Research article

**SuperSAGE: the drought stress-responsive transcriptome of chickpea roots**

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Abstract

**Background:** Drought is the major constraint to increase yield in chickpea (Cicer arietinum). Improving drought tolerance is therefore of outmost importance for breeding. However, the complexity of the trait allowed only marginal progress. A solution to the current stagnation is expected from innovative molecular tools such as transcriptome analyses providing insight into stress-related gene activity, which combined with molecular markers and expression (e)QTL mapping, may accelerate knowledge-based breeding. SuperSAGE, an improved version of the serial analysis of gene expression (SAGE) technique, generating genome-wide, high-quality transcription profiles from any eukaryote, has been employed in the present study. The method produces 26 bp long fragments (26 bp tags) from defined positions in cDNAs, providing sufficient sequence information to unambiguously characterize the mRNAs. Further, SuperSAGE tags may be immediately used to produce microarrays and probes for real-time-PCR, thereby overcoming the lack of genomic tools in non-model organisms.

**Results:** We applied SuperSAGE to the analysis of gene expression in chickpea roots in response to drought. To this end, we sequenced 80,238 26 bp tags representing 17,493 unique transcripts (UniTags) from drought-stressed and non-stressed control roots. A total of 7,532 (43%) UniTags were more than 2.7-fold differentially expressed, and 880 (5.0%) were regulated more than 8-fold upon stress. Their large size enabled the unambiguous annotation of 3,858 (22%) UniTags to genes or proteins in public data bases and thus to stress-response processes. We designed a microarray carrying 3,000 of these 26 bp tags. The chip data confirmed 79% of the tag-based results, whereas RT-PCR confirmed the SuperSAGE data in all cases.

**Conclusion:** This study represents the most comprehensive analysis of the drought-response transcriptome of chickpea available to date. It demonstrates that – inter alia – signal transduction, transcription regulation, osmolyte accumulation, and ROS scavenging undergo strong transcriptional remodelling in chickpea roots already 6 h after drought stress. Certain transcript isoforms characterizing these processes are potential targets for breeding for drought tolerance. We demonstrate that these can be easily accessed by micro-arrays and RT-PCR assays readily produced downstream of SuperSAGE. Our study proves that SuperSAGE owns potential for molecular breeding also in non-model crops.
Background
Chickpea (*Cicer arietinum* L.) is one of the most important grain legume crops worldwide and a major source of protein for millions of families in developing countries. Despite considerable investment in breeding, average chickpea yield in major producer countries such as India stagnates at 0.6–0.7 Mt hectare⁻¹ since many years. This low yield is far below the crop’s potential of 3–5 Mt hectare⁻¹ under optimal conditions. Chickpea is mostly grown in low-input, rain-fed agriculture in Mediterranean-type environments as inter-crop between cereals, and on residual moisture from monsoon rains on the Indian subcontinent. Due to insufficient, untimely and erratic rainfall in these semi-arid and arid areas, the crop often suffers from drought at the end of the cropping season. In future, global warming and soil erosion will even worsen conditions for many crops including chickpea. Thus, drought represents the major constraint to increase chickpea yield, and drought tolerance therefore is a major aim of chickpea breeding. However, drought tolerance is a complex trait and hard to achieve by conventional breeding methods. Understanding of the molecular mechanisms underlying drought tolerance is therefore needed for successful, knowledge-based crop improvement [1].

Molecular genetics and genomics of stress-responses in model plants such as *Arabidopsis* revealed that abiotic stresses such as drought, salinity and cold stress are characterized by ionic- and osmotic-disequilibrium components; eliciting general as well as specific responses and mechanisms of stress-protection [2]. These studies underpinned the importance of early responses to the various stresses for the survival of the plants [3]. Much of our current understanding of stress-response mechanisms comes from genome-wide analysis of gene expression, facilitated by the availability of microarrays carrying a comprehensive set of genes.

In chickpea, like in many other under-researched crops, extensive microarray-based studies are not yet possible, because the necessary EST data are not available. For example, no more than 7,580 chickpea ESTs are publicly available at the National Centre of Biotechnology Information (NCBI, [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)), as compared to at least 1,463,500 ESTs available for *Arabidopsis*. In the absence of such resources, researchers often use less comprehensive approaches as e.g. suppression subtractive hybridisation (SSH) libraries [4], which do not *per se* allow the quantification of expression of differentially expressed genes. SSH results are therefore often used for the generation of macro-arrays for subsequent analysis of gene expression [5]. These authors used this cumbersome approach for the discovery of 101 dehydration-responsive transcripts in chickpea roots.

Open-architecture, whole-genome transcription profiling technologies such as SuperSAGE [6], however, provide a solution to the problem of lacking EST and genomic data. SuperSAGE is an improved version of the Serial Analysis of Gene Expression (SAGE) technique [7]. In the past years, it has demonstrated a high versatility due to its longer tag size (26 bp) [8,9]. In principle, SAGE and all its variants rely on the assumption that a small, defined part of a cDNA, a so-called "tag", characterizes this cDNA, and that counting the number of times a particular tag occurs in the tag population faithfully reflects the abundance of the respective mRNA in the transcriptome. Since 10,000 to 100,000 tags are sequenced in a single experiment, a comprehensive profile of the transcriptome is generated.

Here we report on the stress responses of 80,238 transcripts representing 17,493 unique 26 bp tags (UniTags) from roots of the drought-tolerant chickpea variety ICC588 early after onset of desiccation. We discuss the stress-regulated transcription of genes involved in signal perception and transduction, ROS scavenging and metabolism, osmotic and ionic stress-related pathways, regulation of water and ion homeostasis, as well as several reported effector proteins. To test the reliability of the present results we use microarrays carrying stress-responsive as well as constitutively expressed 26 bp tag sequences. To further confirm the SuperSAGE results with a third method, we use SYBBrGreen and commercially available TaqMan assays produced from 3'-and 5'-RACE sequences from selected chickpea mRNAs. Finally, we compare our transcription profiles from drought-stressed chickpea roots to results obtained from chickpea root SSH libraries [5] and microarray experiments in *Medicago truncatula* [10], and discuss similarities and differences. This study is the first of a series characterizing stress responses of chickpea on a molecular level as a prerequisite for production of expression markers and microarrays for high-throughput germplasm and expression (e)QTL analysis at the onset of knowledge-based breeding for stress-tolerance in this important protein crop.

Results
The combination of high-throughput 454 sequencing with SuperSAGE
Drought libraries are part of a project, which aims at evaluating the transcriptional responses of chickpea upon diverse abiotic stresses, including several other treatments and various tissues (e.g. salt-stressed roots and cold-stressed leaves; data not shown). For the sequencing of all the libraries, a single 454 plate divided into two sections was used, from which a total of 380,000 reads were extracted. After eliminating: i) incomplete reads, ii) twin-ditags, and iii) ditags without complete library-identification DNA linkers, a total of 330,000 26 bp tags were obtained for further analysis. From these, about 50,000
tags were singletons, that were excluded from analysis. Finally, 280,000 tags remained for evaluation (data not shown). Subsequently, sub-datasets were constructed for each experimental situation. For the present study, 82,238 26 bp tags from control and dehydrated roots were analyzed.

**Abundance of UniTags and annotation to public databases**

A total of 82,238 26 bp tags from roots of the drought-tolerant variety ICC588 either subjected to 6 h desiccation (53,141) or from well-watered controls (28,897) were sequenced, and represented 17,493 unique transcripts, so called UniTags. Less than 1% percent of these occurred in very high copy numbers (> 5,000 counts.million⁻¹), whereas 23% and 75% of the transcripts were present between 100 to 1,000 and less than 100 copies.million⁻¹, respectively (Table 1). UniTags from control and stress libraries were deposited in the Gene Expression Omnibus (GEO) public domain under accessions GSM321783 and GSM321790, respectively.

Annotation of the 17,493 UniTags matched 3,858 (22.0%) to well characterized sequences from the Fabaceae family available in public databases. Of these, 53% matched to sequences from *Medicago truncatula*, 29% to *Cicer arietinum*, 6% to *Pisum sativum*, 3% to *Glycine sp.*., 2% to *Medicago sativa*, and 7% to other legume genera. In many cases, TIGR gene index annotations from legumes bridge automatically to characterized Tentative Consensus (TC) sequences mostly from *Arabidopsis*, rice, and maize. Of the remaining 13,635 (78.0%) non-assignable 26 bp tags, 5,685 were significantly homologous to anonymous EST or DNA sequences, whereas 7,950 found no match at all. A summary of the primary data is given in Table 1. Annotation of the 26 bp tags and respective expression ratio values are deposited in the main data matrix [see Additional file 1].

**Annotation of virtual tags generated from chickpea ESTs deposited in public domains**

In order to test the validity of the annotation of chickpea 26 pb tags through sequence homology with other legumes, virtual tags generated from chickpea EST sequences deposited in the NCBI data bank were extracted, and their direct annotation was compared with the annotation of longer homologous ESTs from the model legume *M. truncatula*. After retrieving the complete set of chickpea sequences deposited in the NCBI EST database (7,500 sequences), a total of 3,544 different *in silico*-generated 26 bp tags were selected to be directly BLASTed against the nr NCBI (*Fabaceae*) nucleotide database (Table 2). From these, a total of 998 tags revealed high homology hits. After exclusion of anonymous entries, 253 tags were linked to Uniprot entries or to characterized non-protein coding RNAs (Table 3). In parallel, the same 3,544 tags were BLASTed against the plant EST NCBI (*M. truncatula*) and TIGR (*M. truncatula*) databases separately, where 1,143 and 680 sequences, respectively, found at least one high homology hit. Then, the complete target sequences from each BLAST were retrieved and re-blasted against the nr NCBI (*Fabaceae*) database. A total of 632 (NCBI *M. truncatula* ESTs) and 630 (*M. truncatula* TIGR ESTs) sequences, each representing a different 26 bp tag, revealed high homologies with nr NCBI entries. From these, 369 and 213, respectively, were non anonymous (Table 3). The results from the direct BLAST were compared with each of the *M. truncatula* EST-briged homology searches for commonly annotated sequences, either by

### Table 1: Features of SuperSAGE libraries from control and drought stressed roots

| Library                     | Control | 6 h desiccation | Total (%) |
|-----------------------------|---------|-----------------|-----------|
| Sequenced tags              | 28,897  | 53,141          | 82,238 (100) |
| Number of unique transcripts (UniTags) | 9,110   | 13,899          | 17,493 (100) |

| Differential gene expression (absolute values) | (downregulated*) | (up-regulated*) |
|------------------------------------------------|------------------|-----------------|
| $R_{(n)} > 1$; 2.7-fold differential expression | 4,975            | 2,557           | 7,532 (43) |
| $R_{(n)} > 2$; 8.0-fold differential expression | 589              | 291             | 880 (5)    |

| Abundance classes                |                   |                 |             |
|----------------------------------|-------------------|-----------------|-------------|
| > 5,000 copies.million⁻¹         | -                 | -               | 12 (0.1)    |
| 1,000 – 5,000 copies.million⁻¹   | -                 | -               | 186 (1)     |
| 100–1,000 copies.million⁻¹       | -                 | -               | 4,160 (24)  |
| 2–100 copies.million⁻¹          | -                 | -               | 13,135 (75) |

| Annotation of UniTags            |                   |                 |             |
|----------------------------------|-------------------|-----------------|-------------|
| Match to UniProt entries         | 2,124             | 3,165           | 3,858 (22)  |
| Match to anonymous entries       | -                 | -               | 5,685 (32)  |
| No match                         | -                 | -               | 7,956 (45)  |

* Ratio (ln) values indicating up- or down-regulation calculated with the 6 h desiccation library as reference.
Table 2: Chickpea EST sequences used for in silico extraction of 26 bp tags

| Chickpea ESTs deposited at NCBI | 7584 |
|---------------------------------|------|
| ESTs with more than 30 bp between last CATG and 3'-end | 4754 |
| Different in silico 26 bp tags after virtual NlaIII cutting and elimination of identical sequences | 3544 |

direct ID matching, protein name, or sequence description. For *M. truncatula* ESTs from NCBI, 9 out of 114 (7.8%) annotations were not congruent with the annotation of the corresponding chickpea *in silico* tag. However, only 4 out of the 9 annotations belonged to Uniprot-linked accessions [Table 3, see also Additional file 2]. For *M. truncatula* ESTs from TIGR, 9 out of 86 (10%) common annotations were not congruent. From them, 4 belonged to Uniprot-linked accessions [Table 3, see also Additional file 2]. The present results reveal that in more than 90% of the cases, the assignment of tags to Uniprot-linked accessions is congruent with the annotation of longer ESTs from other legumes. However, databases overloaded with anonymous entries considerably reduce the amount of biologically interpretable data.

#### Differential gene expression in response to drought stress of chickpea roots and assignment of 26 bp tags to Gene Ontology (GO:) functional categories

We calculated the natural logarithm of expression ratios [here denoted as $R_{(ln)}$] of the 26 bp tags from control versus stressed roots as well as significance levels (P) according to Audic and Claverie [11] of up- and down-regulation for each transcript using the software package DiscoverySpace 4.01 (Canada's Michael Smith Genome Sciences Centre). Differences in abundance of tags in control and stressed roots were considered relevant at $R_{(ln)}>1$ (> 2.7-fold change). At this threshold, 7,532 (43%) tags were significantly differentially expressed in stressed as compared to control roots. Of these, 2,557 were up- and 4,975 down-regulated. A total of 880 transcripts (5.0%) showed more than 8-fold difference in expression ($R_{(ln)}>2.0$, $P<0.05$). Of these, 291 were up- and 589 down-regulated under stress. As depicted in the Venn diagram (Figure 1), a considerable number of tags occurred exclusively in either the control tissue or under stress.

The 40 most significantly up- or down-regulated transcripts matching well characterized genes in public databases are listed in Table 4 and Table 5, respectively. However, many transcripts could not be annotated, because they either matched to anonymous ESTs, or did not hit any entry in the database. Therefore, the annotatable transcripts coding for extensin (O65760_CICAR), NADP-dependent isocitrate dehydrogenase 1 (Q6R6M7_PEA), S-receptor kinase-like protein 1 (Q70I30_LOTJA), chalcone isomerase (Q9SXS9_CICAR), UDP-glucose-pyrophosphorylase (Q8W557_9FABA), beta-glucosidase (Q9FSY8_CICAR), specific tissue protein 1 (Q39449_CICAR), S-adenosyl-L-methionine synthetase.

Table 3: Comparison between direct BLASTing of 26 bp chickpea tags and sequence homology search through *M. truncatula* ESTs

| Process | In silico tags direct BLAST (I) | EST-bridged BLAST (II) | EST-bridged BLAST (III) |
|---------|--------------------------------|------------------------|------------------------|
| Screened databases | NCBI nr Fabaceae mRNAs | NCBI *M. truncatula* ESTs | TIGR *M. truncatula* GI ESTs |
| Number of BLASTed in silico chickpea tags | 3544 | 3544 | 3544 |
| Total high homology hits | 998 | 632 | 630 |
| Non-anonymous hits | 253 | 369 | 213 |
| Common hits with NCBI Fabaceae direct BLAST | - | 114 | 86 |
| Direct ID-correlated common hits | - | 48 | 37 |
| Protein name-correlated hits | - | 42 | 23 |
| RNA description-correlated hits | - | 15 | 17 |
| Non-correlated hits | - | 9 | 9 |
(Q9AT56_ELAUM), and cysteine synthase (O65747_CICAR) were the most up-regulated interpretable transcripts under stress though not the most up-regulated in the data set.

Correlation of R_{ln} to defined standard functional gene categories (i.e. biological processes) in the Gene Ontology (GO:) database revealed that the majority of the most up-regulated transcripts are assigned to the GO: biological process "Metabolism" (with the exception of extensin [O65760_CICAR], and S-receptor kinase-like protein [Q70130_LOTJA] transcripts). As depicted in Figure 2, Stress Perception and Signalling (i.e. Intracellular Signalling Cascades, \( P = 0.997 \)), Small GTPase-mediated Signal Transduction (\( P = 0.994 \)), RNA Metabolism (\( P = 0.989 \)), and Cellular Carbohydrate Metabolism (\( P = 0.989 \)) were the most represented GO: functional categories in desiccation-stressed roots. Further, over-representation of transcripts involved in Transport (\( P = 0.943 \)), Proteolysis (\( P = 0.926 \)), Oxidative Phosphorylation (\( P = 0.886 \)), and Stress Response (\( P = 0.878 \)) indicate the mechanisms by which the roots adapt to the stress. Over-representation of tran-
scripts involved in GO: functional category "Oxygen and Reactive Oxygen Species Metabolism" (P = 0.808) suggests that ROS play an important role as side stress, but also as signalling molecules.

Tags from transcripts assigned to the GO: biological process "Protein Biosynthesis" (P = 0.020), such as 40S ribosomal proteins S19 (Q9ZRW2) and S23 (Q9ZRG2), and 60S ribosomal proteins L10 (Q9ZRU3), L18 (R3L18), and L32 (Q43N16), all were significantly down-regulated suggesting a repression of *de novo* protein biosynthesis under drought stress in roots. Additionally, other GO: biological processes like Photosynthesis and Light Reaction (P = 0.045), Chromatin Assembly (P = 0.092), Chromosome Organization and Biogenesis (P = 0.105), Biopolymer Metabolism (P = 0.132), DNA Replication (P = 0.268) were also under-represented (Figure 3).

Regarding GO: Cellular component categories, "Unlocalized Protein Complexes" were most over-represented under drought stress (P = 0.887), followed by Golgi Apparatus (P = 0.861), Endoplasmic Reticulum (P = 0.781), Membrane Integral Genes (P = 0.767) and External Encapsulating Structure (P = 0.747). GO: Cellular components such as "Ubiquitin Ligase Complex" (P = 0.027), Mitochondrial and Inner Membrane (P = 0.040 and 0.044, respectively), and Ribosome (P = 0.063) were amongst the most under-represented. The transcription of genes coding for proteins of the Serine/Threonine Phosphatase Complex (P = 0.44), Cytoplasm (P = 0.55),

Table 4: Top 40 drought stress up-regulated annotatable tags

| Tag code | Protein                                                                 | R_(ln) | GO: Biological process                                                                 | Uniprot ID       |
|----------|--------------------------------------------------------------------------|--------|----------------------------------------------------------------------------------------|------------------|
| STCa-19021 | Extensin                                                                 | 3.694  | Cell wall organization and biogenesis                                                  | O65760_CICAR     |
| STCa-7166  | NADP-dependent isocitrate dehydrogenase I                                | 3.579  | Carbohydrate metabolism                                                                | Q6R6M7_PEA       |
| STCa-7800  | S-receptor kinase-like protein I                                          | 3.579  | Protein amino acid phosphorylation                                                     | Q70130_LOTJA     |
| STCa-10145 | Chalcone isomerase                                                       | 3.517  | No associated term                                                                     | Q9S5X9_CICAR     |
| STCa-8459  | UDP-glucose pyrophosphorylase                                             | 3.341  | Metabolism                                                                             | Q8W5S7_9FABA     |
| STCa-228   | Beta-glucosidase                                                         | 3.261  | Carbohydrate metabolism                                                                | Q9FSY8_CICAR     |
| STCa-20422 | Specific tissue protein I                                                | 3.218  | No associated term                                                                     | Q9R449_CICAR     |
| STCa-23486 | S-adenosyl-L-methionine synthase                                          | 3.127  | One-carbon compound metabolism                                                         | Q9AT56_ELAUM     |
| STCa-2982  | Cysteine synthase, O-acetyl-L-serine (thiol)-lyase                       | 3.079  | Metabolism                                                                             | O65747_CICAR     |
| STCa-22698 | Putative adenosine kinase                                                | 2.916  | Purine ribonucleoside salvage                                                          | Q8L5Q4_CICAR     |
| STCa-17627 | Putative universal stress protein                                        | 2.791  | Response to stress                                                                     | Q700A7_CICAR     |
| STCa-542   | Prolyl 4-hydroxylation, alpha subunit-like protein                       | 2.722  | Protein metabolism                                                                     | Q9FKX6_ARATH     |
| STCa-1589  | Beta-galactosidase                                                       | 2.722  | Carbohydrate metabolism                                                                | O65736_CICAR     |
| STCa-2044  | Fiber protein Fb1 I                                                      | 2.648  | No associated term                                                                     | Q8GT82_GOSBA     |
| STCa-227   | Beta-glucosidase                                                         | 2.568  | Carbohydrate metabolism                                                                | Q9FSY8_CICAR     |
| STCa-286   | Protein kinase Ppi                                                      | 2.568  | Protein amino acid phosphorylation                                                     | Q84P43_SOYBN     |
| STCa-15340 | Alfin-I                                                                  | 2.568  | Regulation of transcription                                                            | Q40359_MEDSA     |
| STCa-16114 | Cytosolic acetocetate-coenzyme A thiolase                                | 2.568  | No associated term                                                                     | Q5XMBB_TOBAC     |
| STCa-16514 | NADH dehydrogenase                                                      | 2.568  | Mitochondrial electron transport                                                       | Q9FNN5_ARATH     |
| STCa-5543  | Epsilon subunit of mitochondrial F1-ATPase                               | 2.525  | ATP synthesis coupled proton transport                                                 | Q8LSQ1_CICAR     |
| STCa-8853  | Ribosomal protein L10 homolog                                           | 2.481  | Protein biosynthesis                                                                   | Q42149_ARATH     |
| STCa-857   | Histone H2B                                                              | 2.386  | Response to DNA damage stimulus                                                       | Q9R3H6_CICAR     |
| STCa-21625 | Serine protease inhibitor-like protein                                   | 2.386  | No associated term                                                                     | Q8R8V99_ORYSA    |
| STCa-24140 | Putative 14-kDa proline-rich protein                                     | 2.386  | lipid transport                                                                       | Q9LEN8_CICAR     |
| STCa-16415 | NADPH-cytochrome P450 oxidoreductase                                    | 2.36   | Electron transport                                                                    | Q7M757_TOBAC     |
| STCa-923   | Ribosomal protein S26                                                   | 2.28   | Protein biosynthesis                                                                   | Q95SW9_PEA       |
| STCa-1343  | Apyrase-like protein                                                     | 2.28   | No associated term                                                                     | Q84UE1_MEDTR     |
| STCa-2122  | Histone H2A                                                              | 2.28   | Chromosome organization                                                                | H2A_CICAR         |
| STCa-6603  | Polygalacturonase PG11 precursor                                        | 2.28   | Carbohydrate metabolism                                                                | Q84TMB_MEDSA     |
| STCa-7388  | Aldolase                                                                 | 2.28   | No associated term                                                                     | Q9PSFZ_CICAR     |
| STCa-8045  | CaM protein                                                             | 2.28   | Calcium related signal transduction                                                    | Q7DLT8_CICAR     |
| STCa-14940 | TGA-type basic leucine zipper protein TGA2.I                             | 2.28   | Regulation of translation                                                             | Q91X0A_PHAU      |
| 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                                                                   | Q81921_CICAR     |
| STCa-24349 | Gibberellin 2-beta-hydroxylase                                           | 2.28   | Antibiotic biosynthesis                                                                | Q20X_PHACN       |
| STCa-24395 | NADPH:isoflavone oxidoreductase                                         | 2.28   | Regulation of nitrogen utilization                                                    | IFR_CICAR        |
| STCa-24543 | Tonoplast intrinsic protein                                             | 2.28   | Transport                                                                              | Q8LSG0_CICAR     |
| STCa-89    | Drought-induced protein                                                 | 2.162  | Response to water stress                                                               | Q941N0_9FABA     |
| STCa-1016  | Protein phosphatase 1, catalytic beta subunit                            | 2.162  | Protein amino acid de-phosphorylation                                                 | O65844_MEDSA     |
Cytosol (P = 0.49), Cytoskeleton (P = 0.48), Thylakoid (P = 0.47), Microtubule Cytoskeleton (P = 0.46), Cell-wall Structure and Modification as well as Cell Surface Protein was constitutive (Figure 4).

Members of a gene family assigned to over- or under-represented GO: categories, respectively, could be up- as well as down-regulated to different extents. For example, whereas UniTagSTCa-6374, annotated to the putative extensin (Q9FSY9_CICAR), was one of the most down-regulated transcripts, UniTag STCa-19021 representing another extensin isoform (O65760_CICAR) was the most Up-regulated tag in our data set. However, both isoforms are grouped in the same GO:category.

| Tag code | Protein                                                                 | $R_{(ln)}$ | Biological process                                    | Uniprot ID               |
|----------|-------------------------------------------------------------------------|------------|-------------------------------------------------------|--------------------------|
| STCa-1804 | Expansin-like protein (fragment)                                        | -3.095     | Sexual reproduction                                    | Q7XHJ2_QUERO             |
| STCa-13652 | 40S ribosomal protein S23                                              | -3.095     | Protein biosynthesis                                   | RS23_EUPES               |
| STCa-4802 | ADP-glucose pyrophosphorylase precursor                                | -2.913     | Glycogen biosynthesis                                 | Q43819_PEA               |
| STCa-5076 | Ribosomal protein L32                                                  | -2.913     | Protein biosynthesis                                   | Q45N61_MEDSA             |
| STCa-7347 | Putative 3-hydroxybutyryl-CoA dehydrogenase                           | -2.913     | Fatty acid metabolism                                 | Q5LD55_ARATH             |
| STCa-8227 | Histone H3                                                             | -2.913     | Chromosome organization                               | H3_ONOV1                 |
| STCa-13267 | Allene oxide synthase precursor                                        | -2.913     | Lipid biosynthesis                                    | Q7X9B4_MEDTR             |
| STCa-17859 | Hypothetical protein 275                                               | -2.913     | No associated term                                    | Q8GTD8_CICAR             |
| STCa-21081 | Vestitone reductase                                                    | -2.862     | Cellular metabolism                                   | Q40316_MEDSA             |
| STCa-3331 | 60S ribosomal protein L18                                               | -2.69      | Protein biosynthesis                                   | RL18_CICAR               |
| STCa-10792 | Calcineurin B-like-interacting protein kinase                          | -2.69      | Signal transduction                                   | Q84XCO_PEA               |
| STCa-12317 | Heat shock protein 70-3                                                | -2.69      | Response to unfolded protein                          | Q47BD0_TOBAC             |
| STCa-18274 | NADPH-ferrithioprotein 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                    | Q9TO58_ARATH             |
| STCa-19785 | Reduced vernalization response I                                       | -2.69      | Regulation of transcription                           | Q8L3W1_ARATH             |
| STCa-19870 | Transaldolase                                                          | -2.556     | Carbohydrate metabolism                               | O04894_SOLU              |
| STCa-18410 | Cytochrome P450                                                        | -2.491     | No associated term                                    | Q9LIN9_ARATH             |
| STCa-18321 | Similar to the auxin-independent growth promoter                       | -2.491     | Translational initiation                              | Q7X7B0_LACSA             |
| STCa-1286 | Eukaryotic translation initiation factor iso4E                          | -2.402     | Carbon utilization                                    | CAPP_PHAVU               |
| STCa-3390 | Phosphoenolpyruvate carboxylase                                       | -2.402     | No associated term                                    | Q5SN38_ORYSA             |
| STCa-3855 | Thf family protein-like                                                | -2.402     | Protein aminoo acid phosphorylation                   | Q5VR67_ORYSA             |
| STCa-3897 | 20S proteasome alpha subunit C                                        | -2.402     | Ubiquitin-dependent protein catabolism                | PSA4_SPIOL               |
| STCa-5074 | Pectin methyl-esterase PER precursor                                   | -2.402     | Cell wall modification                                | Q95C90_MEDTR             |
| STCa-5237 | F-box family protein-like                                              | -2.402     | No associated term                                    | Q5VR67_ORYSA             |
| STCa-5681 | Hydroxyproline-rich glycoprotein                                       | -2.402     | Cell wall organization                                | Q39865_SOYBN             |
| STCa-6267 | Transcription factor MYB5                                              | -2.402     | Regulation of transcription                           | Q8H1D0_ORYSA             |
| STCa-6374 | Putative extensin                                                      | -2.402     | Cell wall organization and biogenesis                 | 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 | Cicer arietinum mRNA for chalcone synthase                             | -2.402     | Biosynthesis                                           | Q39865_SOYBN             |
| STCa-9049 | Translocon-associated subunit alpha precursor                          | -2.402     | No associated term                                    | Q8H1D0_ORYSA             |
| STCa-9308 | Aquoropin-like water channel protein (mpl1 gene)                       | -2.402     | Transport                                             | Q9FSY9_CICAR             |
| STCa-11376 | 60S ribosomal protein L10 (QM protein homolog)                        | -2.402     | Protein biosynthesis                                   | Q9ZRU3_CICAR             |
| STCa-11527 | Putative Bet v I family protein (bet gene)                             | -2.402     | No associated term                                    | Q9Y3Y9_MEDTR             |
| STCa-12919 | I4-3-3-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     | ATP synthesis coupled proton transport                | Q8GET0_CICAR             |

**Table 5: Top 40 drought stress down-regulated annotatable tags**

**Differential expression of drought stress-related sub-transcriptomes in chickpea roots**

Since the present genome-wide expression analysis revealed a plethora of differentially expressed 26 bp tags with and without match to genes of known function, it is impossible to display or discuss all of them in the frame of this paper. Instead, in Figure 5 and Figure 6 we elaborate in more detail on the expression of genes and gene families belonging to the GO: biological processes “Signal Transduction”, “Stress Sensing”, “Regulation of Transcription”, “Transport”, “Post-transcriptional Regulation” and "Pathway Inhibitors", all involved in early responses to stress. In these categories, the gene itself or members of its family have known functions in stress-perception, stress-signalling and stress-responsive regulation of transcript...
Further, we closely look at transcripts encoding proteins involved in Reactive Oxygen Species (ROS) scavenging, and transcripts related to ROS-mediated signal transduction cascades (Figure 6). As examples for the regulation of effector genes downstream of the signalling cascades, we will detail the differential expression of genes coding for water-channel proteins, so-called aquaporins, and of genes coding for proteins involved in osmolyte metabolism (Figure 5, Figure 6).

**Confirmation of SuperSAGE results by qRT-PCR**

Our genome-wide expression analysis results were exemplarily confirmed by quantitative real-time (qRT) PCR using TaqMan probes XPTm-Ca-22356 (O65741_CICAR, mRNA for putative transmembrane channel protein) and XPTm-Ca-7975 (anonymous drought-induced EST) available from GenXPro GmbH, as well as SYBR Green* assays.

Oligonucleotides for SYBR Green assays were deduced from 5'- and 3'-RACE sequences generated with 26 bp tags as primers. The following transcripts were targeted: ST-Ca1921 (O65760_CICAR, extensin), ST-Ca17627 (Q700A7_CICAR, putative universal stress protein), ST-Ca8434 (anonymous drought-induced EST), ST-Ca17859 (AJ515033, *C. arietinum* hypothetical protein [275 gene]), ST-Ca8000 (AI250836, *C. arietinum* PAL gene), and ST-Ca22717 (AI487043, *C. arietinum* CYP450). For SYBR Green* as well as TaqMan assays, the sequence for either the forward or the reverse PCR primer was derived from the 26 bp tag, and the complementary primers from 3'- or 5'-RACE sequences, respectively.

Confirming the SuperSAGE expression levels, amplifications from SYBR Green* assay ST-Ca2271 and TaqMan probe XPTm-Ca22356 revealed constitutive levels of...
expression ($\Delta C_t < 0.5$) (Figure 7). Amplifications for SYBR Green® assays with ST-Ca1921, ST-Ca17627, ST-Ca8434 as well as TagMan probe XPTm-Ca-7975 revealed up-regulation of the respective transcripts under stress ($\Delta \Delta C_t > 0.5$), as already indicated by the differential expression analysis. Stress-induced down-regulation of 26 bp tags was corroborated by SYBR Green® assays for ST-Ca17859 and ST-Ca8000 ($\Delta \Delta C_t < -0.5$). However, for ST-Ca8000, amplification profiles as well as post-qRT-PCR amplicon melting curves suggested partially unspecific priming. Again, in agreement with the SuperSAGE results, the transcript chosen as invariable control indeed displayed almost completely similar expression in control and stressed roots. These experiments confirm our present results and suggest 26 bp tags as reliable sequence information source for other expression profiling techniques.

Profiles confirmation via microarray hybridization of spotted SuperSAGE-derived oligos

To characterize the chickpea transcriptome under drought stress, sequence information from SuperSAGE profiles was used to design an Agilent 16 K microarray, onto which 3,000 selected 26 pb tags were spotted for a comparison of both profiling techniques. On the microarray, the majority of the oligonucleotides with original tag sequences were spotted twice (twin-replicas). Additionally, oligonucleotides with different mismatch numbers from each original tag as well as a small sub-set of longer RACE-derived sequences were also included. After statistical treatment of the different internal twin-replicas, normalization, and mismatch background correction, reproducible signals from different hybridization rounds (i.e. RNA replicas, and dye-swapped samples) were
selected for cluster analyses together with expression ratios of the 26 bp tags. A total of 79.05% probes on the micro-array shared the same tendency of expression with the respective tag when hybridized with cRNAs prepared from drought-treated roots from ICC588. From a total of 1,056 spots showing congruent results among different replicas and dye-swap experiments, 425 and 417 were up- and down-regulated in both techniques, respectively, whereas 214 spots showed in-congruent results (Table 6). UniTag expression ratios from both techniques are deposited in the main data matrix [see Additional file 1].

**Comparison of different transcript profiling techniques: SuperSAGE versus SSH macroarrays and microarrays**

We tested, whether our genome-wide transcription patterns are typical for roots of chickpea and other legumes, even if the profiles were obtained with other methods. Therefore we compared the present results to two studies addressing similar questions. In chickpea, Boominathan and co-workers [5] investigated whether pre-exposure to a dehydration shock improved adaptive responses of the roots during subsequent dehydration treatment. These authors identified 101 dehydration-inducible transcripts by repetitive rounds of cDNA subtraction, differential DNA-array hybridization, and Northern-blot analysis. Additionally, responses to exogenously applied abscisic acid (ABA) were also monitored. Since one analyzed time point was set 5 h after onset of drought, the results of this experiment should be at least partially comparable to our study in which RNA was isolated from roots 6 h after onset of drought stress. However, the results were not 100% congruent. It is important to note, that micro- or macro-arrays do not reliably differentiate between different transcript isoforms from gene families. Hybridization signals may integrate the hybridization intensities over all closely related transcripts, whereas SuperSAGE generates

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**Figure 4**

Drought stress over-represented GO cellular components in chickpea. GO: cellular components assigned to localization as deduced from transcript abundancies (annotated to UniProt entries) in drought-stressed chickpea roots. Representation of GO: terms was calculated by the program ermineJ 2.0. Categories with values above 0.8 are better represented in a given data set.
### Heat map profiles of tags representing genes involved in signalling, and response-related processes

| Metabolic process                                      | Protein                                      | Number of representative SuperTags |
|--------------------------------------------------------|----------------------------------------------|------------------------------------|
| Ca^{2+}-release and signaling                          | Phospholipase C                              | ![Heat map](image)                 |
| Ca^{2+}-release and signaling                          | Ca^{2+} channel protein                      | ![Heat map](image)                 |
| Ca^{2+}-dependent signaling                            | Calcineurin-B like proteins                  | ![Heat map](image)                 |
| Ca^{2+}-dependent signaling                            | CBL-interacting kinases                      | ![Heat map](image)                 |
| Ca^{2+}-dependent signaling                            | Ca^{2+}-dependent kinases                    | ![Heat map](image)                 |
| Signal transduction                                    | G-proteins                                   | ![Heat map](image)                 |
| Signal transduction                                    | Receptor-like protein kinases                 | ![Heat map](image)                 |
| Signal transduction                                    | MAP-kinases                                  | ![Heat map](image)                 |
| Signal transduction                                    | MAP-kinase-kinases                           | ![Heat map](image)                 |
| Signal transduction                                    | MAP-kinase-kinase-kinases                    | ![Heat map](image)                 |
| Transcription activation                               | NAC-domain-protein                           | ![Heat map](image)                 |
| Transcription regulation                               | WRKY transcription factors                   | ![Heat map](image)                 |
| Transcription regulation                               | DREB transcription factors                   | ![Heat map](image)                 |
| Transcription regulation                               | bZIP class transcription factors             | ![Heat map](image)                 |
| Transcription regulation                               | MYB transcription factors                    | ![Heat map](image)                 |
| Transcription regulation                               | Ethylene-responsive factors                  | ![Heat map](image)                 |
| Protein-protein interaction                            | 14-3-3 Proteins                              | ![Heat map](image)                 |
| Stress response (reported)                             | Universal stress response proteins           | ![Heat map](image)                 |
| Stress response (reported)                             | AAA-type ATPase (stress-induced)             | ![Heat map](image)                 |
| Stress response (chaperons)                            | (HSP) Heat shock proteins                    | ![Heat map](image)                 |
| Stress response (chaperons)                            | (HSF) Heat shock factors                     | ![Heat map](image)                 |
| ABA-signaling (ABA biosynthesis)                       | 9-cis-Epoxydocosanoid oxygenase              | ![Heat map](image)                 |
| ABA-signaling (ABA conjugates release)                 | &-glucosidase                                | ![Heat map](image)                 |
| ABA-signaling (negative regulation)                    | Protein phosphatase 2C (AB1)                 | ![Heat map](image)                 |
| Drought response                                        | ABA-responsive proteins                      | ![Heat map](image)                 |
| Drought response                                        | ERD-Proteins                                 | ![Heat map](image)                 |
| Drought response                                        | Dehydrins                                    | ![Heat map](image)                 |
| Drought response                                        | Drought-induced proteins                     | ![Heat map](image)                 |
| Damage and repair responses                             | LEA-Proteins                                 | ![Heat map](image)                 |
| Chromatin organization                                 | Histone H1                                   | ![Heat map](image)                 |
| Chromatin organization                                 | Histone H2                                   | ![Heat map](image)                 |
| Chromatin organization                                 | Histone H3                                   | ![Heat map](image)                 |

**Figure 5**

Heat map profiles of tags representing genes involved in signalling, and response-related processes. Heat map profiles of tags representing genes involved in Ca^{2+}-dependent signalling, general signal transduction, transcription regulation, protein-protein interactions, stress, ABA-dependent signalling, drought-response, damage and repair responses, and chromatin organization.
absolute numbers for each transcript variant (isoform). Due to the much deeper coverage of the transcriptome by this technique, almost all differentially expressed transcripts represented in the SSH libraries have at least one, but usually more counterparts in our 26 bp tags libraries. A cluster analysis of transcription profiles obtained by macroarray hybridization and the differential expression of 26 bp tags from drought-stressed roots 5 h or 6 h after onset of the stress, respectively, is shown in Figure 8.

Similarly up-regulated (category C in Figure 8) and differentially regulated transcripts (i.e. down-regulated in the SuperSAGE experiment, but up-regulated on the macroarray, section D in Figure 8) under both conditions are listed in Table 7.

Investigating regulatory and protective mechanisms leading to desiccation tolerance (DT) in *Medicago truncatula* seeds, Buitink and co-workers [10] published another, at least partially comparable study. These authors used the 16 k *Medicago* microarray to monitor changes in the transcriptome of desiccation-sensitive 3-mm-long radicles at different time points during incubation in a polyethylene glycol (PEG) solution mimicking the effects of desiccation. These experiments identified several specific expression profiles at different time scales. A cluster analysis

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**Figure 6**

Heat map profiles of tags representing genes involved in channelling and detoxification-related processes. Heat map profiles of 26 bp tags representing genes involved in water and ion channelling, ROS detoxification, and compatible osmolyte accumulation, with profiles of 97 26 bp tags annotated to CYP450 genes.
Figure 7
qRT-PCR confirmation of SuperSAGE results. TaqMan™ RT-PCR confirmation of SuperSAGE data by relative curve quantification using primers and TaqMan™ probes designed from 3'- and 5'-RACE sequences, respectively, that are derived from corresponding 26 bp tags. A) TaqMan™ assay with tag primer XPTm-Ca-22356 (derived from a 3'-RACE product; the original tag represents O65741_CICAR, the mRNA for a putative trans-membrane channel protein). Result: no difference between control and desiccated roots, i.e. constitutive expression (black arrow). B) TaqMan™ assay with tag primer XPTm-Ca-7975 (derived from a 3'-RACE product; the original tag represents an anonymous drought-induced EST). Result: earlier C_T for the cDNA from desiccated roots (double headed arrow). Control (red), drought-stressed roots (blue).
Table 6: Shared and contrasting tendencies between SuperSAGE and microarray profiles for transcripts from drought-stressed chickpea roots

| Stress/Organ    | Drought/Roots | Salt/Roots* |
|-----------------|---------------|-------------|
| Total selected spots (tags) | 1,056 | 739 |
| Shared up-regulation | 425 | 349 |
| Shared down-regulation | 417 | 233 |
| Contrasting tendency | 214 | 157 |

*Regulation tendencies for salt-stressed roots in both techniques are included for comparative purposes.

comparing the results from desiccation-stressed Medicago radicles and drought-stressed chickpea roots is depicted in Figure 9. Transcripts up-regulated in both species (category D), regulated in opposite directions in the two species (section B), and transcripts down-regulated in chickpea and Medicago as a reaction to stress (section A) are listed in Table 8.

Differential expression of natural antisense transcripts (NATs)

As expected from an open-architecture technique, tags synthesized on coding strands of template cDNAs and, in addition, tags originating from the opposite (anti-sense) strands were discovered. Here, at least 170 tags matching to the reverse sequences of ESTs in the public databases were detected. These tags could represent potential natural antisense transcripts (NATs). In Lotus japonicus, SAGE tags representing NATs were induced during nodulation [12], and in Arabidopsis endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance [13]. Thus, it is possible that NATs are also involved in stress tolerance in chickpea. However, Galante and co-authors [14] demonstrated that a considerable portion of such NATs found in the databases are artefacts. In drought-stressed chickpea roots, we found – inter alia – up- and down-regulation of NATs for transcripts of several members of the aquaporin gene family and differential expression of NATs for phosphatase transcripts. However, since we did not exclude genomic priming by DNase digestion of the RNA prior to cDNA synthesis, confirmation of the identity of these potential NATs will be subject to further studies.

Discussion

We analysed the expression of approximately 80,000 transcripts from un-stressed control and drought-stressed chickpea roots, respectively. It was previously estimated that the total number of average-sized transcripts per cell in higher plants ranges from 100,000 to 500,000 [15]. Thus, 80,000 26 bp tags provide ~1-fold coverage for transcripts present at a minimum of 6,2 copies per cell. There-
fore our study, which detected > 17,000 unique transcripts, is not comprehensive. However, even for the model legume Medicago truncatula only 36,000 unique ESTs/TCs are deposited in public databases. Therefore, our single study demonstrates that SuperSAGE is suited to overcome the problem of lacking resources in non-model organisms and under-researched crops.

Since, as we have shown, 75% of transcripts are present in less than 100 copies million⁻¹, and a small portion of transcripts is represented by more than 1000 copies million⁻¹, we probably missed a number of transcripts with less than 10–14 copies per cell. These may include transcripts for highly interesting pleiotropic proteins such as transcription factors possibly present at only 0,001 copies per cell [16]. Additionally, sampling larger organ sections with millions of cells obscures the specific transcript profiles of component cells. As a consequence, transcripts found in high copy numbers in just a few specialized cells are under-represented. As elegantly demonstrated for maize [17] and Arabidopsis [18], there are strong differences in transcription profiles between adjacent tissues. Therefore, the detection of rare transcripts should be improved by increasing the number of sequenced transcripts (as can be done with next-generation sequencing platforms such as Illumina’s Solexa or ABI’s SOLiD) in combination with a more selective sampling technique such as laser-capture microdissection [19].

Also, annotation of 55% of the 26 bp tags to the databases was straightforward. Considering that roughly 1,300 chickpea EST sequences are publicly available (at the time of annotation), most tags matched to sequences from the related model legume Medicago truncatula rather than to chickpea ESTs. On the other hand, a large number of significant hits represented fully uncharacterized database accessions, a fact that handicaps functional interpretation of the present chickpea transcription profiles more than the size of the 26 bp tags.

Additionally, a relevant problem arose from the interpretation of the assignment of different distinct 26bp tags to single UniGenes or tentative consensus sequences (TCs;[20]). We decided to classify such tags as isoforms of transcripts from members of gene families rather than assigning them to a particular gene, even if the expression patterns suggested functional homology. In addition, we could not differentiate whether these tags with similar annotation came from alternatively spliced transcripts of the same gene. Since in Arabidopsis and rice at least 21% of genes produce alternatively spliced transcripts [21], evidence beyond sequence homology of the SuperSAGE tags (i.e. 3’- and 5’-RACE sequences) is needed to decide between these two possibilities.

Differential expression of members of large gene families
One major advantage of the differential expression of 26bp tags as compared to macro- and micro-arrays is the very good differentiation between transcripts from different members of large genes families, which comprise the majority of all genes. Gene family members have more or less conserved sequences, and similar or different functions. For example, the cytochrome P450 (CYP) gene superfamily comprises more than 272 members grouped in 44 families in Arabidopsis thaliana, and more than 458 members in rice [22]. In the present study, 97 26bp tags revealed high-homology hits with members of the CYP450 superfamily, from which 33 could be assigned to their respective family through BLASTing against the Arabidopsis-CYP database (http://132.192.64.52/blast/ P450.html, data not shown). The involvement of CYP superfamily members in numerous catalytic reactions on a spectrum of substrates suggests that the transcription profiles of its members reflect this diversity. In accordance with the expectation, 43 (44.3%) of the 97 CYP-annotated 26bp tags changed their expression profiles at least 2.7-fold with R(ln) (absolute value) > 1.0, whereas 47 (55.7%) showed only slight differences or constitutive levels. Among the 26bp tags assigned to drought stress-related CYPs, hits to CYP707A family (STCa-23852) which harbours ABA 8’-hydroxylases (key enzymes in ABA-catabolism;[23]), revealed a very slight up-regulation (almost constitutive levels), indicating that the turnover of ABA is already active under our experimental condition and time-points. UniTag STCa-18410, assigned to a CYP81F4 member, stands out for its extreme down-regulation (15-fold). Although its function is not known, CYP81F4 members generally change their expression profiles dynamically after induction of water stress [24]. Considering the differential expression and diverse functions of individual members of gene families, whole-genome transcription profiling is only useful, if it differentiates between the different genes and their transcript isoforms in such families. Faithful discrimination between, and individual quantification of expression of these isoforms is therefore one advantage of the longer 26 bp tags.

However, also methodological inconsistencies may result in the observation of several different tags from a single gene [25]. To prevent methodological artefacts and assure the validity of the detected transcript variants, confirmative procedures such as double NlaIII digestion were standard in all libraries. Additionally, in silico routines for the exclusion of artefacts were applied (e.g. elimination of twin ditags and singletons [7]).

In the following sections, we discuss the expression of only a subset of drought-regulated genes and gene family members. We focussed on genes known to be involved in stress-perception, signalling and transcription initiation,
Table 7: Comparison between SuperSAGE expression profiles and macroarray-generated data

| Section | Annotation | Uniprot ACC |
|---------|------------|-------------|
| A       | ADP-glucose pyrophosphorylase precursor | Q43819 |
|         | ADP-glucose pyrophosphorylase small subunit CagpS1 | Q9AT06 |
|         | ADP-ribosylation factor I-like protein | Q70XK1 |
|         | ADP-ribosylation factor-like protein | Q9LFJ7 |
|         | AP2/EREBP transcription factor ERF-I | Q5UBL5 |
|         | ATP-dependent Clp protease ATP-binding subunit clpC homolog | CLPC |
|         | Chaperonin 21 precursor | Q9MSA8 |
|         | Cytochrome P450 | Q9GXL7 |
|         | Cytochrome P450 73 cinnamic acid 4-hydroxylase | TCMO |
|         | Cytosolic chaperonin, delta-subunit | Q9ZRX1 |
|         | Dehydration-responsive element binding protein | Q8GZ2F |
|         | Dehydrin-cognate | Q43430 |
|         | DREB-like protein | Q75UI6 |
|         | (RING zinc finger protein, putative, expressed) | Q75I59 |
|         | Fiber protein Fb22 (Fragment) | Q7Y244 |
|         | Fiber protein Fb27 | Q6UA10 |
|         | Fiber protein Fb4 (Fragment) | Q6UA21 |
|         | Glyceraldehyde 3-phosphate dehydrogenase, cytosolic | O81924 |
|         | Histone H2A | H2A2 |
|         | Kinesin (centromere protein)-like heavy chain-like protein | Q9LHL9 |
|         | Lipooxygenase | Q93YA9 |
|         | Metallothionein-like protein 1; MT-1 | MT1 |
|         | PGM; Glucose phosphomutase | PGMC |
|         | Polygalacturonase inhibiting protein | Q6V406 |
|         | Protein kinase | Q41619 |
|         | Protein phosphatase-2C | Q82468 |
|         | RING/C3HC4/PHD zinc finger-like protein | Q8KA9 |
|         | Root-specific metal transporter | Q8LR1 |
|         | S-adenosylmethionine synthase | Q6JR6 |
|         | S-adenosylmethionine synthetase | Q9FUZ1 |
|         | Similarity to zinc metalloproteinase | Q9FKI1 |
|         | Translation initiation factor 5A | Q6PQ38 |
|         | Trehalose-6-phosphate phosphatase | Q64897 |
|         | Zinc finger (C3HC4-type RING finger)protein-like | Q6JQ38 |
|         | Zinc finger (CCCH-type) protein-like | Q657B3 |
|         | Zinc finger protein 5; ZFP5 | Q8LC7 |
|         | Zinc finger-like | Q6K719 |
| B, D    | ADP-glucose pyrophosphorylase precursor | Q43819 |
|         | ADP-glucose pyrophosphorylase small subunit CagpS1 | Q9AT06 |
|         | AP2/EREBP transcription factor ERF-2 | Q5UBL5 |
|         | ATP-dependent Clp protease ATP-binding subunit clpC homolog | CLPC |
|         | Beta-amylase; 1,4-alpha-D-glucan maltohydrolase | AMYB |
|         | Central motor kinesin I | Q6WJ05 |
|         | Cinnamoyl CoA reductase | Q7Y0H8 |
|         | Cytochrome P450 | Q9GXL7 |
|         | Cytochrome P450 73 cinnamic acid 4-hydroxylase | TCMO |
|         | Eukaryotic translation initiation factor 5A isoform II | Q71F50 |
|         | Eukaryotic translation initiation factor 5A-2; elf-5A-2 | IFS5A2 |
|         | Fiber protein Fb19 (Fragment) | Q7X951 |
|         | Glyceraldehyde 3-phosphate dehydrogenase, cytosolic | O81924 |
|         | Glyceraldehyde-3-phosphate-dehydrogenase | Q53S52 |
|         | Histone H2A.6 | H2A6 |
|         | Lipooxygenase (EC 1.13.11.12) | Q9LEA9 |
|         | Metallothionein-like protein 1; MT-1 | MT1 |
|         | Metallothionein-like protein 2; MT-2 | MT2 |
|         | Nine-cis-epoxycarotenoid dioxygenase | Q8P8L7 |
|         | Nodule-enhanced protein phosphatase type 2C | Q9ZPL9 |
|         | P-type H+-ATPase | Q41647 |
|         | PGM; Glucose phosphomutase | PGMC |
Comparison between SuperSAGE expression profiles and macroarray-generated data (Continued)

|                                | Accession |
|--------------------------------|-----------|
| Plasma membrane H\(^+\)-ATPase | Q7Y056    |
| Prolyl 4-hydroxylase alpha subunit-like protein | Q8LAN3 |
| Protein kinase                 | Q41619    |
| Protein phosphatase 2C         | Q8S8Z1    |
| Protein phosphatase 2C-like protein (AT4g31860/F11C18) | Q8S5Z3 |
| Protein phosphatase-2C         | O82469    |
| Putative metallophosphatase    | Q8VXF5    |
| S-adenosylmethionine synthetase| Q9FUZ1    |
| Translation initiation factor 5A | Q6PQ38 |
| Ubiquitin conjugating protein  | Q9M4R0    |
| Vacuolar assembly protein VPS41 homolog | VPS41 |
| Zinc finger (CCCH-type) protein-like | Q657B3 |
| Zinc finger protein-like       | Q5Z8K9    |
| Zinc finger protein-like       | Q69QZ4    |
| Zinc finger protein-like       | Q6K8E9    |
| ADP-glucose pyrophosphorylase precursor | Q43819 |
| ADP-ribosylation factor 1-like protein | Q700X1 |
| Apyrase-like protein           | Q84UE1    |
| Chloroplast chaperonin 21      | Q6B4V4    |
| Cysteine proteinase            | O81930    |
| Cytochrome P450                 | Q9XGL7    |
| Cytochrome P450 73 cinnamic acid 4-hydroxylase | TCMO |
| Dehydration responsive element binding protein | Q7Y0T9 |
| Dehydrin-like protein          | Q94SOL7   |
| DNA binding zinc finger protein; Pspf | Q9ZWJ0 |
| ERD15 protein                  | Q39096    |
| Fiber protein Fb11              | Q8GT82    |
| Fiber protein Fb19 (fragment)   | Q677D1    |
| Fiber protein Fb2               | Q8GT87    |
| Glyceraldehyde 3-phosphate dehydrogenase, cytosolic | Q81824 |
| Histone H2A                     | H2A       |
| Kinesin-like protein; 73641–79546 | Q9CAC9 |
| Late embryogenesis-like protein (Fragment) (LEA-Prot) | O81366 |
| Lipoxigenase (EC 1.13.11.12)    | O04919    |
| Lipoxigenase (EC 1.13.11.12)    | Q93Y18    |
| Metalloendopeptidase            | Q40983    |
| Metallothionein-like protein 1; MT-1 | MT1   |
| Metallothionein-like protein 2; MT-2 | MT2   |
| Nonspecific lipid-transfer protein precursor; LTP | NLTP |
| P-type H\(^+\)-ATPase           | Q9SAW3    |
| P-type H\(^+\)-ATPase            | Q9ARS52   |
| Prolyl 4-hydroxylase, alpha subunit-like protein | Q9FKX6 |
| Protein phosphatase 2C          | O81709    |
| Putative imbibition protein     | Q9M4M2    |
| Root-specific metal transporter  | Q84LR1    |
| Rubisco activase                | Q8GTY4    |
| RuBiC large subunit-binding protein subunit alpha | RUBA |
| Thioprotease                    | Q41064    |
| Transcription factor DRE-binding factor 2 | Q6IVL3 |
| Translation initiation factor 5A | Q6PQ38 |
| Trehalose-6-phosphate phosphatase | O64897 |
| Ubiquitin conjugating enzyme    | O65733    |
| Ubiquitin conjugating enzyme    | O43780    |
| Ubiquitin conjugating protein   | Q9M4R0    |
| UDP-glucose pyrophosphorylase    | Q8VW557   |
| Vignain precursor (EC 3.4.22)   | CYSEP     |
| Vignain precursor; cysteine proteinase EP-C1 | CYSEP |

Comparison between SuperSAGE expression profiles and macroarray-generated data (Boominathan and co-authors [5]). Three main categories from the cluster analysis in Figure 8 are detailed below.

**Section A:** Constitutive tags versus upregulated transcripts on the macroarray.

**Section B, D:** Non-corresponding SuperSAGE and macroarray transcript profiles.

**Section C:** Up-regulated tags and up-regulated transcripts on the macroarray.
| Section | Annotation | Uniprot ACC |
|---------|------------|-------------|
| **A**   | 9/13 hydroperoxide lyase | Q7X9B3 |
|         | Adenosine 5'-phosphosulfate reductase | Q8W1A1 |
|         | Alkaline alpha galactosidase 1 (Fragment) | Q84NI7 |
|         | At1g68060/T23K23 | Q8L7S4 |
|         | AT3g29757/MWEI13 | Q94F39 |
|         | AT4g18030/T6K21 | Q94E6 |
|         | AT5g03040/F15A17 | Q93ZH7 |
|         | Auxin-induced beta-glucosidase | Q7XJH8 |
|         | Calcineurin B-like-interacting protein kinase | Q84XC0 |
|         | ERD3 protein | Q94I3 |
|         | Expansin-like protein (Fragment) | Q7XHJ2 |
|         | Gb| AAD25781.1 | Q9FK34 |
|         | General negative transcription regulator-like | Q9LS9 |
|         | Glucan endo-1,3-beta-d-glucosidase precursor | Q9ZPI2 |
|         | Glucosyltransferase-13 (Fragment) | Q85996 |
|         | Leucine-rich repeat resistance protein-like protein | Q93X72 |
|         | Nine-cis-epoxycarotenoid dioxygenase1 | Q8LP17 |
|         | Photosystem I psaH protein | Q7XAJ6 |
|         | Plasma membrane intrinsic polypeptide | Q9SMK5 |
|         | PS60 protein precursor | Q40473 |
|         | Putative extracellular dermal glycoprotein | Q9FSZ9 |
|         | Putative wound-induced protein | Q95BR5 |
|         | Pyruvate decarboxylase 1 (EC 4.1.1.1) | Q84V95 |
|         | Ubiquitin | Q39257 |
| **B**   | Basic blue copper protein | Q9ZRV5 |
|         | BZIP transcription factor ATB2 | Q8LSW2 |
|         | Cationic peroxidase | Q9FT05 |
|         | CJMDR1 | Q94I6H |
|         | Extensin | O65760 |
|         | Putative ripening related protein | Q8L6V6 |
|         | Putative senescence-associated protein | Q9AVI1 |
|         | S-adenosyl-L-methionine | Q84K6 |
|         | Root-specific metal transporter | Q84LR1 |
|         | Selenium binding protein | Q93WS1 |
| **C**   | Basic blue copper protein | Q9ZRV5 |
|         | BZIP transcription factor ATB2 | Q8LSW2 |
|         | Cationic peroxidase | Q9FT05 |
|         | Extensin | O65760 |
|         | Hydroxycinnamoyl transferase | Q8GS7M |
|         | Putative ripening related protein | Q8L6V6 |
|         | Putative senescence-associated protein | Q9AVI1 |
|         | Putative UDP-glycose | Q9M3H8 |
|         | Root-specific metal transporter | Q84LR1 |
|         | Selenium binding protein | Q93WS1 |
| **D**   | 1-deoxy-D-xylulose 5-phosphate synthase 1 prec. | Q8L693 |
|         | Asparagine synthetase | Q24483 |
|         | Aspartic proteinase | Q95XU0 |
|         | Aspartic proteinase 2 | Q94BP0 |
|         | AT5g17550/K10A8 | Q94E3 |
|         | AT5g64840/MXK3 | Q93ZN6 |
|         | ATP citrate lyase b-subunit | Q93YH3 |
|         | Auxin-induced beta-glucosidase | Q7XJH8 |
|         | CJMDR1 | Q94I6H |
|         | Ferritin | Q92P90 |
|         | Gb| AAD25584.1 | Q9FL6 |
|         | Glutathione S-transferase GST 11 (EC 2.5.1.18) | Q9FQ7E |
|         | Leucine-rich repeat resistance protein-like protein | Q93X72 |
|         | LHY protein | Q8LS5P7 |
because at least some of them are well characterized, and often clear-cut evidence is available for their role in stress-responses [3]. As has been shown for tolerance to salt-stress in rice, these genes or their products regulate early events in drought responses, differentiating between stress-tolerant and -susceptible genotypes [26]. However, genes involved in stress perception and signalling are not necessarily most up- or down-regulated.

Stress perception and signalling in drought-stressed chickpea roots

Dehydration-related stresses such as drought and salinity have ionic as well as osmotic attributes that elicit signal transduction cascades resulting in activation of effector genes to adapt the metabolism of the plant to the stress. In the model arising from research in Arabidopsis or rice, the first step of signalling is the perception of the stress through G-protein coupled receptors (GPCR), inositol polyphosphates, or receptor-like kinases (RLKs[2]). In drought-stressed chickpea roots, 36 RLK transcript variants were detected. One of these increased in abundance more than 20-fold, whereas fourteen were 2 to 8-fold up-regulated under stress, indicating a potential role of these transcripts in stress perception. Calcium transients are major signalling events in plants [27]. Thus, entry points and interconnecting links in major stress-related signal-
ling cascades involve Ca\textsuperscript{2+} sensors and proteins regulated by Ca\textsuperscript{2+}-concentration gradients between apoplast and cytoplasm [28]. In drought-stressed chickpea roots, SuperSAGE revealed moderate to significant changes in expression levels of Ca\textsuperscript{2+}-responsive genes. Transcript isoforms encoding Ca\textsuperscript{2+} sensors (e.g. calcineurin-B-like proteins, CBLs), Ca\textsuperscript{2+}-channel proteins, and inositol-3-phosphate (IP\textsubscript{3})-gated Ca\textsuperscript{2+}-release (e.g. phospholipase C), were generally up-regulated (for an extensive characterization see [29,30]). In contrast, transcript isoforms transcribed from genes involved in downstream events like fine-tuning of, and interconnecting between, signalling cascades were up- as well as down-regulated in reaction to the stress. These included transcripts encoding a wide range of kinases such as Ca\textsuperscript{2+}-dependent protein kinases (CDPKs; [31]), calcineurin-B-interacting protein kinases (CIPKs, [32]), and protein phosphatases class 2C.

Since the interplay between kinases and phosphatases balances activation and inactivation of proteins and with it the cross-talk between signaling cascades and metabolic pathways [33], measurement of transcriptional activity of kinase-encoding genes is important for understanding drought-response homeostasis. Mitogen-activated protein kinases (MAPKs), however, do not seem to interact much with early drought-stress signalling in chickpea roots, since of 6 MAPK transcripts detected, 2 were down-regulated more than 8-fold, and 4 similarly expressed as in control roots. In addition, the only MPKK detected was constitutively expressed, whereas from three 26 bp tags annotated to MPKKKs, two were down-regulated at least 4-fold (STCa-8893, STCa-10844), and one transcript was up-regulated at least 6-fold (STCa-2124).

Regulation of 14-3-3 transcripts
Signal transduction and regulation of metabolism achieved via phosphorylation-mediated transition of protein states require that phosphorylated proteins physically interact with specialized adapter proteins to fulfil their regulatory role. An example for such adapters are the phosphoserine/threonine-binding 14-3-3 proteins [34]. For example, 14-3-3 proteins are phosphorylation targets for SnRK 2.8, a member of the sucrose non-fermenting-related kinase family, that is down-regulated in plants deprived of nutrients and with reduced growth [35]. Plants have large 14-3-3 gene families, and various 14-3-3 isoforms have varying affinities to target proteins. In Arabidopsis, at least 15 expressed members of the 14-3-3 gene family exist and exhibit high cell- and tissue-specificity as well as diverse expression levels [36]. In rice, at least four 14-3-3 transcript isoforms are induced by drought and salt-stress [37].

In drought-stressed chickpea roots, we detected 18 isoforms of 14-3-3 protein transcripts. However, contrary to
results from drought-stressed rice, only three of these were up-regulated more than 2-fold, whereas ten were down-regulated more than 4-fold (Figure 5). Similar differential expression of 14-3-3 transcript isoforms was detected in young tomato roots under normal growth conditions as well as in response to salt stress and potassium and iron deficiencies, suggesting that especially one isoform (TF17) may mediate cross-talk between the salt stress and potassium and iron-deficiency signalling pathways, respectively [38].

Transcription factors and involvement of ABA in early drought stress responses in chickpea roots

Another large and complex class of genes encode transcription factors (TFs). We could identify 124 UniTags from TF transcripts classified into 26 TF families (Figure 10), whereas 8 remained un-classified. The majority of TF-UniTags annotated to the bZIP TF family (18), followed by UniTags matching to the HDZ (14), HMG (13), and WRKY (10) TF families. As described for the bZIP type TF family in Figure 5, expression profiles of transcription factors-encoding transcripts may display diverse regulation tendencies. This is also true for UniTags derived from transcripts encoding MYB family members, though these were reportedly involved in signal transduction under water-deficit [39]. This observation may be related to the constitutive and even slight down-regulation of transcripts for proteins involved in ABA synthesis such as 9-cis-epoxy-carotenoid dioxygenase [40], though Boominathan and co-authors [5] observed a strong up-regulation of a particular mRNA for this enzyme under drought stress, that probably escaped our detection. The difference in expression of genes involved in ABA-dependent signalling such as the isoforms of MYB TFs [41] may suggest that, as in maize, ABA as signal may be restricted to very specific regions of the root [42]. From six MYB transcription factors detected, two were up-regulated, and 3 were down-regulated. Of 18 members of the bZIP TF family (see above) to which AREB factors belong [43], four were up-regulated more than 3-, whereas three were down-regulated more than 4-fold.

In contrast, ABA-independent signalling seems to prevail: two out of five Drought-Responsive-Element-Binding (DREB2) TF isoforms thought to be regulated independently from ABA [44] were at least 3-fold up-regulated (STCa-4170, STCa-4212). Additionally, two further DREB transcript variants revealed constitutive levels, whereas one was 3-fold down-regulated (STCa-13360). Besides DREB2 TFs, WRKY TFs also seem to be involved in drought-stress responses in chickpea, because of the 10 detected family members, two were at least 2-fold (STCa-4132, STCa-10200), and two 4-fold up-regulated (STCa-11618, STCa-11619). Also, UniTag STCa-15340, homologous to an alfin-1-like TF transcript from alfalfa, belonged to the 40 most up-regulated transcripts under drought stress in chickpea (Table 4). In alfalfa, transgenic over-expression of a TF belonging to this class enhances expression of the endogenous MsPRP2 gene and improves salinity tolerance [45]. Thus, up-regulation of STCa-15340 indicates the involvement of alfin-1-like TFs in salt- as well as in drought-stress responses. Though already highly informative, our analysis of TF gene expression in drought-stressed chickpea roots certainly tapped the tip of an iceberg only, since we could assign only 124 UniTags to this class of genes. In fact, there are more than 1500 TF genes in the Arabidopsis genome [46]. Considering that we could assign only 22% of the 26 bp tags to well characterized entries in the public data bases, the number of TFs in our data set should be approx. 6 to 6 times as high as the one we could assign, i.e. approx. 650. This estimate is in good agreement with results from sugarcane, where 237,954 ESTs contained 600 TF sequences [47].

ROS scavenging and ROS-triggered signalling-related genes

Plants generate singlet oxygen-, superoxide-, peroxide-, and hydroxyl-radicals (ROS) that trigger a wide range of partly genetically fixed responses. ROS are released by either NADPH oxidases or peroxidases, that may exist alone or in combination in different plant species. Plant cells perceive changes in the concentrations of ROS as second messengers, and transform them into signals that change the transcription of genes [2]. On the other hand, disturbances in metabolism and photosynthesis by environmental stresses lead to ROS accumulation, which, if not controlled, can rapidly reach toxic levels in the plant cell [48]. Major ROS scavenging enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), mono-dehydroascorbate reductase (MDAR), dehydroascorbate reductase [49], glutathione-S-transferase (GST) [50], glutathione peroxidase (GPX), glutathione reductase (GR), and catalase (CAT) [51]. The balance between the activities of these enzymes could be crucial for determining the steady-state level of ROS.

Our data reflect the complexity of ROS signalling and scavenging. For example, 10 out of 29 peroxidase isoforms were significantly up-regulated (data not shown), but only one NADPH oxidase was detected and moderately up-regulated. However, one transcript each for a hypersensitive-induced response protein and radical-induced cell death 1-1 protein were detected, and both were significantly up-regulated. Of the ROS scavengers, six out of seven SOD transcripts, and one out of two transcripts coding for each catalase and dehydroascorbate reductase were more than 2-fold up-regulated, whereas two of the three ascorbate peroxidase (APX) transcripts were moderately or even significantly down-regulated. Since APX catalyses the H2O2-dependent oxidation of L-
ascorbate (vitamin C;[52]), this potent ROS scavenging mechanism seems to be under-used in early responses of chickpea roots to drought. Considering that STCa-7166 representing an NADP-dependent isocitrate dehydrogenase transcript belonged to the most up-regulated transcripts in our study (R(ln) 3.58, Table 4), scavenging of ROS by glutathione and recycling of oxidized glutathione by this enzyme seems to prevail instead. This assumption is supported by the strong up-regulation (R(ln) 3.08, Table 4) of cysteine synthase (CS)-encoding UniTag STCa-2982.

In rice, as a consequence of CS up-regulation, both the total glutathione pool and reduced glutathione concentration were significantly increased in response to aluminium stress [53].

Still another stress-responsive gene family encodes glutathione-S-transferases (GSTs), key defence enzymes against xenobiotic toxicity, and has at least 56 members in rice [54]. From 14 chickpea 26 bp tags annotated to GSTs, four isoforms (STCa-977, 2175, 20830 and 12384) were at least 2-fold down-regulated, and three isoforms revealed up-regulation (STCa-3042, 12502 and 22470). Apart from ROS-scavenging, GSTs may also function in stress tolerance through signalling [55]. Considering the importance of these proteins for managing ROS-related stress, the GST transcript variants strongly up-regulated in chickpea roots under drought stress are potential targets for molecular breeding for drought tolerance.

**Regulation of aquaporin gene activity under drought stress**

Despite considerable progress in understanding fundamental stress responses in model plants, we know little about the molecular basis of differences between stress-tolerant and susceptible genotypes of crops. Only recently, investigations into drought responses of upland and lowland rice, and genotypic variation for water status under different water regimes in a population of recombinant inbred lines (RILs) of sunflower [56] demonstrated a possible involvement of certain aquaporin genes in differing dehydration-stress response phenotypes of
these crops. For example, the Arabidopsis genome harbours at least 35 genes coding for different aquaporins, that are differentially expressed under different stress conditions and, whereas one family member is up-, the other may well be down-regulated. In chickpea roots, we detected at least 42 different 26 bp tags from aquaporin transcripts, representing the three classes (nodulin-, plasma membrane-, and tonoplast-intrinsic forms). Interestingly, the over-expression of a certain aquaporin isoform in transgenic Arabidopsis resulted in altered expression patterns of other aquaporin isoforms with consequences for seed germination, seedling growth, and stress responses of the plants under various stress conditions [57]. These results suggest a concerted transcriptional regulation of at least a subset of aquaporin genes. Like in Arabidopsis, drought also elicited differential responses in the different members of the aquaporin gene family also in chickpea. It will be interesting to learn, whether the differentially expressed putative NATs corresponding to several of the sense aquaporin isoforms detected in chickpea are involved in the regulation of other gene family members. Also, whether differences exist in the expression of aquaporin isoforms between drought-tolerant and -susceptible genotypes of chickpea needs to be determined.

Changes in transcription of genes regulating compatible osmolyte accumulation

Beyond the broad repertoire of signalling cascades and signalling interactions that plants have on their defence lines against drought stress, the accumulation of sugars, sugar alcohols, amino acids, and polyamines, acting as compatible osmolytes against the osmotic disequilibrium is one of the most widespread strategies of plants to enhance their tolerance against drought stress [58]. Several genes involved in biosynthesis, transport as well as intermediate and catabolic pathways related to this strategy have been genetically and functionally characterized during the past years [59-67]. After screening the C. arietinum UniTags database for the behaviour of genes related to the above processes, transcription profiles and number of isoforms from at least 12 relevant genes were investigated in detail (Figure 6). Related to sugar accumulation, one UniTag annotated to trehalose-6-phosphate synthase (STCa-18759, 2-fold down-regulated), and three 26 bp tags annotated to trehalose-6-phosphate phosphatase (STCa-9149 3-fold upregulated; STCa-11438 3-fold down-regulated; STCa-21065 constitutive) were observed. Trehalose plays an important role as compatible osmolyte and signalling molecule under drought stress [60,62]. However, since we do not observe strong up-regulation of genes encoding threahalose-6-phosphate synthase, we are hesitant to conclude that trehalose accumulated to high concentrations under our assay conditions. Additionally, one significantly up-regulated UniTag STCa-11968 representing a galactinol synthase gene as well as at least three tags representing transcripts related to sucrose metabolism and transport with more than 2-fold expression changes (STCa-19100, STCa-8449, Ca-SS-16426) revealed, that the dynamics of sugar metabolism, transport and accumulation could be altered as a response to drought stress in chickpea. The positive role of galactinol synthase in stress tolerance has already been reported elsewhere [64]. Several 26 bp tags representing amino acid transport- and accumulation-related genes were detected. For proline, a compatible osmolyte [67], one 6-fold up-regulated UniTag with homology to a proline/betain transporter (STCa-24308) as well as moderate down- and up-regulation of two 26 bp tags representing a negative regulator for proline accumulation (proline dehydrogenase; STCa-8454, STCa-8455) were revealed. This suggests that prolin accumulation may occur to some extent under drought stress in chickpea. The transcript levels for betaine aldehyde dehydrogenase UniTag STCa-14752, the key enzyme for glycine betaine synthesis [68], did not markedly change upon drought stress, indicating that this osmolyte, otherwise reported to accumulate under water stress [69], is not important for an early stress response. Since the accumulation of polyamines as compatible osmolytes is discussed as protection against stress, we paid special attention to transcripts encoding arginine decarboxylase and spermidine synthase, that share important roles in putrescine and spermidine accumulation [70]. The detected transcripts were 2-fold (STCa-8875; arginine decarboxylase) and 3-fold (STCa-611; spermidine synthase) up-regulated. Our results indicate a differential influence of drought stress on mechanisms for compatible osmolyte accumulation as an early stress response in chickpea.

Comparison of SuperSAGE versus macro- and micro-arrays

Though drought-stress responses from roots of adult chickpea plants were compared to the roots of much younger seedlings from Medicago ([10], Table 7), the similar expression of several genes (section D, Figure 9) suggests similar reactions of the roots of both legumes to drought stress, independently of the developmental stage of the roots. Genes similarly up-regulated in both species inter alia include a certain extensin isoform, actually the most up-regulated transcript in stressed chickpea roots (O65760_CICAR). Extensins are hydroxyproline-rich proteins strengthening cell walls, and are often activated by mechanical stress [71]. Like other proline-rich cell-wall proteins, extensins require hydroxylation of prolines to 4-hydroxyprolines to form the cell wall matrix [72]. Consistent with extensive hydroxylation of extensins, UniTag STCa-542 representing a prolyl 4-hydroxylase alpha subunit-like protein belonged to the most up-regulated transcripts in drought-stressed chickpea roots ($R_{\text{in}}$ 2,722).
At the same time, STCa-1804, encoding an expansin-like protein, was most down-regulated ($R_{(in)} - 3,095$) in stressed chickpea roots. Expansins weaken cell walls [73], and thus down-regulation of the expansin gene also would contribute to an increased strength of the cell walls of drought-stressed roots. In addition, UniTag STCa-24349, representing a gibberellin 2-beta-hydroxylase transcript, belonged to the most up-regulated transcripts in stressed chickpea roots ($R_{(in)} 2,28$, Table 4). The encoded enzyme inactivates gibberellin through $\beta$-hydroxylation [74] and thus, its up-regulation should result in significantly decreased levels of bioactive hormone and a reduction of cell divisions and extensions. We conclude that in chickpea and *Medicago*, drought impairs division and extension of cells and results in growth-retarded roots with strengthened cell walls.

Other transcripts similarly up-regulated in both chickpea and *Medicago inter alias* code for dehydrin, DREB, 2C protein phosphatase, UDP-glucose phosphorylase and blue copper protein which are discussed elsewhere in this paper. Taken together, a comparison of drought stress responses in chickpea and *Medicago* reveals many interspecies similarities and suggests to exploit the huge resources available for *Medicago* to test the functions of differentially expressed transcripts in chickpea.

**Conclusion**

Applying SuperSAGE to the analysis of abiotic stress responses in chickpea for the first time, our study presents the most comprehensive transcriptome profile of this crop available to date. It increases the number of chickpea ESTs from approximately 1,900 to more than 80,000, and the number of unique transcripts to more than 17,000. The study identified major drought-stress signalling cascades resulting in differential expression of effector genes, and hints to the importance of ROS and N starvation as side stresses resulting from drought. Our study revealed, that (1) genes involved in photosynthesis and energy metabolism were down-regulated, (2) many genes involved in early responses to biotic and abiotic stresses were up-regulated, while (3) other stress-responsive genes were down-regulated, and (4) regulatory genes encoding e.g. transcription factors or signal transduction proteins were both up- and down-regulated. We conclude that follow-up transcription profiling studies of responses to drought in chickpea must take into account the potentially deleterious effect of the stress on SNF and thus, on N supply to the plant in order to prevent mixing up responses to different stresses.

One important fact arising from our study is the unexpectedly high number of differentially expressed isoforms of members of large gene families, that was also observed in SAGE libraries from *Lotus japonicus*, where different levels of transcription induction among leghemoglobin gene paralogs were found [12]. These findings highlight the efficiency of tag-based techniques to discriminate different gene family members. At the same time, they underpin the necessity to – experimentally and linguistically – distinguish between certain transcript isoforms (and the underlying genes) rather than summarizing them under a common term.

With this work, we aimed at identifying candidate genes as targets for molecular breeding for drought tolerance in chickpea. Numerous studies confirmed the polygenic nature of drought tolerance, for which single QTLs have only little individual effect. Considering the large number of genes located at QTLs for drought tolerance and related traits in cereals, comparing our transcription profiles to genes mapped to drought QTLs in these crops may help to decide whether SuperSAGE has identified such potential breeding targets.

**Methods**

**Plant materials and stress treatment**

Surface-sterilized seeds of drought-tolerant chickpea variety ILC588 (Rehman et al. ICARDA, [http://www.pulse.usask.ca/6cprw/poster/reh.pdf](http://www.pulse.usask.ca/6cprw/poster/reh.pdf)) were germinated in germination boxes on filter paper at ICARDA (Syria). The resulting seedlings were grown in a growth chamber at a constant temperature of 22°C, a photoperiod of 12 h light/12 hours 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. Then control plants were removed, and their roots immediately frozen in liquid nitrogen. For desiccation, plants were removed, carefully preventing mechanical damage, and subjected to dehydration for 6 h at room temperature. Light regime, temperature, and humidity were kept constant and strictly monitored during the treatment of the plants. After the desiccation period, the plants showed wilting symptoms (turgor loss), and the roots were separated from the shoots and shock-frozen in liquid nitrogen.

**RNA isolation and construction of SuperSAGE libraries**

Total RNA was isolated from control and stressed roots using a modified CTAB procedure [75] followed by precipitation of the RNA in 3 M LiCl at 4°C overnight. From approximately 1 mg of total RNA, poly(A)⁺RNA was purified using the Oligotex mRNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s batch protocol. Subsequent steps for construction of SuperSAGE libraries were performed as detailed by [76]. However, instead of concatenation of di-tags and subsequent cloning and sequencing, amplified ditags were directly sequenced by 454 Life Sciences, Branford, CT, USA.
**Tags quantification and data analysis**

For each library, 26 bp long 26 bp tags were extracted from the sequences using the GXP-Tag sorter software provided by GenXPro GmbH, Frankfurt am Main, Germany. Library comparison and primary statistical treatment was carried out using the DiscoverySpace 4.01 software (Canada's Michael Simith Genome Sciences Centre, available at [http://www.bcgsc.ca/discoveryspace](http://www.bcgsc.ca/discoveryspace)). Scatter plots of the distribution of the expression ratios ($R_{(ln)}$) and significance of the results were calculated according to Audic and Claverie [11].

**Sequence homology alignment**

Tags sequences were BLASTed [77] against different public databases discriminating the hits in a hierarchical, taxonomical manner using the BLASTN algorithm [http://www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/). First, all 17,493 unique tag sequences were BLASTed against the non-redundant DNA databases, limiting the output hits with the highest priority level to *Cicer arietinum* and members of the Fabaceae, by using the routine BLASTc13 (NCBI, [http://www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). Subsequently, individual local BLAST searches were carried out in Fabaceae sequences, followed by *Arabidopsis*, rice and maize homology searches in the TIGR gene indices [http://compbio.dfci.harvard.edu/tgi/plant.html](http://compbio.dfci.harvard.edu/tgi/plant.html). After each BLAST round, anonymous DNA sequences (e.g. chromosomes, shotgun clones, and ESTs not linked to any characterized protein) were filtered out. Additionally tags assigned to TIGR TCs indicating weak similarity to characterized genes were not selected. For targets from legumes different from chickpea, a maximum of three mismatches was allowed. The expected number of random matches (E value) was kept under 0.009 for individual TIGR databases, and 0.0009 for larger databases (e.g. NCBI nr restricted to fabaceae hits). Low complexity regions were rejected, whereas gap costs were set to 5-2 (NCBI BLAST standard setting).

**Annotation test of in silico generated chickpea 26 bp tags using *M. truncatula* ESTs**

In order to test the validity of the annotation of chickpea tag sequences through homologies with other legumes, 7,500 chickpea EST sequences deposited in the NCBI database were used to generate virtual 26 bp tags. Initially, all ESTs were screened for CATG sites using the BioEdit software, version 7.0.5.3 [http://www.mbio.ncsu.edu/BioEdit/BioEdit.html](http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Subsequently, all ESTs harbouring more than 30 bp between the most 3’ CATG site and the end of the sequence were selected. After virtual 26 bp tag extraction, duplicate fragments were excluded, and the remaining tags BLASTed against public EST/mRNA databases following three main routes: I) BLASTing against the non-redundant (nr) NCBI nucleotide database (*Fabaceae* mRNA accessions), II) against the plant EST database at NCBI (*M. truncatula* accessions), and III) against *M. truncatula* ESTs deposited in the TIGR gene indices. Complete EST/mRNA high homology target sequences derived from BLASTs (II) and (III) were retrieved and reBLASTed against the nr NCBI database (*Fabaceae*). The results obtained by these two BLAST strategies were compared with strategy (I) after exclusion of anonymous entries (e.g. AFLP fragments, shotgun sequencing clones, whole genome entries, whole chromosomes, BAC clones, etc.). BLAST parameters were set as described in the previous section.

**Cluster analysis and functional category distribution analysis**

Cluster analysis of the expression ratios ($R_{(ln)}$) used the software package Cluster 3.0 [http://rana.lbl.gov/EisenSoftware.htm](http://rana.lbl.gov/EisenSoftware.htm). A distance matrix for the $R_{(ln)}$ was calculated with Pearson’s correlation distance method [78]. Transcripts were clustered using the average linkage clustering routine under hierarchical clustering. P values for the most represented GO: biological processes observed after 6 hours of desiccation were calculated and correlated with the UniTag expression ratios ($R_{(ln)}$) using the “Receiver Operator Characteristic” (ROC) routine of the ermineJ 2.0 software package (University of British Columbia, 2006, [http://www.bioinformatics.ubc.ca/ermineJ](http://www.bioinformatics.ubc.ca/ermineJ)). P-values for the representation of GO: categories are calculated according to [79] as indicated by the software developers.

**Rapid amplification of cDNA ends (3’-RACE) using tag sequences as PCR primers**

To test the versatility of the 26 bp tag-derived oligonucleotides for direct use as 3’-RACE PCR primers, 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 26 bp tag-based primer, 10 pmol oligo(dT) (t)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. After amplification, products were separated in 1.5% preparative agarose gels. Bands corresponding to unequivocal amplicons were excised, and DNA extracted with Qiaquick cleanup columns (Qiagen, Hilden, Germany). Cloning of PCR products as well as colony PCR screening followed standard blue-white screening procedures [80]. Positive clones were sequenced via ABPrism multi-colour fluorescence-based DNA analysis system (Applied Biosystems, Foster City CA, USA).

**Confirmation of SuperSAGE expression profiles via qRT-PCR**

Parallel RNA extractions of the same tissue, from which the SuperSAGE libraries were derived, were carried out as
SYBR green oligonucleotide deduced was carried out with the software package Primer Express, version 2.0, provided by Applied Biosystems (Foster City, CA, USA) with 3'or 5'-RACE products from selected 26 bp tags as starting points. The two TaqMan assays used in this study were provided by GenXPro GmbH, Frankfurt, Germany, and used according to the protocol included in the kit.

The real-time PCR reactions for SYBRgreen and TaqMan assays used the Power-SYBRgreen PCR master mix and the TaqMan-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). TaqMan assay temperature amplification 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. Amplexon 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 TaqMan 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 starting copy numbers of the target gene. Relative quantitation of the target genes amplified via SYBRgreen assays was performed by the comparative ΔΔCt method. Genes amplified by TaqMan 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-desiccation as standard templates. The RT-PCR data were normalized with the relative efficiency of each primer pair.

Confirmation of expression profiles via microarrays

SuperSAGE expression profiles were confirmed by direct spotting of a selection of 26 bp tags onto a 16 K Agilent microarray (AGILENT TECHNOLOGIES, Santa Clara CA, USA). Three thousand UniTags with different expression levels under drought, salt, and cold stresses (salt and cold stress expression profiles are not approached in the present paper) were selected. From the 3,000 Tags, a subset of 2,796 oligonucleotides was spotted in duplicate onto different sections of the microarray. Additionally, for each of the 3,000 selected Tags, oligonucleotides with mismatches were spotted onto the microarray in three sets as follows: i) mismatch at position 7; ii) mismatches at positions 7 and 13 and iii) mismatches at positions 7, 13, and 20, respectively. Background correction was achieved by the Feature Extraction softwareTM (Agilent Technologies), subtracting the mismatch intensities for each spotted Tag. Microarray design, spotting and hybridizations were carried out by ARRAY-ON GmbH, Gatersleben, Germany, according to the AgilentTM protocols (AGILENT TECHNOLOGIES, Santa Clara CA, USA).

Authors’ contributions

CM and BR generated the SuperSAGE libraries with the guidance of HM and RT and performed in silico data analyses. RH generated the 3'- and 5'-RACE sequences and designed the primers for qRT-PCR probes. MB and SMU selected the plant material, performed the stress treatments and provided the root material. BB and LB pro- 

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