the chickpea transcriptome data and stress-related physiological processes. Additionally and based on the present chickpea profiles, some new re-arrangements in the scheme of Shinozaki and Yamaguchi-Shinozaki are proposed (**Figure 8-6**).

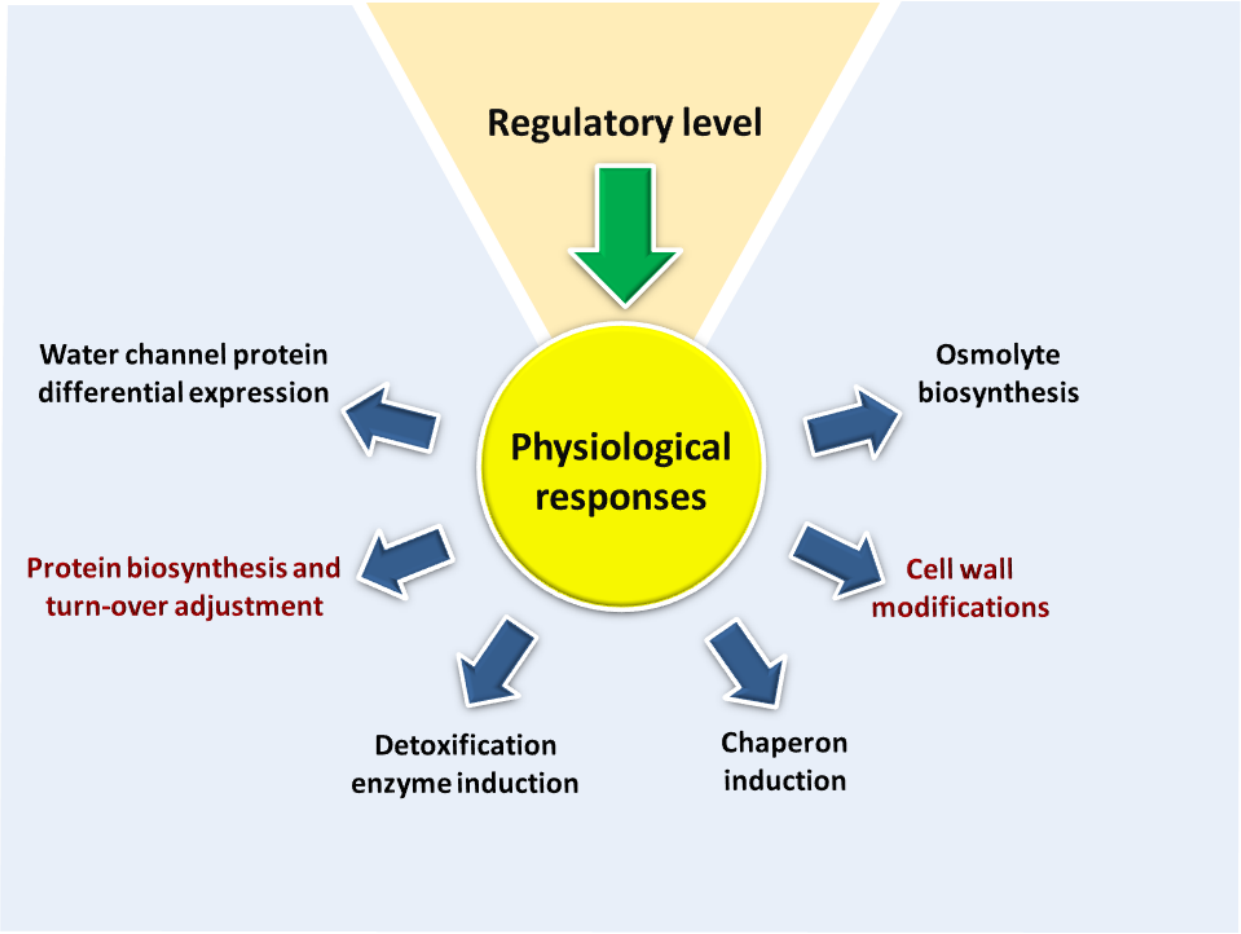


Figure 8-6 Main physiological responses to ionic and osmotic stresses in plants

Main response categories upon ionic and abiotic stresses in plants, based on the general scheme of Yamaguchi-Shinozaki (2007), are depicted. Plant responses are grouped into two major categories: i) regulatory level (orange background) and ii) physiological effector level (light blue background). Additionally, based on the information extracted from chickpea, new physiological level elements are suggested or re-arranged (red writing).

8.6.1 Aquaporin activity and water balance reflected by chickpea transcript profiles

Aquaporins are membrane integral proteins of relatively small size (23-31 kD) belonging to the family known as major intrinsic proteins (MIPs), with members in animals, microbes, and plants (Maurel et al., 2008). Based on sequence homology, MIPs of plants are divided into four groups: plasma-membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs). Physiologically, MIPs serve as channels for other small molecules apart from water, like ammonia (Niemietz and Tyerman, 2000), boric acid (Takano et al., 2006), and gases like CO_2 and NH_3 (Tyerman et al., 2002). In *Arabidopsis*, at least 35 MIPs isoforms are known (not including products from alternative splicing), presenting different expression levels (Alexandersson et al., 2005).

In chickpea roots and nodules, at least 80 UniTags annotated to MIPs were discovered, with expression levels ranging from 30-fold up-regulation under drought or salt stress (STCa-21698; Q8W4T8_MEDTR, PIP2), and 40-fold down-regulation under salt stress (STCa-6786; Q8L5G0_CICAR). The meaning of the differing regulation levels under salt and drought stress relies on the physiological events in which MIPs are involved. These proteins have been associated to processes like general water transport, transpiration, tissue expansion, tissues desiccation, nitrogen fixation, CO_2 transport, and nutrient uptake, processes reacting differentially upon abiotic stresses in plants (Maurel et al., 2008). For example, regulation of turgor under drought and salt stress throughout water re-location in the cytoplasm and the vacuoles is a central issue in which MIPs are involved. This process requires the up-regulation of certain aquaporins (Maurel et al., 1997). On the other hand, adjusting stress-induced osmotic pressure imbalances requires regulation of the water permeability of the plasma-membrane and a reduction of hydraulic conductivity of the cell, all dependent on the down-regulation of other MIPs (Shope and Mott, 2006).

For both salt and drought stress in plants, MIP transcripts keep levels close to constitutive or are down-regulated, with the exception of specific over-expression of certain PIP transcripts (Alexandersson et al., 2005; Guo et al., 2006). These observations are in agreement with the present results. For the entire chickpea dataset, the only MIP-UniTag with $R_{(ln)}$>3.0 under salt and drought stress was annotated to a PIP2 protein ($R_{(ln)}$>3.2; >25-fold up-regulation; **Table 7-2**). Regarding the specific physiological function of PIP2s in roots, a key biochemical study has been published for *Arabidopsis* by Javot and co- authors (2003). According to this report, a single PIP2 isoform (PIP2;2), mostly expressed in the root cortex,

endodermis, and stele, played a crucial role in water uptake of the roots. In chickpea, the particular PIP2 transcript was over-expressed upon both stresses, despite the very different experimental conditions. This fact may link its up-regulation to a common salt and drought response mechanism, which could also be water channelling.

A second MIP transcript commonly up-regulated under drought and salt stresses was annotated to a TIP-type aquaporin (STCa-24453; Q8L5G0_CICAR; **Table 7-2**). The physiological roles of this type of proteins in roots under osmotic and ionic stress involve the equilibration of osmotic pressure (turgor maintenance) and the trapping of toxic ions (e.g. Na^+). Even if their expression levels are kept constitutive upon stress, TIPs are crucial for plant survival. For example, the loss of function of a single TIP isoform in *Arabidopsis* led to plant death (Ma et al., 2004).

Since the activity of MIPs is also regulated by co- and post-translational modifications (Daniels and Yeager, 2005), transcript levels *per se* cannot fully explain the underlying physiological processes in chickpea roots. In summary, the observed results still leave many questions to be answered. However, UniTag STCa-21968 (PIP2), from which the full-length cDNA sequence is known, is a strong candidate for further characterization, since it is highly up-regulated after stress. This observation should be then confirmed with biochemical data. Also, the determination of sub-cellular and tissue-specific localization merit high priority.

8.6.2 Transcripts encoding detoxification enzymes in chickpea roots and nodules: Can transcript levels of proteins tell about their enzymatic activity?

Aside of the mechanisms regulating water balance, the detoxification of by-products derived from the metabolic disequilibrium, like ROS, represents a major field of action in plants under stress (Gechev et al., 2006). In the present study, the transcription profiles of genes coding for proteins involved in ROS scavenging were approached individually for each stress situation (drought and salt) and sampled organs (roots and nodules). As described in each corresponding chapter, the expression levels of several genes suggest that chickpea roots and nodules have already activated their anti-oxidant machineries at the analyzed stress points.

As mentioned before, the transcriptome information delivered in the present work is not supported by biochemical data. Up to now, no report exists in the literature, directly linking the activity of the ROS-scavenging-involved enzymes (e.g. SOD, CAT, APX, AR, DHAR, GR, GPX, and GST) with their transcript levels in plants. However, the enhanced activity of

antioxidant enzymes upon osmotic and ionic stress has already been reported for some species. In potato seedlings, the activities of SOD, APX and CAT were investigated under salt stress in two cultivars with differential NaCl tolerance. The activity of the monitored enzymes increased in direct proportion to the external NaCl concentration and the degree of salt tolerance of the cultivars (Rahnama and Ebrahimzadeh, 2005). In a parallel study in which more than 12,000 potato cDNAs were monitored under salt and cold stress, transcript levels for the above mentioned enzymes revealed stress induction (Rensink et al., 2005). Consequently, transcript levels seem to correlate directly with the activity of antioxidant enzymes, although post-transcriptional regulation events cannot be excluded. This provides supportive evidence, that ROS-scavenging enzymatic mechanisms should be active in drought- and salt-stressed chickpea roots.

This aspect enforces the further characterization of highly expressed UniTags, like for example the SOD transcript isoform STCa-7896, Q9ZNQ4_CICAR, which was differentially expressed already in control nodules ($R_{(ln)}$>2.9, 20-fold), but was highly induced in roots only after salt stress ($R_{(ln)}$>3.7, 40-fold).

8.6.3 Compatible osmolyte accumulation reflected by transcript levels of rate-limiting genes

Complementing water balance and ROS detoxification processes, the accumulation of compatible osmolytes represent one of the strategies of plants to overcome osmotic stress (Munns and Tester, 2008). In the present study, transcript levels of genes encoding proteins involved in compatible osmolyte biosynthesis and transport in salt- and drought-stressed chickpea roots have been presented in **Sections 4.5.5 and 6.3.6**, where many of them revealed to react upon the stress conditions. Despite of the clear responses of these genes at the transcriptome level, the question whether transcript accumulation is positively correlated with the physiological effect (increase in osmolyte concentration), remains unanswered.

For many of these genes, the correlation between transcript levels, enzymatic activities, and concentrations of metabolic products is not clear. In carrot, reduction in the activity of sucrose synthase by antisense expression of the SUS gene did not have effects on sucrose concentration in leaves and roots (Tang and Sturm, 1999). This result agrees well with observations in *Arabidopsis*, in which alternative roles of the SUS genes (i.e. its involvement in signaling events), apart from biosynthesis of sucrose, were correlated with SUS transcript

levels (Baud et al., 2004). Also in *Arabidopsis* the same type of results has been reported for the correlation of trehalose concentration and threhalose 6-phosphate (TPS) gene expression, suggesting that the accumulation of this sugar is not the only physiological role of TPS in plants (Avonce et al., 2004).

Proteins involved in proline accumulation, like proline transporters, have also been targets of functional characterization. In *Arabidopsis*, a transgenic approach monitored the altered expression of a proline transporter (ProT), revealing a correlation between proline accumulation and translocation rates in leaves and roots, and the expression of the ProT gene. Additionally, the expression of transcripts encoding proline dehydrogenase (PDH), an inhibitor of proline accumulation, correlated negatively with the detected proline levles (Ueda et al., 2008).

Regarding the accumulation of polyamines, the reduction of arginine decarboxylase transcripts in rice was accompanied by a reduced putrescine and spermidine pool, indicating a direct correlation between transcript and the osmolyte levels (Trung-Nghia et al., 2003). Also in rice, the over expression of a heterologous S-adenosylmethionine decarboxylase cDNA was linked directly with an increase in spermidine concentration (Thu-Hang et al., 2002). The same type of correlation holds also for spermidine concentration and spermidine synthase (SPDS) transcript levels in *Arabidopsis*. In seeds of mutant plants with impaired transcription of SPDS, the levels of spermidine and its precursor putrescine were drastically reduced.

According to previous literature, the transcription of genes related to sugar metabolism and transport cannot be correlated to an increase in sugar concentration in chickpea. Whereas the transcript levels of proteins related to proline or polyamines accumulation can probably be linked to higher osmolyte concentrations in stressed roots. For example, the 6- and 11-fold up-regulation of arginine decarboxylase (STCa-8875, SPE1_PEA) and S-adenosyl-methionine decarboxylase (STCa-23965, Q8LKJ7_9ROSI) UniTags under salt stress. However, in this case, direct metabolite measures are a must to corroborate the present results.

8.6.4 Activity of chaperons

Among other important physiological reactions, the protection of the internal protein machinery by expression of protein chaperons plays a major role in the avoidance of lethal conditions in plant cells under stress (Parcellier et al., 2003). In this respect, heat shock proteins (HsPs) represent the best-characterized family of plant chaperons (Joe et al., 1981; Waters and Vierling, 1999). Therefore, this type of proteins is taken as an example of the

broad range of expression levels shown by chaperon-encoding genes in chickpea roots and nodules under salt and drought stress (**Table 8-2**).

Table 8-2 Chickpea UniTags annotated to heat-shock proteins

HsPs-annotated UniTags in chickpea along with their expression levels in drought-stressed roots [D], salt-stressed roots [S(rt)], and salt-stressed nodules [S(nd)].

Tag ID	Uniprot ID	Protein name	D	S(rt)	S(nd)
STCa-12549	GSTX6_SOYBN	Heat shock protein 26A (G2-4)			
STCa-3042	GSTX6_SOYBN	Heat shock protein 26A (G2-4)			
STCa-2175	GSTX6_SOYBN	Heat shock protein 26A (G2-4)			
STCa-977	GSTX6_SOYBN	Heat shock protein 26A (G2-4)			
STCa-2920	Q8GSN3_CUCMA	Non-cell-autonomous heat shock cognate protein 70			
STCa-9718	HSP7M_PHAVU	Heat shock 70 kDa protein, mitochondrial precursor			
STCa-3482	Q40323_MEDSA	70 kD heatshockprotein			
STCa-12318	Q67BD0_TOBAC	Heat shock protein 70-3			
STCa-3010	Q84QJ3_TOBAC	Heat shock protein 70			
STCa-19601	HSP7M_PEA	Heat shock 70 kDa protein, mitochondrial precursor			
STCa-9122	Q40323_MEDSA	70 kD heatshockprotein			
STCa-3480	Q40323_MEDSA	70 kD heatshockprotein			
STCa-14016	Q67BD0_TOBAC	Heat shock protein 70-3			
STCa-3481	Q40323_MEDSA	70 kD heatshockprotein			
STCa-12317	Q67BD0_TOBAC	Heat shock protein 70-3			
STCa-3136	HSP80_SOLLC	Heat shock cognate protein 80			
STCa-5806	Q2PEX3_TRIPR	Putative HEAT SHOCK PROTEIN 81-2			
STCa-3558	Q2PEX3_TRIPR	Putative HEAT SHOCK PROTEIN 81-2			
STCa-16614	Q76B83_ORYSA	Heat shock protein 81-2 (HSP81-2)			
STCa-10689	HSP83_IPONI	Heat shock protein 83			
STCa-16643	Q6UJX5_NICBE	Molecular chaperone Hsp90-2			
STCa-8779	Q6UJX5_NICBE	Molecular chaperone Hsp90-2			
STCa-8778	Q6UJX5_NICBE	Molecular chaperone Hsp90-2			
STCa-8313	Q6UJX5_NICBE	Molecular chaperone Hsp90-2			
STCa-5805	Q6UJX5_NICBE	Molecular chaperone Hsp90-2			
STCa-19377	Q8H288_ANACO	Class-1 LMW heat shock protein			
STCa-7084	Q8W0Q8_SORBI	Small heat shock-like protein			
STCa-3195	Q6K2F0_ORYSA	Heat shock protein-like			
STCa-758	O49457_ARATH	Heat-shock protein			

Within the entire chickpea dataset generated by SuperSAGE, at least 29 UniTags were annotated to HsPs, with expression levels ranging between $R_{(ln)}$=2.04 (8-fold up-regulation, STCa-8779; salt stress) and R(ln)=-2.7 (15-fold down-regulation, STCa-12317; drought and salt stress). In plants, HsPs are known to display a complex spectrum of targets, tissue-, and developmental stages-specific expression (Cooper et al., 1984; Kotak et al., 2007). Therefore, any deduction of the activity of HsPs, solely based on transcript levels of whole organs (roots

or nodules) may be very difficult. Additionally, it is to be considered that many of the proteins, from which UniTags have been detected, may also undergo post-transcriptional and -translational regulation. Despite these adversities, transcripts that are highly and differentially expressed upon stress can already be spotted for subsequent studies.

8.6.5 Protein biosynthesis and turnover events are boosted in chickpea plants under salt and drought stress

Apart from displaying a broad battery of proteins that protect, or keep other proteins in functional shape (chaperons), boosting the "protein cycle" is an essential process for the survival of the plant under adverse conditions. The synthesis of new polypeptides and the degradation of pre-existing ones is a crucial mechanism in the plant cell's life (Smalle and Vierstra, 2004). Under stress, fine tuning of rate-limiting enzymes, the continuous renewal of regulatory networks, and biosynthesis and degradation of effector proteins plays a major role for the adaptation of the plant. Therefore, expression changes in genes coding for ribosomal proteins, translation-related polypeptides, and proteins involved in post-translational modifications may reflect adjustments of general protein biosynthesis to new environmental conditions around the plant cell.

In plants, taking *Arabidopsis* as an example, about 5% of the proteome corresponds to pathway components (more than 1,400 proteins and peptides), that can be connected to the functioning of the Ubiquitin/26S proteasome system (Vierstra, 2003). This complex is in charge of disassembling the defect- or the not-needed-proteins, a crucial event in the adaptation of the cell to new conditions. On the other hand, the protein biosynthesis machinery consists of four ribosomal RNAs and 81 ribosomal proteins (r-proteins). Plant r-protein genes exist in multi-member families, showing a high degree of functional redundancy and specificity for tissues, a developmental stages, and stress responses (Degenhardt and Bonham-Smith, 2008).

In the entire chickpea dataset, a total of 30 transcripts were annotated to components of the 26S proteasome sub-units, 60 UniTags to ubiquitin-related proteins, and 215 transcripts to ribosomal proteins, many of them showing differential expression upon drought and salt treatments.

By observing global transcriptome changes, correlation of the expression ratios from the whole UniTag dataset with GO functional categories provided information supporting, that protein cycle-related processes are notoriously reacting upon stress. In drought-stressed roots, **GO biological processes** like Translation (GO:0006412), Proteolysis (GO:0006508), and

Ubiquitin cycle (GO:0006512), as well as **GO cellular components** like Endoplasmic reticulum (GO:0005783) were over-represented with P<0.002. In salt-stressed roots, the same analysis revealed over-representation of **GO biological processes** like Post-translational protein modification (GO:0043687), and Proteolysis (GO:0006508) (P<0.001), as well as **GO cellular components** like Protein complex (GO:0043234), Ribosomal subunit (GO:0033279), and Proteasome complex (GO:0000502) (P<0.06).

The transcription profiles obtained from different chickpea organs allow to conclude, that protein biosynthesis as well as protein turnover undergo major re-adjustments under salt and drought stress. However, the complex networks of multiple and redundant elements catalyzing these two processes makes it impossible to deduce specific protein activities based solely on transcripts levels.

8.6.6 Cell wall rearrangements and growth promotion in salt- and drought-stressed chickpea roots: Gene expression profiles suggest stress-induced responses

Apart from strategies aimed to overcome water misbalance, high toxicity levels, and metabolic disorders in roots under salt and drought stress, some mechanisms are directed towards alleviating mechanical pressures, and to counteract growth inhibition in distinct plant sections. Plants generally can recover and promote further root growth within one day after a short inhibition period induced by osmotic stress (Munns, 2002). As reported by L'Taief and co-authors (2007), the difference in the root growth rate between the salt-succeptible (Amdoum) and -tolerant (INRAT-93) chickpea varieties is significative plants growing in of 25 mM NaCl. This result was also confirmed in the present thesis **(Section 4.1)** by comparing the fresh weight of salt-tolerant (INRAT-93) and -succeptible (ICC4958) chickpea varieties for a growth period of five weeks on 25 mM NaCl. Under drought stress, it is also known that chickpea cultivars with higher root growth rates are more viable in desiccated soils than plants suffering from growth inhibition (Gunes et al. 2006, http://www.cazv.cz/UserFiles/File/PSE%2052_368-376.pdf).

Therefore, the expression levels of transcripts coding for proteins involved in cell wall re-arrangements and plant growth-related processes should be of special interest for the understanding of salt and drought stress-tolerance of chickpea roots. Nevertheless, it should be stressed that the time frame of the present expression profiling does not cover late responses, where growth-related processes could be more active than before (Merchan et al., 2007).

Various examples of chickpea stress-responsive transcripts, which are involved in growth and cell-wall rearrangements, can be extracted from the present results. As observed in **Section 7.2.6** (**Table 7-2**)**,** UniTag STCa-19021, annotated to an extensin protein, was commonly up-regulated in chickpea roots under salt- and drought-stress. Extensin proteins are involved in cell-wall strengthening and counteraction of mechanical pressures in plants (Tire et al., 1994; Ueda et al., 2007). Additionally, the common salt- and drought-stress-induced up-regulation of UniTags annotated to ribosylation factors (ARFs; Song et al., 2006; Matheson et al., 2007) and 4-hydroxyproline-rich glycoproteins (Kieliszewski and Lamport, 1994; Kieliszewski and Shpak, 2001), can be also associated to growth and cell wall re-organization processes (**Table 7-2**).

The mechanisms controlling growth regulation in plants under osmotic and ionic stress are not totally understood (Munns and Tester, 2008). Additionally, as postulated by Poroyko and co-authors (2007), the different root sections exhibit different growth rates, and therefore, very different expression profiles of growth-regulating genes. As discussed in the following section, this fact shadows the power of the present transcriptome survey. Thus, it is unlikely that transcript levels of a few genes can be taken as indicators of growth as a physiological process in chickpea. However, the observed expression changes upon salt and drought stress indicate, that, already at early stages, chickpea roots may promote cell wall rearrangements, which can lead to growth in a later stage.

8.6.7 "Black box" effect: differences in tissue-specific gene expression are masked in the present study leading to loss of resolution

Recent results on cell type-specific gene expression profiling in plants suggest that a major part of the transcriptome in a plant organ is compartmentalized (Galbraith and Birnbaum, 2006). Up to now, only few studies report on tissue- and cell–type-specific expression profiles in plant roots. One of the most complete examples for such a study monitored the expression profiles of 10,500 genes in five different root subzones in *Arabidopsis* (Birnbaum et al., 2003). According to the authors, eight major profile patterns were observed, in which 54% of the genes were differentially expressed between root sections. In a similar study in maize, transcript profiles in millimetre sections away from the root apex revealed a high degree of differential expression between the sampled sections (Poroyko et al., 2007). For a better survey, a typical cross-section of a plant root is depicted in **Figure 8-7**.

In both studies, the differences in gene expression were attributed to the various cell types (including cell type-specific isoforms), the different developmental stages and tissue growth rates. One of the most important results was the discovery that many hormones act localized in so called “cassettes”, which are sections of neighbouring tissues with a specific hormone activity. This latter fact can be reflected by the contrasting profiles of specific hormone-responsive TFs across root tissues. A possible exemplifying explanation for the wide range of bZIP TFs expression levels observed in chickpea roots, a TF class known to be ABA-responsive (Kim et al., 2004).

The transcriptome-wide responses of chickpea plants upon salt and drought stress were studied with whole organs (roots and nodules). In principle, the transcription profiles observed here therefore represent only the average of the expression levels of the different root and nodule sections. This aspect may explain the contrasting expression levels of related transcript isoforms, or the down-regulation of genes expected to be up-regulated and *vice versa*. The present work, however, should be taken as a reference for future studies, in which organ sub-sections rather than whole organs must be approached. To that end, advanced sampling techniques like, for example, laser-capture micro-dissection (LCM; Nakazono et al., 2003) could be of great potential.

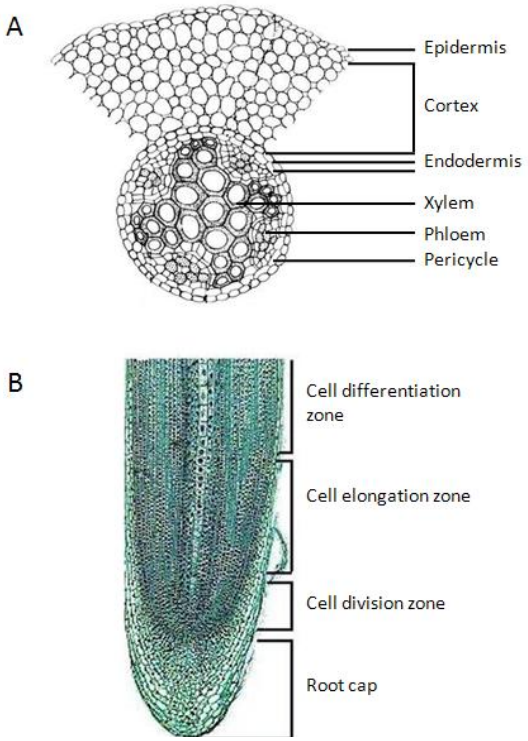


Figure 8-7 Different tissues of a plant root in schematic cross-sections

A) Transversal cross-section
B) Longitudinal cross-section

8.7 Conclusions

8.7.1 High coverage survey of the poly(A)$^+$-mRNAome in chickpea roots

As critically discussed in the previous sections, the transcriptome of chickpea roots is not yet complete, because non-polyadenylated RNAs and other small non-coding, but transcribed RNA species are still missing in the samples. However, an important first step has been achieved with the present work. More than 30,000 UniTags representing most probably 30,000 different transcripts have been detected, some even discovered for the first time, and monitored under salt or drought stress. The amount of processed information in the present work is 30-fold larger than the previously published data of salt- and drought-stress responses in this species.

8.7.2 SuperSAGE expression profiling combined with 454-sequencing: a strong analysis tool

Thanks to various optimizations of the SuperSAGE technique in the present work; the amount of processed information has been boosted. In other genome-wide expression profile analyses carried out with the improvements presented here, the analysis of far more than a million tags per sequencing round has been achieved (GenXPro GmbH, personal information). Even the analysis of 270,000 tags presented here already exceeds many of the previously published SAGE-based data, not only in plants**.** As discussed in previous sections, the procedure still has several drawbacks. However, the constraints imposed by the relation of tag-size and potentially processed information could be much reduced.

8.7.3 Low abundance transcripts as major information source in transcriptome analysis

As discussed in Section **8.2.4**, low abundance transcripts may represent a big portion of the transcriptome. The present work reveals the dynamics of thousands of low-abundant transcripts in chickpea. Probably some of them are no “genuine transcripts”. However, it is fair to say that the level of resolution possible by SuperSAGE cannot be reached by most, if not all, other profiling techniques.

8.7.4 Chickpea transcriptome responses to salt and drought stress imply the activity of stress-related genes and many genes previously not stress-associated

The high expression of transcripts annotated to genes not previously associated with stress, together with stress-related genes, allow to think of stress reactions specific for chickpea and legumes generally. Probably, the differences between the results of the present work and gene expression studies carried out in non-crop model organisms (e.g. *Arabidopsis,* but also the legumes *Medicago truncatula* and *Lotus japonicus*) can be explained by the dynamics of crop-related traits (i.e. growth, yield). However, this hypothesis requires supporting information, ideally derived from studies designed as continuation of the present research.

To satisfy the needs of the breeders and growers in terms of stress tolerance and stable crop yield in chickpea, future bioassays should be carried out monitoring several abiotic, but also biotic threats. It is not enough to only know genes that confer stress tolerance. Expression levels of these genes have to be correlated with nutritional values, plant growth, and plant metabolism. For such a comprehensive view, the thousands of non-stress related genes identified in this thesis may gain importance. Up to now, we are not aware of the biological meaning of many of them.

8.8 Future steps : perspectives for abiotic stress research in chickpea

The present work represents a step forward towards a better understanding of the transcriptome dynamics in drought- and salt-stressed chickpea plants. Hitherto, the available information merely comprised about 1,000 transcripts upregulated under drought and salt stress. With the results documented in the present thesis, information of at least 30,000 unique transcripts derived from 270,000 sequenced tags is now available. What should be done with this huge amount of new information? What to do with 30,000 pieces of genes? In the following sub-sections some suggestions to exploit this huge information content will be highlighted.

8.8.1 Screening of substantially more varieties/cultivars for promising candidate transcripts (genes)

One of the immediate obligations extending from the present line of research is an evaluation of different chickpea cultivars/accessions with diverse degrees of tolerance or sensitivity to drought and salt in multiple environments and at multiple time points after

onset of stress. Time and costs are presently still limiting the application of SuperSAGE on hundreds of cultivars in parallel in diverse situations after stress treatment. However, already selected candidate transcripts can be screened via qRT-PCR instead. Today, already more than 2,500 transcription profiles can be analyzed in parallel via this technique, including weakly expressed genes (Caldana et al., 2007). For this purpose, massive 3' and 5'-RACE amplifications are needed, which is demanding, but absolutely more efficient than the process of blind EST sequencing.

8.8.2 Generation of segregating chickpea populations with different stress tolerance levels and eQTL-mapping of candidate genes

Following the screening of different chickpea varieties/cultivars for the reactions of candidate transcripts (genes) upon diverse stresses, the logic next step is the segregation analysis of such candidates in chickpea populations derived from parents with differential stress tolerance. The objective should be the conversion of expression markers into genomic markers. One of the potential methods for such purpose is expression QTL (eQTL) mapping, which uses the expression levels of transcripts as quantitative traits (QTLs). Subsequently, these quantitative traits can be linked to other traits of interest such as drought or salt tolerance for the identification and localization of master regulators. In *Arabidopsis*, this approach has already shown success, delivering information on thousands of genes, their regulation dynamics, and possible linkages with phenotypic traits (West et al., 2007).

9 Zusammenfassung

Genomweite Genexpressionsanalyse einer Nichtmodell-Pflanze im Hochdurchsatz: Das Transkriptom der Wurzel und Wurzelknöllchen der Kichererbsen-pflanze unter Salz- und Trockenstress

Dürre in Pflanzen

Der Begriff "Dürre" bezeichnet eine außergewöhnliche Trockenperiode durch Niederschlagsmangel bei gleichzeitig hohen Temperaturen. Dürre wirkt sich schädigend auf die Vegetation aus, da die Pflanzen die abgegebene Feuchtigkeit nicht mehr durch Feuchtigkeitsaufnahme aus dem Boden ausgleichen können. Daher ist für das Auftreten einer Dürre auch die Verdunstungsrate und die Wasserrücklage im Boden von Bedeutung. Die Folgen der Dürre sind Ernteausfälle und Trinkwasserknappheit. Als Dürreperiode bezeichnet man einen Zeitraum von mindestens vier Tagen, an denen die Temperatur über dem langjährigen mittleren Höchstwert liegt und die Luftfeuchtigkeit am Mittag nur noch bis zu 40 % beträgt. Dürreperioden sind in semiariden, also halbtrockenen Gebieten normal und werden durch die hohe Niederschlagsvariabilität dieser Regionen bedingt. Erst durch eine nichtangepasste Lebensweise der Menschen wird eine Dürre zur Dürrekatastrophe.

- **Salzstress in Pflanzen**

Salzstress: bei den meisten Pflanzen (Glykophyten) die durch einen hohen Bodensalzgehalt bedingte Beeinträchtigung von physiologischen, biochemischen und molekularen Prozessen, die sich z. B. durch vermindertes Wachstum oder Verfärbung der Blätter bemerkbar macht. Dadurch, dass gelöste Substanzen im Wurzelbereich das Wasserpotenzial des Bodens absenken, treten zudem ähnliche Stress-Symptome wie bei Dürrestress auf (osmotische Einstellung). Salzstress ist nicht an Standorten, die von Natur aus hohe Salzkonzentrationen aufweisen (z. B. Meeresküsten) anzutreffen, deren Vegetation zudem eine Reihe von Anpassungen aufweist (Halophyten), sondern vor allem in Regionen mit künstlicher Bewässerung, wo es durch Verdunstung von Wasser zu einer Versalzung der Böden kommt.

- **Die SuperSAGE Methode**

SuperSAGE ist die am weitesten entwickelte Version der *Seriellen Analyse der Genexpression* (SAGE) zur qualitativen und quantitativen Analyse von exprimierten Genen. Wie bei SAGE werden von jedem Transkript (aus mRNA, die in cDNA umgeschrieben wurde) enzymatisch ein Sequenzabschnitt herausgeschnitten und so ein sogenannter *Tag* (engl. Etikett) gewonnen. Sequenziert man möglichst viele dieser Tags und zählt die verschiedenen Tags, erhält man eine Antwort auf die Frage, welches Gen wie häufig abgelesen wurde, beziehungsweise wie viele Transkripte welchen Gens in der Probe vorliegen.

Bei SuperSAGE werden mit dem Restriktionsenzym *Eco*P15I besonders spezifische Tags erzeugt, die 26bp lang sind, im Gegensatz zu den Vorgängertechniken SAGE und LongSAGE mit nur 14 und 18bp langen Tags. Die wesentlich längeren Tags erlauben eine sehr viel präzisere Zuordnung des Tags zum zugehörigen Transkript und ermöglichen es, mehr Transkripte zu erkennen. Die Genauigkeit der Tags erlaubt es auch, die Trankripte verschiedener Organismen exakt zu unterscheiden, so dass Transkriptionsanalysen von mehreren Organismen im Wechselspiel möglich werden, zum Beispiel von Parasit und Wirt ohne deren physische Trennung, wie in der konventionellen Pathologie bis heute verbreitet. Wie im SAGE-Protokoll werden aus je zwei Tags sogenannte Ditags erzeugt, die vor der Sequenzierung mittels PCR amplifiziert werden.

Mit modernen Hochdurchsatz-Sequenziermethoden können heute Hunderttausende dieser Ditags sehr schnell und günstig sequenziert werden, so dass ein sehr genaues Transkriptionsprofil entsteht, bei dem auch die vielen seltenen Transkripte, wie etwa von Transkriptions-Faktoren genau erfasst und gezählt werden können. Die Genauigkeit und Reproduzierbarkeit der Quantifizierung bei ausreichender Menge von sequenzierten Tags übertrifft die von Microarrays bei weitem. Zudem können mit SuperSAGE neue Transkripte identifiziert werden, und auch Proben von Eukaryonten mit noch unbekannten, oder nur wenig bekannten Genomen sehr genau untersucht werden.

- **Hauptergebnisse der vorliegenden Dissertation**

Die langen 26-bp-Tags können für weitere Analysen von neuen Transkripten als hochspezifische Primer eingesetzt werden, (z.B. für RACE) als Sonden zur Identifikation von Klonen in einer Genbank oder sogar für Analysen mit höherem Durchsatz direkt auf einen Microarray gespottet werden und somit auch der Kostenvorteil der Microarrays genutzt werden.

Die vorliegende Dissertationsschrift präsentiert die erste **Hochdurchsatz-Transkriptom-Analyse** der Kichererbse (*Cicer arietinum* L.), einer Kulturpflanze, die von der Forschung bisher weitgehend vernachlässigt worden ist. Dazu wurden mehr als 270,000 cDNA-Sequenzen, jede 26 Basenpaare (Bp) lang (als „Tags“ bezeichnet), die mehr als 30,000 einzigartige Transkripte (sog. UniTags) repräsentieren, sequenziert, und ihre Reaktionen auf Salz- und Trockenstress hin untersucht. Die wichtigsten Ergebnisse werden hier kurz aufgelistet:

(1) SuperSAGE als eine Technik zur Charakterisierung des Transkriptoms. Im Rahmen dieser Dissertation wurde die SuperSAGE-Technik erheblich verbessert. Zusätzlich zur Vereinfachung des ursprünglichen Protokolls wurde SuperSAGE mit einer Sequenziertechnologie der zweiten Generation, der Pyrosequenzierung von 454 Life Sciences (USA), kombiniert, was den Informationsgehalt der Ergebnisse um das 20-fache steigerte (bezogen auf die originären SAGE- und LongSAGE-Protokolle).

(2) Das Wurzeltranskriptom unter Salzstress. In Wurzeln des **salz-toleranten Kultivars INRAT-93** wurden insgesamt 86,919 Tags identifiziert, die sich in 17,918 UniTags gruppieren ließen. Von diesen UniTags wurden durch Salzstreß 2,055 (11%) induziert bzw. 346 (1,93%) reprimiert (jeweils mindestens 8-fach). Ein Transkript mit Sequenzähnlichkeit zu einem Enod 40-Protein wurde dabei am stärksten (>250-fach) induziert, während Transkripte für Superoxyd-Dismutase, Trypsin-Inhibitor und Extensin immerhin um das 30-fache aufreguliert wurden. Als Stoffwechselwege, die unter Salzstreß vorwiegend mit Transkripten versorgt werden, wurden RNA-Biosynthese, post-translationelle Proteinmodifikationen,

zelluläre Organisation und Proteinfaltung identifiziert (sog. Gene Ontology Categories, GO-Katagorien).

(3) Das Wurzelknöllchentranskriptome unter Salzstreß. In Wurzelknöllchen der gleichen Pflanzen wurden 57,281 26 Bp-Tags sequenziert, die von insgesamt 13,115 UniTags stammen. Auch hier war das Transkript für das Enod4-Protein am stärksten induziert (60-fach). Dennoch reagierten Wurzeln und Wurzelknöllchen sehr verschieden auf den gleichen Salzstreß. Zum Beispiel waren von 2,207 bzw. 2,162 mehr als 3-fach induzierten UniTags aus Wurzeln und Knöllchen nur 363 beiden Organen gemeinsam.

(4) Das Wurzeltranskriptom unter Trockenstress. In Wurzeln des **dürre-toleranten Kultivars ICC588** waren von 80,012 sequenzierten Transkripten (entsprechend 17,498 UniTags) sechs Stunden nach Beginn des Trockenstresses 388 (2,22%) mindestens 8-fach induziert bzw. 589 (3.37%) reprimiert. Ein Transkript, das für ein 14-3-3-Protein kodiert, war am stärksten induziert (45-fach). Weiterhin war die Zahl der Transkripte für einExtensin und eine NADP-abhängige Isocitrat-Dehydrogenase um mehr als das 30-fache erhöht. Die GO-Kategorien Translation, Reizbeantwortung, Produktion von Vorläufer-Metaboliten und Energie, und Reaktion auf biotischen Streß waren eindeutig überrepräsentiert.

(5) Transkript-Isoformen. Im Rahmen dieser Untersuchungen wurden verschiedenste Transkript-Isoformen von Genen entdeckt, die nach Streß aktiviert werden. Zum Beispiel waren Genfamilien, wie etwa die Genfamilie für Rezeptor-ähnliche Kinasen (receptor-like kinases, RLKs) durch mehr als 36 UniTags vertreten, die zudem noch eine differentielle Organ- und Streß-spezifische Regulation aufwiesen.

(6) Übertragbarkeit von Transkriptomdaten. Die durch SuperSAGE gewonnenen Resultate waren mit verschiedenen anderen Plattformen wie z.B. quantitativer Echtzeit-PCR (qRT-PCR) oder Microarrays kompatibel, was weitere Anwendungen

impliziert, wie z.B. eine funktionelle Genanalyse mit small interfering RNAs (siRNAs), oder eine Expressionskartierung (eQTL mapping).

(7) Eine ***in silico*-Analyse der vorliegenden Daten** ergab, dass

i) Kichererbsenpflanzen auf Salz-und Trockenstreß hin starkem osmotischen und ionischen Streß und darüber hinaus einer Überproduktion von Sauerstoffradikalen (reactive oxygen radicals, ROSs) ausgesetzt sind.

ii) in Wurzelknöllchen der Kichererbse vor Einsetzen eines Stresses bereits Transkripte für Proteine der ROS-Kontrolle stärkstens induziert sind, was auf eine vorgebildete ROS-Detoxifizierung schließen lässt.

iii) die in dieser Arbeit beobachteten Transkriptionsprofile nach Einsetzen beider Streßformen keine aktive Neusynthese des Streßhormons Abscissinsäure (abscissic acid, ABA) vermuten lassen. Jedoch wurden einige ABA-aktivierte Gene induziert, was wiederum auf eine Rolle alternativer ABA-Quellen in den betroffenen Pflanzen (wie z. B. die Freisetzung von ABA aus Konjugaten) hinweist.

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12 Appendix

High-throughput transcriptomics generates massive amounts of data which are not handable in their printed form. The present work has generated a main data matrix consisting of at least 30,000 rows, what would require about 200 printed pages. Therefore, additional data is presented here in electronic form. Detailed information about the files comprised in the attached CD (**Electronic Appendix**) is depicted in **Table A-1**.

File	Description	data type
File-1	Main data matrix	MS excel 2003
File-2	cDNA sequences of 3'- and 5'-RACE products	MS word 2003

Table A-1 Description of files included as Electronic Appendix

Erklärung

Ich erkläre hiermit, dass ich die vorliegende Dissertation selbstständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Frankfurt am Main, im August 2008 _____

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Studies

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Research / work experience

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05/2003 – up to date	**University of Frankfurt** Doctoral candidate researcher in Plant molecular biology Thesis description: "High throughput genome-wide expression analysis on a non model organism: Salt, drought, and cold abiotic stresses in Chickpea. Deep SuperSAGE screening via 454-technology by sequencing of more than 270'000 root and nodules transcripts"
06/1999 – 04/2003	**Colombian Institute of Agricultural Research / University of Frankfurt** International Atomic Energy Agency (IAEA) and UNESCO Biotechnology research council research fellow

Scientific stays

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Publications

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