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Exploring genetic and expression differences between physiologically extreme ecotypes: comparative genomic hybridization and gene expression studies of Kas-1 and Tsu-1 accessions of *Arabidopsis thaliana*

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ABSTRACT

Recent studies have documented remarkable genetic variation among *Arabidopsis thaliana* accessions collected from diverse habitats. Of particular interest are accessions with putatively locally adapted phenotypes – that is, accessions with attributes that are likely adaptive at their sites of origin. These genotypes may provide insight into the genetic basis of adaptive evolution as well as allow the discovery of genes of ecological importance. We studied the physiology, genome content and gene expression of two physiologically extreme accessions (Tsu-1 from Tsushima, Japan and Kas-1 from Kashmir, India). Our study was conducted under two levels of soil moisture and accompanied by physiological measurements to characterize early responses to soil drying. Genomic hybridizations identified 42 503 single feature polymorphisms (SFP) between accessions, providing an initial screen for genetic differences. Transcript profiling identified a large number (5996) of genes exhibiting constitutive differences in expression including genes involved in many biological pathways. Mild soil drying resulted in only subtle physiological responses but resulted in gene expression changes in hundreds of transcripts, including 352 genes exhibiting differential responses between accessions. Our results highlight the value of genomic studies of natural accessions as well as identify a number of candidate genes underlying physiological differences between Tsu-1 and Kas-1.

Key-words: Acclimation; affymetrix; drought; expression polymorphism; hybridization polymorphism; soil moisture; water-use efficiency.

INTRODUCTION

As sessile organisms, plants are constantly challenged by a variety of abiotic stresses from which they cannot flee. As such, they are a fascinating system for studying the physiological mechanisms underlying abiotic stress tolerance, including both acclimation responses and local adaptation. An especially critical environmental stress for plants is the availability of soil moisture, as water availability is fundamental to almost all aspects of plant physiology. Plant water status has a strong and direct impact on C3 photosynthesis and carbon fixation through stomatal regulation of gas exchange, as well as important consequences for plant growth, phenology and susceptibility to other abiotic and biotic stresses (Rizhsky *et al*. 2003). More broadly, water availability and atmospheric demand, interacting with temperature, are fundamental determinants of plant distribution abundance, and productivity worldwide (Walter 1964; Whittaker 1975). As a result, crop yields are commonly reduced by water limitations to less than half of potential yields (Boyer 1982; Gleick 1998). Therefore, research exploring the molecular genetic and physiological basis of tolerance to soil drying is critical to understanding and improving plant function under stressful conditions.

The model angiosperm *Arabidopsis thaliana* has emerged as a valuable tool in deciphering how plants respond to environmental stresses, including drought and desiccation (Zhang, Creelman & Zhu 2004). Decades of research has shown that cell signalling and gene expression networks underlie physiological adjustment and are likely to affect plant performance under stress. As an example, a host of studies in *Arabidopsis* have documented gene expression changes induced by dehydration-related treatments. Two *cis*-acting DNA sequence elements clearly contribute to water deficit-induced gene expression in the Columbia accession, suggesting that regulatory evolution may be a key process in drought adaptation in *Arabidopsis*. The

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abscisic acid (ABA)-response element (ABRE) is important for ABA-dependent changes in gene expression (Uno *et al*. 2000; Bray 2004), and many ABA-responsive genes are likely to play a role in drought acclimation. Moreover, ABA functions in stomatal regulation, control of growth and osmolyte accumulation (Verslues & Zhu 2007). The dehydration-response element/C-repeat (DRE/CRT) is essential for ABA-independent induction of many desiccation-responsive genes (Yamaguchi-Shinozaki & Shinozaki 1994). Several of the transcription factors (DREB/CBF) involved in DRE/CRT-responsive gene expression have been cloned and a number of target genes characterized (Shinozaki & Yamaguchi-Shinozaki 2000; Seki *et al*. 2001, 2002; Bray 2002a; Shinozaki *et al*. 2003). Genetic manipulation of drought-responsive transcription factors and/or their downstream targets has resulted in increased plant performance under specific stress treatment conditions (reviewed in Umezawa *et al*. 2006).

Despite this progress, we still have much to learn. Although the existing literature on stress induction has been critical for benchmarking gene function (Bray 2002a,b, 2004; Kilian *et al*. 2007), lab treatments (desiccation of cut leaves on benchtop environments), short response times (1–24 h) and limited genetic diversity (wild-type, knockout or overexpression lines) may limit the scope of inference from these experiments. In particular, it is possible that many of the effects observed are better considered 'stress shock', given the timescales of stress imposition and the lack of acclimation. It is likely that 'shock' studies have touched only the surface of how plants respond to diverse abiotic stresses. As such, we have initiated a number of studies implementing more realistic progressive soil-drying manipulations and utilizing a diversity of accessions.

A. thaliana provides a unique opportunity to explore adaptive evolutionary responses to drought as it has an extensive geographical distribution and has experienced a wide-range of climatic selective regimes for thousands of generations (McKay, Richards & Mitchell-Olds 2003; Koornneef, Alonso-Blanco & Vreugdenhil 2004; Bouchabke *et al*. 2008). We have completed several common garden and quantitative genetic and quantitative trait loci (QTL) mapping experiments focused on plant–water relations and integrative water-use efficiency measures in *Arabidopsis* (McKay *et al*. 2003; Hausmann *et al*. 2005; Juenger *et al*. 2005, 2006; Christman *et al*. 2008; McKay *et al*. 2008). These studies have characterized the general range of variability as well as identified a number of accessions with extreme physiological traits. We have centred our recent efforts on the Kas-1 and Tsu-1 accessions, as they represent the highest and lowest identified water-use efficiencies in diversity panels, respectively (McKay *et al*. 2003), and the climate of the accession sites of origin differs greatly in both precipitation and temperature. The site of origin of Tsu (Tsushima, Japan) has high water availability throughout the growing season and the Kas site of origin (Kashmir, India) has very limited precipitation inputs during the growing season (McKay *et al*. 2008). To provide a tool to understand the genetic basis of this putative adaptive differentiation, Kas-1 and Tsu-1 were reciprocally crossed to create a new recombinant-inbred mapping population (McKay *et al*. 2008). The Kas-1 × Tsu-1 mapping population provides a powerful new resource for mapping QTL underlying natural variation and for dissecting the genetic basis of water-use efficiency differences.

Here, we extend previous studies of drought-induced gene expression to incorporate more realistic stress treatments and putatively locally adapted plant material. We utilize genomic hybridization and gene expression approaches using Affymetrix GeneChip microarrays (Santa Clara, CA, USA) and the Tsu-1 and Kas-1 accessions. Our goals are to characterize natural physiological responses to soil drying in *Arabidopsis*, identify putative sequence differences between physiologically extreme accessions and explore patterns of transcript responses to initial and mild stress conditions.

MATERIALS AND METHODS

Plant growth and drydown

Tsu-1 (CS 1640) and Kas-1 (CS 903) were grown in 6 × 6 × 5 cm plastic pots filled with ~150 mL Profile porous ceramic rooting media (Profile Products LLC, Buffalo Grove, IL, USA). Prior to planting, dry weight of each pot and soil (DWpot & soil) was measured and the media was saturated with a complete nutrient solution (Epstein & Bloom 2005) for several days. Saturated pots were covered and allowed to drain by gravity for >24 h until a constant field capacity weight (FCpot & soil) was obtained for each pot. Three seeds were planted in each pot and dark stratified at 4 °C for 3 days. Germination was on 9–10 August 2006. Plants were thinned to one per pot and were grown and treatments were applied in a controlled environment chamber with 10 h photoperiod of 330 μmol m^{-2} s^{-1} photosynthetic photon flux density. Daytime/night-time temperatures were 23/18 °C with 60% relative humidity. During growth, all plants were fertilized with the nutrient solution every two days and watered daily. The drydown began on 26 August 2006 with all plants in rosette stage. Plants were grown in three replicate blocks with treatments and accessions randomly located within each block. There were six subsample plants each of Tsu and Kas in each treatment in each block (72 plants total).

During the drydown treatment, each pot was weighed daily at 'pre-dawn' (WTpot & soil) and distilled water was added by pipette at the base of the rosette to bring the percent water remaining (WatRem%) in each pot to the target level for that day (e.g. 90% on day 1, 80% on day 2, etc.) until a final drying treatment level of ~50% was reached by all pots on the fifth day. A major goal of the slow drydown treatment was to allow the intact, growing plants to acclimate to the imposed soil moisture stress. WatRem% was calculated as 100 × (WTpot & soil – DWpot & soil)/(FCpot & soil – DWpot & soil). Wet (control) pots were treated identically, except WatRem% was maintained near 90% for the entire drydown period. At 'dusk', prior to the

harvest day, plants were enclosed in covered trays to minimize evapotranspiration. Covered trays were transported to the lab the following morning, final pot and soil weights were taken, WatRem% at harvest were calculated and plants were harvested. Harvest occurred during the first several hours of the normal daily light period.

Harvest and physiological measurements

Plants of both lines and both treatments in each block were harvested together. Rosettes were excised and fresh weight of rosette and any bolt stem were determined separately. Immediately, ~80 mg (~40% of the leaves on the rosette) of healthy, fully expanded leaves were detached from the rosette and placed in RNA*later* (Ambion, Inc., Austin, TX, USA), several additional leaves were removed and placed in a psychrometer chamber for water potential measurement (below) and 2–3 leaves were removed for water content (WC) and relative water content (RWC) measurements. For WC and RWC measurements, leaf fresh weight was immediately determined on a microbalance, leaf bases were placed in distilled water in a microfuge vial and the vial and leaves were enclosed in a larger sealed vial to allow leaves to reach full hydration in the dark for ~18 h. Turgid weight of the hydrated leaves was then determined, leaves were dried and dry weight determined using the same microbalance. WC was calculated as $100 \times$ (fresh weight – dry weight)/(dry weight) and RWC as $100 \times$ (fresh weight – dry weight)/(turgid weight – dry weight) (Boyer 1995). Remaining leaves were weighed fresh and after drying so that total rosette and total leaf weight, both fresh and dry, could be calculated by summing values from material used for each type of measurement. After drying, leaves were used for C and N content and stable isotope analyses at the UC Davis Stable Isotope Facility (http://stableisotopefacility.ucdavis.edu). Carbon isotope composition ($\delta^{13}C$) is given relative to the PeeDee Belemnite standard and composition is used rather than discrimination (Δ) because the isotopic composition of carbon dioxide in the ambient air, which is required to compute Δ, was extremely variable (see McKay *et al*. 2003).

Leaf water potential (Ψ_{tot}) was measured using the excised leaves, individually calibrated (Brown & Bartos 1982) thermocouple psychrometers (Merrill Specialty Equip., Logan, UT, USA) and stainless steel chambers (see Donovan, Linton & Richards 2001; Donovan, Richards & Linton 2003). Entire leaves were placed in chambers within 30 s of excision and chambers were sealed and suspended in a water bath to minimize temperature gradients during measurement. Mature leaf tissue was used to minimize any growth effects on leaf Ψ_{tot} (Boyer 1995). Psychrometer outputs were logged hourly (CR7 data logger; Campbell Scientific, Logan, UT, USA) and leaf Ψ_{tot} was determined after equilibration (~24 h). After Ψ_{tot} was measured, the sealed chambers were immersed in liquid nitrogen to rupture cell membranes of the leaves and an estimate of bulk leaf osmotic potential (Ψ_{sol}) was determined during a second 24 h equilibration period. For each subsample plant, leaf turgor (Ψ_p) was calculated as $\Psi_{tot} - \Psi_{sol}$. Similar to leaf measurements, soil water potential (Ψ_{soil}) was determined on a sample of soil, with roots, taken from the centre of a subset of the pots and placed in stainless steel chambers with thermocouple psychrometers. All water potentials were calculated from psychrometer μV outputs using the Brown & Bartos (1982) model that accounts for zero offset (always between 0.2 and 0.2 μV) and temperature.

Genomic DNA hybridizations to ATH1 array

Comparative genomic hybridizations were completed following the protocols outlined in Borevitz (2006) and using the Invitrogen (Carslbad, CA, USA) BioPrime Labeling System. Genomic DNA was extracted from seedlings of Tsu-1, Kas-1 and Col-0 (CS 60000) accessions using the Qiagen DNAeasy (Germantown, MD, USA) plant kit. Approximately 300 ng of genomic DNA was added, on ice, to 60 μL of $2.5 \times$ random primer solution and made to a final volume of 132 μL with distilled water. The mixture was denatured by incubation at 99 °C for 10 min and immediately placed on ice for 5 min. Next, 15 μL of $10 \times$ deoxyribonucleotide triphosphate solution (with biotin-deoxycytidine triphosphate) and 3 μL Klenow were added to the denatured DNA mixture. The reaction was incubated in a PCR block at 25 °C for 16 h and the reaction was terminated by the addition of 15 μL stop solution. Labelled DNA was precipitated by the addition of 20 μL of 3 mol L^{-1} sodium acetate and 400 μL cold 100% EtOH. This solution was incubated on ice for 2 h and centrifuged at 15 000 g for 10 min followed by a wash with 70% EtOH. The pelleted DNA was dried and resuspended in deionized distilled H_20. The labelled DNA was then treated the same as labelled cRNA using standard hybridization protocols for gene expression (see methods below). Genomic hybridizations to the Affymetrix ATH1 array were completed for six samples of each accession for a total of 18 arrays. Hybridizations were completed in two separate batches of nine arrays.

The genomic hybridization data from this study are accessible through GEO Series accession number GSE20340 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20340).

Gene expression studies

We used a subset of plants from the drydown experiment in our studies of soil water deficit-regulated gene expression. In brief, our experimental design was a fully factorial experiment involving two accession (Tsu-1 and Kas-1), two treatments (control and soil drying), with six biological replicates ($2 \times 2 \times 6 = 24$ arrays) from a single experimental block. As described above, fully expanded rosette leaves were sampled on RNA*later* (Ambion, Inc.) and total RNA was extracted using Qiagen RNAeasy kits. Samples for mRNA profiling studies were processed by Asuragen, Inc. (Austin, TX, USA) according to the company's standard

operating procedures. The integrity of total RNA was qualified by Agilent Bioanalyzer 2100 capillary electrophoresis (Palo Alto Santa Clara, CA, USA) and used for preparation of biotin-labelled targets (cRNA) using a MessageAmp II-based protocol (Ambion Inc.). The cRNA yields were quantified by ultraviolet spectrophotometry and the distribution of transcript sizes was assessed using the Agilent Bioanalyzer 2100 capillary electrophoresis system. Labelled cRNA was fragmented and used for array hybridization and washing, according to the standard Affymetrix protocol. In brief, labelled cRNA was resuspended in 5× fragmentation buffer and incubated at 94 °C for 35 min then stored on ice. The hybridization cocktail and the fragmented cRNA mixture were heated to 99 °C for 5 min, and incubated at 45 °C for 5 min. After a final spin to collect the samples, hybridization to arrays was carried out at 45 °C for 16 h in an Affymetrix Model 640 hybridization oven. Arrays were washed and stained on an Affymetrix FS450 Fluidics station. The arrays were scanned on an Affymetrix GeneChip Scanner 3000. A summary of the image signal data for every gene interrogated on the array was generated using the Affymetrix Statistical Algorithm MAS 5.0 (GCOS v1.3) algorithm.

The cRNA hybridization data from this study are accessible through GEO Series accession number GSE20339 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20339).

Statistical analyses

Physiological studies

Two-way (accession and treatment) factorial analysis of variance (ANOVA) was used for analysis of all individual soil and plant response variables following tests to assure assumptions of normality and homoscedasticity were met. For these univariate analyses, there were generally data for each variable from six subsample plants for each line and treatment in each of the three replicate blocks; subsample values within each block were averaged before analysis. Soil moisture variables and absolute value of Ψ_{sol} were ln transformed to meet ANOVA assumptions. All results presented are least-squares (LS) means, back-transformed as needed. Multivariate analysis of water relations responses including leaf RWC, Ψ_{tot}, Ψ_{sol} and $\delta^{13}C$ were conducted with multivariate analysis of variance to assess response patterns by Tsu-1 and Kas-1 to the treatments. Statistical analyses of physiological data were conducted with JMP7.0 (SAS Institute Inc., Cary, NC, USA).

Comparative genomic hybridizations

The Affymetrix ATH1 array was constructed from the full genome sequence of the Col-0 accession of *Arabidopsis*. Statistical analyses were performed using SAS procedures as called by JMP Genomics or using several procedures implemented in the R statistical package (R Development Core Team 2008). Original CEL files from gDNA hybridizations were imported and processed at the probe level using a Robust Multichip Average (RMA) based background correction, log2 transformation and quantile normalization of raw intensity values (Irizarry *et al*. 2003). We utilized a custom CDF file (ATH1_AT_TAIR.cdf) constructed from the Arabidopsis Information Resource (TAIR) version 7 of the *Arabidopsis* genome (available for download at http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/.asp) (Dai *et al*. 2005). This custom CDF file was created using a series of searches to identify unique probes and filter probes with cross-hybridization to multiple genomic sites. We confirmed sample labelling by performing a hierarchical clustering of intensity data across all arrays. As expected, replicates of each accession consistently clustered.

One-way ANOVA models were subsequently fit for each probe with *t*-test contrasts between either Tsu-1 or Kas-1 and the Col-0 control line using JMP Genomics 3.2. An empirical Bayes approach was used to shrink the residual variance for each probe based on a prior distribution of the variance estimated from all probes using an inverted-gamma distribution. This approach resulted in increased power and sensitivity by improving the stability of the residual variance estimates. We controlled for multiple testing using a positive false discovery rate (pFDR) of 0.05 (Storey 2003).

Gene expression analyses

We completed analyses of gene expression data using filtered RMA expression values (Irizarry *et al*. 2003). CEL files were imported into the R environment using the Affy package (Irizarry, Gautier & Cope 2002) and gene expression measures generated using the RMA function (background corrected, log2 transformed, quantile normalized, median-polished summary) with the custom CDF file described above. However, we filtered the processing CDF file of probes that were identified as having significantly different genomic hybridization intensities when compared to the control line Col-0. Filtering was completed with the R package CustomCDF (http://arrayanalysis.mbni.med.umich.edu/MBNIUM.html#CustomCDF), using a 0.05 pFDR criteria, and a requirement of at least three probes per probeset. The removal of probes containing sequence polymorphisms, including single nucleotide polymorphisms (SNPs) and copy number differences, should result in robust gene expression measures that are minimally impacted by sequence divergence among test accessions. We also completed CEL file processing without probe filtering to assess the impact of hybridization polymorphisms on differentially expressed gene lists.

Expression measures were subsequently imported into JMP Genomics 3.2 and processed using the ANOVA procedure. In this case, we fit a fixed-effect general linear model including a term for 'accession', 'treatment' and their interaction using SAS Proc Mixed and an empirical Bayes shrinkage of the residual variance for each probeset. In this case, the shrinkage analysis was completed using the

method of Ledoit & Wolf (2004) as described by Schafer & Strimmer (2005) with a custom R script. We controlled for multiple testing using a pFDR of 0.05.

Quantitative PCR studies

In addition to our array studies, we used hybridization based quantitative PCR assays (QPCR) of 31 candidate genes (Table 2) picked from prior studies and a screening of the literature (Bray 2004) to further evaluate gene expression responses. This list contains genes that were found to be consistently regulated by abiotic stress in the microarray studies of Seki *et al*. (2002) (ABA treatment), Kreps *et al*. (2002) (mannitol imposed osmotic stress) and Kawaguchi *et al*. (2004) (progressive soil-water deficit) (Bray 2004) with the addition of several transcription factors of interest (CBF4, DREB2a, DREB1a, DREB1b). Our list is diverse and includes genes involved in metabolism, transport, signal transduction, transcription, hydrophilic/heat-soluble proteins as well as several transcripts of 'unknown' function.

As described above, rosette leaf material of replicate plants was harvested on RNA*later* and extracted as described above for each experimental plant (six biological replicates per accession, per treatment, per block: $6 \times 2 \times 2 \times 3 = 72$ plant samples total). We used ProbeLibrary and ABI Taqman assays (genes AT1G01470 and AT2G43570) for our QPCR experiments with ABgene one-step QPCR reagents and the ABI 7900 HT real-time PCR machine. Samples were screened with duplicate technical replicates and subsequent analyses were completed on the replicate averages.

Relative mRNA abundance was determined on the basis of the threshold cycle (C_T) value for each reaction. We utilized three reference genes (ACTIN, AT3G18780; SAND, AT2G28390; TIP41-LIKE, AT4G34270) as endogenous controls to normalize the quantity of input RNA in reactions. The C_T-value for each target gene was subtracted from the geometric mean C_T-value for the control genes to obtain ΔC_T-values, which were used in subsequent statistical analyses. ΔC_T-values were analysed using fixed-factor ANOVA with Proc Mixed in SAS (Littel *et al*. 1996) with block, accession, treatment and accession–treatment interaction effects. We sequenced the probes and primers for each QPCR assay in both Tsu-1 and Kas-1 to evaluate the impact of sequence polymorphisms on expression measures. We found SNPs in assays for three genes (AT1G62570, AT2G40000, AT5G06760), and therefore, the results from these should be taken with caution as polymorphisms in these assays may impact expression estimates.

Annotation analyses

We used several annotation-based analyses to explore the biological underpinnings of the observed gene expression differences. We searched for overrepresented promoter motifs in various gene lists using the web-based software Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) (O'Conner, Dyreson & Wyrick 2005). In addition, we utilized the software package, Mapman (http://gabi.rzpd.de/projects/MapMan) to explore broad physiological patterns in our transcript abundance data. MapMan converts data values to a false colour scale and paints them onto diagrams representing current models of metabolic, physiological and regulatory pathways (Thimm *et al*. 2004). We used differences in LS mean RMA values as data input [log2(fold-change values)]. Specifically, we used contrasts between Kas and Tsu (mean Kas-1 minus mean Tsu-1) to evaluate constitutive differences, and contrasts between accession specific responses to the treatment (mean Kas-1 in dry condition minus mean Kas-1 in wet condition) to explore plastic or inducible responses. Functional categories of the MapMan annotation (Thimm *et al*. 2004; Usadel *et al*. 2005) were tested for significance of expression change by applying a two-sided Wilcoxon rank tests with a Benjamin–Hochberg correction for multiple tests. In this test, the log2 ratios for all genes in a particular MapMan annotation BIN were compared to the ratios of all remaining genes on the chip – a significant value indicates an altered expression of a particular BIN relative to all BINs represented in the MapMan annotation. We visually explored enriched pathways as well as completed a heuristic evaluation of central pathways (e.g. metabolism_overview, cellular_responses, regulation, transcription). We used the ATH_AFFY_TAIR7 mapping.

RESULTS

Physiological responses

Even though soil gravimetric WC was reduced by more than 50% over the 5 days of the drydown [WatRem% at harvest = 46.1 ± 0.7% dry, 87.1 ± 0.7% wet; LS means ± 1 standard error (SE); $n = 6$; $P < 0.001$], Ψ_{soil} was just slightly lower in the dry than the wet treatments (–0.14 ± 0.07 MPa dry; –0.04 ± 0.003 MPa wet; LS means ± 1 SE; $n = 6$; $P = 0.058$).

Constitutive differences (all $P \leq 0.002$) between Tsu-1 and Kas-1, but no treatment effects, were evident in slightly advanced bolt production at the end of the drydown by Tsu-1, higher leaf N concentration in Kas-1 and lower leaf C : N ratio in Tsu-1 (Table 1). In addition, as expected based on previous results (see McKay *et al*. 2003, 2008), Kas-1 had constitutively less negative $\delta^{13}C$ in leaves (correlated with higher water-use efficiency) than Tsu-1 ($P < 0.001$), but there was no significant effect of the very mild soil-drying treatment ($P = 0.083$) on this parameter in the univariate analyses (Fig. 1).

None of the leaf water potential measurements (Ψ_{tot}, Ψ_{sol} and Ψ_p) were significantly different by line, treatment or the interaction in the univariate analyses (all $P > 0.132$; Fig. 1). Among all the water relations parameters, only leaf RWC of Tsu-1 and Kas-1 responded differently to the treatment (interaction $P = 0.042$; Fig. 1). Despite the few significant differences suggested by these univariate analyses, the multivariate pattern of response by accessions and to the treatments appeared to be different and this was confirmed with

Table 1. Growth and leaf composition parameters [least-square (LS) means and 1 standard error (SE); $n = 3$ each cell] for Tsu-1 and Kas-1 at harvest in control (wet) and soil moisture deficit (dry) treatments

Accession	Treatment	Fresh weight bolt (g)	Fresh weight rosette (g)	Fresh weight leaf (g)	Dry weight rosette (g)	Dry weight leaf (g)	Leaf WC (%)	Leaf N (%)	Leaf C : N ratio
Kas	**Dry** LS mean	0.001**a**	0.272	0.232	0.034	0.029	807	3.4**a**	10.9**a**
	SE	0.001	0.019	0.021	0.000	0.001	56	0.1	0.4
	Wet LS mean	0.002**a**	0.241	0.206	0.028	0.024	881	3.6**a**	10.5**a**
	SE	0.002	0.035	0.025	0.005	0.004	64	0.4	1.0
Tsu	**Dry** LS mean	0.028**b**	0.267	0.214	0.033	0.027	805	2.6**b**	14.5**b**
	SE	0.005	0.021	0.019	0.002	0.002	24	0.1	0.8
	Wet LS mean	0.022**b**	0.203	0.166	0.027	0.022	797	2.5**b**	15.0**b**
	SE	0.004	0.024	0.013	0.006	0.004	147	0.2	0.9
***P* values from ANOVA**									
Accession; *df 1,8*		**<0.001**	0.421	0.186	0.858	0.523	0.630	**0.002**	**0.001**
Treatment; *df 1,8*		0.446	0.102	0.100	0.176	0.146	0.710	0.778	0.923
Accession × Treatment; *df 1,8*		0.369	0.538	0.597	0.972	0.958	0.650	0.533	0.626

ANOVA results are also given, with significant differences in bold and differences between means indicated by different letters.

multivariate analysis that included four key traits: $\delta^{13}C$, RWC, Ψ_{tot} and Ψ_{sol} (Fig. 1). Leaf WC was not included because of correlation with RWC and Ψ_p was not included because it was calculated from Ψ_{tot} and Ψ_{sol}. Results of the multivariate analysis indicated a significant difference in the pattern of response of Tsu-1 and Kas-1 to the mild drying treatment ($P = 0.036$, accession–treatment interaction). Tsu had consistently higher RWC (also true for WC; see Table 1) and Ψ_{tot} in the dry than in the wet treatment, whereas Kas had the opposite pattern. This suggests that stomata of Tsu-1 were more closed in the dry than the wet treatment and this is consistent with the shift to less negative $\delta^{13}C$-values by Tsu-1 in the dry treatment, while there was no change in $\delta^{13}C$ in Kas-1. These three parameters (RWC, Ψ_{tot} and $\delta^{13}C$) are completely different kinds of measurements on different leaves. Overall, these data suggest that Tsu-1 was beginning to close stomata more than Kas-1 in response to the very mild soil-drying treatment.

The very mild soil moisture stress imposed in the dry treatment had no detectable effect on plant growth and some of the leaf composition parameters. There were no significant differences between Tsu-1 and Kas-1 or by treatment in rosette fresh weight, total leaf fresh weight, rosette dry weight, total leaf dry weight, leaf WC (Table 1) or leaf C content (data not shown). This slow and mild soil drying should allow the detection of early signalling components in stress sensing prior to major expression and physiological changes in response to severe stress.

Genomic hybridizations

We utilized the Affymetrix ATH1 array to explore the genomic characteristics of two physiologically extreme accessions of *Arabidopsis*. A total of 18 arrays were hybridized with labelled gDNA, intensities for each of the perfect match (PM) probes were obtained from the CEL files and these data analysed using a *t*-test contrast with the Col-0 reference accession used to design the microarray. Overall, 42 503 single feature polymorphisms (SFP) (Borevitz *et al*. 2003) were identified at less than a 5% false discovery rate (pFDR) (Supporting Information Table S1, Fig. 2). These polymorphisms were found in 16 508 Affymetrix probesets. Of these probesets, 10 681 contained more than one significant probe, with a mean of 3.43 (±1.87 standard deviation) polymorphic probes per probeset. Of these significant probes, 11 290 (29%) were identified from both Tsu-1 and Kas-1 contrasts, while the remaining probes (Kas, 19 011; Tsu, 12 202) were unique to particular accession contrasts with Col-0.

The magnitude and sign of the difference in mean intensity between the accessions and Col-0 (e.g. Kas minus Col) indicated possible patterns of divergence in relation to the Col-0 reference genome. Positive values indicated larger intensities for either Tsu-1 or Kas-1 compared to Col-0 and likely indicate copy number differences resulting from duplicated loci of unknown genomic position (Borevitz *et al*. 2007). Negative values indicated lower intensities for either accession compared to Col-0 and are most likely the result of hybridization differences generated from sequence polymorphisms. Thus, probes with negative values are a potential confounding factor in estimation of transcript abundance and were removed as explained below. Of the identified SFP, only 48% of the Kas-1 identified and 41% of the Tsu-1 identified probes were the result of lower intensity values in the Col-0 reference, suggesting considerable copy number evolution.

We used gDNA hybridization data to filter our expression studies with a requirement of at least three 'good' probes per probeset (gene) for the calculation of gene expression. As a result of this criterion, we removed 512 genes from the array (see Supporting Information Table S2). These genes likely contain many SNPs or differ in copy number between the study accessions and Col-0. We manually inspected the pattern of probe signal and statistical significance to make preliminary predictions of gene loss (consistent significant negative signal for all probes), gain (consistent significant positive signal for all probes) or more complex patterns (likely caused by SNPs or complex

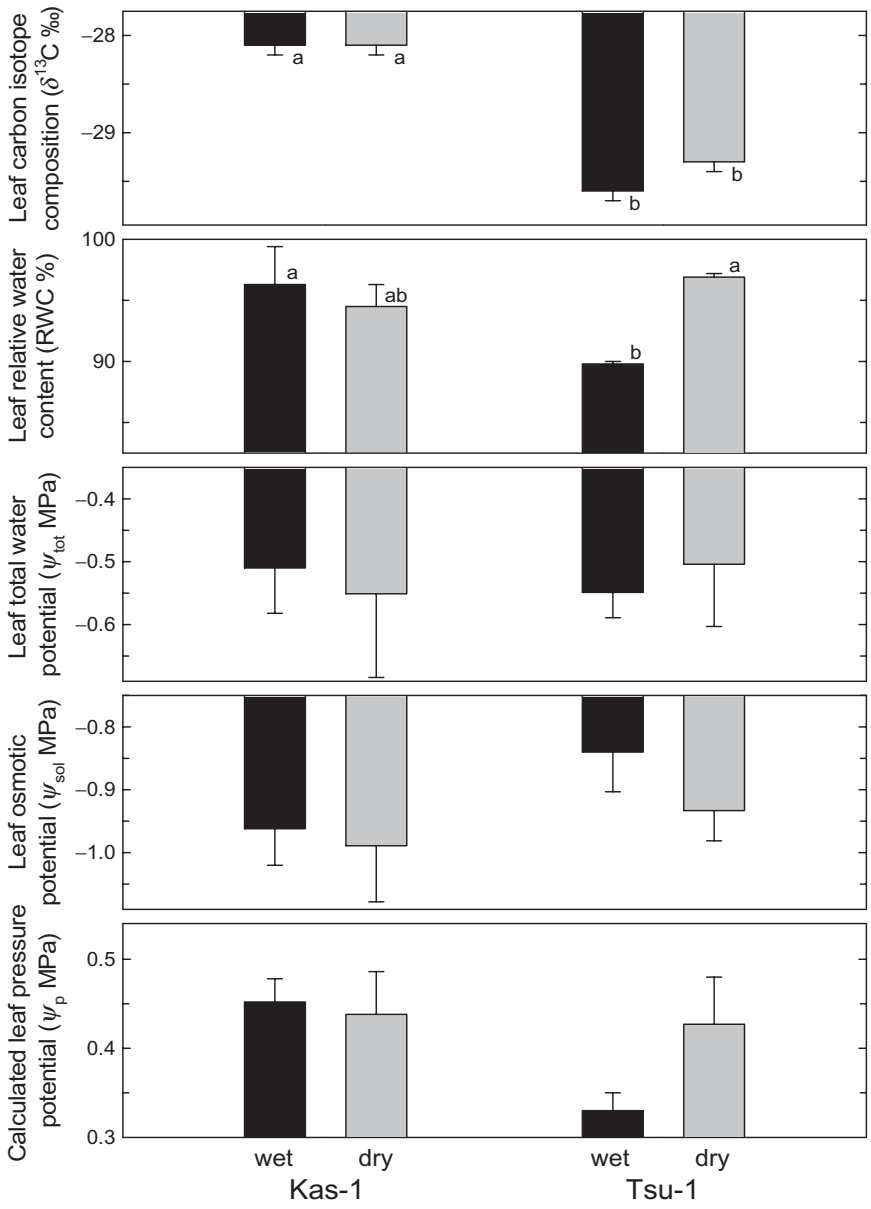

Figure 1. Water relations parameters for Tsu-1 and Kas-1 at harvest in control (wet) and soil moisture deficit (dry) treatments (LS means ± 1 SE; $n = 3$ each bar). Significant differences among means, from univariate ANOVA, are indicated by different letters (see text for P values). MANOVA with δ^{13}C, RWC, Ψ_{tot}, and Ψ_{sol} indicated a significant difference ($P = 0.036$) in the pattern of response of Tsu-1 and Kas-1 to the treatments.

indels). Overall, we find evidence for 60 Tsu-1-specific losses, 59 Kas-1-specific losses and 88 joint losses in both Tsu-1 and Kas-1 relative to Col-0. In contrast, we infer 16 Tsu-1-specific gains, 25 Kas-1-specific gains and only five joint gains relative to Col-0. A number of interesting genes appear on this list, include physiological candidates such as ABA INSENSITIVE 2 (ABI2, AT5G57500; involved in ABA signal transduction, possible gain in Tsu-1), a drought stress-induced histone (e.g. HIS4, AT2G28740, possible Kas-1 loss), EARLY-RESPONSIVE TO DEHYDRATION 9 (ERD9, AT1G10370, possible Kas-1 gain), a metabolic gene affecting plant water status (DEDOL-PP synthase, AT2G23400, wilty mutant, Kas-1 loss), an osmotically responsive RNA-binding protein (ATGRP2, AT4G13850, possible Kas-1 loss) as well as a large number of unknown or unannotated genes. These genes are good candidates for some of the constitutive and induced physiological differences observed at the whole-plant level among Tsu-1, Kas-1 and Col-0 accessions.

Differentially expressed genes

We explored constitutive and water deficit-induced patterns of gene expression using a factorial manipulation of the soil-drying treatment and Tsu-1 and Kas-1 plant material. At a false discovery rate of 5%, we detected 5996 (~29% of the genes measured) genes that were constitutively differentially expressed between Tsu-1 and Kas-1, 388 genes with expression altered by the soil-water deficit treatment and 191 genes that exhibited a significant accession by soil-drying treatment interaction (Supporting Information Table S3, worksheets 1–3). Of the 388 soil-water-deficit-regulated genes, 172 were also identified as constitutively

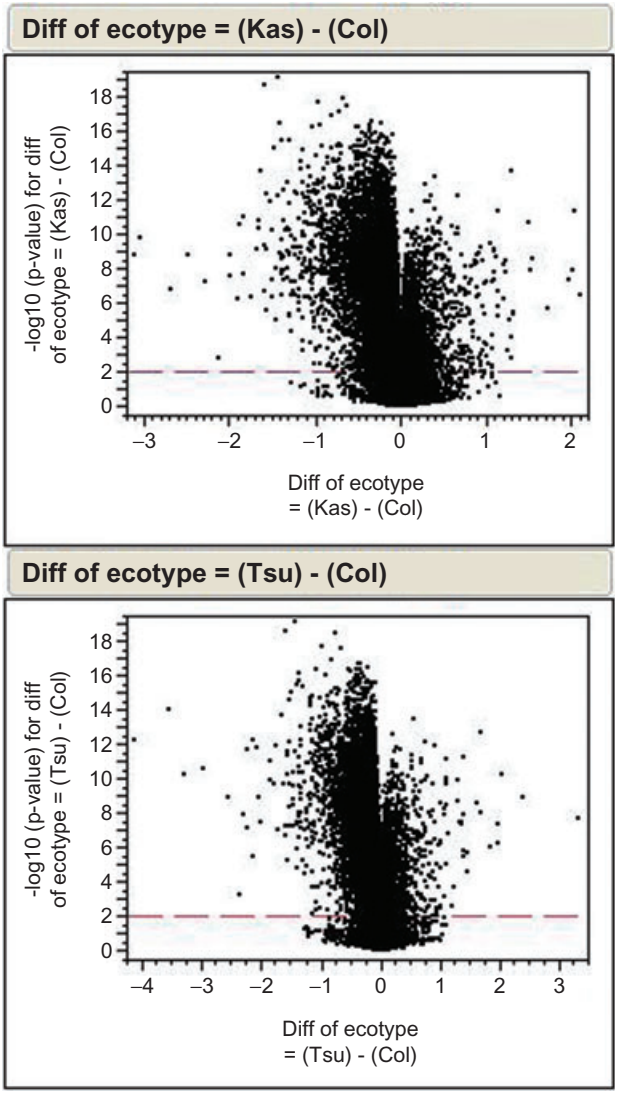

Figure 2. Volcano plots depicting the identification of SFP by gDNA hybridization at a pFDR level of 0.05 for Tsu-1 and Kas-1 contrasts with Col-0.

differentially expressed between Kas-1 and Tsu-1 (Fig. 3). A representative sample of these expression differences is presented in Figs 4 and 5.

A major result of our study is the detection of a large number of constitutive differences in gene expression between accessions. Of these genes, roughly an equal number of genes were more highly expressed in either accession: 49% (2924 genes) were more highly expressed in Tsu-1, and 51% (3072 genes) were more highly expressed in Kas-1. In general, the distribution of differential expression was skewed with a long tail associated with genes that were vastly differentially expressed. The mean expression difference was similar (approx. 1.4-fold difference) regardless of whether the more highly expressed accession was Tsu-1 or Kas-1. However, the distribution of expression differences was more skewed for genes with higher expression in Kas-1 (e.g. a maximum of a 44-fold increase versus a maximum 13-fold increase in Tsu-1). This differentially expressed gene list is large and diverse. With respect to physiological roles of genes, some interesting candidates include abiotic response transcription factors of the AP2-EREBP/DREB family (e.g. DREB1A, DREB2A, DREB2B, CBF2, CBF4), a number of genes involved in two-component response signalling (e.g. AHP1-5, APRR, ATHK1), many genes annotated as 'early response to dehydration' (e.g. RD2, RD19, RD22, ERD1, ERD3, ERD4, ERD5, ERD7, LEA14), many potassium transporters (e.g. KAT1, KAT2, KEA1, KEA6, KUP3, KUP6, KUP10, KUP11), water channels (PIP1, PIP1A, PIP2, PIP2AP), several genes involved in ABA biosynthesis or signalling (ABA1, ABF1, ABIL1, ABIL3, ABO1, NCED2-5, CYP7072A), genes involved in guard cell regulation (GPA1, ATRBOHF, OST1), and many histones, histone deacetylases and chromatin-related genes (e.g. HD1, HD2a, HD2b, HDA05, HDA9, HDT4, HAC1, MSL1). In addition, there were a number of significantly differentially expressed flowering time genes (e.g. *FLC*, *CO*, *GI*, *FD*, *FY*, *CRY2*) and many members of the MYB and ATHB families of transcription factors. For convenience, we included a list of candidate genes identified in Supporting Information Table S4 – these genes were largely identified from background knowledge and interest in our research group, literature searches or TAIR annotation. We acknowledge that this is a limited screening and emphasize that 18% (1055 genes) of the constitutively expressed differences are unannotated. Clearly, a major challenge is exploring and synthesizing the biological significance of these consistent expression differences between accessions.

Genes that were significantly affected by the soil drying were both induced (35%, 135 genes) and repressed (65%, 253 genes). Drought-induced genes had an average fold-increase of 1.4 (maximum fold-increase 8.2), while drought-repressed genes had an average fold-reduction of 0.82

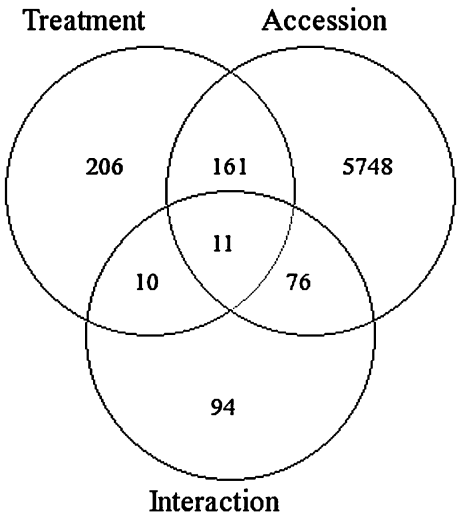

Figure 3. Venn diagram representing the overlap of gene lists for various experimental factors (Accession, Treatment, Accession × Treatment Interaction) identified from factorial ANOVA.

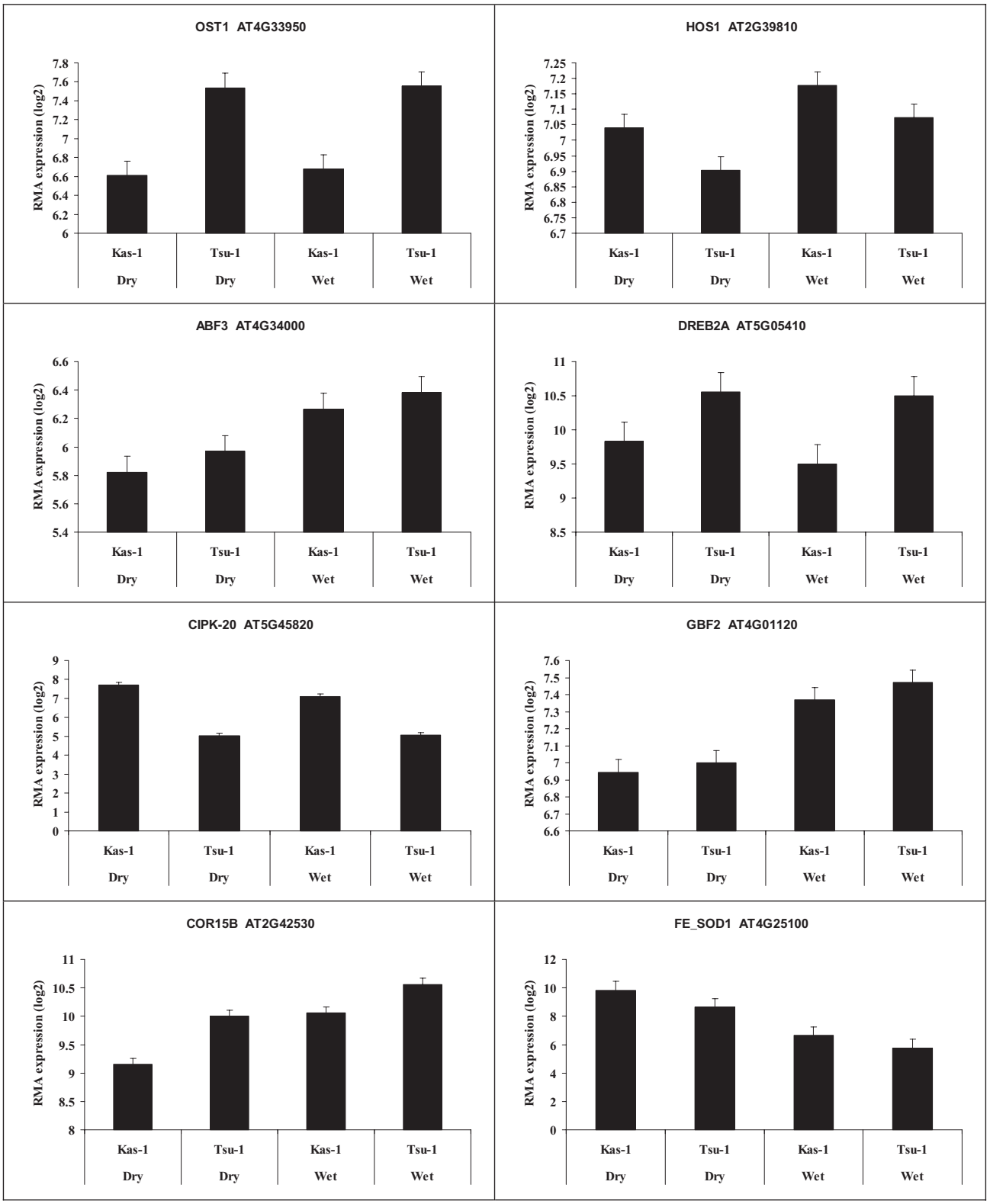

Figure 4. Bar plots displaying constitutive or soil moisture deficit-regulated expression differences for eight representative genes detected through ANOVA. Mean expression measures are presented on the RMA scale (log2) with ± 1 SE. In this case, a 1 unit difference corresponds to a twofold change in expression.

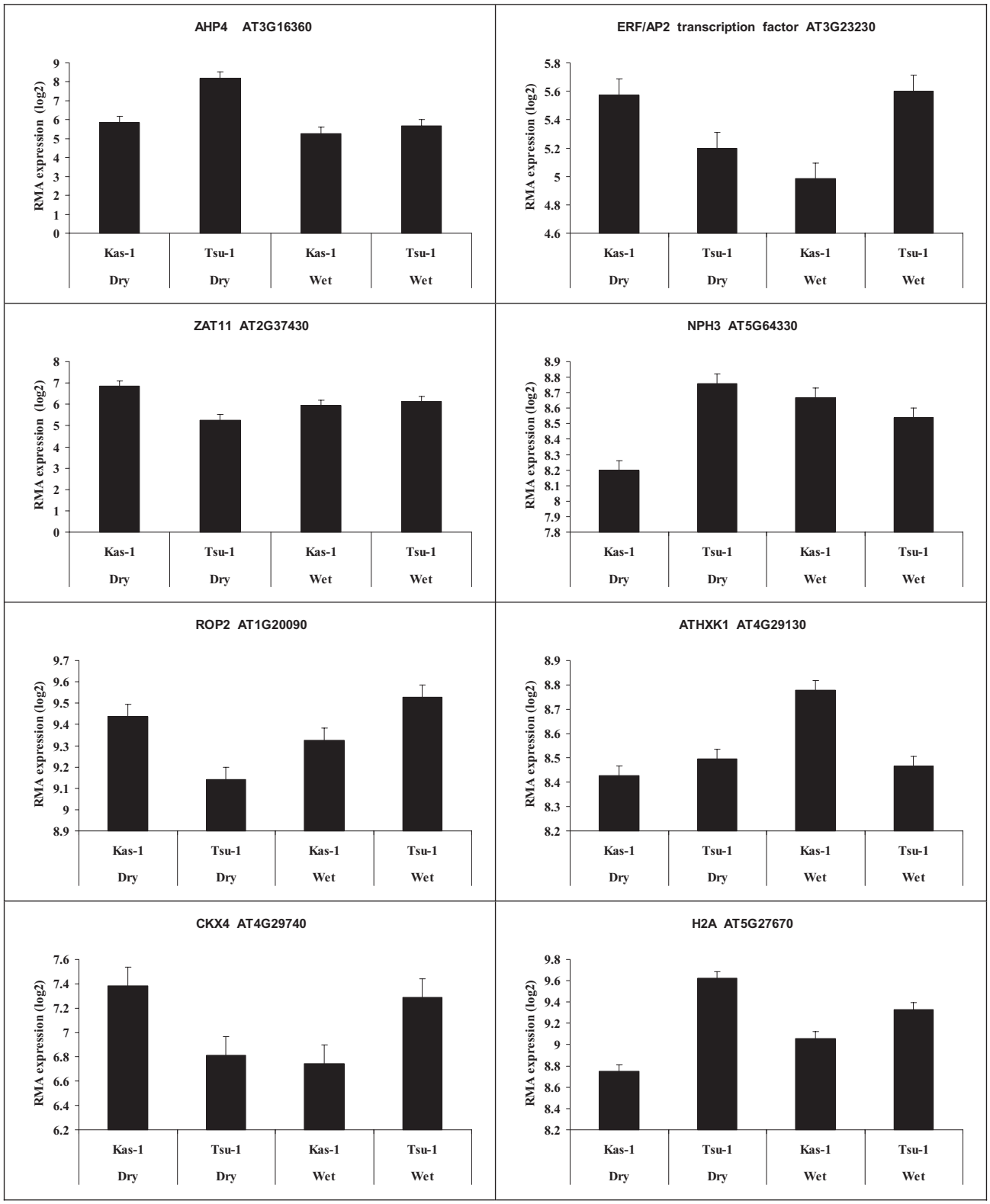

Figure 5. Bar plots displaying accession specific responses to the soil moisture deficit treatment (dry) compared to control (wet) for eight representative genes detected through ANOVA. Mean expression measures are presented on the RMA scale (log2) with ±1 SE. In this case, a 1 unit difference corresponds to a twofold change in expression.

(maximum fold-reduction 0.41). We point out that these gene expression changes are relatively small in magnitude, likely reflecting the relatively minor soil drying of the treatment, but were nevertheless highly significant as a result of our well-replicated experimental design. Drought-induced genes included several abiotic stress-related transcription factors such *DREB1A* (AT4G25480), *CBF2/DREB1C* (AT4G25470), *STH* (AT2G31380), as well as several genes involved in ABA biosynthesis including UGT73B1 (AT4G34138) and CYP707A2 (AT2G29090). Drought-repressed genes included those involved in ABA signalling such as ABF3 (AT4G34000) and GPA1 (AT2G26300), a number of genes annotated as cold- or dehydration-regulated (AT2G42530, AT2G15970, AT2G43200), and HOS1 (high expression of osmotically regulated genes) (AT2G39810). Nineteen percent (73 genes) of the drought-regulated genes were unannotated.

The interpretation and study of interactions in factorial ANOVA is determined by the scale of the data (e.g. interactions on the raw scale are not the same as interactions on the log transformed scale) (Fitzmaurice 2000). On the original scale, treatment effects and interactions are expressed in terms of differences or absolute reductions, while on the logarithmic scale, they are expressed in terms of proportional reductions. Our primary analyses were completed on the RMA (log2) scale and 191 genes meet our significance threshold for accession-by-treatment interactions in this model. However, we detected an additional 161 genes that exhibited both significant accession and treatment effects on the log2 scale but lacked a significant interaction term. The occurrence of both main effects suggests an additive relationship on the log2 scale, but an interaction (multiplicative relationship) on the raw scale. Considering both classes of interaction, we detected 352 genes with accession specific responses to soil drying. Genes exhibiting accession-specific responses to the soil drying were diverse in their pattern, including genes with responses restricted to one accession as well as responses of differing direction or magnitude. A small number of the interaction effects are likely the result of differential variability (heterogeneous variances) in expression depending on the treatment. For genes with significant interactions on the log2 scale, Tsu-1 and Kas-1 had very similar response distributions with a slight skew to high drought induction. Interestingly, the fold-change response to soil drying of the two accessions was significantly negatively correlated (Spearman's $r = -0.61$, $P = 0.0001$): there was a tendency for Tsu-1-induced genes to be repressed in Kas-1 and vice versa. The interaction gene list contained a number of intriguing physiological candidates including, for example, the stress-responsive transcription factor ZAT11 (AT2G37430; drought repressed in Kas-1, drought-induced in Tsu-1), a two-component signal transducer AHP4 (histidine-containing phosphotransmitter) (AT3G16360; drought-induced in Tsu-1, but not in Kas-1), CYP707A2 (involved in ABA catabolism) (AT2G29090; differentially repressed by drought), AFP4 (a negative regulator of ABA responses) (AT3G02140; drought-induced in Kas-1, drought-repressed in Tsu-1) and PYL4 and PYL5 (putative ABA receptors) (AT23G38310, AT5G05440; drought induced in Kas-1, drought repressed in Tsu-1). Eighteen percent (63 genes) of the differentially responding genes are unannotated.

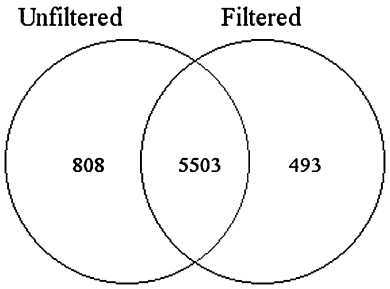

Figure 6. Venn diagram representing the overlap of constitutively differentially expressed genes identified using 'Unfiltered' and 'Filtered' expression measures.

We explored the impact of sequence divergence on gene expression estimates using two simple approaches. Firstly, we completed ANOVA analyses using data that were filtered for hybridization polymorphisms (probes with different hybridization intensity contrasts with the reference Col-0 were removed) or not. In this case, we found that the constitutive gene list generated from the filtered expression data was largely a subset of the unfiltered list (Fig. 6). The unfiltered analysis identified 5503 genes that overlapped with the filtered analysis, but there were an additional 1301 genes that were identified in only one or the other analyses. Genes identified solely in the unfiltered analyses were likely caused by substantial hybridization differences between Kas-1 and Tsu-1 resulting in differing signals in cRNA hybridizations. In contrast, genes identified solely in the filtered analyses were likely missed in the unfiltered analyses because of SNPs that caused heterogeneity in probes resulting in poor estimates of gene expression. Importantly, our filtering based on lost probes may not have controlled for certain types of sequence divergence affecting expression – for example, single (or few) indels resulting in loss of protein due to early stops in transcripts likely underlie some of the constitutive expression difference observed (Plantegenet *et al.* 2009). Assuming the 5503 genes identified from both analyses are 'true' expression differences, unfiltered analyses can lead to a 9% false-positive and 15% false-negative rate for the identification of expression level polymorphisms.

We emphasize that both expression and sequence divergence are of interest and are likely important in understanding the adaptive evolution of *Arabidopsis* physiology. Nevertheless, we consider that it is important to differentiate between the underlying mechanisms using robust analyses when possible.

To complement the whole genome expression array data, we completed QPCR for 31 candidate genes in our soil-drying experiment. We found that 75% of these study genes exhibited strong constitutive differences in gene expression

between Tsu-1 and Kas-1 (Supporting Information Fig. S1). In contrast, only 13% (four genes) exhibited expression responses to soil drying and only one gene (AT1G05340) exhibited accession-specific responses to the treatment on the log2 scale (three genes likely exhibit accession-specific responses on the raw scale based on main effects). In the case of response to soil drying, all of the identified genes responded in the direction predicted from the literature. In the case of constitutive expression, differences were varied but in some cases amounted to many fold differences in expression between Tsu-1 and Kas-1. Our QPCR results are in strong agreement with our array studies. In 60% of the assays, QPCR and array results were identical. In 30% of the assays, QPCR identified additional significant effects, primarily accession differences, suggesting false-negatives on the array due to differences in sample size or measurement accuracy. In 10% of the assays, significant effects were identified from the array studies that were not discovered in subsequent QPCR. These 'false discoveries' may represent true errors under our FDR threshold of 5%, or genetic- and treatment-induced variation may be masked by the large block effects identified in our QPCR studies.

Annotation analyses

We explored the lists of drought-regulated genes using a series of annotation analyses. One approach focused on exploring the 1500 base pairs upstream of each gene for known promoter-binding elements and on asking whether the frequency of occurrence of these motifs is greater than expected by chance. For promoter enrichment analyses, we used the web-based data analysis features of Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl). For the 537 drought-regulated genes (identified in both 'treatment' and/or 'treatment × accession interactions'), Athena identified five enriched motifs including an ABRE-like binding element (consensus sequence, BACGTGKM) ($P < 10^{-10}$, 183 promoters), the ABRE-like response element found in *Arabidopsis* drought-responsive gene RD22 (consensus sequence, RYACGTGGYR) ($P < 10^{-10}$, 31 promoters), the binding site of GBF4 (CACGTG) ($P < 10^{-10}$, 150 promoters), the experimentally determined core of the ABRE-motif (consensus, ACGTGKC) ($P < 10^{-7}$, 131 promoters) and a sequence associated with the GA-down regulated cluster [consensus, ACGTGTC (similar to the ABRE)] ($P < 10^{-5}$, 82 promoters). These results suggest an important role of ABA signalling, and ABRE-binding protein transcription factors, in early responses to soil drying before growth changes and many physiological responses could be detected. Athena detected no significant enrichment of GO annotation terms for this list of drought-regulated genes.

We utilized Mapman annotation (Thimm *et al*. 2004) to further explore constitutive and drought-induced changes in regulation, physiology and metabolism associated with Kas-1 and Tsu-1 accessions. We present *P*-values for bin enrichment tests in Supporting Information Table S5 and discuss significant patterns observed at the corrected *P*-value threshold of 0.01. These analyses identified a number of striking constitutive differences between Tsu-1 and Kas-1 (Supporting Information Fig. S2). These differences include, to name but a few, changes in the expression of genes involved in protein dynamics (including changes in synthesis, degradation, post-translational modification, activation, folding and targeting bins; generally all up-regulated in Kas-1 relative to Tsu-1) as well as genes involved in transcriptional regulation, transport, hormone metabolism (especially auxin), light signalling, development, and DNA synthesis and chromatin structure (generally up-regulated in Kas-1 relative to Tsu-1). With regard to regulation, MYB-related, bHLH, Homeobox, Aux/IAA and Pseudo ARR transcription factor families were identified as significantly enriched for differences between Kas-1 and Tsu-1 (generally up-regulated in Tsu relative to Kas). The bins that are up-regulated in Kas relative to Tsu generally represent growth (cell wall, mRNA, amino acid and lipid synthesis, and cell repair of proteins, DNA and oxidatively damaged components). The bins that are up-regulated in Tsu relative to Kas represent osmotic changes, protein damage and gene expression that indicate transitions in development state such as hormones, signalling and development. Again, these results suggest fundamental divergence in the standing physiology and metabolism of these accessions.

In addition, we identified a number of accession-specific responses to soil drying. We present plots of the accession-specific responses in Supporting Informations Figs S3–5. In the case of Kas-1, we identified an enrichment of gene expression responses related to protein dynamics (including synthesis, targeting, degradation and glycosylation, generally drought repression), cell-related processes (including cell organization and vesicle transport), photosynthesis (especially strong drought induction of light reactions and photosystem II), hormone metabolism (especially drought-induced ethylene metabolism and signal transduction), regulation involving several transcription factor families [HAP2 (drought repressed), ARF (drought repressed), PHOR1 (drought induced)] and signalling through receptor kinases (including a mixture of drought repression and induction). In the case of Tsu-1, we identified enrichment of photosynthesis-related gene expression (especially up-regulation of light reactions), signalling by receptor kinases (especially drought repression of gene expression), redox regulation (especially drought-induced regulation of thioredoxin, ascorbate and glutathione), amino acid metabolism, stress-related genes (especially biotic) and regulation via DOF zinc finger transcription factors (drought repressed).

DISCUSSION

Water status is a central component in plant physiological and metabolic processes including cellular osmoregulation, transpiration and water/nutrient translocation, photosynthetic carbon fixation and general growth and development. Moreover, poor water status resulting from drought is one of the most important stresses affecting agricultural yields

and has immense influence on the distribution and productivity of plants globally and locally (Boyer 1982; Gleick 1998). As such, a better understanding of how plants respond to soil drying and the mechanisms of adaptation and acclimation that allow the maintenance of good water status are critical for our better understanding of plant function. Here, we examine the interaction of soil drying with two 'climatically' extreme accessions of *A. thaliana*, focusing on physiological and transcriptome responses.

Our experimental protocol centred on slow soil drying, occurring across a 5 day period, and a very mild final level of 'stress'. This approach was motivated by a specific interest in the acclimation responses of accessions as well as the expected importance of early signalling components in stress sensing. As expected from our mild soil drying, univariate analyses of a set of growth and leaf-level physiological measurements found few statistically significant soil-drying effects and no striking patterns among Tsu-1 and Kas-1. Nevertheless, multivariate analyses revealed consistent treatment–accession interaction ($P < 0.05$) when jointly considering leaf-level responses. The overall pattern suggests that Tsu-1 (collected from a region with low VPD and putatively 'low' drought stress) was more responsive to the soil drying than Kas-1 (collected from a region with high VPD and putatively 'high' levels of drought stress) (Christman *et al*. 2008; McKay *et al*. 2008). One possible interpretation is that Kas-1 is simply constitutively better adapted to soil drying, and from the perspective of Kas-1, the treatment was not yet a stressful environment requiring physiological responses. Moreover, Kas-1 generally maintains a more conservative physiology in the context of water use (McKay *et al*. 2003, 2008), and as such may not require physiological adjustments to mild stress levels.

Despite detecting only subtle physiological changes, we observed considerable regulation of gene expression as a result of soil drying. These gene expression changes included both up and down regulation, but on average, relatively small fold-changes. The list of genes showing soil-drying responses is diverse and involves a number of intriguing molecular pathways, including a number of transcription factor and ABA biosynthesis genes that may be important in early signalling (see Supporting Information Table S3). These genes may provide new insight into the early 'generalized' response of *Arabidopsis* to soil drying. In addition, several hundred genes were identified through their accession specific response – for example, two genes identified as putative ABA receptors (PYL4/PLY5) (Ma *et al*. 2009, Park *et al*. 2009) show differential responses with drought induction in Kas-1 and drought repression in Tsu-1. In addition, two genes involved in cytokinin biosynthesis (CKX4) and cytokinin signalling (AHP4) show contrasting responses in Tsu-1 and Kas-1 (Fig. 5) along with constitutive differences in expression of a cytokinin signalling histidine kinase (ATHK1). Recent studies have implicated new roles for cytokinin signalling in responses to abiotic stresses (Argueso, Ferreira & Keiber 2009), possibly upstream of many traditional stress-responsive transcription factors (e.g. AREB1, DREB2A, NAC) (Tran *et al*. 2007). Moreover, overexpression of the first committed step to cytokinin production in tobacco has resulted in remarkable drought tolerance (Rivero *et al*. 2007), possibly through cytokinin effects on leaf senescence. These results suggest the possible importance of natural variation in cytokinin signalling or ABA reception in physiological responses of accessions to soil drying.

One surprising outcome of our study is the discovery of a large number of genes exhibiting putative constitutive expression differences between accessions identified in both array and QPCR studies. These genes include loci from virtually every possible functional annotation, including many implicated in general growth, development and flowering time as well as a large number of interesting physiological candidates. There are several possible explanations for this observation including extensive sequence divergence and hybridization polymorphism, the sampling of heterogeneous tissues and cell types (e.g. whole rosette leaf tissue with epidermis, guard cells, mesophyll cells, and vasculature) and general divergence in developmental phenologies. We do not believe that the bulk of these expression differences are caused by hybridization polymorphism, despite the fact that our study identified a large number of SFP through genomic hybridization comparisons. These expression patterns are robust as they appear to be largely insensitive to filtering of probes with differential DNA hybridization, are confirmed in independent QPCR assays (Table 2), and many have been observed in subsequent array studies completed in our lab (T. Juenger, unpublished data). The pattern is more likely the result of evolved shifts in development, physiology and phenology. Although striking, the detection of considerable genetic variation in expression observed here is similar to that detected in a number of studies exploring expression divergences in other model systems (Morley *et al*. 2004; Brem & Kruglyak 2005; Li *et al*. 2006; Ayroles *et al*. 2009) and has been previously observed in studies of natural expression divergence in *Arabidopsis* (Kliebenstein *et al*. 2006; van Leeuwen *et al*. 2007; Zhang *et al*. 2008). A major challenge will be disentangling the impact of protein divergence and constitutive expression differences as causally related to the interesting phenotypes observed among accessions. One promising avenue in this regard is the linking of genetic mapping with genome-wide studies of gene expression and physiological phenotypes (Jansen & Nap 2001). This approach is potentially very powerful as it centres on the identification of *cis*-regulators and *trans*-regulators of expression as well as facilitates the linking of expression, metabolomic and phenotypic data through the identification of shared regulators.

One additional avenue for elucidating mechanism, especially based on broad genomic scale data, is the use of annotation level analyses. In our case, we used both searches for enrichment of promoter motifs and functional binning of genes identified from our microarray analyses. In each case, a number of suggestive patterns emerge. In the case of promoter enrichment, we generally observed an overrepresentation of genes containing abscisic

Table 2. Results of QPCR studies of candidate stress genes in soil-drying experiments including response predicted *a priori* from literature, *P*-values from factorial ANOVA, and correspondence with results form our array studies

Gene	Name	Response	Block	Accession (A)	Treatment (T)	A × T	Array pattern
Metabolism							
AT2G38400	AGA	↑	0.3627	<0.0001 (T > K)	0.0003 (D > W)	0.9344	Confirmed
AT1G09500	Cinnainl-alcohol dehvdrogenase	↑	<0.0001	<0.0001 (T > K)	0.0962	0.3693	Treatment on array
AT1G154100	ALDH7B4	↑	<0.0001	<0.0001 (T > K)	0.0121 (D > W)	0.9766	No treatment effect
AT1G62570[a]	FMO GS-OX4	↑	<0.0001	<0.0001 (T > K)	0.5871	0.9657	Confirmed
AT2G43570[b]	Chitinase	↑	<0.0001	<0.0001 (K > T)	0.5488	0.8007	No accession effect
Transporters							
AT2G41190	Amino acid transporter protein	↑	0.0034	0.9215	0.3154	0.5888	Confirmed
AT2G22500	PUMP-5	↑	0.9200	<0.0001 (T > K)	0.2478	0.5746	Confirmed
Signal transduction							
ATIG49450	Transducin protein *I* WD-40 repeat	↑	0.0908	<0.0001 (T > K)	0.5453	0.3745	Confirmed
AT3G11410	ATPP2CA	↑	0.9609	<0.0001 (T > K)	0.1580	0.8885	No accession effect
AT4026080	ABI1	↑	0.9253	0.0014 (T > K)	0.1454	0.3935	No accession dicer
Transcription							
AT1G27200	C3HC4-type RING finger	↑	0.0402	<0.0001 (T > K)	0.4391	0.6074	Confirmed
AT2G46680	ATHB-7	↑	0.5457	0.5662	0.6846	0.1279	Confirmed
AT3G61890	ATHB-12	↑	0.8221	<0.0001 (T > K)	0.9425	0.7731	Confirmed
AT4G27410	RD26	↑	0.0006	0.0004 (T > K)	0.1103	0.9307	No accession effect
AT2G18050	HIS1-3	↑	0.0008	0.406	0.3133	0.4889	Accession effect
AT5G51990	CBF4	varied	<0.0001	<0.0001 (T > K)	0.7761	0.2666	Confirmed
AT4G25480	DREB1a	↑	0.0031	<0.0001 (T > K)	0.1018	0.2856	Treatment on array
AT4G25490	DREB1b	varied	0.0201	0.0026 (T > K)	0.0044 (W > D)	0.6193	No accession effect
AT5G05410	DREB2u	↑	0.0072	<0.0001 (T > K)	0.859	0.4935	Confirmed
Hvdrophilic/heat soluble							
AT1G01470[b]	LEA14	↑	0.063	<0.0001 (K > T)	0.4726	0.7321	Confirmed
AT5G06760[a]	LEA group1	↑	0.0132	0.0072 (K > T)	0.7871	0.2011	No accession effect
AT5G66400	RAB18	↑	0.0035	<0.0001 (T > K)	0.5290	0.4154	Confirmed
AT5G52310	COR78	↑	0.1091	<0.0001 (T > K)	0.1203	0.2676	Confirmed
AT1G20440	COR47	↑	0.016	<0.0001 (K > T)	0.7020	0.8796	Confirmed
Unknown function							
AT1G05340	Unknown protein	↑	0.0001	0.6107	0.1850	0.005	No interaction
AT2G17840	ERD7	↑	0.0100	<0.0001 (T > K)	0.5777	0.2038	Confirmed
AT2G47770	Benzodiazepine receptor-related	↑	0.5127	0.1614	0.1861	0.9816	Confirmed
AT2G40000[a]	ATHSPRO2	↑	0.1174	<0.0001 (K > T)	0.9733	0.5761	No accession effect
Cell wall synthesis							
AT1G72610	Germin-like protein (GLP1)	↓	0.0003	0.6223	0.7922	0.9881	Confirmed
AT2G06850	Endoxyloglucan transferase	↓	0.0008	0.4060	0.3133	0.4889	Confirmed
AT5G20630	Germin 3	↓	<0.0001	0.1806	0.0031 (W > D)	0.5122	No treatment effect

[a]Indicates polymorphism in probe or primer.
[b]Indicates tagman assay.

acid-binding element (ABRE) or ABRE-like motifs in the upstream 1500 bp of sequence of genes regulated by our soil-drying manipulation. This finding supports a general importance of ABA signalling in early responses to drought, and possibly in adaptive divergence of Tsu-1 and Kas-1. With respect to functional binning, we observed significant constitutive differences between Tsu-1 and Kas-1 for a host of general processes (e.g. protein dynamics, patterns of transcription, regulation, photosynthesis, redox pathway), and more subtle patterns for accession-specific responses. The most striking pattern in this regard relates to the repression of protein dynamics following soil drying in Kas-1 in comparison to Tsu-1. Overall, Kas-1 appears to exhibit a general down-regulation of many protein processes in response to stress, perhaps related to its overall conservative physiological response.

There are a number of limitations to our current study. In particular, our drying manipulation does not provide a dynamic range nor did it lead to a severe level of stress. Furthermore, our transcriptome profiling has focused on only a single tissue type, necessarily limiting our inferences to above-ground processes. The lack of a dynamic stress series is especially restrictive as no doubt some of our responsive genes could be representative of a shift from 'wet' conditions to equally favourable 'moist' conditions, rather than the appearance of stress per se. This may be especially true for the Kas-1 accession that was presumably grown under more favourable conditions in our lab experiments than it likely experiences in the field. Nevertheless, the experiments here provide a key first step to more extensive studies of responses under dynamic soil drying.

In summary, we used experimental soil drying and two physiologically extreme accessions to explore genomic characteristics and transcriptome responses in *Arabidopsis*. We observed considerable evidence for sequence divergence among our study accessions, as well as remarkable constitutive differences in gene expression. In addition, we detected a small set of candidate genes exhibiting altered transcript abundance under soil drying. These loci are likely candidates for the basic differences in plant–water relations observed between our study accessions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Plots presenting least-square means (±1 SE) of ΔC_T values for each accession*treatment combination for candidate genes studied with QPCR.

Figure S2. Mapman presentation of constitutive differences in leaves between Kas-1 and Tsu-1 accessions for general process bins. Data are plotted as log2 (fold-change) of difference between accessions (Kas-1 minus Tsu-1) – red indicates higher expression in Kas-1 relative to Tsu-1.

Figure S3. Mapman presentation of responses in leaves to mild soil drying in Kas-1 and Tsu-1 for general process bins. Data are plotted as log2 (fold-change) of response to the soil moisture deficit treatment relative to control (red indicates induction by soil moisture deficit and blue indicates repression by soil moisture deficit).

Figures S4 and S5. Mapman presentation of transcription factor responses in leaves to mild soil drying in Tsu-1 and Kas-1 accessions. Data are plotted as log2 (fold-change) of response to the soil moisture deficit treatment relative to control (red indicates induction by soil moisture deficit and blue indicates repression by soil moisture deficit).

Table S1. Excel file containing Affymetrix ATH1 probes identified as significantly different in intensity for contrasts of gDNA hybridizations of either Kas-1 or Tsu-1 with Col-0 using at an FDR level of 5%.

Table S2. Excel file containing genes removed from expression analyses because they failed to meet a minimum criteria of 3 'good' probes defining the gene probeset following filtering by genomic hybridization. These genes contain numerous SNPs or copy number differences between Tsu-1/Kas-1 and Col-0.

Table S3. Excel file containing genes identified as differentially expressed at a FDR of 5% either constitutively between Kas-1 and Tsu-1 (worksheet 1), as a response to the drought treatment (worksheet 2), or as exhibiting accession specific responses to the drought treatment (worksheet 3). Each worksheet contains information on gene identifiers, short gene descriptions, least-square means for various experimental levels, raw *P*-values, and FDR adjusted q-values.

Table S4. Excel file containing constitutive candidate genes of interest identified from TAIR annotation, literature searches, or author interests. Genes are grouped in functional categories, and include gene identifiers, raw *P*-values, q-values, and $\log_2$ (fold-change) values.

Table S5. Excel file containing Wilcoxon tests of differential expression for Mapman bins and presenting Benjamini–Hockberg FDR corrected *P*-values for constitutive and accession specific responses to the drought treatment.

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