Transcriptome analysis reveals the mechanism of improving erect-plant-type peanut yield by single-seeding precision sowing

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Background: Double-seed sowing (i.e., sowing two seeds per hole) is the conventional pattern of erect-plant-type peanut sowing in China used to ensure high germination rates, which can be limited by poor seed preservation conditions. However, within-hole plant competition usually limits subsequent growth and yield formation. Accordingly, we developed a high-yield cultivation system of single-seed precision sowing that can save 20% of seeds and increase yields by more than 10% relative to conventional double-seed sowing. Methods: In order to explore the mechanisms of these two different cropping patterns in peanut yields, transcriptomic and physiological comparisons between single-seed precision sowing and standard double-seed sowing treatments were conducted. **Results:**After assembly, each library contained an average of 43 million reads, and a total of 523,800,338 clean reads were generated. By combining GO with KEGG pathway analysis, key genes for biotic and abiotic stress, such as genes encoding disease resistance, oxidation–reduction, hormone related, and stress response transcription factors and signaling regulation proteins showed high expression in roots of plants grown under the single-seed precision sowing treatment. In particular, the resveratrol synthesis genes related to stress and disease resistance induced in roots under the single-seed sowing treatment. **Conclusion:** These data indicated that *Aspergillus flavus* resistance and stress tolerance in roots under single-seed precision sowing were enhanced compared with roots under the double-seed sowing treatment. This work benefits the development of underground pods and thus increasing peanut yields.

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Abstract

Background: Double-seed sowing (i.e., sowing two seeds per hole) is the conventional pattern of erect-plant-type peanut sowing in China used to ensure high germination rates, which can be limited by poor seed preservation conditions. However, within-hole plant competition usually limits subsequent growth and yield formation. Accordingly, we developed a high-yield cultivation system of single-seed precision sowing that can save 20% of seeds and increase yields by more than 10% relative to conventional double-seed sowing.

Methods: In order to explore the mechanisms of these two different cropping patterns in peanut yields, transcriptomic and physiological comparisons between single-seed precision sowing and standard double-seed sowing treatments were conducted.

Results: After assembly, each library contained an average of 43 million reads, and a total of 523,800,338 clean reads were generated. By combining GO with KEGG pathway analysis, key genes for biotic and abiotic stress, such as genes encoding disease resistance, oxidation–reduction, hormone related, and stress response transcription factors and signaling regulation proteins showed high expression in roots of plants grown under the single-seed precision sowing treatment. In particular, the resveratrol synthesis genes related to stress and disease resistance appeared induced in roots under the single-seed sowing treatment.

Conclusion: These data indicated that *Aspergillus flavus* resistance and stress tolerance in roots under single-seed precision sowing were enhanced compared with roots under the double-seed sowing treatment. This work benefits the development of underground pods and thus increasing peanut yields.

Keywords: Peanut (*Arachis hypogaea* L.), RNA-Seq, Stress tolerance, Resveratrol synthase,

Yield

Introduction

Before 1960s, agricultural production in China was labor intensive due to the lack of modern farming conditions. Thus, erect-plant-type peanuts, which are easier for manual labor relative to prostrate-plant-type peanuts, were successfully bred and cultivated widely in China. Because the seed germination rate of peanut is usually unable to meet the necessary high field emergence percentage owing to uneven seed quality, conventionally, the sowing patterns with double-seed (DS) and multi-seed in one hole were widely adopted by farmers as the main planting patterns, which generally leads to two issues: a requirement of a large number of seeds and poor population quality. The total amount of peanut seeds per year is 1.5 million tons, accounting for about 10% of the total peanut yield per year of the country. Poor population quality is mainly attributed to increased competition under double-seed sowing, which hinders the increases of peanut yield (*Zhang et al., 2020*).

Our team introduced the principle of competitive exclusion, and proposed the technical idea of "single-seed (SS) precision sowing of erect-plant-type peanuts, robust individual, optimized population", and created a high-yield cultivation technique of single-seed precision sowing. SS precision sowing can alleviate inter-plant competition, save 20% of seeds used for planting, and increase yields by more than 10% in common fields relative to DS precision sowing (*Liang et al., 2019*). However, the molecular mechanisms regulating individual development under SS are still unknown.

Crop yield is usually affected by biotic and abiotic stress. In recent years, biotic stress research

has mainly focused on disease resistance in wheat, rice, maize, and soybean. It has been reported that *Fhb1* was associated with resistance to wheat scab and has important breeding potential (*Rawat et al., 2016*). Two grape stilbene synthase (STS) genes, *VST1* and *VST2*, were transformed into tobacco for the first time, which improved the resistance of tobacco to *Botrytis cinerea* (*Hain et al., 1993*). "MutRenSeq", a new efficient method for the discovery of disease-resistant genes in crops was also proposed by a British research team (*Steuernagel et al., 2016*). These advances lay a solid foundation for the further study of crop disease resistance.

Aflatoxin contamination caused by *Aspergillus flavus* is a major obstacle to the development of peanuts (*Fajardo et al., 1994*). It was indicated that the resveratrol content could be greatly induced when *Aspergillus flavus* infects the seeds of peanut, and resistance of peanut to *Aspergillus flavus* infection can be conferred by increasing the content of resveratrol in peanut seeds as well as the speed of its synthesis (*Tian et al., 2008*). Resveratrol (Res), known as 3,4,5-trihydroxy-stilbene, was first isolated from *Veratrum grandiflorum* root in 1940 (*Takaoka 1940*). At present, Res has been identified in many different plant species, and current research is mainly focusing on grapes (*Wang et al., 2010*), peanuts (*Tang et al., 2010*), and a few other species. Furthermore, there are also differences among plant tissues; for example, grape pericarps are the main grape tissue in which the compound is synthesized, while the content of Res in peanut pericarps is higher than that in peanut kernels (*Sanders et al., 2000*). Accumulat of Res, a non-flavonoid polyphenol, could improve abiotic stress resistance. Although there has remained query of the specific mechanisms of anti-stress abilities of Res, a recent report showed that Res resistance to ROS is one aspect involved in the stress resistant processes (*Zheng et al., 2011*).

Abiotic stresses, including drought, salt, and extreme temperature, remain the most challenging frontier in the field of plant abiotic interaction research, as the search continues for primary sensors and important stress-resistant genes that respond to abiotic stresses (*Zhu, 2016*). Significant progress has been made in the past few years. The cold tolerant rice protein COLD1 has been identified to mediate extracellular Ca^{2+} influx and net cytosolic Ca^{2+} concentration in response to chilling stress (*Ma et al., 2015*). The basic leucine zipper bZIP and NAC

transcription factors may sense or contribute tolerance to salt stress through their interaction with corresponding proteins (*Liu et al., 2016*). The chloroplast is the main site of the production of reactive oxygen species (ROS), including hydrogen peroxide, superoxide anions, singlet oxygen, and hydroxyl radicals (*Mignolet-Spruyt et al., 2016*). Various abiotic stresses, particularly high light stress, aggravate ROS production, which destroys ROS-scavenging systems and generates various secondary messengers. The protective mechanisms that scavenge ROS in plants can be divided into two categories: enzymatic (e.g., superoxide dismutase [SOD], catalase [CAT], and peroxidase [POD]) and non-enzymatic (e.g., glutathione, mannitol, and flavonoids). Improving the content of these substances in plants by genetic engineering can effectively remove excessive ROS in plants and thus improve stress tolerance. Whether the yield advantage of SS precision sowing is related to the stress resistance genes remains to be studied.

With the development of high-throughput sequencing technologies, more attention is being paid to combining genomic methods such as genome sequencing with transcriptome, proteome, and metabolome analyses in order to reveal the molecular mechanism of life science research. This integrative research offers synergies that can be used to uncover the molecular mechanism of different phenomena in crop production. The publication of reference transcripts for *Arachis_duranensis* (*Chopra et al., 2014*) facilitated a better understanding of agronomically important phenomena and genetic improvement of peanut. In this study, we will also use these new techniques to reveal the mechanism of high yield of SS precision sowing.

Materials & Methods

Plant material and growth condition

Peanut (*Arachis hypogaea* L.) cultivar ‘Huayu 22’ (provided by the Shandong Peanut Research Institute, China), a large-grain peanut cultivar, was used in this study. Peanut seeds were cultivated under field pot conditions at the Shandong Academy of Agricultural Sciences Station (117°5′ E, 36°43′ N), Ji’nan, China. The soil is sandy loam, containing 1.1% organic matter (W/W), 82.7 mg kg^{-1} alkali-hydrolyzed nitrogen, 36.2 mg kg^{-1} available phosphorus, 94.5 mg kg^{-1} available potassium, and 14.9 g kg^{-1} exchangeable calcium. The single-seed (SS) sowing

treatment consisted of 237,000 holes per hectare with 1 grain per hole, while the double-seed (DS) sowing consisted of 138,000 holes per hectare with two grains per hole as the control; the distances between two adjacent holes were 10.5 cm and 18 cm in the SS and DS treatments, respectively. Three biological replicates were used in this study. The mixed tissues were used in transcriptome sequencing.

RNA extraction and cDNA library construction

When the peanut plants reached the seedling stage, the top third leaf and the whole root systems of peanut plants were collected for RNA extraction and library construction. Total RNA was isolated from the leaves and roots respectively using the total RNAiso Reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. The quality and purity of RNA samples were detected using the Agilent 2100 Bioanalyzer platform and Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, CA, USA). Qualified RNA samples were used to isolate mRNA with the oligo (dT) method, and the mRNAs were fragmented. Then, first strand cDNA and second strand cDNA were synthesized. Next, the purified cDNA fragments were linked with adapters, and sequencing was performed by a commercial service provider (BGI Tech, Shenzhen, China). The sequenced reads that contained adaptor sequences, low-quality bases, and high contents of unknown bases (i.e., N calls) were removed before downstream analyses. After read filtering, clean reads were mapped to the *Arachis_duranensis* reference genome (BioSample: SAMN02982871, BioProject: PRJNA258023) using Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT). Then, Genome Analysis Toolkit (GATK) was used to call single SNPs and INDELs (*McKenna et al., 2010*), and RNA-Seq by Expectation-Maximization (RSEM) was used to calculate gene expression levels for each sample, which were then determined using the fragments per kb per million (FPKM) mapped fragments method developed by Li et al. (2011) (*Li et al., 2011*). DEGseq algorithms was used to assess the DEGs. Based on gene expression level, DEG was identified between samples or groups. Based on these DEGs, Gene Ontology (GO) was performed classification and Kyoto Encyclopedia of Genes and

Genomes (KEGG) pathway classification. The GO framework includes three ontologies: molecular biological function, cellular component, and biological process. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies. Coexpression analysis was performed using the WGCNA R package according to the methods detailed by previously published studies (*Gao et al., 2018; Song et al., 2018*).

Yield composition per plant and measurement

At maturity, 10 representative plants were selected to investigate the number of pods, full pods, and double kernels as well as pod weight per plant. The pods and plants were dried to constant weights, and the economic coefficient was calculated as follows: economic coefficient = pod dry weight / (plant dry weight + pod dry weight).

Antioxidant enzyme activities and root activity

Leaf tissues (0.5 g) were ground up with phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 1% (g ml^{-1}) PVP and centrifuged at 4°C for 10 min. Then, the supernatants were used as enzyme extracts. The nitroblue tetrazole (NBT) method was used to detect SOD activity. The absorbance value was determined at a 560 mm wavelength after the reaction; 50% inhibition of NBT reduction was regarded as the unit of enzyme activity (U), expressed as U g^{-1} FW. The guaiacol method was used to determine the POD activity; the increase in OD_{470} per min was used as the unit of enzyme activity (U), expressed as $\Delta 470\ g^{-1}$. CAT activity was also determined by the guaiacol method. The decreased in OD_{240} per min was used as the unit of enzyme activity (U) and expressed as $mg \cdot g^{-1} \cdot min^{-1}$ (*Giannopolitis et al., 1977; Aebi et al., 1984; Jimenez et al., 1977*). Root activity was measured by the TTC method; 0.5 g of root tip samples were revolved in a mixture of 0.4% TTC and phosphoric acid buffer solution and kept in the dark for 1–3 h at 37°C. After that, 2 ml of 1 mol L^{-1} sulfuric acid was added to stop the reaction. Simultaneously, a blank

experiment was conducted. The root samples and sulfuric acid were added first, and 10 min later, the other reagents were added. The operation was the same as that described above. The roots were removed, dried, and ground together with 3–4 ml or quartz sand and ethyl acetate in a mortar. The supernatants were transferred to a test tube, and the residue was washed with a small amount of ethyl acetate two or three times, with all ethyl acetate collected into the test tube. Finally, ethyl acetate was added to bring the total volume up to 10 ml. Blank experiments were used as the control, and the OD value of the reaction solution was measured at $\lambda = 485$ nm.

qRT-PCR verification analysis

Leaves and roots of plants grown under the SS and DS treatments, as in the RNA-seq experiment, were selected randomly and analyzed using qRT-PCR. The qRT-PCR amplification instrument (ABI 7500 fast; Applied Biosystems, Foster City, CA, USA) was used to amplify the related genes with SYBR Premix Ex Taq™ (TaKaRa) following the manufacturer's instructions (*Yang et al., 2013*). The relative gene expression was calculated using the $2^{-\triangle\triangle CT}$ method as described by Livak and Schmittgen (2001) (*Livak & Schmittgen, 2011*). For $\log_2$-transformed FPKM values, the maximum expression level of each selected gene was considered to be 100, and the expression levels of the other genes were transformed accordingly (*Zhang et al., 2016*).

Determination of resveratrol content

The Res extraction processes were carried out according to the method detailed by Tang et al. (*Tang et al., 2010*) with minor modification; 0.25 g of leaves or roots were collected and ground into a fine powder, which was dissolved with 20 ml of 95% ethanol in a flask and oscillated at room temperature overnight. After centrifugation at 8000 rpm for 10 min, the supernatants were subjected to HPLC analysis. The extracts were filtered through a 0.45-μm membrane and then tested with the Rigol L3000 HPLC system (Rigol Technologies, Beaverton, OR, USA) using a Kromasil C18 reversed phase column (4 μm, 250 mm × 4.6 mm; Kromasil, Bohus, Sweden) at room temperature. Mobile phase A consisted of 0.1% phosphoric acid–H_2O, while phase B

consisted of acetonitrile (A:B = 70:30, flow rate 1 ml min^{-1}); the determination wavelength was 280 nm.

Results

Transcriptome sequencing and gene analysis

The peanut plants grown under different planting patterns differed little in their above-ground portions, while great differences were observed between their root systems. The basis of high and stable crop yields is high biological yields, which depends largely on the development of root systems. The capillary lateral root is the main type of root exhibited under SS, and thus, the capillary lateral root length and its proportion within the root system were promoted accordingly, thereby increasing the total root length and root absorption area of peanut plants. This phenomenon was verified by the mean absorbing area and dry weight of root tissue observed under SS (Fig. 1).

Twelve cDNA libraries were constructed from leaf and root tissues of peanut plants grown under SS and DS sowing treatments. All 12 samples were sequenced on the Illumina HiSeq Platform in total, generating about 6.55 Gb per sample. The reads that contained adaptor sequences, were low-quality, or had high contents of unknown base (i.e., N) calls were removed, resulting in a total of 523,800,338 clean reads that were acquired, with an average of 43 million reads per libraries (Table 1). Approximately 77% and 80% of reads from leaf and root tissues were mapped to the reference genome, and 36,778 genes were identified, of which 34,529 are known genes and 2,322 are novel genes. The RNA-seq sequencing data for the present work has been uploaded in NCBI Sequence Read Archive under BioProjects, PRJNA497502 (SRA: SRP166140). This is the raw data (9 Illumina); where is the assembled data?

Single nucleotide polymorphisms (SNP), which include transitions or transversions of single bases, refer to the difference of a single nucleotide (A, T, C or G) between homologous DNA sequences, a key element of the diversity of genomes among species or individuals. There were more transitions than transversions among all the samples. Among SNPs, transition, transversion, A-G and C-T were the most abundant terms (Fig. S1). Insertion-Deletion (INDEL) refers to the

insertion or deletion of the small fragments (one or more, less than 50 bp) that occur in a sample relative to the reference genome. Most INDELs occurred in exonic and intronic sequences, with the proportion differing among samples (Fig. S2). Based on the results of SNP, INDEL and gene expression, our study was presented in the form of a ring diagram with Circos software (Fig. S3).

Differentially expressed gene detection

A total of 2,567 and 2,706 differentially expressed genes (DEGs) were identified from leaves and roots, respectively. Under SS sowing treatment 544 and 1,771 genes were expressed at a higher level, while 2.023 and 935 genes were expressed at lower levels compared with the DS sowing treatment in leaves and roots, respectively (Fig. 2). The expression patterns of the majority of DEGs differed between roots and leaves. For example, only 83 (15.2%) of the 1,771 genes up-regulated in roots were also up-regulated in leaves (Fig. 3). Notably, some genes in roots and leaves even showed opposite expression patterns; for example, forty DEGs were up-regulated in leaves, but down-regulated in roots. However, 245 DEGs in roots and leaves had the same trends in expression, including 83 up-regulated DEGs and 162 down-regulated DEGs (Fig. 3).

qRT-PCR verification of RNA-Seq results

To verify the RNA-seq data, quantitative real-time PCR (qRT-PCR) was used to test the expression of 24 genes with different functional assignments (Fig. 4a, 4b). Among them, six and five genes were up-regulated under the SS sowing treatment in leaves and roots, respectively. UDP-glycosyltransferase, chalcone synthase, and GPI mannosyltransferase were each encoded by one of these genes, while two genes encoding hypothetical proteins were also used for qRT-PCR detection. The other eleven selected genes were downregulated both in roots and leaves. These genes included one phospholipase, one seed linoleate 9S-lipoxygenase, one zinc finger SWIM domain-containing protein, and one pectinesterase. The RNA-seq data were consistent with the qRT-PCR results for these 24 genes. The correlation between the relative expression ($\log_2$ SS/DS) estimated by RNA-seq and the qRT-PCR results were rather high in leaves (R^2 = 0.9772) and a little lower in roots (R^2 = 0.8884; Fig. 4c,4d).

298

299 **Functional analysis of DEGs**

300 Blast2GO was used to perform Gene Ontology (GO) classification and functional enrichment
301 (*Conesa et al., 2005*) on the identified DEGs. The sequences were categorized into 45 functional
302 groups according to sequence homology. Then, the main categories of biological process,
303 molecular function, and cellular component were visualized employing WEGO (Fig. 5). In the
304 biological process category, "metabolic process," "single-organism process," and "cellular
305 process" terms were enriched, suggesting metabolic activity is higher under SS sowing. In the
306 cellular component category, "cell," "cell part," and "membrane" were the most abundant terms.
307 The most enriched category was molecular function, with enrichment for "binding," "catalytic
308 activity," and "transporter activity" in particular, suggesting a high level of metabolic activity
309 changes under SS sowing (Fig. 5).

310 KEGG pathway classification was performed to acquire biological information for
311 understanding the regulatory networks and molecular mechanisms associated with the SS sowing
312 treatment. DEGs were mainly enriched in MAPK signaling pathway, glycerolipid metabolism,
313 phenylalanine metabolism, sphingolipid metabolism, isoflavonoid biosynthesis, flavonoid
314 biosynthesis, and tryptophan metabolism in leaves. Meanwhile, phenylpropanoid biosynthesis,
315 biosynthesis of secondary metabolites, MAPK signaling pathway, flavonoid biosynthesis, zeatin
316 biosynthesis, and flavone and flavonol biosynthesis were mainly enriched among DEGs in roots
317 (Fig. S4). Interestingly, all of these pathways participated in biosynthesis associated with
318 particular metabolic processes, suggesting that these processes were activated. Weighted gene
319 correlation network analysis (WGCNA) is a systematic biological method for describing the
320 pattern of gene association among different samples. Information about nearly 10,000 genes,
321 corresponding to the genes with the greatest changes in expression, was used to identify gene
322 sets of interest and to analyze associations with phenotypes. In our study, the genes from all the
323 samples were divided into modules for analysis, with twelve colors representing each of the
324 different modules in Fig. 6.

325

326 **Changes of resveratrol synthesis related pathway between cultivation techniques**

327 In our study, a total of 20 resveratrol synthesis-related genes were dramatically induced while no
328 resveratrol synthesis-related genes were down-regulated in the roots under the SS sowing
329 treatment. In contrast, a total of 10 DEGs related to resveratrol synthesis were down-regulated in
330 the leaves (Table 2). We also measured the resveratrol content, and the results were in agreement
331 with the gene expression levels in leaf and root tissues (Fig. 7). Accordingly, compared with the
332 roots of plants grown under DS sowing, the higher resveratrol content under SS precision sowing
333 appeared to support initiation of the defense response against toxins produced by *Aspergillus*
334 *flavus*; pathogenesis-related protein, transcription factors (e.g., WRKY, bZIP, ERF), and 4CL
335 were up-regulated (Table 3).

336

337 **Key genes related to stress tolerance under SS**

338 Transcription factors (TFs), which bind to the *cis*-elements upstream of promoters, have been
339 reported to orchestrate abiotic stress responses (*Joshi et al., 2016*). Under the SS 6 precision
340 sowing treatment, the higher expression of these TF genes (WRKY, MYB, bZIP, ERF) may
341 improve stress tolerance compared with that observed in the DS treatment. In plants, redox
342 processes also play an important role in stress tolerance. Several oxidoreductase genes encoding
343 ascorbate oxidase, glutaredoxin, and cytochrome P450 monooxygenases (CYP) exhibited
344 increased expression in roots under SS relative to DS. Specifically, compared with DS, about
345 thirty genes encoding CYP were up-regulated under SS (Table 3). In addition, four genes
346 encoding L-ascorbate oxidase homologs, which take part in ascorbate recycling, were enriched in
347 the SS sowing treatment. In plants, ascorbate contributes to improving tolerance against various
348 stresses by regulating the levels of cellular H_2O_2 (*Ishikawa et al., 2008*). Higher enzyme
349 activities of POD, SOD, and CAT were also observed in roots under SS compared to that under
350 DS (Table S1). Four genes encoding glutaredoxin, which might promote reducing disulfide
351 bridges, were slightly induced under SS compared with DS (Table 3).

352

353 **Peanut pod yields and associated genes under the SS sowing pattern**

354 The number of pods and pod weight per plant are the direct factors affecting peanut yield. In this
355 study, yield estimation indicated that the number of pods, full pods, and double kernels per plant
356 was higher under SS than under DS. The theoretical pod yield of SS reached 1.225 kg, which
357 was 12.9% higher than that of DS (1.085) (Table 4). Hormones, such as indole-3-acetic acid
358 (IAA), abscisic acid (ABA), gibberellin (GA), and brassinosteroid (BR), play important roles in
359 the regulation of growth and development in plants (*Tian et al., 2017*). Auxin response factor
360 (ARF), a transcription factor involved in auxin and regulating plant root growth and seed
361 development (*Salmon et al., 2008*), showed increased expression levels under SS. Nine genes
362 that participate in auxin and BR biosynthesis were abundantly expressed under SS compared
363 with under DS (Table 3). Two lipoxygenase family genes also had increased expression levels
364 under SS.

365

366 **Discussion**

367 In recent years, with the rapid development of sequencing technology, genomes and
368 transcriptomes have been used to explore the mechanisms mediating various problems in
369 agricultural production. However, few studies have focused on the mechanisms by which single-
370 seed **precision** sowing improves yields in erect-plant-type peanut cultivars. Aerial flowering
371 combined with underground fruit development are special characteristics of peanut plants, thus,
372 the growth and development of root tissues are particularly important for peanut yields. Robust
373 roots are the basis of crop growth and high yields (*Zheng et al., 2013*).

374 Various biotic and abiotic stresses under adverse environmental conditions severely reduce
375 global crop production and food security (*Mengiste et al., 2003*). Peanut is one of the most
376 susceptible crops to *Aspergillus flavus*, and abiotic stresses such as salinity, heat, mechanical
377 damage, and drought, seriously affect the growth and development of peanut crops (*Amin et al.,*
378 *2019*). In our study, families of TFs including Zinc finger protein, MYB, bZIP, ERF, and WRKY

were increased in the SS sowing treatment compared with the DS sowing treatment. Many studies have identified these TFs as being involved in abiotic stress responses (*Wei et al., 2017; Wang et al., 2016; Banerjee et al., 2015*). In addition, secondary messengers, like Ca^{2+} and ROS, trigger signaling proteins downstream, such as calcium-dependent protein kinases (CDPKs), calmodulin (CaM), calcineurin-B-like proteins (CBLs), mitogen-activated protein kinase (MAPK) cascades, and ROS-modulated protein kinases (PKs), and these can also respond to numerous plant development and environmental challenges (*Yang et al., 2019*). Genes encoded calcium-binding protein CML, calcium-binding protein KIC-like, and MAPKKK, which is part of the Ca^{2+} signaling pathway, were expressed at higher levels under SS (Table 3). Genes involved in the MAPK cascade include interlinked MAPK, MAPKK, and MAPKKK, and such cascades play important roles in signal transduction of plant hormones, biotic stresses, and abiotic stresses (*Wang et al., 2017*).

Resveratrol (Res), an important phytoalexin, can be induced by pathogenic bacteria or other stimuli and has strong bactericidal and defensive activity in plants. So far, heterogeneous transformation of Res genes from rice, barley, wheat, tomato, tobacco, and other crops has been reported. All these transgenic crops exhibit improved resistance to diseases such as rice blast (*Stark et al., 1997*), powdery mildew in barley and wheat (*Fettig et al., 1999*), and gray mold in tomato and tobacco (*Hain et al., 1990*). The rate of Res induction in peanut seeds has also been linked to resistance to *Aspergillus flavus* infection (*Fajardo et al., 1994*). Res is synthesized by the phenylalanine metabolic pathway in plants, which includes four key enzymes: cinnamate-4-hydroxylase (C4H), phenylalanine ammonia lyase (PAL), 4-coumarate-CoA ligase (4CL), and STS. STS genes can be divided into two types, one of which is resveratrol synthase (RS), which synthesizes Res using malonyl-CoA and coumaric acid-CoA as substrates, and this type mainly exists in *Arachis hypogaea* and *Vitis vinifera*. The other is pinosylvin synthase (PS), which utilizes malonyl-CoA and cinnamyl-CoA as substrates, and has mainly been identified in *Pinus sylvestris* and *Pinus strobus*. RS is the major rate-limiting enzyme for the synthesis of resveratrol, which is reported to participate in ROS resistance and is a key element in stress resistance

processes (*Chang et al., 2011*). Endogenous or exogenous Res can also reduce ROS content in plants (*Zheng et al., 2015*). These effects of Res are likely to promote disease resistance and antioxidant ability in peanut roots, thus supporting high quality harvests and high yields of peanuts.

In this study, high activities of the enzymes POD, SOD, and CAT in the ROS scavenging pathway were observed in roots of plants grown under SS (Table S1). SOD, as a key element of enzymatic defense systems, catalyzes the disproportionation of radicals $O_2^{\bullet-}$ to O_2 and H_2O_2, the latter of which can then be scavenged by POD or CAT. The elevated activity of antioxidant enzymes can be regarded as an effective mechanism for resisting oxidative stress. The balance of the redox state of plant cells contributes to the improvement of plant resistance to abiotic stress (*Duan et al., 2012*). It has been reported that overexpression of glutaredoxin in tomato can confer drought, oxidation, and salt resistance (*Guo et al., 2010*). These results suggested oxidoreductases are involved in protecting plants from various abiotic stresses through maintaining oxidation–reduction homeostasis and scavenging surplus ROS. CYP catalyzes the biological oxidation of various substrates through the activation of molecular oxygen and acts a pivotal part of stress responses and metabolic processes, exhibited as differential transcription levels under SS and DS treatments (*Xiong et al., 2017*).

Based on previous studies, auxin and BR are known to play important roles in root development but the root tip phenotypes of auxin mutants differ from those of BR mutants (*González-García et al., 2011*), indicating that the auxin and BR pathways acting on the root are not the same. In *Arabidopsis*, the BR encoding gene *AtDWF4* was found to regulate leaf growth by promoting cell expansion (*Hur et al., 2015*), and its overexpression in *Brassica napus* can increase seed production (*Sahni et al., 2016*). Many unigenes encoding auxin and BR biosynthesis-related genes are expressed at a high level under SS. Lipoxygenase, which exhibited different expression levels between SS and DS treatments, is an enzyme that is important to the synthesis of jasmonic acid, which plays a vital role in stored lipid migration during seed germination (*Rahimi et al., 2016*). The up-regulated expression of lipoxygenases under SS is

likely to provide sufficient nutrients essential for pod development. ABA also plays crucial roles in seed dormancy and development (*Chauffour et al., 2019*). These results strongly suggested that the biotic and abiotic stress tolerance in roots of peanut plants grown from single-seed precision sowing were enhanced compared with those under the double-seed sowing treatment. These mechanisms may explain the higher yields achieved under SS **precision** sowing based on patterns at the transcriptional level.

Conclusions

In this study, we performed a comparative transcriptomic analysis of peanut leaves and roots between precision SS sowing and standard DS sowing treatments. Genes involved in resveratrol synthesis were found to be dramatically induced in roots under the SS treatment. Accordingly, a higher content of resveratrol was detected under SS precision sowing. In addition, genes involved in calcium signaling transduction and phytohormone metabolism were identified to be differentially expressed in the roots of SS and DS. Redox process genes and transcription factors, including WRKY, MYB, MADS-box, and zinc finger proteins, were up-regulated in roots of plants grown under the SS precision sowing treatment. These genes participate in resistance to biotic and abiotic stresses, which thus function to protect plants from disease and drought, salinity, and chilling stress. These results may inform breeding to enhance the activity and growth of roots, providing a basis for achieving higher yields in peanuts. To the best of our knowledge, we have provided a novel mechanism by which SS precision sowing improves erect-plant-type peanut yields.

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List of abbreviations

ABA, Abscisic acid; BR, Brassinosteroid; ARF, Auxin response factor; CAT, Catalase; CBLs, Calcineurin-B-like proteins; CDPKs, Calcium-dependent protein kinases; C4H, Cinnamate-4-hydroxylase; CIPK, CBL-interacting protein kinase; 4CL, 4-coumarate-CoA ligase; CYP, Cytochrome P450; DEGs, Differentially expressed genes; FPKM, Fragments per kb per million; GA, Gibberellin; GATK, Genome analysis toolkit; GO, Gene ontology; HISAT, Hierarchical indexing for spliced alignment of transcripts; IAA, Indole-3-acetic acid; INDEL, Insertion-Deletion; MAPK, Mitogen-activated protein kinase; NBT, Nitroblue tetrazole; PAL, Phenylalanine ammonialyase; POD, Peroxidase; PS, Pinosylvin synthase; qRT-PCR, Quantitative real-time PCR; Res, Resveratrol; RSEM, RNA-Seq by expectation-maximization; SNP, Single nucleotide polymorphisms; SOD, Superoxide dismutase; STS, Stilbene synthase; WGCNA, Weighted gene correlation network analysis.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

SW and XL designed the study, SY and JZ carried out most of the experiments and data analysis and wrote part of the manuscript. YG wrote part of the manuscript, and finalized the figures and tables. ZT, FG, and JW performed part of experiments and JM and QW tended the plants. All authors have read and approved the manuscript.

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624 **Figure Legends**

625 Fig. 1 Phenotypical comparisons of peanut in SS and DS sowing treatment. (a) The phenotypes,
626 (b) absorbing area of root in SS and DS, and (c) Dry weight of root in SS and DS.
627 Fig. 2 Scatter plot of DEGs in leaf and root between SS and DS treatments.
628 Fig. 3 Differentially expressed genes analysis in roots and leaves. (a) Venn diagram
629 demonstrated the common and specific differentially expressed genes (DEGs), (b) Heat map
630 demonstrated the expression profile DEGs.
631 Fig. 4 qRT-PCR verification of randomly selected genes. Heatmaps represent the expression of
632 the 22 DEGs in (a) leaves and (b) roots between SS and DS treatments. For each heatmap, the
633 FPKM values increased from green to red. The correlation of the fold change in (c) leaves and (d)
634 roots analyzed by RNA-Seq (x-axis) with data obtained using qRT-PCR (y-axis).
635 Fig. 5 GO annotation among DEGs identified in leaf of SS treatment compared with DS
636 treatment and root from the two different treatments.
637 Fig. 6 Co-expression of DEGs between SS and DS treatments. (a) Hierarchical of 10,000 genes
638 based on topological overlap and visualization of gene modules with assigned colors. Branches
639 in the hierarchical clustering dendrograms correspond to modules. Color-coded module
640 membership is displayed in the colored bars below and to the right of the dendrograms. (b)
641 Network of co-expressed modules. The colored clustering bars directly correspond to the module
642 (color) designation for the clusters of genes.
643 Fig. 7 Determination of resveratrol contents according to the method detailed by Tang (*Tang et*
644 *al., 2010*) in leaf and root between SS and DS treatments.
645

646 **Additional files**

647 **Supplementary Information**

648 **Fig. S1** The SNP variants were summarized for each sample. A-G: The amount of A-G variant

649 type; C-T: The amount of C-T variant type; Transition: The amount of A-G and C-T variant type;
650 A-C: The amount of A-C variant type; A-T: The amount of A-T variant type; C-G: The amount
651 of C-G variant type; G-T: The amount of G-T variant type; Transversion: The amount of A-C,
652 A-T, C-G and G-T variant type.
653 **Fig. S2** The distributions of INDEL location for each sample were analyzed. Up2k means
654 upstream 2,000 bp area of a gene. Down2k means downstream 2,000 bp area of a gene.
655 **Fig. S3** Based on the SNP, INDEL, and gene expression result, Circos were used to perform the
656 analysis.
657 **Fig. S4** Pathway functional enrichment of DEGs. X axis represents enrichment factor. Y axis
658 represents pathway name. The color indicates the q-value (high: white, low: blue), the lower q-
659 value indicates the more significant enrichment. Point size indicates DEG number (The bigger
660 dots refer to larger amount). Rich Factor refers to the value of enrichment factor, which is the
661 quotient of foreground value (the number of DEGs) and background value (total Gene amount).
662 **Table S1** Variety of the activities of SOD, POD, CAT, and TTC in SS and DS sowing treatments,
663 respectively. The data presented are the mean values ± SD of three individual experiments.
664
665
666
667

Figure 1

Phenotypical comparisons of peanut in SS and DS sowing treatment.

(a) The phenotypes, (b) absorbing area of root in SS and DS, and (c) Dry weight of root in SS and DS. Fig. 2 Scatter plot of DEGs in leaf and root between SS and DS treatments.

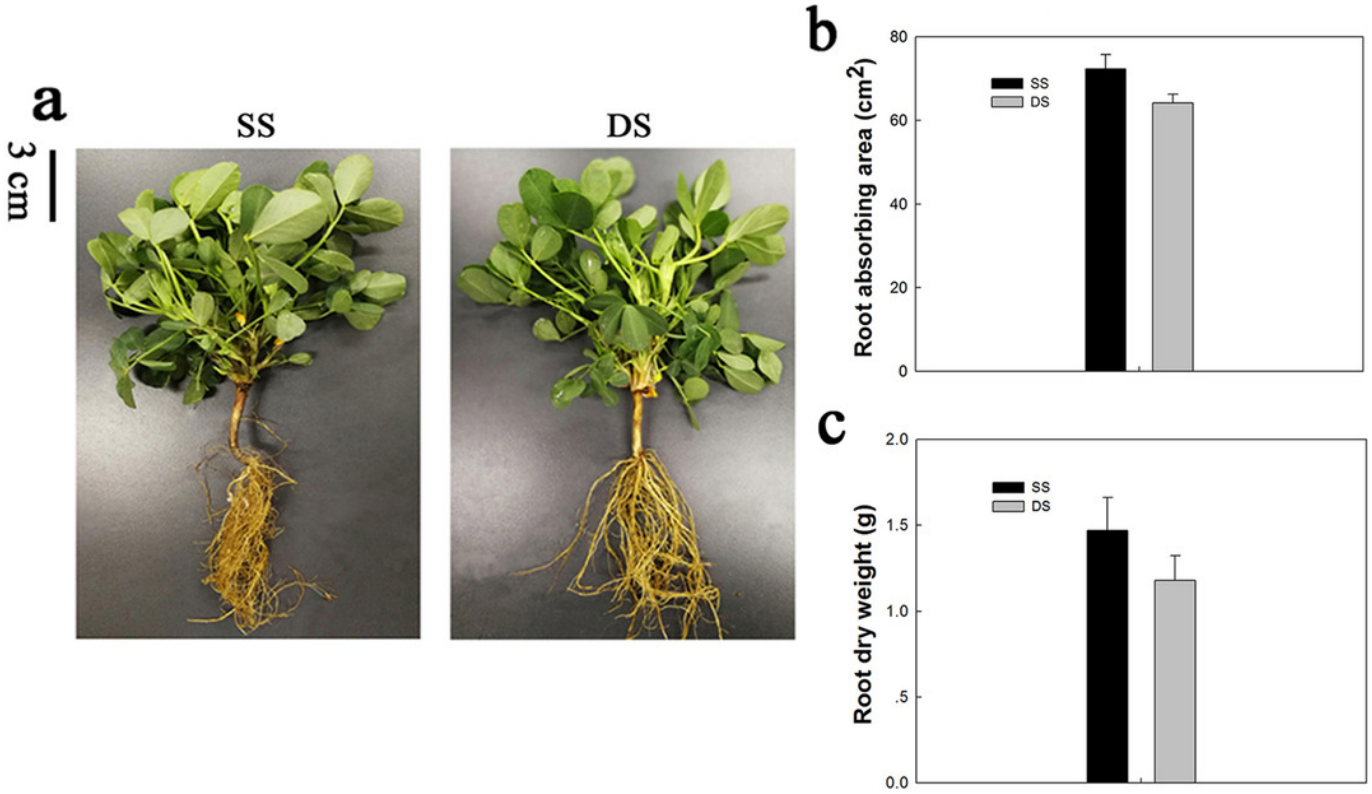


Figure 2

Fig. 2 Scatter plot of DEGs in leaf and root between SS and DS treatments.

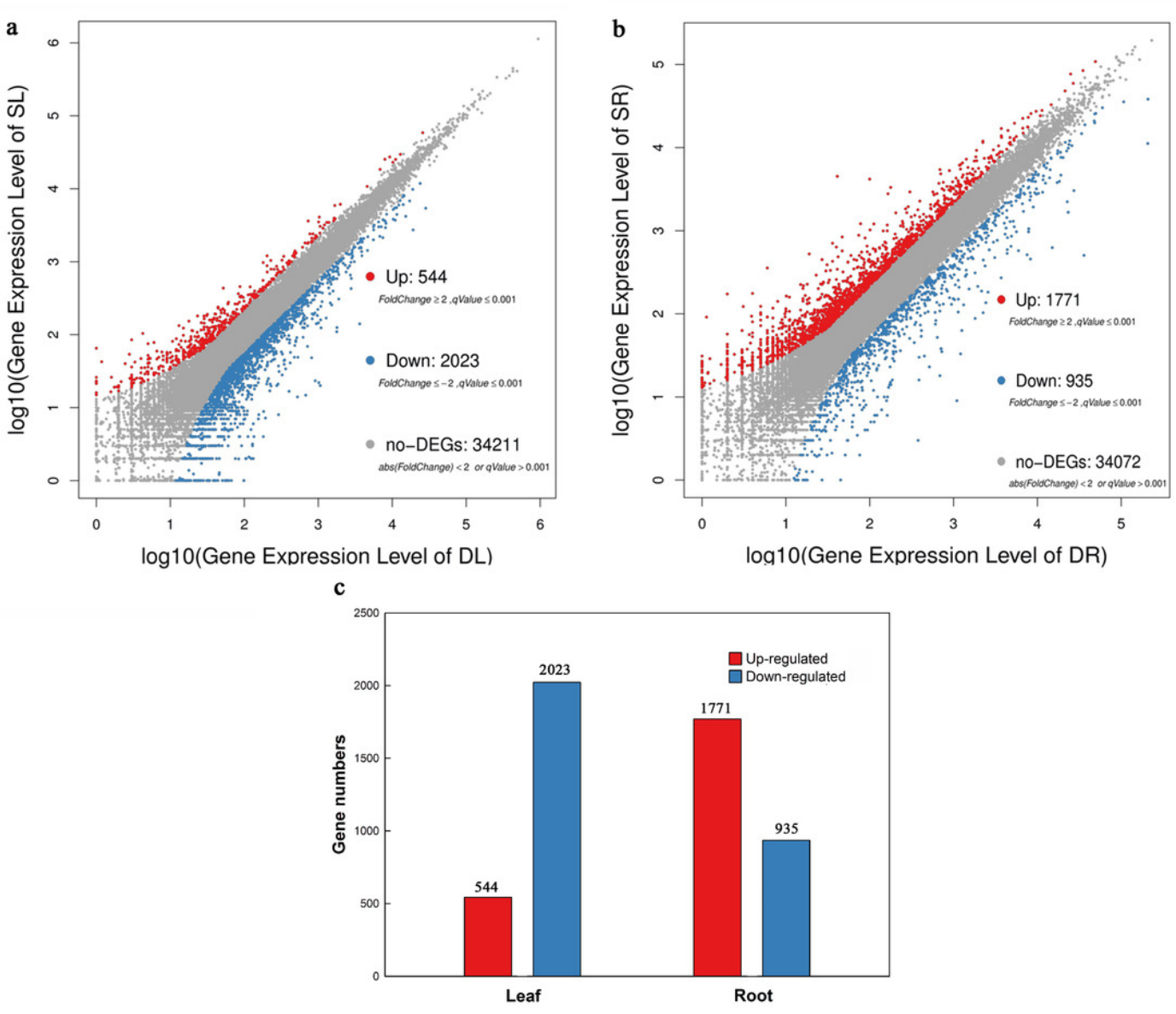


Figure 3

Fig. 3 Differentially expressed genes analysis in roots and leaves.

(a) Venn diagram demonstrated the common and specific differentially expressed genes (DEGs), (b) Heat map demonstrated the expression profile DEGs.

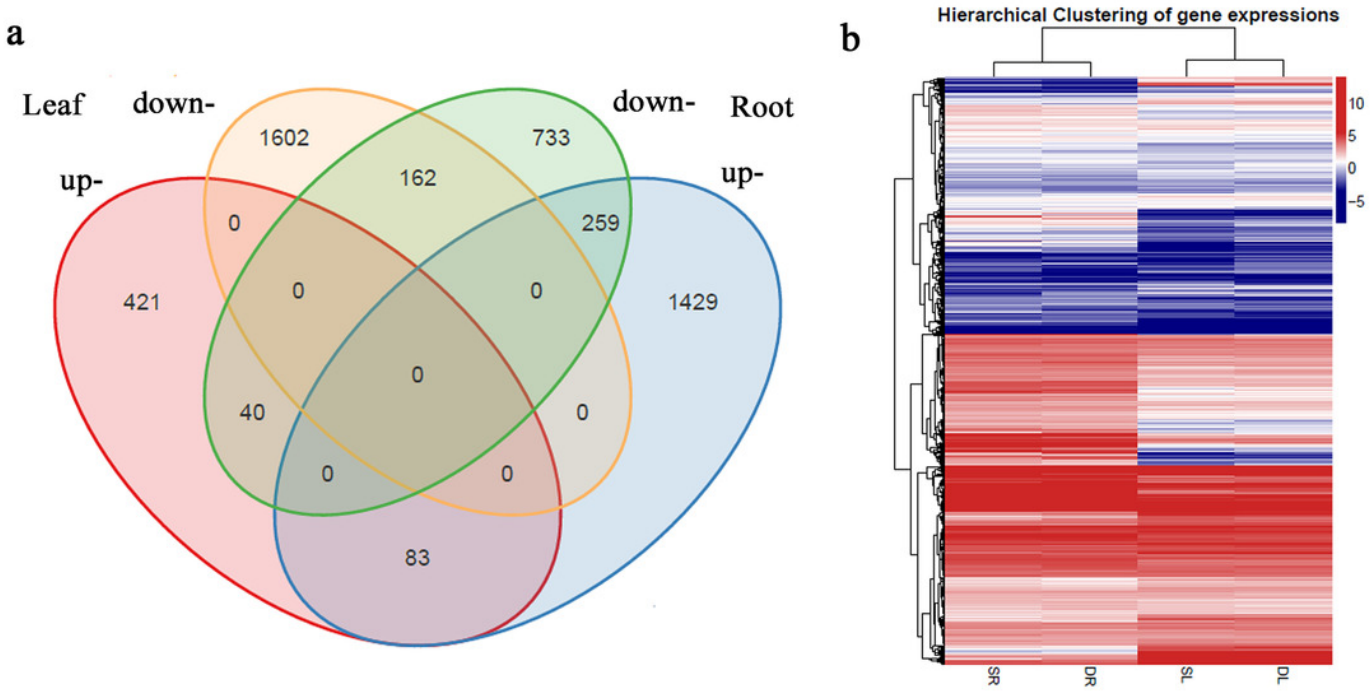
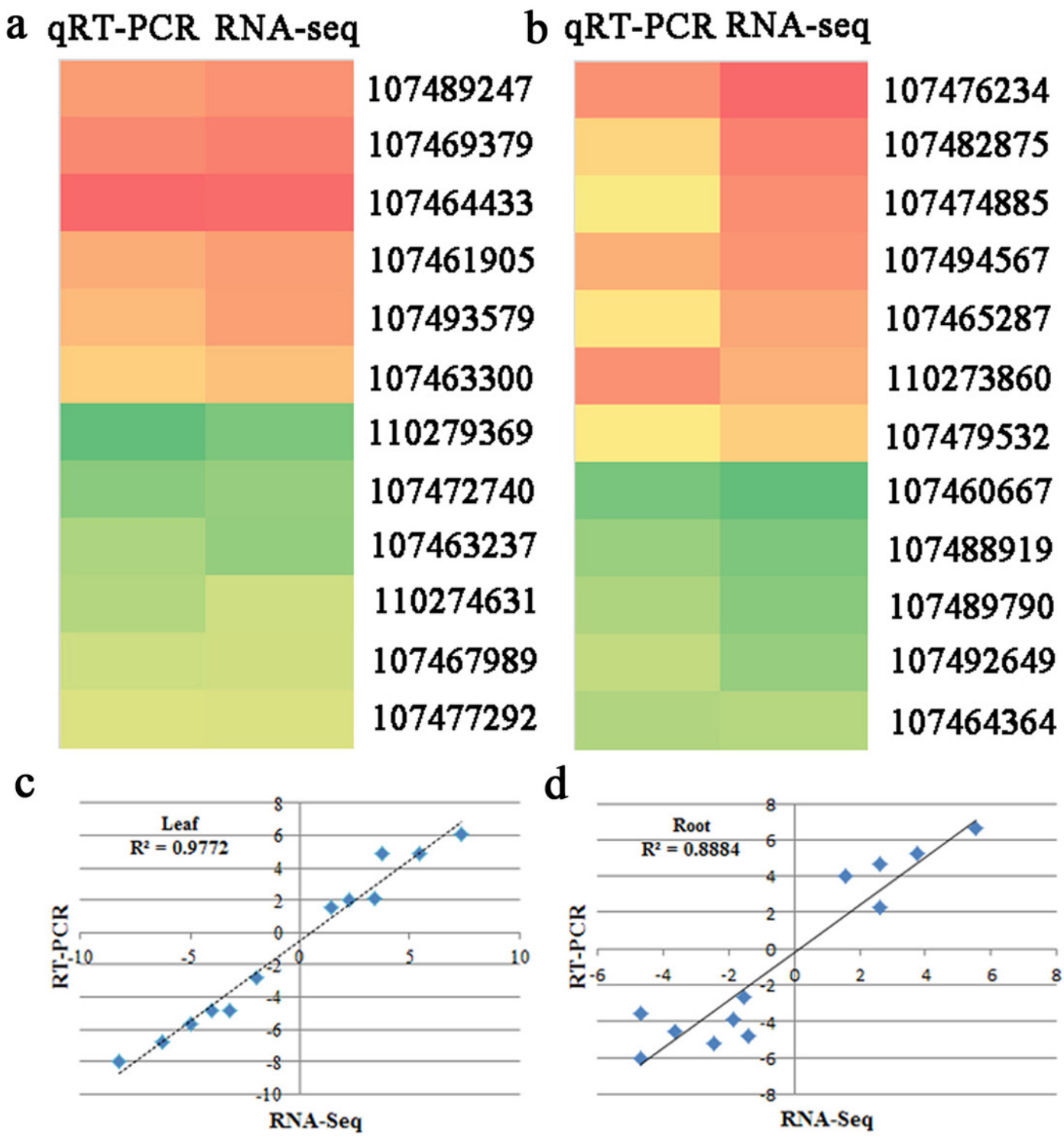


Figure 4

Fig. 4 qRT-PCR verification of randomly selected genes.

Heatmaps represent the expression of the 22 DEGs in (a) leaves and (b) roots between SS and DS treatments. For each heatmap, the FPKM values increased from green to red. The correlation of the fold change in (c) leaves and (d) roots analyzed by RNA-Seq (x-axis) with data obtained using qRT-PCR (y-axis).


a qRT-PCR RNA-seq
107489247
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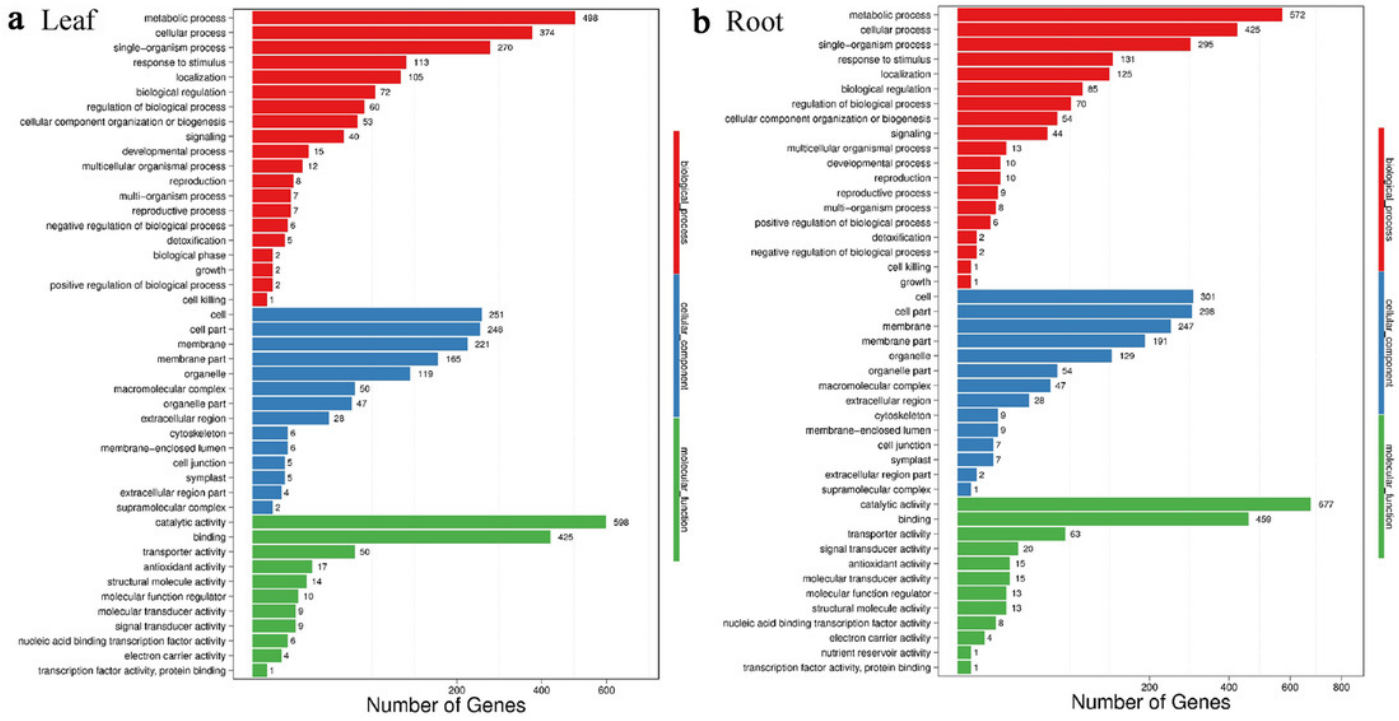
110274631
107467989
107477292
b qRT-PCR RNA-seq
107476234
107482875
107474885
107494567
107465287
110273860
107479532
107460667
107488919
107489790
107492649
107464364
c
Leaf
R² = 0.9772
RT-PCR
RNA-Seq
d
Root
R² = 0.8884
RT-PCR
RNA-Seq

Figure 5

Fig. 5 GO annotation among DEGs identified in leaf of SS treatment compared with DS treatment and root from the two different treatments.



There is no clarity between this figure and the Table S2!

Figure 6

Fig. 6 Co-expression of DEGs between SS and DS treatments.

(a) Hierarchical of 10,000 genes based on topological overlap and visualization of gene modules with assigned colors. Branches in the hierarchical clustering dendrograms correspond to modules. Color-coded module membership is displayed in the colored bars below and to the right of the dendrograms. (b) Network of co-expressed modules. The colored clustering bars directly correspond to the module (color) designation for the clusters of genes.

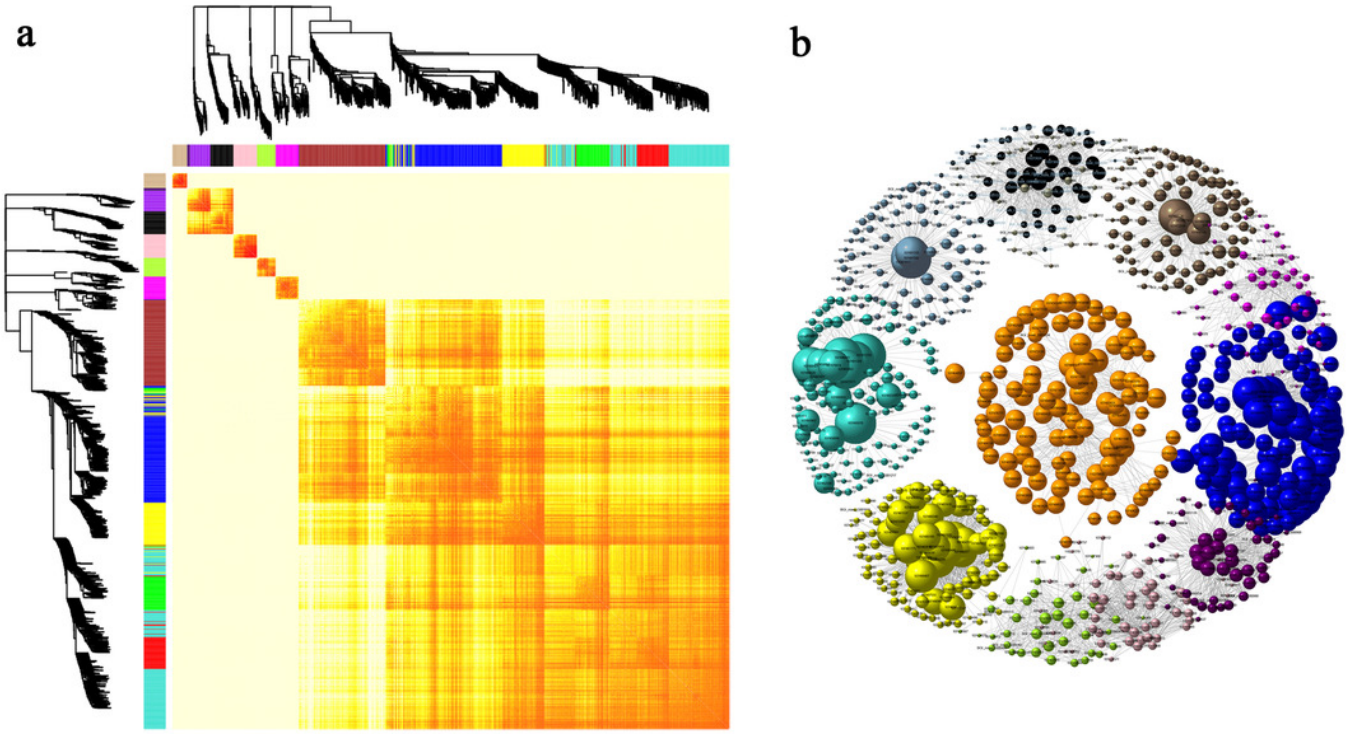


Figure 7

Fig. 7 Determination of resveratrol contents according to the method detailed by Tang (*Tang et al., 2010*) in leaf and root between SS and DS treatments.

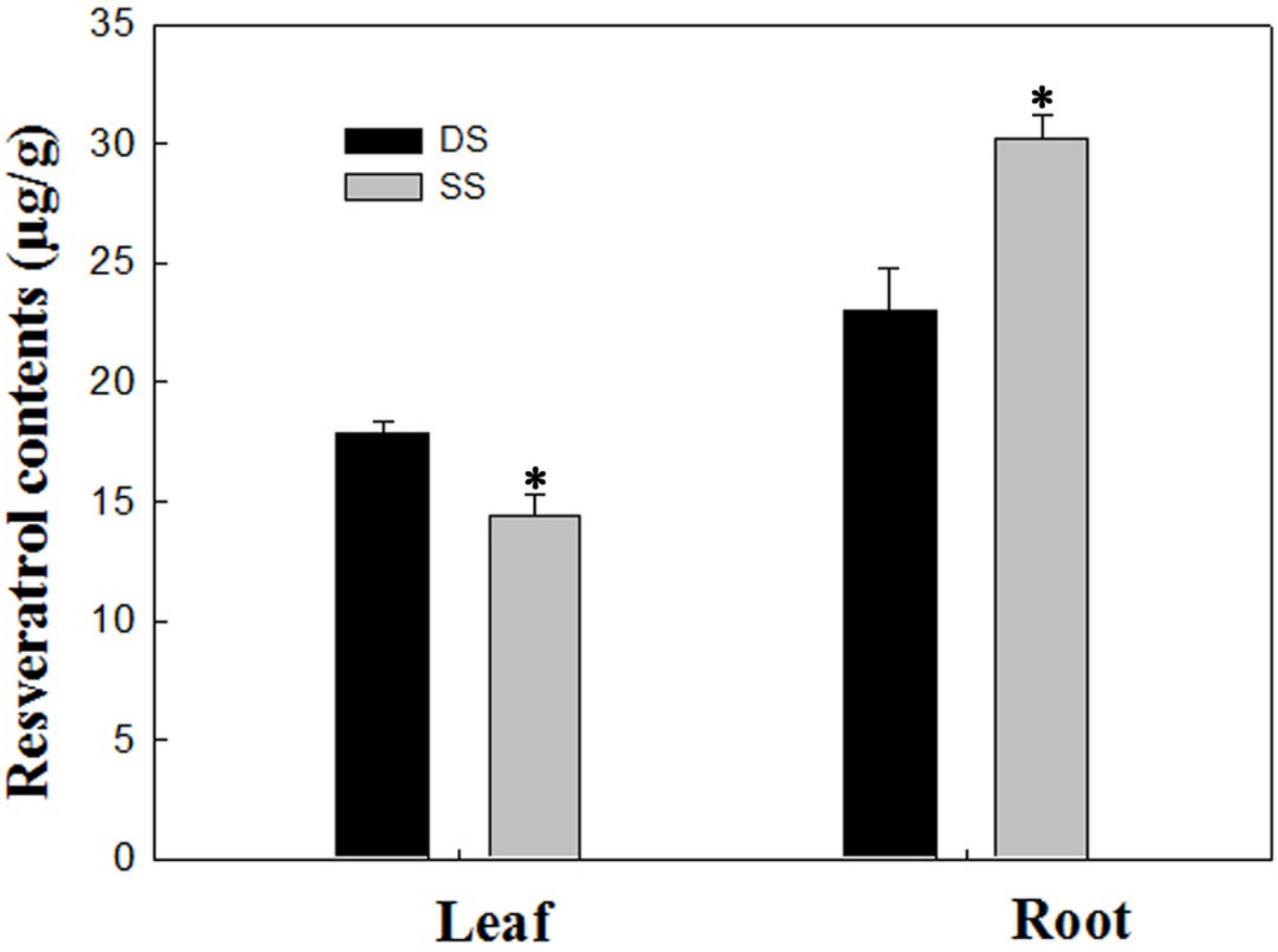


Table 1(on next page)

Table 1 Summary of read numbers from leaves and roots.

SL: Leaf in single-seed (SS) precision sowing; DL: Leaf in double-seed (DS) sowing; SR: Root in single-seed (SS) precision sowing; DR: Root in double-seed (DS) sowing.

Table 1 Summary of read numbers from leaves and roots. SL: Leaf in single-seed (SS) precision sowing; DL: Leaf in double-seed (DS) sowing; SR: Root in single-seed (SS) precision sowing; DR: Root in double-seed (DS) sowing.

Samples	Reads in leaf						Reads in Root					
	SL1	SL2	SL3	DL1	DL2	DL3	SR1	SR2	SR3	DR1	DR2	DR3
Total clean reads	45,001,686	44,475,256	44,262,552	42,022,054	42,294,220	42,570,982	42,569,308	42,180,566	44,533,150	44,784,894	44,608,344	44,497,326
Total Mapping Ratio	75.27%	74.87%	74.88%	78.26%	80.52%	80.60%	80.73%	80.88%	82.71%	78.44%	78.15%	80.95%
Unique match Ratio	55.15%	53.51%	54.34%	49.41%	51.29%	54.53%	51.93%	52.18%	53.61%	46.73%	43.23%	52.30%
Unmapped reads	24.73%	25.13%	25.12%	21.74%	19.48%	19.40%	19.27%	19.12%	17.29%	21.56%	21.85%	19.05%
Novel transcript number	13,152	12,615	13,149	13,218	13,030	12,895	13,139	13,136	13,316	13,021	12,911	12,909

Table 2(on next page)

Table 2 Analysis of resveratrol synthesis related genes in leaves and roots.

The ratio was obtained from the transcriptome results. SL: Leaf in single-seed (SS) precision sowing; DL: Leaf in double-seed (DS) sowing; SR: Root in single-seed (SS) precision sowing; DR: Root in double-seed (DS) sowing.

Table 2 Analysis of resveratrol synthesis related genes in leaves and roots. The ratio was obtained from the transcriptome results. SL: Leaf in single-seed (SS) precision sowing; DL: Leaf in double-seed (DS) sowing; SR: Root in single-seed (SS) precision sowing; DR: Root in double-seed (DS) sowing.

	Annotation	$\log_2^{(SL/DL)}$	Gene expression change
Leaf	resveratrol synthase	-1.43	Down-regulated
	resveratrol synthase	-1.92	Down-regulated
	resveratrol synthase	-2.03	Down-regulated
	resveratrol synthase	-2.31	Down-regulated
	resveratrol synthase	-2.66	Down-regulated
	resveratrol synthase	-2.77	Down-regulated
	resveratrol synthase	-3.36	Down-regulated
	resveratrol synthase	-6.66	Down-regulated
	stilbene synthase	-1.18	Down-regulated
	stilbene synthase	-1.38	Down-regulated
	Annotation	$\log_2^{(SR/DR)}$	Gene expression change
Root	resveratrol synthase	2.67	Up-regulated
	resveratrol synthase	2.59	Up-regulated
	resveratrol synthase	2.37	Up-regulated
	resveratrol synthase	2.27	Up-regulated
	resveratrol synthase	2.24	Up-regulated
	resveratrol synthase	2.13	Up-regulated
	resveratrol synthase	2.12	Up-regulated
	resveratrol synthase	2.05	Up-regulated
	resveratrol synthase	1.86	Up-regulated
	resveratrol synthase	1.80	Up-regulated
	resveratrol synthase	1.68	Up-regulated
	resveratrol synthase	1.67	Up-regulated
	resveratrol synthase	1.61	Up-regulated

resveratrol synthase	1.56	Up-regulated
resveratrol synthase	1.55	Up-regulated
resveratrol synthase	1.47	Up-regulated
resveratrol synthase	1.34	Up-regulated
resveratrol synthase	1.27	Up-regulated
stilbene synthase	2.47	Up-regulated
stilbene synthase	2.08	Up-regulated
stilbene synthase	1.19	Up-regulated

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Table 3(on next page)

Table 3 List of putative candidate genes for high yields in SS sowing treatment.

These candidate genes were up-regulated in SR vs DR comparison groups with the corrected *p* value < 0.05 and are here classified according to predicted gene function. The values in the columns are the log2 Fold Change values for the SR vs DR comparison groups obtained from the transcriptome results. SR: Root in single-seed (SS) precision sowing; DR: Root in double-seed (DS) sowing.

Table 3 List of putative candidate genes for high yields in SS sowing treatment. These candidate genes were up-regulated in SR vs DR comparison groups with the corrected *p* value < 0.05 and are here classified according to predicted gene function. The values in the columns are the log2 Fold Change values for the SR vs DR comparison groups obtained from the transcriptome results. SR: Root in single-seed (SS) precision sowing; DR: Root in double-seed (DS) sowing.

Gene_id	**Gene description**	SR vs DR
Oxidation-reduction		
107460187	L-ascorbate oxidase homolog	1.4
110279516	L-ascorbate oxidase	1.4
107482474	L-ascorbate oxidase	1.2
107480398	L-ascorbate oxidase	1.1
107471312	glutaredoxin	2.8
107475918	glutaredoxin 3	2.4
107494759	glutaredoxin-C1-like	1.5
107461150	glutaredoxin 3	1.1
107475477	cytochrome P450 83B1-like	2.2
107491506	cytochrome P450 71A1-like	2.1
107475482	cytochrome P450 83B1-like	2.0
107475708	cytochrome P450 83B1-like	3.5
107478833	cytochrome P450 84A1-like	1.1
107475710	cytochrome P450 71A1-like	1.2
107487896	cytochrome P450 71D8-like	1.2
107492914	cytochrome P450 93A3	1.3
Hormone related		
107491535	Protein brassinosteroid	1.7
107486286	gibberellin-regulated protein	2.4
BGI_novel_G000490	gibberellin receptor GID1	2.0
107495262	gibberellin receptor GID1	2.6
107476131	gibberellin receptor GID1	1.9
107464975	gibberellin 2-beta-dioxygenase 2-like	1.7
107477445	DELLA protein	1.7
107491113	DELLA protein	1.1
107476118	auxin responsive GH3 gene family	1.9
107465048	auxin-responsive protein IAA	1.8
107492413	auxin response factor	1.9
107463844	auxin-responsive protein IAA	1.4
107462192	auxin responsive GH3 gene family	1.8
107459619	auxin-responsive protein IAA	1.5
107478268	auxin responsive GH3 gene family	1.0
107464096	auxin-responsive protein IAA	1.2

107495645	lipoxygenase	2.4
107464479	linoleate 9S-lipoxygenase	1.1
Transcription factor and signaling regulation		
107491013	MADS-box transcription factor	3.0
107476111	MADS-box transcription factor	5.7
107458618	zinc finger protein CONSTANS-LIKE 4-like	2.8
107492404	Zinc finger protein	2.4
110279598	zinc finger protein 3-like	2.3
107493757	zinc finger protein-like protein	1.8
107481180	Zinc finger protein 6	1.5
107460097	C2H2-like zinc finger protein	1.3
107483222	MYB family transcription factor	4.8
107461398	MYB86 Transcription factor	1.5
107475194	MYB transcription factor MYB51	1.2
107491849	R2R3 MYB protein 2	1.2
107459300	WRKY transcription factor 70	2.4
107472118	WRKY transcription factor	1.8
107472768	WRKY transcription factor 22-like	1.5
107481590	WRKY transcription factor 14	1.3
107463444	ERF114-like	2.4
107488356	ERF022	1.6
107464842	ERF13	1.5
107471058	ERF WRI1	1.0
107486141	ERF 1-like	1.0
BGI_novel_G002321	bZIP	7.3
107487341	bZIP	1.5
107466101	Calcium-binding protein CML	1.4
107491924	Calcium-binding protein KIC-like	1.3
107460313	Calcium-binding protein CML	1.2
107479434	MAPKKK	1.5
Disease resistance		
107468687	pathogenesis-related protein 1	2.2
107468499	pathogenesis-related protein 1	1.8
107468493	pathogenesis-related protein 1	1.4
107474846	pathogenesis-related protein 1	1.0
107460041	4-coumarate--CoA ligase	4.2
107472893	4-coumarate--CoA ligase	3.1
107480465	4-coumarate--CoA ligase	2.6
107458085	4-coumarate--CoA ligase	2.0
107466660	4-coumarate--CoA ligase-like 5	1.7

107481001	4-coumarate--CoA ligase	1.6

Table 4(on next page)

Table 4 Pod per plant at maturity stage of peanut under different planting patterns

1 Table 4 Pod per plant at maturity stage of peanut under different planting patterns

Treatment	Pods number per plant	Full pods number per plant	Double kernel number per plant	Pod weight per plant (g)	Economic coefficient
SS	39.1a	22.6a	24.4a	51.7a	0.49a
DS	31.8b	16.3b	16.9b	39.3b	0.45b

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