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Digital expression analysis of the genes associated with salinity resistance after overexpression of a stress-responsive small GTP-binding RabG protein in peanut

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ABSTRACT. The Rab protein family is the largest family of the small GTP-binding proteins. Among them, the RabG genes are known to be responsive to abiotic stresses, but the molecular mechanisms of the stress responses mediated by RabG genes in plants is poorly understood. To investigate the molecular mechanism of *AhRabG* gene in peanut, transgenic plants overexpressing the *AhRabG* gene (S6) with relatively higher salinity resistance than the non-transgenic plants (S7) were obtained. Digital gene expression (DGE) sequencing was performed with the leaves of S6 and S7 plants before and after

salinity-stress treatment. The *AhRabG* gene in peanut was found to be involved in a few pathways such as “photosynthesis”, “oxidative phosphorylation”, “AMPK signaling pathway”, “plant hormone signal transduction”, etc. A total of 298 differentially expressed genes (DEGs) were found to be upregulated or downregulated at five sampling time points based on the comparison between S6 and S7 plants. Among them, 132 DEGs were responsive to salinity stress in S6 and/or S7 after salinity-stress treatment. These 132 DEGs included genes encoding various transcription factors and proteins involved in resistance to salinity stress such as MYB, AP2, RING-H2 zinc finger proteins, late embryogenesis abundant (LEA) proteins, dehydration-responsive protein RD22, peroxidases, CBL-interacting protein kinases, calcium-binding proteins, and others. The information from this study will be useful for further studies on elucidating the mechanism of salinity resistance conferred by *RabG* genein peanut.

Key words: Peanut (*Arachis hypogaea* L.); RabG; Overexpression; Salinity stress; Digital gene expression; Differentially expressed genes

INTRODUCTION

The small GTP-binding proteins with a molecular mass ranging from 20 to 30 kDa are ubiquitous among eukaryotes (Lowy and Willumsen, 1993). They can be divided into Ras, Rho/Rac, Ypt/Rab, Ran/TC4, and Arf/Sar subfamilies, and each of them plays a distinct role in various cellular functions. Among these, the Rab proteins constitute the largest subfamily of the small GTP-binding proteins (Brighouse et al., 2010).

The Rab proteins participate in the vesicle formation, transportation, and docking during exocytic and endocytic cycles (Pereira-Leal and Seabra, 2001). Furthermore, they are involved in signal transduction, cytoskeletal organization (Nuoffer and Balch, 1994), pathogen response (Sano et al., 1994; Zhao et al., 2015), and responses to various environmental stimuli (Xing et al., 1997; Nicolás et al., 1998; O’Mahony and Oliver, 1999; Bolte et al., 2000). Among different Rab proteins, the Rab7 (G)-related proteins regulate the delivery of the internalized materials into the degradative compartments and the acquisition of lysosomal hydrolases (Bogdanovic et al., 2000). In yeast, a homologue of Rab7, namely Ypt7p, localizes to vacuoles and regulates the homotypic fusion between vacuolar compartments (Schimmöller and Riezman, 1993). The Ypt7 mutant is characterized by highly fragmented vacuoles and differential defects in vacuolar protein transport and maturation (Wichmann et al., 1992).

Interestingly, some of the Rab7 genes are found to be responsive to various environmental stimuli including drought, salt, extreme temperature, abscisic acid (ABA) application, and elicitors (Nahm et al., 2003; Mazel et al., 2004; Agarwal et al., 2008; Rajan et al., 2015). For example, the rice Rab7 homolog, *OsRab7* transcript, was strongly induced by dehydration and ABA treatment, but not by salt treatment. Transgenic plants overexpressing the *OsRab7* gene showed enhanced growth and increased proline content at the seedling stage after salt stress treatment. Moreover, an increase in the number of vesicles was observed in the root tip of transgenic rice overexpressing the *OsRab7* gene (Nahm et al., 2003). The *AtRabG3e* gene from *Arabidopsis* was induced during the programmed cell death following

treatment with superoxide or salicylic acid, or infection with necrogenic pathogens. The transgenic plants expressing the *AtRabG3e* gene exhibited accelerated endocytosis, increased tolerance to salt and osmotic stresses, and reduced accumulation of reactive oxygen species under salt-treated conditions (Mazel et al., 2004). The expression of *PgRab7* transcript from *Pennisetum glaucum* increased drastically by various environmental stimuli such as salt, cold, dehydration, and a plant hormone indole-3-acetic acid (IAA). Transgenic tobacco overexpressing *PgRab7* gene showed enhanced tolerance to NaCl and mannitol as well as increased alkaline phosphatase activity (Agarwal et al., 2008). The transcript expression of the *AlRab7* gene from *Aeluropus lagopoides*was differentially regulated by dehydration, salinity, and ABA. The recombinant *Escherichia coli* harboring the *AlRab7* gene showed improved growth in the medium containing salt, mannitol, ABA, and IAA (Rajan et al., 2015).

In our previous study, we isolated an important small GTP-binding protein from peanut; this protein was found to be responsive to different stress treatments and plant hormone ABA, and exhibited enhanced tolerance to different stresses in *E. coli* and transgenic peanut (Song et al., 2012). To elucidate the mechanism of *AhRabG* in peanut, we prepared transgenic plants overexpressing the *AhRabG* gene and non-transgenic plants (as control), and determined the dynamic differences in their digital gene expression (DGE) profiles before and after salinity stress treatment. The specific pathways regulated by *AhRabG* were identified. The possible roles of the differentially expressed genes (DEGs) were discussed.

MATERIAL AND METHODS

Plant growth and stress treatments

Transgenic peanut plants overexpressing the *AhRabG* gene (S6, the level of *AhRabG* gene was 1.5 times that of control) and peanut cultivars ‘Xuzhou68-4’ used as control plants (S7) were obtained from Qingdao Agricultural University, China. The seeds of each genotype were grown in a growth chamber with dark/light cycle of 8/16 h at a temperature of 28°C for six weeks. Subsequently, the seedlings of the S6 and S7 plants were irrigated with 250 mM NaCl for salinity stress under the same conditions. After exposure to a salinity stress for 0, 6, 12, 24, and 48 h, the leaves of the S6 and S7 seedlings were removed and placed in liquid nitrogen until further use.

Library construction for transcriptome sequencing and functional annotation

Total RNA was isolated from the sampled leaves with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA from each combination of seedling type (S6 or S7) after salt treatment was pooled, and then analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA sequencing was carried out by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) on an Illumina HiSeq2000 sequencer. The 125-bp paired-end reads were generated. All clean sequence read data were deposited in the sequence read archive (SRA) database (accession number SRR3114511) of the National Centre for Biotechnology Information (NCBI). Subsequently, they were assembled into comprehensive unigenes by means of the Trinity and TGICL programs. The functional annotation of these genes was conducted with reference to various online databases such as NR, NT, SwissProt, PFAM, KOG, GO, and KEGG (Sui et al., unpublished data).

DGE sequencing and mapping

The RNA samples from the S6 and S7 genotypes were labeled with the sampling times (0, 6, 12, 24, and 48 h after NaCl treatment) as follows: S6_0, S6_6, S6_12, S6_24, and S6_48 for the S6 genotype, and S7_0, S7_6, S7_12, S7_24, and S7_48 for the S7 genotype. Each combination of genotype and sampling time after salt treatment was represented by two replicate RNA samples. DGE sequencing was carried out by Novogene Institute (Beijing, China) on an Illumina HiSeq2000 sequencer with a single 50-bp end read for each reaction. All reads of each library were deposited in the NCBI SRA database (accession number SRR3210688 and SRR3204350) and mapped onto the unigenes. The unigene expression was normalized with the value of RPKM (reads per kilobase per million mapped reads).

Identification of differentially expressed genes (DEGs)

Differential expression analysis was compared both within each genotype and between the two genotypes using the DESeq R package (1.10.1). The DESeq package provides statistical routines for determining differential expression in the digital gene expression data using a model based on the negative binomial distribution (Anders and Huber, 2010). The resulting P values were adjusted using the Benjamini-Hochberg approach for controlling the false discovery rate (Benjamini and Hochberg,1995). Between the genotypes S6 and S7, the gene expression was compared at 0, 6, 12, 24, and 48 h. If the level of expression was significantly different (the adjusted P value < 0.05) in a comparison, then the gene was considered to be differentially expressed. Within the genotypes S6 and S7, the gene expression was compared between two sampling times. If the level of expression was significantly up- or downregulated (the adjusted P value < 0.05) in a comparison, then the gene was considered to be responsive to salinity stress. Pathways that were statistically significant (false discovery rate (FDR) ≤ 0.05) were enriched with KEGG pathways.

RT-qPCR analysis

Reverse transcription was performed using the SuperScript Reverse Transcriptase Kit (Invitrogen). The primers were designed using the Oligo6 software. For RT-qPCR, the SYBR® Premix Ex Taq™ (TAKARA) was used on a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Gene expression was analyzed for S6 and S7 samples at 6, 12, 24, and 48 h after the application of the salinity-stress treatment. All reactions for each gene were performed in three biological replicates with a 20-μL reaction volume. The parameters of the thermal cycle were: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 40 s and annealing at 50°-56°C for 40 s. The relative expression levels were calculated using the $2^{-r\triangle Ct}$ method, with normalization to the internal reference *β-actin* gene from peanut as described previously (Livak and Schmittgen, 2001).

RESULTS

To investigate the gene expression patterns of each genotype before and after the salinity-stress treatment, 20 DGE libraries were constructed and sequenced with the method of Illumina deep sequencing technology. For each sample, it generated 11,037,884-13,409,746

clean reads and 0.55-0.67 G clean bases. In the S6 genotype, the number of unigenes (FPKM>0.3) ranged from 49,755 to 53,353 with an average value of 51,371, whereas in the S7 genotype, the number of unigenes (FPKM>0.3) ranged from 49,695 to 54,337 with an average value of 51,479 (**Table S1**).

Clustering analysis of samples and identification of DEGs

To obtain a global view of the gene expression profiles of the S6 and S7 genotypes, we analyzed the gene expression profiles for 10 samples from both genotypes using the method of clustering algorithms and treeview. Generally, similar gene expression patterns were found between S6 and S7 before and after salinity stress at paired time points. The DEG profiles at the 6 and 12 h were significantly different from those before the stress treatment. With an increase in the treatment time, the tendency of DEGs became similar (Figure 1).

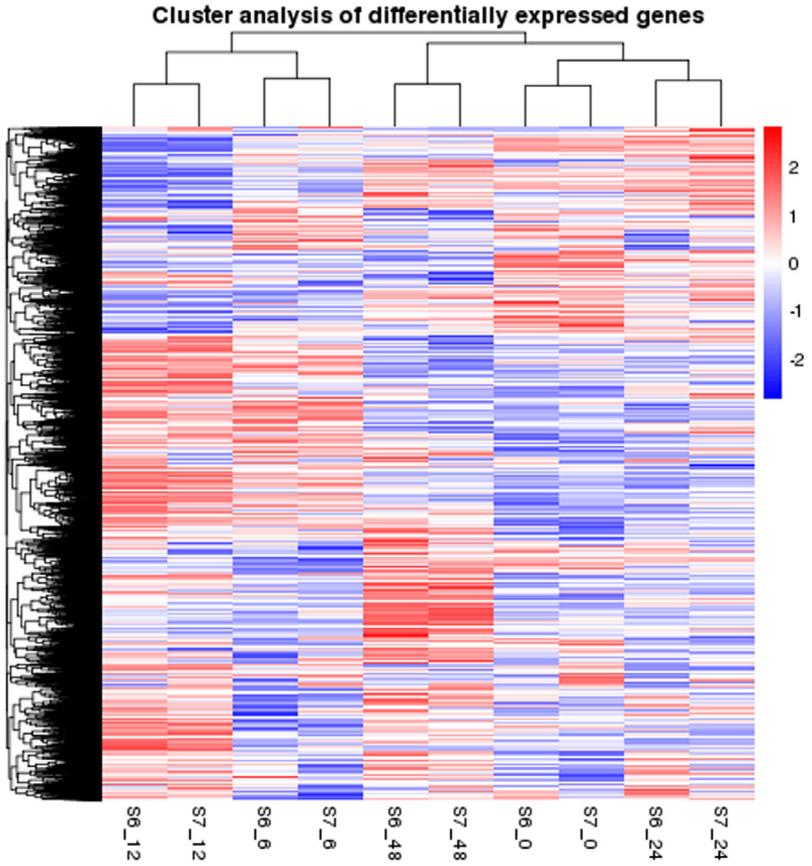

Figure 1. Hierarchical clustering analysis of salinity-induced changes in gene expression in peanut leaves. S6, transgenic peanut plants overexpressing *AhRabG* gene; S7, Xuzhou68-4 (control); the first letter plus number refers to the S6 or S7 sample and the second number refers to the time (from 0 to 48 h) after salinity stress treatment. Each value of gene expression is an average of the two replications. Red bar indicates upregulated expression, and blue bar indicates downregulated expression.

In the present study, the DEGs were identified with the criteria of the adjusted P value less than 0.05. The differential comparisons between the control and salinity stress-treated samples were shown in the S6 and S7 series data sets. These 10 series data sets represented the DEGs of both genotypes before and after salinity stress treatment.

The dynamic trends of DEGs in the S6 and S7 data sets were investigated. Compared with control at 0 h, the differentially expressed genes showed peaks after treatment with salinity stress, and the number of downregulated DEGs was markedly higher than the upregulated ones at 12 and 48 h in S6 and S7 genotypes. The total DEGs, downregulated as well as upregulated, showed similar changing trends at 0, 6, 12, 24 and 48 h in S6 and S7 genotypes (Figure 2). A total of 217 DEGs in the S6 genotype (Figure 3A) and 655 DEGs in the S7 genotype (Figure 3B) were found to be common at four different time points after salinity stress treatment.

	DEGs in S6					DEGs in S7				
Time (h)	0	6	12	24	48	0	6	12	24	48
0	0	1581	1859	516	869	0	2321	2889	1402	1683
6	1624	0	231	748	751	2443	0	838	1682	1650
12	2667	680	0	2012	1056	3190	1166	0	2661	1958
24	407	775	1340	0	323	1420	1797	2190	0	1111
48	1113	880	859	380	0	1904	1794	1874	1143	0

Figure 2. Changes in the numbers of differentially expressed gene (DEGs) in the S6 and S7 genotypes of peanut following salinity-stress treatment. S6 overexpresses the *AhRabG* gene, while S7 does not. Orange indicates upregulated expression, and blue indicates downregulated expression.

DEGs were also identified by comparison of gene expression between S6 and S7 at each sampling time. The data set at 6 h showed the least exclusive DEGs, and they were mostly exclusive in the data sets at 0 and 24 h. Interestingly, three DEGs were found to be common in all five data sets (Figure 3C).

Annotation and KEGG enrichment analyses of DEGs based on S6 *vs.* S7 comparison

For functional annotation of the DEGs that were determined by comparison of gene expression in S6 *vs.* S7, the KEGG enrichment analyses were mainly referenced. At 0 h (before salinity stress treatment), 13 downregulated and 12 upregulated DEGs were screened. The main pathways included "photosynthesis", "oxidative phosphorylation", "AMPK signaling pathway", and "plant hormone signal transduction" (Figure 4A). Interestingly, the enrichment analyses indicated that all differentially regulated DEGs involved in "photosynthesis" and "oxidative phosphorylation" pathways were upregulated after overexpression of the RabG genes. After 6 h of salt stress, five downregulated and two upregulated DEGs were assigned the KEGG pathways. Among the five downregulated DEGs, three DEGs were involved in the "circadian rhythm" pathway and two DEGs (c39883_g1 and c35382_g1) were involved in the "flavonoid biosynthesis" pathway. One DEG (c34015_g1) was found to be involved in three pathways, including "GABAergic synapse", "synaptic vesicle cycle", and "retrograde endocannabinoid signaling" (Figure 4B). After 12 h of salt stress, five Tand Gs ously. bacamef treatment time, downregulated DEGs were assigned the KEGG pathways (Figure 4C). Among them, two DEGs, which were also found to be downregulated at 6h, were involved in the "circadian rhythm" and "flavonoid biosynthesis" pathways. A DEG (c19905_g1), which encoded homogentisate phytyltransferase, was also found to be downregulated at 0 and 6 h. Another DGE (c32262_g1), which was involved in the "thiamine metabolism" and "terpenoid

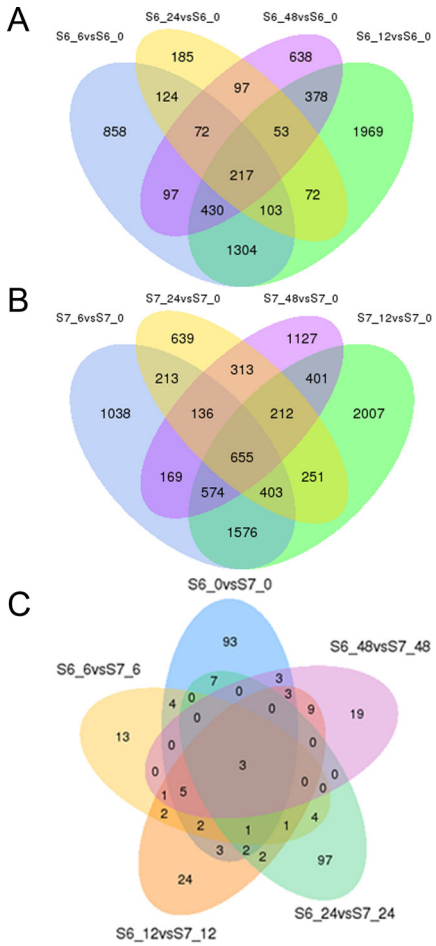

Figure 3. Venn diagrams indicating the numbers of differentially expressed genes (DEGs) in: **A.** the S6 genotype (after *vs.* before the salinity-stress treatment; **B.** the S7 genotype (after *vs.* before the salinity-stress treatment); and **C.** S6 *vs.* S7 (after the salinity-stress treatment). The numbers of DEGs exclusively expressed in one comparison are shown in the non-overlapping regions. The numbers of DEGs with a common tendency of expression change in the comparisons are shown in the overlapping regions.

backbone biosynthesis" pathways, was also found to be downregulated at 6 and 12h. After 24 h of salt stress, four upregulated and 22 downregulated DEGs were assigned the KEGG pathways, and they were mainly involved in the "plant-pathogen interaction," "PI3K-Akt signaling pathway", "protein processing in endoplasmic reticulum", "plant hormone signal transduction", "thyroid hormone synthesis", "circadian rhythm-plant", "antigen processing and presentation", "signaling pathways regulating pluripotency of stem cells", etc. (Figure 4D). One DGE (C34875_g1), which encoded the vacuolar-processing enzyme (dynein heavy chain of dynein motor), was involved in the "antigen processing and presentation" and "lysosome" pathways. After 48 h of salt stress, 43 DEGs were screened, but none of them were found in the KEGG enrichment analysis.

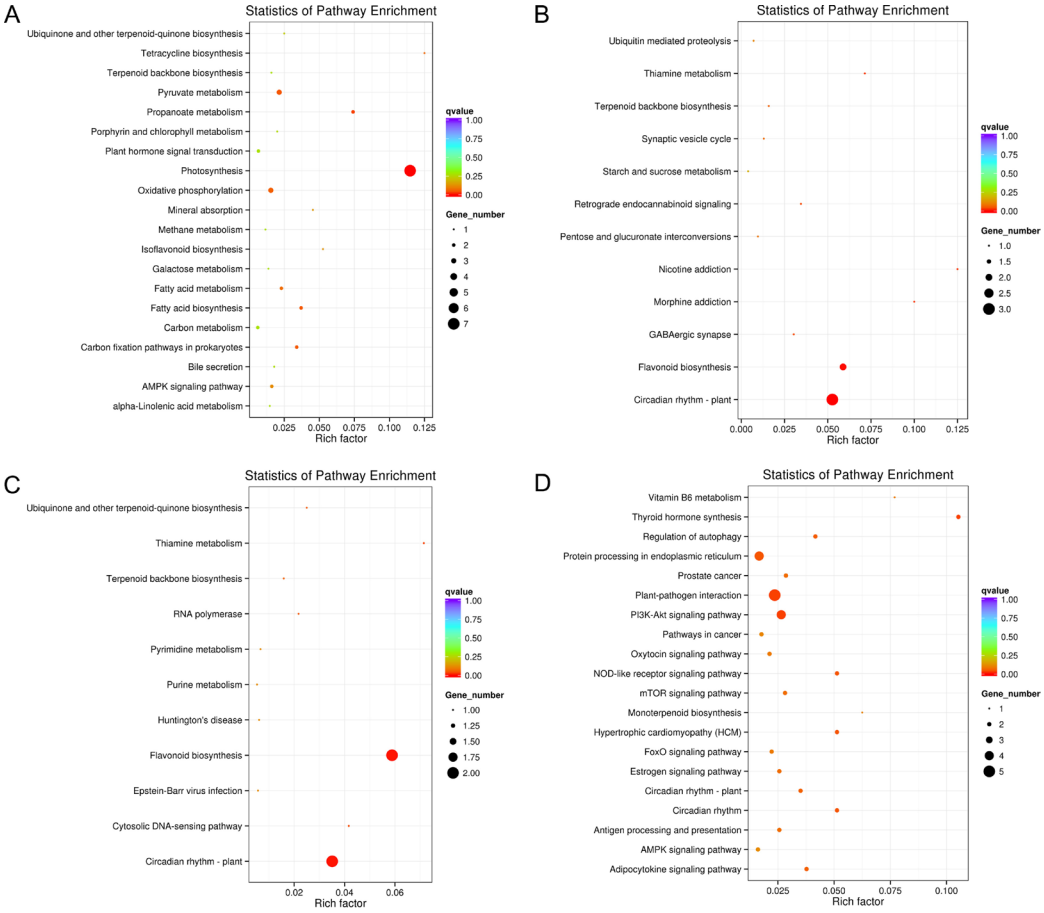

Figure 4. KEGG Pathway enrichment analyses of differentially expressed genes (DEGs) in D_6 (**A**), D_12 (**B**), D_24 (**C**), and D_48 (**D**) based on the comparison of S6 *vs* S7 genotypes of peanut.

Screening of salinity-responsive DEGs based on S6 vs. S7 comparison

To further elucidate the salinity-resistant mechanism of the *RabG* gene in peanut, the salinity-responsive DEGs were screened on the basis of S6 *vs* S7 comparison. Of all the 298 DEGs at five sampling times (**Table S2** and Figure 3), 132 DEGs, whose expression were significantly up- or downregulated in S6 relative to S7, were responsive to the salinity stress at 6, 12, 24, and 48 h (**Table S3**). The expression patterns of these genes taking key roles in plant resistance to salinity stress were investigated. Among them, 66 DEGs (9 in the S6 genotype and 57 in the S7 genotype) were sensitive to the salinity stress treatment, and 15 among them were sensitive at two sampling times.

Experimental verification of DEGs

To determine whether our RNA-seq identification in peanut was reliable, we used

RT-qPCR to monitor the expression pattern of eight DEGs according to the result of S6 *vs* S7 comparison at 0, 6, 12, 24, and 48 h sampling times; the expression of these genes was quantified in both S6 and S7 genotypes. The primers of the selected genes are listed in **Table S4**. Their expression levels, as determined by RT-qPCR, were correlated with the results of the RNA-seq (**Table S5**).

DISCUSSION

Our DGE sequencing identified some previously reported stress-related transcription factors (TFs) and genes after overexpressing the *AhRabG* gene from peanut. Five TFs, including the ethylene-responsive transcription factor AP2, MYB, and RING-H2 zinc finger protein, were differentially expressed under salinity stress between S6 and S7 genotypes. The MYB TFs have been found to play key roles in both the repression and derepression of the responses to salt stress as well as other abiotic stresses (Lu et al., 2014). An R2R3-type MYB TF from rice, OsMPS, which might simultaneously play roles in multiple stress-induced signaling pathways, was found to regulate rice tolerance to salinity, plant hormones, and cell wall synthesis (Schmidt et al., 2013). We found thatone MYB TF (c38087_g1) was upregulated at 6 h, while the other MYB TF (c41425_g1) was downregulated at 24 h. One AP2 TF (32271_g1) was found to be upregulated at 6 h [the log2 (fold-change) >3]. The AP2 TFs have been proved to be involved as cross-talk factors in various biotic and abiotic stresses and plant hormone signal transduction (Fujita et al., 2006). For example, an ethylene response factor (ERF)-typeAP2 TF from soybean (GmERF3) was induced by various kinds of stress treatments mentioned above (Zhang et al., 2009). Two DEGs encoding RING-H2 zinc finger proteins (c37125_g2 and c35368_g1) were differentially expressed between the S6 and S7 genotypes after salinity stress.

Some genes that were differentially expressed in S6 *vs* S7 comparison were screened to be responsive to the salinity stress. These included DEGs encoding LEA proteins, dehydration-responsive protein RD22, and peroxidase. Among these genes, three *LEA* genes (c32599_g1, c30685_g1, and c35561_g1) were significantly upregulated in S6 relative to S7 [the log2 (fold-change) > 2]. In plants, the LEA proteins can accumulate to high levels during the last stage of seed maturation and under abiotic stresses, playing a protective role during the damage caused by environmental stresses (Battaglia et al., 2008). The gene encoding RD22 (c65965_g1) was downregulated in S6 relative to the S7 genotype at 12 h. Its expression level was extremely downregulated after the salinity-stress treatment as compared to its expression before treatment in S6 and S7, especially at 48 h, and this was in agreement with the findings of *HbRD22* gene from *Hevea brasiliensis* exposed to drought stress (Gao et al., 2010), but the opposite result was obtained in the other species such as *Arabidopsis*. The result showed that the *RD22* gene (c65965_g1) from peanut might be a negative regulator under salinity stress. Peroxidases are considered to be bifunctional enzymes that not only oxidize various substrates in the presence of H_2O_2, but also generate H_2O_2 (Passardi et al., 2004). Apoplastic peroxidases can either restrict or promote cell expansion (Tsukagoshi et al., 2010). In our study, one DEG encoding peroxidase (c33518_g1) was found to be differentially expressed in S6 relative to the S7 genotype at 24 h.

Other DEGs that might be related to stress were also detected in our study of S6 and S7. These DEGs included genes encoding the two-component response regulator (c20693_g1) and cytochrome P450 (c31385_g1), serine/ threonine-protein kinase (c38177_g1 and

c43920_g1), expansin (c49431_g1), DNA repair protein (c40074_g1), low-temperature-induced protein (c41310_g1), Ca^{2+}/H^{+} exchange protein (c39501_g1), protein phosphatase 2C (c35944_g1), CBL-interacting protein kinase (c26640_g1 and c32118_g1), calcium-binding proteins (c31008_g1), and synaptobrevin, a member of the vesicle-associated membrane protein (VAMP) family(c40050_g1), etc.

Moreover, the functional classification of DEGs showed that out of 132 DEGs, approximately 30 unclassified and unknown-function genes were responsive to salinity stress. These 30 genes represented a larger set of genes, which might be the novel genes involved in salinity stress responses after the overexpression of *AhRabG* gene in peanut (**Table S3**). However, some genes with relatively lower transcript levels might not be detected using the RNA-seq analysis.

Collectively, our results indicate that the regulation of salinity stress resistance after overexpression of the *AhRabG* gene in peanut involves many aspects which are mentioned above. The information from this study can help us understand the mechanism of salinity resistance conferred by the expression of *RabG* gene in peanut.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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Supplementary material

Table S1. The summary of differential gene expression (DGE) library sequencing and analysis.

Table S2. Differentially expressed genes (DEGs) at five time points between S6 and S7 genotypes.

Table S3. Selected differentially expressed genes (DEGs) responsive to salinity stress based on comparison of S6 *vs* S7.

Table S4. Primers for quantitative real-time PCR (qRT-PCR) analysis.

Table S5. Comparison of the expression levels of 8 differentially expressed genes (DEGs) as determined by RNA-seq and real-time PCR analysis.