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Isolation and analysis of differentially expressed genes from peanut in response to challenge with *Ralstonia solanacearum*

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Abstract

Background: Bacterial wilt caused by *Ralstonia solanacearum* is the most devastating disease in peanut. Planting resistant peanut cultivars is deemed as the sole economically viable means for effective control of the disease. To understand the molecular mechanism underlying resistance and facilitate breeding process, differences in gene expression between seeds of Rihua 1 (a Virginia type peanut variety resistant to bacterial wilt) inoculated with the bacterial pathogen suspension (10^9 cfu ml^{-1}) and seeds of the same cultivar treated with water (control), were studied using the Genefishing™ technology. **Results:** A total of 25 differentially expressed genes were isolated. Expression of genes encoding cyclophilin and ADP-ribosylation factor, respectively, were further studied by real time RT-PCR, and full length cDNAs of both genes were obtained by rapid amplification of cDNA ends. **Conclusions:** The study provided candidate genes potentially useful for breeding peanut cultivars with both high yield and bacterial wilt resistance, although confirmation of their functions through transgenic studies is still needed.

Keywords: *Arachis hypogaea*, ARF, cyclophilin, differentially expressed genes, genefishing, *Ralstonia solanacearum*

INTRODUCTION

Being a rich source of cooking oil and dietary protein, the cultivated peanut (*Arachis hypogaea* L.) is regarded as one of the most important cash crops in the world. Bacterial wilt (BW) disease caused by *Ralstonia solanacearum* (Yabuuchi et al. 1995) poses a great threat to peanut production in China and Southeast Asia. More than 10% of the area under peanut is affected in China (Yu et al. 2011). Yield reduction generally ranges from 10% to 20%; however, in heavily infested field, over 50% yield losses are not uncommon. In extreme cases, the disease may even cause total crop failure (Yu et al. 2011).

Like any other plant BW diseases, peanut BW is difficult to control. No desirable chemical management measure is currently available. Planting resistant peanut cultivars is deemed as the sole economically viable means for effective control (Yu et al. 2011). Unfortunately, most of the resistant germplasm lines identified are small-seeded genotypes with low yield potential; transferring BW resistance to high yielding adapted peanut cultivars has therefore become an urgent task (Yu et al. 2011).

Understanding the mechanism underlying BW resistance at molecular level may hasten the breeding process. Thus far, in peanut, there have been several reports regarding identification of DNA markers related to BW resistance (Yu et al. 2011), and transcripts involved in response to biotic and abiotic stresses other than BW have been identified. These included, transcripts related to resistance to late spot disease (Luo et al. 2005; Nobile et al. 2008; Kumar and Kirti, 2011), *Aspergillus flavus* and *A. parasiticus* infection (Guo et al. 2008; Guo et al. 2011), and root-knot nematode (*Meloidogyne arenaria*) parasitization (Guimãraes et al. 2010; Tirumalaraju et al. 2011), and desiccation (Jain et al. 2001; Gopalakrishna et al. 2001) and chilling responsive genes (Tang et al. 2011); in contrast, there is only one report on differentially expressed genes (DEGs) between BW resistant and susceptible peanut genotypes. Peng et al. (2011) identified 119 transcription-derived fragments (TDFs) after root wounding inoculation with *R. solanacearum*, from Yuanza 9102 (a Spanish type peanut cultivar with BW resistance) and Zhonghua 12 (a susceptible Spanish type peanut cultivar) using cDNA-AFLP and further studied their expression patterns. 98 TDFs were cloned and sequenced, 40 of which were found to have homology to sequences deposited in non-redundant (nr) database of NCBI with known function, while 15 had homology to sequences with unknown function and 43 had no homology.

In the present communication, we reported isolation of DEGs in response to challenge with *Ralstonia solanacearum* from a large-seeded peanut cultivar Rihua 1 after treatment with the bacterial pathogen suspension using Genefishing^{TM} technology and cloning of full length cDNAs of genes coding for cyclophilin (CyP) and ADP-ribosylation factor (ARF), respectively, by rapid amplification of cDNA ends (RACE).

MATERIALS AND METHODS

Material

Peanut variety and bacterial strain. Rihua 1, a Virginia type peanut cultivar with verified high resistance to BW both in field and at laboratory, was kindly provided by Mr. Dian Wen Zhang, a peanut breeder from San Zhuang Town (119º8'E, 35º30'N), Rizhao City, Shandong Province, China. The causal pathogen, *R. solanacearum* RZ strain was isolated from diseased peanut plants collected in Rizhao field by the first author and maintained at Laboratory of Biotech Division, Shandong Peanut Research Institute.

Methods

Inoculum preparation and inoculation. The bacterial strain was streaked onto TZC Agar (nutrient agar supplemented with 0.05% tetrazolium chloride) (Kelman, 1954) and incubated at 28ºC for 48 to 72 hrs. The bacterial strain was then flushed off the surface of the culture media with sterile double distilled water to prepare a suspension of 10^9 cfu per millilitre for inoculation.

The peanut seeds were surface sterilized with 75% (v/v) ethanol, and soaked in the bacterial suspension in a Petri dish in a growth chamber (28ºC). No light was provided. Sterile water treatment was used as control.

Isolation of total RNA. Total RNA was isolated from the peanut seeds treated with sterile double distilled water or bacterial suspension for 2, 3, 4 and 5 days, respectively, using RNAprep pure Plant Kit (Tiangen, Beijing, China). Concentration and integrity of the RNA were determined by spectrophotometry and relative intensity of brightness of GelRed (Biotium, CA, USA) stained bands resolved on a 1.2% agarose gel.

Cloning and sequencing of DEGs. DEGs from seeds of Rihua 1 treated with bacterial suspension or water (control) were identified using Genefishing^{TM} DEG Premix Kit (Seegene, Korea) following manufacturer's instructions. The reaction mixture for reverse transcription (RT) (20 µl total volume) consisted of 3 µg of total RNA isolated from control or pathogen infected samples, 1 µl of 10 µM dT-ACP1, 4 µl of 5 x RT buffer, 2 µl of 10 mM dNTP mix, 0.5 µl of RNase inhibitor (40 U l^{-1}) (Tiangen, Beijing, China) along with 200 U of M-MLV reverse transcriptase (TaKaRa, Japan). RT was conducted at 42ºC for 90 min, followed by incubation at 70ºC for 15 min to terminate the reaction. First strand cDNA products were then diluted with 80 µl of DNase-free water and directly used in subsequent

Genefishing PCR. PCR mixture (20 µl) contained 50 ng of first strand cDNA, 0.5 µM arbitrary ACP, 0.5 µM dT-ACP2 and 2 x SeeAmp ACP Master-mix. PCR program was 94ºC for 5 min, 50ºC for 3 min and 72ºC for 1 min, followed by 40 cycles of 94ºC for 40 sec, 65ºC for 40 sec and 72ºC for 40 sec, and a final extension of 72ºC for 5 min. PCR products were separated on a 2% agarose gel, stained with Gelred and visualized under UV light. Amplicons of interest from treated samples were cloned into a pGM-T vector (Tiangen, Beijing, China), and sequenced by Genscript Inc., Nanjing, China.

Sequence analysis and annotation of DEGs. Cloned DEGs were analyzed using Blastn in search of homologous sequences in nr and EST databases of NCBI. Annotation was performed based on the best match identified by Blastn against nucleonic acid databases at NCBI.

Real time RT-PCR. First strand cDNA was synthesized using total RNA, M-MLV reverse transcriptase (TaKaRa, Japan), oligo-dT, dNTPs and reverse transcriptase buffer. PCR primer pairs were designed with Beacon Designer 7.91 (Premier Biosoft International, Palo Alto, CA, USA) (Table 1). Real time RT-PCR was run on a Lightcycler 2.0 PCR machine (Roche Diagnostics, Penzberg, Germany). Both the PCR program and the component of reaction mixture were the same as previously described by Tang et al. (2011). The PCR program was followed by a melting program of 65ºC to 95ºC at a transition rate of 0.1ºC s^{-1} with the fluorescence continuously monitored. In each run, a negative control without cDNA template was included to evaluate overall specificity. Reactions were performed in triplicate, and the averages presented. Fold changes in RNA transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with β-actin gene as an internal control (Table 1).

Table 1. List of PCR primers used in this study.

Primer ID	Forward primer(5'-3')	Reverse primer(5'-3')
3d-A13	TTGATGTATGGTACTAAGC	TAAATGACGAATGGGAAA
4d-A9	CGGATGTTGAATGAGGATGAA	GGCGGAGTGAATGAAGTC
β-actin	TTGGAATGGGTCAGAAGGATGC	AGTGGTGCCTCAGTAAGAAGC
DE-ARF	GTGTTTTTCCGTATCTCGAATCGCT	CCATCCTTCTCCACGAACCGCCACA
DE-CYP	ATGGCTAACCCTAAGGTTTACTT	CTAAGAGAGTTGACCGCAATC

5' RACE and isolation of full length cDNA of cyclophilin and ADP-ribosylation factor. 5' cDNA ends were amplified with gene specific primers designed with Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) using SMARTer™ RACE cDNA Amplification Kit (Clontech, California, USA) according to manufacturer's instructions. The full length cDNA sequence of cyclophilin from Rihua 1, designated as CyP, was deduced by aligning 5'RACE product and 3d-A13 cDNA sequence. The primer pair, DE-CYP (Table 1), was used to amplify the open reading frame (ORF). Similarly, the full length cDNA sequence of ADP-ribosylation factor from Rihua 1, designated as ARF, was obtained by aligning 5 'RACE product and 4d-A9 cDNA sequence. The primer pair, DE-ARF (Table 1), was synthesized to amplify the corresponding ORF.

To obtain the ORFs of the two genes, total RNA was used for reverse transcription in the presence of Oligo-dT, dNTPs and M-MLV reverse transcriptase enzyme (TaKaRa, Japan). First strand cDNA thus synthesized was used as template for subsequent PCR using *Taq* Platinum Master Polymerase (Tiangen, Beijing, China). PCR products were cloned into a pGM-T vector (Tiangen, Beijing, China) and sequenced by Genscript Inc., Nanjing, China.

Analysis of full length cDNA sequences. After vector and the primer sequences were removed, the cloned sequences were subjected to further analysis. Blastn similarity searches were performed at NCBI website (http://www.ncbi.nlm.nih.gov). Open reading frame (ORF) was analyzed with BioxM, and multiple sequence alignment with DNAStar Lasergene MegAlign 7.1.0 (DNASTAR, Inc., WI, USA). Annotation was performed using BLAST2GO (http://www.blast2go.com/b2ghome).

Table 2. Blast results of the 25 DEGs from Rihua 1 upon interaction with *R. solanacearum.*

Sequence name	Corresponding ACP primer	Length	Closest accession hit	Description	E value	Max identity
3d-1-3	ACP43	627	EU331154.1	*Arachis diogoi* 60S ribosomal protein L1 mRNA, partial cds	0	96%
3d-A13	ACP13	652	EU170616.1	*Arachis diogoi* cyclophilin mRNA, complete cds	0	98%
4d-5-3	ACP43	482	AK285364.1	*Glycine max* cDNA, clone: GMFL01-08-B07	3.00E-62	73%
4d-6-3	ACP44	382	FR687022.1	*Cancer pagurus* partial mRNA for putative DEAD box ATP-dependent RNA helicase (ddx gene)	5.00E-13	100%
4d-9-2	ACP49	867	AJ938036.1	*Lupinus albus* mRNA for glyceraldehyde-3-phosphate-dehydrogenase (gapdh gene)	0	86%
4d-10-1	ACP49	452	X51764.1	*L. polyphyllus* mRNA for peroxidase	2.00E-63	78%
4d-11-1	ACP50	755	AK285672.1	*Glycine max* cDNA, clone: GMFL01-14-G11	2.00E-155	79%
4d-12-2	ACP50	485	AC235404.1	*Glycine max* strain Williams 82 clone GM_WBb0124F21, complete sequence	2.00E-20	80%
4d-13-3	ACP50	251	EU780119.1	*Clonorchis sinensis* clone ACP-1U-117-2	1.00E-13	100%
4d-21-2	ACP56	329	AK285662.1	*Glycine max* cDNA, clone: GMFL01-14-F07	3.00E-15	75%
4d-21-3	ACP56	312	GU480450.1	*Arachis duranensis* clone BAC ADUR185P1 retrotransposons FIDEL, complete sequence	1.00E-84	90%
4d-A4-3	ACP4	231	FR687022.1	*Cancer pagurus* partial mRNA for putative DEAD box ATP-dependent RNA helicase (ddx gene)	2.00E-08	100%
4d-A9	ACP9	745	DQ861992.1	*Arachis diogoi* ADP-ribosylation factor mRNA, partial cds	0	98%
A9-1-2	ACP9	333	DQ889568.1	*Arachis hypogaea* clone UU9 putative L24 ribosomal protein mRNA, complete cds	1.00E-109	96%
4d-A10-2-2	ACP10	330	AK337483.1	*Lotus japonicus* cDNA, clone: LjFL1-064-BB10, HTC	9.00E-35	94%
4d-A13-2-1	ACP13	518	BT097186.1	Soybean clone JCVI-FLGm-26G1 unknown mRNA	7.00E-96	85%
4d-A14-1	ACP14	758	AK338234.1	*Lotus japonicus* cDNA, clone: LjFL2-015-BA03, HTC	3.00E-133	82%
4d-A17-1-1	ACP17	639	AK286939.1	*Glycine max* cDNA, clone: GMFL01-40-J01	3.00E-145	86%
4d-A51	ACP51	392	DQ296045.1	*Arachis hypogaea* disease resistance response protein mRNA, partial cds	3.00E-36	100%
5d-3-1	ACP48	121	FR687023.1	*Cancer pagurus* partial mRNA for putative phenoloxidase activating factor (ppaf gene)	2.00E-11	100%
5d-4-3	ACP51	588	BT053375.1	*Medicago truncatula* clone MTYFP_FQ_FR_FS1G-I-8 unknown mRNA	5.00E-111	80%
5d-7-1	ACP54	415	NM_001034680.1	*Bos taurus asparagine* synthetase domain containing 1 (ASNSD1), mRNA	5.00E-20	95%
5d-7-4	ACP54	358	FJ597734.1	*Harmonia axyridis* antibacterial peptide mRNA, complete cds	2.00E-12	96%
5d-A19-1	ACP19	466	AB489995.1	*Citrullus lanatus* subsp. *vulgaris* CitBC mRNA for blue copper protein precursor, complete cds	7.00E-13	100%
5d-A19-3	ACP19	518	BT096639.1	Soybean clone JCVI-FLGm-20P16 unknown mRNA	1.00E-74	80%

RESULTS

Isolation and annotation of DEGs from peanut in response to challenge with *R. solanacearum*

DEGs in Rihua 1 upon infection with *R. solanacearum* were isolated using Seegene's Genefishing DEG Premix Kit. Partial results were shown in Figure 1. The kit utilizes Annealing Control Primer (ACP) technology (Hwang et al. 2003) suitable for high annealing temperature, thereby essentially eliminating false positives (Kumar and Kirti, 2011). Twenty-five partial cDNAs were cloned and sequenced from Rihua 1 in response to bacterial inoculation (Table 2).

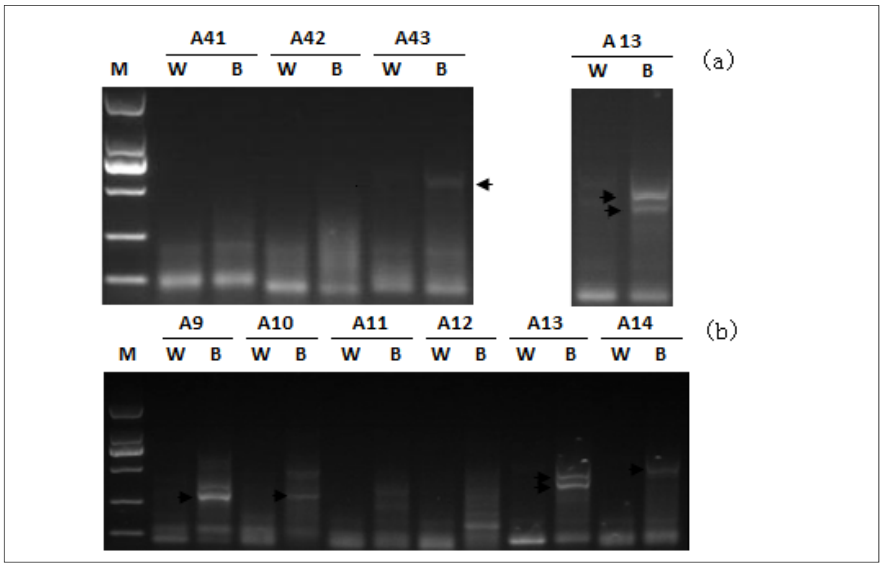

Fig. 1 DEGs between treated and control peanut seeds on the 3rd (a) and 4th (b) day as indicated by arrows (only partial DEGs were shown). M: DL2000; W: seeds soaked in water (control); B: seeds treated with bacterial suspension; A: ACP arbitrary primers.

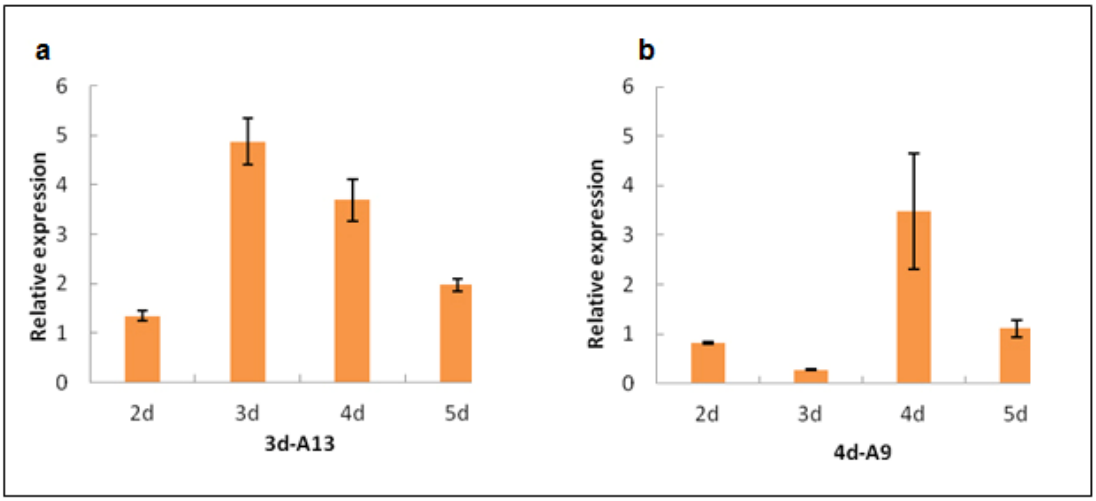

Fig. 2 Relative expression of 2 genes, 3d-A13 (a); and 4d-A9 (b), in peanut seeds treated with bacterial suspension and water for 2, 3, 4 and 5 d, respectively. Error bar indicating standard deviation of mean.

Verification of DEGs

Of the 25 unique cDNA sequences, 11 (44%) had no known function, 4 (16%) were potentially related to diseases defence, 2 (8%), and 1 (4%), 2 (8%), 3 (12%) and 2 (8%) were involved in signal transduction, transcription, protein synthesis and basic metabolism, respectively.

Two of the four transcripts encoding putative diseases defence related proteins, 3d-A13 and 4d-A9 (Table 2) were verified by real time RT-PCR. The results showed that both genes expressed differentially in stressed and control peanut seeds, with relative expression of 1.34 and 0.82 in the 2nd day, 4.88 and 0.28 in 3rd day, 3.69 and 3.49 in 4th day, and 1.97 and 1.11 in 5th day, respectively (Figure 2), indicating that a marked increase in relative expression appeared in the 3rd day for 3d-A13 and 4th day for 4d-A9.

Changes in gene expression in bacterial suspension treated seeds over time were investigated. The expression of the 2 genes in the 3rd, 4th and 5th day relative to that in the 2nd day was shown in Figure 3. Both genes reached peak relative expression in the 4th day.

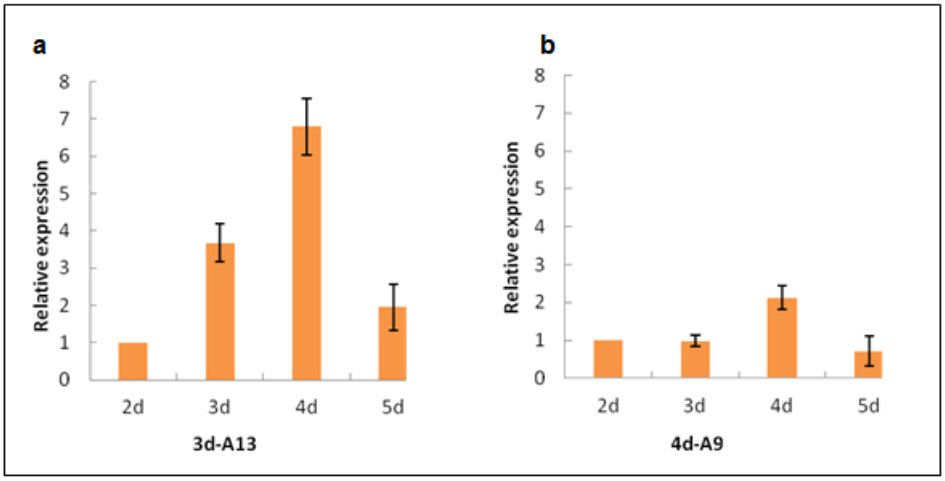

Fig. 3 Relative expression of 3d-A13 (a) and 4d-A9 (b) in peanut seeds treated with bacterial suspension for 2, 3, 4 and 5 d, respectively. Error bar indicating standard deviation of mean. Relative expression (3, 4, 5 d) is computed based on the corresponding gene expression in the 2nd day.

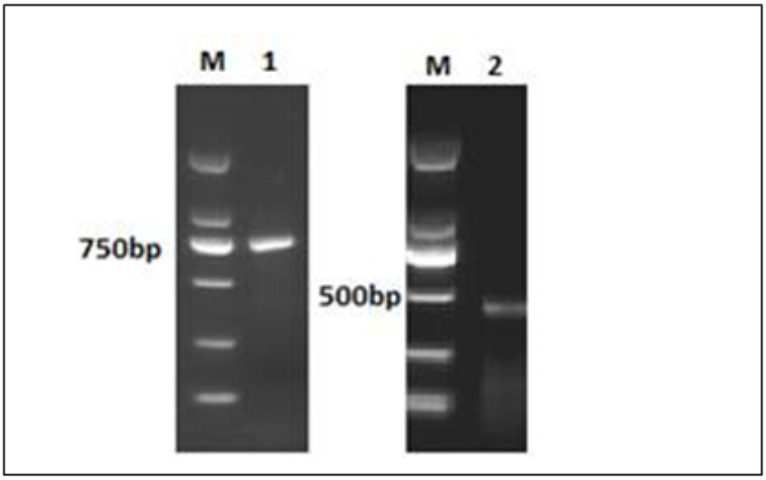

Fig. 4 Amplification of 5' cDNA sequences of CyP and ARF. M: DL2000, Lane 1 and 2: 5' sequence from CyP and ARF.

Isolation and analysis of full length cDNAs of cyclophilin and ADP-ribosylation factor

Considering the fact that the sequence 3d-A13 encoded a cyclophilin-like protein potentially related to diseases defence, 5' cDNA sequence of the gene was amplified with 5'RACE (Lane 1 in Figure 4), and full length cDNA obtained. The full length cDNA was 907 bp in length potentially encoding a single polypeptide of 172 amino acids. An 87 bp 5'UTR and a 296 bp 3'UTR including the poly-A tail were present flanking the ORF. The full length cDNA of *cyclophilin* (CyP) was submitted to the Genbank under the accession number JN379456. The corresponding ORF amplified with primer pair DE-CYP showed high sequence identity to *A. diogoi* (data not shown). Multiple sequences alignment of the deduced amino acid sequence of cyclophilin from Rihua 1 in the present study and those from *Arabidopsis thaliana*, *Glycine max* and *Populus trichocarpa* revealed high sequence homology (Figure 5).

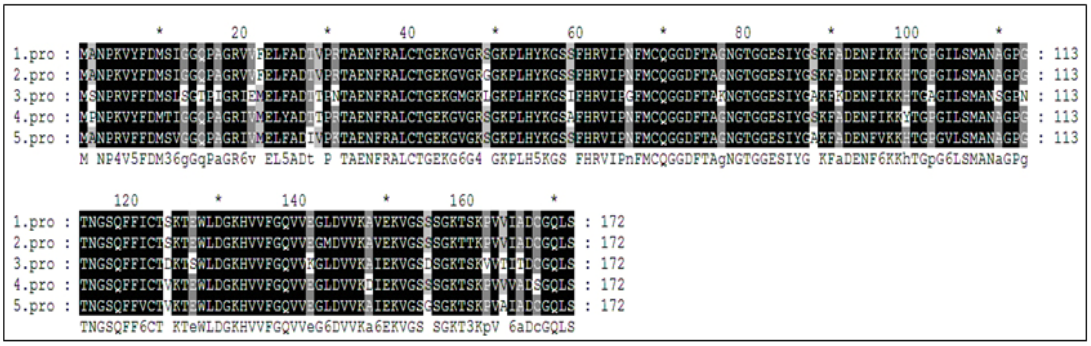

Fig. 5 Alignment of deduced amino acid sequences of cyclophilin from Rihua 1 (1), *A. diogoi* (ABY579461) (2), *Arabidopsis thaliana* (NP1792511) (3), *Glycine max* (ACU164311)(4) and *Populus trichocarpa* (XP0023137361) (5). GenBank acc# included in parenthesis.

4d-A9, the sequence that potentially coded for ADP-ribosylation factor (ARF), was used to obtain the full length cDNA of ARF with 5'RACE (Lane 2 in Figure 4). The full length cDNA was 923bp long with a 114 bp 5'UTR and a 266 bp 3'UTR, potentially encoding a single polypeptide of 118 amino acids. It was submitted to the Genbank under the accession number JN379456. Alignment of the deduced amino acid sequence of ARF from Rihua 1 in the present study with those from other organisms like *Arabidopsis thaliana*, *Populus trichocarpa* and *Medicago sativa* demonstrated that the protein was evolutionarily conserved (Figure 6).

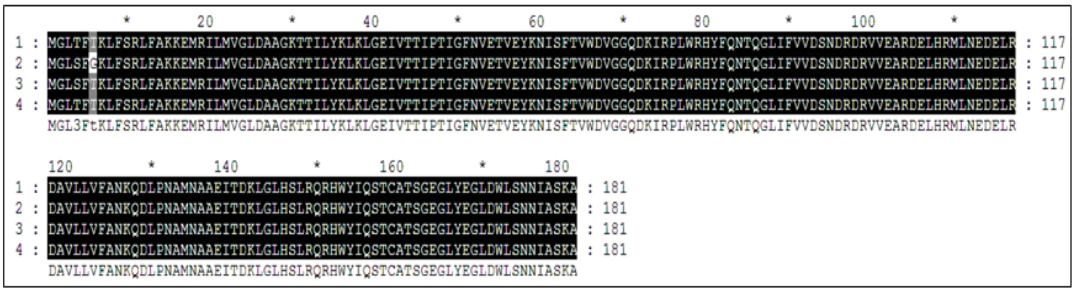

Fig. 6 Alignment of deduced amino acid sequences of ARF from Rihua 1 (1), *Arabidopsis thaliana* (NP1822391) (2), *Populus trichocarpa* (XP0023189351) (3), *Medicago sativa* (AAR292931) (4). GenBank acc# included in parenthesis.

The annotation results of both genes using BLAST2GO were shown in Table 3.

Table 3. Annotation of CyP and ARF using BLAST2GO.

Sequence name	Sequence description	#Hits	Min. e value	Mean similarity	#GOs	GOs
gi|359770106|gb|JN379456.1|	adp-ribosylation factor	20	5.82E-125	99.45%	14	F:GTP binding; P:small GTPase mediated signal transduction; F:hydrolase activity; F:nucleotide binding; C:intracellular; P:intracellular protein transport; F:transporter activity; C:plastid; P:protein amino acid ADP-ribosylation; C:Golgi apparatus; P:vesicle-mediated transport; C:mitochondrion; P:protein transport; P:transport
gi|359770108|gb|JN379457.1|	cyclophilin	20	2.77E-109	96.95%	4	F:peptidyl-prolyl cis-trans isomerase activity; P:protein folding; P:protein peptidyl-prolyl isomerization; F:isomerase activity

DISCUSSION AND CONCLUSION

Although several DNA markers related to BW resistance were identified in previous studies, the map distances were too large to be used in peanut breeding programs (Yu et al. 2011). In contrast, differential expression analysis, which can be performed without a population in a short period of time, may result in some genes usable in peanut breeding, as long as their functions are further confirmed by transgenic studies. In the report of Peng et al. (2011), artificially wounded peanut roots were used for inoculation. In the present study, however, the seeds were soaked in the bacterial suspension instead. BW resistance used to be identified by sowing seeds directly in diseased field or by soaking seeds in the bacterial suspension prior to sowing. In either case, calculation of the survival percentage of each genotype at harvest was needed. Consequently, both methods were time-consuming. Recent years, root wounding or cuttings inoculation has been frequently used for evaluation of BW resistance in vegetable crops, and has proved to be rapid and reliable. The root wounding method is also applicable to peanut for evaluation of BW resistance, but it might be inappropriate for isolation of resistance related genes through transcriptome profiling, as roots wounding inevitably leads to the expression of wound-inducible genes unrelated to BW resistance. Up-regulated genes from the present study, however, may contain genes conferring BW resistance, as well as genes unrelated to resistance. In some cases, up-regulation may be a consequence of plant-pathogen interaction, rather than a determinant in resistance (Seevers et al. 1971). If there is a susceptible control for comparison, the unrelated genes may be excluded.

In this report, gene expression patterns of CyP and ARF were studied and full length cDNAs of both genes obtained. Huang et al. (2011) studied the dynamics of *R. solanacearum* population by stem injection and real time RT-PCR, and concluded that 3-5 day post inoculation was of vital importance for peanut and *R. solanacearum* interaction; likewise, in our study, expression of the 2 genes during this period was generally higher than that in 2 day, providing supports to Huang's observation.

CyPs are ubiquitous and constitutively expressed. However, they are also stress-responsive proteins, and up-regulated gene expression have been reported in response to abiotic/biotic stresses including heat, cold, salt, wounding, and virus infection (Dubery, 2007). For example, Dubery (2007) observed accumulation of potato CyPs mRNA in response to salicylic acid, *Phytophthora infestans* elicitor and *P. infestans* infection, and concluded that CyPs played an important role in plant stress responses. Kumar and Kirti (2011) isolated *cyclophilin* from *A. diogoi* inoculated with peanut late spot pathogen, *Phaeoisariopsis personata*. Constitutive heterogonous expression of the gene in transgenic tobacco enhanced resistance to *R. solanacearum* (Kumar and Kirti, 2011). We speculated that the CyP isolated from BW resistant cultivar Rihua 1 was also of importance to BW resistance in peanut.

ARFs have been isolated from *Arabidopsis*, rice, maize and wheat. Their function is mainly involved in mitosis and cell cycle control during seed development and regulation of intracellular transport (Lee et al. 2003, Table 3). Several reports have indicated that ARFs have a role in stress resistance in plants. Lee et al. (2003) reported that over-expression of rice ARF1 gene induced pathogenesis-related (PR) genes and pathogen resistance in tobacco plants, and they deduced that ARF1 might be a component of various plant defense signaling pathways in inducing the expression of a subset of PR genes. Coemans et al. (2008) identified an ARF1 involved in non-host resistance to bacteria and N gene-mediated resistance in *Nicotiana benthamiana* through high-throughput in planta expression screening. The ARF identified in our study might also have some implications in peanut resistance to the bacterial pathogen, *R. solanacearum*, whose function still deserves further confirmation.

To summarize, in this study, a total of 25 DEGs upon inoculation of seeds with *R. solanacearum* suspension were isolated from Rihua 1, a Virginia type BW resistant peanut cultivar by using Genefishing^{TM} technology, and gene expression patterns of CyPand ARF were studied. However, the detailed relationship of these genes with BW resistance in peanut still remains unknown. Further research is under way to elucidate their functions in peanut by antisense/RNAi technology or over-expression, as a high-efficiency genotype-independent transgenic protocol has already been developed at our lab (Li et al. 2011). The outcome of the present study may therefore provide candidate genes potentially useful for breeding high yielding BW resistant peanut cultivars.

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