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Transcriptomic drivers of plant adaptation to the desert in *Nicotiana* sect. *Suaveolentes* (Solanaceae)

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Content

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Abstract

The paleopolyploid *Nicotiana* sect. *Suaveolentes* emerged approximately 6 million years ago (mya) in South America, from where it reached Australia via long distance dispersal. There it underwent extensive radiation, becoming the most diverse section within *Nicotiana*. The ancestral chromosome count of the section is n = 24, which is encountered in species inhabiting humid coastal environments. The colonization of the arid interior of the continent, which occurred only about 2 mya, was accompanied by independent gradual chromosome reduction in at least four clades. Notably, species in open desert environments exhibit the lowest chromosome numbers (n = 15, 16, 18, or 19), while closely related species in protected areas display higher chromosome numbers. This thesis aims to start to identify the molecular basis of the adaptation to extreme habitats and explore possible links to diploidization. To achieve this, two pairs of sister species with contrasting chromosome numbers and habitat preferences were exposed to artificial drought conditions. Messenger-RNA profiles during drought recovery after subsequent rewatering in leaf and root tissues were captured using RNA-seq. Resulting Illumina reads were mapped against a high-quality reference genome for *N. benthamiana*, another member of sect. *Suaveolentes*. Gene expression levels of drought-exposed plants were compared to those of well-watered (control) plants for each species to determine differentially expressed genes (DEGs). This thesis focused on the analysis of leaf samples from one species pair. For the drought susceptible *N. excelsior* (n = 20) 458 significant DEGs were identified, whereas the drought adapted *N. truncata* (n = 18) yielded 670 DEGs. The distribution of candidate genes was mapped on the reference genome, to reveal potential clustering that would indicate linkage groups of drought-adapted genes. Finally, a gene ontology (GO) enrichment analysis based on DEGs, revealed similar pathways to be overrepresented in both species, but many of them show regulation in opposite direction. Among others, pathways related to phytohormone signaling, biotic defense and cell-wall organization were upregulated in *N. excelsior* and downregulated in *N. truncata*. Pathways related to storage metabolites and photosynthesis were mutually downregulated. These results indicate persistent stress response even after rewatering and potential irreversible damage in *N. excelsior*. In contrast, the results for *N. truncata* suggest a fast recovery, which might be required to take advantage of rare and unpredictable rainfall.

Zusammenfassung

Die paleopolyploide *Nicotiana* Sektion *Suaveolentes* entstand vor ungefähr 6 Millionen Jahren in Südamerika. Nachdem sie Australien erreichte, diversifizierte sich die Gruppe zu der artenreichsten Sektion innerhalb der Gattung *Nicotiana*. In humiden Küstenregionen Australiens sind heute hauptsächlich Arten mit der ursprünglichen Chromosomenzahl n = 24 zu finden, während die Besiedlung des ariden Zentrums vor ca. 2 Millionen Jahren mit einer graduellen Chromosomenreduktion in mindestens vier Kladen einher ging. Auffällig ist, dass die Arten, mit den niedrigsten Chromosomenzahlen (n = 15, 16, 18 oder 19) ungeschützt in der offenen Wüste wachsen, während nahverwandte Arten mit höheren Chromosomenzahlen auf geschützte Habitate beschränkt sind. Das Ziel dieser Masterarbeit ist es, molekulare Mechanismen aufzuzeigen, die die Anpassung an extreme Lebensräume ermöglichen. Außerdem wird versucht, einen Zusammenhang zwischen diesen Anpassungen und dem Diploidisierungs-Prozess aufzuzeigen. Dazu wurden zwei Paare von Schwesterarten mit gegensätzlichen Chromosomenzahlen und Habitatpräferenzen, künstlichem Trockenstress ausgesetzt. Die mRNA-Profile während der Dürre-Erholung nach anschließender Wiederbewässerung in Blatt- und Wurzelgewebe wurden mithilfe von RNA-seq erfasst. Daraus resultierende “Illumina reads” wurden auf dem Referenzgenom von *N. benthamiana* angeordnet, um Expressionsmuster von gestressten (Drought) mit denen von regelmäßig gegossenen (Control) Pflanzen für jeder Art zu vergleichen, wodurch differentiell exprimierte Gene (DEGs) identifiziert werden konnten. Diese Arbeit konzentriert sich auf die Analyse von Blattproben einer Artengruppe. Für die anfällige Art *N. excelsior* (n = 20) wurden 458 signifikante DEGs identifiziert, während die an Trockenheit angepasste *N. truncata* (n = 18) 670 DEGs zeigte. Die Verteilung dieser Gene wurde auf dem Referenzgenom kartiert, um potenzielle Kopplungsgruppen zu identifizieren. Schließlich zeigte eine “Gene Ontology (GO)" Analyse, dass Gene im Zusammenhang mit Phytohormonen, biotischer Abwehr und Zellwand Organisation in *N. excelsior* hochreguliert und in *N. truncata* herunterreguliert waren. Diese Ergebnisse deuten, trotz Wiederbewässerung auf eine anhaltende Stressreaktion und mögliche irreversible Schäden bei *N. excelsior* hin. Im Gegensatz dazu, legen die Ergebnisse für *N. truncata* eine schnelle Erholung nahe, die möglicherweise erforderlich ist, um vom seltenen und unvorhersehbaren Regen in offenen Wüstenhabitaten zu profitieren.

Introduction

Drought adaptation and desert plants

Drylands make up more than 40% of the total land area on Earth. According to Burrell *et al.* (2020) 6% became deserts between 1982 and 2015. The consequences are soil degradation, loss of biodiversity, migration, and human conflicts over scarce resources. Desertification, the spread of deserts into arid and semiarid areas, is further exacerbated by climate change and human activities such as excessive irrigation, deforestation, and the expansion of agricultural lands (Martínez-Valderrama *et al.,* 2020; Alsharif *et al*., 2020). Efforts to limit further desertification through reforestation have been largely unsuccessful, mostly due to ignorance of the complex biology of desert plants or simply use of inappropriate plants (Blilou & Hirt 2023). With expansion of arid lands, rising temperatures, intensifying drought periods and a still growing global population, research on drought-adapted plant species is gaining importance (Martínez-Valderrama *et al.,* 2020).

Deserts represent a hostile environment to the majority of living organisms, allowing only specialized species to thrive. To endure the harsh conditions of scarce water, drastic temperature fluctuations, relentless solar exposure, nutrient depletion, and often high salinity, desert plants have developed a range of morphological and physiological adaptations. Across the plant kingdom, several lineages evolved strategies to either endure these harsh conditions or avoid unfavorable seasons by adjusting their phenology (Alsharif *et al.,* 2020; Johnson *et al.*, 2022).

Perennial drought-adapted plants tend to expose reduced surface area with a low stomatal density and thick layers of cuticular wax, serving the purpose of minimizing water loss through transpiration (Kruse *et al.,* 2019). Additionally, leaf and stem succulence in combination with an expansive root system can be observed to varying degrees in many desert plants (Shishkova *et al*., 2013; Alsharif *et al.,* 2020). Physiological adaptations include accumulation of antioxidants and osmolytes as well as C4 or CAM photosynthesis instead of the less water efficient C3 pathway (Talbi *et al*., 2015; Iovieno *et al*., 2016; Li *et al*., 2019).

This master's thesis will be part of a project that studies drought adaptation in the Australian *Nicotiana* sect*. Suaveolentes*. The species examined in this study exhibit minimal

morphological adaptation for drought resistance, despite being distributed in the arid interior of Australia. Instead, they adopt an ephemeral strategy. Prompted by an undiscovered combination of rainfall and temperature, these plants swiftly initiate germination and growth, channeling resources predominantly first to roots and then to leaves, stems, and flowers. As water availability decreases, they flower, desiccate and leave seeds in the soil until subsequent appropriate environmental events (Chase *et al*., 2021a). Morphologically, desert dwelling plants in the *Nicotiana* sect. *Suaveolentes* are very similar to their relatives inhabiting wetter coastal regions. Shared features include herbaceous and often tall growth in addition to relatively broad, thin leaves.

How these plants can endure extreme, exposed conditions of Australian deserts from germination to seed set is still mostly unknown and offers a unique perspective on survival in arid environments. The ephemeral lifestyle not only underscores flexibility of plants to respond to unpredictable weather events but also sheds light on the balance between resource allocation, growth and reproduction.

Plant genome evolution

In an evolutionary context, plant genomes are dynamic systems constantly undergoing transformations that, permit plants to evolve and diversify in different environments over time. These changes include alterations in genome size, shifts in chromosome numbers, and chromosomal rearrangements. Within the plant kingdom, genome sizes can range from 10 Mbp in algae up to an astonishing 149 Gbp in angiosperms. Notably, genome size and chromosome number are not inherently associated with the complexity of the organism and can even vary significantly between closely related species (Blommaert 2020). Although the number of genes increases gradually with the accumulation of duplicated genes as a result of whole-genome duplication (Prooste *et al*., 2009), genome size is predominantly influenced by accumulation of repetitive DNA, particularly transposable elements (TEs) and tandem repeats (Lim *et al*., 2007; Lynch & Conery 2003). Although the prevailing evidence suggests that the accumulation of non-coding DNA has a neutral or only mildly negative impact on fitness (Petrov 2002; Grover & Wendel 2010; Arkhipova 2018), observations indicate that in environments characterized by extreme nutrient limitation, the evolution of small, streamlined genomes is favored (e.g., Kang *et al*., 2015; Bales & Hersch-Green 2019).

Another major factor responsible for genome alteration among plants are whole-genome duplications (WGD) or polyploidy. Throughout plant evolution, there have been recurrent cycles of WGD, succeeded by diploidization (Dodsworth *et al*., 2016b; Landis *et al*., 2018; Escudero & Wendel 2020). In higher plants approximately 64-79% of the protein-coding genes are part of multigene families, in contrast to around 40% for green algae (Prooste *et al*., 2009). This can be explained by the selective retention of duplicated genes post-WGD, coupled with neofunctionalization and subfunctionalization. Therefore, polyploidization is closely tied to subsequent innovations in physiology, ecology, and morphology, enabling the colonization of previously unoccupied niches (Van de Peer *et al*., 2017; Soltis *et al.,* 2010). Many instances of whole-genome duplication (WGD), linked to adaptive radiations, have occurred during periods of environmental instability and are often closely linked to mass extinction events (Arrigo & Barker 2012). Polyploidization is commonly directly associated with speciation, as gene flow between diploid progenitors and their polyploid descendants rarely occurs (Bowers & Patterson 2021; Soltis *et al.,* 2010). This reproductive isolation frequently coincides with shifts from cross-pollination to self-pollination (Van de Peer *et al*., 2017). Although newly formed polyploid populations frequently experience severe bottlenecks associated with high extinction rates, they often outcompete their diploid relatives in unstable or extreme environments (Arrigo & Barker 2012).

Allopolyploidy, resulting from hybridization and WGD, introduces an additional layer of complexity. In allopolyploids, homologous genes originate from species that have already undergone divergence, providing a higher adaptive potential compared to autopolyploidy. The merging of two genomes can lead to the creation of novel allelic combinations, often resulting in more extreme phenotypes due to heterosis (Dodsworth *et al*., 2020a; Van de Peer *et al*., 2017; Soltis *et al*., 2010). Following polyploidization, genome restructuring because of genomic shock (Dodsworth *et al*., 2020a).

Diploidization, the process of reducing chromosome numbers and genome downsizing to the diploid state, as well as genome downsizing, represent a widespread long-term biological response to WGD (Escudero & Wendel 2020; Parisod *et al*., 2012). The increased loss of DNA in polyploids can be predominantly attributed to higher rates of illegitimate recombination compared to diploid relatives (Leitch *et al*., 2008). Genome upsizing has also been observed in some cases, especially in the short term (Dodsworth *et al*., 2016a; 2020a).

RNA sequencing

RNA sequencing is a technique utilized for the analysis and quantification of the transcriptome, providing insights into gene expression levels, alternative splicing, and identification of novel transcripts (Geraci *et al*., 2020). It is especially useful for obtaining insights within experiment designs that include rearing plants with distinct genotypes (i.e., biological replicates) in different conditions, such as drought stress versus control conditions. With its capability to offer a comprehensive and high-throughput analysis of the transcriptome, RNA sequencing has advanced the understanding of drought resistance in recent years (e.g., Ye *et al*., 2018; Szukala *et al*. 2023).

This master's thesis employs an experimental approach, subjecting plants to controlled drought followed by rewatering and capturing their gene expression profiles through RNA sequencing in both root and leaf tissues. The goal is to reveal crucial genetic pathways associated with drought and subsequent recovery, including water conservation, stress signaling, and metabolic adjustments. Notably, the analysis of both root and leaf tissues is essential, as expression patterns between tissues often exhibit greater variation than those observed between different species.

Similar drought experiments have been conducted for economically important crop plants like rice, sorghum, cotton and maize (e.g., Thatcher *et al*., 2016; Bowman *et al*., 2013; Fracasso *et al*., 2016; Yoo *et al*., 2017). Although insights gained from these studies already form a solid basis for better understanding of drought resistance, only few actual desert plants have been analyzed in this manner. Studies that did work on desert plants focused on perennial, woody plants like date palm, sea buckthorn or welwitschia (Ye *et al*., 2018; Kruse *et al*., 2019; Wan *et al*., 2021; Ksouri *et al.,* 2016). Therefore, strategies that ephemeral plants employ to withstand the harsh desert climate and how they react to renewed availability of water after a prolonged drought period, are mostly unknown.

Nicotiana

Nicotiana is the fifth largest genus of Solanaceae with its center of origin in South America (Chase *et al*., 2018; Ladiges *et al.* 2011). Beyond the economic importance of tobacco, members of the genus are model organisms for plant-herbivore (e.g., Bandoly *et al*., 2016) and plant-pathogen interactions (e.g., Ishihama *et al.*, 2011), as well as for genome evolution, polyploidization, and evolution of the repeatome (e.g., Dodsworth *et al*., 2016a; Chase *et al*., 2023; Kelly *et al*., 2012). *Nicotiana* comprises eight diploid and five allotetraploid sections, which encompass the majority of species diversity (Knapp *et al.,* 2004; Marks *et al.,* 2011). Twelve of the sections are endemic to the New World. Only the largest section *Suaveolentes*,which originated in South America, is currently not native there (Chase *et al*., 2018).

Diploid sections of the genus predominantly possess a haploid chromosome number of n = 12 with few exceptions of n = 9 or 10 (Marks *et al.,* 2011). Allopolyploidization has resulted in the emergence of five extant sections from multiple diploid progenitors at different times between 20 000 years - 6 myr ago (Leitch *et al*., 2008). The presence of old to relatively recent allopolyploids that are at different stages during the post-polyploidization processes makes *Nicotiana* an ideal study system (Parisod *et al*., 2012; Dodsworth *et al.*, 2020a).

Despite a stable chromosome number in all sections apart from sects. Alatae and *Suaveolentes*, allopolyploidy in *Nicotiana* has been accompanied by a multitude of genetic alterations (Clarkson *et al*., 2005). Alterations include rDNA homogenization and the loss of loci, intergenomic translocations, as well as modifications in the copy number and organization of TEs up to near complete genome turnover (Leitch *et al.,* 2008; Lim *et al*., 2007; Renny-Byfield *et al*., 2011; Dodsworth *et al*., 2020a).

Nicotiana section Suaveolentes

Early cytological and taxonomic investigations of the section were initiated by Goodspeed in 1945. He observed variation in chromosome numbers and interpreted them as an indication for potential multiple origins of the section. However, recent studies indicate a clear monophyletic status of this section (e.g., Marks *et al.,* 2011). Over the past two decades, *Nicotiana* sect. *Suaveolentes* has captured the interest of evolutionary biologists.

Advancements in molecular genetic techniques have revealed various aspects of their evolution, genomics and ecology, although there are still many open questions.

In the past, species-level taxonomy has posed challenges for this group of, white-flowered, mostly annual herbs, as species exhibit limited distinct morphological characteristics and different DNA datasets yielded conflicting or unresolved results (Chase *et* al., 2003; Marks *et al.,* 2011; Dodsworth *et al.*, 2020b). Only recently a well resolved/supported phylogenetic hypothesis for the section was established using RADseq (Chase *et al.,* 2023a). Through extensive field expeditions spanning ten years and ex-situ cultivation coupled with DNA analysis, species that were previously overlooked have been delineated. In less than a decade, the number of recognized species within the section has doubled (Chase *et al.,* 2018; 2021a; 2022; 2023b). Out of the 91 currently acknowledged *Nicotiana* species (POWO 2023), a notable 42 belong to the section *Suaveolentes*, with ongoing research suggesting the presence of even more undescribed species (Chase *et al*., 2023b). Consequently, more than half of the species in the genus are members of section *Suaveolentes.*

Allotetraploid *Nicotiana* sect. *Suaveolentes* is hypothesized to have originated in South America approximately 6 million years ago. It later reached Australia through long-distance dispersal, after the aridification of the continent, where it appears to have diversified mostly through dispersal (Cauz *et al*., submitted). This expansion was likely initiated by the occupation of wetter coastal regions, with the arid interior of the continent being colonized later (~2 mya) (Dodsworth *et al.*, 2020b; Chase *et al*., 2023a). Members of the section are now widely distributed across Australia, especially in the arid interior. Additionally, the section includes three species found on Pacific islands and a singular African taxon in Namibia (Marks *et al*., 2011).

Kelly *et al.* (2012) attempted to reconstruct the evolutionary origin of the section and identified the pollen parent as a member of sect. *Sylvestres*. The maternal parent was likely of hybrid origin (now extinct) between species of *sects. Petunioides* and *Noctiflorae*. An allotetraploid descendant of these two parental lineages (both = 12) should exhibit a haploid chromosome count of n = 24. In several cytological studies (e.g., Marks *et al.,* 2011; Chase *et al*., 2023), this or numbers close to that ancestral chromosome count (i.e., n = 24-22) were predominantly found in early diverging taxa of *sect. Suaveolentes*, which are confined to wet coastal regions of Australia, Pacific islands and Namibia (Ladiges *et al.*, 2011; Chase *et al*.,

2023). Within the major clades of the section, independent decreases of chromosome numbers are observable. The lowest chromosome numbers (n = 19, 18, 16 or 15) are only found in the most derived lineages, that curiously mostly occur in the arid interior of Australia (Chase *et al*., 2023). This decrease in chromosome number is a product of dysploid reductions and is putatively related to adaptation to desert habitats. Theories suggest that the diploidization helps to reduce expenses for the organism while protecting favorable allele combinations from recombination with other alleles, and maintaining the advantages of multiple sets of genes, through processes like neofunctionalization. Thus, diploidization hypothetically increasing adaptive potential (e.g., Kelly *et al*., 2012; Chase *et al*., 2023a). Lower chromosome number in plants was shown to be generally favored in drought prone habitats and in general in resource-poor environments (Carta *et al.*, 2018).

According to Chase *et al*. (2021a), a key characteristic many desert-dwelling species within this section is their rigorous control over germination. This ensures that germination occurs only when favorable conditions for flowering and seed set are present. Notably, this strict germination control is absent in related species inhabiting wetter habitats in the northern and eastern coasts and mesic habitats in the interior (gorges, south-facing cliffs and other shaded sites). Furthermore, the dry-adapted species can adjust their reproductive efforts in response to unfavorable conditions, producing fewer, cleistogamous flowers to conserve energy (Chase *et al*., 2022).

Another contributing factor to divergence is the prevalence of self-pollination (Cauz-Santos *et al*., 2021). Self-pollination and limited gene-flow predispose populations in *N.* sect. *Suaveolentes* to diverge rapidly from each other. Coupled with the availability of unoccupied niches in the expansive landscape of Australian deserts, these factors likely contribute to the rapid radiation of the desert clades of the section (Chase *et al.,* 2021a; 2022).

Study objects and aims

Although karyological analyses in combination with phylogenetic and biogeographic inferences have already given insights into the evolutionary processes behind WGD and diploidization in the section, the advantages that individuals gain from these processes remain largely unexplored. The project in which this master's thesis takes place aims to shed light on the transcriptomic response to drought stress in two pairs of closely related Nicotiana species

with contrasting chromosome numbers and drought responses. The compared species *N. gossei* with *N. velutina* (n = 16 and n = 18, respectively) and *N. excelsior* with *N. truncata* (n = 18 and n = 20, respectively; Chase *et al*. 2023) were chosen because both pairs exhibit variation in chromosome numbers and habitat preferences. *Nicotiana gossei* and *N. excelsior* both inhabit mesic sites in the (partial) shade of cliffs or trees, while their respective sister species grow in highly exposed habitats in open desert.

Nicotiana excelsior is found in central southern Northern Territory and northern South Australia, close to the geographical center of Australia. There it often grows in rocky gullies and streambeds, protected by various *Acacia* species. It typically grows in sandy soil, benefiting from fertilization by animals that shelter under *Acacia* trees. In this region of Australia, the annual rainfall averages approximately 250 mm, predominantly occurring during the winter months. *Nicotiana excelsior* is a short-lived perennial reaching heights of up to 250 cm. It initially forms a loose rosette and then swiftly transitions to a more developed state with a main stem with long branches. Leaves are arranged along the stems and reach lengths of up to 17 cm (Chase & Christenhusz 2018).

Nicotiana truncata has a narrow distribution in open areas within dry creek beds characterized by gypseous, slightly saline soils of northern South Australia. The yearly precipitation there averages 175 mm, making it one of the driest regions of Australia. In the wild, the species is an ephemeral, but in cultivation it can be a short-lived perennial. *Nicotiana truncata* forms a well-developed rosette and produces several nearly leafless inflorescences simultaneously. The leaf lamina is lanceolate and can reach 25 cm (Chase *et al*., 2021b).

Through comparison of gene expression between drought-exposed and control plants of each species, we aim to identify those genes that play a role in drought resistance, and in particular in the phase of recovery after drought periods. For the purpose of this thesis, only leaf samples from *N. excelsior* and *N. truncata* were analyzed. Given the contrasting habitat preference *N. truncata* is expected to show a well coordinated response to drought, followed by rapid recovery upon rewatering. In contrast *Nicotiana excelsior* is expected to react in a more chaotic way with massive transcriptional changes during drought and a slow recovery afterwards. Beyond the scope of this MSc thesis, a better understanding of how changes in chromosome number and genome architecture drive the colonization of extreme habitats will be achieved through the comparison of the two pairs of sister species.

The specific aims of this thesis are to: 1) compare the quantity and ratio of up- and downregulated differentially expressed genes identified in each species, with the hypothesis that the drought-resistant species will show more rapid recovery after drought and therefore less DEGs compared to the drought sensitive species; and 2) identify sets of genes and functions that exhibit opposite expression trends between the two species during recovery from drought, given their expected differential physiological reactions during and after drought stress.

Material and Methods

Drought experiment

The four investigated *Nicotiana* species were grown for 10 weeks starting from wild-collected seeds in a PERCIVAL climate cabinet under the same conditions (12-hour light cycle, with 20 °C during day and 16 °C during the night). Before harvest, individuals under drought conditions were no longer watered for 10 days, while control plants were watered normally every 3 days with sufficient amounts of water (30ml per pot) to maintain soil moisture. Tissue fixations were undertaken mid morning, 24 hours after watering all accessions, to investigate the recovery capacity of each species after drought stress. Per species and treatment leaf and root samples of six individuals were snap frozen in liquid nitrogen, and stored at -80°C, for a total of 96 samples.

RNA-extraction

For the extraction of total RNA, the RNeasy Plant Mini Kit (QIAGEN) was employed following the manufacturer's manual, with an input of 70-90 mg of frozen leaf/root tissue per accession. The tissue was ground into a fine powder using a mortar and pestle while frozen by repeatedly adding liquid nitrogen. Subsequently, the frozen tissue powder was transferred to a tube with RLT-Buffer and β-mercaptoethanol. The lysate was vortexed and transferred to a QIAshredder spin column for debris removal. Ethanol (96%) was added to the cleared lysate to facilitate RNA precipitation, and the sample was transferred to an RNeasy Mini spin column. On-column washings were performed using RW1 and RPE Buffers, and RNA was finally eluted with 30 µl RNase-free water. To increase the RNA concentration, the elution step was repeated once. The estimation of RNA concentration and purity was carried out with a Nanodrop Photospectrometer, and RNA integrity was determined with a Bioanalyzer (Agilent). Only extracts with an RNA integrity number (RIN) ≥ 7 were used for library preparation.

Library preparation

The NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) was used for isolation of mRNA from total RNA extracts. Preparation of libraries was performed using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760) with Unique Dual Index UMI Adaptors RNA Set 1 (NEB #E7416). The protocol was adjusted for larger inserts (450 bp instead of the standard 200 bp), and thus fragmentation time at 94°C was decreased from 15 to 7 minutes. Also, during "first strand cDNA synthesis", the incubation time at 42°C was increased from 15 to 50 minutes. After the ligation reaction, 25 µl SPRI beads were used for size selection instead of 70 µl. Only 4 µl NEBNext Primer Mix was added to the PCR reaction instead of 5 µl. To compensate for the decrease in yield of cDNA through size selection 17 PCR cycles were performed. For the purification of the PCR reaction, 40 µl SPRI-beads were used instead of 45 µl.

The final libraries were checked on a Bioanalyzer and quantified with a Qubit. After normalization, the libraries were pooled in four pools, each sequenced on one lane of an Illumina NovaSeq S4 machine as 150 bp pair-end reads. NGS sequencing was performed at the Genome Centre of Queen Mary University of London, UK.

Data analysis

The preparation of raw Illumina reads for subsequent analysis adhered to a pipeline outlined on the website of the Plant Ecological Genomics group at the University of Vienna: https://plantgenomics.univie.ac.at/rnaseq-introcourse/. Steps requiring high computational power were executed on the LISC cluster of the same University: https://lisc.univie.ac.at/.

Quality assessment and filtering

To identify potential sequencing errors and contamination, quality assessment using FastQC (Brown *et al*., 2017) was performed on the sequence data. The filtering with Trimmomatic v. 0.36 (Bolger *et al*., 2014) was performed with the following parameters: LEADING:13; TRAILING:13; SLIDINGWINDOW:4:20; MINLEN:36.

Mapping and differential expression analysis

The mapping of RNAseq reads is a critical step in analyzing gene expression profiles. For this purpose, a dedicated splice-aware aligner STAR v. 2.7.11a (Dobin *et al*., 2013), was employed. The alignment of sequences was conducted with the *N. benthamiana* draft genome v.3.6, available from https://www.nbenth.com (Ranawaka *et al.*, 2023). This reference genome comprises 20 scaffolds, in which 19 are the pseudomolecules designated as the species chromosomes 1-19, and the remaining small sequences concatenated and denoted as "chromosome 0."

The FeatureCounts tool within the Subread package v. 2.0.6 (Liao *et al*., 2014) was employed to quantify the number of reads mapped to exons. Subsequently, exons counts were summarized to give the read count per gene. Despite using the -p option to declare the data as pair-end, the counting was performed for reads rather than pairs. The output, a count matrix, with the number of reads associated with each gene was then used for the differential expression (DE) analysis. The R-package edgeR v. 4.0.14 (Robinson *et al*., 2010) was employed to calculate the log2 fold changes, p-values, and adjusted p-values (FDR) for each gene in comparison between control and drought samples per species and tissue. Log2 fold changes were calculated using the control group as a baseline.

The R-library ggplot2 v. 3.4.4 (Wickham 2011) was used to visualize differential gene expression analysis results as a Volcano plot. Only genes with log2 fold change above or equal to 2 and FDR-values below 0.05 were used for further downstream analysis. The R-package DESeq2 v. 3.18 (Love *et al*., 2014) was used for creating PCA-plots.

Venn diagram

The Venn diagram was used to visualize the overlap of differentially expressed genes (DEGs) between the two species. Therefore the VennDiagram package v. 1.7.3 (Chen & Boutros 2011) was utilized in R.

Gene position heatmap

A heatmap-style plot visualizing the positions of genes on the chromosomes of *N. benthamiana* along with their log-fold change values was created in R, using ggplot2 v. 3.4.4. Only differentially expressed genes with a logFC > 2 and FDR < 0.05 were plotted.

Gene Ontology enrichments

The gene ontology (GO) enrichment analysis, performed with TopGO v. 2.42.0 (Alexa *et al.,* 2006) involves comparing the functions (i.e., GO terms) of a list of differentially expressed genes to those of the background gene list of the entire genome. This process determines if specific pathways are particularly affected by the drought treatment by checking the fraction of genes annotated to a particular GO-term and comparing it to the proportion of genes in the entire genome annotated to the same term. The resulting p-value indicates the probability that the pathway is overrepresented in the list of differentially expressed genes (Alexa *et al.,* 2006). Terms were considered significant when the adjusted p-value was < 0.1. The graphical visualization was performed in ggplot2 v. 3.4.4 in R. Depicted GO-terms from the biological process (BP) category were chosen from the entire table based on their relation to drought response.

Results

After quality filtering on average 74 649 551 pairs of reads per sample were kept (Table 1) for the leaf samples of *N. excelsior* and *N. truncata.* Reads aligning to the reference genome were classified into two groups: uniquely mapped reads, indicating those mapping to a unique position in the reference genome, and multi-position matches, representing reads mapping to more than one position in the reference genome. Per sample on average approximately 65 966 246 pairs of reads (~88% of the filtered input pairs) were uniquely mapping to the reference genome. Only these reads were used for further analysis. Mapping rates of the two species have similar values, indicating no mapping bias resulting from different genetic divergence towards the reference genome of *N. benthamiana*. The "Mismatch rate per base %" represents the percentage of bases that deviate from expected in the reference genome. A higher mismatch rate suggests more discrepancies between the sequenced sample and the reference, potentially indicating sequencing errors or genetic divergence. In the leaf samples of the two investigated species the mismatch rate was on average 1.25%. In featureCounts per sample on average 117 892 533 reads were uniquely assigned to features of the reference genome (see Table 1).

Table 1: *List of the 24 samples analyzed and their respective results. Sample ID consists of species:* N. excelsior *(E) or* N. truncata *(T); treatment: control (C) or drought (D); tissue: leaf (L); and sample number.*

Sample ID	No. reads after filtering	Uniquely mapped reads %	Mismatch rate per base %	Assigned alignments to genomic features
ECL49	118 896 390	88.89%	1.25%	97 753 739
ECL50	157 753 970	88.97%	1.23%	129 394 010
EDL51	115 025 668	89.32%	1.22%	95 266 241
EDL52	165 609 202	88.94%	1.28%	136 955 494
EDL53	133 231 694	89.70%	1.21%	111 707 432
EDL54	110 552 402	90.10%	1.23%	93 081 365
ECL55	134 602 844	89.02%	1.23%	110 953 245
ECL56	121 614 600	88.77%	1.22%	99 726 632
ECL57	139 249 936	86.17%	1.18%	110 353 625
EDL58	171 896 964	86.97%	1.16%	138 411 507
ECL59	162 528 804	89.14%	1.22%	133 032 969
EDL60	147 609 182	89.57%	1.16%	123 474 349
TDL73	140 880 080	87.70%	1.34%	113 770 496
TCL74	171 419 288	87.89%	1.28%	138 329 342
TDL75	115 937 926	88.85%	1.31%	95 492 425
TCL76	202 785 654	86.23%	1.26%	161 059 165
TDL77	157 540 816	88.28%	1.21%	129 189 349
TCL78	125 716 410	88.03%	1.27%	101 585 969
TDL79	117 886 014	87.55%	1.24%	95 247 660
TCL80	144 249 916	87.38%	1.30%	115 406 371
TCL81	129 918 188	88.47%	1.25%	106 272 487
TDL82	149 606 594	89.02%	1.27%	123 552 644
TDL83	128 652 082	86.52%	1.25%	102 839 807
TCL84	210 752 054	86.10%	1.25%	166 564 468

Principal component analysis (PCA)

The PCA plot (Figure 1) shows a prominent difference in expression patterns between the two species. However, for both species an effect of the treatment could be observed when they were plotted separately.

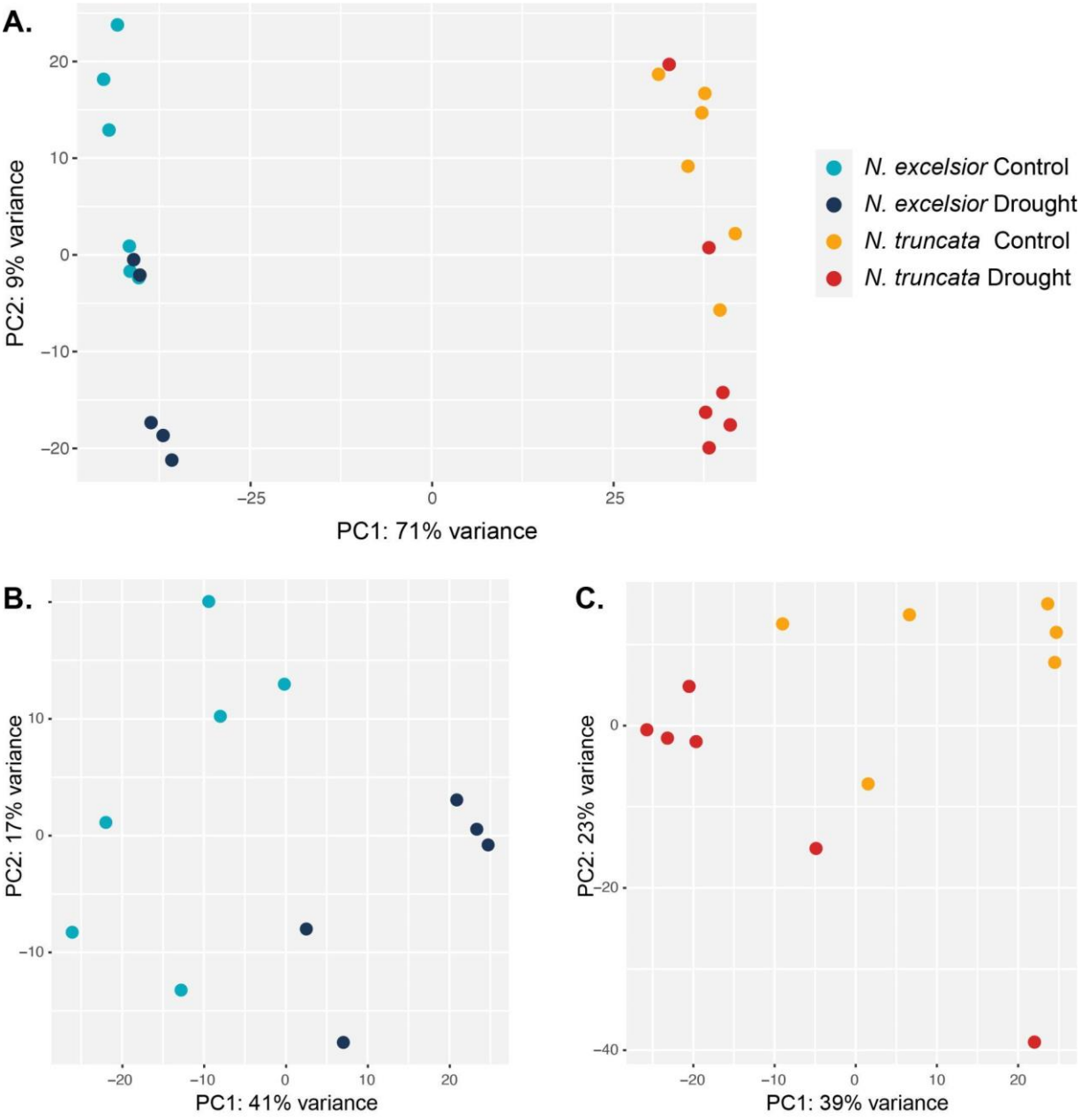


Figure 1: *PCA plots comparing expression patterns for all samples of both species (A), only samples of* N. excelsior *(B), and only samples of* N. truncata *(C).*

Differentially expressed genes (DEGs)

After applying stringent filtering by removing all genes with adjusted p > 0.05 and log2FC < 2 from the 30,296 genes, we retained 670 genes as confidently differentially expressed (DE) between the treatments in *N. truncata* (Figure 2). Of the 670 DEGs, 513 were downregulated for the drought-treated samples, while only 157 genes were upregulated compared to controls. In *N. excelsior*, from 30,290 genes that had mapped reads only 458 DEGs were retained after filtering. Of those, 209 were downregulated for the accessions that recovered from drought in comparison to controls, while 249 were upregulated. The log2 Fold Change (FC) in *N. excelsior* ranged from -8.4 to 9.6. In *N. truncata* the log2FC even ranged from -11 to 9.8 as seen in the volcano plot (Figure 2). A log2FC means a four-fold change between control and drought, while a log2FC of -11 translates to a 2,048 times higher read count mapping to exonic regions of a gene in control than in drought-treated samples.

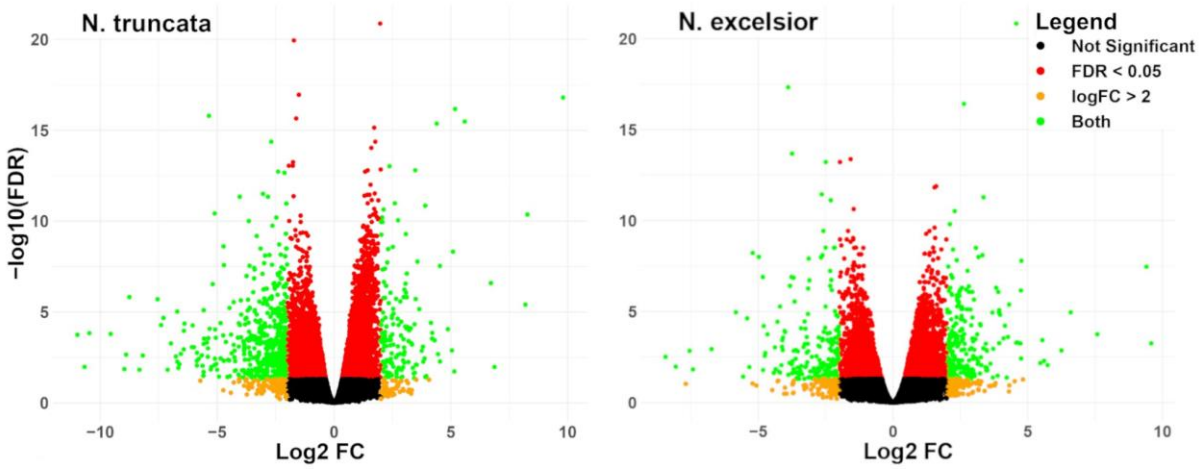
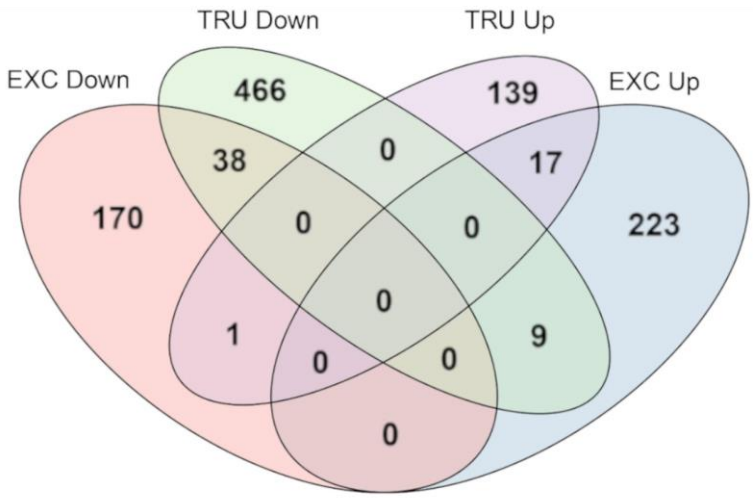


Figure 2: *Volcano plot of DEGs between drought-treated and control samples in* N. truncata *(left) and* N. excelsior *(right). Green: candidate DEGs; Red: significant but log2FC < 2; Orange: logFC > 2 but not significant; Black: log2 FC < 2 and not significant.*

The Venn diagram (Figure 3) shows the overlap of up- and down-regulated DEGs between the two species. Several areas with zero genes in the diagram were expected since a gene could be either “Up” or “Down” within a species. Ten genes were regulated in opposite directions between species. Seven of the nine genes that were upregulated in *N. excelsior* and downregulated in *N. truncata* were transcription factors (Figure 3). From the other two genes one is related to iron homeostasis and the other one has no functional annotation. The single gene that had the opposite expression pattern is related to Ca^{2+} signaling.



ExcUp_TruDown

gene.ID	Protein
NbL18g17540	cytochrome b561 containing protein
NbL07g16620	WRKY transcription factor 40
NbL03g20130	WRKY transcription factor 40
NbL17g05520	WRKY transcription factor 50
NbL04g09340	WRKY transcription factor 51
NbL19g07440	NAC domain-containing protein 68
NbL14g09290	uncharacterized protein
NbL02g19900	pathogenesis-related tf PTI5
NbL10g05370	ethylene-responsive tf ERF027

TruUp_ExcDown

NbL19g04880	phospholipase A1-gamma-like

Figure 3: *Venn diagram indicating the overlap of up- and downregulated DEGs between* N. truncata *and* N. excelsior*. The table shows gene IDs and predicted functions of DEGs that were upregulated in one species and downregulated in the other.*

Figure 4 shows the distribution of up- and downregulated DEGs on the 19 chromosomes of the reference genome of *N. benthamiana*. Just visually, there were more clusters in *N. truncata,* whereas the genes in *N. excelsior* seemed more evenly distributed, also considering the different amounts of DEGs. However, statistical tests, comparing distribution (in genes/Mbp per chromosome) of DEGs with all annotated genes from the reference genome yielded no significant clustering.

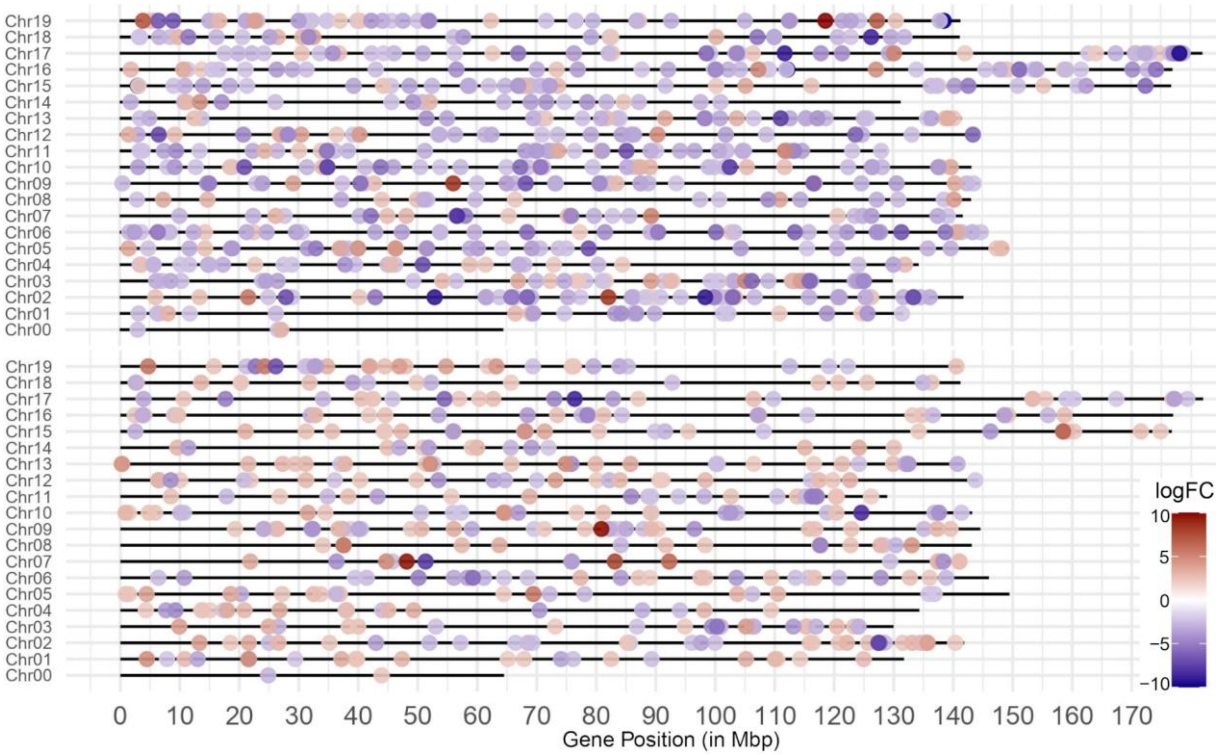


Figure 4: *Distribution of candidate DEGs of* N. truncata *(top) and* N. excelsior *(bottom) across chromosomes of* N. benthamiana*. Shades of red indicate upregulation, whereas shades of blue indicate downregulation according to the legend.*

Gene Ontology (GO) enrichment

Of the 670 DEGs in *N. truncate*, 458 genes were functionally annotated and had assigned BP:GO-terms. Of those only 325 were involved in significantly enriched terms. In the 448 DEGs of *N. excelsior*, 319 genes had corresponding BP:GO-terms, of which 275 were annotated to significantly overrepresented GO terms.

Go terms, depicted in the Figures 5-6 were selected to represent the entire data set from Supplementary Table S1-3. Terms related to oxidative stress were predominantly differential upregulated in both species. Most terms related to photosynthesis, storage metabolites (i.e., carbohydrates and lipids) and secondary metabolites (mainly terpenoids) were mutually downregulated. All other major categories showed opposite regulation between the two species.

Phytohormone signaling was among the categories with the highest number of DEGs, encompassing 61 genes in both species. Terms associated with abscisic acid, ethylene,

cytokinin and jasmonic acid were predominantly upregulated in *N. excelsior* but downregulated in *N. truncata*. Salicylic acid related genes were upregulated in *N. excelsior* and not enriched in *N. truncata*. Jasmonic acid signaling was downregulated in both species. Moreover, genes in the term regulation of DNA-templated transcription were mostly upregulated in *N. excelsior* and downregulated *N. truncata* with 51 and 88 DEGs respectively.

Terms related to cell wall organization and modification collectively contained 55 DEGs in *N. truncata.* Among these genes, there were xyloglucan endotransglucosylase/hydrolases (XTHs), COBRA-like proteins, pectin esterases and extensins, which were mostly downregulated. Expansins were predominantly upregulated. The 38 DEGs involved in cell wall related processes in *N. excelsior* were mostly upregulated XTHs. Lignin related genes were downregulated in both species.

Defense response related terms had exclusively negative Z-scores in *N. truncata*. In *N. excelsior* apart from the response to wounding, which was also downregulated, the functions related to defense response were mostly upregulated.

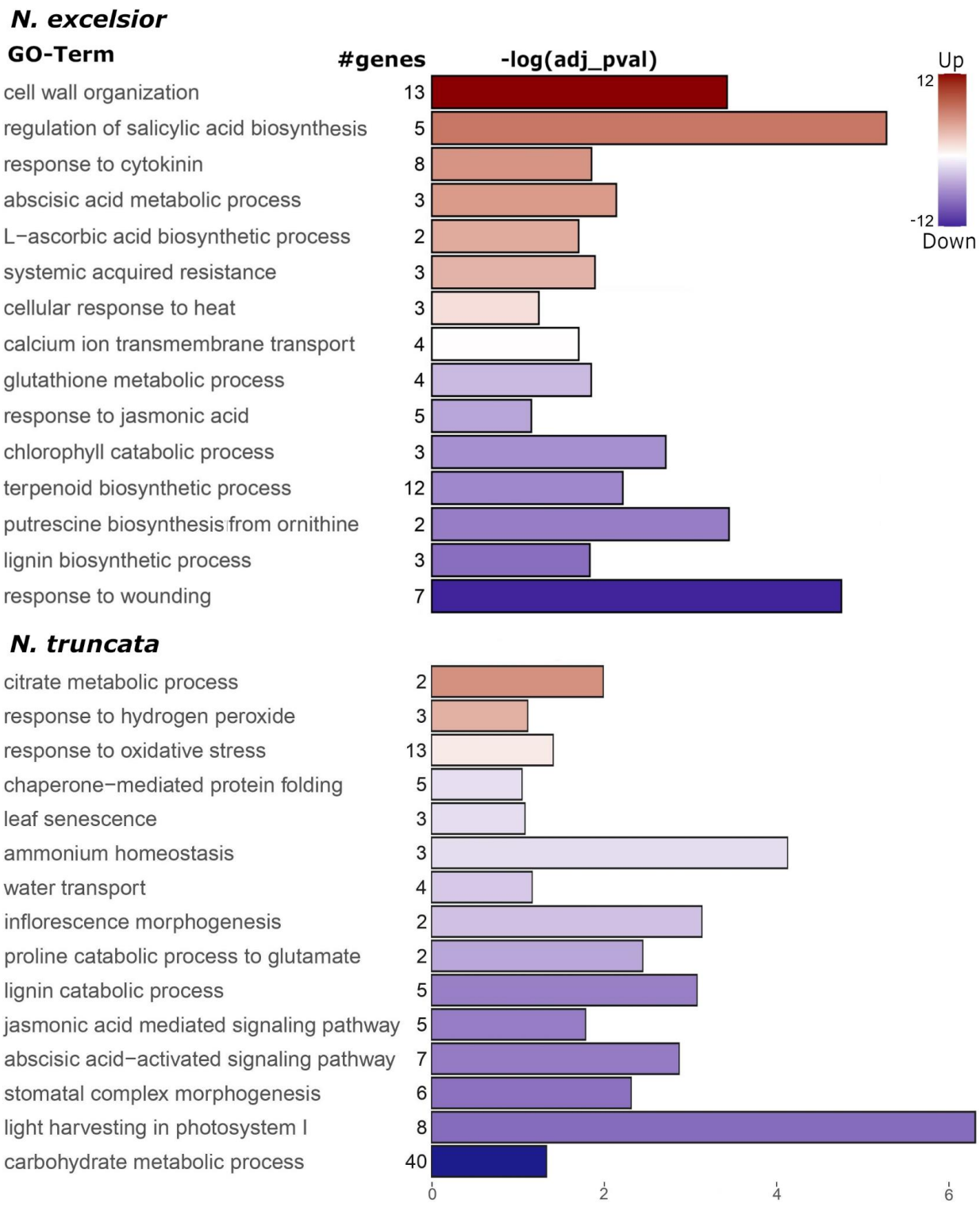


Figure 5: *Enriched GO categories that are differentially expressed and unique to* N. excelsior *(top) and unique to* N. tuncata *(bottom). Bar length (X-axis) corresponds to the -log(adjusted p-value) of category enrichment, while color indicates the Z-score according to the legend. Red signifies categories with more genes upregulated for drought-treated accessions compared to controls, whereas blue indicates categories with more genes downregulated. Pale colors indicate categories with a more balanced ratio of genes exhibiting up- and downregulation.*

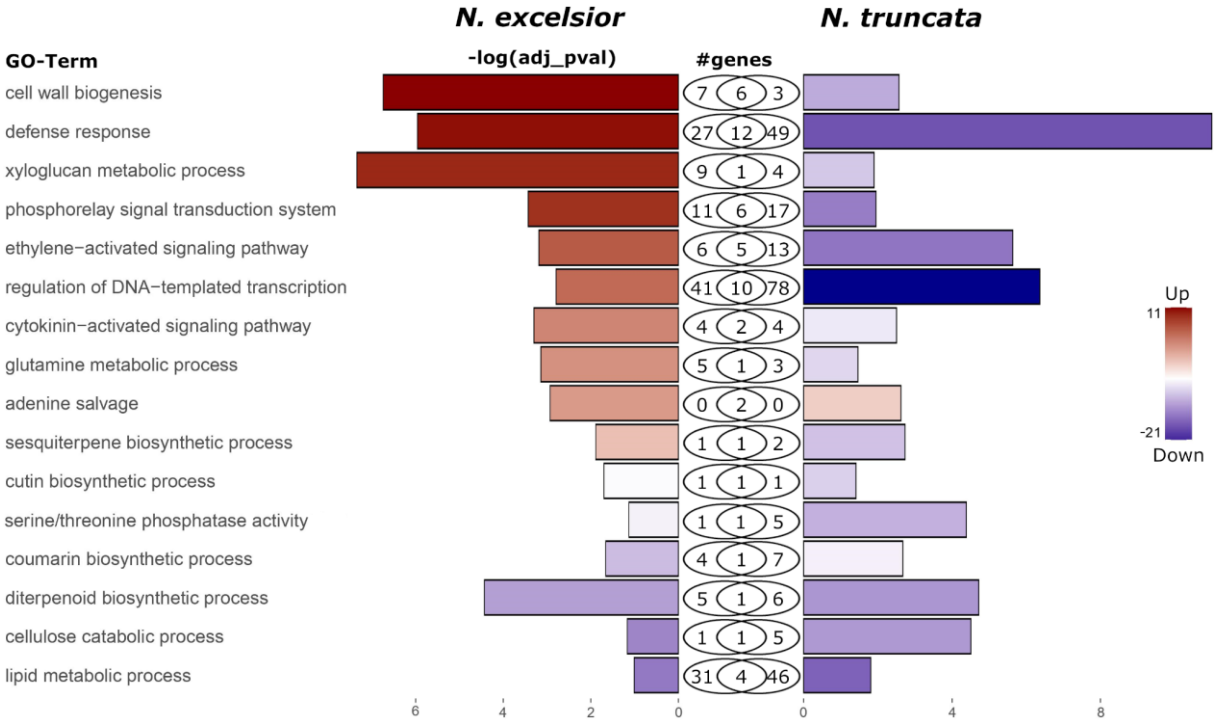


Figure 6: *Representation of enriched differentially expressed GO categories that are shared by both species. "Venn diagrams" contain the count of differentially expressed genes unique to* N. excelsior *(left) and* N. truncata *(right) and shared (middle) within each term. Bar length corresponds to the -log(adjusted p-value) of category enrichment, while color indicates the Z-score according to the legend. Red signifies categories with more genes upregulated in drought exposed samples compared to controls, whereas blue indicates categories with more genes downregulated. Pale colors indicate categories with a more balanced ratio of genes exhibiting up- and downregulation.*

Transcription factors

Genes in the GO-term regulation of DNA-templated transcription, which almost exclusively contained transcription factors (TFs) were assigned to their respective families. Most TF-families contained genes that showed an opposite regulation between the two species (Figure 7). Most notable NAC, WRKY and ERF factors were predominantly upregulated in *N. excelsior* and downregulated in *N. truncata*. Auxin responsive factors (ARF) were only downregulated in *N. truncata* and not found among DEGs of *N. excelsior*. The downregulation of bHLH transcription factors was found in both species.

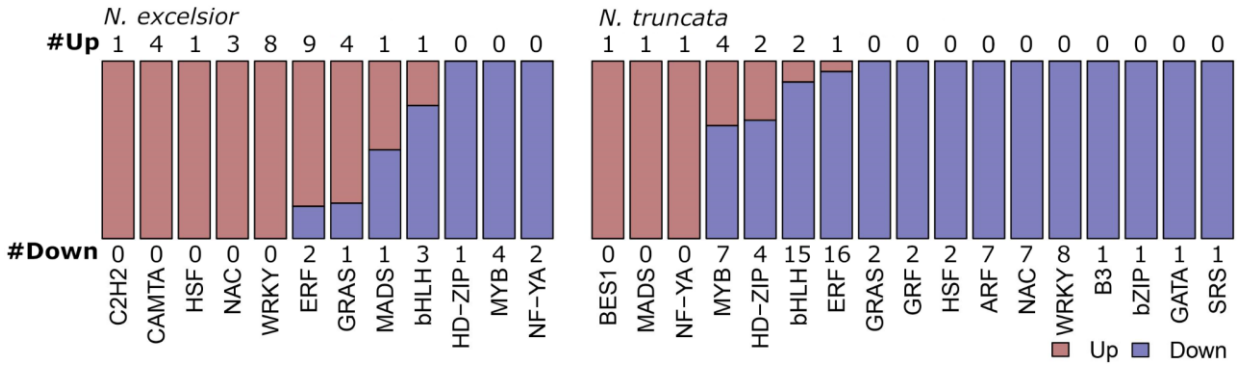


Figure 7: *Number and proportion of up- (red) and downregulated (blue) TFs within their respective families in* N. excelsior *(left) and* N. truncata *(right) between the drought-exposed and control accessions.*

Discussion

Remarks to data evaluation

Compared to other drought experiments with similar fold-change thresholds (e.g, Li *et al*., 2019: *Oryza sativa*; Arjmand *et al*., 2021: *Arabidopsis thaliana*) the number of DEGs in the presented results was relatively low, despite a high number of input reads. However, as plants were rewatered prior to sampling, the data more accurately reflect recovery from drought instead of direct effects of drought stress. The results from other studies, performing drought experiments followed by rehydration showed that mRNA profiles were approximated to control plants within few hours, as seen in Guo *et al*. (2023) and drought response has been vastly reverted (Huang *et al*., 2008; Iovieno *et al*., 2016). In the above-mentioned studies, recovering plants yielded only a fraction of DEGs compared to drought stressed plants.

The stringent nature of filtering steps during the analysis is also responsible for the low number of candidate DEGs. Studies have suggested (e.g., Shaar-Moshe *et al.,* 2015) that even genes with low logFC can have a big impact on the response to drought. A log2FC >2 might therefore filter out important pathways that could draw a broader picture of the mechanisms behind drought adaptation and acclimatization. That is why some RNA-seq studies use a log2FC threshold of 1 (e.g., Wang *et al*., 2015; Khan *et al*., 2019), even though a less strict approach also increases the “background noise” of drought-unrelated genes.

The biological interpretation of significantly enriched gene ontology (GO) terms and corresponding Z-scores should be approached with caution, as it does not lend itself to exact statements but rather implies general trends. A highly negative z-score, for instance, does not necessarily indicate downregulation of a specific function during recovery after drought. GO terms often provide broad functional descriptions, encompassing diverse pathways, some of which may be unrelated. Consequently, the specific functions performed by these genes and how their up- or downregulation affects the plant remain unknown. For instance, the downregulation of a protein serving as an inhibitor could potentially result in the upregulation of a particular function. Without knowledge over the identities of gene products within a GO term, any interpretation regarding their effects in relation to drought should be treated with caution.

Drought stress vs. recovery

The availability of water is crucial for almost every step of plant metabolism. Nonetheless, water is constantly lost through transpiration to facilitate photosynthesis. Consequently, a high number of genes associated with water deficit are conserved across the plant kingdom. As shown in a cross-species meta-analysis approach by Shaar-Moshe *et al.* (2015) drought response is a combination of adjustments of primary metabolism with lineage specific fine-tuning. Evolutionary conserved functions include production of cuticular wax, cell wall modifications and prolonged stomatal closure. Specific drought related alterations, coupled with limited resource availability, also result in differential regulation of housekeeping functions (Wang *et al*., 2017; Li *et al*., 2019; Alsharif *et al.,* 2020).

Irrigating water-deprived plants has been shown to promptly reverse most changes induced by drought stress, within a few hours (Huang *et al*., 2008; Guo *et al*., 2023). Although the present study does not provide results for actual drought stress, the observed drought recovery aligns well with findings reported in other studies, and therefore also allows conclusions about the drought response. After rewatering, many differentially expressed functions show opposite regulation than during drought, while the number of DEGs is much lower (e.g., Borràs *et al*., 2021; Teshome *et al*., 2023; Guo *et al*., 2023). After severe drought stress, physiological recovery lags transcriptomic changes (Iovieno *et al*., 2016). Fast recovery is especially important in environments with rare and unpredictable rainfall, where every second of water-availability is valuable. In mesic habitats, moisture is retained much longer, so plants are adapted to a constant supply of water.

Nicotiana truncata should react to drought in a more coordinated manner than *N. excelsior*. Fast and complete recovery was expected to cause expression profiles closer to well-watered plants and therefore yield fewer DEGs. Our results, however, reveals an opposite trend, which shows that the number of DEGs after rewatering does not accurately reflect the degree of drought resistance. Instead, the distinction of transcriptomic responses between resistant and susceptible species might be resembled by opposite up- and downregulation of DEGs *i.e*., more upregulated genes in *N. excelsior* and more downregulated genes in *N. truncata*. Similar trends can be observed in other RNA-seq studies on the response to rewatering after drought. Crop plants and model species not inherently adapted to extreme drought conditions had a similar ratio of up- and downregulated genes after rewatering as *N*. *excelsior* (e.g., Iovieno *et*

al., 2016: *Solanum lycopersicon*; Borràs *et al*., 2021: *Capsicum annuum*; Guo *et al*., 2023: *Vigna radiata*). Studies of presumably drought adapted plants showed patterns closer to *N. truncata* (e.g., Sun *et al.,* 2022: *Tamarix taklamakanensis*; Teshome *et al., 2023: Eucalyptus grandis*). A higher number of upregulated DEGs could be explained by a prolonged stress reaction that aims to prevent lethal damage in species that are not adapted to severe drought stress. Species anticipating low-water conditions might in contrast react with growth stagnation and are therefore less prone to drought damage, which also speeds up the post-drought recovery process. Accumulation of drought-related metabolites might suppress further biosynthesis after rewatering. In some cases, these genes then exhibit a higher level of expression in well-watered plants. Thus, there is opposite regulation between plants before and after rewatering (see Huang *et al*., 2008; Borràs *et al*., 2021; Teshome *et al*., 2023; Guo *et al*., 2023).

Regulatory functions

Independent of the stressor, in plants the initial perception of stress is mediated through the accumulation of reactive oxygen species (ROS) (Arjmand *et al*., 2021), which are produced in various cellular organelles result from the metabolic imbalance caused by environmental stress (Qi *et al*., 2018). ROS can damage proteins and membranes, which is why an almost uniform response to most stresses is the production of radical scavenging compounds and enzymes (Meyer *et al*., 2014; Sun *et al*., 2022; Guo *et al*., 2023). ROS accumulation and Ca^{2}+ spiking, lead to biosynthesis of phytohormones, which induces further downstream transcriptional changes, and thereby the response to a specific stimulus (Choi *et al*., 2017). Phytohormones are the key signaling molecules in regulatory networks of plants. Different hormones act simultaneously and often antagonistically (Huang *et al.*, 2007; Ullah *et al*., 2018). Phytohormone signaling is transduced through various mechanisms like transcription factors, protein kinases and the phosphorelay signal transduction system (Choi *et al*., 2017; Pamei & Makandar 2022). The presented results indicate an elevated expression of stress-signaling related terms in *N. excelsior* as opposed to *N. truncata*, suggesting the presence of heightened stress levels even after watering in the former. The main hormones responsible for drought response and recovery that were also enriched in the present results are abscisic acid (ABA), ethylene, cytokinins, auxin, jasmonic acid (JA) and salicylic acid (SA) (Ullah *et al*., 2018).

Abscisic acid has been shown to be the most important phytohormone in relation to drought (Antofie & Sand 2021). According to Huang *et al.* (2007) at least 14% of the total transcriptome in aerial parts of *Arabidopsis thaliana* is influenced by ABA signaling. Huang *et al*. (2008) showed that over 90% of the drought-responsive genes in leaves of *A. thaliana* are significantly influenced by ABA. Abscisic acid signaling is involved in numerous physiological processes related to the water household like regulation of stomatal closure, senescence and suppression of root growth, just to name a few (Huang *et al.*, 2007; Choi *et al*., 2017; Ullah *et al*., 2018; Antofie & Sand 2021; Borràs *et al*., 2021).

Iovieno *et al*. (2016) measured a 10-fold higher leaf-ABA content in tomato plants after 16 days of water withholding than after rewatering. In that experiment, hydrated plants had an even lower ABA content than regularly watered control plants. These results indicate that the renewed availability of water leads to a complete reset of phytohormone signaling. In *N. truncata* significant GO-terms related to ABA signaling had highly negative Z-scores, which matches with these findings. *Nicotiana excelsior* on the other hand showed only upregulated DEGs (notably only three genes) related to ABA metabolism. Two of them were annotated as "abscisic acid 8'-hydroxylase", which is involved in ABA-degradation. This suggests the presence of a residual leaf ABA content that was about to be depleted, indicating a slower recovery from drought, as also observed by Teshome *et al*. (2023).

Genes related to ethylene signaling showed a similar pattern of upregulation in *N. excelsior* and downregulation in *N. truncata.* Like ABA, ethylene is accumulated under severe stress conditions. It inhibits growth and accelerates tissue maturation, ultimately leading to senescence. Accelerated ethylene degradation has been shown to greatly enhance drought resistance (Errickson *et al*. 2023). Yao *et al.* (2021) showed that during drought recovery the re-establishment of gas exchange was shown to be influenced more strongly by ethylene degradation than by decreasing ABA-levels. The high number of predominantly downregulated DEGs related to ethylene signaling and senescence underscores the ability for rapid recovery in *N. truncata*. As for ABA, the upregulation of ethylene related DEGs in *N. excelsior* indicates high levels of retained ethylene, again suggesting a much slower recovery and probable permanent drought damage.

Although ABA and ethylene primarily orchestrate stress response, auxin and cytokinins contribute to the complex regulatory network governing growth and development.

Differentially expressed genes related to cytokinin in both investigated species mostly revolved around the “phosphorelay signal transduction system”. These cytokinin mediated signal transduction pathways can affect growth and other functions in multiple ways. Studies found both positive and negative effects of cytokinins on drought resistance. Acclimation to drought has also been shown to be affected by cytokinin (Ullah *et al*., 2018). Although DEGs related to cytokinin signaling were regulated differently in the two investigated *Nicotiana* species, solely based on the presented results obtained from mature leaves, drawing conclusions about differential growth rates proves difficult.

Adjustments of cellular concentration of phytohormones, in response to external stimuli, leads to the activation of transcription factors (TFs) (Errickson *et al*. 2023). Based on their conserved DNA-binding domain, TFs are categorized in different families. Especially bHLH, MYB, NAC, WRKY transcription factors have been shown to be key regulators of drought response pathways (Yang *et al*., 2022). During drought recovery the majority of observed differentially expressed transcription factors also belonged to these families, although mostly differential expressed was in the opposite direction (also observed by Borràs *et al*., 2021; Teshome *et al*., 2023). In the results of the present study, ethylene responsive factors (ERF) and auxin responsive factors (ARF) were also highly differentially expressed (see Figure 7). Auxin responsive TFs were only differentially downregulated in *N. truncata* and not differentially expressed in *N. excelsior*. Suppression of ARF-genes was shown to increase the content of soluble sugars and proline, which improves osmotic potential and thereby drought resistance. In roots, auxin signaling induces growth (Li *et al*., 2023). Rapid root growth in response to rewatering might be required to take advantage of precipitation for a plant that grows in a powdery soil (Chase *et al*., 2021b) with low water-retention, as in *N. truncata*. Rapid and extensive root growth is also commonly observed in desert-dwelling Cactaceae in response to rainfall (Shishkova *et al*., 2013). This trait will be more thoroughly explored at a later stage of the project with the evaluation of root samples.

In these two *Nicotiana* species, an opposite regulation of many TF-families was visible as seen in the GO-term “regulation of DNA-templated transcription”. The almost exclusive downregulation of ERF factors in *N. truncata* again indicates low ethylene levels, which implies complete recovery from stress. The upregulation of ERF factors in *N. excelsior* on the other hand suggests presence of ethylene, and therefore suppression of gas exchange, leading to

premature leaf senescence (Yao *et al.*, 2021; Errickson *et al*., 2023). The same pattern of up/down regulation was visible in WRKY, BhLH and NAC transcription factors. These are involved in response to different stresses and developmental processes. Most of them are regulated by ABA, JA and SA signaling but some also by ROS and other cellular signals (Nuruzzaman *et al.*, 2013; Bakshi & Oelmüller 2014; Borràs *et al*., 2021) again demonstrating the varying levels of stress and opposite hormone signaling between treatments and species.

In an RNA-seq experiment investigating the response of *N. benthamiana* to pathogen infection (Shen *et al*., 2016), a great number of differentially upregulated WRKY and ERF TFs were identified. This suggests that many DEGs observed in *N. excelsior* might be linked to pathogen infection rather than drought, providing an alternative explanation for prevailing stress after rewatering. However no phenotypic signals of pathogen infection could be observed on the plants at tissue fixation. In *N. excelsior*, prolonged drought may increase susceptibility to pathogens through exhaustion, leading to upregulation of pathways related to biotic defense. Conversely, in *N. truncata*, drought might even enhance pathogen resistance, as indicated by the differential downregulation of similar DEGs related to biotic defense. Alternatively, these patterns may be linked to physical damage of leaf tissue that induced expression patterns related to wounding and biotic response.

Cellular response to drought and stress alleviation

Due to its high consumption of water, photosynthesis is among the first functions impaired by drought stress. Errors through insufficient consumption of reduction potential and supply of water lead to photoinhibition and production of ROS (Iovieno *et al*., 2016; Zhang *et al*., 2021). Free radicals must be detoxified to stop them from damaging cellular constituents. Even during the recovery process, increased activity of antioxidant compounds is visible, as seen in the results of this thesis. In both species, processes revolving around photosynthesis and chlorophyll biosynthesis were downregulated. Pathways involved in accumulation of storage metabolites like carbohydrates and lipids had highly negative Z-scores, also indicating reduced photosynthetic efficiency in both species. Changes of lipid metabolism might, however, also be related to changes in membrane composition. In *N. truncata* upregulation of the “citrate metabolic pathway” and downregulation of “carbohydrate metabolic process” indicate increased respiratory activity and starch breakdown (Teshome *et al*., 2023). Positive Z-scores for the terms “response to hydrogen peroxide” and “response to oxidative stress” suggest

high activity of radical scavenging pathways, in response to increased ROS-levels. Biosynthesis of ascorbic acid, a strong antioxidant, was upregulated in *N. excelsior.* The term “glutathione metabolic”, which is also involved in ROS detoxification (Hasanuzzaman *et al*., 2017) was downregulated. This could be explained through already high glutathione levels in drought treated plants compared to the control group, while ROS content is decreasing. This decreasing (compared to drought), but still high ROS levels (compared to well-watered) was also observed in *Tamarix taklamakanensis* after rewatering (Sun *et al*., 2022).

Several studies observed increased production of terpenoids in response to drought and herbivory (e.g., Tattini *et al*., 2014; Li *et al*., 2015; Sun *et al*., 2022). Terpenoids have been demonstrated to protect the photosynthetic apparatus from drought-induced damage by two key mechanisms. They actively prevent the production of ROS, while also exhibiting effective scavenging activity (Tattini *et al*., 2014). In the presented results, terpenoid biosynthesis was downregulated in both species during the recovery process, likely due to the accumulation during drought. Only sesquiterpene biosynthesis was upregulated in *N. excelsior*. Given the high number of differentially upregulated genes related to defense response, sesquiterpenes are likely involved in defense activity. This suggests a potential mechanism of repurposing terpenoids, originally employed for drought protection, into defense compounds.

During drought, cellular processes are not only impaired by oxidation, but also through osmotic stress. The most obvious effect of water deficit is seen in loss of turgor pressure and thereby decreased stability, especially pronounced in herbaceous plants. To counteract this, non-toxic osmolytes, also known as compatible solutes, are accumulated (Iovieno *et al*., 2016; Sun *et al*., 2022). Since rewatering enables the re-establishment of turgor pressure, only few terms related to osmolyte accumulation were significantly enriched. The downregulation of “proline catabolic process to glutamate” and “glutamine metabolic process” could be related to proline accumulation but might also be interpreted as breakdown of proline in response to rewatering in *N. truncata*. Downregulation of genes in the term “putrescine biosynthetic process from ornithine” in *N. excelsior* also indicates breakdown of drought related compounds induced by rewatering. Both ornithine and putrescine play roles in pathways associated with growth and development and act in mitigating abiotic stress (Liu *et al*., 2022; González-Hernández *et al*., 2022). Differential expression of carbohydrate metabolism can

also be related to osmotic adjustments because many sugars act as osmolytes (Meyer *et al*., 2014; Sun *et al*., 2022).

High cellular salt concentration in combination with heat that often coincide with drought can lead to denaturation of proteins (Iovieno *et al*., 2016). The terms “chaperon mediated protein folding” in *N. truncata* and “cellular response to heat” in *N. excelsior* both include genes coding for heat-shock proteins and other chaperons that mediate protein refolding.

Cell wall fortifications are another mechanism to re-establish structural integrity in response to decreasing turgor pressure in herbaceous plants (Borràs *et al*., 2021). Due to the flat arrangement of leaves in a basal rosette, cell wall modification might not be necessary in *N. truncata*, which is seen in the predominant downregulation of DEGs related to that function. The upright growth in *N. excelsior* might in contrast necessitate additional stability, as implied by differential upregulation of cellulose synthase-like genes. Downregulation of functions related to lignin biosynthesis was observed in both species. Under cultivation *N. truncata* can survive for several seasons (Chase *et al*., 2021b). *Nicotiana excelsior* can exhibit a perennial life cycle even in nature (Chase & Christenhusz 2018). Since stable control conditions enable perennial growth, differential lignification between treatments was not surprising.

Genes annotated as xyloglucan endotransglucosylase/hydrolases (XTHs) and extensins were differentially downregulated in *N. truncata*. These genes are involved in cell wall loosening, which facilitates cell size expansion, and growth (Tenhaken 2015; Iurlaro *et al*., 2016). Downregulation of these genes indicates stagnation in *N. truncata*. In a drought experiment with subsequent watering with maize seedlings Zhang *et al*. (2018) observed that genes related to cell expansion were downregulated during drought and upregulated after rewatering (also shown by Claeys & Inzé 2013). Contradicting these results, suppression of leaf growth in the already recovering *N. truncata*, might be a mechanism to prepare for subsequent droughts. In contrast to XTHs and extensins, expansins were mostly upregulated in *N. truncata*. Overexpression of expansins in *Arabidopsis* has been shown to enhance drought resistance and recovery through cell wall remodeling (Tenhaken 2015). In *N. excelsior* most DEGs related to cell wall modification were annotated as XTHs and were predominantly upregulated, indicating growth, despite the still prevailing stress.

Response to biotic stress

Plant response mechanisms are categorized as constitutive or induced. Constitutive defenses, such as the accumulation of toxic substances, are known to be energy-intensive processes (Wit *et al*., 2013) and tend to be suppressed during periods of severe drought. Upon rewatering, it is likely that the production of defense compounds would be predominantly restored to levels approximating those observed in well-watered control plants. Consequently, "coumarin biosynthetic process" was the only significantly enriched GO-term associated with constitutive defense in both species.

This trade-off between energy conservation and defense comes with the downside of increased susceptibility to biotic attack, which is seen in upregulation of induced defense (Teshome *et al*., 2023). The main signaling molecules for mediating the response to biotic stress are jasmonic acid (JA) and salicylic acid (SA). Both hormones have mutually antagonistic interactions and induce contrasting downstream pathways, depending on the stressor (Wit *et al*., 2013; Ilyas *et al*., 2017). Terms related to JA were downregulated in both *Nicotiana* species indicating suppression through SA, which was significantly enriched and upregulated in *N. excelsior* but not *N. truncata*. The high number of upregulated DEGs related to biotic defense in *N. excelsior*, including genes related to “systemic acquired resistance”, could be explained through exhaustion and an overall weak state in drought affected-plants, resulting in high susceptibility to pathogens. The opposite regulation of DEGs related to defense response between the two species is probably also due to varying stress levels. As mentioned above, drought could have led to increased pathogen resistance in *N. truncata*, due to activation of effective stress coping mechanisms that act against both biotic and abiotic stresses. In addition to their crucial role in biotic defense, JA and SA are regulators of growth and development. Moreover, they play a significant role in abiotic stress response, including drought (Ullah *et al*., 2018; Khalvandi *et al*., 2021; Sun *et al*., 2022).

Flexibility enables desert survival

Based on the severity of drought stress and the developmental state of a plant, resources are balanced between survival, growth and reproduction (Claeys & Inzé 2013). The allocation of resources during severe drought stress is, however, highly dependent on the life form of a given species. Perennial species for instance aim to withstand stress conditions and reproduce

when water becomes available again. In turn, in ephemeral annuals, flowering is often even induced by drought conditions, which leads to exhaustion of the plant afterwards (Johnson *et al.*, 2022). The results of the present study show that the allocation of resources in response to severe drought and subsequent rehydration differs even between closely related ephemeral species.

By drawing comparisons with other RNA-seq studies (Iovieno *et al*., 2016; Choi *et al*., 2017; Ullah *et al*., 2018; Borràs *et al*., 2021; Zhang *et al*., 2021) and considering field observations (Chase & Christenhusz 2018; Chase *et al*., 2021b; 2022; 2023b), the results of drought recovery reveal notable differences in drought adaptations in the two species. We propose the following strategies: *Nicotiana truncata* limits vegetative growth, with the aim of saving resources and reducing exposed leaf surface and stomatal density. Meanwhile, resources are channeled into reproduction. In unfavorable years, these species can react by producing dwarf plants with cleistogamous flowers (Chase *et al*., 2022). Reduced photosynthetic efficiency and increased energy demand through repair mechanisms is compensated by respiration (Zhang *et al*., 2021). Due to the hostile environment with little competition and few herbivores, the species is adapted to suppress defense responses in favor of conserving energy and water. If rewatering occurs before complete exhaustion, *N. truncata* can react with rapid recovery and reinitiation of reproduction. The basal rosette with leaves close to the ground reduces the need for stability through cell-wall modifications. Additionally, lower airflow directly at ground level might result in reduced transpirational water loss (Bang *et al*., 2010).

Nicotiana excelsior in contrast grows in comparatively mesic sites, which facilitates an upright phenotype. Height is even required to prevail against competitors. This species is adapted to a stable environment with an overall greater water-availability and is therefore able to exhibit a more constant growth. Exhaustion through growth even during drought is indicated by a much slower recovery after rewatering. Upregulation of genes related to biotic stress was probably caused by the weakened state of the plant after drought but also contribute to further stress.

Overall, the most important trait that enables survival in the open desert is a high flexibility to allocate resources between growth and survival under unstable environmental conditions. This plasticity, on the other hand, makes *N. truncata* less competitive in more stable ecosystems. Consequently, the species is confined to extreme habitats, which explains its

narrow distribution range and habitat preference. The most significant adaptation to open desert habitats might, however, be the strict control over germination in *N. truncata* (Chase *et al*. 2021a). Sufficient availability of water under the correct conditions in the first place is necessary for these plants to germinate, mature and accumulate enough resources to endure drought in later stages of their development.

Chromosome condensation in context of drought adaptation

The structure and function of plant genomes has been marked by a history of repeated cycles of whole genome duplications followed by diploidization (Dodsworth *et al*., 2016b; Escudero & Wendel 2020). Despite the number of duplications in their ancestry, most extant plants, particularly herbaceous groups, exhibit the low chromosome numbers typical of diploids (Landis *et al*., 2018). In *N.* sect. *Suaveolentes* reduction of chromosome number coincides with the colonization of extreme habitats. However, the drivers of these rapid genome reconstructions and how they are linked to adaptation to the desert are still largely unknown (Kelly *et al*., 2012; Chase *et al*., 2023a).

A study by Kang *et al.* (2015) showed that selected species growing in habitats with extreme nutrient limitation evolved smaller genomes than their relatives in more fertile environments. Likewise, a study by Bales and Hersch-Green (2019) suggested that diploid species of a genus have an adaptive advantage in nutrient-poor soil compared to their polyploid counterparts. Underscoring these findings, the “large genome constraint” hypothesis (Knight *et al*., 2005) states that plants with large genomes diversify more slowly and are therefore less likely to be highly species rich and are also under-represented in extreme environments. Studies testing this hypothesis in various extreme environments mostly support it (e.g., Escudero & Wendel 2020; Guignard *et al*., 2016; Zaveska *et al*., 2023), with few exceptions.

The selection towards more streamlined genomes may reflect an optimization to better suit the nutrient constraints of extreme habitats. The reduction of expenses for DNA could be a driver of diploidization in *N.* sect. *Suaveolentes*, but it cannot be the sole reason, because the species with the lowest chromosome numbers (n <20) exhibit genomes up to 30% larger than those with intermediate numbers (n >20; Chase *et al*., 2023). However nutrient limitation might still be a major constraint during drought for the species investigated in this thesis. Possibly, fusion of chromosomes, which could lead to genome size reduction in the short term,

could have played a role for the initial colonization of desert habitats. After adaptation to these environments through other factors, bursts of TE-proliferation (Arkhipova 2018), could then have led to increase in genome size beyond the initial sizes (Chase *et al*. 2023a).

An hypothesis proposed by Chase *et al*. (2023a), grounded in Darlington's (1937) law, posits that environmental instability and stress drives chromosomal reorganization by creating linkage groups of adaptive alleles. Alterations in chromosome structure in return reduces gene flow and thereby recombination and loss of adapted, linked alleles, while favoring sympatric speciation. The formation of a reproductive barrier through alteration in chromosome structure might explain the great number of species in the subgenus. This theory could provide an explanation for the parallel dysploid decrease in chromosome number in desert-dwelling species within *N.* sect. *Suaveolentes*.

A study on mediterranean plants facing environmental instability and seasonality conducted by Carta *et al.* (2018) supports the hypothesis that low chromosome number, which causes reduced recombination through suppression of crossover-rates, is favored in barren habitats. As in *N.* sect. *Suaveolentes*, Mediterranean species in open, disturbed and drought-prone habitats tend to exhibit lower chromosome numbers. On the contrary, plant species with higher chromosome numbers, and therefore increased recombination rates, prefer shaded, stable environments with abundant water and nutrient availability. Results of that study also indicated that polyploids are favored in stable environments, implying that diploidization as in Australian *Nicotiana* is required for colonization of extreme, unstable habitats.

To test the hypothesis that formation of linkage groups of drought-adapted genes drives the adaptation to the open desert, DEGs were mapped on the reference genome of *N. benthamiana* (see Figure 4) to see whether genes associated with adaptation to drought cluster more than would be expected by chance. Although the results of this analysis reveal a general trend, drawing definitive conclusions proves difficult for several reasons: the reference genome of *N. benthamiana* comprises 19 chromosomes, in contrast to the 18 chromosomes found in *N. truncata* and 20 in *N. excelsior*. Consequently, comparing the position of differentially expressed genes from one species to the genome structure of another makes this analysis an in-appropriate test of this hypothesis. Also performing meaningful statistical tests becomes challenging. This is because gene density (genes/Mbp) per chromosome does not accurately capture the clustering of genes within a much smaller

genomic frame. On the other hand, testing for smaller frames would be complex and carries the risk of introducing biases. Another issue could be the experimental setup. Genes involved in drought recovery might have a different distribution than genes involved in acute drought response and may not play a role in more general adaptations to xeric habitats (e.g., more effective water use at all times, more common resorts to cleistogamous flowers on dwarf plants, etc.).

To address some of those issues, PacBio sequencing for each of the four species will be performed in the future. Thereby their structural variation can be investigated. If loss or condensation of chromosomes influences formation of linkage groups as predicted by Chase *et al*. (2023a), structural variation data will probably reveal statistically measurable clusters of DEGs and other drought-related genes under selection but not specifically involved in drought response and recovery.

Nicotiana truncata is adapted to a more extreme habitat than *N. excelsior* while also possessing a lower chromosome number, therefore the clustering was expected to be more pronounced. Based on the results mapped on the reference genome presented in Figure 4, this was not the case. Since the lab accession of *N. benthamiana*, from which the genome was derived, is a mesically adapted species, these results were not surprising (Chase *et al*., 2022). Drought responsive genes are evolutionarily deeply rooted and usually not clustered at specific chromosomal positions (Wang *et al*. 2017). Nonetheless, chromosomal rearrangements might still cause significant clustering, and thereby enhance drought adaptation, and this remains to be specifically tested in the future.

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Appendix

Table S1: List of all significantly enriched (adj_pvalue < 0.1) GO-terms of N. excelsior with respective number of genes (count), adjusted p-values and Z-scores. *Excluding shared terms*.

GO-term	count	adj_pval	Z-score
glutamine metabolic process	6	0.00072	5.54
putrescine biosynthetic process from ornithine	2	0.00036	-5.59
S-methylmethionine cycle	1	0.0535	2.35
asparagine biosynthetic process	1	0.08423	2.10
threonine catabolic process	1	0.09424	3.50
cellular response to heat	3	0.05756	1.46
negative regulation of response to salt stress	1	0.0741	6.25
systemic acquired resistance	3	0.01288	3.58
response to wounding	7	0.000018	-9.79
response to herbivore	3	0.0000051	-0.03
defense response to oomycetes	4	0.00113	0.63
response to insect	2	0.00859	3.90
extracellular ATP signaling	1	0.01094	2.02
tyramine biosynthetic process	1	0.01094	-3.76
defense response to bacterium	5	0.0326	3.44
response to bacterium	6	0.08329	0.87
defense response	39	0.0000011	11.58
response to molecule of fungal origin	5	0.02615	-2.92
cell wall organization	13	0.00038	11.29
xyloglucan metabolic process	10	0.000000045	10.73
cell wall biogenesis	13	0.00000018	11.88
cellulose catabolic process	2	0.06781	-5.57
cutin biosynthetic process	2	0.0199	-0.19
DNA protection	1	0.08423	9.59
negative regulation of response to water deprivation	1	0.02175	6.25
inositol catabolic process	2	0.00408	4.05
thiamine biosynthetic process	2	0.01803	-4.08
1-deoxy-D-xylulose 5-phosphate biosynthetic process	2	0.0032	-4.08
oxaloacetate(2-) transmembrane transport	1	0.04304	2.64
succinate transmembrane transport	1	0.06386	2.64
galactomannan catabolic process	1	0.02175	-2.50
cellular response to hypoxia	1	0.06386	2.58
calcium ion transmembrane transport	4	0.01985	0.05
zinc ion transmembrane transport	2	0.04502	-4.39
lignin biosynthetic process	3	0.01474	-6.33
lipid catabolic process	4	0.05114	-5.33
lipid metabolic process	35	0.09774	-6.09
guard mother cell differentiation	1	0.03245	2.52
'de novo' CTP biosynthetic process	3	0.0002	3.97
pyrimidine nucleobase biosynthetic process	3	0.00249	3.97

dCTP catabolic process	1	0.08423	9.59
AMP salvage	2	0.00408	5.04
purine ribonucleoside salvage	2	0.00506	5.04
adenine salvage	2	0.00117	5.04
chlorophyll catabolic process	3	0.00195	-4.79
chloroplast disassembly	1	0.03245	-3.20
regulation of salicylic acid biosynthetic process	5	0.0000054	6.54
abscisic acid metabolic process	3	0.00728	4.78
response to cytokinin	8	0.01412	5.06
response to jasmonic acid	5	0.07018	-3.80
transmembrane receptor protein serine/threonine kinase signaling pathway	3	0.0011	2.00
regulation of abscisic acid biosynthetic process	1	0.0741	2.05
regulation of jasmonic acid mediated signaling pathway	2	0.08359	-1.10
phosphorelay signal transduction system	17	0.00037	10.33
cytokinin-activated signaling pathway	6	0.0005	6.12
ethylene-activated signaling pathway	11	0.00065	8.32
protein dephosphorylation	10	0.03917	3.98
protein homotetramerization	1	0.02175	3.50
regulation of protein dephosphorylation	4	0.02787	1.90
protein sumoylation	2	0.03261	5.35
fucose metabolic process	3	0.0647	-1.92
protein stabilization	2	0.08035	-0.03
regulation of protein serine/threonine phosphatase activity	2	0.07399	-0.57
regulation of inflorescence meristem growth	1	0.02175	2.50
pollen exine formation	2	0.01293	-3.14
regulation of floral meristem growth	1	0.02175	2.50
syncytium formation	1	0.03245	2.33
glutathione metabolic process	4	0.01422	-2.87
L-ascorbic acid biosynthetic process	2	0.0199	4.05
glutathione transport	1	0.02175	-2.21
response to singlet oxygen	1	0.08423	-5.56
terpenoid biosynthetic process	12	0.00612	-5.10
polyketide biosynthetic process	1	0.0741	2.65
xenobiotic metabolic process	1	0.08423	-2.27
triterpenoid biosynthetic process	1	0.08423	-3.16
coumarin biosynthetic process	5	0.02188	-2.92
diterpenoid biosynthetic process	6	0.000037	-4.31
sesquiterpene biosynthetic process	2	0.01293	3.13
thiosulfate transport	1	0.04304	2.64
tRNA transcription by RNA polymerase III	2	0.00859	0.34
regulation of DNA-templated transcription	51	0.0016	7.51
metabolic process	234	0.0004	5.51
response to stress	62	0.02851	6.95
regulation of biosynthetic process	53	0.09118	8.04

Table S2: List of all significantly enriched (adj_pvalue < 0.1) GO-terms of N. truncata *with respective number of genes (count), adjusted p-values and Z-scores. Excluding shared terms with* N. excelsior

GO-term	count	adj_pval	Z-score
glutamine metabolic process	4	0.03448	-3.03
proline catabolic process to glutamate	2	0.00354	-4.23
tyrosine biosynthetic process	1	0.07609	-5.04
cellular heat acclimation	1	0.0906	-2.00
positive regulation of circadian rhythm	1	0.06135	2.68
response to low light intensity stimulus	2	0.00647	-3.10
response to high light intensity	2	0.04244	-3.10
response to cold	6	0.06668	3.25
detection of hypoxia	2	0.00823	-4.79
defense response	61	0.00000000001	-14.02
response to molecule of fungal origin	9	0.0007	-2.11
response to biotic stimulus	39	0.00774	-8.57
plant-type hypersensitive response	5	0.00433	-1.86
defense response to Gram-negative bacterium	1	0.07609	-2.03
positive regulation of defense response to insect	1	0.06135	-2.04
positive regulation of defense response to virus by host	1	0.03116	-3.17
pattern recognition receptor signaling pathway	1	0.0906	-3.59
xyloglucan metabolic process	5	0.01262	-4.22
cell wall biogenesis	9	0.00268	-6.59
cellulose catabolic process	6	0.000031	-8.11
plant-type cell wall organization	11	0.0000078	-4.07
lignin catabolic process	5	0.00083	-6.31
cell wall modification	6	0.05277	-10.32
regulation of cell adhesion	1	0.06135	-2.00
galacturonate biosynthetic process	1	0.03116	-2.03
plant-type cell wall modification	1	0.06135	-2.04
regulation of cell wall pectin metabolic process	1	0.04637	-2.04
cellulose microfibril organization	3	0.0085	-4.30
pectin catabolic process	6	0.03606	-8.60
cutin biosynthetic process	2	0.03879	-3.68
wax biosynthetic process	2	0.01711	-3.21
DNA double-strand break processing involved in repair via single-strand annealing	1	0.03116	-2.35
histone H3-K36 demethylation	1	0.0157	3.31
galactomannan catabolic process	1	0.03116	-2.14
carbohydrate metabolic process	40	0.04637	-12.58
carbohydrate transmembrane transport	7	0.00134	-2.58
citrate metabolic process	2	0.01018	6.01
triglyceride biosynthetic process	3	0.02639	-4.48

mitochondrial electron transport. succinate to ubiquinone	1	0.07609	3.95
mitochondrial respiratory chain complex II assembly	1	0.0906	3.95
lipid metabolic process	50	0.01553	-12.76
sterol metabolic process	6	0.0038	-2.75
phosphatidylcholine metabolic process	3	0.04233	-4.84
phospholipid catabolic process	4	0.00456	-5.46
leaf senescence	3	0.08247	-1.54
stomatal complex morphogenesis	6	0.00483	-7.06
negative regulation of cell size	1	0.0157	-2.21
primary shoot apical meristem specification	1	0.07609	-2.47
regulation of meristem structural organization	1	0.0906	-2.47
somatic stem cell population maintenance	1	0.03116	-2.47
anatomical structure morphogenesis	17	0.06995	-2.95
polarity specification of adaxial/abaxial axis	2	0.01463	-3.02
regulation of monopolar cell growth	2	0.0049	-3.11
regulation of shoot apical meristem development	2	0.00823	-3.35
xylem development	2	0.08965	-3.39
establishment or maintenance of cell polarity	2	0.04244	3.43
phloem development	2	0.00823	-4.46
guard cell differentiation	4	0.00237	-6.54
phloem or xylem histogenesis	6	0.07468	-6.55
ammonium homeostasis	3	0.000074	-1.62
ammonium transmembrane transport	3	0.00222	-1.62
nitrate assimilation	3	0.01968	-1.97
regulation of nitrate assimilation	1	0.03116	2.43
response to nitrate	2	0.0541	-3.07
nitrite transport	1	0.0157	-3.75
nitrate transport	3	0.01361	-4.67
AMP salvage	2	0.00823	4.27
purine ribonucleoside salvage	2	0.01018	4.27
adenine salvage	2	0.00238	4.27
snoRNA guided rRNA pseudouridine synthesis	1	0.04637	2.18
snRNA pseudouridine synthesis	1	0.06135	2.23
chloroplast disassembly	1	0.04637	-2.51
light harvesting in photosystem I	8	0.00000031	-7.37
positive regulation of chlorophyll biosynthetic process	1	0.07609	2.43
siroheme biosynthetic process	1	0.06135	2.43
phosphorelay signal transduction system	23	0.01123	-10.32
cytokinin-activated signaling pathway	6	0.00314	-1.69
ethylene-activated signaling pathway	18	0.0000023	-11.16
jasmonic acid mediated signaling pathway	5	0.01632	-6.42
abscisic acid-activated signaling pathway	7	0.00134	-6.57
1-aminocyclopropane-1-carboxylate biosynthetic process	2	0.02257	-4.09

regulation of protein serine/threonine phosphatase activity	6	0.000041	-6.12
chaperone-mediated protein folding	5	0.08918	-1.48
self-proteolysis	1	0.0157	-2.04
protein refolding	4	0.05713	-2.84
negative regulation of protein transport	1	0.03116	-3.17
protein-FAD linkage	1	0.04637	3.95
negative regulation of catalytic activity	4	0.02329	-6.03
proteolysis involved in cellular protein catabolic process	8	0.03829	-6.89
inflorescence morphogenesis	2	0.00073	-2.98
fruit ripening	2	0.02553	-3.73
recognition of pollen	6	0.01423	-4.24
response to oxidative stress	13	0.03879	1.05
response to hydrogen peroxide	3	0.07667	4.20
coumarin biosynthetic process	8	0.00213	-1.13
diterpenoid biosynthetic process	7	0.000019	-8.28
sesquiterpene biosynthetic process	3	0.00185	-4.79
regulation of sulfate assimilation	1	0.03116	2.43
regulation of DNA-templated transcription	88	0.00000043	-20.90
termination of mitochondrial transcription	1	0.03116	2.10
positive regulation of DNA-templated transcription	10	0.03637	-3.26
tRNA export from nucleus	1	0.06135	2.02
asparaginyl-tRNA aminoacylation	1	0.0906	-2.35
water transport	4	0.06798	-2.58
transpiration	1	0.03116	-2.00
oxygen transport	1	0.07609	-3.67

Table S3: List of shared significantly enriched (adj_pvalue < 0.1) GO-terms between N. excelsior *and* N. truncata with number of genes (count), adjusted p-values and Z-scores for both species respectively.

GO-term	**count Exc**	**adj_pval Exc**	**Z-score Exc**	**count Tru**	**adj_pval Tru**	**Z-score Tru**
glutamine metabolic process	6	0.00072	5.54	4	0.03448	-3.03
defense response	39	0.0000011	11.58	61	1E-11	-14.02
response to molecule of fungal origin	5	0.02615	-2.92	9	0.0007	-2.11
xyloglucan metabolic process	10	0.000000045	10.73	5	0.01262	-4.22
cell wall biogenesis	13	0.00000018	11.88	9	0.00268	-6.59
cellulose catabolic process	2	0.06781	-5.57	6	0.000031	-8.11
galactomannan catabolic process	1	0.02175	-2.50	1	0.03116	-2.14
lipid metabolic process	35	0.09774	-6.09	50	0.01553	-12.76

cutin biosynthetic process	2	0.0199	-0.19	2	0.03879	-3.68
AMP salvage	2	0.00408	5.04	2	0.00823	4.27
purine ribonucleoside salvage	2	0.00506	5.04	2	0.01018	4.27
adenine salvage	2	0.00117	5.04	2	0.00238	4.27
chloroplast disassembly	1	0.03245	-3.20	1	0.04637	-2.51
phosphorelay signal transduction system	17	0.00037	10.33	23	0.01123	-10.32
cytokinin-activated signaling pathway	6	0.0005	6.12	6	0.00314	-1.69
ethylene-activated signaling pathway	11	0.00065	8.32	18	0.0000023	-11.16
regulation of protein serine/threonine phosphatase activity	2	0.07399	-0.57	6	0.000041	-6.12
coumarin biosynthetic process	5	0.02188	-2.92	8	0.00213	-1.13
diterpenoid biosynthetic process	6	0.000037	-4.31	7	0.000019	-8.28
sesquiterpene biosynthetic process	2	0.01293	3.13	3	0.00185	-4.79
regulation of DNA-templated transcription	51	0.0016	7.51	88	0.00000043	-20.90