Salt Stress Tolerance in *Casuarina glauca*: Insights from the Branchlets Transcriptome

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1. Introduction

Climate change is unequivocally driving major environmental struggles associated with greenhouse gas (GHG) emissions and their impact on global warming [1,2] and sea-level rise [3]. Together with the accelerated rate of population growth, these changes are imposing a substantial loss of biodiversity and arable land [4,5]. Salinization is one of the major global challenges for agricultural systems. Besides the rising seawater levels, prolonged drought events cause secondary soil salinization as a result of an imbalance between water input (irrigation or rainfall) and use (transpiration) [6]. In this context, the...
use of saline water and halophytes is an alternative strategy to overcome the projected food
and environmental crisis [7].

High salinity is strongly associated with ionic and osmotic stresses [8], which disturb
a range of key metabolic functions, primarily associated with photosynthesis, respiration,
and mineral absorption [9]. During the first phase of stress, the capacity of the plant to
take up water is reduced, with a concomitant reduction of the relative water content (RWC)
and growth rate. In the second phase, the accumulation of salt ions in the leaves hampers
the distribution of photosynthates, and concomitantly plant growth and survival [10]. The
extent of salinity impact depends on several aspects such as, the intensity and duration of
the stress, leaf and plant age, as well as plant species and ecotype [6,10].

Actinorhizal plants are a group of perennial dicotyledonous angiosperms covering
three orders (Fagales, Cucurbitales and Rosales), eight families, and 25 genera [11]. This
group is widely distributed worldwide, being able to thrive under extremely harsh con-
ditions, from polar to desert environments. Most of the tree species are pioneer elements
in several ecosystems and have been successfully used in land reclamation for a long
time [12–17]. The environmental resilience of actinorhizal tree species has been associ-
ated with their capacity to establish root-nodule symbiosis with N\textsubscript{2}-fixing \textit{Frankia}
bacteria, which promotes biomass accumulation and soil fertility [16,18]. Therefore, they constitute
a powerful tool to restore degraded lands and at the same time an intriguing model of
evolutionary adaptation.

Within the actinorhizal group, \textit{Casuarina glauca} Sieb. ex Spreng. (family Casuarinaceae),
a fast-growing tropical tree originating in the southeastern coastal regions of Australia,
is considered the model species [19]. Its small genome and the availability of a genetic
transformation system make \textit{C. glauca} well suited for basic research. In addition, the biol-
ogy of its root-nodule symbiosis with \textit{Frankia} has been studied extensively [20]. Moreover,
\textit{C. glauca} has an outstanding capacity to endure extreme environments, particularly saline
conditions, as it tolerates seawater levels of salt and is commonly planted in areas with a
shallow, saline water table [12,19,21–23]. Therefore, during the last decade our lab has been
dedicated to the elucidation of the mechanisms underlying salt tolerance in this species,
as well as the extent of the contribution of symbiotic \textit{Frankia}. Such studies revealed that
\textit{C. glauca} withstands up to 400 mM NaCl with remarkably low tissue dehydration, associ-
ated with significant osmotic adjustments that minimize the impact of salt on cell water
content and the photosynthetic machinery [22]. Besides that, salt tolerance is closely related
to the maintenance of cellular membranes’ stability and to a robust anti-oxidative response
owing to the accumulation of ROS scavenging components [24], osmolytes, such as neutral
sugars, proline, and ornithine [25,26], and flavonoid-based metabolites, i.e., flavonols and
proanthocyanidins [27]. Moreover, depending on the plant and bacterial genotype, the
ability of \textit{C. glauca} to endure high NaCl concentrations might be coupled or not to the
presence of symbiotic \textit{Frankia} [7,28–30].

The availability of high-throughput techniques, with a high level of precision and
at increasingly affordable prices is currently one of the most straightforward strategies
to understand molecular changes of plants in response to salinity at the system level.
Among them, RNA-Seq is the main choice for studying phenotypic variations, through
the detection of differentially expressed genes (DEGs) between specific environmental
conditions [31]. Additionally, a comprehensive annotation of the genome allows the
analysis of the functions and activities of all types of transcripts [32], offering a solid
basis for the prediction of biomolecular functions and the reconstruction of metabolic
pathways [33,34]. Recent transcriptomic studies provided relevant information about the
mechanisms underlying the ability of halophyte plants to endure high salt concentrations.
Although gene interactions are complex and vary according to plant organ, age, and species,
key genes related to salt-tolerance are often related to hormone signaling pathways that
regulate cell homeostasis and prevent oxidative damage [35–37].

In the present work, we have analyzed the transcriptome of the photosynthetic organs
of \textit{C. glauca} (branchlets), one of the first stress targets, to unravel the molecular mechanisms
underlying stress tolerance, in complement to previous studies in this species, thus contributing to halophyte research. For that, C. glauca plants either supplied with chemical nitrogen (KNO$_3$) or nodulated by Frankia (NOD) were exposed to a gradient of salt concentrations (200, 400, and 600 mM NaCl) and RNA-Seq was performed.

2. Results

2.1. Overall Transcriptome Profiling and De Novo Assembly

Quality assessment, data filtering and trimming generated between 22.9 and 26.6 million clean reads (from 33.4 and 37.0 million raw reads) in NOD plants and between 24.5 and 27.4 million clean reads (from 33.5 to 38.7 million raw reads) in KNO$_3$ plants (Table S1). Minimum per base sequence quality was improved from 2–28 in raw reads to 31–33 in clean reads. No reads were flagged as poor-quality reads. Since FastQ Screen showed no relevant contaminants, all trimmed reads were used in the assembly. De novo assembly showed 41% GC content within a total of 181,484 contigs, 86,202 genes and a contig N50 size of 2792 bp (Table S2). More than 96% of the reads were mapped back to the transcriptome (Table S3) with almost 95% completeness (Table S4).

2.2. Differential Gene Expression in Response to Increasing NaCl Concentrations

Principal Component Analyses (PCA) showed a clear separation of samples in four different quadrants: (i) control (0 mM NaCl) and 200 mM NaCl KNO$_3$ plants (thereafter referred as 200- KNO$_3$); (ii) control and 200 mM NaCl NOD plants (thereafter referred as 200- NOD); (iii) 400 and 600 mM NaCl KNO$_3$ plants (thereafter referred as 400- and 600- KNO$_3$); and (iv) 400 and 600 mM NaCl NOD plants (thereafter referred as 400- and 600- NOD). PC1 accounted for 86% of the total variance, with a clear distinction between the control and 200 mM NaCl samples from the 400 and 600 mM NaCl samples. PC2 (9% variance) distinguished KNO$_3$ from NOD plants (Figure S1).

Overall, the number of Differentially Expressed Genes (DEGs) increased with increasing salinity (Figure 1A). All DEGs (FDR < 0.05) showed a log2FC lower than −2 or higher than 2 (Figure S2). KNO$_3$ plants expressed a total of 19,913 genes in the control and an average of 19,645 genes in samples exposed to salinity (Table 1). From these, an average of 15,328 genes was expressed in both control and salinity-exposed plants. The percentage of significant DEGs was extremely low, i.e., 0.04% (9), 1% (238) and 2% (359) in 200-, 400- and 600- KNO$_3$, respectively. All DEGs were downregulated at 200 mM NaCl. At 400 mM NaCl and 600 mM NaCl, ca. 88% (210 and 314, respectively) DEGs were downregulated and ca. 13% (28 and 45, respectively) upregulated (Figure 1A and Table 1).

NOD plants expressed a higher number of genes compared to KNO$_3$ plants, with a total of 20,278 genes at 0 mM NaCl and an average of 19,780 genes in samples exposed to salinity (Table 1). From those, an average of 16,023 genes was common to control and salinity-stressed plants. Similar to KNO$_3$ plants, a decreasing number of common genes and an increasing number of DEGs were observed with increasing salinity. The percentage of significant DEGs at all salinity conditions was slightly lower than in KNO$_3$ plants: 0.03% (6), 0.5% (104), and 1.3% (254) in 200-, 400- and 600- KNO$_3$, respectively. All DEGs were downregulated at 200 mM NaCl. At 400 mM NaCl and 600 mM NaCl, the percentage of downregulated vs. upregulated DEGs was 60% (62) vs. 40% (42), and 85% (217) vs. 15% (37), respectively.

The number of DEGs specific to only one salt concentration (200, 400, or 600 mM NaCl), increased with the salinity in both plant groups (Figure 1B). In KNO$_3$ plants, the number of treatment-specific downregulated DEGs was 1 (0.3%), 69 (18%), and 175 (45%) at 200, 400, and 600 mM NaCl, respectively. Additionally, two (0.5%) DEGs were commonly found in 200- and 400 KNO$_3$ plants, six (2%) in all set of conditions, and 133 (35%) in 400- and 600 KNO$_3$. Together, the two highest salt concentrations accounted for ca. 98% (175 + 133 + 69) of all downregulated DEGs. Among the upregulated DEGs, 11 (18%) were activated in both 400- and 600- KNO$_3$, 17 (27%) were specific to 400 mM NaCl and 34 (55%) were specific to 600 mM NaCl (Figure 2B). In NOD plants, treatment-specific downregulated
DEGs increased with the salinity level: three (200 mM), 62 (400 mM), and 218 (600 mM). Among these, 27 (11%) were specifically found in 400 NOD⁺, and 183 (75%) in 600 NOD⁺ (Figure 2C). Specific downregulated DEGs were not found in 200-NOD⁺. Only 1 (0.4%) DEG was commonly found in 200- and 400 NOD⁺, 2 (0.8%) in all sets of conditions, and 32 (13%) in 400- and 600 NOD⁺. Together, the number of specific DEGs in 400- and 600- NOD⁺ accounted for 99% (27 + 32 + 183) of the downregulated pool. Among the upregulated DEGs, only 1 (1.5%) was found in 200 NOD⁺, 30 (44%) in 400 NOD⁺, and 25 (37%) in 600 NOD⁺ (Figure 2D). The transcriptional activity of two (3%) DEGs was activated in samples from all set of conditions and 10 (15%) in 400- and 60 NOD⁺. No common DEGs were identified between the other set of conditions. Together, 400- and 600 mM NOD⁺ accounted for 96% (25 + 10 + 30) of the treatment-specific DEGs.

![Figure 1. Differential gene expression in branchlets of *Casuarina glauca* plants nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD⁺) or supplied with KNO₃ (KNO₃⁺). 200: 200 vs. 0 mM NaCl; 400: 400 vs. 0 mM NaCl; and 600: 600 vs. 0 mM NaCl, with a False Discovery Rate < 0.05. (A) Total number of DEGs. (B) Number of DEGs specific to each salinity condition relative to control.](image-url)
Table 1. Differential gene expression quantification in branchlets of *Casuarina glauca* nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD⁺) or non-nodulated plants supplied with KNO₃ (KNO₃⁺), grown at control (0 mM NaCl) and three salinity conditions (200 mM, 400 mM and 600 mM NaCl). Number of total expressed genes by each plant, at each condition, number of genes expressed by both treatment and control of each comparison (common genes percentage relative to the respective average gene expression between brackets), number of differential expressed genes (DEGs) detected by DESeq, NOISeq and edgeR analyses, number of overlapping DEGs in the three analyses (DEGs percentages relative to the respective average gene expression between brackets) and number of respective annotated DEGs, enumerated by type of regulation. Detected and overlapping DEGs correspond to the number of significant DEGs in each salinity-stress treatment in comparison with the control NaCl (respectively, 200: 200 mM vs. 0 mM, 400: 400 mM vs. 0 mM and 600: 600 mM vs. 0 mM), with a False Discovery Rate < 0.05 and a normalized log2 fold change (log2FC) > 2 or < −2.

| Plant Series | [NaCl] mM | Expressed Genes | Detected DEGs | Overlapping DEGs |
|--------------|-----------|-----------------|---------------|------------------|
|              |           | Stressed        | Control       | Common           | DESeq | NOISeq | edgeR | All | Annotated |
| KNO₃⁺        | 200       | 20,765          | 19,913        | 15,928 (78%)     | 58    | 330    | 22    | 9   | (0.04%)  |
|              | 400       | 19,078          | 19,913        | 15,079 (77%)     | 524   | 1213   | 381   | 23  | (1%)     |
|              | 600       | 19,092          | 19,913        | 14,976 (77%)     | 650   | 1450   | 612   | 359| (2%)     |
| NOD⁺         | 200       | 19,397          | 20,278        | 16,386 (83%)     | 44    | 204    | 20    | 6   | (0.03%)  |
|              | 400       | 20,470          | 20,278        | 16,137 (79%)     | 359   | 962    | 184   | 104| (0.5%)   |
|              | 600       | 19,472          | 20,278        | 15,547 (78%)     | 548   | 1261   | 373   | 254| (1%)     |

Figure 2. Differential gene expression in *Casuarina glauca* plants supplied with KNO₃ (KNO₃⁺) or nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD⁺), grown at 200 mM, 400 mM, and 600 mM NaCl. Total number of treatment-specific differentially expressed genes (DEGs) for each salt treatment relative to the control (0 mM NaCl). 200: 200 vs. 0 mM NaCl; 400: 400 vs. 0 mM NaCl; and 600: 600 vs. 0 mM NaCl. (A) KNO₃⁺ downregulated DEGs. (B) KNO₃⁺ upregulated DEGs. (C) NOD⁺ downregulated DEGs. (D) NOD⁺ upregulated DEGs.

2.3. Differential Gene Expression between KNO₃⁺ and NOD⁺ Plants

Comparing KNO₃⁺ and NOD⁺ plants, no common DEGs were observed in samples exposed to 200 mM NaCl (Figure 3B), while 32 (10%) and 135 (28%) were observed in samples subjected to 400 and 600 mM NaCl, respectively (Figure 3B,C). The number of specific DEGs was higher in KNO₃⁺ than in NOD⁺: nine vs. six, 206 (67%) vs. 72 (23%),...
and 224 (47%) vs. 119 (25%), at 200, 400, and 600 mM NaCl, respectively (Figure 3, Tables S5 and S6).

![Figure 3. Specific and overlapping genes between Casuarina glauca plants supplied with KNO$_3$ (KNO$_3^+$) or nodulated by nitrogen-fixing Frankia caesariiue Thr (NOD$^+$). Significant differentially expressed genes (DEGs), with a False Discovery Rate (FDR) < 0.05, were detected between the control (0 mM NaCl) and salinity-exposed plants: (A) 200 mM NaCl; (B) 400 mM NaCl; (C) 600 mM NaCl.](image)

2.4. Top NaCl-Responsive Genes in KNO$_3^+$ and NOD$^+$ Plants

In KNO$_3^+$ plants, only 46% (208 out of 448) DEGs were uniquely mapped to UniProtKB/Swiss-Prot database proteins, two of which corresponded to yet uncharacterized proteins (Figure S3A). Considering each salt condition, 2 (22%), 125 (53%) and 164 (46%) mapped DEGs were identified in samples exposed to 200, 400, and 600 mM NaCl, respectively (Table 1). In 200 KNO$_3^+$, the most downregulated DEGs were ERF020 (log2FC: -4.06), related to stress signal transduction pathways, and GT-3B (log2FC: -3.81), a salt responsive transcription factor. In 400 KNO$_3^+$ the top responsive DEGs were WAXY (log2FC: 3.46) and XCP1 (log2FC: -6.88), respectively. The first is associated with starch biosynthesis, while the second is related to programmed cell death and proteolysis. In 600-KNO$_3^+$, XCP1 (log2FC: -6.88) was also the most downregulated DEG, and PLT6, which is related to glucose import, the most upregulated one (log2FC: 4.55). The top 10 most down- or upregulated DEGs for KNO$_3^+$ plants are summarized in Table 2 (400 mM NaCl) and Table 3 (600 mM NaCl), while the full list of DEGs can be found in Table S7.

Heatmaps were used to analyze the regulation pattern of the top 10 salt-responsive DEGs from 600 mM NaCl relative to the control. For each treatment, the normalized read counts of these genes were also plotted to allow the comparison of gene regulation over the salinity gradient (Figures S4 and S5). Two main clusters were formed, one with samples from 0 mM and 200 mM NaCl, and another with samples from 400 mM NaCl and 600 mM NaCl.

The top downregulated DEGs from KNO$_3^+$ plants exposed to 600 mM NaCl included an association with response to hypoxia (GO:0001666), DNA (GO:0003677), carbohydrate metabolism (GO:0030246), metal ion- (GO:0046872) and mannose binding (GO:0005537), leaf senescence (GO:0010150), cell death (GO:0008219), cell wall organization biogenesis (GO:0071554), and iron homeostasis (GO:0055072) and transport (GO:0006810). The top upregulated DEGs were related to the biosynthesis of (+)-secologanin, the precurser of monoterpene indole alkaloids (GO:1900994), abscisic acid-activated signaling (GO:00097389), alkaloid metabolism (GO:009820), plant-type hypersensitive response (GO:009626), regulation of stomatal opening (GO:1902456), glucose import (GO:0046323), UDP-glycosyltransferase (GO:0008194), antiporter activities
(GO:0015297), chitin catabolism (GO:0006032), and chaperone cofactor-dependent protein refolding (GO:0051085) (Table 3; Figure S4).

**Table 2.** Top 10 most down and upregulated differentially expressed genes (DEGs) in non-nodulated *Casuarina glauca* plants supplied with KNO$_3$ (KNO$_3^+$), grown at 400 mM NaCl relative to the control (0 mM NaCl), ordered by log$_2$ fold-change (log$2$FC). NBCI gene symbol, protein name, log$2$FC and respective Gene Ontology (GO) molecular function(s) and biological process(es) were retrieved from UniprotKB database. Protein names coloured in blue are also among the top 10 most down or upregulated DEGs, respectively, at 400 mM NaCl in plants nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD$^+$.)

| Gene      | Protein Name                                      | Log$_2$FC | Molecular Function/Biological Process                                      |
|-----------|--------------------------------------------------|-----------|---------------------------------------------------------------------------|
| **Downregulated** |                                                 |           |                                                                           |
| XCP1      | Cysteine protease XCP1                            | −6.883    | Programmed cell death; proteolysis                                          |
| AT3G10080 | Germin-like protein subfamily 3 member 2          | −6.658    | Manganese ion binding; nutrient reservoir activity                         |
| ACA13     | Putative calcium-transporting ATPase 13, plasma   | −6.476    | ATPase-coupled cation and calcium transmembrane transporter activity       |
| membrane-type|                                               |           |                                                                           |
| RL5       | Protein RADIALIS-like 5                          | −5.931    | DNA binding                                                               |
| ERF020    | Ethylene-responsive transcription factor ERF020   | −5.644    | Ethylene-activated signaling pathway; response to chitin                   |
| YAH3      | Histone H3.2                                      | −5.476    | DNA binding; protein heterodimerization activity                           |
| LRX2      | Leucine-rich repeat extensin-like protein 2       | −5.157    | Cell wall organization                                                    |
| PATL4     | Patellin                                          | −5.129    | Cell cycle and division; cellular response to auxin stimulus; auxin polar transport |
| PGL3      | Polyalacturonase 1 beta-like protein 3            | −5.073    | Cell size determination                                                   |
| FLA12     | Fasciclin-like arabinogalactan protein 12         | −5.055    | Plant-type secondary cell wall biogenesis                                  |
| **Upregulated** |                                               |           |                                                                           |
| WAXY      | Granule-bound starch synthase 1, chloroplastic/amyloplastic | 3.459    | Starch biosynthetic process                                               |
| HSP70     | Heat shock 70 kDa protein                         | 3.363     | Cellular response to unfolded protein; chaperone cofactor-dependent protein refolding |
| N/A       | Myrcene synthase, chloroplastic                   | 3.129     | Magnesium ion binding; myrcene synthase activity                           |
| SD11      | Protein sulfur deficiency-induced 1              | 3.000     | Cellular response to sulfur starvation; regulation of glucosinolate biosynthetic process and sulfur utilization |
| UGT87A1   | UDP-glycosyltransferase 87A1                      | 2.824     | UDP-glycosyltransferase activity                                           |
| ARF16     | Auxin response factor 16                          | 2.644     | Auxin-activated signaling pathway; cell division; pattern specification process; response to auxin; root cap development |
| LECKR11   | Putative L-type lectin-domain containing receptor | 2.369     | Defense response to bacterium and oomycetes                               |
| TOGT1     | Scopoletin glucosyltransferase                    | 2.290     | Identiﬁcal protein binding; scopoletin glucosyltransferase activity        |

In NOD$^+$ plants, 43% (136 out of 313) DEGs were uniquely mapped to known proteins, four of which uncharacterized (Figure S3B). Considering each salt condition, four (67%), 49 (47%) and 111 (44%) of mapped DEGs were identiﬁed in 200-, 400-, and 600- NOD$^+$, respectively (Table 1). Among the annotated DEGs from 200- NOD$^+$, the most upregulated gene was PEPR1 (log$2$FC: 3.59) which is involved in PAMP-triggered immunity (PTI) signaling, followed by GDH2 (log$2$FC: 3.32) that catalyzes the production of GABA, and CYP78A5 (log$2$FC: 2.89) required for the promotion of leaf and floral growth and the prolongation of the plastochron. The only downregulated gene was GLPR2.8 (log$2$FC: −3.25), involved in light signal transduction and calcium homeostasis via the regulation of calcium influx into cells. In 400 NOD$^+$, the most responsive DEGs were GDH2 (log$2$FC: 5.09) encoding a glutamate dehydrogenase, and RL5 (log$2$FC: −7.79) related to DNA binding. In 600 NOD$^+$, the most up- or downregulated DEGs were PEPR1 (log$2$FC: 4.59) encoding a Leucine-rich repeat receptor-like kinase involved in PTI signaling, and RL5 (log$2$FC: −7.79),
respectively. The top 10 most responsive DEGs in the NOD+ group are summarized in Tables 4 and 5 and the full list of DEGs can be found in Table S8.

**Table 3.** Top 10 most down and upregulated differentially expressed genes (DEGs) in non-nodulated *Casuarina glauca* plants supplied with KNO\(_3\) (KNO\(_3^+\)), grown at 600 mM NaCl relative to the control (0 mM NaCl), ordered by log2 fold-change (log2FC). NBCI gene symbol, protein name, log2FC and respective Gene Ontology (GO) molecular function(s) and biological process(es) retrieved from UniprotKB database. Protein names coloured in blue are also on the top 10 most down or upregulated DEGs, respectively, at 600 mM NaCl in plants nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD+).

| Gene           | Protein Name                                | Log2FC | Molecular Function/Biological Process                                                                 |
|----------------|---------------------------------------------|--------|--------------------------------------------------------------------------------------------------------|
| XCP1           | Cysteine protease XCP1                      | −6.883 | Programmed cell death; proteolysis                                                                   |
| AT3G61750      | Cytochrome b561 and DOMON domain-containing protein AT3g61750 | −6.775 | Metal ion binding                                                                                   |
| N/A            | Lectin                                      | −6.600 | Mannose binding                                                                                     |
| LAC22          | Laccase-22                                   | −6.459 | Iron ion homeostasis and transport; lignin catabolic process                                         |
| N/A            | Seed lectin                                 | −6.392 | Carbohydrate binding; metal ion binding                                                             |
| CESA9          | Cellulose synthase A catalytic subunit 9 [UDP-forming] | −6.109 | Cell wall organization and biogenesis                                                               |
| SERK4          | Somatic embryogenesis receptor kinase 4     | −5.954 | Cell death; phosphorylation; response to hypoxia and chitin; leaf senescence and seedling development |
| RL5            | Protein RADIALIS-like 5                    | −5.931 | DNA binding                                                                                          |
| LECKR91        | L-type lectin-domain containing receptor kinase IX.1 | −5.781 | Defense response to bacterium and oomycetes; positive regulation of cell death and hydrogen peroxide metabolic process |
| LRK10L-1.4     | Leaf rust 10 disease-resistance locus receptor-like protein kinase-like 1.4 | −5.700 | Cellular response to hypoxia; phosphorylation                                                        |

**Table 3 (continued).** For the top 10 upregulated DEGs, two main clusters were also formed in NOD+, one including the 0 mM and the 200 mM NaCl groups, and the other including the 400 mM and 600 mM NaCl groups. However, unlike the situation in KNO\(_3^+\) plants, a clear separation of control and 200- NOD+ was observed in the first cluster. Similar to KNO\(_3^+\) plants, the top downregulated DEGs in NOD+ plants exposed to 600 mM NaCl were associated with response to hypoxia, DNA- and mannose binding, cell death and cell wall biogenesis and organization (Table 3 vs. Table 5; Figure S4 vs. Figure S5). Additionally, these DEGs were also related to defense responses (GO:0009738), leaf morphogenesis (GO:0009965), response to oxidative stress (GO:0006979), cellular response to amino acid stimulus (GO:0071230), phosphorylation (GO:0016310), defense responses to bacteria (GO:0042742), signal transduction (GO:0007165) and metabolic processes (GO:0008152) (Table 5; Figure S5). Also comparable to KNO\(_3^+\) plants, in NOD+ plants the top upregulated DEGs at 600 mM NaCl were related to abscisic acid-activated signaling (GO:0009738), alkaloid metabolism (GO:0009820) and regulation of stomatal opening (GO:1902456) (Table 3 vs.
Table 5; Figure S4 vs. Figure S5). Additionally, they were also associated with response to sulphur starvation (GO:0010438), jasmonic acid (GO:0009753) and wounding (GO:0009611), protein transport (GO:0015031), chaperone cofactor-dependent protein refolding, transmembrane transporter activity (GO:0022857), carbohydrate metabolism (GO:0005975), cell growth (GO:0016049), and lumen of the endoplasmic reticulum (GO:0005788) (Table 5; Figure S5).

Table 4. Top 10 most down and upregulated differentially expressed genes (DEGs) in Casuarina glauca nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD+), grown at 400 mM NaCl relative to the control (0 mM NaCl), ordered by log2 fold-change (log2FC). NBCI gene symbol, protein name, log2FC and respective Gene Ontology (GO) molecular function(s) and biological process(es) retrieved from UniprotKB database. Protein names coloured in blue are also among the top 10 most down or upregulated DEGs, respectively, at 400 mM NaCl in non-nodulated plants supplied with KNO3 (KNO3+).

| Gene    | Protein Name                      | Log2FC | Molecular Function/Biological Process                                                                 |
|---------|-----------------------------------|--------|-------------------------------------------------------------------------------------------------------|
| **Downregulated**                                                                 |
| RL5     | Protein RADIALIS-like 5           | −7.794 | DNA binding                                                                                          |
|         |                                   |        | Auxin polar transport; carotene catabolic process; leaf morphogenesis; response to auxin; secondary shoot formation; strigolactone biosynthetic process; xanthophyll catabolic process |
| CCD8    | Carotenoid cleavage dioxygenase 8, chloroplastic | −7.249 | Hydrogen peroxide catabolic process; response to oxidative stress                                     |
| PER64   | Peroxidase 64                     | −5.977 | Cell wall modification; pectin catabolic process                                                       |
| PME6    | Probable pectinesterase/pectinesterase inhibitor 6 | −5.274 | Calcium ion transport; calcium-mediated signaling; cellular response to amino acid stimulus              |
| GLR2.8  | Glutamate receptor 2.8            | −5.247 | DNA binding; DNA-binding transcription factor activity                                                 |
| FLA12   | Fasciclin-like arabinogalactan protein 12 | −4.542 | Cellulose microfibril organization; plant-type cell wall biogenesis; plant-type cell wall cellulose biosynthetic process; plant-type cell wall organization; plant-type secondary cell wall biogenesis |
| RL3     | Protein RADIALIS-like 3           | −4.261 | DNA binding; protein heterodimerization activity                                                       |
| COBL4   | COBRA-like protein 4              | −4.087 | Aromatic compound biosynthetic process; methylation                                                   |
| YAH3    | Histone H3.2                      | −3.906 | DNA binding; protein heterodimerization activity                                                       |
| ROMT    | Trans-resveratrol di-O-methyltransferase | −3.729 |                                                                                                       |
| **Upregulated**                                                                 |
| GDH2    | Glutamate dehydrogenase 2         | 5.087  | Glutamate catabolic process; response to cadmium ion                                                 |
|         |                                   |        | Abscisic acid catabolic process; abscisic acid metabolic process; oxidation-reduction process; release of seed from dormancy; response to red or far red light; sterol metabolic process |
| CYP707A2| Abscisic acid 8'-hydroxylase 2    | 4.321  | Regulation of transcription, DNA-templated                                                          |
| MYBAS1  | Myb-related protein MYBAS1        | 4.129  | Defense response; response to abscisic acid; response to biotic stimulus                             |
| Pathogenesis-related thaumatin-like protein 3.5 | 3.954 |                                                                                                       |
| DTX27   | Protein DETOXIFICATION 27         | 3.762  | Antipporter activity; transmembrane transporter activity; xenobiotic transmembrane transporter activity |
|         |                                   |        | Defense response; regulation of defense response to bacterium; regulation of defense response to fungus; response to bacterium; response to jasmonic acid; salicylic acid biosynthetic process |
| EPS1    | Protein enhanced pseudomonas susceptibility 1 | 3.654 |                                                                                                       |
| PEPR1   | Leucine-rich repeat receptor-like protein kinase PEPR1 | 3.584 | Immune response; innate immune response; response to jasmonic acid; Response to wounding               |
| UGT73E1 | UDP-glycosyltransferase 73E1      | 3.523  | Transf erase activity, transferring hexosyl groups                                                  |
| SDI1    | Protein sulfur deficiency-induced 1 | 3.459 | Cellular response to sulfur starvation; regulation of glucosinolate biosynthetic process; regulation of sulfur utilization |
| HSP70   | Heat shock 70 kDa protein         | 3.301  | Cellular response to unfolded protein; chaperone cofactor-dependent protein refolding; protein refolding; response to unfolded protein |
Table 5. Top 10 most down and upregulated differentially expressed genes (DEGs) in *Casuarina glauca* nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD\(^+\)), grown at 600 mM NaCl relative to the control (0 mM NaCl), ordered by log2 fold-change (log2FC). NBCI gene symbol, protein name, log2FC and respective Gene Ontology (GO) molecular function(s) and biological process(es) retrieved from UniprotKB database. Protein names coloured in blue are also among the top 10 most down or upregulated DEGs, respectively, at 600 mM NaCl in non-nodulated plants supplied with KNO\(_3\) (KNO\(_3^+\)).

| Gene          | Protein Name                        | Log\(_2\)FC | Biological Process                                                                 |
|---------------|-------------------------------------|-------------|------------------------------------------------------------------------------------|
| **Downregulated** |                                     |             |                                                                                   |
| RL5           | Protein RADIALIS-like 5             | −7.794      | DNA binding; auxin polar transport; carotene catabolic process; leaf morphogenesis; response to auxin; secondary shoot formation; strigolactone biosynthetic process Xanthophyll catabolic process defense response to bacterium; defense response to bacterium, incompatible Interaction; signal transduction |
| CCD8          | Carotenoid cleavage dioxygenase 8, chloroplastic | −6.134      |                                                                                   |
| TAO1          | Disease resistance protein TAO1     | −5.727      |                                                                                   |
| N/A           | Lectin                             | −5.643      |                                                                                   |
| pod           | Peroxidase 15                       | −5.529      |                                                                                   |
| GLR2.8        | Glutamate receptor 2.8              | −5.247      | calcium ion transport; calcium-mediated signaling; cellular response to amino Acid stimulus |
| LAC22         | Laccase-22                          | −5.183      | Iron ion homeostasis; iron ion transport; lignin catabolic process cellulose microfilibr organization; plant-type cell wall biogenesis; plant-type cell wall cellulose biosynthetic process; plant-type cell wall organization; plant-type Secondary cell wall biogenesis defense response; defense response to bacterium; defense response to oomycetes; positive regulation of cell death; positive regulation of hydrogen peroxide Metabolic process |
| COBL4         | COBRA-like protein 4                | −5.087      |                                                                                   |
| LECRK91       | L-type lectin-domain containing receptor kinase IX.1 | −5.087      |                                                                                   |
| LRK10L-1.4    | Leaf rust 10 disease-resistance locus receptor-like protein kinase-like 1.4 | −5.058      | Cellular response to hypoxia; phosphorylation                                    |
| **Upregulated** |                                     |             |                                                                                   |
| At3g18200     | WAT1-related protein At3g18200      | 2.906       | Transmembrane transporter activity                                                 |
| TIC214        | Protein TIC 214                     | 2.906       | Protein transport                                                                 |
| SAT           | Stemmadene O-acetyltransferase      | 2.700       | Alkaloid metabolic process                                                        |
| DTX56         | Protein DETOXIFICATION 56           | 2.929       | Cellular response to carbon dioxide; regulation of stomatal opening                |
| MAKR6         | Probable membrane-associated kinase regulator 6 | 2.861       | Abscisic acid-activated signaling pathway Cellarular response to unfolded protein; chaperone cofactor-dependent protein refolding; protein refolding; response to unfolded protein |
| HSP70         | Heat shock 70 kDa protein           | 2.728       | Endoplasmic reticulum lumen                                                        |
| HSP22.7       | 22.7 kDa class IV heat shock protein | 2.357       | Cellarular response to sulfur starvation; regulation of glucosinolate biosynthetic process; regulation of sulfur utilization |
| SDI1          | Protein sulfur deficiency-induced 1 | 4.087       | Immune response; innate immune response; response to jasmonic acid; response to wounding |
| PEPR1         | Leucine-rich repeat receptor-like protein kinase PEPR1 | 4.584       | Carbohydrate metabolic process; plant-type cell wall modification involved in multidimensional cell growth |
| At1g48100     | Polygalacturonase At1g48100         | 2.495       |                                                                                   |

Only two out of the top 10 most downregulated DEGs in samples exposed to 400 mM NaCl were found in both plant groups, namely RL5 and FLA12, both related to plant-type secondary cell wall biogenesis. Among the top upregulated DEGs, only the sulphur deficiency induced gene SDI1 was common to both groups. At 600 mM NaCl, half of the top downregulated DEGs were common to both KNO\(_3^+\) and NOD\(^+\), namely RL5, Lectin, LAC22, LECRK91, and LRK10L-1.4. These genes were respectively involved in the following biological processes: DNA binding, iron ion homeostasis and transport, lignin catabolic process, defense response to bacterium and oomycetes, positive regulation of cell death, positive regulation of hydrogen peroxide metabolic process, cellular response to hypoxia.
and phosphorylation. Regarding the top upregulated DEGs, only two were common to KNO$_3^+$ and NOD$^+$ plants, namely DTX56, associated with the cellular response to carbon dioxide and regulation of stomatal opening, and HSP70, involved in cellular response to unfolded protein and chaperone cofactor-dependent protein refolding. Overall, differential expression values, estimated by log2FC, were always higher in downregulated than in upregulated DEGs in both plant series.

2.5. Differentially Top-Expressed Genes in KNO$_3^+$ and in NOD$^+$ Plants

In KNO$_3^+$ plants, Clust tool showed 10 different clusters (Figure 4, Table S9). Clusters C0 to C3, C8, and C9 consistently presented a clear downregulation of DEGs at 600 mM NaCl relative to the control, with changes in the regulation pattern at 200- and 400 mM NaCl. Cluster C0 showed a progressive downregulation with increasing salinity from 200 to 600 mM NaCl, while C1 displayed an accentuated downregulation only after 400 mM NaCl. Although clusters C2 and C3 presented a sharp downregulation between 400 and 600 mM, they also showed an increasing upregulation of DEGs from control to 400 mM NaCl, which is much more evident in C3. The downregulation pattern in clusters C8 and C9 was sharp until 400 mM NaCl, nearly maintaining the expression levels at the highest salinity condition. In contrast, clusters C4 and C5 consistently presented a strong upregulation of DEGs from 0 to 600 mM NaCl. Overall, in clusters C6 and C7, a downregulation was observed at 400 mM NaCl, more accentuated in C7, and reverted at 600 mM NaCl in both cases.

Figure 4. Pattern of expression of differentially expressed genes (DEGs) in non-nodulated Casuarina glauca plants supplied with KNO$_3$ (KNO$_3^+$), grouped by predicted co-expressed gene clusters. Clustering analysis was performed by Clust tool with normalized reads from samples grown at 0, 200, 400, and 600 mM NaCl.

DEGs in almost all clusters were associated with DNA-, metal-, or sugar-binding, catalytic activity, transport, signaling, defense against biotic and abiotic stress and response to stimuli. In cluster C0, they were also related to chloroplast components, cell death, aging, oxidation-reduction process, and response to osmotic stress and water deprivation. Cluster C1 was more associated with membrane and mitochondrial components, and response to cold and salt stress. Although presenting similar expression patterns, while C2 was mainly associated with membrane components and defense responses, C3 was related to stomatal movement and signaling pathways involving brassinosteroids, ethylene and abscisic acid. Cluster C4 was associated with membrane and chloroplastic components, stomatal opening, glucose import, response to carbon dioxide, jasmonic acid and auxin, heat acclimation,
regulation of growth. However, cluster C5, which presented a similar expression pattern, was mainly related to the response to metabolic processes and oxidative stress. Clusters C6 and C7 were both related to membrane, Golgi apparatus, mitochondria, and chloroplast components. However, while C7 was associated with the cell cycle, response to cold, root hair elongation and cell wall organization, C6 was related to cellular respiration, defense responses and the response to stress, including cold and salt stress. Clusters C8 and C9 were associated with defense responses and membrane, but while C8 was deeply related to the microtubule, auxin homeostasis, and signaling, C9 was more related to cell wall, leaf abscission, and cell death. The full list of GO terms of the three main categories associated with each cluster of DEGs from KNO$_3^+$ plants can be seen in Table S10.

In NOD$^+$ plants, the Clust tool showed 11 different clusters (Figure 5, Table S11). Clusters C0 to C5 and C10 presented a noticeable downregulation of DEGs at 600 mM NaCl relative to the control, with different patterns of regulation at 200- and 400 mM NaCl. Clusters C0 and C1 showed a behavior similar to that of the corresponding clusters in KNO$_3^+$ plants, where the first had a progressive downregulation with increasing salinity and the second only showed an accentuated downregulation at 600 mM NaCl. Clusters C2 and C3 showed upregulation at 200 mM NaCl, which overall was reverted at higher salinity levels. In clusters, C4 and C5 the major downregulation happened rapidly between 200 and 400 mM NaCl, slightly higher in C4 and moderately reverted in C5 at 600 mM NaCl. In cluster C10, the downregulation occurred in two marked steps, between the control and 200 mM NaCl and between 400 and 600 mM NaCl. Cluster C6 presented a strong downregulation at 400 mM NaCl, which was inverted at the highest salinity concentration to levels close to the control. Cluster C7 showed a somewhat similar behavior. However, the upregulation at 600 mM was much more evident and the transcript levels were higher than those in the control.

![Figure 5](image_url)

**Figure 5.** Pattern of expression of differentially expressed genes (DEGs) in *Casuarina glauca* nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD$^+$), grouped by predicted co-expressed gene clusters. Clustering analysis was performed by Clust tool, with normalized reads from samples grown at 0, 200, 400, and 600 mM NaCl.

Overall, like in the case of KNO$_3^+$ plants, the majority of clusters were associated with binding, catalytic activity, transport, signaling, the defense response and responses
to stimuli and stress. Cluster C0 was related to membranes and mitochondria, and highly associated with responses to stimuli, namely salicylic, jasmonic, and abscisic acids, ethylene and chitin, and stress responses, specifically to wounding, hypoxia, water deprivation and salt stress. Cluster C1 included a set of genes associated with chloroplasts, defense response, oxidation-reduction process and cell death. Cluster C2 was mainly associated with the defense response and responses to stimuli and stress, namely cold, hypoxia, wounding, and auxin, also including some genes related to cell differentiation and membrane components. Clusters C3 and C4 were enriched in genes involved in cell death and redox homeostasis. Additionally, C4 was also related to chloroplast, aging, cell wall biosynthesis and organization, response to oxidative stress, wounding, and biotic stimuli. Cluster C5, which was associated with chloroplasts and membrane components, included also genes involved in response to auxin and biotic stimuli, leaf morphogenesis and metabolic processes. Cluster C6 represented DEGs associated with regulation of growth, cell differentiation, and responses to oxidative and salt stress. Cluster C7 was mainly related to cell cycle, protein phosphorylation and the Golgi apparatus. Cluster C8 included genes involved in oxidation-reduction processes, chloroplasts, stomatal opening, and cellular responses to carbon dioxide. Cluster C9, which was related to membrane components, contained also genes related to the response to jasmonic acid and biotic stimuli, DNA protection, and defense against biotic stress. Finally, Cluster C10 included genes associated with membrane components, response to ozone, phloem development, response to hypoxia and abscisic acid. The full list of GO terms of the three main categories associated with each cluster of DEGs from NOD+ plants can be seen in Table S12.

2.6. Significantly Enriched GO Terms, Metabolic Pathways and PPIs

Over-representation analysis (ORA) performed in gProfiler for both plants, found a few sets of enriched GO terms including DEGs in samples exposed to 400- and 600 mM NaCl with FDR < 0.05. Due to their low number, no enriched GO terms were found for DEGs in samples exposed to 200 mM NaCl. In 400 KNO3+ plants, enriched DEGs were related to UDP-glycosyltransferase activity (GO:0008194), cell wall organization (GO:0071555), external encapsulating structure organization (GO:0045229), defense against pathogenic bacteria (GO:0050829), polysaccharide biosynthetic process (GO:0000271), leaf abscission (GO:0060866), cell periphery (GO:0071944), and anchored component of plasma membrane (GO:0046658). In NOD+ plants enriched DEGs corresponded to glutamate dehydrogenase (NAD+) (GO:0004352), terpenoid catabolic process (GO:0016115), and cell periphery (GO:0005886) (Table 6). In 600- NOD+, DEGs from both plant groups were enriched in protein serine/threonine kinase activity (GO:0004674), ATP binding (GO:0005524), purine ribonucleoside triphosphate binding (GO:0035639), adenyl ribonucleotide binding (GO:0032559) and drug binding (GO:0008144), protein phosphorylation (GO:0006468) and defense response, incompatible interaction (GO:0009814). In KNO3+ plants, DEGs were also enriched in carbohydrate derivative binding (GO:0097367) and abscission (GO:0009838). In NOD+ plants, there was an enrichment in all-trans-beta-apo-10’-carotenal cleavage oxygenase transcription (GO:0102251), 9-cis-10’-apo-beta-carotenal cleavage oxygenase activity (GO:0102396), phospholipase transcription (GO:0004620), purine ribonucleotide binding (GO:0032555), as well as processes involving the interaction between organisms (GO:0051704), response to hypoxia (GO:0001666), defense response to bacteria (GO:0042742), positive regulation of defense responses (GO:0031349), lipid catabolic process (GO:0016042), leaf abscission (GO:0060866), and cell death (GO:0008219) (Table 7).
Table 6. Significantly enriched Gene Ontology (GO) terms, identified by Gene Set Enrichment Analysis (GSEA) of downregulated differentially expressed genes (DEGs) in non-nodulated *Casuarina glauca* plants supplied with KNO$_3$ (KNO$_3^+$) or nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD$^+$), grown at 400 mM NaCl, relative to control (0 mM NaCl). GO aspect, GO ID, GO Name, False Discovery Rate (FDR), intersection size, effective domain size and main gene intersections were retrieved from g:GOSt functional profiling of the gProfiler website, within a threshold of FDR < 0.05.

| Aspect | GO ID        | GO Name                                           | FDR    | Intersection Size | Effective Domain Size | Intersections |
|--------|--------------|---------------------------------------------------|--------|-------------------|-----------------------|--------------|
|        |              | **KNO$_3^+$**                                      |        |                   |                       |              |
| MF     | GO:0008194   | UDP-glycosyltransferase activity                  | 0.02845| 6                 | 21,106                | GATL2        |
|        | GO:0071555   | cell wall organization                            | 0.00081| 9                 | 23,362                | LRX2         |
|        | GO:0045229   | encapsulating structure organization defense response to Gram-negative bacteria | 0.00134| 9                 | 23,362                | LRX2         |
| BP     | GO:0050829   |                                                   | 0.00667| 3                 | 23,362                | EDS1         |
|        | GO:0060866   | leaf abscission polysaccharide biosynthetic process | 0.02170| 2                 | 23,362                | EDS1         |
|        | GO:0000271   |                                                   | 0.04131| 5                 | 23,362                | GATL2        |
| CC     | GO:0071944   | cell periphery anchored component of plasma membrane | 0.00013| 24                | 21,026                | XCP1         |
|        | GO:0046658   |                                                   | 0.01625| 4                 | 21,026                | FLA10        |
|        |              | **NOD$^+$**                                       |        |                   |                       |              |
| MF     | GO:0004352   | glutamate dehydrogenase (NAD$^+$) activity        | 0.01952| 1                 | 21,106                | GDH2         |
| BP     | GO:0016115   | terpenoid catabolic process                        | 0.02074| 2                 | 23,362                | CYP707A2     |
| CC     | GO:0005886   | plasma membrane                                    | 0.00174| 10                | 21,026                | GDH2         |
|        | GO:0071944   | cell periphery                                    | 0.00842| 10                | 21,026                | GDH2         |

Through ShinyGO, enriched DEGs were observed only at 600 mM NaCl in both plant groups. In both InterPro and Pfam databases, KNO$_3^+$ plants were enriched for the transcription of genes involved in the following pathways: glycosyltransferase family 8, GIY-YIG catalytic domain/nuclease superfamily, UDP-glucorosyl and UDP-glucosyl transferase, KOW motif, and the unknown function domain DUF2828. Furthermore, a set of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was also enriched for this condition, namely cyanoamino acid metabolism, amino sugar and nucleotide sugar metabolism, and starch and sucrose metabolism. NOD$^+$ plants were enriched in transcripts of AAA-type ATPase, chalcone/stilbene synthase, flavinoxireductase/NADH oxidase, glutamate/phenylalanine/leucine/valine dehydrogenase, and phosphatase 2C family. All enriched pathways are listed in Table S13.

Likewise, PPI networks of DEGs were found in the STRING database only at 600 mM NaCl in both plant groups. In 600- KNO$_3^+$ plants, three DEGs associated with enriched biological processes were mapped in the PPI network, namely SAG101, CRT3 and TAO1 (Figure S6). The first two, which were related to the regulation of response to stress and positive regulation of response to stimuli, were directly linked in the network. Moreover, in NOD$^+$ plants, these two genes were indirectly linked to RBOHD, which was associated with cell death. Another four DEGs in NOD$^+$ plants were mapped in the PPI network, namely SAG101, CRT3, TAO1 and PUB17 (Figure S7). Again, the first two were directly linked in the network. However, in this case, while CRT3 is associated with cell death, SAG101 is related to defense signaling, response to bacteria, regulation of response to stress and positive regulation of response to stimuli. Moreover, PUB17, which was associated with the defense response, was indirectly linked to both of these genes.
Table 7. Significantly enriched Gene Ontology (GO) terms, identified by Gene Set Enrichment Analysis (GSEA) of downregulated differentially expressed genes (DEGs) in non-nodulated *Casuarina glauca* plants supplied with KNO\(^3\) (KNO\(^3^+\)) or nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD\(^+\)), grown at 600 mM NaCl, relative to control (0 mM NaCl). GO aspect, GO ID, GO Name, False Discovery Rate (FDR), intersection size, effective domain size and main gene intersections were retrieved from g:GOSt functional profiling of the gProfiler website, within a threshold of FDR < 0.05. GO Names coloured in blue were found to be enriched for DEGs at 600 mM NaCl in both plant series.

| Aspect | GO ID      | GO Name                                      | FDR    | Intersection Size | Effective Domain Size | Intersections |
|--------|------------|----------------------------------------------|--------|-------------------|-----------------------|--------------|
| MF     | GO:0004674 | protein serine/threonine kinase activity      | 0.00289| 11                | 21,106                | STN8         |
|        | GO:0005524 | ATP binding                                   | 0.01405| 17                | 21,106                | AT2G40270    |
|        | GO:0035639 | purine ribonucleoside triphosphate binding    | 0.01480| 18                | 21,106                | AT2G40270    |
|        | GO:0032559 | adenylyl ribonucleotide binding               | 0.02552| 17                | 21,106                | AT2G40270    |
|        | GO:0008144 | drug binding                                  | 0.03319| 17                | 21,106                | AT2G40270    |
|        | GO:097367  | carbohydrate derivative binding              | 0.03632| 18                | 21,106                | AT2G40270    |
| BP     | GO:0006468 | protein phosphorylation                       | 0.00152| 13                | 23,362                | AT2G40270    |
|        | GO:0009814 | defense response, incompatible interaction    | 0.02407| 5                 | 23,362                | RPP1         |
|        | GO:0009838 | abscission                                    | 0.04514| 3                 | 23,362                | SAG101       |
| MF     | GO:0004674 | protein serine/threonine kinase activity      | 0.00439| 9                 | 21,106                | CCR4         |
|        | GO:0005524 | ATP binding                                   | 0.00930| 14                | 21,106                | TAO1         |
|        | GO:0102251 | all-trans-beta-apo-10'-carotenal cleavage    | 0.01331| 1                 | 21,106                | CCD8         |
|        |            | oxygenase activity 9-cis-10'-apo-beta-10'-    |        |                   |                       |              |
|        |            | carotenal cleavage oxygenase activity        |        |                   |                       |              |
|        | GO:0032559 | adenylyl ribonucleotide binding               | 0.01575| 14                | 21,106                | TAO1         |
|        | GO:0008144 | drug binding                                  | 0.01988| 14                | 21,106                | TAO1         |
|        | GO:0004620 | phospholipase activity                        | 0.02127| 3                 | 21,106                | ATIG008000   |
|        | GO:0035639 | purine ribonucleoside triphosphate binding    | 0.03000| 8                 | 21,106                | TAO1         |
|        | GO:0032555 | purine ribonucleotide binding                 | 0.04092| 8                 | 21,106                | TAO1         |
| BP     | GO:00351704| multi-organism process                        | 0.00033| 13                | 23,362                | TAO1         |
|        | GO:0042742 | defense response to bacterium                 | 0.00076| 7                 | 23,362                | TAO1         |
|        | GO:0006468 | protein phosphorylation                       | 0.00869| 10                | 23,362                | CCR4         |
|        | GO:0031349 | positive regulation of defense response       | 0.00903| 4                 | 23,362                | SOBIR1       |
|        | GO:0016042 | lipid catabolic process                       | 0.01363| 5                 | 23,362                | CCD8         |
|        | GO:0068166 | leaf abscission                               | 0.01451| 2                 | 23,362                | SAG101       |
|        | GO:008219 | cell death                                    | 0.01457| 5                 | 23,362                | SOBIR1       |
|        | GO:0009814 | defense response, incompatible interaction    | 0.03085| 4                 | 23,362                | TAO1         |
|        | GO:0001666 | response to hypoxia                           | 0.04357| 5                 | 23,362                | PLP2         |

2.7. Genetic Stability and Volatility among DEGs

CodonW analysis was performed to assess the genetic stability or volatility among DEGs. It was observed that genes upregulated in NOD\(^+\) plants had a GC content of 69.35% with 58.79% GC content at synonymous sites (GC3). A significant positive correlation between % GC and the frequency of optimal codons (Fop) was found ($r = 0.57$), whereas a significant negative correlation was obtained between GC3 and the effective number of codons (Nc) ($r = -0.62$). On the contrary, the upregulated genes in KNO\(^3^+\) plants showed different features. In this group, GC was 50.02% with 51.03% GC3. Moreover, statistical analyses between GC and Fop along with GC3 and Nc did not demonstrate statistical significance. This indicated distinct genetic volatility among the upregulated genes present in KNO\(^3^+\) plants. Moreover, COG analysis revealed “cellular processing and signaling”
as a major functional category followed by “information storage and processing” and “metabolism” in NOD\(^+\) plants (Figure 6A), whereas “information storage and processing” was found to be the major COG family in non-nodulated KNO\(_3\)\(^+\) plants (Figure 6B). The upregulated proteins in NOD\(^+\) plants were more energy-consuming than those in non-nodulated plants.

Figure 6. COG distribution pattern in NOD\(^+\) plants (A) and non-nodulated KNO\(_3\)\(^+\) plants (B).

3. Discussion

The impact of salt stress in *Casuarina glauca* has been extensively studied in depth at the morpho-physiological and biochemical levels, as well as at the proteome and metabolome scale [7]. Altogether, these studies revealed the robustness of the stress-responsive mechanisms in this species, particularly regarding protective traits at the photosynthetic and membrane level, whose activation was triggered at early stages of stress. In order to conclude this broad analysis, here, we report on the effects of increasing salt concentrations (200, 400 and 600 mM NaCl) on the transcriptome of branchlets from non-nodulated (KNO\(_3\)\(^+\)) and nodulated (NOD\(^+\)) *C. glauca* plants. RNA-Seq analysis yielded an average of ca. 25 and ca. 26 million clean reads for KNO\(_3\)\(^+\) and NOD\(^+\) plants, respectively, corresponding to 86,202 unigenes with a N50 size of 2792 bp and 41% GC content. These values are within the range of those reported in similar studies in *Casuarina*, which vary according to the species, environmental condition, and plant organ. For example, in *Casuarina equisetifolia* seedlings exposed to cold, ca. 21 million reads corresponding to ca. 118,000 unigenes and a N50 value of 2827 bp have been reported [38]. Developing secondary wood tissue of the same species
presented ca. 43 million clean reads, comprising ca. 27,000 unigenes with a N50 contig size of 750 bp [39]. On the other hand, in *C. equisetifolia* roots from plants exposed to salt, these values were ca. 75–95 million clean reads comprising ca. 53,000–72,000 unigenes [37].

Finally, Wang et al. [40] reported ca. 41 million clean reads comprising ca. 60,000 unigenes with a N50 value of 2832 bp in arbuscular mycorrhiza roots of *C. glauca* exposed to salt. These results, together with the high mapping coverage of our data (almost 95%) indicates that a high-quality transcriptome assembly has been generated for downstream analyses.

In both plant groups, the patterns of differentially expressed genes (DEGs) clearly separate two groups, (i) control and 200 mM NaCl-treated plants, and (ii) 400 and 600 mM NaCl-treated plants (Figure 4, Figure 5, Figures S1, S4 and S5). With a few exceptions, this separation from low-to-moderate impacts (0, 200 mM) and heavy impacts (400, 600 mM) was also reported for several morpho-ecophysiological parameters, like plant growth and water use efficiency, mineral contents, leaf gas exchanges, chlorophyll a fluorescence, thylakoid electron transport rates, photosynthetic enzymes, and structural carbohydrates [22], as well for differentially expressed proteins (DEPs) of the same experimental set [30]. Notably, although the number of total transcripts was relatively high in both plant groups (9930 in the control and an average of 19,645 genes in NaCl-treated KNO$_3^+$ plants, and 20,278 in the control and an average of 19,780 genes in NaCl-treated NOD$^+$ plants), the percentage of significant DEGs was remarkably low, ranging from 6 (200 NOD$^+$) to 314 (600 KNO$_3^+$), mostly downregulated (Figure 1; Table 1). The results are within the range of those reported for the invasive halophyte, *Phragmites karka*, exposed to 150 mM NaCl: 305 and 289 DEGs in leaves and roots, respectively [41]. Nevertheless, in this case, the fraction of positively regulated genes was higher than that of the negatively regulated ones. On the other hand, the number of DEGs reported in *C. equisetifolia* seedlings was considerably higher: >6000 DEGs in roots from plants exposed to 200 mM NaCl for seven days [37] and >4000 DEGs in leaves of plants exposed to cold [38]. However, in both cases the fold-change, rather than the statistical significance, has been privileged. Such observations highlight the complexity of the molecular interactions leading to salinity tolerance in *C. glauca* which might be related to a rapid and robust stress-responsive system [7], probably linked to long-term ecological adaptation [38].

A minor proportion of DEGs was common to both plant groups, in response to 400 (10%) and 600 mM NaCl (28%) (Figure 3), while no common DEGs were identified at the lowest salt concentration. The fact that the nodulation process triggers a set of defense-responses [42,43] and that at 200 mM NaCl the N2 fixation by *Frankia* is residual [23] may explain this difference. Nevertheless, in both plant groups, the increase of the number of DEGs was gradual along the salt gradient, likely reflecting changes towards acclimation to high salt levels [30].

Considering the two key stress concentrations, i.e., 400 and 600 mM NaCl, the stress-responsive genes were in general related to regulatory processes in both plant groups. The results are rather different from those reported for the *C. glauca* branchlets’ proteome [30], and metabolome [25–27], where changes were associated to major physiological changes related to photosynthesis, membrane stability and osmoprotection mechanisms [22,24–27]. This reinforces the hypothesis that *C. glauca* has a strong stress-responsive system with an extensive set of constitutive defense mechanisms [7,43] complemented by rapid induction of transcriptional changes at the initial stages of salt exposure [37,40]. Despite the differences associated with the molecular changes imposed during the nodulation process (highlighted above), in both plant groups the top-ten up-regulated DEGs were related to signaling, transport, refolding, and stomatal control (Tables 1–5; Figures S4 and S5). These included auxin- (400 KNO$_3^+$) and abscisic acid (ABA)-activated signaling (600 KNO$_3^+$; 600 NOD$^+$), Myb transcription factors and jasmonic acid signaling (400 NOD$^+$), regulation of sulphur utilization, transporter activity, and protein refolding (400- and 600- KNO$_3^+$; 400- and 600 NOD$^+$), and response to cellular CO2/regulation of stomatal opening (600 KNO$_3^+$; 600 NOD$^+$). It is widely known that the mechanisms used by plants to cope with salt stress are highly dependent on a coordinated set of events that regulates growth, metabolism, and
homeostasis \cite{37,44,45}. In halophytic plants, gene regulatory networks are an essential part of salt tolerance. For example, in \textit{C. equisetifolia} \cite{37}, \textit{Spartina alterniflora} \cite{46}, and \textit{Puccinellia nuttalliana} \cite{47}, transcription factors (TFs), and ion transporters were identified as key drivers of salt stress tolerance, playing an essential role in ion homeostasis, regulation of ROS scavenging and detoxification, water balance, and water transport.

The top up-regulated genes common to both plant groups were the sulphur deficiency induced gene, SDI1, the gene encoding protein detoxification 56, DTX56, and the gene encoding the 70 KDa heat shock protein, HSP70 (Tables 2–5). The accumulation of SDI transcripts is related to sulphur deficiency, utilization of stored sulphate pools and sulphur partitioning \cite{48–50}. It has already been shown that the expression of the sulfate uptake transporter SULTR3 is induced in \textit{Arabidopsis} and \textit{Medicago} in response to various abiotic stresses \cite{51} and that this induction depends on ABA \cite{52}. Given that levels of cysteine and glutathione tend to increase significantly under abiotic stress conditions, which was also confirmed for glutathione in \textit{C. glauca} \cite{24,30}, an upregulation of sulphate use is a plausible response to long-term salinity stress. On the other hand, DTX belongs to the group of transporters involved in detoxification in plants \cite{53–55}, as well as in turgor-regulation and ABA efflux in drought tolerant \textit{Arabidopsis} \cite{56}. Thus, it seems reasonable to hypothesize that DTX56 is a key multifunctional gene involved in the modulation of \textit{C. glauca} responses to high salinity through the regulation of signal transduction pathways and associated mechanisms leading to stress tolerance \cite{57}. Finally, the induction of the expression of the chaperone HSP70 is likely related to the maintenance of protein folding/unfolding as well as degradation of misfolded and denatured proteins, a common mechanism observed in plants under stress, e.g., osmotic \cite{58}, drought \cite{59}, and heat \cite{60}.

To be highlighted also is the fact that in NOD\textsuperscript{+} plants, glutamate dehydrogenase (GDH) 2 is upregulated under all three salinity levels tested (Tables 5 and 6, Supplementary Table S8). This enzyme is generally involved in stress adaptation \cite{61} and plays an important role in replenishment of the tricarboxylic acid cycle and generally at the interface between carbon and nitrogen metabolism \cite{62}. In fact, these authors showed that during short term salt stress, post-transcriptional regulation of GDH led to increased enzyme activity, and overexpression of the corresponding gene, improving biomass accumulation. Interestingly GDH is upregulated in NOD\textsuperscript{+}, but not in KNO\textsubscript{3}\textsuperscript{+} plants.

Abscisic acid 8’-hydroxylase initiates the degradation of ABA and is thus important for maintaining ABA homeostasis \cite{63,64}. The upregulation of its gene in NOD\textsuperscript{+} plants exposed to 400 mM NaCl is interesting as during salt stress, an upregulation of ABA synthesis, not degradation, would have been expected \cite{65}, supporting a gradual stomatal closure that reached negligible values at 600 mM NaCl \cite{22}. Another result that might seem surprising is the upregulation of WAXY, the starch synthase responsible for the synthesis of amylose, in 400 KNO\textsubscript{3}\textsuperscript{+} plants. It was previously established for rice seeds that salt stress leads to a decrease in amylose biosynthesis \cite{66,67}. However, another study \cite{68} found a relative increase of amylose for seeds of triticale wheat under salt stress. In any case, despite the differences between the two plant groups, overall, these results suggest that the robustness of the stress response system in \textit{C. glauca} is regulated by a limited number of genes that tightly regulate detoxification and protein/enzyme stability.

One of the mechanisms used by \textit{C. glauca} to cope with salinity is the reduction of growth ensuring relative high values of hydration (as evaluated by the branchlets relative water content, RWC), ion homeostasis, and photosynthetic functioning \cite{22}. In this context, the down-regulation of genes involved in growth and developmental processes, e.g., histones (DNA), patellins (cell polarity and patterning) \cite{69}, or cysteine proteases such as XCP1 (differentiation of xylem vessels) \cite{70}, is not surprising. In addition, previous studies on the effects of salinity stress have often shown negative regulation of genes involved in cell wall biosynthesis, a part of the growth process, or up-regulation of genes involved in cell wall degradation \cite{40,71}. Accordingly, in this study, we found down-regulation of a set of cell-wall related genes, e.g., pectin esterase/pectin esterase inhibitor (400 NOD\textsuperscript{+}), a polygalacturonase (400 KNO\textsubscript{3}\textsuperscript{+}), an arabinogalactan (400 NOD\textsuperscript{+} and 400 KNO\textsubscript{3}\textsuperscript{+}), a
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...cellulose synthase (600 KNO$_3^+$), a laccase, (600 NOD$^+$ and 600 KNO$_3^+$), and a regulator of cell expansion, cobra-like protein (600 NOD$^+$).

As mentioned above, the expression of several genes encoding TFs putatively involved in signal transduction was differentially regulated under salt stress. The striking effect here, however, was the consistent downregulation of RADIALIS-LIKE SANT/MYB TFs (RADIALIS-LIKE 3 and RADIALIS-LIKE5) in both NOD$^+$ and KNO$_3^+$ plants (400 and 600 mM NaCl). These TFs form a small family in Arabidopsis [72], and one of them (RSM1), which is down-regulated under salinity, was recently shown to modulate seedling development in response to ABA and salinity [73]. The function of RSM1 in the response to long-term salinity stress was confirmed in Castor Bean (Ricinus communis) by Han et al. [35], who suggested that the downregulation of RSM1 by salt was regulated via epigenetic modifications of the promoter.

Hierarchical cluster analysis identified 10 (KNO$_3^+$) and 11 (NOD$^+$) gene clusters (Figures 4 and 5), all including genes associated with binding, catalytic activity, transport, signaling, defense against biotic and abiotic stress, and response to stimuli. Among these, five clusters (C0–C2, C8–C9) were sharply downregulated in KNO$_3^+$ plants exposed to 600 mM NaCl, while in NOD$^+$ plants the number of negatively regulated gene clusters was seven (C0-C5, and C10). Despite the differences between the two plant groups, likely related to the nitrogen source [23, 24], the overall set of results shows that C. glauca is able to balance the transcriptional activity at 200 mM NaCl to levels similar to that of the control (Figures 4 and 5). At 400 mM NaCl the expression patterns tend to be more variable, while at 600 mM NaCl transcript levels are severely affected, i.e., ca. 60% of the DEGs are downregulated in both KNO$_3^+$ and NOD$^+$ plants. Indeed, PPI networks of DEGs were found only at 600 mM NaCl in both plant groups, mainly related to the response to stress and stimuli, as well as to cell death. In our view, despite the positive regulation of some stress-related genes, at the highest salt concentration the plant is unable to sustain the necessary transcriptional activity to cope with salinity. Comparing this trend with the overall impact of 600 mM NaCl on plant growth, eco-physiological performance, proteome, and metabolome [22, 24–27, 30], it seems clear that under the experimental conditions used, this C. glauca ecotype tolerates at least 400 mM. Such tolerance is related to a constitutive defense system, an enhanced antioxidative and osmoprotectant status, as well as a tight mechanism of transcription regulation and post-transcriptional modification.

Finally, codon and amino acid usage of DEGs among KNO$_3^+$ and NOD$^+$ plants revealed a distinct pattern. Compositional constrain and translational efficiency were found to be the major driving force in maintaining the codon usage indices among DEGs of NOD$^+$ plants. In this group, the presence of costly proteins with higher amounts of aromatic amino acids highlights the importance of energy rich amino acids. Moreover, COG analysis made it evident that in NOD$^+$ plants, the signaling proteins were mostly functional. This may be due to a strong influence exerted by the symbiosis with Frankia casuarinae Thr. While the bacteria inside nodules are mostly dead at 200 mM NaCl [23], the effect of nodulation on the plant should still be in place. In legumes, extensive research has revealed complex local and systemic control of root architecture during nodulation [74, 75]. Consistent with these data, legume studies have shown that nodulation affects the response to salt stress with regard to pathways involved in photosynthesis, respiration, anion transport, and plant defense [40]. Similar processes have evolved in actinorhizal plants [76]. However, they were not yet analyzed in Casuarina spp. In short, it is not surprising that even if the microsymbionts in nodules do not survive salt stress, the symbiosis still affects the stress response.

In conclusion, the results highlight the complexity of the molecular interactions leading to salinity tolerance in C. glauca likely linked to long-term ecological adaptation and, in this case, independent of symbiotic Frankia.
4. Materials and Methods

4.1. Growing Conditions and Experimental Setup

Casuarina glauca clones were grown in Broughton and Dillworth’s (BD) medium under the conditions previously described by Zhong et al. [77] and Tromas et al. [78]. Six-month-old plants were either nodulated by Frankia casuarinae Thr (NOD⁺) or supplemented with mineral nitrogen (KNO₃⁺) and maintained in a walk-in growth chamber (10,000 EHHF, ARALAB, Portugal) under environmental controlled conditions of temperature (26/22 °C), photoperiod (12 h), relative humidity (70%), external CO₂ levels (380 µL L⁻¹), and irradiance (ca. 500 µmol m⁻² s⁻¹). Salt stress was gradually imposed through the addition of 50 mM NaCl per week to the nutrient solution until concentrations of 200, 400 and 600 mM were obtained, respectively. Control plants were supplemented only with mineral nutrients without the addition of NaCl. In all cases, the nutrient solution was renewed twice per week. To ensure the same age of all plants at the time of sample collection, i.e., one week after the exposure to each NaCl concentration, stress implementation was imposed sequentially from the highest to the lowest salt condition, i.e., the 600 mM NaCl group was treated first, followed by the 400 mM group (when the 600 mM group surpassed 200 mM NaCl), and the 200 mM group (when the 400 mM group surpassed 200 mM NaCl). Branchlets from four plants per treatment were harvested, frozen immediately in liquid nitrogen, and stored at –80 ºC until RNA extraction.

4.2. Total RNA Extraction and Library Preparation

Total RNA from branchlets of NOD⁺ and KNO₃⁺ plants exposed to the three stress levels, as well as from control plants was extracted using the GeneJet Plant RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) and digested with dsDNAse (Thermo Scientific, USA) to remove genomic DNA, as per manufacturer’s instructions. The quality of the RNA samples was verified by electrophoresis in 1% agarose–Tris acetate EDTA buffer containing GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA), by evaluating the integrity of the 28S and 18S ribosomal RNA bands and absence of smears, as well as with a bioanalyzer (Agilent 2100, Agilent, Santa Clara, CA, USA), by determining the RNA integrity number (RIN > 8.5). Libraries were prepared with the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) and sequenced on an Illumina HiSeq 2000 platform (2 × 125 bp pair-end reads; 30 million reads per sample) at Macrogen (Seul, South Korea). Raw data is available in NCBI Sequence Read Archive (SRA) BioProject SymbSaltStress under accession PRJNA706159 (https://www.ncbi.nlm.nih.gov/sra/PRJNA706159, accessed on 1 September 2022).

4.3. Processing and Mapping of Illumina Reads

The sequenced raw reads were assessed for integrity, quality and contamination through the application of the following tools: FastQC version 0.11.8 to analyze reads quality [79] and FastQ Screen version 0.13 to survey putative contaminants, run against the genome of 14 default pre-indexed species and adaptors [80]. Trimomatic version 0.39.1 was then used to eliminate the remaining adaptors and low-quality or small reads [81], using ILLUMINAACLIP with keepBothReads option, SLIDINGWINDOW:4:15, MAXINFO:36:0.5 and MINLEN:36. After filtering and trimming, Trinity version 0.39.1 was used to perform de novo transcriptome assembly, combining all samples to generate one single assembly [82]. This software was developed specifically for short reads and is advantageous for non-model plant sequence assemblies [83]. The assembled transcriptome was assessed for completeness through the gVolante [84] online interface with BUSCO v2.0.1 option [85]. To align the reads against the assembled transcriptome, the sequences were processed with Trinity tool Bowtie2 version 2.3.5 [86,87] and the aligned reads for each condition were quantified at gene expression level with RSEM version 1.3.2. [88]. The normalized expression of all samples was estimated using the Trimmed Mean of M values (TMM). A principal component analysis (PCA) was performed to survey the relatedness of all samples using the function plotPCA in R studio version 3.6.0 [89].
4.4. Identification of Differentially Expressed Genes

To identify differentially expressed genes (DEGs), a set of R packages were applied, namely edgeR version 3.26.8 [90], DESeq version 1.36.0 [91] and NOISeq version 2.28.0 [92]. DESeq fits a generalized linear model to estimate variance-mean dependence in count data, testing for differential expression based on the negative binomial distribution. The dispersion was estimated through blind mode [91]. On the other hand, edgeR uses weighted likelihood methods to implement a flexible empirical Bayes approach to allow gene-specific variation estimates even with very few or no replicates [93]. The dispersion value, which must be fixed manually in the absence of replicates, was set to 0.1. By contrast, NOISeq is an exploratory analysis that tests for differential expression between two experimental conditions without parametric assumptions that can simulate technical replicates. This method relies on the premise that read counts follow a multinomial distribution, where probabilities for each feature are the probability of a read to map to it [92]. Differential expression was computed using a stringent threshold of \( q = 0.9 \) along with the following parameters: \( \text{pnr} = 0.2, \text{nss} = 5, \text{v} = 0.02 \).

To study the effect of salinity, these three tools were used to identify DEGs at each salinity level (200, 400, and 600 mM NaCl) compared to the control (0 mM NaCl), for KNO\(_3^+\) and NOD\(^+\) plants, respectively. The results were adjusted with the Benjamini and Hochberg’s approach for controlling the false discovery rate (FDR) [94]. A filter of FDR < 0.05 and normalized log2 fold change (log2FC) > 121 were set to define DEGs. DEGs commonly detected by edgeR, DESeq and NOISeq were combined to increase the accuracy of results, and only the genes detected as differentially expressed by all the three tools were used in downstream analyses. To visualize the resulting expression profiles, volcano plots, heatmaps, barplots, and Venn diagrams were prepared for each combination, using stats and graphics core R packages and Python’s Matplotlib 3.2.1 library [95].

The Clust version 1.8.10 command-line tool was applied to visualize the expression patterns of the detected DEGs and to find co-expressed genes [96]. This is a fully automated method for the identification of gene clusters that are co-expressed in heterogeneous datasets that automatically normalizes the data and determines the best number of clusters, based on a selected tightness, which in this case was five (5). Then, the results were filtered to keep only the DEGs that were found uniquely in one of the clusters and to find terms related to salt stress response.

4.5. Functional Annotation and Enrichment Analysis

The Basic Local Alignment Search Tool (BLAST) version 2.9.0 command line application from the NCBI C++ Toolkit [97] was used for functional annotation of DEGs, using homology searches with the latest references, comprising only the expertly curated component of UniProtKB. Results were filtered by maximum E-Value of 1.0E-3 and minimum Identities of 40% [98]. The resultant annotated proteins were then characterized by Gene Ontology (GO) terms: Cellular Component (CC), Molecular Function (MF), and Biological Process (BP), using the Uniprot and QuickGO APIs [99] to retrieve direct terms and GO term ancestors.

An over-representation analysis (ORA) was implemented by g:GOst functional profiling tool from gProfiler website, which was applied using g:SCS tailored algorithm that uses a minimum hypergeometric test (Fisher’s exact test). ShinyGO version 0.61 webtool, which is also based on hypergeometric distribution followed by FDR correction was used to retrieve Protein-Protein Interactions (PPIs) from the STRING database [100]. In both tools, Arabidopsis thaliana was selected as the organism of interest and separate log2PC ranked lists of DEGs for each salinity condition were used as inputs.

4.6. Downstream Bioinformatics Analysis

The downstream analysis included the codon and amino acid usage analysis of DEGs. Compositional constrain (GC and GC3), effective number of codons (ENC), and frequency of optimal codons (Fop) were determined using CodonW software. Biosyn-
thetic energy costs of DEGs were obtained through DAMBE software. The Cluster of Orthologue analysis (COG) was performed using an in-house python-based program where information regarding different COG families was taken from the COG database (http://www.ncbi.nlm.nih.gov/COG, accessed on 1 September 2022). A detailed study on biological networks maintained by DEGs was performed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11212942/s1, Table S1: Sequencing data from Casuarina glauca nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD⁺) or non-nodulated plants supplied with KNO₃ (KNO₃⁺), grown in three different salinity conditions (200, 400, and 600 mM NaCl), plus the control (0 mM NaCl). Raw reads, obtained after sequencing, generated trimmed reads after submission to quality filters with Trimmomatic software; Table S2: Quantification of basic quality metrics de novo transcriptome assembly of Casuarina glauca. Samples of C. glauca nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD⁺) and non-nodulated plants supplied with KNO₃ (KNO₃⁺), grown at 0, 200, 400, and 600 mM NaCl, were combined to perform a de novo transcriptome assembly using Trinity software; Table S3: Alignment statistics of Casuarina glauca nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD⁺) and non-nodulated plants supplied with KNO₃ (KNO₃⁺) reads, from samples grown at 0, 200, 400, and 600 mM NaCl, against a de novo transcriptome assembly, performed with Trinity software using these same reads; Table S4: Quality assessment of a de novo transcriptome assembly, using gVolante website with BUSCO v2.0.1 option. The transcriptome was performed with Trinity software, using Casuarina glauca nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD⁺) and non-nodulated plants supplied with KNO₃ (KNO₃⁺) reads, from samples grown at 0, 200, 400, and 600 mM NaCl; Table S5: List of differentially expressed genes (DEGs) in both non-nodulated Casuarina glauca plants supplied with KNO₃ (KNO₃⁺) and nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD⁺), grown in 400 mM NaCl relative to the control (0 mM NaCl). NCBI gene symbol and Protein Name, retrieved from NCBI blastx command line tool using UniprotKB/Swissprot database, and Log₂FC. DEGs were detected by DESeq, NOISeq and edgeR analysis with a False Discovery Rate < 0.05; Table S6: List of differentially expressed genes (DEGs) in both non-nodulated Casuarina glauca plants supplied with KNO₃ (KNO₃⁺) and nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD⁺), grown in 600 mM NaCl relative to control (0 mM NaCl). NCBI gene symbol and Protein Name, retrieved from NCBI blastx command line tool using UniprotKB/Swissprot database, and Log₂FC. DEGs were detected by DESeq, NOISeq and edgeR analysis with a False Discovery Rate < 0.05; Table S7: Full list of significantly differentially expressed genes (DEGs) in branchlets of Casuarina glauca nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD⁺), grown in 200, 400, and 600 mM NaCl. NCBI gene symbol, protein ID, Protein Name, E-value and Identities retrieved from NCBI blastx command line tool using UniprotKB/Swissprot database. DEGs refers to each salinity-treatment in comparison with the control NaCl, respectively, 200: 200 vs. 0 mM NaCl, 400: 400 vs. 0 mM NaCl and 600: 600 vs. 0 mM NaCl), detected by DESeq. NOISeq and edgeR analysis with a False Discovery Rate < 0.05; Table S8: Full list of significantly differentially expressed genes (DEGs) in branchlets of Casuarina glauca nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD⁺), grown in 200, 400, and 600 mM NaCl. NCBI gene symbol and protein names were retrieved from NCBI blastx command line tool; Table S9: Full list of Gene Ontology (GO) biological processes, molecular functions and cellular components associated with each cluster of potentially co-expressed differentially expressed genes (DEGs) in branchlets of non-nodulated Casuarina glauca plants supplied with KNO₃ (KNO₃⁺), grown at 200, 400, and 600 mM NaCl. Clusters were formed by Clust software and GO names were retrieved from UniprotKB database; Table S10: Full list of Gene Ontology (GO) biological processes, molecular functions and cellular components associated with each cluster of potentially co-expressed differentially expressed genes (DEGs) in branchlets of non-nodulated Casuarina glauca plants supplied with KNO₃ (KNO₃⁺), grown at 200, 400, and 600 mM NaCl. Clusters were formed by Clust software and GO names were retrieved from NCBI blastx command line tool; Table S11: Clusters of potentially co-expressed differentially expressed genes (DEGs) in branchlets of Casuarina glauca nodulated by nitrogen-fixing Frankia casuarinae Thr (NOD⁺), grown at 200, 400, and 600 mM NaCl. Clusters were formed by Clust software and NCBI gene symbol and protein names were retrieved using NCBI blastx command line tool; Table S12: Full list of Gene Ontology (GO) biological processes, molecular functions and cellular components
associated with each cluster of potentially co-expressed differentially expressed genes (DEGs) in branchlets of *Casuarina glauca* nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD+), grown at 200, 400, and 600 mM NaCl. Clusters were formed by Clust software and GO names were retrieved from UniprotKB database; Table S13: Enriched pathways among differentially expressed genes (DEGs) in branchlets of non-nodulated *Casuarina glauca* plants supplied with KNO3 (KNO3+), grown at 600 mM NaCl relative to control (0 mM NaCl). Pathways were retrieved from InterPro, Pfam and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, through ShinyGO webtool, with a False Discovery Rate (FDR) threshold < 0.05; Figure S1: Principal component analysis of gene expression counts from *Casuarina glauca* nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD+) and non-nodulated plants supplied with KNO3 (KNO3+), grown at control (0 mM NaCl) and three salinity conditions (200, 400, and 600 mM NaCl). PCA was plotted using R function plotPCA using normalized expression matrices; Figure S2: Gene expression in branchlets of non-nodulated *Casuarina glauca* plants supplied with KNO3 (KNO3+) and nodulated with nitrogen-fixing *Frankia casuarinae* Thr (NOD+), grown at control (0 mM NaCl) and three salinity conditions (200, 400, and 600 mM NaCl). Black dots represent genes without differential expression and red dots represent differentially expressed genes (DEGs) between control (0 mM NaCl) and salinity-exposed plants: (A) KNO3+ plants at 200 mM NaCl; (B) KNO3+ plants at 400 mM NaCl; (C) KNO3+ plants at 600 mM NaCl; (D) NOD+ plants at 200 mM NaCl; (E) NOD+ plants at 400 mM NaCl and (F) NOD+ plants at 600 mM NaCl; Figure S3: Percentage of unknown and annotated differentially expressed genes (DEGs) in *Casuarina glauca*: (A) non-nodulated plants supplied with KNO3 (KNO3+); (B) nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD+). Plants were grown at control (0 mM NaCl) and three salinity conditions (200, 400, and 600 mM NaCl). Annotations were retrieved through NCBI blastx command line tool, using UniprotKB/Swissprot database; Figure S4: Heatmap of top 10 most up and downregulated differentially expressed genes (DEGs) in non-nodulated *Casuarina glauca* plants supplied with KNO3 (KNO3+), grown at 600 mM NaCl relative to the control (0 mM NaCl). Comparative gene expression of trimmed mean of M values (TMM) normalized read counts between 600 mM samples and plants grown at 0, 200, and 400 mM NaCl in both KNO3+ and NOD+ series. Counts were scaled by row for improved visualization; Figure S5: Heatmap of top 10 most up and downregulated differentially expressed genes (DEGs) in *Casuarina glauca* nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD+), grown at 600 mM NaCl relative to control (0 mM NaCl). Comparative gene expression of trimmed mean of M values (TMM) normalized read counts between 600 mM samples and plants grown at 0, 200, and 400 mM NaCl in both NOD+ and KNO3+ series. Counts were scaled by row for improved visualization; Figure S6: Protein-Protein interactions (PPIs) network of differentially expressed genes (DEGs) in non-nodulated *Casuarina glauca* plants supplied with KNO3 (KNO3+), grown at 600 mM NaCl relative to control (0 mM NaCl). PPIs network was retrieved through ShinyGO tool, based on STRING database; Figure S7: Protein-Protein interactions (PPIs) network of differentially expressed genes (DEGs) in *Casuarina glauca* nodulated by nitrogen-fixing *Frankia casuarinae* Thr (NOD+), grown at 600 mM NaCl relative to control (0 mM NaCl). PPIs network was retrieved through ShinyGO tool, based on STRING database.

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