Evolution of Drought Resilience: Comparative Transcription Expression Analysis in Australian *Nicotiana* Species

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The study investigates how the *Nicotiana truncata/excelsior* clade adapts to arid conditions in Australia by analyzing root transcriptomes under drought stress. Differential expression analysis using edgeR and DESeq2 revealed distinct responses between the closely related *N.truncata* and *N.excelsior*. Notably, *N.truncata* exhibits upregulation of aquaporin PIP1-2 genes, suggesting a specific adaptation to drought. Both species show decreased expression of genes associated with cell wall biogenesis and oxidative stress response. Additionally, *N.excelsior* prioritizes flower and seed development, while *N.truncata* enters a dormancy-like state with reduced metabolic activity. However, further research is needed to validate these findings and elucidate the underlying mechanisms. Improved gene annotations from extracted genomes will enhance understanding of drought response strategies in this clade.

Abbreviations

[C]	Condition	[TRU]	*N. truncata*	[logFC]	logarithmized fold change by 2
		[EXC]	*N. excelsior*	[FDR]	False Discovery Rate

Introduction

The rapid increase in global surface temperatures, driven by the greenhouse effect and human activities, is causing significant shifts in Earth's climate dynamics, leading to intensified water evaporation and resulting in varied precipitation patterns and prolonged droughts (IPCC, 2021; Seneviratne et al., 2021). Hence, there is an urgent necessity to study plant adaptation to drought stress in order to enhance the resilience of crop plants. The allotetraploid *Nicotiana* section *Suaveolentes* serves as an excellent case study for this purpose. Recent radiation of the Australian species in this section is frequently found in extremely arid habitats of Australia compared to other members inhabiting wet tropical forests of the same continent and the New World (Chase et al., 2023).

Genomic studies of 46 *Nicotiana* species revealed increases and decreases of genomic and chromosomal size. Reductions in both found in several clades. For instance, *N.truncata/excelsior* and *N.velutina* are associated with their recent radiation (approximately 2 million years ago) in harsh conditions of the arid zone in Australia (Chase et al., 2023).

Representative species chosen for the clade *N.truncata/excelsior* are *N.truncata* and *N.excelsior*, and for *N.velutina* *N.velutina* and *N.gossei*.

Drought adaptation in *N. truncata* and *N.velutina* is hypothesized, as these species are frequently appearing in very arid regions of Australia (Chase et al., 2021; Symon, 2005). They are compared to their closely related counterparts growing in tropical forests to test for drought beneficial different expression (Chase & Christenhusz, 2018a, 2018b).

To ascertain the extent and mechanisms of drought adaptation in these aforementioned clades, a drought stress experiment was conducted using 96 representative samples was conducted. Each species had 24 plants, with 12 grown under high water availability as control and the other 12 subjected to very limited water supply. After 2 weeks of stress, either all leaves or all roots were collected from 12 replicates of each species for transcriptome analysis to identify differential expression under drought conditions.

This study presents an in-depth analysis of root sample transcriptomes from *N.truncata* and *N.excelsior*. Through the application of cutting-edfe bioinformatics methodologies such as edgeR and DESeq2, a comprehensive examination of gene expression patterns in these closely related plant species was conducted. Utilization of topGO allowed for exploration of Gene Ontology terms, providing crucial insights into their distinct responses to drought stress.

Furthermore, this work provides optimized pipelines for the differential expression analysis, enabling the efficient identification of significant genes and streamlining the data

processing workflow.

Genomic DNA extraction was performed on flowers from all four representative species: *N.excelsior*, *N.truncata*, *N.velutina*, and *N.gossei*, to enable genome sequencing. This step is important for improving the precision of gene annotations and deepening our comprehension of the chromosomal structure in these species in future research. Presently, gene annotation relied on the genome of the model plant *Nicotiana benthamiana* (Ranawaka et al., 2023).

The findings of this study are significant as they contribute to our understanding of how plants, particularly the Nicotiana clades, adapt to drought stress. This research elucidates the hypothesized drought adaptation in these clades, offering valuable insights for further exploration in the field of plant biology. Understanding these mechanisms is vital for developing resilient crops and sustainable agricultural practices in response to changing environmental conditions, including the challenges posed by climate change.

Materials and Methods

Experimental setup and conditions

96 collected plants from Australia, with 24 representatives of each species, were cultivated in a glasshouse. Of these, 48 plants of each species were subjected to limited water availability, while the remaining 48 received sufficient water supply. Following a grouth period of 2 weeks, tissue samples were collected from either all roots or leaves of 12 samples from each Treatment condition for each species. Prior to sampling, the plants subjected to drought conditions were watered to ensure tissues with comparable turgor. The tissue samples were frozen with nitrogen until RNA extraction for sequencing.

Extraction and Sequencing of plant genome

The shock-frozen flower samples were extracted following the attached protocol A, with the following mentioned washing and adjustments. The extraction material was grinded by **Retsch grinder at 30sec with 300 hertz**. The tissue powder was then transferred into **40mL** of washing solution (1% Sorbitol solution). 160 μL ß-Mercaptoethanol was added to prevent oxidization. The sample were then incubated for 10 min in the washing solution, prior to centrifugation at 4000 rpm. The pellets were resuspended in 30mL washing solution mixed with 120 μL ß-Mercaptoethanol. The samples were then incubated for 10 min again, prior to centrifugation at 1000 rpm. The washing steps repeated until the centrifugate liquid was clear. Afterwards, the sample pellet was resuspended and lysed with the mentioned 20mL Carlson lysis buffer. 50μL ß-Mercaptoethanol and **50 μL** RNase A (20mg/mL) were added to the sample and mixed by tube inversion only. After mentioned incubation, the samples were extracted with the mentioned amount of chloroform/isoamylalcohol by tube inversion mixing only. The solution was then centrifuged at 3220g at 15 min with slow acceleration/deceleration. Further steps followed the protocol completely. The DNA was extracted by anion exchange columns from QIAGEN.

The quality of the extracted DNA was determined via Nanodrop photometry and gel electrophoresis. 3 μL samples were separated with 1.5 % agarose gel under 100 volts for 28 minutes.

Genome reference

The genome reference NbLab360.v103 of *Nicotiana benthamiana* and its gene annotations were retrieved from the website https://bioweb01.qut.edu.au/benthTPM/download.html, provided by the work of Ranawaka et al., 2023. The genome size was around 283 billion basepairs (Gbp) and consisted of 20 chromosomes.

Bioinformatical Analysis of the Transcriptome Data

All SLURM scripts and R notebooks are provided in the github repository https://github.com/Kactaceapengu/EvoecoNico.

Software Used

Software	Version	Citation
FastQC	0.12.1	(Andrews, Accessed: 2024)
Trimmomatic	0.39	(Bolger et al., 2014)
STAR	2.7.11b	(Dobin et al., 2013)
SubRead - FeatureCounts	2.0.6	(Liao et al., 2014)
R Studio	1.3.1093	(RStudio Team, 2020)
edgeR	3.40.2	(Chen et al., 2024)
DESeq2	1.38.3	(Love et al., 2014)
plot_ly	4.10.14	(Inc., 2015)
topGO	2.50.0	(A & J, 2023)

Quality trimming of sequencing data

Adapters were already removed at data retrieval. Following to that, the sequences were trimmed at a phred score of 33 using Trimmomatic:

```
trimmomatic SE -phred33 -threads 4 input output
    SLIDINGWINDOW:4:28
```

STAR indexing

The reference genome NbLab360 was indexed via STAR:

```
nice -n 19 STAR --runMode genomeGenerate \
          --genomeDir /lisc/project/evoeco/trinh/
              reference \
          --genomeFastaFiles /lisc/project/evoeco/
              trinh/reference/NbLab360.genome.
              fasta \
          --runThreadN 4 \
          --sjdbGTFfile ./NbLab360.v103.gff3 \
          --sjdbGTFtagExonParentTranscript Parent
              --sjdbOverhang 149
```

STAR alignment

Alignment with STAR was conducted:

```
STAR --runThreadN 4 \
       --genomeDir /lisc/user/trinh/Nicotiana/
           starindex/NbLab360starindex \
       --readFilesCommand gunzip -c \
       --readFilesIn /lisc/project/evoeco/trinh/
           data/61_S81_R1.fastq.gz
```

FeatureCount counting

Fragment counting of paired end reads for annotated genes was perfomed with FeatureCount:

```
featureCounts -a /lisc/project/evoeco/trinh/
     reference/NbLab360.v103.gff3 \
       -g ID -t gene -p --countReadPairs -o 61_S81_
            R1_phred28.txt \
       /lisc/user/trinh/Nicotiana/starindex/61_S81_
            R1_phred28.sam
```

Differential Expression Analysis

Outlier detection

PCA plots were made to investigate clustering behaviour of the samples in species (*N.excelsior, N.truncata* and experimental condition (Drought, Control). For this the count data was normalized with regularized logarithmic, as provided as function "rlog()" by DESeq2. Outliers were identified by wrong clustering. They were further inspected via 3D PCA and zero counts per sample retrieval.

Differential expression analysis by DESeq2

Differential expression analysis was conducted using DESeq2, which automatically filtered the count data by applying Cook's outlier removal and identifying the lowest mean of normalized count filter threshold. This threshold was determined such that the number of rejections fell within 1 residual standard deviation to the peak of a curve fit to the number of rejections over the filter quantiles. The logFC of the un-normalized count data was shrunken, such that it is associated with the Control. Positive values indicate higher expression in drought condition and vice versa. The dispersion model was chosen and fit automatically as well. Significant genes were determined with a FDR smaller or equal to 0.05 and a logFC larger or equal to 1.5 . All plots can be found in the R notebook provided in the github repository, mentioned above.

Differential expression analysis by edgeR

Differential expression analysis performed by edgeR started with the identification of a pre-filtering threshold. For that counts per million (CPM) thresholds ranging from 0 to 1 were compared to the number of rejections and number of retrieved significant genes in 10 incremental steps (0.0, 0.11, 0.22, and so forth). A CPM threshold of 0.33 was chosen for both plant species (TRU and EXC).

The count data was normalized by trimmed mean of m-values, as provided by edgeR with "calcNormFactors()". The dispersion model (trended and tag-wise) was fit with "glmQLFit()". "glmQLFTest()" calculated the significance values and the logFC. Significant genes were retrieved with a FDR smaller or equal to 0.05 and a logFC larger or equal to 1.5. All plots can be found in the R notebook provided in the github repository, mentioned above.

GO-Term Retrieval with TopGO and Statistical Analysis via R

TopGO utilized provided GO annotations to associate gene counts with corresponding GO terms, grouping genes and computing Z-scores for each term. Subsequently, it conducted significance testing for the GO terms using Fisher statistics.

GO-terms, appearing in both species were retrieved and their counts were plotted. Their respective counts were evaluated with two-way ANOVA, considering both species and treatment conditions. For GO-terms appearing in only one species, the calculated FDR-values of the differential expression analysis was provided in the GO-term barplots to determine significance.

Results

0.1 Outlier Removal

Overall clustering of the samples is shown in Figure 1. TRU appears to be better separated than EXC condition-wise. However, "TCR92" a control sample of TRU was mixed into the drought conditions.

Inspection of TRU-specific PCA lead to the same observation of the outlier "TCR92" of TRU in control condition, which clusters in the drought condition closely to "TDR94". This is shown in Figure 2.

3D PCA of only TRU specific samples show the same behaviour, with "TCR92" and "TDR94" clustering very closely. Their values are the following: "TCR92" with (PC1, PC2, PC3) had (12.09, -7.86, -11.90) and "TDR94" had (12.00, -7.57, -11.57).

Furthermore, the zero counts of the samples were for "TCR92" 7021 and for "TDR94" 6544; with the minimum zero count over all samples being 6204 and maximum being 8875.

"TCR92" was removed due to the different zero counts, even though it may be a technical replicate of "TDR94" with the high resemblance of the first three PCs.

It has to be noted, that removal of "ECR61" and "ECR65" lead to different significant GO-terms f.ex. "response to water deprivation" in *N. excelsior*, which would have been filtered by the given thresholds. The EXC specific PCA in Figure 4.

0.2 Differential Expression analysis of edgeR

33079 genes in total were tested in TRU, with 505 being significantly expressed with the given thresholds.

33785 genes were tested in EXC, with 185 being detected as significantly different. With removal of "ECR61" and "ECR65" as outliers, 375 genes are detected as being significant.

Their significant GO-terms are demonstrated in barplots in Figure 10. TRU shows high fold change in the regulation of cellular respiration. Response to water deprivation and ethylene-activated signaling pathways are also high in logFC. There are many low logFC in TRU, which are associated with the cell wall, plasma membrane, the extracellular region and plasmodesma. High significance was also found in hydrogen peroxide catabolic process, which is very low in logFC.

In contrast, EXC shows more abiotic stress related responses for instance cellular response to heat, response to wounding, regulation of DNA-templated transcription (Khan et al., 2018). Glutathione is also known to act as an reactive oxygen species scavenger (Hameed et al., 2014). Furthermore, cutin, fucose and sesquiterpene biosynthetic processes are low in logFC indicating a resource saving mechanisms of the root tissues as these compounds are needing a high demand of carbon for synthesis.

Both plants show similar stress GO-terms and also similar reaction in low logFC of cell wall associated processes. Lignin associated processes are shown to be low in logFC in both. Both plant root tissues also show low logFC in various transmembrane transport associated GO-terms.

In Figure 11 with "EDR61" and "ECR65" being removed as outliers, both plants are shown to be significant in their high logFC response to water deprivation. Protein dephosphorylation has been added to the GO-terms of EXC, indicating another stress related response. Response to abscisic acid, which is a sesquiterpene, has also been added, which plays an important role to prevent further water loss and also in growth regulation of plant roots (Harris, 2015).

0.3 Differential Expression analysis of DESeq2

37605 genes in total were tested in TRU, with 283 being significantly expressed with the given thresholds.

36907 genes were tested in EXC, with 197 being detected as significantly different. With removal of "ECR61" and "ECR65" as outliers, 375 genes are detected as being significant.

The GO-terms shown in Figure 12 show similar responses to edgeR retrieved GO-terms.

Interestingly, DESeq2 resulted in way more high logFC changes between the conditions. For TRU carbohydrate transmembrane transport and protelolysis are now high in logFC. There are also methylation/demethylation processes shown, indicated by S-methylmetionine cycle and histone H3-K36 demethylation. Xyloglucan and galactose are also metabolized significantly more than in control.

In contrast, phosphate ion transport and dephosphorylation of proteins are low in logFC. There are also many biosynthetic processes being very low.

New GO-terms in EXC were also added by DESeq2. For example flower development and embryo development are high in logFC. Fructose metabolism is also shown to be very high, with a corresponding high logFC in carbohydrate transport.

Phosphate and protein dephosphorylation are similarly low in logFC compare to TRU. However, response to nitrate is very low compared to the control condition samples.

LogFC and Z-score in response to **water deprivation** were found to be similar in EXC (2.06, 2.83) and in TRU (2.06, 2.83). However, FDR shows a very high significance in EXC (0.00005) compared to TRU (0.0011). The corresponding eight genes were retrieved and the significant ones under two-way ANOVA were summarized in Table 1. Their log-scale count plots are shown in Figures 5, 6, 7, 8 and 9.

Discussion

Comparison between edgeR and DESeq2 for drought adaptation analysis

The differential expression analysis conducted using edgeR and DESeq2 yielded quite distinct sets of significant Gene Ontology (GO) terms. Discrepancies in the results could be attributed to differences in their pre-filtering, dispersion estimation, and statistical testing methodologies.

The GO-terms combined have lead to an interesting interpretation of the given expression data from the root samples of *N.truncata* and *N.excelsior*.

First of all, the two plant species were similarly responding to water deprivation with the same logFC change and also the same Z score. This indicates that, the applied drought stress was equal for both species. More detailed analysis revealed, that chloroplastik like beta amylase and an abscisic acid inducible protein were significantly higher in the drought stressed samples. The NbLab360 gene annotation shows duplications for chloroplastic like beta amylase and probable aquaporin PIP1-2. For the latter, *N.truncata* is very significantly higher in fragment counts than *N.excelsior*. Both transcript are high in counts in control condition, but the second gene NbL17g15210 is also very highly transcribed in drought compared to the EXC counts. This indicates, that the roots of *N.truncata* overexpresses aquaporin transcripts to better deal with drought stress.

Drought stress leads to an overall similar response for both species, which is expected given their close relationship. Both plants exhibit decreased fold changes associated in cell wall biogenesis, catabolism and biosynthesis of various substances ssuch as lignin, fucose and cutin. Additionally, the importance of oxidative stress for the root cells is diminished, as indicated by low fold changes of hydrogen peroxide catabolic processes.

Root tissues appear to be undergoing a reduction in essential pathways associated with growth. Notably, transcripts related to phosphate and nitrate transport are also decreased in root tissue cells of both plants.

With compromised growth and functionality, root cells signal stress through a significant increase in the log fold change (logFC) of the ethylene-activated signaling pathway and the dephosphorylation of proteins (in EXC, Figure 11).

EXC drought response strategy is well depicted in Figure 12, in which it demonstrates high expression of genes important for flower and seed development. Carbohydrates are metabolized (fructose 1,6 - bisphosphate metabolic process) and is indicated by high logFC in carbohydrate transmembrane transport to be transported away from the roots.

Similarly, TRU demonstrates higher carbohydrate transmembrane transport expression levels. However, in contrast to EXC, TRU clearly expresses consistent upregulation of genes regulating cellular respiration across all barplots. This pattern suggests a potential metabolic shift towards dormancy in TRU root cells, supported by the downregulation of metabolic processes observed in approximately 207 genes (Figure 10 and 11). Furthermore, the higher log fold change in methylation/demethylation processes in TRU roots may indicate an epigenetic adaptation associated with this dormancy state (Figure 12). Interestingly, despite the dormancy-like state, TRU roots also metabolize carbohydrates such as xyloglucan and galactose for transport, as depicted in Figure 12.

Conclusive remarks

The study's findings suggest interesting possibilities about how the *N.truncata/excelsior* clade may have adapted to the arid zone of Australia. The gene expression shows a significantly different response in the probable aquaporin PIP1-2 genes for TRU compared to EXC. Furthermore, the drought response strategies differ greatly in both plants while they seem to be similarly affected by drought stress.

EXC focuses into flowering and reproduction, possibly hoping for the next generation to find better survival conditions. On the other hand, TRU root cells seem to enter a dormancy state, reducing respiration and metabolism and conserving resources like phospate and nitrate. It also pro-

cesses and moves carbohydrates away from the roots.

As of now, this work needs better research into the literature to further prove these interpretations of the data. The exact experimental design and sequencing background of the data was not provided yet as well. Having this data would make the interpretations stronger and better support the conclusions drawn from the study. Better gene annotations, obtained from the extraced genomes of all plant species, would also help clarify how these plants respond to drought.

Figures

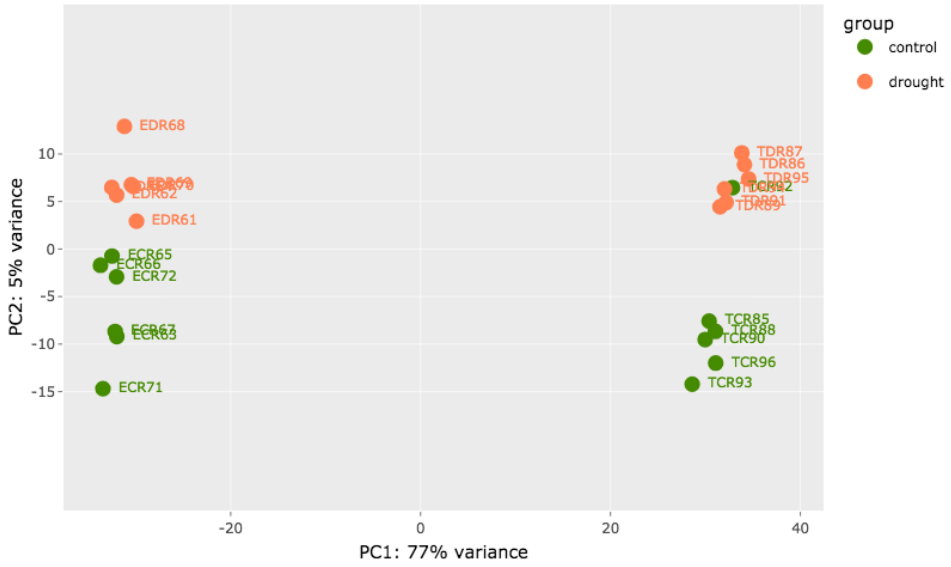

Figure 1: 2D PCA plot of all TRU and EXC samples

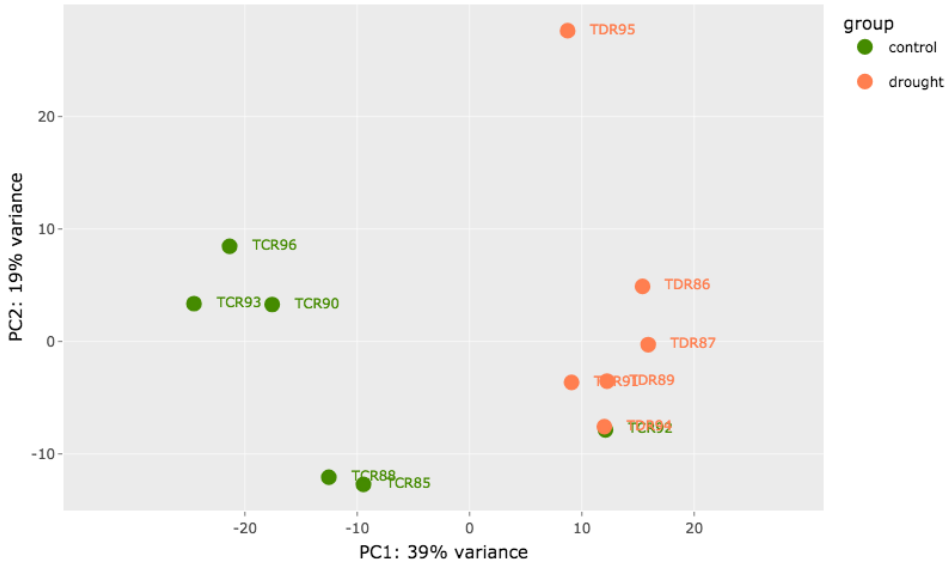

Figure 2: 2D PCA plot of only TRU samples, showing a false clustering of the sample "TCR92"

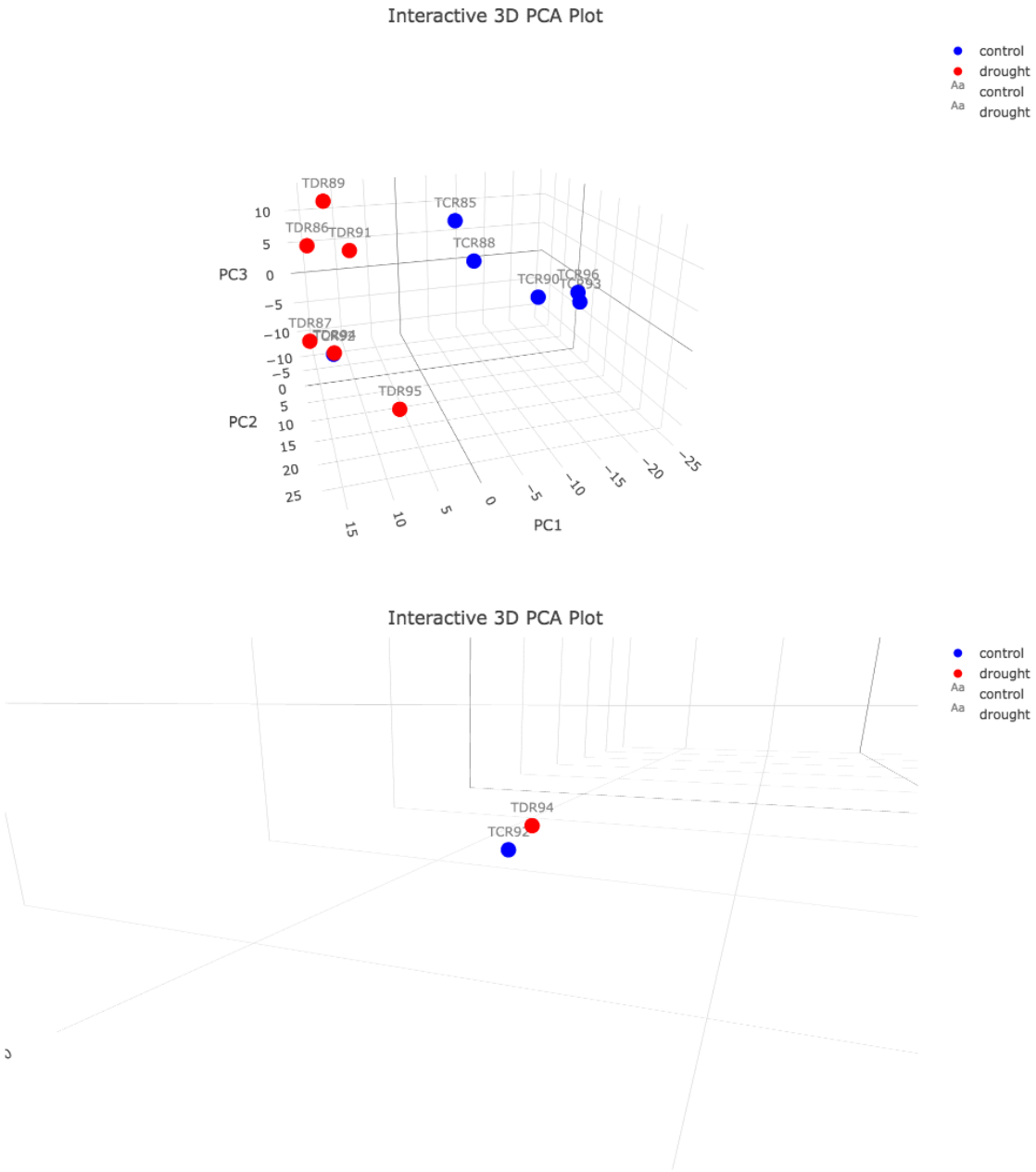

Figure 3: 3D PCA plot of TRU samples for outlier "TCR92" detection, in close vicinity of "TDR94".

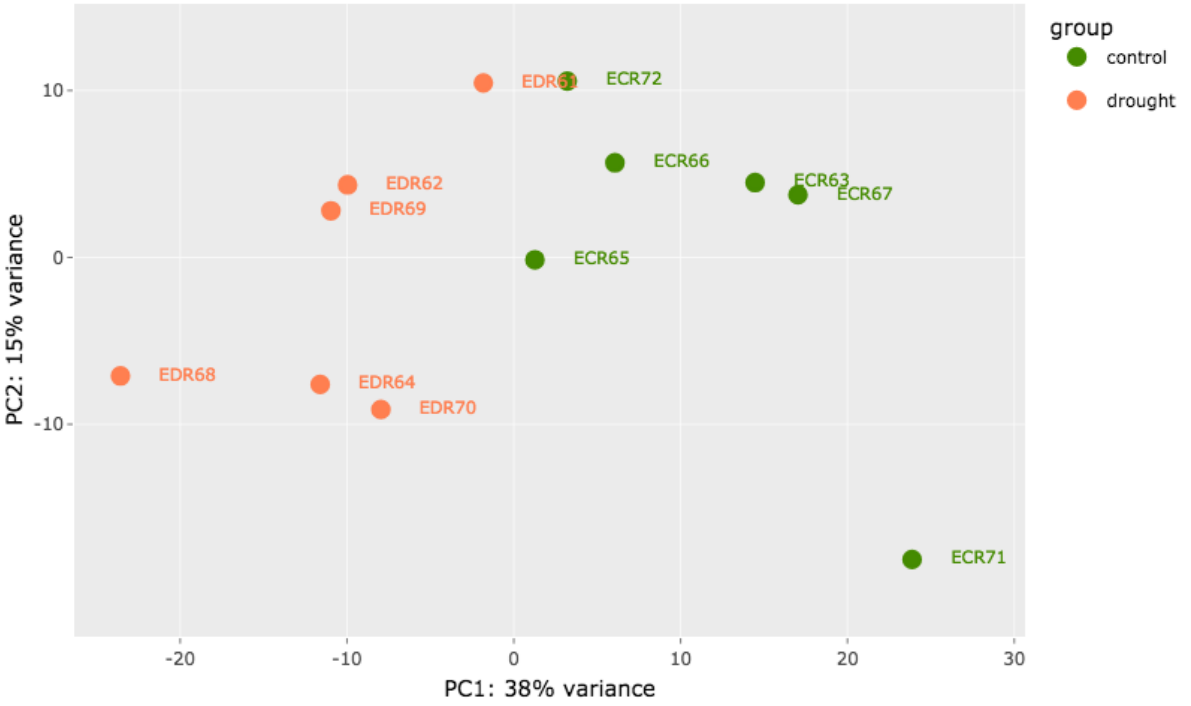

Figure 4: 2D PCA plot of only EXC samples, showing "ECR65" and "EDR61" in close vicinity

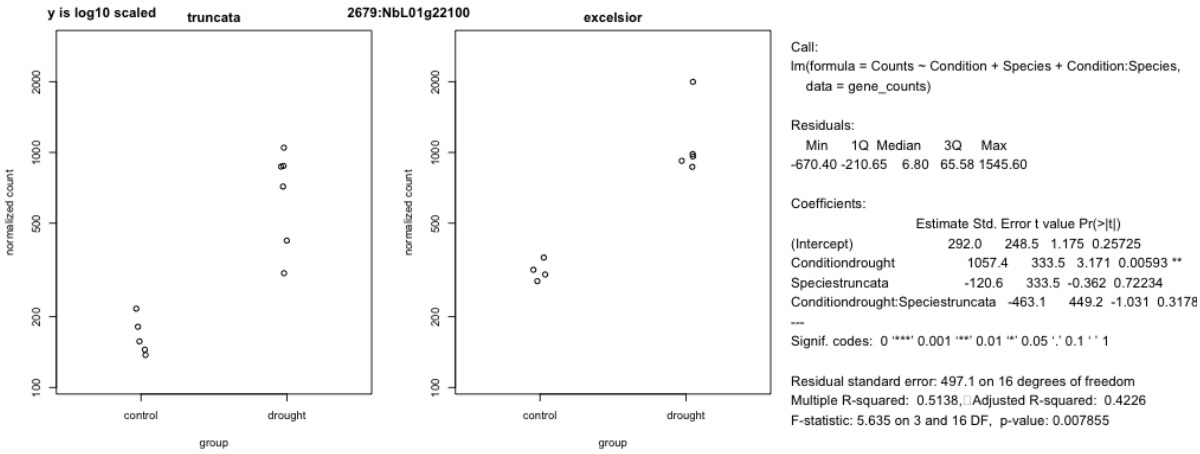

Figure 5: Log10-scaled count plot of the gene NbL01g22100 associated with water deprivation response

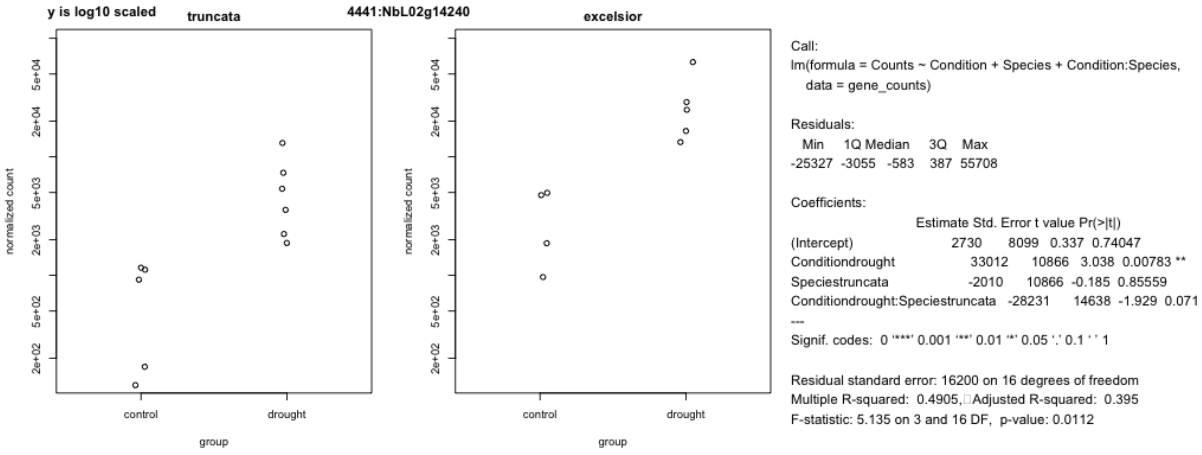

Figure 6: Log10-scaled count plot of the gene NbL02g14240 associated with water deprivation response

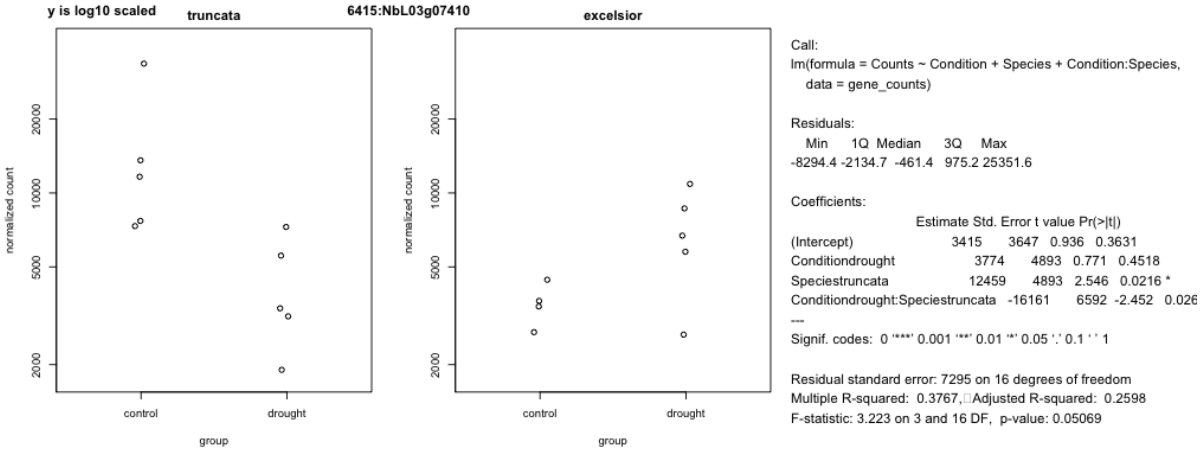

Figure 7: Log10-scaled count plot of the gene NbL03g072410 associated with water deprivation response

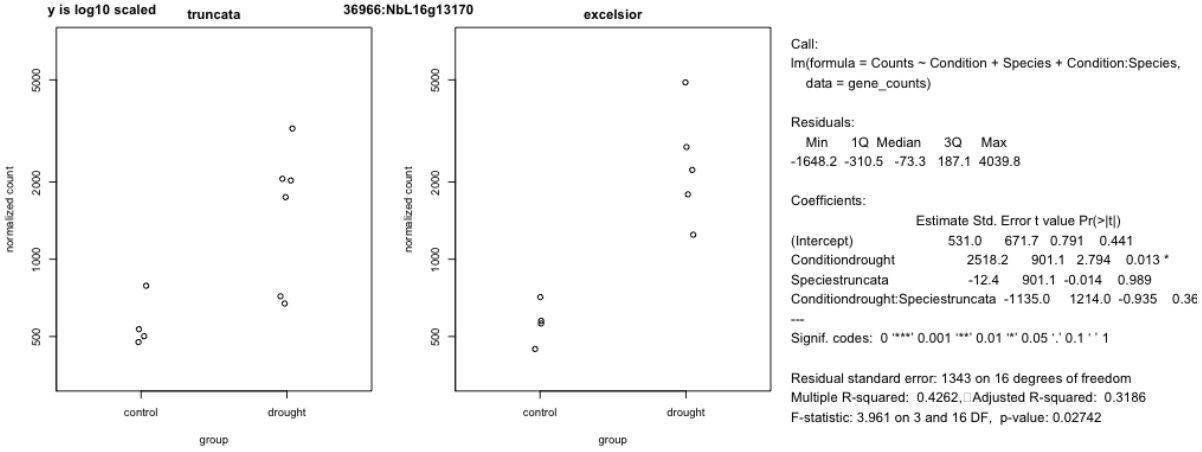

Figure 8: Log10-scaled count plot of the gene NbL16g13170 associated with water deprivation response

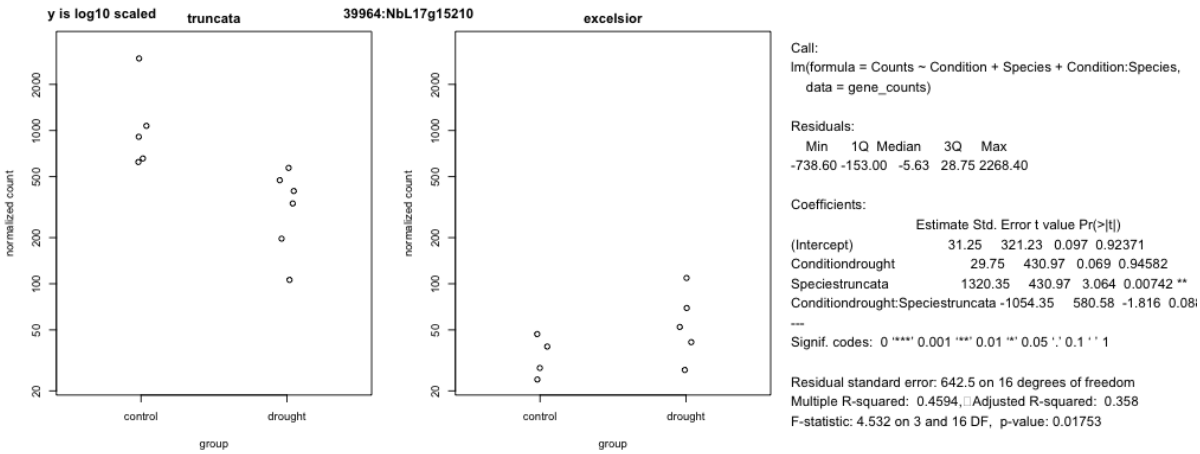

Figure 9: Log10-scaled count plot of the gene NbL17g15210 associated with water deprivation response

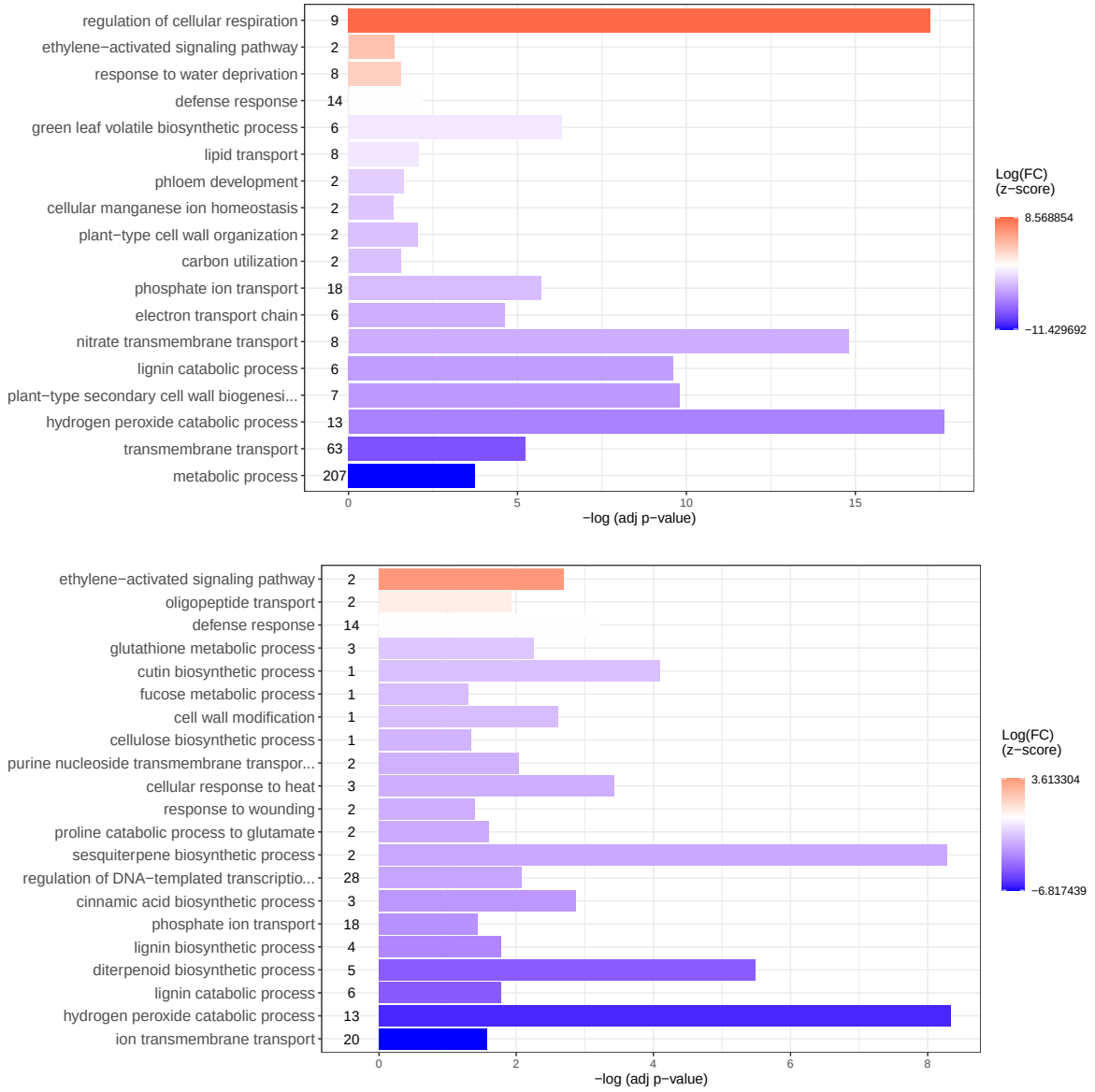

Figure 10: Barplots of the significant GO-terms found in (above:) TRU and (below:) EXC

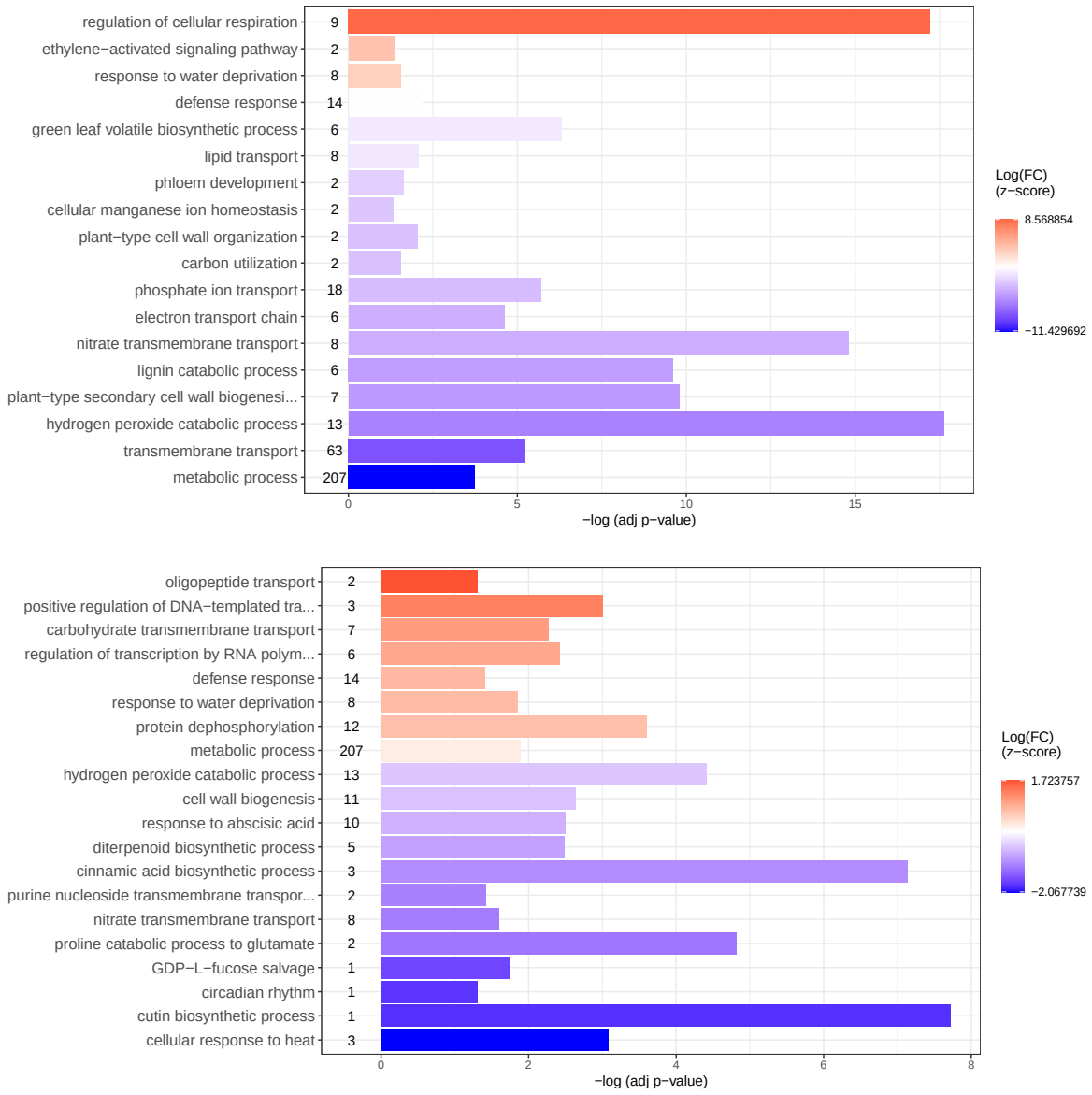

Figure 11: edgeR retrieved barplots of the significant GO-terms found in (above:) TRU and (below:) EXC, with "ECR61" and "and ECR65" removed as outliers

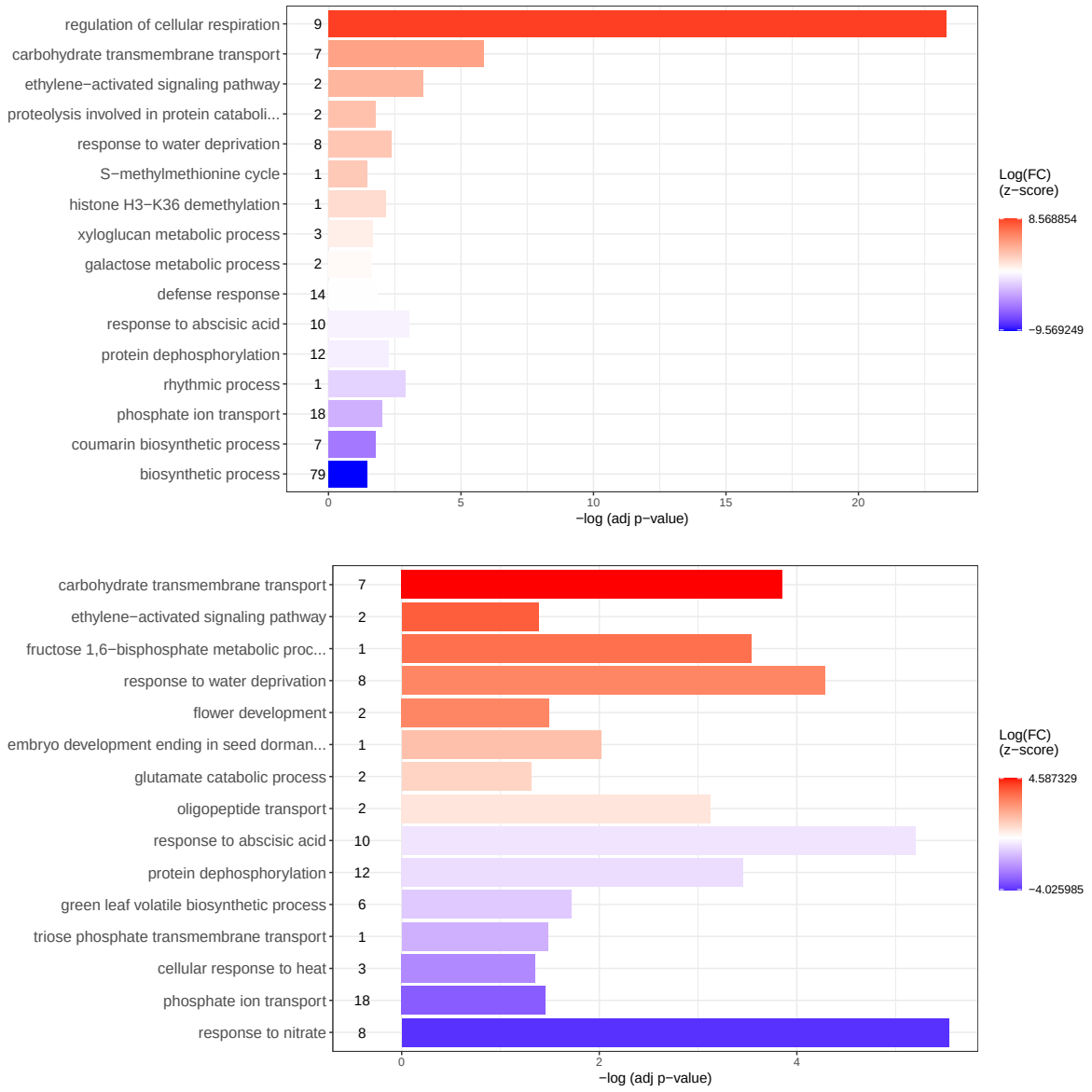

Figure 12: DESeq2 retrieved barplots of the significant GO-terms found in (above:) TRU and (below:) EXC

Tables

[C]	Condition	[TRU]	*N. truncata*	[logFC]	logarithmized fold change by 2
		[EXC]	*N. excelsior*	[FDR]	False Discovery Rate

Table 1: Summary of significant responses to water deprivation found in the root tissue of TRU and EXC

Gene id	Annotation (NbLab360)	Species pvalue	Condition pvalue	Species&Condition pvalue	**High count in...**
NbL01g22100	(LOC107776343) chloroplastic like beta amylase	0.72	0.006	0.32	EXC (both C)
NbL02g14240	(LOC107825693) abscisic acid and environmental stress inducible protein	0.85	0.008	0.07	EXC (both C)
NbL03g07410	**(LOC109237954) probable aquaporin PIP1-2**	0.02	0.45	0.03	**TRU (control)**
NbL16g13170	(LOC107776343) chloroplastic like beta amylase	0.99	0.013	0.36	EXC (drought)
NbL17g15210	**(LOC109237954) probable aquaporin PIP1-2**	0.007	0.94	0.08	**TRU (both C)**

Acknowledgements

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A Supplemental Information

User-Developed Protocol:

Isolation of genomic DNA from plants using the QIAGEN® Genomic-tip

This procedure has been adapted by customers from the QIAGEN® Genomic-tip Protocols, and is for use with QIAGEN Genomic-tips**. It has not been thoroughly tested or optimized by QIAGEN.**

The protocol has been successfully used by customers for genomic DNA isolation from tobacco, *Arabidopsis*, maize, cotton, tomato, pine, rhododendron, oak, fir, elm, and poplar. Genomic DNA prepared by this method is generally suitable for restriction enzyme digestions, Southern blotting, and PCR.

The protocol below is designed for isolation of genomic DNA from 1–2 g of starting material with a QIAGEN Genomic-tip 500/G. However, it can be scaled down for use with QIAGEN Genomic-tip 100/G.

Please be sure to read the *QIAGEN Genomic DNA Handbook* and the detailed Protocol for Isolation of Genomic DNA from Blood, Cultured Cells, Tissue, Yeast, or Bacteria.

Procedure

1. **Grind up to 1–2 g of leaf material in liquid nitrogen with a mortar and pestle.**
2. **Transfer tissue powder into a 50 ml screw-cap tube.**
3. **Add 20 ml of Carlson lysis buffer pre-warmed to 74°C.**

   Carlson lysis buffer:

100 mM	Tris ·Cl, pH 9.5
2 %	CTAB
1.4 M	NaCl
1 %	PEG 6000 or 8000
20 mM	EDTA

4. **Add 50 µl ß-mercaptoethanol and 200 µl of RNase A (20 mg/ml). Vortex at full speed for 5–10 s**

   Note: ß-mercaptoethanol is toxic; dispense in a fume hood and wear appropriate protective clothing.

5. **Incubate at 74°C for 20 min in a shaking water bath.**

   Note: If a shaking water bath is not available, gently shake the samples every 5 min during incubation.

6. **Cool the samples to room temperature, add 1 volume of chloroform/isoamylalcohol (24:1), and vortex at full speed for 5–10 sec.**

7. **Centrifuge at 5000 x *g* for 10 min at 4°C.**
8. **Transfer aqueous upper phase to a fresh 50 ml screw-cap tube.**
9. **Add 1 volume of distilled water and adjust the pH to 7.0 using HCl.**
   Note: Generally 100–200 µl of 25% HCl is required.
10. **Follow the QIAGEN Genomic-tip 500/G procedure in the *QIAGEN Genomic DNA Handbook*.**

Reference

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