Activated charcoal and promotion of growth regulation in wheat seedlings: transcriptome analysis

Fu-shaung Dong
Institute of Genetics and Physiology,Hebei Academy of Agriculture and Forestry Sciences https://orcid.org/0000-0001-7474-5062

Meng-yu lv
Insitute of Genetics and Physiology,Hebei Academy of Agriculture and Forestry Sciences

Jin-ping Wang
Institute of Millet Crops,Hebei Academy of Agriculture and Forestry Sciences

Xue-ping Shi
The Semi-arid Agriculture Engineering§ Technology Research Center of P.R.

Yong-wei Liu
Institute of Genetics and Physiology,Hebei Academy Agriculture and Forestry Sciences

He zhao
Institute of Genetics and Physiology,Hebei Academy of Agriculture and Forestry Sciences

Shou Zhou (zhoushuobio@163.com)
https://orcid.org/0000-0002-2047-5885

Jian-Fang Chai
Institute of Genetics and Physiology,Hebei Agriculture and Forestry Sciences

Research article

Keywords: RNA sequencing; wheat; immature embryo culture; phenylpropanoid biosynthesis; plant hormone signal transduction

Posted Date: August 30th, 2019

DOI: https://doi.org/10.21203/rs.2.13743/v1

Version of Record: A version of this preprint was published on July 6th, 2020. See the published version at https://doi.org/10.1186/s12863-020-00877-9.

Abstract

Background Activated charcoal (AC) is highly adsorbent and is often used to promote seedling growth in plant tissue culture, although the molecular mechanism remains unclear. In this study, global transcriptome analysis by RNA sequencing was performed on 10 day old seedlings prepared from both underground and aboveground growing conditions to provide new insights into seedling growth in the presence of AC.Results A total of 18,555 differentially expressed genes (DEGs) were identified, in which 11,182 were found in roots and 7,373 were found in shoots. In seedlings grown with AC in immature embryo culture, 9,460 DEGs were up-regulated and 7,483 DEGs were down-regulated compared to the control. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that 254 KEGG pathways were enriched in DEGs, 226 of which were common in roots and shoots. Further analysis of major metabolic pathways showed that AC stimulated the expression of nine genes in the phenylpropanoid biosynthesis pathway, including PLA, CYP73A, COMT, CYP84A, and 4CL , which promoted cell differentiation and seedling growth. In the plant hormone signal transduction pathway, AC up-regulated genes related to stress resistance and disease resistance including EIN3, BZR1, JAR1, JAZ, and PR1 , and down-regulated genes related to plant growth inhibition, including BKI1, ARR-B, DELLA, and ABF .Conclusions Growth medium containing AC not only promoted seedling growth, but also enhanced resistance to stress and disease.

Background

Activated charcoal (AC) is a porous carbonized substance with a large inner surface area on which many substances can be adsorbed. AC is often used in plant tissue culture to improve cell growth and development [1]. It can adsorb harmful substances in culture media including impurities in agar, 5-hydroxymethylfurfural produced by sucrose during high-pressure sterilization, phenoquinones secreted by explants during culture, as well as beneficial substances in culture media such as growth regulators, vitamin B6, folic acid, and nicotinic acid [2]. There are many reports on its effects such as anti-browning, improvement of primary culture survival rates, promotion of bud proliferation and seedling growth in a dark environment, and promotion of rooting [3]. In recent years, high-throughput sequencing technology has been widely used in plant mechanism research and its efficiency has dramatically improved [4-7]. In this study, the difference in gene expression of 10 d old wheat seedlings cultured on two media types (with and without AC) was analyzed by transcriptome sequencing technology, which laid a foundation for further study of gene expression and the mechanism used by AC to promote the development of immature wheat embryos. Genes that promote wheat growth were analyzed and excavated to provide a theoretical basis for breeding high-yielding wheat varieties.

Methods

Plant material

The middle part of young spikes of wheat (*Triticum aestivum* liang xing 99 from Liang xing seed institue) at 15 d post-bloom was peeled, sterilized with 1.5 % NaClO for 15 min, and rinsed thoroughly with distilled water. Then, immature embryos were peeled off and the scutella were downward inoculated in base medium (N6 supplemented with 0.02 mg/L NAA, 0.05 mg/L 6-BA) and NAC. Two biological replicates were prepared for each group, with 10 immature embryos in each replicate. Ten of the 5 d and 10 of the 10 d seedlings were taken to determine leaf length, root length, leaf number, root number, fresh weight of stem and leaf, and fresh weight of roots of single plants. Trait differences were analyzed by SPSS18.0 software (IBM, USA). Forty of the 10 d cultured seedlings were removed from N6 and NAC

media, and roots and shoots of seedlings from each tank were collected separately. Each sample comprised 20 independent shoots or 20 independent roots; two biological replicates were paired for each sample, immediately frozen in liquid nitrogen, and stored at -80 ℃.

RNA isolation and cDNA library construction

Total RNA was isolated using a TRIzol total RNA extraction kit (Invitrogen, USA), which yielded ~10 μg of total RNA from each sample. RNA quality was examined by 0.8 % agarose gel electrophoresis and spectrophotometry. High quality RNA with 28S:18S > 1.5 and 260/280 absorbance ratios between 1.8 and 2.2 was used for library construction and sequencing. Illumina HiSeq library construction was performed according to the manufacturer's instructions (Illumina, USA). Magnetic beads with poly T oligos attached were used to purify mRNA from total RNA. RNA was broken into 200-300 bp fragments using ion interruption. Using RNA as the template, 6-base random primers and reverse transcriptase were used to synthesize the cDNA chain, and the first chain of cDNA was used as a template for the synthesis of the second chain of cDNA, where the base T was replaced by the base U. After construction of the library, polymerase chain reaction (PCR) amplification was used to enrich the library fragments, and then the library was selected according to fragment size, which was 300-400 bp. The library was then accessed using an Agilent 2100 Bioanalyzer (Agilent, USA). After RNA extraction, purification, and library building, based on the Illumina HiSeq sequencing platform, the samples were subjected to double-end sequencing using next-generation sequencing (NGS) to obtain raw reads (Shanghai Personal Biotechnology Co., Ltd., China).

RNA sequencing and transcript analysis

Raw reads were filtered before data analysis; high quality clean reads with Q > 20 were obtained for subsequent analysis. Reference genome data were derived from the Ensembl database (http://www.ensembl.org/). The reference genome index was created by Bowtie2 software [19], and the clean reads were compared to the reference index using Tophat2 (http://tophat.cbcb.umd.edu/). The Read Count value on each gene was compared using HTSeq0.6.1p2 (https://github.com/genepattern/HTSeq.Count) as the original gene expression level. Expression levels were normalized using RPKM (Reads Per Kilo bases per Million reads) with RPKM values > 1 as the gene expression standard [20]. Differential analysis of gene expression was performed using DESeq with more than a two-fold change of expression level (log2 fold change value > 1) and the expression with significant difference (P-value < 0.05) as the differential gene screening condition [21]. KEGG pathway analysis was used to analyze the metabolic pathways and signaling pathways in which DEGs were primarily involved.

Quantitative real-time PCR analysis

To validate the expressions of DEGs, 15 candidate DEGs were randomly selected for a quantitative real-time PCR (qRT-PCR) analysis. Selected gene names and primer information are listed in Table 1. The housekeeping gene in wheat,

TaRP15, was used as an internal control [22]. Using 1 μg RNA from each sample as a template, cDNA was synthesized using a PrimeScript^{TM} RT reagent Kit with gDNA Eraser (TakaRa, JPN). RT-PCR was performed using a SYBR Premix Ex Taq kit (TakaRa, JPN) on an ABI ViiATM7 instrument (Applied Biosystems, USA). The comparative 2-ΔΔCT method of quantification was used to quantify the relative expression of specific genes [23].

Results

Effect of AC on wheat seedling growth

Results from 5 d and 10 d old seedling measurement in both media treatments showed that seedling growth potential on base medium with 4 g/L AC (NAC) was significantly higher than on N6. Leaf and root length, root number, plant height, and fresh weight of 10 d old seedlings were significantly higher than those of 5 d old seedlings grown on NAC medium. On N6 medium, shoot length and fresh weight of the 10 d old seedlings were significantly higher than those of the 5 d old seedlings, but roots stopped growing and showed atrophy (t-test, $P < 0.05$) (Figure 1, 2).

Illumina sequence analysis and validation of selected differentially expressed genes (DEGs) using quantitative real-time polymerase chain reaction (qRT-PCR)

Two biological replicates were processed for each treatment, and a total of 10 roots and 10 stems from the 10 d old seedlings were analyzed for correlation with the cDNA libraries. Results showed that correlation between biological replicates was high, indicating that the sequencing data was reliable and repeatable (Figure 3). After removing joints and low quality sequences in the raw reads, high quality clean reads of Q > 20 were obtained, where 255,820,114 clean reads were found in the aboveground treatment and 283,192,836 clean reads were found in the underground treatment. A total of 461,062,200 filtered clean reads were compared to reference genomes using Tophat2. The percentage of clean reads (85.6 %) (452,832,933) was compared to gene regions, 97.7 % (442,365,747) of which were compared with exon regions, indicating that clean reads were mostly effective.

To confirm the DEGs identified from sequencing and computational analysis, 15 DEGs were randomly selected for qRT-PCR. Correlation analysis between differential gene expression levels in RNA-seq and qRT-PCR was performed after log2 transformation. The Pearson correlation coefficient was 0.992, which was significantly correlated at the 0.01 level (Figure 4), indicating that expression of these genes was consistent across RNA-seq and qRT-PCR data.

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs

A total of 18,555 genes with unchanged expression were found by DESeq (Version 1.18.0) including 1,182 DEGs in roots and 7,373 DEGs in shoots, with a total of 1,612 DEGs common between roots and shoots. For genes affected by AC, there were 9,460 up-regulated DEGs and 7,483 down-regulated DEGs identified between the AC group and control (Figure 5). To further identify functional pathways, DEGs were found in 254 KEGG functional pathways by KEGG pathway analysis; there were 226 common KEGG pathways between roots and shoots. Among these pathways, the

Metabolic category (105, 39.10 %) represented the largest group, followed by Organismal Systems (58, 25.66 %), Environmental Information Processing (24, 10.62 %), Genetic Information Processing (21, 9.29 %), and Cellular Processes (18, 7.96 %). A p value < 0.05 was considered as the screening condition. KEGG enrichment analysis showed that the AC treatment regulated genes were enriched in 37 KEGG pathways in roots and 30 KEGG pathways in shoots, which included phenylpropanoid biosynthesis, plant hormone signal transduction, starch and sucrose metabolism, biosynthesis of amino acids, and other metabolic pathways (Figure 6). We analyzed three major metabolic pathways: (1) phenylpropanoid biosynthesis, (2) plant hormone signal transduction, and (3) starch and sucrose metabolism. In these three major pathways, there were 29 genes with changed expression between the AC group and control; 21 genes were up-regulated, which promoted cell differentiation, seedling growth, or enhanced stress and disease resistance (e.g., *PLA*, *HCT*, *ZIM*, and *JAC*), and eight genes were down-regulated, which were related to the inhibition of plant growth (e.g., *BKI1, ARR-B*, *DELLA*, and *ABF*) (Table 2).

Discussion

Using KEGG enrichment analysis, 169 DEGs were mapped to the plant hormone signal transduction pathway, which represents the second largest group among the mapped functional pathways. Ninety-six DEGs mapped in this pathway were up-regulated and 73 DEGs were down-regulated in the AC group. In this study, the addition of AC to seedling culture medium increased the expression of *EIN3*, *BZR1*, *JAR1*, *JAZ*, and *PR1* genes. These genes were previously found to be involved in the plant hormone signal transduction pathway, which directly or indirectly play an important role in regulating plant stress resistance or disease resistance [12-16]. For example, PR1 is a type of water-soluble protein that is produced by plants infected by pathogens or stimulated by a biotic factor. Its main functions include attacking pathogens, degrading cell wall macromolecules, degrading pathogen toxins, and binding viral coat protein to plant receptor molecules [17]. Alternatively, the expression of inhibited plant growth regulating genes (*BKI1*, *ARR-B*, *DELLA*, and *ABF*) was reduced (Table 3). For example, DELLA protein is a transcription factor that acts as an inhibitor of the GA (gibberellin) signal transduction pathway; it is a type of growth inhibitory protein located in the nucleus, which can directly interact with proteins of key transcription factors in plants and plays a central role in many plant signaling activities [18]. The addition of AC to the seedling culture medium causes changes in the internal hormones to enhance both stress and disease resistance of seedlings.

Conclusions

AC can significantly promote wheat seedling growth while enhancing resistance to stress and disease. Our transcriptome data provide new insights into gene expression influenced by AC. Results show that AC stimulates gene expression in phenylpropanoid biosynthesis to promote cell differentiation and seedling growth; both stress and disease resistance genes increased expression, and the growth inhibiting genes decreased expression through the regulation of plant hormone signal transduction. Results of this study preliminarily show that AC can significantly promote the molecular mechanisms of wheat seedling growth, which is helpful for further growth studies.

Abbreviations

AC, activated charcoal; DEGS, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; NAC, base medium with 4 g/L AC; PAL, phenylalanine ammonia-lyase; qRT-PCR, quantitative real-time polymerase chain reaction

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Key Research and Development Program of China [Grant No. 2016YFD0101802], Key Research and Development Program of Hebei [Grant No. 19226322D], and Innovation Project of Hebei Academy of Agriculture and Forestry Sciences.

Authors' contributions

SZ and FSD designed the experiments and wrote the manuscript. MYL, FSD, and JPW conducted the immature embryo culture and tissue sampling. XPS, YWL, and FY performed RNA extraction and qRT-PCR. FSD, HZ, and JFC analyzed the data. All authors read and approved the final manuscript.

Acknowledgments

We acknowledge Institute of Genetics and Physiology, Hebei Academy of Agriculture and Forestry Sciences for providing necessary resources to conduct this study

Appendix A.Supplementary data

Supplementary data to this article can be found online at https://www.ncbi.nlm.nih.gov/sra/PRJN556084

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Tables

Table 1. Primer information for qRT-PCR

Gene ID	Forward (5′→3′)	Reverse (5′→3′)
TaRP15	GCACACGTGCTTTGCAGATAAG	GCCCTCAAGCTCAACCATAACT
TRIAE_CS42_2DL_TGACv1_159322_AA0536590	CCCTGGGAGACTTACGATGGA	CCCGTGCTTGCTCTTGTGGAT
TRIAE_CS42_7DS_TGACv1_622571_AA2042030	GCCAACCGCGTGGACGAGTA	CCATCCCTGCCGTATGACCT
TRIAE_CS42_1DL_TGACv1_062003_AA0207290	TGTTCCACATCGGTGACTTCTTC	CCCGCTGATTGGGTTTGC
TRIAE_CS42_4AS_TGACv1_307691_AA1022770	ATACGGGTTCATATCCTTACCG	CCCAGCCACGCTTCACA
TRIAE_CS42_2AL_TGACv1_096957_AA0322250	AGGTGAACAACGGCAAGGTG	AGGATGAGGTCGCTGATTGG
TRIAE_CS42_2AS_TGACv1_114776_AA0369340	GGATGCCCTGGTCCAGAAGA	AGGTGGTCGAGCGGGTTGTC
TRIAE_CS42_U_TGACv1_641881_AA2106610	GGACGGGAACTTCATCGC	TGGTCGGAGTAGGTCGTACATT
TRIAE_CS42_4BL_TGACv1_320441_AA1039150	CCTCCGCTCTGCCAATA	CCAATACGATCTGCCACC
TRIAE_CS42_1DL_TGACv1_061642_AA0200700	AAGTCGTGGATAGTGCCTGGAT	GGTTGCTGGGTCCGTTGA
TRIAE_CS42_5AL_TGACv1_374196_AA1193340	TGAACTCCGTCATCATCGCACAG	CGGCGTTGGCAAACTCCTCT
TRIAE_CS42_4DL_TGACv1_344866_AA1150230	CCCTTGTAACTCCTTCCTC	TTCATAGTCGCCATCACC
TRIAE_CS42_1BL_TGACv1_031704_AA0119280	TTCAACAAGCTGGAGGTTCG	GCCAAATGCTCGTAGGAGTAAA
TRIAE_CS42_5DL_TGACv1_436333_AA1460060	GTGACCGTGGACGAAGTGAT	GCTGTTGGTGATGCGAAAGT
TRIAE_CS42_U_TGACv1_642434_AA2117550	TGGAACACCGACGACCGC	CTGCTCGCTGGAGAAGCTGAC
TRIAE_CS42_2AS_TGACv1_112552_AA0340650	ATGAGGCAAGTATGGAGAACA	GCAATGAGCCGAGTAATAGAA

Table 2. Part of three major pathways and related genes identified by KEGG enrichment analysis

Pathway	Gene ID	Fold change (NAC/N6)	Expression in NAC	Description
Phenylpropanoid biosynthesis	TRIAE_CS42_1BS_TGACv1_049914_AA0164150	47.15	up	(PAL) phenylalanine ammonia-lyase
	TRIAE_CS42_1DS_TGACv1_080107_AA0239320	12.22	up	(PAL) phenylalanine ammonia-lyase
	TRIAE_CS42_1BS_TGACv1_049965_AA0164870	10.79	up	(PAL) phenylalanine ammonia-lyase
	TRIAE_CS42_2AL_TGACv1_096113_AA0317230	10.21	up	(PAL) phenylalanine ammonia-lyase
	TRIAE_CS42_6DL_TGACv1_527273_AA1701630	8.24	up	(PAL) phenylalanine ammonia-lyase
	TRIAE_CS42_1AS_TGACv1_019041_AA0058710	7.14	up	(PAL) phenylalanine ammonia-lyase
	TRIAE_CS42_3AL_TGACv1_194598_AA0636520	9.78	up	(CYP73A) trans-cinnamate 4-monooxygenase
	TRIAE_CS42_3B_TGACv1_220699_AA0715850	6.61	up	(CYP73A) trans-cinnamate 4-monooxygenase
	TRIAE_CS42_2BS_TGACv1_148390_AA0492590	6.15	up	(CYP73A) trans-cinnamate 4-monooxygenase
	TRIAE_CS42_5AL_TGACv1_378388_AA1253080	5.77	up	(CYP73A) trans-cinnamate 4-monooxygenase
	TRIAE_CS42_3DS_TGACv1_271628_AA0904230	5.50	up	(CYP73A) trans-cinnamate 4-monooxygenase
	TRIAE_CS42_6DS_TGACv1_543204_AA1737020	4.28	up	(COMT) caffeic acid 3-O-methyltransferase
	TRIAE_CS42_6BS_TGACv1_514476_AA1660340	2.19	up	(COMT) caffeic acid 3-O-methyltransferase
	TRIAE_CS42_2BL_TGACv1_132718_AA0439360	7.96	up	(CYP84A, F5H) ferulate-5-hydroxylase
	TRIAE_CS42_2AS_TGACv1_113803_AA0360840	6.15	up	(4CL) 4-coumarate--CoA ligase
	TRIAE_CS42_6BL_TGACv1_502904_AA1626620	5.14	up	(4CL) 4-coumarate--CoA ligase
	TRIAE_CS42_7BS_TGACv1_591841_AA1923260	8.26	up	(HCT) shikimate O-hydroxycinnamoyltransferase
	TRIAE_CS42_2DS_TGACv1_178855_AA0601830	5.80	up	(HCT) shikimate O-hydroxycinnamoyltransferase
	TRIAE_CS42_7AS_TGACv1_569782_AA1824070	5.73	up	(HCT) shikimate O-hydroxycinnamoyltransferase
	TRIAE_CS42_3AL_TGACv1_194329_AA0631150	2.42	up	(CYP98A, C3'H) 5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase
	TRIAE_CS42_7BS_TGACv1_592306_AA1935390	3.59	up	caffeoyl-CoA O-methyltransferase
	TRIAE_CS42_7DS_TGACv1_621454_AA2016210	4.05	up	caffeoyl-CoA O-methyltransferase
	TRIAE_CS42_5DL_TGACv1_436308_AA1459900	6.25	up	(CCR) cinnamoyl-CoA reductase
	TRIAE_CS42_5BL_TGACv1_406204_AA1342180	5.56	up	(CCR) cinnamoyl-CoA reductase
	TRIAE_CS42_5AL_TGACv1_375041_AA1214580	5.15	up	(CCR) cinnamoyl-CoA reductase
Plant hormone signal transduction	TRIAE_CS42_4BL_TGACv1_321177_AA1056660	2.09	up	(ARR-A) two-component response regulator ARR-A family
	TRIAE_CS42_3B_TGACv1_221378_AA0738750	20.04	up	(PYL) abscisic acid receptor PYR/PYL family
	TRIAE_CS42_7DL_TGACv1_602538_AA1959790	2.44	up	(EBF1_2) EIN6-binding F-box protein
	TRIAE_CS42_6BS_TGACv1_514535_AA1661150	2.40	up	(EBF1_2) EIN3-binding F-box protein
	TRIAE_CS42_3DL_TGACv1_251912_AA0885890	3.87	up	(EIN3) ethylene-insensitive protein 3

	TRIAE_CS42_2DS_TGACv1_178626_AA0598480	2.61	up	(BZR1_2) brassinosteroid resistant 1/2
	TRIAE_CS42_1BL_TGACv1_030488_AA0092220	5.34	up	(JAR1_4_6) jasmonic acid-amino synthetase
	TRIAE_CS42_4BL_TGACv1_320580_AA1043710	6.24	up	(JAZ) jasmonate ZIM domain-containing protein
	TRIAE_CS42_5BL_TGACv1_405157_AA1321310	24.45	up	(PR1) pathogenesis-related protein 1
	TRIAE_CS42_7DS_TGACv1_625472_AA2065280	8.73	up	(PR1) pathogenesis-related protein 1
	TRIAE_CS42_3B_TGACv1_221831_AA0750870	0.32	down	(AHP) histidine-containing phosphotransfer protein
	TRIAE_CS42_7AS_TGACv1_569714_AA1822400	0.31	down	(ARR-B) two-component response regulator ARR-B family
	TRIAE_CS42_7AS_TGACv1_569714_AA1822400	0.31	down	(DELLA) DELLA protein
	TRIAE_CS42_3AL_TGACv1_197036_AA0664480	0.19	down	(ABF) ABA responsive element binding factor
	TRIAE_CS42_5BL_TGACv1_404247_AA1292100	0.22	down	(BKI1) BRI1 kinase inhibitor 1
	TRIAE_CS42_3DL_TGACv1_250531_AA0869810	0.48	down	(NPR1) regulatory protein NPR1
Starch and sucrose metabolism	TRIAE_CS42_2AS_TGACv1_114089_AA0363940	10.52	up	(otsB) trehalose 7-phosphate phosphatase
	TRIAE_CS42_2DS_TGACv1_178535_AA0597240	4.84	up	(otsB) trehalose 8-phosphate phosphatase
	TRIAE_CS42_1AL_TGACv1_003899_AA0051890	2.29	up	(TREH, treA, treF) alpha,alpha-trehalase
	TRIAE_CS42_1DL_TGACv1_061138_AA0186610	2.03	up	(TREH, treA, treF) alpha,alpha-trehalase
	TRIAE_CS42_3DL_TGACv1_249164_AA0840030	2.79	up	(scrK) fructokinase
	TRIAE_CS42_7DS_TGACv1_624145_AA2059200	Inf	up	(glgA) starch synthase
	TRIAE_CS42_4DS_TGACv1_361541_AA1169860	0.31	down	sucrose-phosphate synthase
	TRIAE_CS42_6DL_TGACv1_526359_AA1680390	0.08	down	(AMY, amyA, malS) alpha-amylase
	TRIAE_CS42_2DL_TGACv1_158310_AA0515330	0.43	down	(GBE1, glgB) 1,5-alpha-glucan branching enzyme

Figures

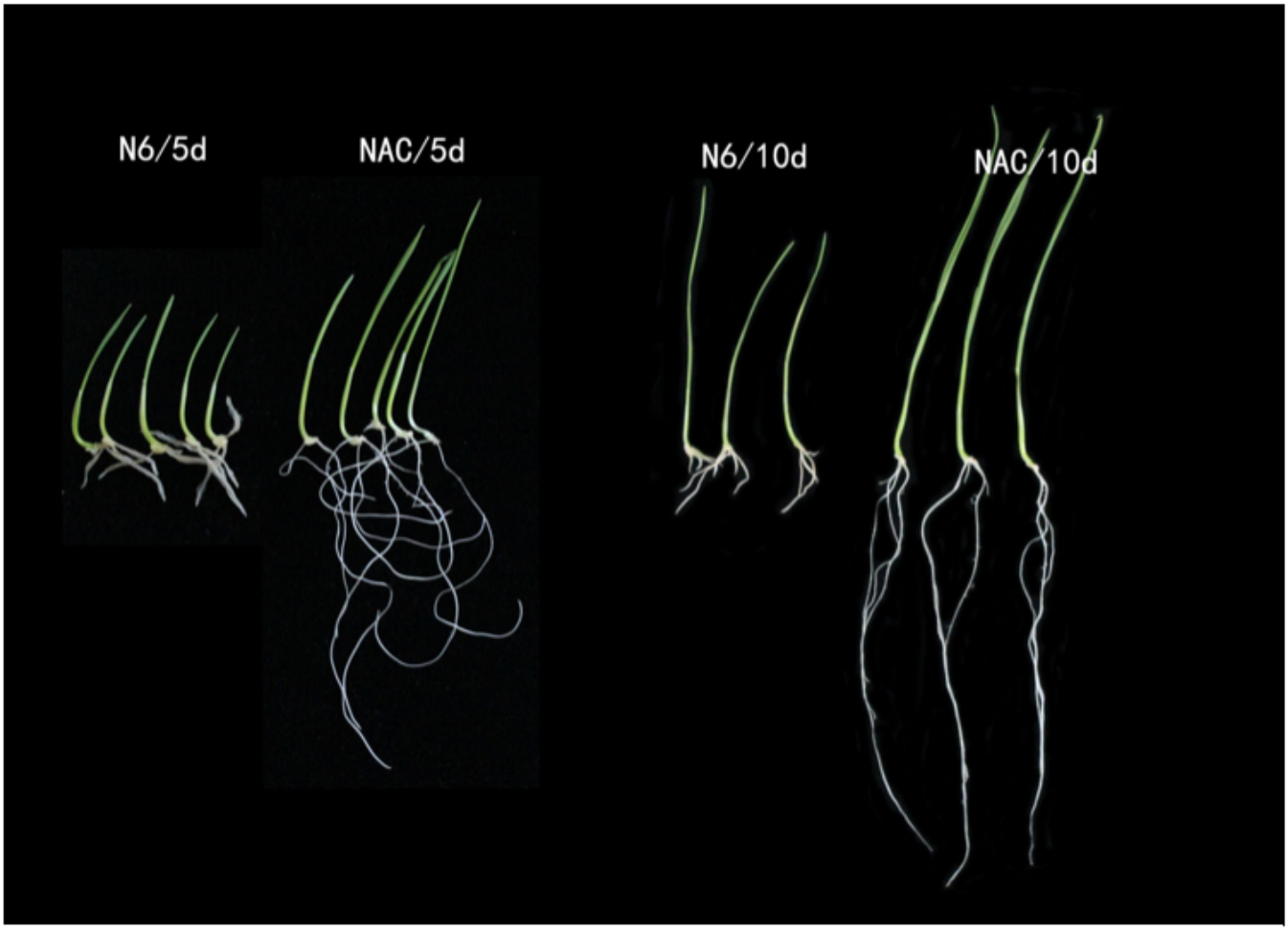

Figure 1

The growth potential of seedlings cultured for 5 and 10 days compared to the control.

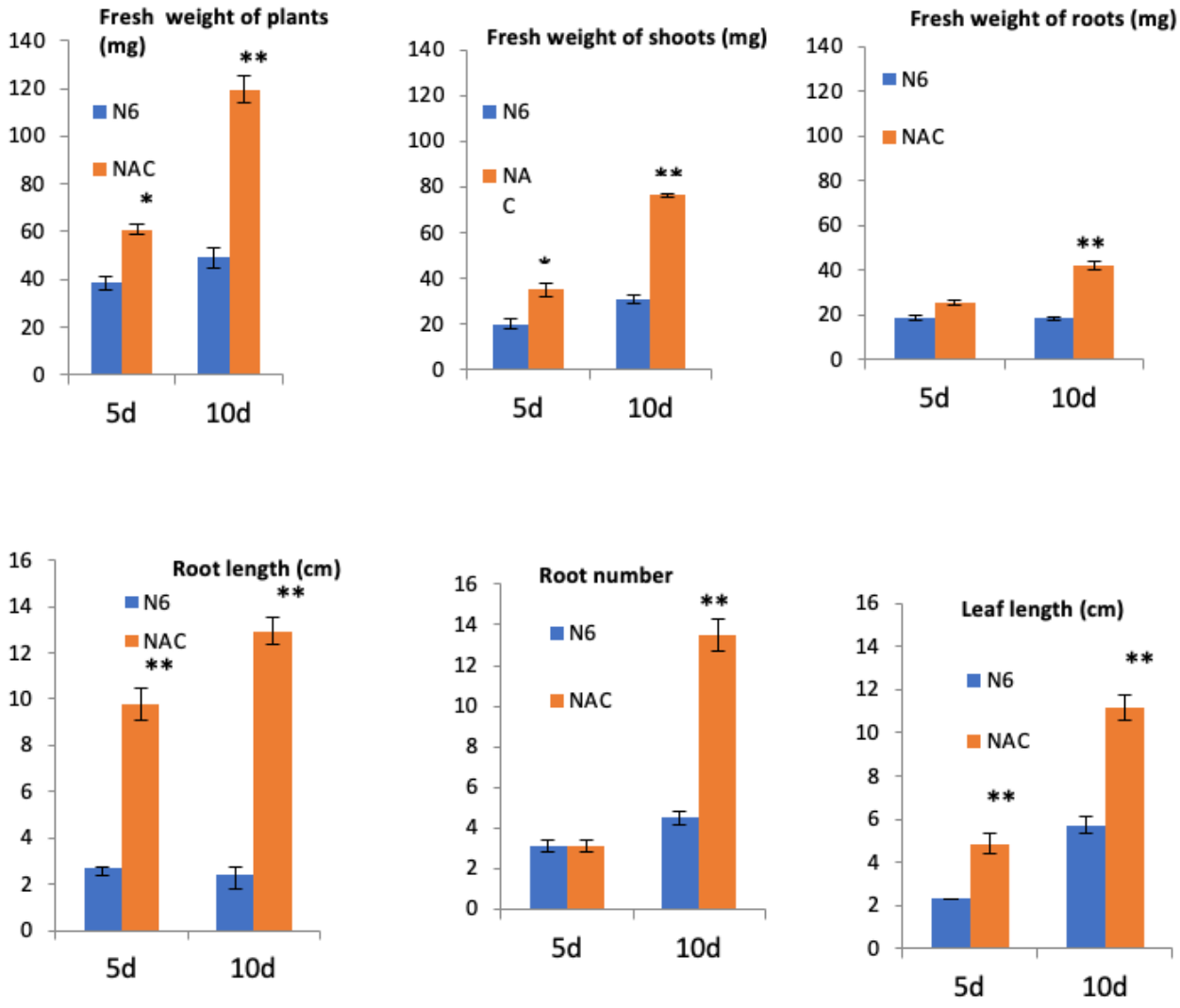

Figure 2

Differences in 5 and 10 d seeding growth potential between NAC and N6 medium with regard to leaf length, root length, leaf number, root number, fresh weight of stem and leaf, and fresh weight of roots (t test,*: $p < 0.05$, **: $p < 0.01$, respectively).

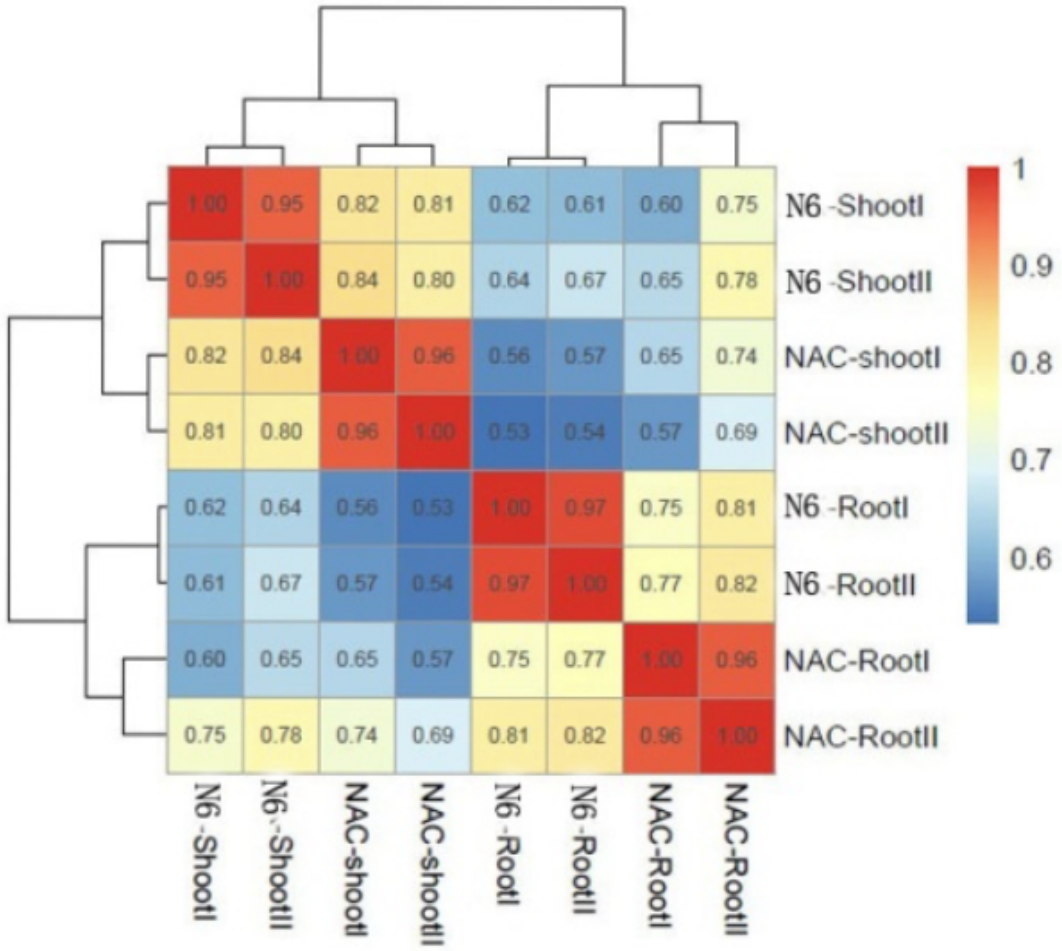

Figure 3

Correlation of sample detection.

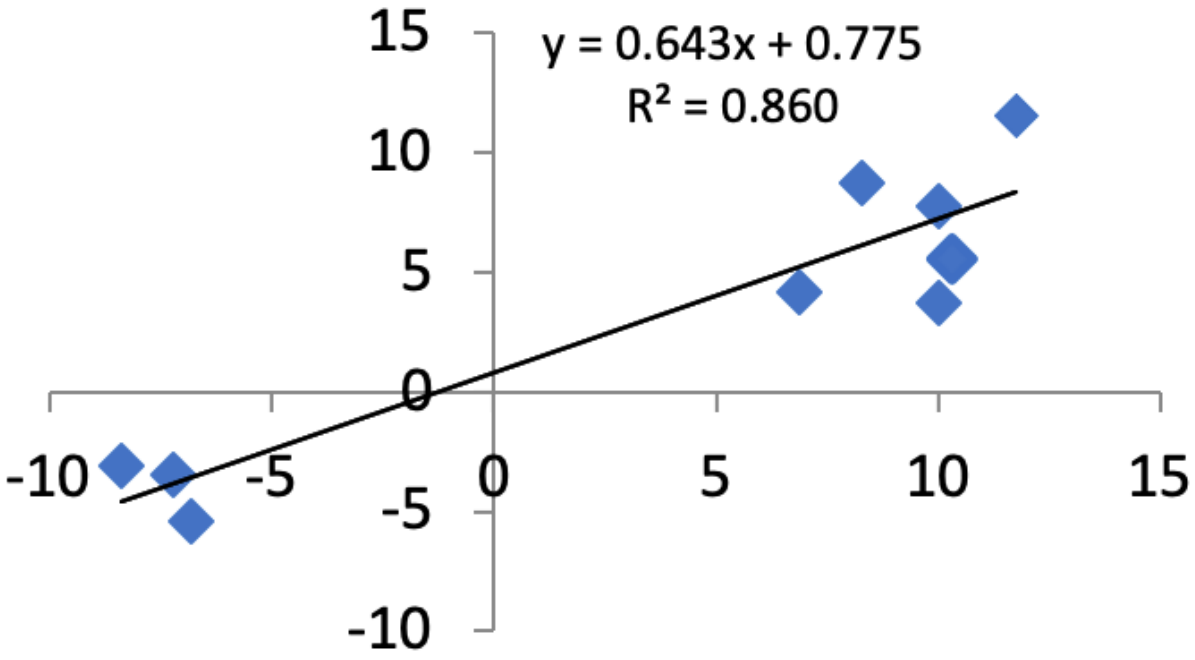

Figure 4

Correlation analysis of DEGs between RNA-seq and qRT-PCR. Scatter plot indicates the log2 transformed gene expression values in RNA-seq and qRT-PCR.

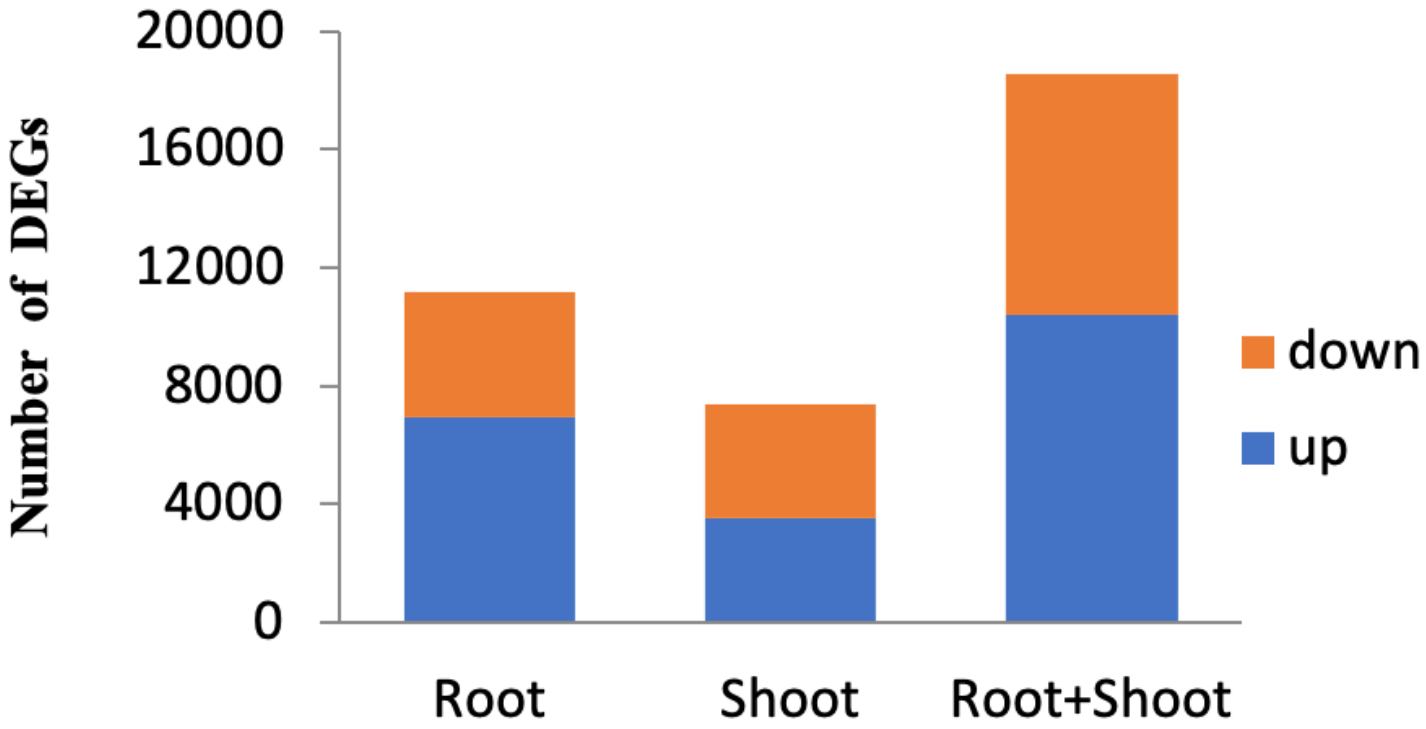

Figure 5

DEGs in wheat roots and shoots under added AC conditions compared to the control condition.

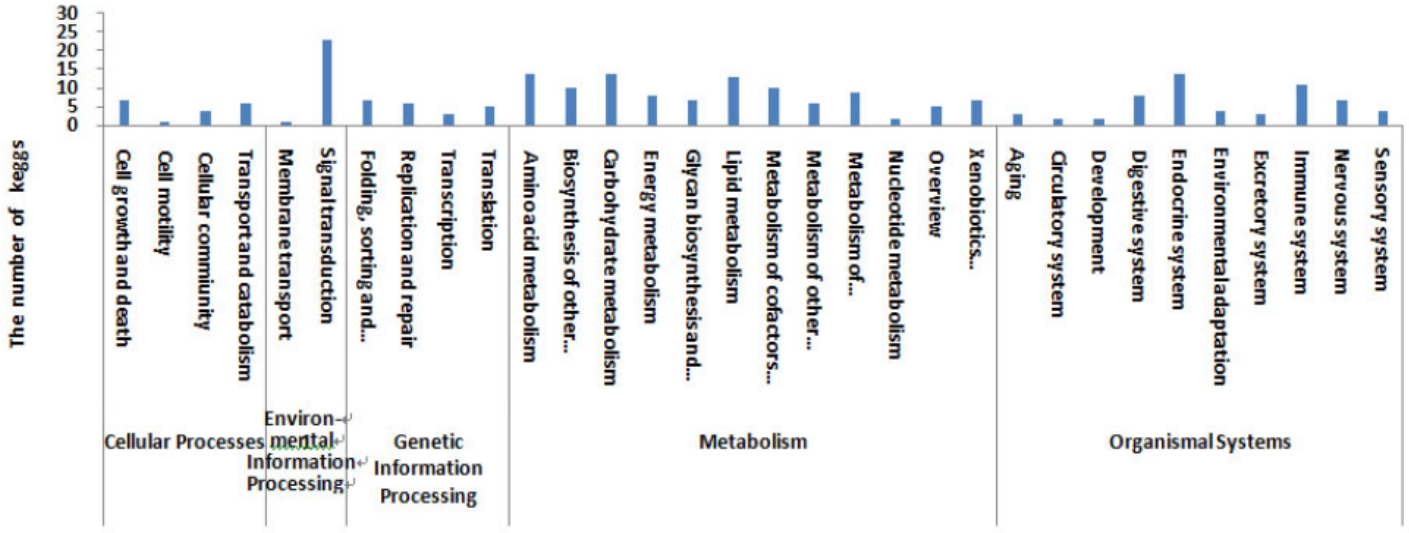

Figure 6

The major pathways under AC by KEGG enrichment analysis.