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UNIVERSITET
INSTITUT FOR MOLEKYLÆRBIOLOGI OG GENETIK

FAUPE – Forbedring af Afgrødernes Udbytte og Produktionsmæssige Egenskaber

Arbejdspakke 2 – tørkestress genekspressionsanalyser i rajgræs

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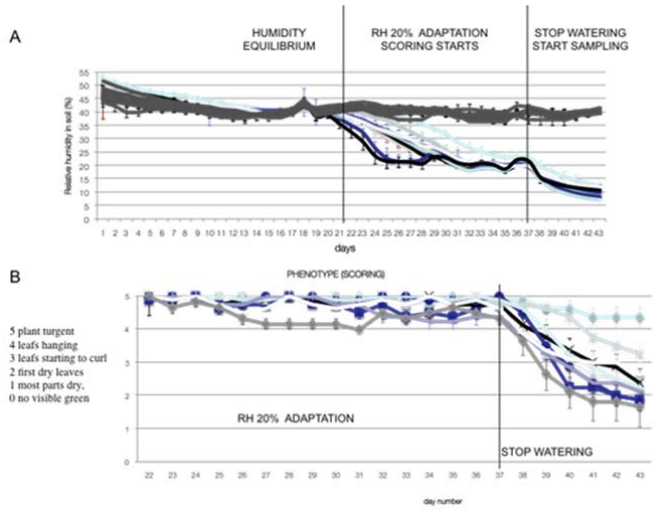
promilleafgiftsfonden
for landbrug

Transcriptome analysis in drought-sensitive and tolerant lines in perennial ryegrass (Lolium perenne).

Plant material and experimental setup

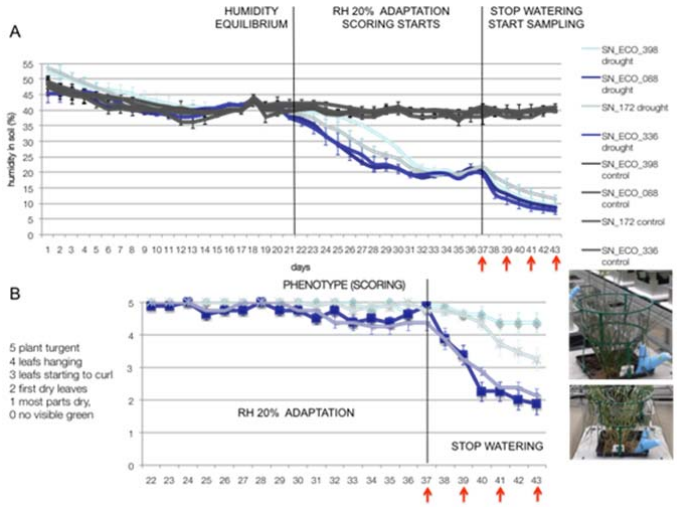
The Lolium SuperNAM GWAS population consisting of 556 genotypes representing ecotypes and breeding lines was phenotyped for response to water deficiency and regrowth after drought.
Ten genotypes showing the highest level of drought tolerance and the lowest level of drought tolerance were selected for subsequent transcriptome analysis.
Vegetatively propagated material (clonal shoots) from these 10 lines were growing in greenhouse under controlled conditions (20ºC, 16/8 h day/light) in 15 cm pots containing 500 g growing substrate which was a mixture of peat, clay and natural field soil. Five lateral shoots of similar size taken from cloned donor plants were planted in each pot. Pots (12 replicates/genotype) were randomized and grown for 50 days before starting the experiment. The relative humidity of the soil (RH) was recorded in regular intervals in each individual pot using chip-based sensors (Flowerpower, Parrot). The built-in watering system of the greenhouse was switched off and the pots were watered manually an individually according to actual soil humidity in each pot. The purpose of the first phase of the experiment was to equilibrate soil humidity (Figure 1A).

Figure 1. (A): Relative soil humidity (control plants in grey; drought stressed plants in colors). (B): Phenotyping for drought response. Plants were scored at the time corresponding to the humidity values. The legend describes the scoring scale.



After soil humidity equilibration 4 pots from each genotype were treated as controls and further 8 pots were subjected to reduced water supply for a few days until the soil in the pots reached a relative humidity (RH) of 20%. From this point on, all plants were scored every daily (Figure 1 B). Plants that already have adapted to 20% RH did not show drought symptoms when scoring was started. Samples from the second and the third leaves were taken from adapted and control plants. After this, manual watering was stopped (except for the control plants) and sampling was repeated in every second day.
Based on mean scoring four lines with contrasting drought response were selected (two lines with highest and further two lines with the lowest average scores, Figure 2.).

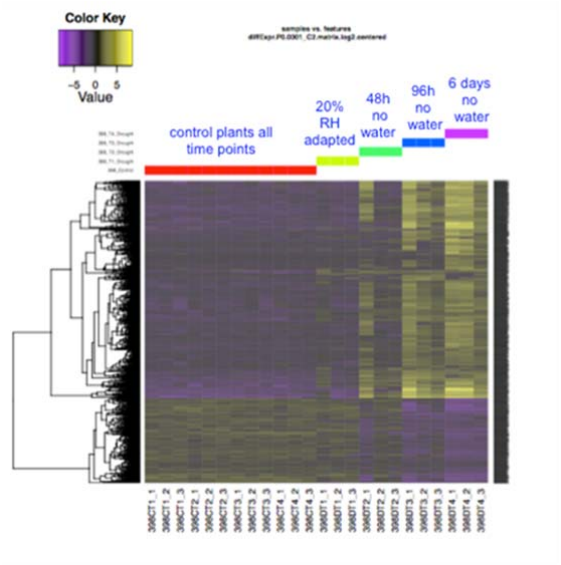
Figure 2. Tolerant and sensitive genotypes. (A): Average relative soil humidity of the plants selected for RNAseq analysis (control plants in grey; drought stressed plants in colors). (B): Phenotyping for drought response of the selected genotypes.
Red arrows indicate the sampling days. Pictures are representative for one tolerant genotype and one sensitive genotype, respectively, at the end of the experiment.



Quantitative expression profiling

Total RNA was extracted from leaf samples in 3 biological replicates from drought-treated and control plants. Paired-end Illumina HiSeq sequencing data were obtained for 96 total RNA samples. Raw sequence reads (2x100 nt) were subjected to adapter trimming and quality filtering, resulting in 2,122,588,324 cleaned paired-end reads. Cleaned paired-end reads were mapped onto reference sequences from a comprehensive transcript database of perennial ryegrass (Byrne et al., 2015).
Differential expression analysis was carried out using the Trinity software pipeline. Expression profiles proved to be consistent among all the controls at all the time points (Figure 3).

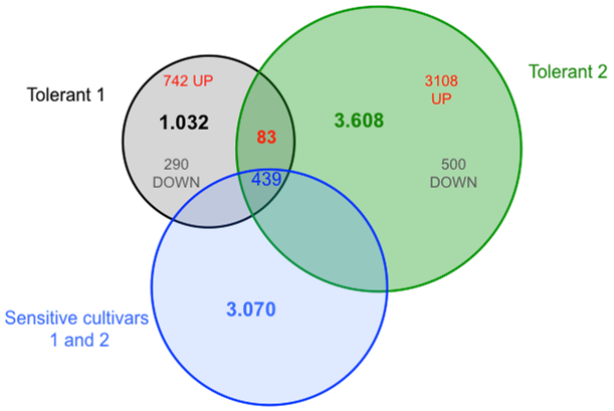
Figure 3. Gene expression heat map for genotype “Tolerant 1”.



Expression profiles in each treatment (time point) were compared to standardized values of control samples. Plants didn't show significant expression profiles changes immediately after adapting to 20% RH and there were no noticeable drought symptoms at this stage. However, the expression differences gradually increased with the progressing of water deprivation time.
All in all, we found 5,321 differentially expressed genes (DEGs) throughout the whole extent of the drought experiments. In case of genotype “Tolerant 1” 1.032 DEGs were identified (742 up-regulated and 290 down-regulated genes Figure 3, Figure 4). In case of the line “Tolerant 2” 3,608

DEGs (3,108 induced, 500 repressed)could be found. The line “Sensitive 1” exhibited 2,303 DEGs (induced 1.823, repressed 480), while 1.291 DEGs (933 up-regulated and 358 down-regulated) were found in case of the line “Sensitive 2”. Some DEGs genes were common between tolerant and sensitive genotypes (Figure 4).

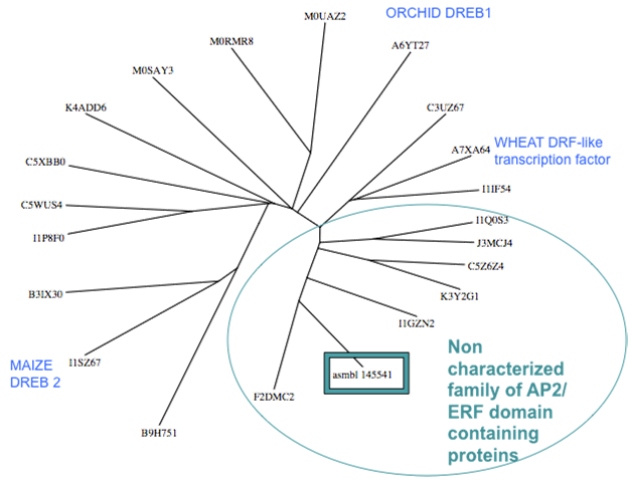
Figure 4. Venn diagram representing the number of DE genes.



The quantitative expression profiling results proved to be highly consistent between drought tolerant and drought sensitive lines: No DEGs were found with opposing expression profile in parallel genotypes (i.e. tolerant vs. tolerant or resistant vs resistant comparisons).
Altogether, 522 genes were differentially expressed in both tolerant lines and 439 genes were differentially expressed in both sensitive lines. We could identify 83 genes that were exclusively up-regulated in both tolerant genotypes, without showing expression differences in the sensitive lines.
Functional annotations data for 82% of all DEGs could be retrieved from the UniProt database using the sma3s software. Among the 83 genes specifically up-regulated in tolerant genotypes, two orthologs of a barley glutatione reductase were found. Similar antioxidative stress enzymes have previously been shown to be associated with drought tolerance (Talbi et al., 2015). We also found a differentially expressed ortholog of a wheat cytochrome P450 (CYP709C1 family) gene. The members of this family usually are associated with other types of abiotic and biotic stress. We further characterized CytP450-like genes under the DEGs. It is known that another CYPP450 family (CYP707A) is involved in ABA catabolism in *Arabidopsis* and members of the CYP96B were shown to be involved in drought stress response in rice. It is very likely that similar CYPP450 genes are involved in in drought tolerance in ryegrass as well.
There are further DEGs of interest with potential function in drought response, like two genes coding for proteins similar to *abi1* and *abi2* ABA (abscisic acid) receptors. A further interesting candidate is a DEAD box RNA helicase1. Helicases are involved in almost every aspect of RNA metabolism. In tomato, two helicases have been identified recently as key regulators in salt and drought tolerance (SlDEAD31, Zhu et al., 2015). In tomato the water- and salt-stress-regulated ASR1 (Abscisic Acid Stress Ripening) gene encodes a transcription factor that confers drought resistance. A wheat ASR1 confers drought resistance in transgenic tobacco (Hu et al., 2013).
One of our DEGs is a ASR1-like protein. In grape, an ASR1 protein was found to interact with an AP2-type transcription factor, the dehydration responsive binding element of the DREB2 family (Saummoneau et al., 2008). We found also an AP2 domain containing gene DREB2-like protein gene. We used blastp to find similar protein sequences containing DREB elements in related species and conducted sequence comparisons and phylogenetic analysis using the Clustal Omega software (Figure 5). It is likely that the identified AP2-type gene represent a key factor in drought tolerance. As this family is not yet well characterized. Our findings could provide novel information about the function of this family of transcription factors .
In addition to this DREB-like gene, which is up-regulated in tolerant genotypes only, we identified

3 further DREB-like genes that are up-regulated in the sensitive genotypes only. The detailed characterization of these genes is in progress.

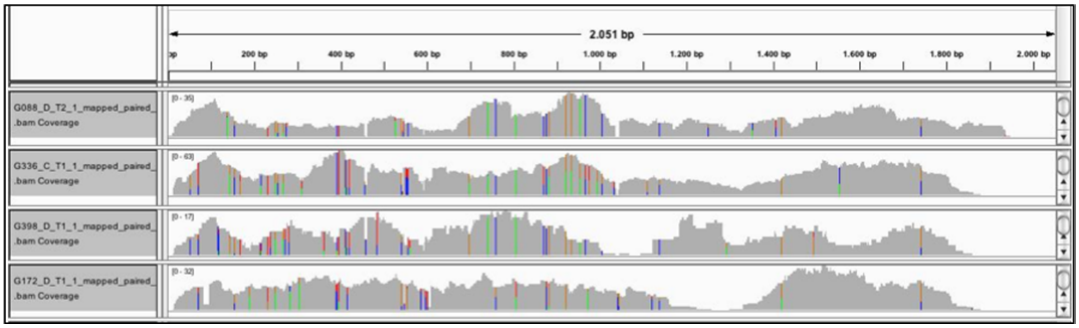
Figure 5. Phylogenetic tree for the DREB-like protein identified up-regulated in the tolerant genotypes.



Variant discovery in differentially expressed genes

Illumina reads from the drought resistant and drought tolerant genotypes were mapped to transcript reference sequences using the bwa-mem algorithm. Variants were detected in the alignments using Freebayes.
All in all, we found 1.059.541 SNPs in 56,240 coding sequences.
Out of 83 genes exclusively upregulated in tolerant genotypes alignments, 64 genes were suitable for SNP discovery. On these genes 2018 SNPs could be identified (30 SNPs/gene, 10 SNPs/Kb, Figure 6).

Figure 6. SNP discovery results visualized for the AP2-type gene. Colors highlight the SNP polymorphisms in the four genotypes.



Classification and characterization of SNPs distinguishing between drought tolerant and drought resistant genotypes might provide valuable plant breeding tools in producing new varieties with enhanced drought tolerance.

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