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Research article

Comparative transcriptome analysis of the garden asparagus (*Asparagus officinalis* L.) reveals the molecular mechanism for growth with arbuscular mycorrhizal fungi under salinity stress

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ABSTRACT

Soil salinity is one of the most abiotic stress factors that severely affects the growth and development of many plants, which can ultimately threaten crop yield. Arbuscular mycorrhiza fungi (AMF) has been proven to be effective in mitigating salinity stress by symbiosis in many crops. *Asparagus officinalis* are perennial plants grown in saline-alkaline soil, however, limited information on their molecular mechanisms has restricted efficient application of AMF to garden asparagus under salinity stress. In this study, we conducted a transcriptome analysis on the leaves of garden asparagus to identify gene expression under salinity stress. Seedlings were grown in 4 treatments, including non-inoculated AMF using distilled water (NI), inoculated AMF using distilled water (AMF), non-inoculated with salinity stress (NI + S), and inoculated with salinity stress (AMF + S). A total of 6019 novel genes were obtained based on the reference-guided assembly of the garden asparagus transcriptome. Results revealed that 455 differentially expressed genes (DEGs) were identified when comparing NI + S to AMF + S. However, among the up-regulated DEGs, 41 DEGs were down-regulated, while 242 DEGs had no differences in their expression levels when comparing NI to NI + S. These DEGs' expression patterns may be key induced by AMF under salinity stress. Additionally, the GO and KEGG enrichment analyses of 455 DEGs revealed that these genes mainly participate in the improvement of the internal environment in plant cells, nitrogen metabolic-related processes, and possible photoprotection mechanisms. These findings provide insight into enhanced salinity stress adaptation by AMF inoculation, as well as salt-tolerant candidate genes for further functional analyses.

1. Introduction

Soil is an important factor for plant growth and development; however, more than 6% of the world's land has been affected by soil salinity (Munns and Tester, 2008). Additionally, salinity stress has been one of the most severe environmental stressors that has detrimentally affected crop yield, and the underlying molecular mechanism may be related to several biological pathways and processes, including osmotic, ionic, and oxidative pathways, as well as nutritional disorders, alteration of metabolic processes, reduction of cell division and expansion, and even genotoxicity (Yu et al., 2016). Previously, multiple signal transduction pathways have been identified in plants, such as salt overly sensitive (SOS), mitogen-activated protein kinase (MAPK) cascades, phosphatidylinositol (PI), and ABA-mediated signal transduction pathways, which help plants to adapt to salinity stress (Colcombet and Hirt, 2008; Xue et al., 2009; Ji et al., 2013; Fernando and Schroeder, 2016). Moreover, in response to salinity stress, molecular signaling can activate other transcription factors (TFs), which then amplify the signals for gene regulation and re-establish homeostasis of cellular ionic, osmotic, and reactive oxygen species (ROS) as a way to adapt (Yang and Guo, 2018).

Arbuscular mycorrhizal fungi (AMF) are a type of beneficial microorganism that engages in symbiosis with other plants, thus allowing further amelioration of the negative effects of a stressful environment (Levy et al., 1983). These AMF can form mutualistic associations with roots in more than 80% of terrestrial plants all over the world (Smith and Read, 1996). This association enhances plant growth and changes the morphological, physiological, and nutritional levels of plants,

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thereby improving resistance against multiple abiotic stressors, including high temperature, cold, drought, and salinity stress (Silong et al., 2009; Porcel et al., 2012; Pedranzani et al., 2016). Under salinity stress conditions, plants with AMF inoculation can ameliorate negative effects by enhancing regulation on ion homeostasis, osmotic balance, and anti-oxidant enzymes activity (Porcel et al., 2016; Hashem et al., 2018), as well as photosynthetic activity, nutrient acquisition, and water uptake in plants (Ruiz-Lozano et al., 1996; Sheng et al., 2008; Çekiç et al., 2012; Porcel et al., 2015). However, previous studies on amelioration mechanisms are limited to morphology and physiology, and few studies have been conducted on gene expression in AMF-inoculated plants on a genome-wide scale, which restricts the application of AMF to salt resistance in plants. Therefore, further molecular analyses on the AMF amelioration mechanism under salinity stress conditions are required.

The garden asparagus (*Asparagus officinalis* L.) is a species belonging to the *Asparagus* (Liliaceae) genus and is a perennial herb with important economic and pharmacological value; it is also a moderately salt-sensitive crop. In our previous studies (Yanpo et al., 2015, 2017), *A. officinalis* with AMF inoculation effectively ameliorated 100 mM salinity stress and was found to play an important physiological role in the amelioration of salt-induced osmotic stress, oxidative damage, and ionic stress. These results lay a solid foundation for further investigation of the molecular mechanisms underlying salt tolerance in *A. officinalis* inoculated by AMF. Additionally, with the release of the *A. officinalis* draft genome, transcriptome sequencing and functional genomics have greatly facilitated *A. officinalis* research (Murase et al., 2017; Dong et al., 2019).

In this study, we investigated global gene transcription changes in the leaf tissues of *A. officinalis* using the Illumina HiSeq™ 2500 sequencing platform in order to explore the molecular mechanisms underlying salt tolerance initiated by AMF. Through the analysis of mRNA sequencing (RNA-Seq) data, we aimed to identify key pathways and genes induced by AMF under salinity stress conditions. Our results may provide evidence toward a more complete understanding of the role of AMF in enhanced salt tolerance and facilitate effective genetic engineering strategies for enhancing *A. officinalis* salinity stress tolerance.

2. Materials and methods

2.1. Plant materials and treatments

A. officinalis L. cv. No. 08–2 was used as a host plant and was retrieved from the Institute of Cash Crops, Hebei Academy of Agriculture and Forestry Sciences. The AMF *R. irregularis* inoculum, which consisted of a dried cultivation substrate containing spores (approximately 42 g^{-1}), extraradical hyphae, and chopped roots (Ma et al., 2018), was retrieved from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences. *A. officinalis* seeds were germinated on 3 layers of wetted filter in petri dishes at 26 °C in the dark for 4 days. Then, healthy seedlings were selected for sowing in plastic pots (10 cm diameter, 1 seedling/pot). During sowing, half of the selected seedlings were inoculated with 10 g of *R. irregularis* inoculum per pot and mixed with perlite and sand (1/1, v/v, mycorrhizal treatment); the second-half received the same amount of inoculum, which was sterilized by autoclaving (4 h at 160 °C, non-mycorrhizal treatment). All seedlings were cultured in a greenhouse at an ambient temperature of 28 °C/19 °C (day/night). After 2 months, seedlings from each treatment were subjected to 2 different salinity treatments: (1) normal salinity; and (2) salinity stress. Salinity treatments were imposed by irrigating *A. officinalis* with 200 mL distilled water and NaCl solution (100 mM) to ensure that the soil EC values of normal salinity and salinity stress treatments reached to roughly 1 mS cm^{-1} and 4 mS cm^{-1}, respectively. Thus, our experiment consisted of 4 total treatments: (1) non-inoculated *A. officinalis* plants without salinity stress (NI), (2) inoculated *A. officinalis* plants without salinity stress (AMF), (3) non-inoculated *A. officinalis* plants subjected to salinity stress (NI + S), and (4) inoculated *A. officinalis* plants subjected to salinity stress (AMF + S). Leaves were harvested 10 days later with 3 biological replicates each. A total of 12 samples were kept at −80 °C after frozen in liquid nitrogen for further analysis. Growth parameters, including plant height, fresh weight, and dry weight on the ground and underground, were measured for the calculation of increments over time. Root samples were collected to determine root colonization by AMF at 0, 5, and 10 days after salinity treatments following the method outlined by Ma et al. (2018).

2.2. RNA extraction, cDNA library construction, and RNA-Seq

Total RNA of the 12 leaf samples was isolated using an RNA prep Pure Plant Kit (Tiangen Biotech Co., Ltd., Beijing, China). The library construction and RNA-Seq assay were performed by the Biomarker Biotechnology Corporation (Beijing, China). First, the enrichments of poly(A) mRNA from total RNA were performed using the NEBNext® poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA), in which poly(A) mRNA was chemically fragmented into shorter segments. These interrupted fragments were used as templates for first- and second-strand cDNA synthesis. The resulting cDNAs were then subjected to end-repair, phosphorylation, and ligation to sequencing adapters. Afterward, the suitable products were amplified by PCR to construct the cDNA library. Finally, the 12 purified libraries were sequenced using Illumina HiSeq™ 2500 (San Diego, CA, USA). The sequencing data were deposited to the Sequence Read Archive (SRA), National Centre for Biotechnology Information (NCBI) with accession number SRP188664.

2.3. RNA-Seq reads mapping and functional annotation

After the RNA-Seq assays were performed, clean reads were obtained by trimming adapters and removing empty and low-quality raw reads. Clean reads were mapped to the garden asparagus reference genome (GCA_001876935.1, 2017) using HISAT2. Mapped reads were assembled and quantitatively analyzed as fragments per kilobase of exon per million fragments mapped (FPKM) values using the StringTie software. The untranslated region (UTR) of the original genes from the reference genome, with support of successive mapped reads, was extended upstream and downstream to optimize the gene structure. Additionally, by comparing the original annotation information of the genome, previously unannotated transcription regions could be found [i.e., novel genes after filtering the short polypeptide product (length $<$ 50 amino acids) and single exon regions]. For annotations of new genes, they were aligned to the publicly available protein databases, including Nr, Swiss-Prot, GO, COGs, KOG, Pfam, eggNOG, and KEGG, using the BLASTx algorithm with a cut-off E-value of 10^{-5}.

2.4. Analysis of differentially expressed genes

The expression level of each gene was determined by the FPKM value. Differentially expressed genes (DEGs) analysis in pair-wise comparisons was conducted using the DESeq software. Through calculating the false discovery rate (FDR) and log of fold change ($\log_2$FC) for all genes, DEGs were identified for subsequent analysis through comparisons with FDR ≤ 0.01 and an absolute value of $\log_2 FC \geq 1$. Furthermore, GO and KEGG enrichment analyses were carried out in the key comparisons.

2.5. Confirmation of DEGs by quantitative real-time PCR

The expression profiles of 9 *A. officinalis* DEGs in NI + S and AMF + S treatments were confirmed by quantitative real-time PCR (qRT-PCR) analysis with 3 biological replicates. These DEGs were selected based on their putative functions involved in scavenging ROS, osmotic adjustment, ion homeostasis, synthesis of secondary metabolites, and nitrogen metabolism, among others. First, total RNA was converted into single-stranded cDNA using the TransScript II All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech, Beijing, China), following the manufacturer's protocol. Gene-specific primers were designed using the Primer Premier v5.0 (Premier Biosoft, Palo Alto, CA, USA) (Table S1). Then, qRT-PCR was performed using TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) and ran on the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, USA). Each reaction contained 10 μL 2 × TransStart Top Green qPCR SuperMix, 2.0 μL cDNA sample, and 0.2 μM gene-specific primers with a final volume of 20 μL. According to the output data, relative expressions of the mRNA of each sample were calculated in relation to the reference gene ubiquitin-long tail fusion (GenBank: X66875.1) using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). Finally, the changing tendency of gene expression in the NI + S and AMF + S treatments was compared after qRT-PCR and RNA-Seq analyses to evaluate whether the sequencing results were reliable.

3. Results

3.1. Mycorrhizal colonization and growth parameters

Before salt treatment, root colonization after AMF inoculation was observed in the hyphae, arbuscules, and vesicles of plant roots at the following rates: 54.17%, 52.3%, and 38.97%, respectively; there was no colonization in the treatment without AMF inoculation. After salt treatment, all colonization parameters of the AMF + S treatment were significantly ($p < 0.05$) reduced compared to the AMF treatment (Fig. 1); the root structures decreased by 19.43%, 19.63%, and 20.25%, after 10 days of salt treatment. Although salt treatment reduced AMF colonization in *A. officinalis* roots, the possible mitigation of negative effects induced by salt stress cannot be ignored.

Increments of plant height in salinity stress conditions were significantly ($p < 0.05$) lower compared to plants under normal conditions, with or without AMF inoculation, although the difference was more obvious without AMF inoculation (Table 1). Moreover, AMF inoculation significantly ($p < 0.05$) increased plant height increments compared to non-inoculated *A. officinalis* under salinity stress and normal conditions.

Response patterns of other biomass parameters in the 4 treatment conditions were reflective of the plant height results, suggesting that AMF inoculation plays a role in enhancing growth and salt tolerance in *A. officinalis*. Additionally, AMF inoculation ameliorated the symptoms of salinity stress. Over time, although the symptoms of etiolated and wilted plants exposed to salinity stress gradually worsened in both inoculated and non-inoculated treatments, the differences between the treatments became more obvious after 10 days of salinity stress. For instance, AMF inoculated seedlings showed less etiolation and wilting and had much better development than seedlings without AMF inoculation.

3.2. Overview of transcriptome data by RNA-Seq analysis

To develop a comprehensive overview of AMF effects on *A. officinalis* leaves' transcriptomes under salinity stress, 12 cDNA libraries were designed for RNA-Seq. After removing adapters, low-quality regions, and all possible contamination, a total of 90.25 Gb of clean data with Q30 > 93.64% and a GC percentage between 47.58% and 48.71% were obtained (Table 2). The ratio of reads mapping to the *A. officinalis* genome was high, ranging from 81.58% to 83.80%. These results indicate that there were sequencing data available for further analysis.

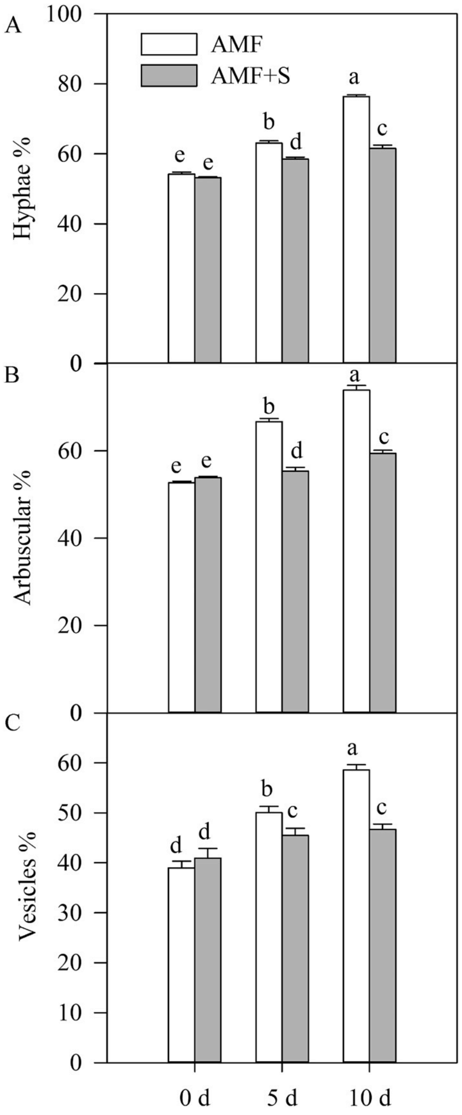

Fig. 1. Colonization of AMF on *A. officinalis* roots with and without salt treatment at 0, 5, and 10 days: (A) Colonization intensity in hyphae; (B) Colonization intensity in arbuscules; and (C) Colonization intensity in vesicles. Bars with different lowercase letters in each colonization parameter mean significant difference (Duncan multiple comparison test, $P < 0.05$).

3.3. Optimization of gene structures

The optimization of gene structures relies on transcriptome reads mapped to the genome sequences. In this study, the reads were realigned, and the structures of 502 genes were optimized (Table S2).

3.4. Identification of new genes and functional annotations

Based on the RNA-Seq results, a total of 6019 new transcripts in the *A. officinalis* genome were identified and were evenly distributed across all chromosomes. As for the functional annotation, a total of 4672 new genes were annotated in at least 1 of the aforementioned databases (Table 3).

3.5. Identification of DEGs related to salt tolerance

When subjected to the salt treatment, almost 2 times the number of DEGs was down-regulated than up-regulated, with or without inoculation (Table 4). However, for AMF inoculated treatments, the

Table 1
Increments of plant growth parameters. Lowercase letters indicant significant differences (Duncan's multiple range test, $p < 0.05$).

Increments/Treatments	Plant height (cm)	Fresh weight on the ground (g·plant^{-1})	Fresh weight underground (g·plant^{-1})	Dry weight on the ground (g·plant^{-1})	Dry weight underground (g·plant^{-1})
NI	6.45 ± 0.43 b	2.21 ± 0.12 b	2.00 ± 0.11 a	0.49 ± 0.07 a	0.26 ± 0.01 b
AMF	7.25 ± 0.32 a	2.41 ± 0.08 a	2.04 ± 0.12 a	0.54 ± 0.06 a	0.30 ± 0.02 a
NI + S	3.71 ± 0.62 d	1.11 ± 0.18 d	0.95 ± 0.21 c	0.29 ± 0.06 c	0.10 ± 0.02 d
AMF + S	5.52 ± 0.55 c	1.85 ± 0.16 c	1.54 ± 0.15 b	0.40 ± 0.04 b	0.20 ± 0.06 c

Table 2
Summary of sequences analysis of 12 libraries.

Samples	Number of clean reads	Number of clean bases	GC content	Q30
NI-1	24,092,922	7,204,250,660	48.69%	93.64%
NI-2	20,600,209	6,159,570,306	48.40%	93.84%
NI-3	23,819,921	7,122,154,880	48.43%	93.97%
AMF-1	29,151,194	8,718,959,652	48.50%	93.86%
AMF-2	23,668,718	7,088,165,762	48.71%	93.86%
AMF-3	27,224,266	8,149,477,994	48.19%	93.73%
NI + S-1	26,062,151	7,801,640,136	48.52%	94.15%
NI + S-2	23,836,228	7,131,915,478	47.93%	94.38%
NI + S-3	24,848,127	7,439,308,186	47.98%	94.47%
AMF + S-1	23,847,943	7,141,683,710	48.47%	93.99%
AMF + S-2	25,777,631	7,718,628,700	47.68%	94.28%
AMF + S-3	28,635,814	8,571,491,466	47.58%	94.31%

Table 3
Results of the BLAST analysis of new genes compared to public databases.

Annotated databases	Number of new genes
COG	626
GO	1751
KEGG	1181
KOG	1920
Pfam	1660
Swiss-Prot	2190
eggNOG	3057
Nr	4644
Total new genes	4672

Table 4
The number of up-regulated and down-regulated DEGs in the 4 comparisons.

Treatments	All DEG	Up-regulated DEG	Down-regulated DEG
AMF/AMF + S	1630	515	1115
NI/NI + S	2428	768	1660
NI/AMF	11	7	4
NI + S/AMF + S	455	286	169

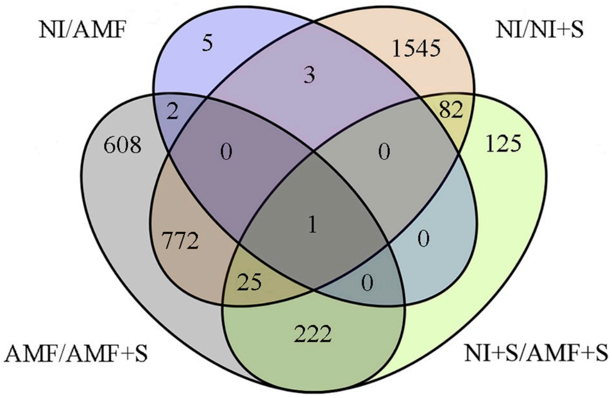

Fig. 2. Venn diagrams showing specific and common DEGs in the 4 comparisons.

number of up-regulated DEGs was almost 2 times the number of down-regulated DEGs under both normal and salinity stress conditions. The above results demonstrate that more *A. officinalis* DEGs were down-regulated in response to salinity stress, whereas more DEGs were up-regulated in response to AMF inoculation. Interestingly, 41 genes were found to be down-regulated when comparing the NI and NI + S treatments but up-regulated when comparing the NI + S and AMF + S treatments (Table S3). Additionally, 242 genes were up-regulated when comparing the NI + S and AMF + S treatments, but had no differences in their expression levels when comparing the NI and NI + S treatments (Table S4). Therefore, it can be inferred that DEGs with these expression patterns may be key genes induced by AMF under salinity stress conditions.

To further explore the relationships among DEGs from the 4 different comparisons, Venn diagrams were constructed using a total of 3390 DEGs (Fig. 2). A total of 798 DEGs were regulated in conditions with and without AMF-inoculation (i.e., the control against salinity stress) according to the comparisons. Moreover, non-inoculated plants had 1630 specific DEGs, and AMF-inoculated plants had 832 specific DEGs. These results indicate that the molecular responses to salinity stress between conditions with and without AMF inoculation were strikingly different. Furthermore, in order to identify DEGs regulated specifically by AMF under salinity stress, DEGs in the NI + S and AMF + S comparison, and NI and AMF comparison were found to have only 1 DEG in common, which indicates that most of the DEGs in the NI + S and AMF + S comparison are possibly linked to salinity stress regulated by AMF. These DEGs in the NI + S and AMF + S comparison were selected for subsequent gene function characterization to explore a possible molecular mechanism for salt tolerance initiated by AMF.

3.6. Functional annotation of DEGs induced by AMF under salinity stress

Based on the analysis above, in order to interpret the role of AMF in salinity stress amelioration, the function of 455 DEGs in the NI + S and AMF + S comparison was examined. For the GO analysis, 247 out of 455 DEGs were assigned to at least 1 GO term in order to describe their biological processes, molecular functions, and cellular components (Fig. 3); DEGs were significantly (KS value ≤ 0.05) enriched in 32 terms of biological processes (Table 5). These enriched terms were highly linked to biological processes in the adaptive response to salinity stress, including protein biosynthesis-related processes (GO:0006383, GO:0006452, GO:0006449, GO:0045901, GO:0045905, GO:0006414, and GO:0006415), the ATP biosynthetic process (GO:0006754), protein glutathionylation (GO:0010731), regulation of cell differentiation (GO:0045595), nitrogen assimilation related processes (GO:0010167, GO:0009399, and GO:0009084), the respiratory electron transport chain (GO:0022904), and the apocarotenoid metabolic process (GO:0043288).

There were 98 out of 455 DEGs that were assigned a KEGG ID and categorized into 59 pathways (Fig. 4). Additionally, the top 20 enriched pathways (based on q-values) were highlighted (Fig. 5). Among them, DEGs were significantly (q-value ≤ 0.05) enriched in 3 pathways

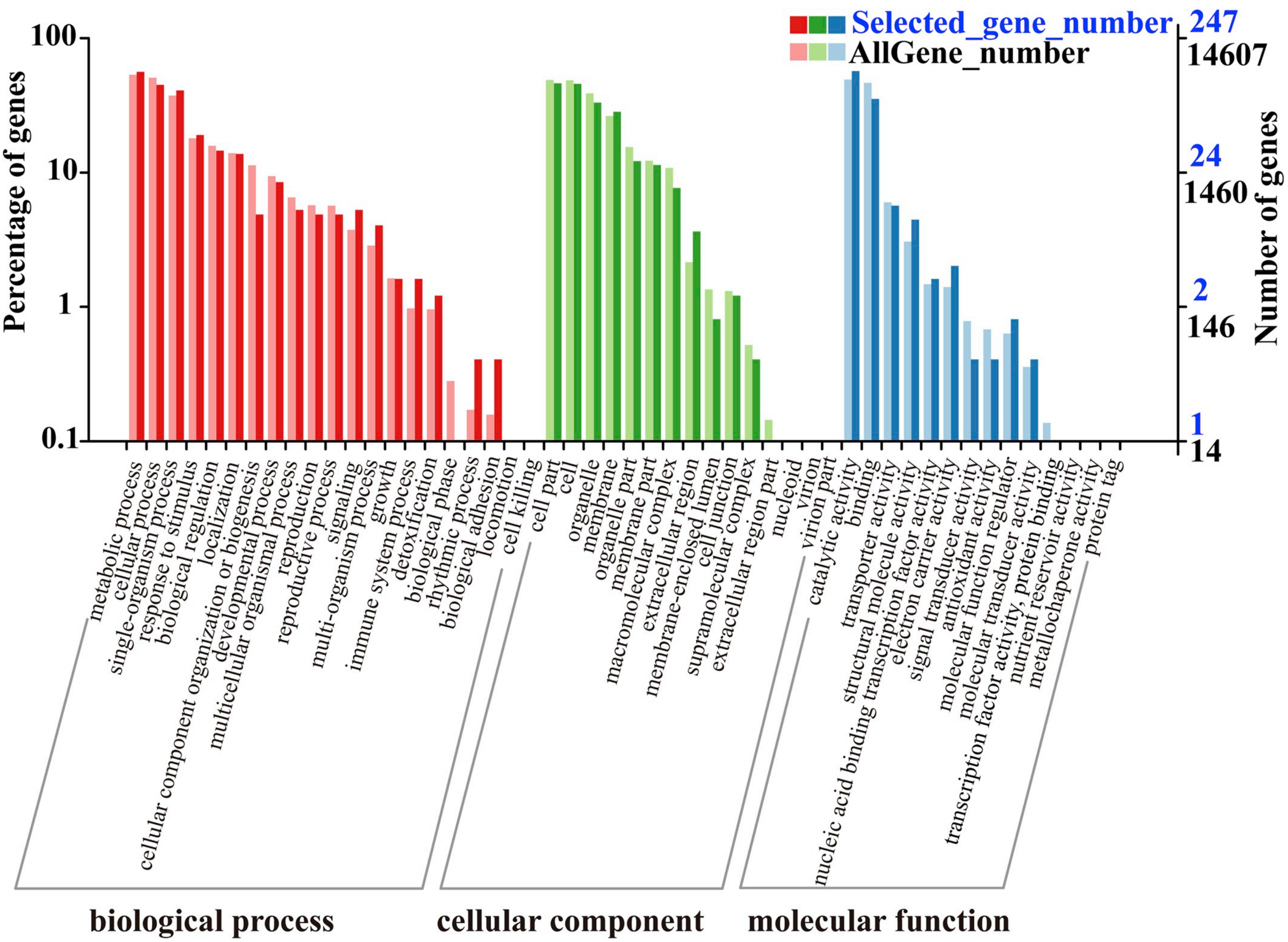

Fig. 3. Functional annotation of DEGs based on gene ontology (GO) categorization. The chart was plotted against the reference *A. officinalis* genome, which showed each significant GO term and the amount (percentage) of sequences annotated with this term.

related to secondary metabolite biosynthesis, including phenylpropanoid, flavonoid, stilbenoid, diarylheptanoid, and gingerol biosynthesis, which may be linked to salt tolerance.

3.7. qRT-PCR validation

In order to validate RNA-Seq data, 9 DEGs in the NI + S and AMF + S comparison were selected for qRT-PCR analysis. The expression tendency of these 9 genes by qRT-PCR was found to be highly consistent with the RNA-Seq results (Fig. 6). Thus, these results indicate that the DEG analyses were highly reliable.

4. Discussion

The potential for salinity stress amelioration by AMF has been demonstrated in various plants, including cucumber, maize, and wheat (Sheng et al., 2008; Talaat and Shawky, 2014; Hashem et al., 2018). Combined with the results of our previous studies (Yanpo et al., 2015, 2017), salt tolerance initiated by AMF was further confirmed in *A. officinalis*, characterized by increased biomass, activities of antioxidant enzymes, content of soluble protein, ion balance, reduced electrolytic leakage, and malondialdehyde content. To better understand the underlying molecular mechanism of salt tolerance, we have provided a snapshot of transcriptome regulation in AMF-inoculated *A. officinalis* under salinity stress conditions. Beginning with the 12 sequenced libraries, we identified 6019 new genes and from them annotated 4672 new genes, which contribute to a better annotation of the *A. officinalis* genome. Furthermore, genes encoding calcium-binding protein and mannose-specific lectin were induced by AMF in the controls, which may involve signal transduction in this symbiotic relationship. Additionally, the up-regulation of genes encoding chlorophyll a-b binding proteins of LHCII and thylakoid soluble phosphoprotein, which participate in the process of photosynthesis, may be directly related to the enhanced growth of plants with AMF inoculation under control conditions. Complicating things further, 455 DEGs initiated by AMF under salinity stress conditions were significantly enriched in several GO and KEGG pathways that are involved in core salt tolerance mechanisms, including improvement of the internal environment in plant cells, nitrogen metabolic-related processes, and possibly photoprotection mechanisms (Table S5). These pathways further confirm that AMF ameliorates the negative effects of osmotic, ionic, and oxidative stress and enriches nitrogen metabolic and photoprotection mechanisms under salinity stress.

4.1. The improvement of the internal environment in plant cells

First, it was evident that the induced expression of antioxidant enzymes, along with the synthesis of non-enzymatic compounds, under

Table 5
The GO classification of enriched DEGs using the topGO tool.

GO ID	GO term	Annotated	Significant	KS
GO:0015713	phosphoglycerate transport	5	2	0.00940
GO:0006383	transcription from RNA polymerase III promoter RNA	4	1	0.01195
GO:0009399	nitrogen fixation	3	1	0.01299
GO:0045901	positive regulation of translational elongation	9	1	0.01644
GO:0045905	positive regulation of translational termination	9	1	0.01644
GO:0006452	translational frameshifting	9	1	0.01644
GO:0006449	regulation of translational termination	9	1	0.01644
GO:0009084	glutamine family amino acid biosynthetic process	25	3	0.01710
GO:0018958	phenol-containing compound metabolic process	33	2	0.01969
GO:0009145	purine nucleoside triphosphate biosynthetic process	58	1	0.02028
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	58	1	0.02028
GO:0022904	respiratory electron transport chain	30	1	0.02126
GO:0006754	ATP biosynthetic process	53	1	0.02470
GO:0043241	protein complex disassembly	41	2	0.02476
GO:0032984	macromolecular complex disassembly	41	2	0.02476
GO:0048571	long-day photoperiodism	8	1	0.02502
GO:0048574	long-day photoperiodism, flowering	8	1	0.02502
GO:0009696	salicylic acid metabolic process	30	2	0.02576
GO:0051336	regulation of hydrolase activity	41	1	0.02853
GO:0006414	translational elongation	101	2	0.03063
GO:0006415	translational termination	18	1	0.03065
GO:0000103	sulfate assimilation	12	1	0.03167
GO:0045595	regulation of cell differentiation	46	1	0.03167
GO:0071900	regulation of protein serine/threonine kinase activity	31	1	0.03433
GO:0022411	cellular component disassembly	42	2	0.03455
GO:0098656	anion transmembrane transport	51	2	0.0355
GO:0043288	apocarotenoid metabolic process	9	1	0.03584
GO:0043624	cellular protein complex disassembly	40	2	0.04058
GO:0010167	response to nitrate	19	1	0.04376
GO:0046189	phenol-containing compound biosynthetic process	31	2	0.04414
GO:0010731	protein glutathionylation	7	1	0.0473
GO:0043244	regulation of protein complex disassembly	20	1	0.04981

salinity stress conditions plays an important role in scavenging excessive ROS to maintain oxidative balance and reduce the effects of abiotic stress in the cell, such as peroxidase (POD), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR), adenosylhomocysteinase (AHCY), glutathione S-transferase (GST), ascorbic acid (AsA), glutathione (GSH), and thioredoxin (TRX) (Foyer and Noctor, 2011; Chan and Lam, 2014; Das et al., 2015). In this study, numerous DEGs encoding POD, GST, MDHAR, AHCY, and TRX were up-regulated and participated in multiple antioxidant systems, including the GPX pathway, ascorbate-GSH cycle, and peroxiredoxin/thioredoxin (PrxR/Trx) pathway (Foyer and Noctor, 2011; Zhang et al., 2012).

Second, the primary strategy for ameliorating osmotic stress was the accumulation of compatible osmolytes, which lowered the osmotic potential in plant cells and stabilized protein and cellular structures (Yancey et al., 1982). It has been reported that AMF strongly alters leaf proline metabolism through regulating proline-metabolized enzyme activities, which is important for osmotic adjustment in the host plant (Wu et al., 2017). In *A. officinalis*, salinity stress may induce increased proline accumulation by reducing the expression levels of genes that encode proline dehydrogenase (ProDH), which is the first enzyme found in the proline degradation pathway (Szabados and Savouré, 2010). Whereas, a gene encoding Δ 1-pyrroline-5-carboxylate synthetase (P5CS), which is the key rate-limiting enzyme in proline biosynthesis (Kishor et al., 1995), has been found to be up-regulated in AMF-inoculated plants under salinity stress conditions. These results suggest that proline accumulation through regulation of different metabolic directions induced by AMF may be linked to enhanced salt tolerance in *A. officinalis*. In addition to organic solute proline, plants experiencing salinity stress also accumulate inorganic solutes, such as potassium in order to maintain osmotic balance (Yang et al., 2009). In this study, 2 genes encoding the K^+ channel were found to up-regulate AKT genes, which are involved in the translocation of K^+ in shoots (Marten et al., 1999).

Lastly, SOS signaling pathway plays an important role in ion homeostasis by regulating Na^+ transport in the plasma membrane and tonoplast. Under salinity stress, a cytosolic Ca^{2+} signal is triggered by excessive intracellular or extracellular Na^+ (Zhu, 2000). The EF-hand Ca^{2+} binding proteins (SOS3) and SCaBP8/CBL10 further decode the Ca^{2+} signal and translate it to a serine/threonine protein kinase (SOS2) (Ishitani et al., 2000; Quan et al., 2007). Then a plasma membrane Na^+/H^+ antiporter (SOS1) and a vacuolar Na^+/H^+ exchanger (NHX1) are activated and transport Na^+ from the cytoplasm to the apoplast and vacuoles, a process driven by the H^+ gradient established by H^+-ATPase (Yang and Guo, 2018). In this study, several genes involved in the SOS pathway were found to be up-regulated, including DEGs encoding calcium-binding protein (SOS3) and CBL-interacting protein kinase 19-like (SOS2). Additionally, based on the increased expression levels of a gene encoding V-type H^+-transporting ATPase in the NI and NI + S comparison, a gene encoding plasma membrane ATPase was also up-regulated in the NI + S and AMF + S comparison, which are essential for establishing an electrochemical H^+-gradient across tonoplasts and the plasma membrane. This suggests that AMF inoculation contributed to ion homeostasis by adjusting the driving forces behind excessive Na^+ ion transport in a more comprehensive, balanced model. Moreover, excessive Na^+ often leads to K^+ deficiency in glycophytes under salinity stress. Therefore, the up-regulated expression of AKT genes, as demonstrated in this study, may also maintain a higher K^+/Na^+ ratio in plant cells as a way to adapt to salinity stress.

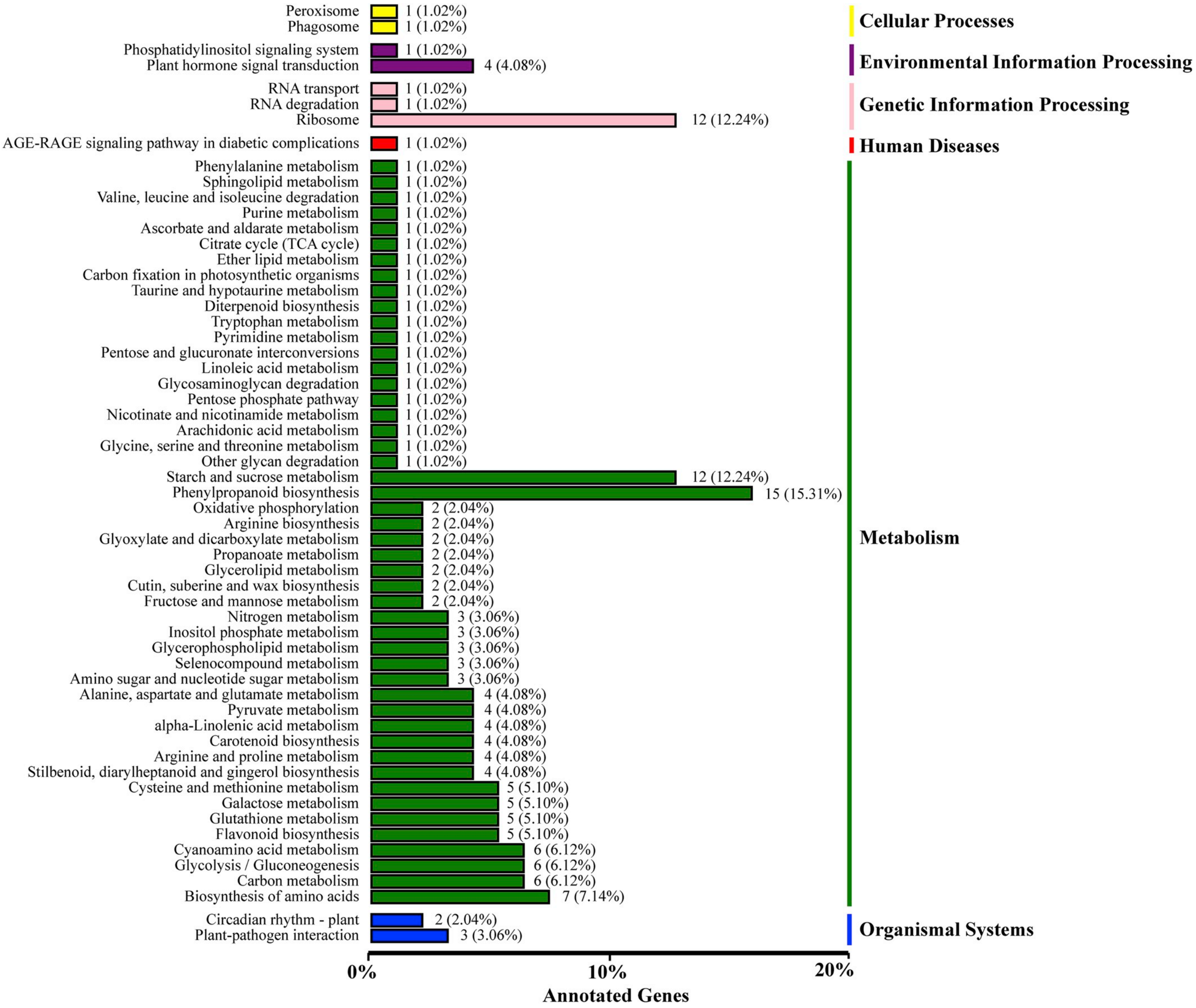

Fig. 4. The KEGG metabolism pathway categories of 455 DEGs.

4.2. Nitrogen metabolic-related processes

Nitrogen (N) is a macronutrient that limits plant growth and development. Several studies have demonstrated that metabolic processes involving N, including N uptake, transport, reduction, and assimilation, as well as amino acid metabolism, are affected by salinity stress (Gu et al., 2018). Enhanced metabolism of N could be one of the most important mechanisms activated by AMF in terms of plant adaptation to saline soils (Talaat and Shawky, 2014). Moreover, it is well known that glutamine synthetase (GS), glutamate dehydrogenase (GDH), and glutamate synthase (GOGAT) are key enzymes involved in N assimilation. In this study, the DEGs encoding GS and GDH were down-regulated under salinity stress. However, with AMF inoculation the DEGs encoding GS, GDH, and GOGAT, along with 2 DEGs encoding proteins in the NRT1/PTR families, which play multifunctional roles in N uptake and transport (Bai et al., 2013), were up-regulated under salinity stress. This demonstrates that enhanced salt tolerance of *A. officinalis* may be related to improvements on N-utilization efficiency by AMF inoculation. Furthermore, enhanced nitrogen assimilation could provide essential substrates, such as amino acids for secondary metabolism. Several studies have implicated that AMF also causes increased expression levels of genes encoding enzymes related to secondary metabolisms, such as the metabolism of phenolic compounds, flavonoids, and lignin (Dixon et al., 2002; Costa et al., 2003), which are involved in antioxygenation, defense systems, and salt tolerance (Rice-Evans et al., 1996; Li et al., 2017). In *A. officinalis*, the 455 identified DEGs were significantly enriched in 3 pathways involved in the biosynthesis of secondary metabolites. Consistent with previous reports, AMF inoculation in this study was demonstrated to induce an increase in the expression levels of genes encoding enzymes associated with flavonoid and lignin synthesis, including CAD, HCT, CSE, COMT, ALDH2C4, CCR, POD, laccase, and F3H (Whetten et al., 1998; Nair et al., 2004; Vanholme et al., 2013; Li et al., 2017). These results suggest that the activation of secondary metabolism genes by AMF inoculation may play an important role in enhanced salt tolerance.

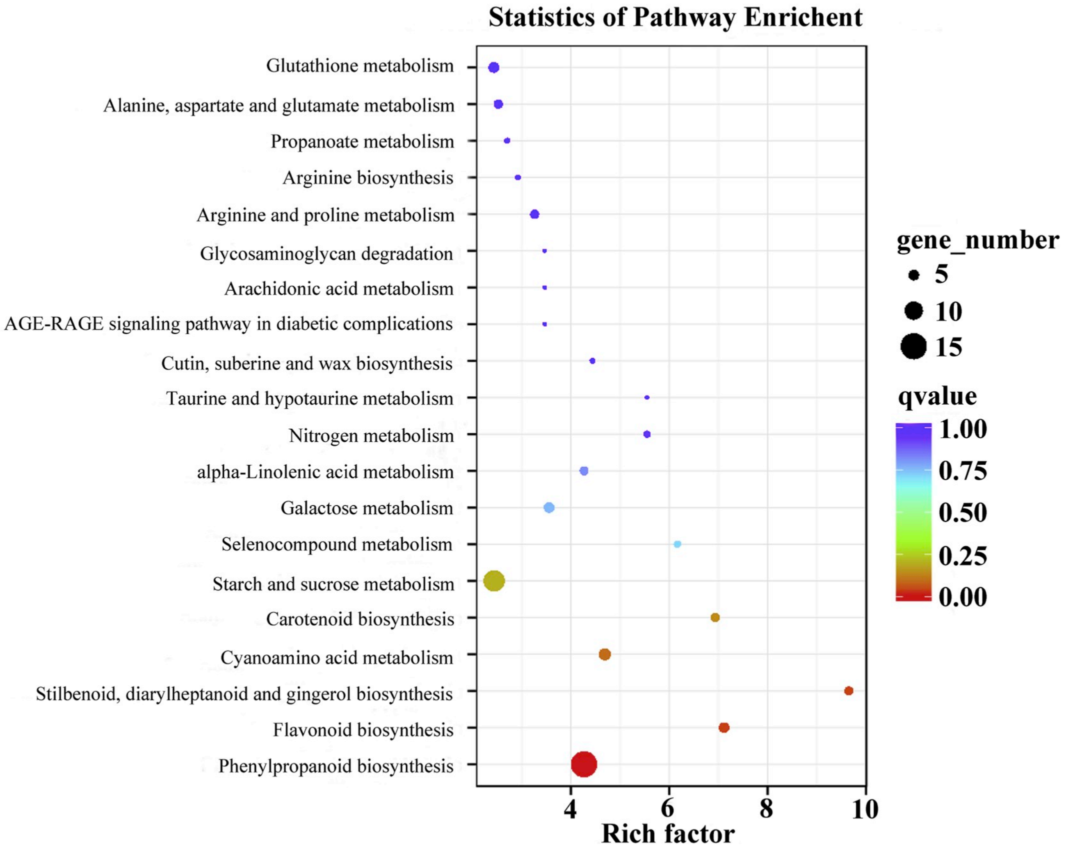

Fig. 5. The KEGG pathway enrichment scatter map.

4.3. Possible photoprotection mechanisms

Photosynthesis is one of the primary processes affected by salinity stress and could lead to excess light energy generation, which causes photoinhibition of the photosynthetic apparatus or even light damage in plants (Reinbothe and Reinbothe, 1996). However, plants have evolved several mechanisms to protect themselves from photodamage. For instance, in this study, *A. officinalis* avoided photoinhibition by balancing the absorption and utilization of light energy, accompanied by the repair of the cell environment. Under salinity stress, *A. officinalis* may reduce the light energy absorption by down-regulating the expression levels of genes encoding light harvesting chlorophyll a/b binding proteins (LHCII), which are the most abundant light harvester in plants (Turan, 2012). Moreover, state transition of LHCII migration from PSII to PSI may be initiated by the up-regulated expression levels of genes encoding serine/threonine-protein kinase (STN7) to balance excitation energy distribution between the photosystems (Turan, 2012). Furthermore, with AMF inoculation, the improvement of the internal environment in plant cells contributed to the repair of the photosynthetic apparatus under salinity stress. Additionally, the enhanced assimilation of mineral nutrition N, as a necessary component of amino acids, proteins, nucleic acids, coenzymes, photosynthetic pigments, and chloroplasts, could improve photosynthesis by regulating chloroplast development, chlorophyll biosynthesis, and photosynthetic enzyme activity.

Another noticeable change was of four DEGs involved in carotenoid biosynthesis, which were up-regulated and might contribute to enhanced salt tolerance due to their ROS scavenging ability, photoprotection, and membrane stabilization (Jin et al., 2015). Excess light energy can also be exported from the chloroplasts and dissipated by the mitochondrial respiratory chain (Noguchi and Yoshida, 2008). The alternative respiratory pathway, an important bypass of the energy-conserving classical electron transport (ETC) pathway (Millar et al., 2011), which is involved in type II NAD(P)H dehydrogenases (NDs) and cyanide-resistant alternative oxidase (AOX) (Plaxton and Podestá, 2006), plays a key role in efficient photoprotection (Noguchi and Yoshida, 2008). Under salinity stress, a gene encoding AOX was down-regulated in this study. With the addition of AMF inoculation, a gene encoding NDB2, a type II ND, was up-regulated. These results indicate that AMF-inoculated garden asparagus use a multi-pronged approach to protect themselves from photodamage and overcome salinity stress.

5. Conclusion

In this study, a total of 6019 new transcripts in the garden asparagus genome were identified and 455 DEGs were identified in the NI + S and AMF + S comparison. Furthermore, among the 455 DEGs, some were identified as salt-resistance genes, as they are involved in the improvement of the internal environment in plant cells, nitrogen metabolic-related processes, and possible photoprotection mechanisms. Our findings provide insight into enhanced salinity stress adaptation by AMF inoculation, as well as numerous salt-tolerant candidate genes for further investigation in functional analyses.

Rapid development of biotechnology has advanced the conditions for which deep and systematic studies on the salt tolerance mechanism of *A. officinalis* initiated by AMF can be conducted. Based on the important roles of photosynthesis and metabolism, research on photosynthetic rate measurements on the unique needles of *A. officinalis* and

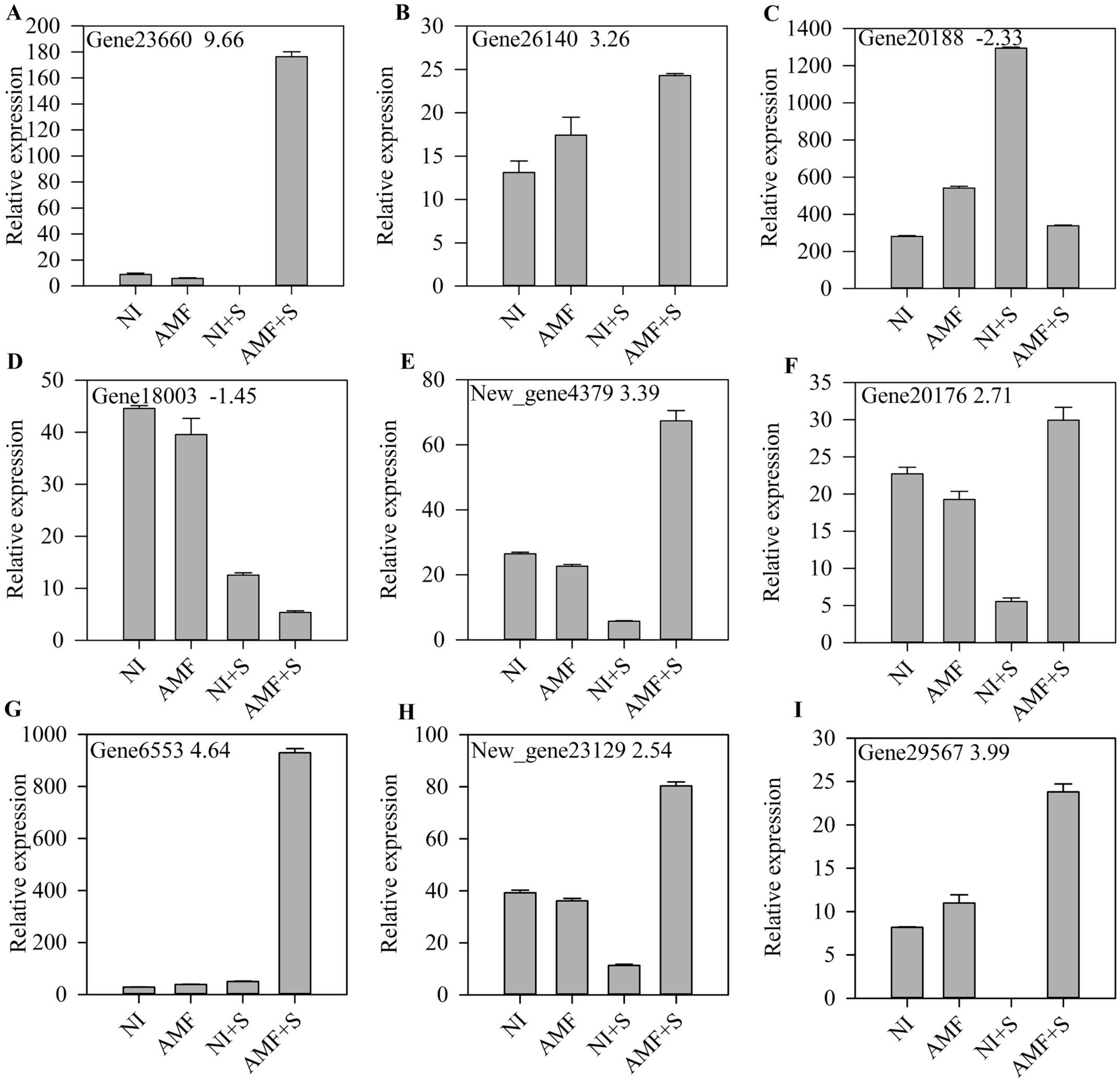

Fig. 6. qRT-PCR confirmation of DEGs identified by transcriptome analysis. Numbers flanking the gene ID are the $\log_2$FC values of expression between NI + S/AMF + S.

metabolomics analysis is urgently needed. Due to this lack of research, these voids are the focus of our future work. Such research would supplement and enhance the understanding of the salt tolerance mechanism of *A. officinalis* initiated by AMF. Lastly, research on the function verification of key genes and cultivating new salt-tolerant germplasms will allow for the effective use of saline soil and increase the yield of *A. officinalis* in order to meet market demands.

Author contributions

Yanpo Cao designed the project and performed the experiments. Xuhong Zhang and Changzhi Han analyzed the data and wrote the manuscript. All authors contributed to the revision of this manuscript and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.013.

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