



High Salinity Induces Changes in the Leaf Transcriptome of *Withania somnifera*

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Abstract

Withania somnifera, commonly referred to as Indian Ginseng, is a medicinally important plant that can be found in many regions of the world, such as South Africa and India. Withanolides are key secondary metabolites responsible for conferring the plant its unique medicinal properties. The reaction of *W. somnifera* to salt stress, in particular the possible effects on its withanolide production, has yet to be explored. Under salt stress, germination efficiency dropped by 17.8% and caused physiological damage to emerging roots. A transcriptome analysis of salt-stressed leaves revealed the differential regulation of 3048 transcripts, with gene ontology analysis indicating an increased transcript abundance associated with phenylpropanoid, plant hormone signaling, detoxification response, concomitant with reduced growth, energy metabolism, and protein modification. Most withanolide pathway genes were significantly downregulated. Notably, 26 transcription factor families, particularly AP2/ERF, NAC, GRAS, and HB-WOX members, as well as 10 Late Embryogenesis-Abundant members, predominantly dehydrins, were significantly differentially regulated, suggesting their potential involvement in the salt stress response of *W. somnifera*. This study provides valuable insights into the molecular responses of *W. somnifera* to salt stress, highlighting potential targets for future research and subsequently for withanolide production, which has vast potential in the pharmaceutical industry.

Keywords *Withania somnifera* · Salt stress · Withanolide · Transcriptomics · LEA genes · Transcription factors

Introduction

Withania somnifera, commonly known as Indian Ginseng or ashwagandha, is a xerophytic, evergreen, Solanaceous plant (Paul et al. 2021). The plant is prominent in African (Afewerky et al. 2021) and Indian traditional medicine (Mirjalili et al. 2009), where the leaves, bark, stem, seeds, and berries are used in the form of decoctions, powders, infusions, and ointments to treat various ailments. It is the

roots, however, that are most valued and are used alone or in combination with other ingredients in a vast number of formulations of folk medicine (Afewerky et al. 2021; Paul et al. 2021). *W. somnifera* produces a wide range of secondary metabolites such as tannins, alkaloids, flavonoids, and terpenoids (Saleem et al. 2020). Nonetheless, it is the presence of pharmacologically active steroidal lactones, collectively known as withanolides, that confers this plant its remedying properties and its popularity (Grover et al. 2013; Mirjalili et al. 2009; Dhar et al. 2015; Hakim et al. 2025).

Evidence suggests that isoprenogenesis, resulting in the formation of isoprene precursors required for withanogenesis, occurs through two routes in plants. The methylerythritol phosphate (MEP) pathway takes place in plastids and results in the formation of mono- and diterpenoids, whereas the cytosolic mevalonate (MVA) pathway produces tri- and sesquiterpenes, although exceptions to the contribution of each pathway exist (Chaurasiya et al. 2012). The availability and amount of particular withanolides differ with tissue type, although in general, a withanolide gradient from the

Key Message

- Reduced germination efficiency and root damage symptoms were observed in salt-treated seedlings.
- Transcriptomic analysis of *Withania somnifera* salt stressed leaf tissue is reported.
- The transcript abundance of genes encoding the enzymes in the MVA and MEP/DOXP pathway was reduced in salt-treated plants.
- Increased detoxification and altered regulation of transcription factor families and LEA gene family members.

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leaves to the roots exists (Dhar et al. 2015). Studies have shown that, while withanolides may be shuttled from the leaves to the roots, the roots are capable of *de novo* withanolide synthesis, resulting in a qualitative overlap of leaf and root withanolides (Sangwan et al. 2008).

The current supply of *W. somnifera* for the production of Withania-based therapies mainly relies on the harvesting of wild plants, such as in Africa, where formal cultivation of *W. somnifera* is still not practiced (Afewerky et al. 2021). In India, wild-plant harvested supplies are mixed with plants cultivated in limited areas from several regions in North-Western states (Anandakumar et al. 2022). The ease of cultivation, high price for the roots, and an insufficient supply to meet the growing demand for pharmaceutical requirements create the opportunity for large-scale cultivation under irrigation (Rajeswara Rao et al. 2012; Mir et al. 2014). However, withanolide biosynthesis is a plant tissue-specific process (Gupta et al. 2015), where yield is dependent on the growing conditions of the plant and is affected by biotic and abiotic stresses (Mishra et al. 2020).

A commonly featured, unfavorable growth condition for plants under cultivation is that of soil salinity (Sabir et al. 2012). Soil salinity imposes its effects on plants in two phases, namely ion toxicity and osmotic stress (Shrivastava and Kumar 2015). In sodic soils, the presence of exchangeable sodium ions (Na^+) results in ion toxicity through its interaction with K^+ (Kamran et al. 2019), and the penetration of hydration shells of proteins by Na^+ and Cl^- ions, resulting in disruption of their function (Tuteja et al. 2012). Additionally, the accumulation of Na^+ causes an ion imbalance resulting in a nutrient deficiency (Hu and Schmidhalter 2005), as well as affecting photosynthetic components such as enzymes, chlorophylls, and carotenoids (Demetriou et al. 2007). In parallel, salt stress induces reactive oxygen species (ROS) production, causing oxidative damage to many cellular components by oxidizing proteins, lipids, DNA, and interrupting essential cellular processes in plants (Gupta and Huang 2014). These factors negatively affect plant growth and development at the physiological, biochemical, and molecular levels, potentially reducing secondary metabolite production (Selmar 2008). Recently, Thorat et al. (2023) showed the metabolic and physio-biochemical adjustments which subsequently facilitate the enhanced withanolide contents in the shoots and roots of *W. somnifera* under saline conditions.

In *W. somnifera*, salt stress has been shown to reduce seed germination, plant growth, and chlorophyll content as well as induce drastic changes in antioxidant metabolism. These changes included reduced levels of ROS scavengers such as catalase, reduced glutathione, superoxide dismutase, polyphenol oxidase, and peroxidase activities, impairing the plant's ability to cope with salt stress (Jaleel et al. 2009a, b). Conversely, salt stress has been utilized as a

beneficial abiotic elicitor to enhance the production of secondary compounds *in planta* in multiple species (Said-Al Ahl and Omer 2011), including *W. somnifera* *in vitro* grown shoots (Sharma et al. 2023). Though encouraging results were available from conventional breeding to molecular aspects, studies to date have not determined the effect that salt stress has on adult *W. somnifera* in terms of its transcriptional responses. Therefore, the present study aims to address this through transcriptome analysis of salt-stressed plants, in particular assessing those genes that are putatively involved in the production of withanolides. These findings will prove important in determining if withanolide biosynthesis is enhanced in salt-stressed adult plant tissues grown in soil, as well as managing and optimizing large-scale cultivation of *W. somnifera* for commercialization.

Materials and Methods

Plant Material

W. somnifera seeds were planted in sand-filled pots and watered daily with a fertilizer that consisted of 2 g Microplex (Ocean Agriculture (Pty) Ltd, South Africa), 164 g Sol-u-fert (Kynoch Fertilizers (Pty) Ltd, South Africa), and 77 ml potassium nitrate per 100 L of water. After 95 days, plants were exposed to salinity stress by substituting the fertilizer mixture during watering with a Hoagland growth medium (Hoagland and Arnon 1950) or Hoagland growth medium containing 100 mM NaCl. All experiments were conducted in triplicate. A total volume of 50 ml of Hoagland growth medium or Hoagland growth medium containing 100 mM NaCl was added to each pot of “Control” plants or “Stressed” plants, respectively. The respective solutions were administered twice weekly for a total of 2 weeks.

Phenotypic Data

For seed germination tests, seeds were surface sterilized (0.2% H_2O_2 solution), placed on filter paper within petri dishes, and watered with 4 ml of Hoagland growth medium with or without 100 mM NaCl. The petri dishes were then placed in a dark growth chamber at 26 °C for 3 weeks and allowed to germinate. After the first week, the amount of Hoagland growth medium used was reduced to 2 ml per plate. Seeds were monitored to evaluate germination rate, and this was expressed as percentage germination over time. Chi-square calculations of germination rates were performed using the GraphPad Software (USA) with Yates correction. A 2×2 contingency table was used to compare stressed and control samples with germination or no-germination as outcomes. A probability value less than 0.05 was deemed to be significant.

Scanning Electron Microscopy of Roots

Stressed and control plants were grown for 86 days to maturity. Both control and stressed plants were either administered 50 ml of Hoagland's solution or 50 ml of the solution with a 100 mM NaCl concentration, respectively, twice a week for 3 weeks. The roots were then stored in 100% ethanol before being sent for hexamethyldisilazane (HMDS) drying at Stellenbosch University's Central Analytical Facility (CAF) Imaging Unit. Scanning electron microscopy (SEM) was performed on control and stressed roots using a Zeiss Merlin SEM.

Transcriptome Analysis Using RNA Sequencing

Mature *W. somnifera* plants (95 days-old) were exposed to 100 mM NaCl (reconstituted in Hoagland growth medium) for seven consecutive days. Each plant received 250 ml of the liquid throughout the exposure period. Mature leaf samples (first five leaves from the bottom) were collected at 0 h and 168 h after exposure commenced and homogenized in liquid nitrogen using a mortar and pestle. RNA was isolated from approximately 100 mg of leaf tissue using a RNeasy Mini kit in combination with QIAshredder columns according to the manufacturer's instructions (Qiagen, DE) with on-column DNaseI digestion. The quantity and integrity of RNA were assessed using Qubit® 2.0 fluorometer (Thermo Fisher Scientific, USA) and Bioanalyzer (Agilent Technologies, Inc., USA) analyses, respectively. All RNA samples were stored at -80°C until sequencing library preparation could commence.

Paired-end sequencing libraries were prepared using the Illumina TruSeq Stranded Total RNA kit (LT Sample Preparation Guide Part #15031048 Rev. E.) and sequenced on the Illumina NovaSeq 6000 platform by Macrogen Inc. (South Korea) for three replicates of both the control ($n=3$) and 100 mM NaCl-treated ($n=3$) *W. somnifera* samples. Reads obtained from the NovaSeq 6000 system were analyzed using FASTQC v0.11.8 (Andrews 2010) and quality trimmed using Trimmomatic v0.39 (Bolger et al. 2014). The trimmed reads were used to perform a strand-specific *de novo* assembly through use of the Trinity software suite v2.14.0 (Haas et al. 2013). Quality assessment of the assembled transcriptome was assessed through mapping the reads back to the assembled transcripts using Bowtie2 (Langmead and Salzberg 2012) and using BUSCO v4.2 (Simão et al. 2015) with the Solanales (odb10) orthologue set to determine the number of single-copy orthologues represented.

Descriptive statistics such as the number of transcripts, transcript average length, transcript average %GC content, transcript N50, and transcript Ex90N50 were also calculated. Gene ontology assignment of the transcripts was performed through the use of OmicsBox v1.2 (OmicsBox

– Bioinformatics Made Easy, BioBam Bioinformatics, March 3, 2019, <https://www.biobam.com/omicsbox>) by performing a BLASTx search against the NCBI's nr database (accessed on 2023/07). Transcription factors and transcriptional regulators were identified and classified based on the rules of the iTAK pipeline v1.7 (<http://itak.feilab.net/cgi-bin/itak/index.cgi>) (Zheng et al. 2016).

Transcript abundance was quantified using kallisto (Bray et al. 2016) and differentially expressed (DE) genes between the control and 100 mM NaCl-treated samples were determined using DESeq2 (Love et al. 2014). Only genes with greater than two counts-per-million in at least three samples were retained for DE analysis and we considered genes DE if they had a $\text{Log}_2\text{FC} \geq 1$ and $\text{padj} < 0.05$ after adjusting for multiple testing using the Benjamini–Hochberg (BH) procedure (Benjamini and Hochberg 1995).

Functional Annotation

Differentially expressed genes (DEGs) from *W. somnifera* were functionally annotated using eggNOG-mapper (v2.1.13). KEGG Orthology (KO) identifiers were extracted and used to perform orthology-based pathway analysis against tomato (*Solanum lycopersicum*).

Tomato-Specific KEGG Pathway Enrichment

W. somnifera KO identifiers were mapped to their corresponding tomato gene IDs by retrieving the gene mapping file from the KEGG API (<http://rest.kegg.jp/link/sly/ko>). Pathway enrichment analysis was then conducted using the R clusterProfiler package (v4.6.2). A hypergeometric test was performed against tomato pathways using the enrichKEGG function with the tomato gene IDs derived from the *W. somnifera* DEGs' KO IDs (gene list) and all *W. somnifera* annotated unigenes' KO IDs (background). Pathways were considered significantly enriched if the Benjamini–Hochberg adjusted p-value was less than 0.05. Significant results were visualized using a dot plot to display the top 10 pathways, ordered by gene ratio (the proportion of input genes in the pathway).

Identification of Late Embryogenesis-Abundant (LEA) Genes from *W. somnifera* Transcriptome Data.

Using Pfam IDs PF03760 (LEA-1), PF03168 (LEA-2), PF03242 (LEA-3), PF02987 (LEA-4), PF00477 (LEA-5), PF10714 (LEA-6), PF02496 (ASR, LEA-7), PF00257 (DHN), and PF04927 (SMP), the LEA amino acid sequences of potato were searched and downloaded from the Spud DB database (<http://solanaceae.plantbiology.msu.edu/>; Date accessed: 2023/08). The obtained potato amino acid sequences were used as a query and, through use of

BLASTp, were searched against a translated nucleotide database of the *W. somnifera* transcriptome. BLAST matches with a score of > 60% identity and > 60% query coverage were considered homologous.

Real-Time qPCR Analysis

Plants were grown for 21 days after germination, followed by 7 days of salt stress. The salt stress consisted of watering plants every second day with Hoaglands containing 100 mM NaCl or Hoaglands for the control plants. The first true leaf of five plants per treatment was sampled, and using liquid nitrogen, was ground to powder. 50 mg of powdered leaf material was used to extract RNA with the RNeasy mini kit (Qiagen) using on-column DNase (Qiagen). cDNA was synthesized with SensiFAST™ cDNA Synthesis Kit (Bio-line) using 1 µg RNA, following the manufacturer's protocol. cDNA was quantified fluorometrically using Qubit 4 (Thermo Scientific).

Primers for RT-qPCR were designed on the *W. somnifera* *de novo* assembled transcriptome to amplify all transcripts of a given unigene (Online Resource10). RT-qPCR reactions consisted of 1.2 ng cDNA template, 5 µl QuantiNova SYBR Green Mastermix (Qiagen), and 0.5 mM of each primer in a total reaction volume of 10 µl. Reactions were performed using a CFX96 thermocycler (BioRad) following the QuantiNova SYBR Green (Qiagen) temperature cycling protocol. Relative gene expression was calculated using the Pfaffl method (Pfaffl, 2001), taking into account the reaction efficiency for every plate. Ribosomal protein L2 (*RPL2*) was used as a reference gene. To determine statistically significant differences in gene expression, the relative gene expression data were log2 transformed followed by an unpaired *t*-test performed using Prism 10 for macOS (GraphPad).

Results

Physiological Response to Salt Stress

Germination efficiency was generally low (36.7%) when compared to other members of the Solanaceae (Commander et al. 2008) and decreased significantly (17.8%, $p < 0.05$) after the 100 mM NaCl treatment (Fig. 1a). Root tips visually displayed signs of dehydration after the 100 mM NaCl treatment when compared to control plant roots (Fig. 1b–e) and micrographs further revealed a decrease in micro-organisms associated with the root tips (Fig. 1f–g).

RNA-Seq Transcript Analysis

Sequencing using the Illumina NovaSeq 6000 system delivered a total of 24.8 Gb of *W. somnifera* sequences of

good quality (Q20 = 98.7%) with an AT richness of ~58% (Online Resource 1). After *de novo* transcript assembly, 235,635 transcripts were obtained totalling 204.8 Mb (Online Resource 1) which were clustered into 156,397 unigenes (supertranscripts representative of all isoforms) totalling 117.1 Mb (Online Resource 2). BUSCO analysis, using the transcriptome mode, was then performed using the BUSCO core gene sets of the Liliopsida, Solanales, and Chlorophyta as sources to establish the number of represented orthologues captured in the *de novo* RNA-seq assembly. The Chlorophyta orthologue set resulted in the most matched complete single copy orthologues (79.8%), followed by the Solanales orthologue set (72.3%) and lastly the Liliopsida orthologue set (67.3%). In the absence of a documented genome size to evaluate depth of genome coverage, we analyzed the number of transcripts per percentage of total expression and found that approximately 30% of our captured expression was represented by rare/low abundance transcripts, which suggested that our depth of sequence allowed for attaining rare/less abundant transcripts and not only the ones that are highly transcribed (Online Resource 7). To assess the influence of salt stress on gene expression, differentially expressed transcripts were identified between the control and salt-treated samples. A total of 3,048 (1,057 upregulated and 1,991 downregulated) transcripts were identified as significantly differentially expressed ($\text{Log}_2\text{FC} \geq 1$, $\text{padj} \leq 0.05$) in the comparison between control and salt-stressed leaf tissue (Online Resource 3).

Gene ontology Analysis of Differentially Expressed Genes DEGs

To explore the functions of differentially expressed genes (DEGs) between control and salt-stressed plants, an analysis of DEGs from *W. somnifera* was functionally annotated using eggNOG-mapper (Fig. 2). The analysis revealed significant regulation of DEGs involved in phenylpropanoid biosynthesis, hormonal signaling, stress, and other metabolic functions. We then investigated the top one hundred significantly upregulated and downregulated transcripts, which were categorized into broad functional categories (i.e., biological processes, cellular component, and molecular function). The DEGs upregulated in response to salt treatment (Fig. 3) were assigned gene ontology (GO) terms attributed to enhanced defence and detoxification ("Defence against abiotic stress" and "Detoxification"), cellular remodeling and transport ("Plasma membrane," "Golgi apparatus," "transmembrane-," and "vesicle-mediated transport"), molecular adaptations and regulations ("RNA binding" along with diverse molecular functions such as enzymes, binding proteins, and signaling pathway genes), as well as cellular maintenance ("Endosome" and "nucleolus"). The largest downregulated classes (Fig. 4)

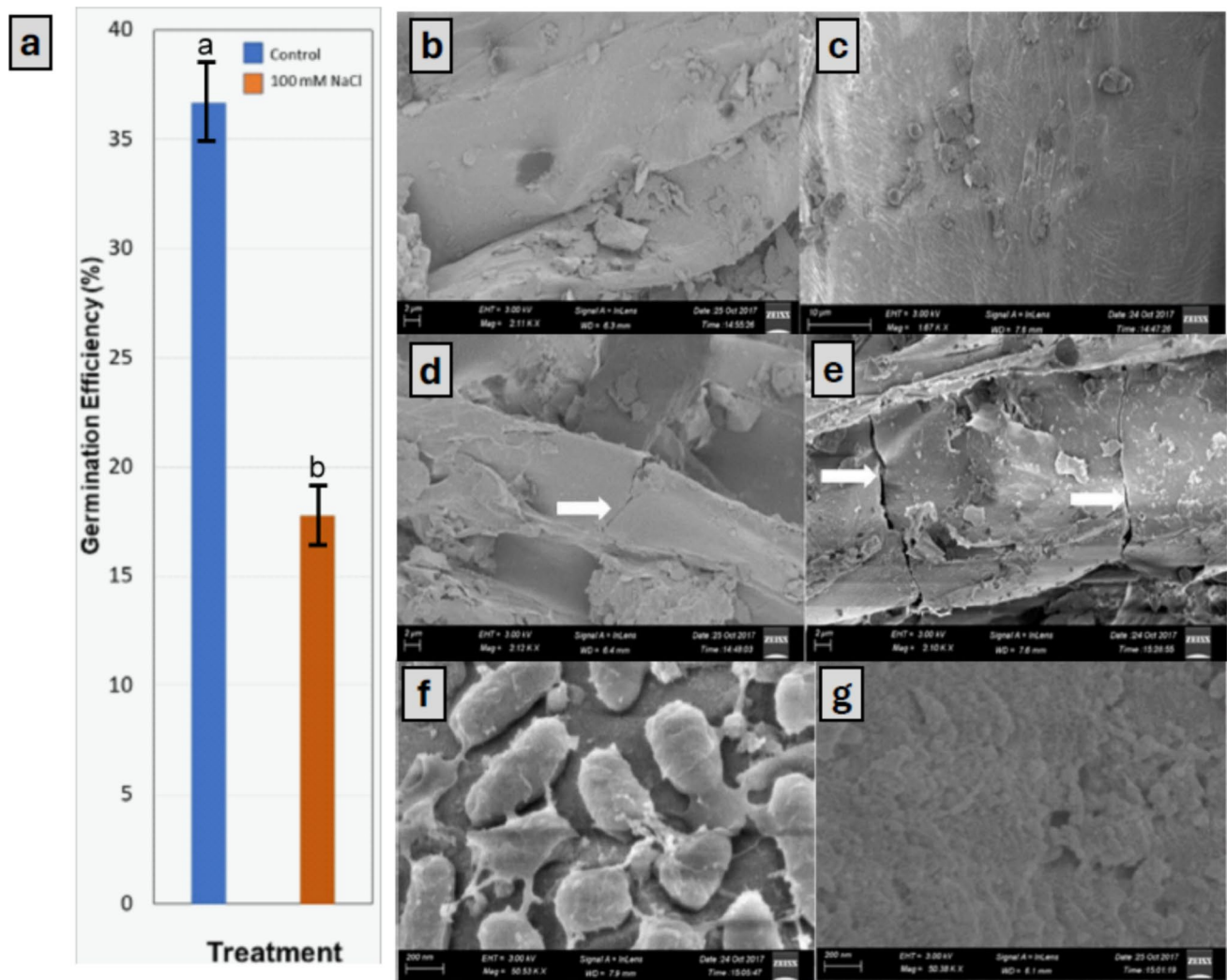


Fig. 1 Germination and damage observed in the root tips of *Withania somnifera* before and after treatment with 100 mM NaCl. **a** Germination efficiency of *W. somnifera* control (blue bar) and 100 mM NaCl-treated (orange bar) seeds. The germination efficiency of *W. somnifera* seed was significantly reduced (17.8%, $p < 0.05$) following NaCl treatment. The error bars indicate standard error, while different alphabetical letters indicate significant difference at $p < 0.05$. **b–e**

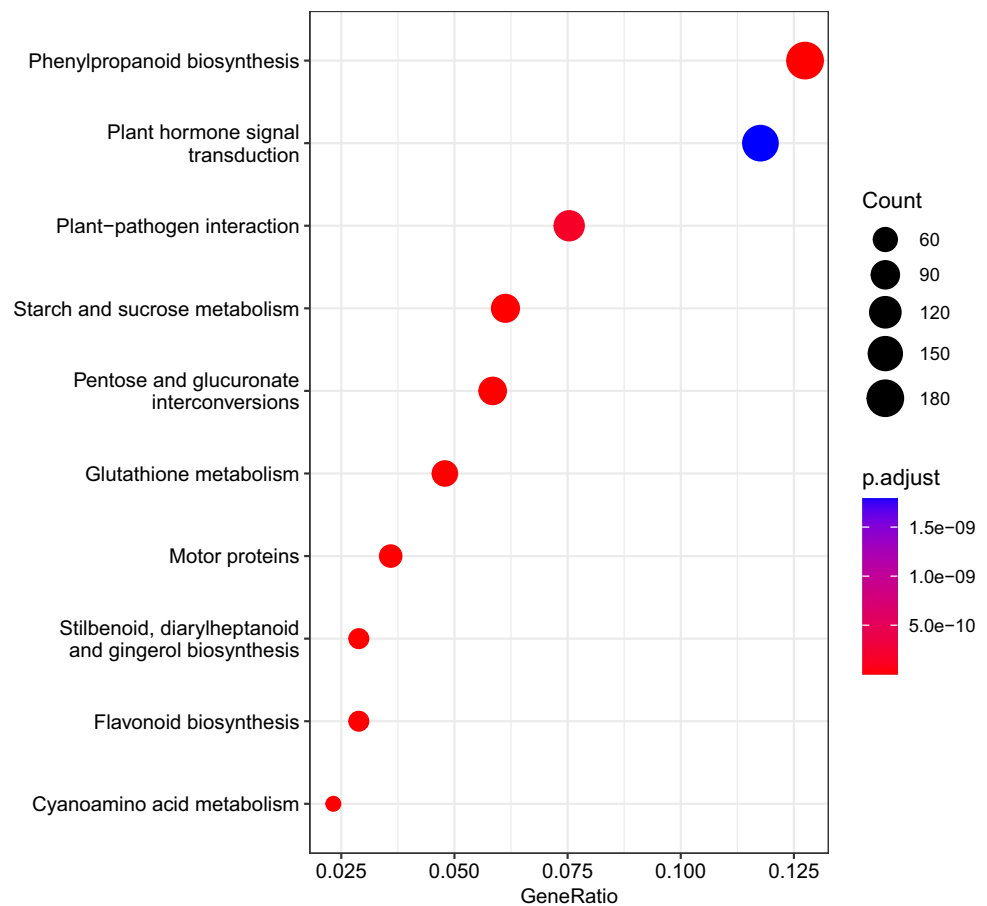
SEM micrographs of *W. somnifera* root tips. **b, c** Control plants and **d, e** plants exposed to 100 mM NaCl. Root damage and/or dehydration is visible as cracks in **d** and **e** as indicated by the arrows. Scale bar = 2 μ m. **f, g** SEM micrographs of *W. somnifera* root tips. **f** Control plants and plants exposed to 100 mM NaCl. Microorganisms are visible on the root surface of control plants (**f**) but not on roots of 100 mM NaCl stressed plants. (**g**) Scale bar = 200 μ m

in response to salt treatment indicated potential changes in gene expression and protein processing (“Nucleus” and “transferase activity”). DEGs downregulated in response to salt treatment also indicated a reduction in growth and development (“Anatomical structure development,” “cell differentiation,” and “cytoskeleton” organization), cellular metabolism, and energy production (“Carbohydrate”- and “lipid-metabolic processes” as well as “intracellular protein transport” and “protein glycosylation”), DNA replication and cell division (“DNA replication,” “repair,” and “recombination” along with “mitotic nuclear division”).

Salt Treatment Effects on the Withanolide Pathway

To investigate the effect of the salt treatment on genes encoding enzymes involved in the biosynthesis of withanolides (MVA and MEP-DOXP pathways) was investigated (Figs. 5 and 6, Online Resource 8). A comprehensive list of genes associated with withanolide production from previous studies was compiled (Online Resource 3), including genes encoding enzymes involved in the MVA pathway and the MEP/DOXP pathway. Of these 57 genes associated with withanolide production, several were

Fig. 2 Dotplot of tomato-specific KEGG pathway enrichment after *W. somnifera* KO identifiers were mapped to their corresponding tomato gene. Pathways were considered significantly enriched if the Benjamini–Hochberg adjusted p-value was less than 0.05. Significant results were visualized using a dot plot to display the top 10 pathways, ordered by gene ratio (the proportion of input genes in the pathway).



found to be significantly differentially expressed (down-regulated) between control and salt-stressed leaf tissue (Figs. 5 and 6, Online Resource 8).

Following salt stress of 21-day-old plants with 100 mM NaCl for 7 days, no significant difference in *HMGR* expression was observed (difference between means of relative expression: 0.3090; $p = 0.0530$) (Figs. 5 and 6).

Identification of Differentially Expressed Transcription Factors (TFs)

Through iTAK analysis of the transcriptome data, a total of 1,506 transcripts were assigned to 64 TF families and 25 transcriptional regulators. Of these transcripts, 72 were significantly differentially expressed ($\text{Log}_2\text{FC} \geq 1$), with 60 transcripts assigned to 26 TF families and 12 transcripts assigned to 10 transcriptional regulator families. The most regulated transcription factor families in response to salt stress included the AP2/ERF (9), NAC (7), MYB-related (5), C2H2 (4), and HSF (4) TF families (Fig. 7, Online Resource 4).

Identification of LEA Gene Transcripts Regulated in Response to Salt Stress

A total of 77 transcripts with similarity to LEA genes were identified from the transcriptome data (Online Resource 5). These transcripts were classified into eight families, based on the 60% identity and query-coverage cut-off, where 59 belonged to LEA-2 and a single transcript each to LEA-5, LEA-6, and Seed maturation protein (SMP), the largest and smallest represented families, respectively. Of the 77 transcripts, only 10 transcripts were significantly regulated ($\text{Log}_2\text{FC} \geq 1$), with seven transcripts upregulated and three downregulated. Most upregulated LEA transcripts (4 transcripts) belonged to the LEA-2 subgroup of dehydrins, whereas all three downregulated transcripts belonged to the LEA-2 family.

Discussion

Environmental factors and stresses cause plants to respond by inducing several morphological, physiological, and metabolic responses. Included in these responses are the changes

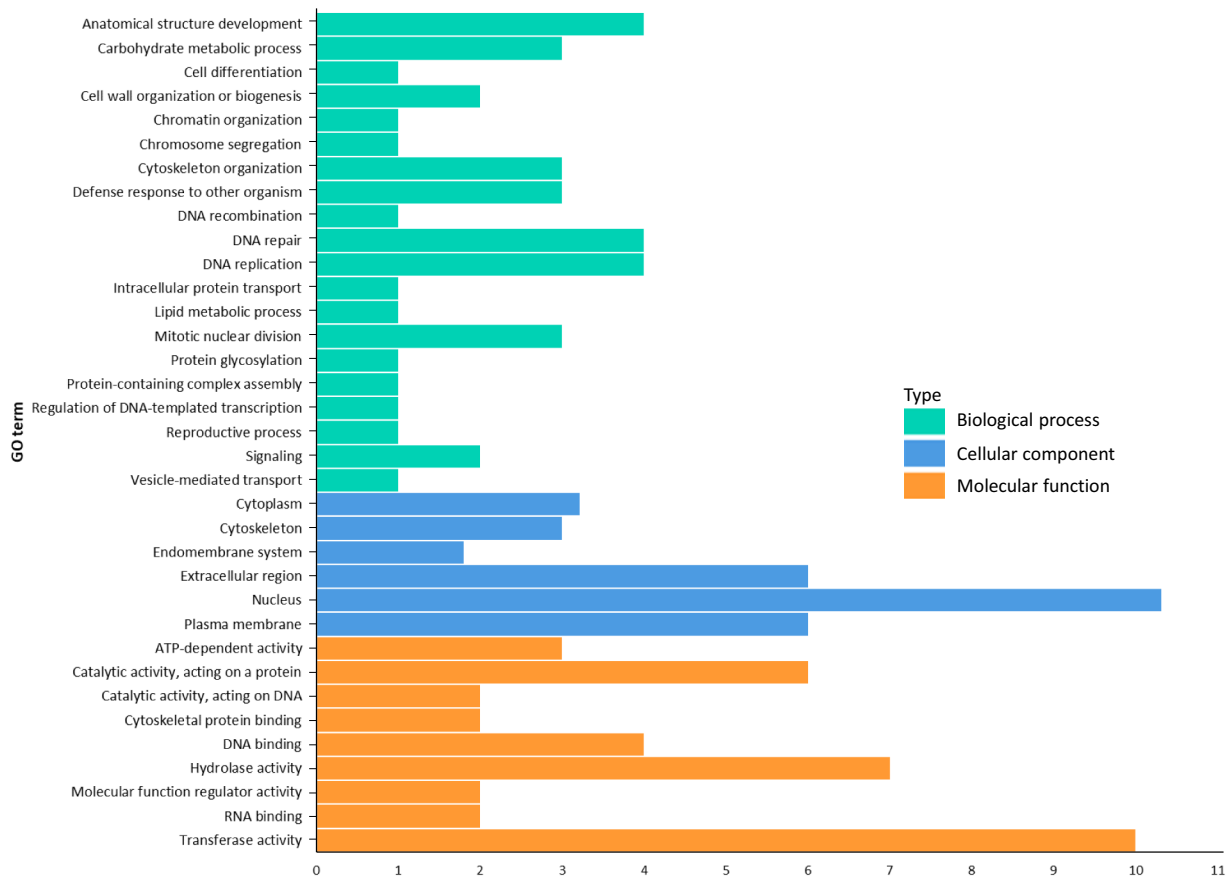


Fig. 3 Gene ontology analysis of downregulated DEGs. Bar graph representing level 2 GO annotation into three categories: biological processes, molecular functions, and cellular components of the 100-

most significantly downregulated transcripts in response to the 100 mM NaCl-induced stress

in metabolic pathways resulting in altered production and accumulation of secondary metabolites (Mareri et al. 2022), such as withanolides. Withanolide biosynthesis is therefore dependent on the growing conditions of the plants, with soil salinization being a major environmental factor to consider if large-scale, formal cultivation of *W. somnifera* is needed to meet the growing global requirement for withanolides. Yet, the cultivation of this plant may be a viable strategy for exploiting saline soils, provided that an increase in the production of withanolides in response to high soil salinity is observed. The response by plants to salt stress is a complex mechanism, involving various physiological and biochemical processes. However, there is a lack of molecular characterization of *W. somnifera* in response to salt treatment, and therefore this study serves as an initial investigation to address this imperative approach.

Germination Data

Plant species exhibit varying degrees of sensitivity to salt stress across different growth stages, with the seed

germination period being the most vulnerable to salt stress. During this phase, exposure to salt stress results in a decrease in germination percentage, a delay in the germination rate, and an inhibition of plant tissue elongation (Tarchoun et al. 2022). This is attributed to ion toxicity, oxidative stress, and increased external osmotic potential, resulting in the reduction of water uptake during imbibition (Shahid et al. 2020). Salt treatment of *W. somnifera* seeds significantly reduced their germination rate compared to untreated control groups (Fig. 1a). This concurs with previous studies, showing a reduction in the germination rate of *W. somnifera* seeds exposed to as little as 10 mM NaCl (Jaleel et al. 2009a, 2009b). Furthermore, salt stress appeared to induce damage to the root surface and prevent the colonization of the root by rhizospheric microbiota in comparison to untreated controls (Fig. 1b–g). While the mechanisms and implications of these induced changes on *W. somnifera* roots are yet to be investigated, damage to or changes in root structure in other plant species have been observed following salinity stress (Hasan et al. 2018; Silva et al. 2021; Tan et al. 2020).

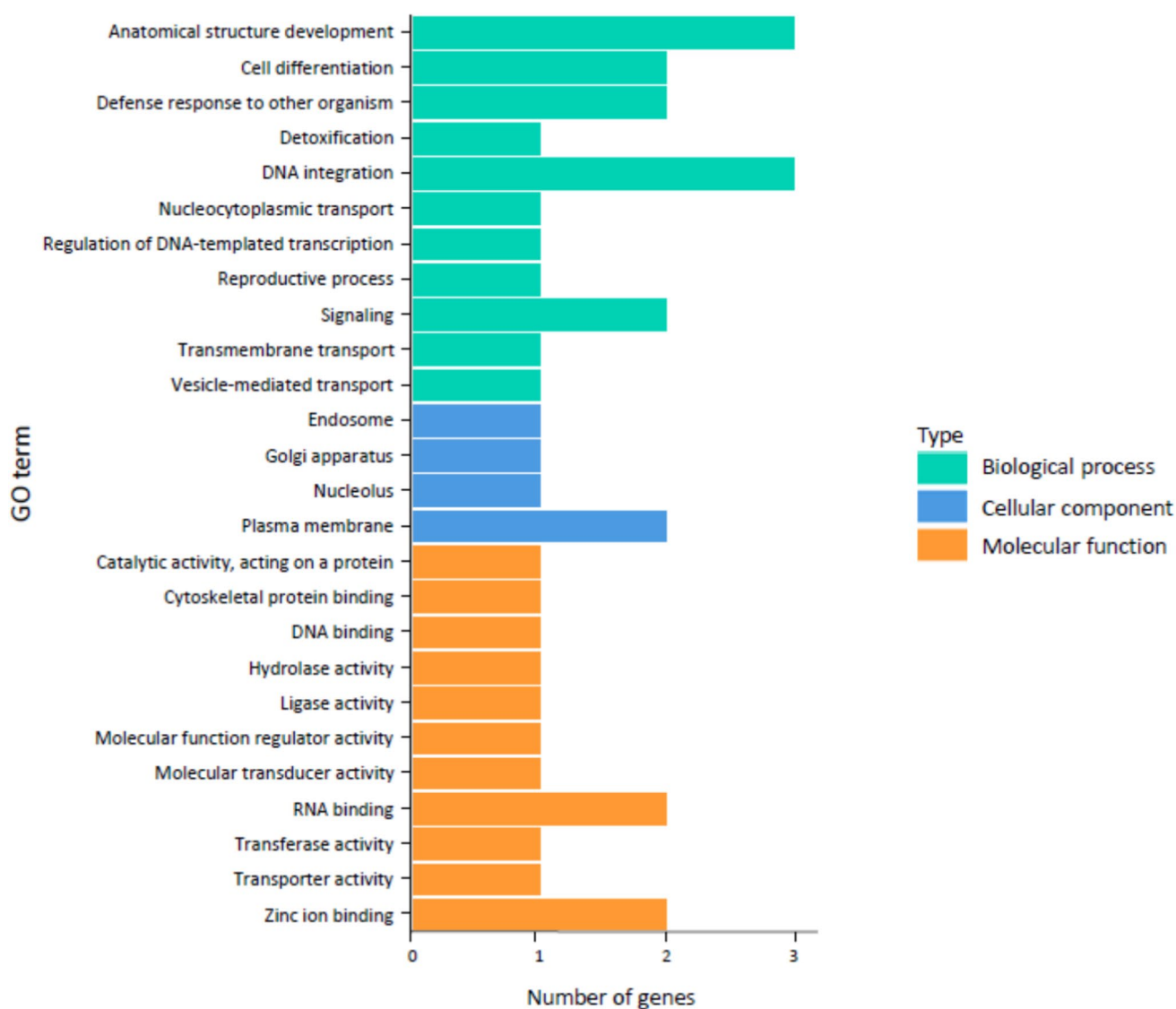


Fig. 4 Gene ontology analysis of upregulated DEGs. Bar graph representing level 2 GO annotation into three categories: biological processes, molecular functions, and cellular components of the 100-most

significantly upregulated transcripts in response to the 100 mM NaCl-induced stress.

Differentially Expressed Genes and Gene Ontology Analysis

Salt stress resulted in the significant regulation of many genes in (Online Resource 3) *W. somnifera* leaf tissue. However, of those genes submitted for GO analysis, few were returned with descriptions (Figs. 2, 3, and 4), highlighting the lack of annotated genes for this medicinally important plant species in the publicly available database. In response to salt stress, significantly upregulated genes with annotated descriptions indicate molecular-level adaptations aimed at mitigating stress. Increased detoxification pathways facilitate the removal of toxic ions (Puccio et al. 2023), while the upregulation of

genes related to transmembrane, vesicle-mediated transport, and specific components like plasma membrane and Golgi apparatus points toward adjustments in compartmentalization to discharge or isolate salt ions (Heydarian et al. 2018; Sun et al. 2021). Additionally, increased expression of genes related to DNA integration, RNA binding, and diverse molecular functions (enzymes, binding proteins, regulatory activities) suggests adaptations to manage protein synthesis, gene expression, and overall cellular processes under stress (Diray-Arce et al. 2015).

The significant downregulation of genes related to anatomical structure development, cell differentiation, cell wall organization, and mitotic nuclear division suggests the plant

prioritizes immediate survival over growth and expansion under stress. This is supported by the observed decrease in activity within carbohydrate and lipid metabolic processes, DNA replication, repair, recombination, and chromatin organization, indicating a reallocation of resources toward stress response mechanisms and a reduction in cell division (Kitavi et al. 2023). Furthermore, reductions in intracellular protein transport and protein glycosylation, as well as large reductions in transferase activity, imply adjustments in protein movement and modifications and metabolic pathways, thereby conserving resources (Han et al. 2020).

Overall, analysis of the highly differentially expressed genes in *W. somnifera* under salt stress reveals an upregulation of detoxification pathways and an adjustment in transmembrane transport mechanisms to manage toxic ion concentrations. Concurrently, a notable downregulation of energy-related processes, growth factors, and protein modification pathways underscores a prioritization of internal cellular maintenance over growth. These findings underscore the intricate molecular adaptations employed by *W. somnifera* to navigate salt stress conditions, highlighting a strategic balance between cellular homeostasis and environmental adaptation.

Transcription Factor Genes Responding to Salt Stress

Salt-tolerance mechanisms strongly rely on transcription factors (TFs) to link salt-sensory pathways to stress-responsive gene expression. As such, the expression levels of these various TFs and the genes they regulate may impact the degree of salt tolerance of plants. In this study, the expression levels of 60 TF genes, from 26 TF families, were markedly altered concomitant with salt stress (Fig. 7 and Online Resource 4). Several of the upregulated TF families have been intensively studied, such as WRKY, MYB, and C2H2 Zinc finger proteins (reviewed in Baillo et al. 2019; Liu et al. 2022) and are associated with enhanced stress tolerance in plants. In salt-stressed *W. somnifera* leaf tissue, the TF family with the greatest number of altered members was the AP2/ERF-ERF family (Online Resource 4). The AP2/ERF TF family comprises a family of plant-specific transcription factors extensively engaged in diverse biological processes, including growth, development, as well as responses to both biotic and abiotic stresses (Licausi et al. 2013). Transcriptome analysis of other Solanaceous species, namely tomato and pepper, indicates that AP2/ERF are involved in stress response mechanisms, including salt stress. In particular, the AP2/ERF-ERF subgroup members were enriched in differentially expressed genes, both up- and downregulated, in Solanaceous species indicating their key role in salt-stress tolerance (Choi et al. 2023). Six members of the NAC transcription factor family were also upregulated in *W. somnifera*

following salt stress (Online Resource 4). NAC TFs are a class of stress-related TFs that directly regulate the expression of abiotic stress-related genes and have received much attention as they have been found to play a positive role in drought and salt stress tolerance through modulating an ABA-mediated pathway (Hong et al. 2016; Nuruzzaman et al. 2013; Su et al. 2023). The transcript exhibiting the highest level of induction following salt stress in this study ($\log_2\text{FC} = 2.75$, Online Resource 4) has similarity to a TF belonging to the GRAS family. GRAS TF members have been found to participate in the response to abiotic stress, including salt stress, where their overexpression or silencing generated gain- and loss-of salt-tolerant phenotypes in *Betula platyphylla* (He et al. 2022). The most strongly downregulated transcript with similarity to a TF in response to salt stress belonged to the HB-WOX family ($\log_2\text{FC} = 3.94$, Online Resource 4). While typically associated with plant developmental processes, recent studies have revealed their importance in regulating responses to salt stress (Hao et al. 2019; Li et al. 2022), with several observed to be downregulated (Akbulut et al. 2022).

Withanolide Biosynthesis Pathway

Withanolides, the signature and major active secondary metabolites of *W. somnifera*, are synthesized via the triterpenoid pathway. As such, transcripts of genes involved in the isoprenogenesis precursor pathway were investigated for altered expression in salt stressed plant leaf tissue. The transcript of nine and eleven genes implicated in the MEP and MVA pathways, respectively, of isoprenogenesis were examined (Online Resource 3). The transcript levels of the cytosolic MVA pathway gene 3-hydroxy-3-methylglutaryl-CoA synthase (*HMGs*) were significantly reduced ($\log_2\text{FC} = -1.4$, Fig. 6, Online Resource 3) in salt-stressed leaf tissue compared to control plants. This may indicate a reduced HMG-CoA pool for the downstream conversion into mevalonic acid, a key regulatory step of isoprenogenesis (Akhtar et al. 2013). However, no other statistically significant differences in the transcript levels of MEP or MVA pathway genes were observed between control and salt-stressed leaf tissue. These results suggest that the salt treatment applied in this study has little effect on isoprenogenesis, implying that the metabolic flux of precursor molecules toward withanolide biosynthesis was unaltered.

Additional key regulatory genes implicated in withanolide biosynthesis were investigated in this study for altered expression following salt stress. Within the withanolide biosynthesis pathway, genes such as farnesyl pyrophosphate synthase (*FPPS*), squalene synthase (*SQS*), cycloartenol synthase (*CAS*), and sterol methyl transferase (*SMT*) lie at critical branch-points of several pathways leading to the synthesis of compounds essential for plant growth and

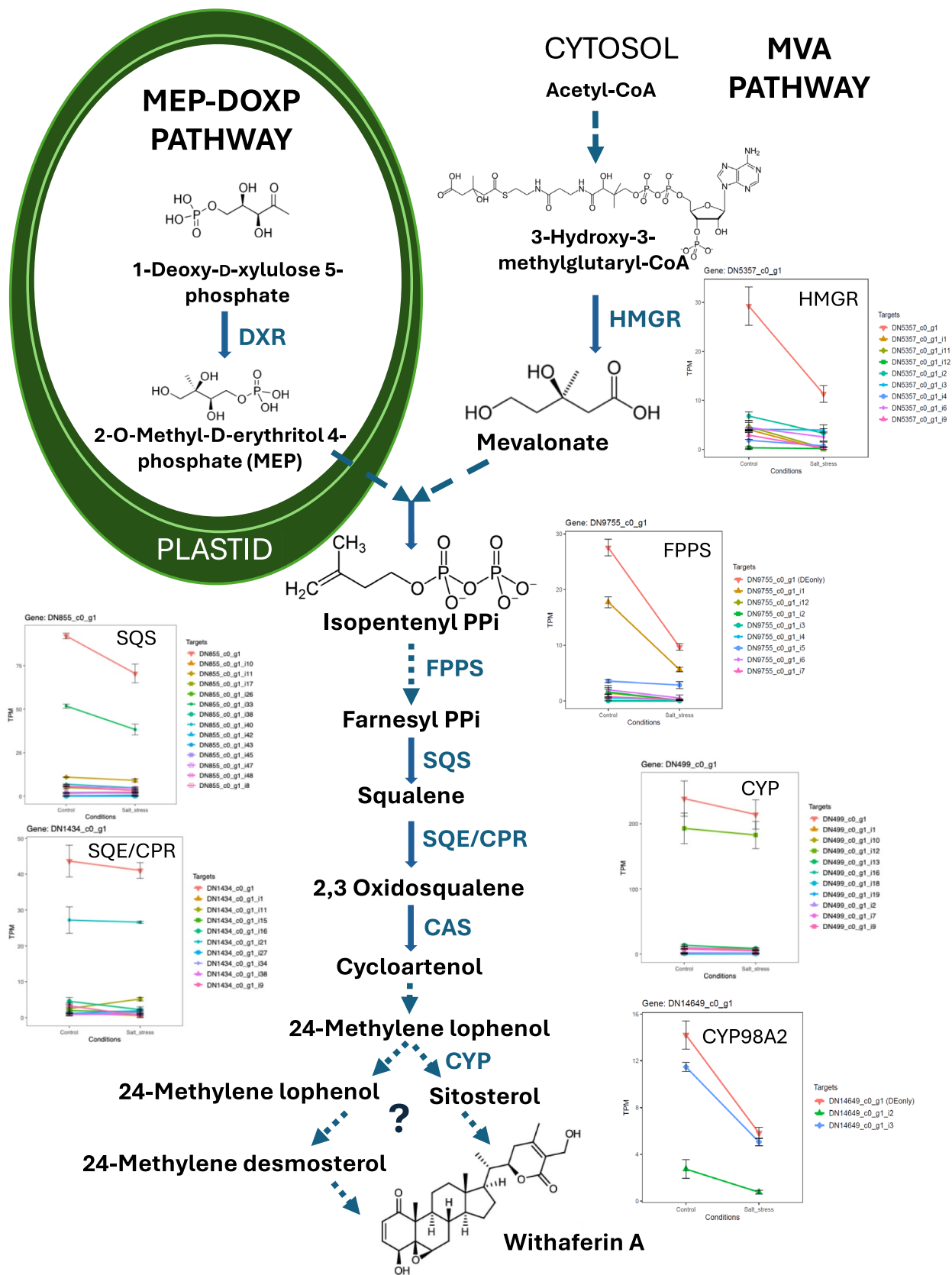


Fig. 5 The withanolide biosynthetic pathway. Indicated are some of the most important steps in the withanolide biosynthetic pathway, as well as the regulation of transcripts (*W. somnifera* orthologs, Online Resource 6) encoding the synthesis of selected enzymes. Genes were considered significantly regulated if the Benjamini–Hochberg adjusted *p*-value was less than 0.05. Where MEP-DOXP=plastidial methylerythritol phosphate; MVA=cytosolic mevalonic acid; DXR=1-deoxy-D-xylulose 5-phosphate; HMGR=hydroxy-3-methylglutaryl coenzyme A reductase; FPPS=farnesyl diphosphate synthase; SQS=Squalene synthase; SQE/CPR=Squalene epoxidase/cytochrome P450 reductase; CAS=cycloartenol synthase; CYP=cytochrome P450 monooxygenase; CoA=acetyl coenzyme A

development as well as withanolides (Singh et al. 2014). Changes in the expression of these genes caused by salt stress may result in a deregulation of downstream metabolic flux toward 24-methylene cholesterol and subsequent withanolides (Figs. 5 and 6).

A common downstream enzyme of precursor biosynthesis, FPPS catalyzes the formation of farnesyl diphosphate (FPP), a short-chain prenyl diphosphate important in various branched pathways (Senthil et al. 2015). As

such, FPPS is considered a branch-point enzyme capable of directing carbon flow away from central isoprenoid biosynthesis (Dhar et al. 2013). This implies that changes in the levels of FPPS would alter the flux of isoprenoids toward various branches of this pathway (Dhar et al., 2013). Furthermore, FPPS acts as a key enzyme in isoprenoid biosynthesis, particularly in its role in initiating the triterpenoid precursor synthesis that contributes to withanolide biosynthesis (Thirugnanasambantham and Senthil 2016). This may indicate a perturbation in downstream withanolide biosynthesis; however, as FPPS is positioned at the intersection of different sterol biosynthesis pathways and is involved in the synthesis of sesquiterpene precursors for several classes of essential metabolites, changes in *FPPS* expression may not directly modulate withanolide content. As such, direct measurement of the leaf withanolide content would be necessary to determine if downregulation of these genes in response to salt stress resulted in reduced withanolide concentrations.

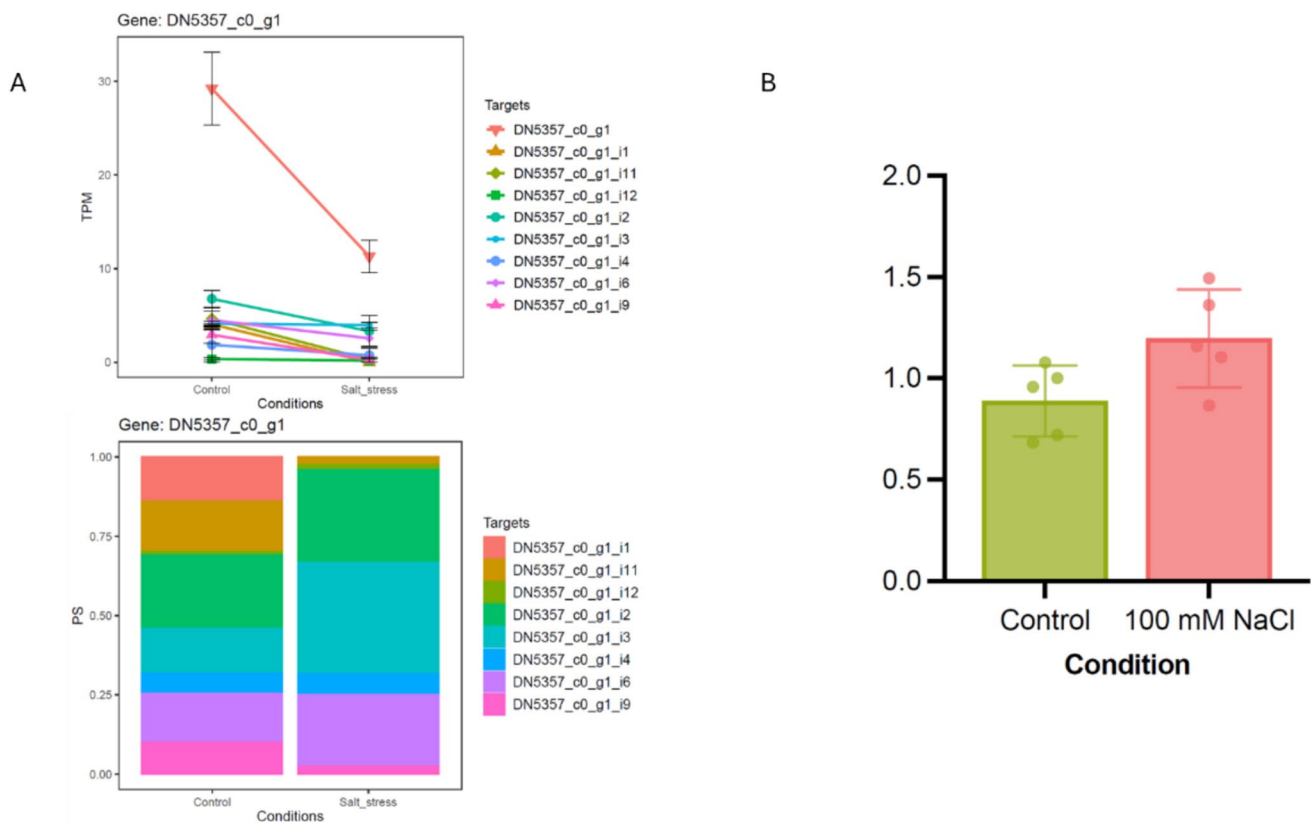


Fig. 6 Comparison between the abundance in hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*) transcripts obtained after RNAseq analysis and the relative gene expression as measured using qRT-PCR. A gene encoding a key regulator in the Mevalonate (MVA) pathway, hydroxy-3-methylglutaryl coenzyme A reductase

(*HMGR*), was significantly downregulated ($\text{padj} \leq 0.05$) in the leaves of *W. somnifera* compared to control plants following the 100 mM NaCl treatment. **a** Abundance in transcripts in transcripts per million (TPM) and **b** relative *HMGR* expression as quantified using qRT-PCR analysis and using *RPL2* as the reference gene

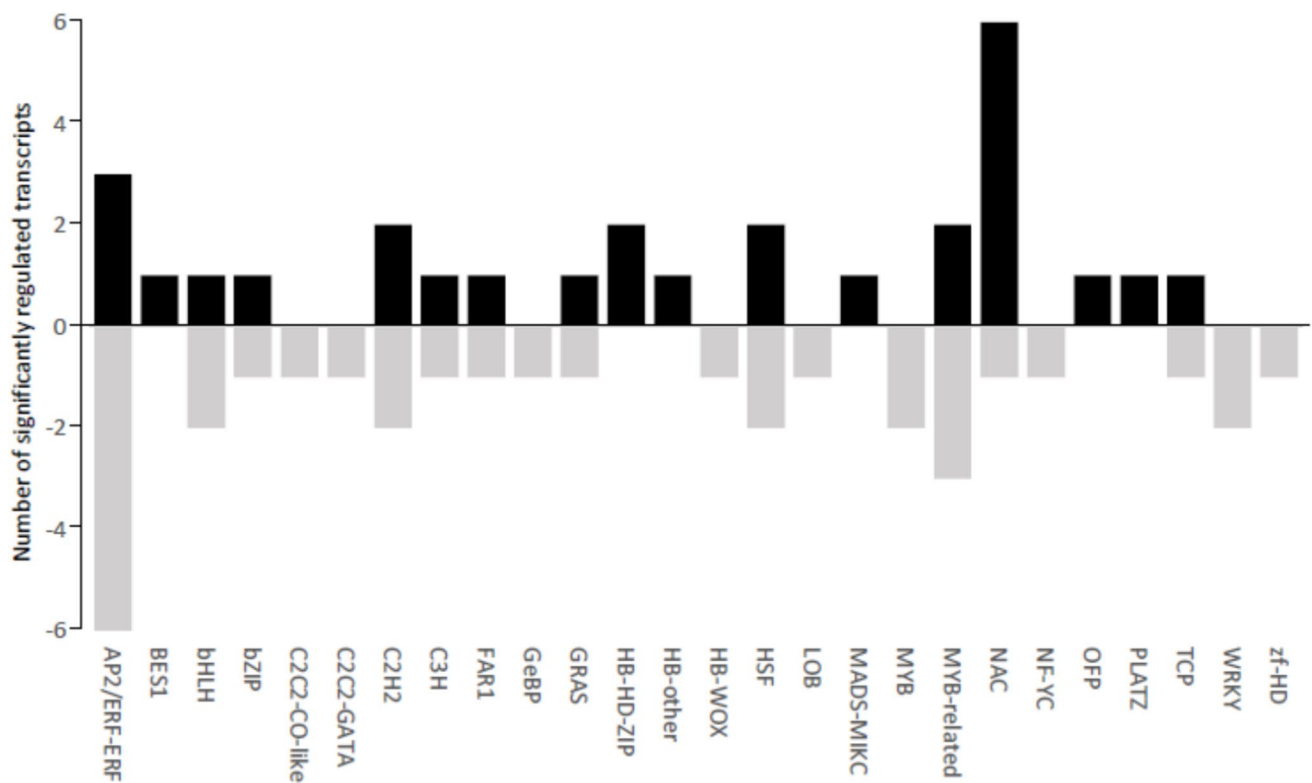


Fig. 7 Analysis of transcription factors responding to salt stress in leaves of *W. somnifera*. The number distribution of up/downregulated TF families in leaves of *W. somnifera* in response to 100 mM NaCl-

induced stress. Black bars indicate the upregulated genes, and the grey bars indicate the downregulated genes

Contribution of LEA Proteins in *W. somnifera* in Response to Salt Stress.

LEA proteins are low molecular weight, hydrophilic proteins that accumulate in plant cells as a functional adaptation to stress, primarily in response to drought conditions, but also in reaction to salinity and low temperatures (Hong-Bo et al. 2005; Tunnacliffe and Wise 2007). While LEA proteins have been identified from various plant species, no inventory of LEA proteins in *W. somnifera* has as yet been performed. In this study, *W. somnifera* transcriptome sequences with similarity to potato LEA gene families resulted in the identification of 77 transcripts with similarity to LEA genes, where the majority of transcripts were classified as part of the LEA-2 family (Online Resource 5). Through genome-wide analysis studies, the LEA-2 group has been identified as the largest LEA family in several plant species (Huang et al. 2022; Ibrahime et al. 2019; Liu et al. 2023; Magwanga et al. 2018; Nagaraju et al. 2019), including the halophyte *E. salsugineum* (Li et al. 2021). Among the LEA-2 protein sub-family, dehydrins were the most upregulated LEA family members, with the expression of four *DHN* transcripts markedly induced in response to salt stress (Online Resource 5). Dehydrins, which accumulate late in embryogenesis and in nearly all vegetative

tissues during normal growth conditions and in response to stress-induced cellular dehydration, have their expression influenced by various abiotic factors and phytohormones. Notably, certain dehydrins, known as Response to ABA (RAB) proteins, exhibit responsiveness to abscisic acid (ABA). This association is significant in the context of ABA's pivotal role as a plant hormone, regulating salt stress responses. Exposure to stressors such as salinity induces ABA accumulation and therefore dehydrins (Allagulova et al. 2003; Hundertmark and Hinch 2008; Rorat 2006), thereby enhancing the plant's salt tolerance by stabilizing membranes, enzymes, and nucleotides in cells under abiotic stresses (Yu et al. 2018). The increased expression of dehydrins and other members of the LEA family observed in this study suggests that the accumulation of LEA proteins may represent a significant strategy employed by *W. somnifera* to mitigate the effects of salt stress.

Conclusion

Exposure of *W. somnifera* to salt stress resulted in reduced germination rates and physiological damage symptoms on the emerging roots of seedlings. Even though the abundance of transcripts encoding the enzymes within the withanolide

pathway was significantly lower in salt-stressed tissue when compared with control plants, we never quantified the production of withanolide content of the leaves. While it will be necessary to ascertain whether the downregulation of these genes results in measurable effects on the withanolide content of the leaves, such an outcome appears unlikely. Furthermore, it appears evident that cultivating *W. somnifera* on saline soils to capitalize on an augmented production of pharmaceutically valuable withanolides represents an impractical strategy. Rather, *W. somnifera* prioritizes immediate survival over growth and expansion under stress conditions, downregulating energy-related processes and protein modification pathways while upregulating detoxification processes. Furthermore, transcription factor genes, particularly those belonging to families like AP2/ERF and GRAS, exhibit significant regulation, suggesting their crucial role in *W. somnifera*'s salt stress response. Furthermore, the regulation of *LEA* genes, particularly the upregulation of dehydrins, indicates a protective mechanism against salt stress-induced cellular dehydration. Overall, this study sheds light on the complex molecular mechanisms underlying *W. somnifera*'s response to salt stress and highlights potential targets for engineering improved salt-tolerance mechanisms in this important medicinal plant species.

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Author Contributions AMB, AB, AM, PSR and NFVB planned the experimental layout. AB and RP were responsible for germination tests and SEM microscopy. NFVB and HWS analyzed the transcripts using software tools. HWS conducted the pathway analysis. VFN wrote the manuscript draft. HWS conducted the RT-qPCR analysis. AMB, NFVB, HWS and VFN assisted with writing and edited the manuscript before submission. All authors read and approved the final manuscript.

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Data Availability The RAW RNAseq data were deposited at the Bio-Project database: accession number PRJNA603753.

Declarations

Competing Interests The authors declare no competing interests.

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