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Scientific Research

Cloning, Expression Pattern Analysis and Subcellular Localization of Resveratrol Synthase Gene in Peanut (*Arachis hypogaea* L.)

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Abstract

Resveratrol synthase (RS) is a key enzyme that plays a critical role in the resveratrol synthesis pathway. In this study, six RS genes were isolated and characterized from peanut variety "Zhenzhu Hong" by silico cloning and RT-PCR. Bioinformatics analysis showed that deduced amino acid sequences of the six cloned RS genes were highly conserved with a similarity from 95% to 99% when compared to the RS genes which had been deposited at the GenBank. The results of amino acid sequences analysis showed six RS proteins contained the Chal_Sti_Synt_N and ACP_Syn_III_C domains and can be classified to same family but with different evolutionary distance. Expression pattern analysis by QRT-PCR provided evidence indicating that the mRNA of six RS genes were primarily expressed in the peanut shell at different developmental stages with different expression levels, but only lower levels of them were evident in the peanut kernel. The subcellular localization of RS protein in onion epidermal cell was performed by *Agrobacterium tumefaciens*-mediated transformation and the green fluorescent was monitored by confocal fluorescence microscopy. The results indicated that, RS1 and RS5 were located in the nucleus and plasma membrane respectively, while the RS2, RS3, RS4 and RS6 were located in both nucleus inner membrane and plasma membrane. The data will provide basic information for elucidating the regulatory mechanisms and enzyme kinetics underlying the RS genes in the resveratrol synthase pathway.

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Keywords

Peanut (*Arachis hypogaea* L.), Resveratrol Synthase Gene, Expression Pattern Analysis, Subcellular Localization, Development

1. Introduction

Resveratrol (*Trans*-3,5,4'-trihydroxy stilbene) is a natural plant phytoalexin which produced by plants in response to bacterium, fungal and other biotic or UV irradiation, wounding and other abiotic stress. It is present in more than 70 dietary plant species and with high concentration reported in grapes, berries and peanuts [1] [2]. Resveratrol exhibits several biological properties which include anti-microbial [3] [4], anti-oxidant [5], anti-mutagen [6], anti-inflammatory [7], anti-neoplastic [8] [9], and as a cholesterol reducing agent [10]. It also contributes to inhibition, delay, reversion of cellar events associated with heart disease and tumorigenesis [11]-[15]. The concentrations of resveratrol in most of plants is at the low level under ambient environmental conditions, however the compound is accumulated under stressful conditions which include pathogenic infection, but and other biotic stresses or UV irradiation, wounding and other abiotic stresses and functions to protect the plants from attacks [16]-[20].

Resveratrol is synthesized by the catalysis of resveratrol synthase (RS) using one molecule p-coumaroyl-CoA and three molecules of malonyl-coenzyme A [1] [21]. A number of RS genes have been cloned and identified from several plant species, including peanut, pines and grape. The first two RS genes cloned from peanut cell cultures was at 1988 [22] and four RS genes were identified at 1990 [23], and a range of RS genes have been cloned from peanut [24] [25]. Genetic engineering of plants with the objective of increasing the expression of RS can be exploited in order to develop plants with a higher level of resistance to pathogens [26]-[28]. The first reported transformation of foreign phytoalexin expression in a novel plant resulted disease resistance, was performed with a peanut RS gene introduced into tobacco, resulted in the rapid accumulation of resveratrol following treatment of cell suspension cultures with fungal elicitor [29]. From then on, RS genes have been introduced into a number of plants, including rice, barley and wheat, alfalfa, kiwifruit, grapevine, apple, aspen, papaya, white poplar, oilseed rape, banana, Rehmannia, tomato, *Arabidopsis*, lettuce, pea, and hop [30]-[33].

Although the resveratrol biosynthesis and regulation mechanism of RS gene at transcriptional level especially under the biotic and abiotic stress in peanut has been extensively studied in recent years [21] [34], but the regulation mechanism at transcriptional and translational level and the mechanism to antimicrobial still remains obscure. Here, we cloned and characterized six RS genes from peanut variety "ZhenZhuHong" by used the silico cloning and RT-PCR methods, and the sequences characters were analyzed by bioinformatics methods. To primarily study the distribution of six RS genes, QRT-PCR was applied to determine the mRNA expression pattern of them in peanut kernel and shell at different development stages. Analysis the subcellular location of the RS protein was carried out by Agrobacterium mediated transformation of onion epidermal cells with gene constructs representing each of the RS genes followed by—monitoring using confocal fluorescence microscopy. The study provides an important molecular basis for conducing further analysis on the functions, regulatory mechanisms and enzyme kinetics underlying the RS genes in resveratrol synthase process.

2. Materials and Methods

2.1. Plant Materials

The peanut variety "ZhenZhuHong" was planted in the experimental field of Guangdong Academy of Agricultural Sciences with 18˚C - 31˚C, **long-day photoperiod and acid soil** Seven samples at different developmental stages (**Figure 1**) were retrieved and total RNA was extracted. These were: peanut pods of tissue differentiation stage (I-P), kernel (II-K) and shell (II-S) of cotyledon elongation stage, kernel (III-K) and shell (III-S) of main leaf elongation stage, kernel (IV-K) and shell (IV-S) of mature stage (IV-K). Samples from six individual plants were pooled and frozen immediately in liquid nitrogen and then stored at −80˚C prior to total RNA extraction.

2.2. RNA Extraction and Reverse Transcription

Total RNA was extracted from the seven collected samples using TRIzol reagent (Invitrogen, USA) following

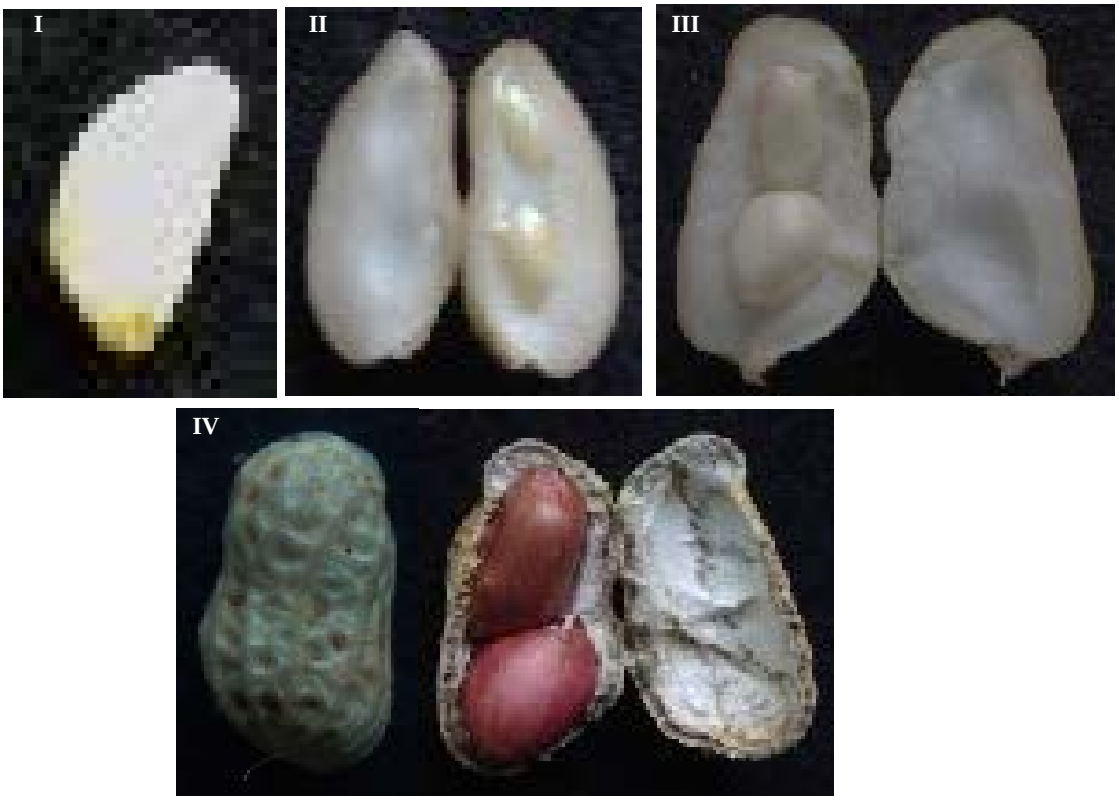


Figure 1. The pods characters of "Zhenzhu Hong" at different development stage. I: Tissue differentiation stag, II: Cotyledon elongation stage, III: Main leaf elongation stage, IV: Mature stage.

the manufacture's instruction and then treated with RNase-free DNase I (Promega, USA) to remove contaminating genomic DNA. First-strand cDNAs were synthesized from each sample by using 50 pmol of poly(T)$_{12\text{-}18}$ primer and SuperScript™ III First-Strand Synthesis System according the standard protocol (Invitrogen, USA). For each sample, two separated reverse transcription reactions were pooled.

2.3. cDNA Cloning

The full length reference cDNA sequence of peanut RS genes firstly were cloned by silico cloning methods. The primary peanut EST sequence in previously study were used in a search of the available peanut EST database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST), the selected ESTs were assembled by using the DNAStar program (Madison, USA). The homology or similarity searches of the assemble sequences by using Nucleotide BLAST and protein BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against public databases (GenBank), the putative encoding RS genes were selected to further study (as showed in **Table 1**). Then RT-PCR amplification was performed to cloning and validated the ORF of six RS genes. The gene-specific primers (**Table 1**: RS-ORF) were designed by using the Primer Premier 5.0 (http://www.premierbiosoft.com). The mixture cDNA of seven samples was used to RS genes cDNA cloning, the PCR program were performed according to the standard condition and protocols by Ex-Taq PCR Kit (Takara, Japan). PCR products were subsequently fractionated on 1.0% agarose gel and the DNA fragments were purified by DNA Gel/PCR purification Miniprep kit (Biomega, USA), then subcloned into pMD18-T Vector (Takara, Japan). Clones containing target DNA inserts were screened by PCR with the M13 forward and reverse primers (M13F:5'-GTAAAACGACGTCCAGT-3', M13R: 5'-CAGGAAACGACTATGAC-3'). The DNA sequencing were performed with the M13 forward and reverse primers by the BGI corporation (China).

2.4. Bioinformatics Analysis

All the individual sequences by raw sequencing data were removed from the vector Sequences by the VecScreen

Table 1. Primers used in this study.

Code	Sequence (5' - 3')	Use	Accession NO.
RS1-ORF-F	ATGGTGTCTGTGAGTGGAATTCG	ORF Cloning	AY170347
RS1-ORF-R	TTATATGGCCATGCTGCGGAG		
RS2-ORF-F	ATGTATTTAACAGAAGAAATACTCA	ORF Cloning	EU384706
RS2-ORF-R	TTATATGGCCACACTGCG		
RS3-ORF-F	ATGTACTTAACGGAAGAGATACTGA	ORF Cloning	HM130568
RS3-ORF-R	GAGTCCAGGACCAAATCCAA		
RS4-ORF-F	ATGGTGTCTGTGAGTGAGATCC	ORF Cloning	JQ728483F
RS4-ORF-R	TCAGATGGTCACACTACGAAGA		
RS5-ORF-F	ATGGTGTCTGTGAGTGGAATTCG	ORF Cloning	L00952
RS5-ORF-R	TTATATGGCCACACTGCGGAG		
RS6-ORF-F	ATGTGCGCATACAAAGCACC	ORF Cloning	X62300
RS6-ORF-R	TCATATGGCCACGCTGCG		
RS1-QRT-F	CCTACCTTTGAGATCGTTTCAACC	QRT-PCR	
RS1-QRT-R	AGTGCGTCGTTGATGTTTTGTG		
RS2-QRT-F	AAGGCCCTGCAACCGTATTAG	QRT-PCR	
RS2-QRT-R	TCTTGAGGTCGGTCATGTGCT		
RS3-QRT-F	AAGTGAACTTAAAACCAGAGAAGATG	QRT-PCR	
RS3-QRT-R	CTTCAAGGGATTTTTTCCTCATT		
RS4-QRT-F	ATCCGCAACGTTCAAAGAGC	QRT-PCR	
RS4-QRT-R	GTTTGATGGATTTGCCGTGC		
RS5-QRT-F	GACAAGCATTGTTTGCCGAT	QRT-PCR	
RS5-QRT-R	TTGAGCGCGTCATTGATATTT		
RS6-QRT-F	ATTAGATATCCCTCCATATATGCG	QRT-PCR	
RS6-QRT-R	ATAATATATAGGCCAATTAGGACCTT		
Actin-F	GATTGGAATGGAAGCTGCTG	Normalization for QRT-PCR	
Actin-R	CGGTCAGCAATACCAGGGAA		
RS1-SCL-F	CGCGGATCCGCGATGGTGTCTGTGAGTGGAATTCG	Subcellular Localization	
RS1-SCL-R	AAAACTGCAGTATGGCCATGCTGCGGAG		
RS2-SCL-F	CGCGGATCCGCGATGTATTTAACAGAAGAAATACTCA	Subcellular Localization	
RS2-SCL-R	AAAACTGCAGTATGGCCACACTGCG		
RS3-SCL-F	CGCGGATCCGCGATGTACTTAACGGAAGAGATACTGA	Subcellular Localization	
RS3-SCL-R	AAAACTGCAGGAGTCCAGGACCAAATCCAA		
RS4-SCL-F	CGCGGATCCGCGATGGTGTCTGTGAGTGAGATCC	Subcellular Localization	
RS4-SCL-R	AAAACTGCAGGATGGTCACACTACGAAGA		
RS5-SCL-F	CGCGGATCCGCGATGGTGTCTGTGAGTGGAATTCG	Subcellular Localization	
RS5-SCL-R	AAAACTGCAGTATGGCCACACTGCGGAG		
RS6-SCL-F	CGCGGATCCGCGATGTGCGCATACAAAGCACC	Subcellular Localization	
RS6-SCL-R	AAAACTGCAGTATGGCCACGCTGCG		

The sequences underlined are the corresponding restriction enzyme cutting sites. GGATCC represent BamH I cutting site, CTGCAG represent Pst I cutting site.

in NCBI (http://blast.ncbi.nlm.nih.gov/), and then the sequence reads were assembled into contiguous sequences with the Seqman and translate into Amino-acid sequence with the Editseq from the Lasergene package (http://www.dnastar.com/). The homology or similarity searched by using nucleotide BLAST and protein BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The protein molecular weight prediction and isoelectric point were calculated by ExPASY (http://www.expasy.ch/). The motif search was performed online with SMART protein analysis program (http://smart.embl-heidelberg.de/). Multiple amino-acid sequences alignment and phylogenetic tree (by NJT and the number of bootstrap replicates is 500) of the RS genes translated polypeptide sequences were done using CLUSTALW, constructed by MEGA 4.0 program, the alignment picture was showed by GeneDoc.

2.5. Expression Pattern Analysis

The cDNA of seven samples as described in previously were used to expression pattern analysis by Light Cycler 480 system with the "LC Fast Start DNA Master SYBER GREEN I kit" (Roche, Germany). Gene-specific primers were designed base on the cloned RS genes sequence, peanut Actin1 gene which was expected to show a constitutive expression pattern was used as the control to normalize the expression of RS genes (**Table 1**: RS-QRT, Actin1). The QRT-PCR reactions were run for two-step PCR as follows: 95˚C for 10 s; 40 amplification cycles at 95˚C for 5 s, 60˚C for 10 s and 72˚C for 10 s. At the end of each reaction, the fluorescence signal was detected, and used to generate an amplification profile. All PCR amplifications were performed in triplicate for each RNA sample and gene expression levels were quantified relative to Actin1 expression using Light-Cycler Software version 1.5 (Roche) based on the manufacturer's instructions. All tests were based on the same pooled cDNA to insure uniformity of the results. Difference in gene expression between groups were evaluated using Student's-test and were considered statistically significant at $P < 0.05$.

2.6. Subcellular Localization

Gene-specific primers for RS were designed to incorporate BamHI and PstI retriction sites at the N-terminus of the forward and reverse primers (**Table 1**: RS-SCL). The products of PCR amplions were then cloned into the modified pCAMBIA1302 vectors (CAMBIA, Australia), to generate the pCAMBIA1302-$P_{CAMV35S}$::RS:mGFP and then verified by restriction and sequencing analysis. Six recombinant plasmid and pCAMBIA1302 vectors were introduced into onion epidermal cells by an *Agrobacterium*-mediated system respectivly, incubated on 1/2 MS medium for 24 h at 26˚C in darkness, and the fluorescence of GFP was visualized through a fluorescence microscope. All transient expression assays were repeated at least three times

3. Results

3.1. Molecular Cloning of RS Genes from Peanut

BLAST searches by using the EST sequences which obtained in previously, six RS genes candidates plus putative sequences were selected for further analysis (**Table 1**). Primers were designed based on these reference sequences and six cDNA fragments corresponding to the RS genes from peanut "Zhenzhu Hong" variety were generated by RT-PCR (**Figure 2**). By cloned and sequenced of six RS genes, the complete ORF capable of varying length and encoding protein of different residues length were conducted, the molecular mass and isoelectric point (pI) were also calculated as showed in **Table 2**.

3.2. Bioinformatics Analysis

Base on the deduced amino acid sequence, six cloned RS genes share with 95% - 99% identity by BLASTP searches, and showing 92% - 98% identity with those peanut RS genes in the public database. The amino acid alignment results also show the high conservation of RS gene, amino acid variation only at a few sites (**Figure 3**). And all of them contain two conserved domains Chal_Sti_Synt_N and ACP_Syn_III_C domain supported by SMART protein analysis program. The high similarities mean that these RS proteins share significant structural similarity in a superfamily.

To investigate the evolutionary relationship between RS genes and other peanut RS genes sequences in public database, a phylogenetic tree was constructed with neighbor-joining algorithms by using the translated polypeptide

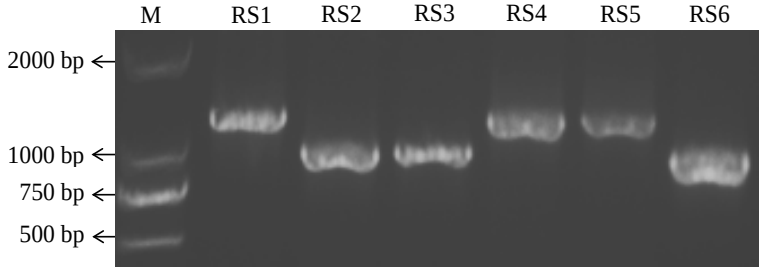


Figure 2. Amplification six RS genes from peanut "ZhenZhuHong" variety. M: Marker 2000. RS1-RS6: the PCR products of six RS genes amplified from the cDNA of peanut "Zhenzhu Hong" variety.

Table 2. Bioinformatics analysis of six RS genes.

RS gene	ORF in length (bp)	Encoding protein residues	Molecular mass (Da)	Isoelectric point (pI)
RS1	1170	389	42784.5	6.62
RS2	963	320	34856.2	5.08
RS3	927	308	33611.8	5.17
RS4	1170	389	42897.4	5.80
RS5	1170	389	42741.2	5.58
RS6	924	307	33345.5	5.25

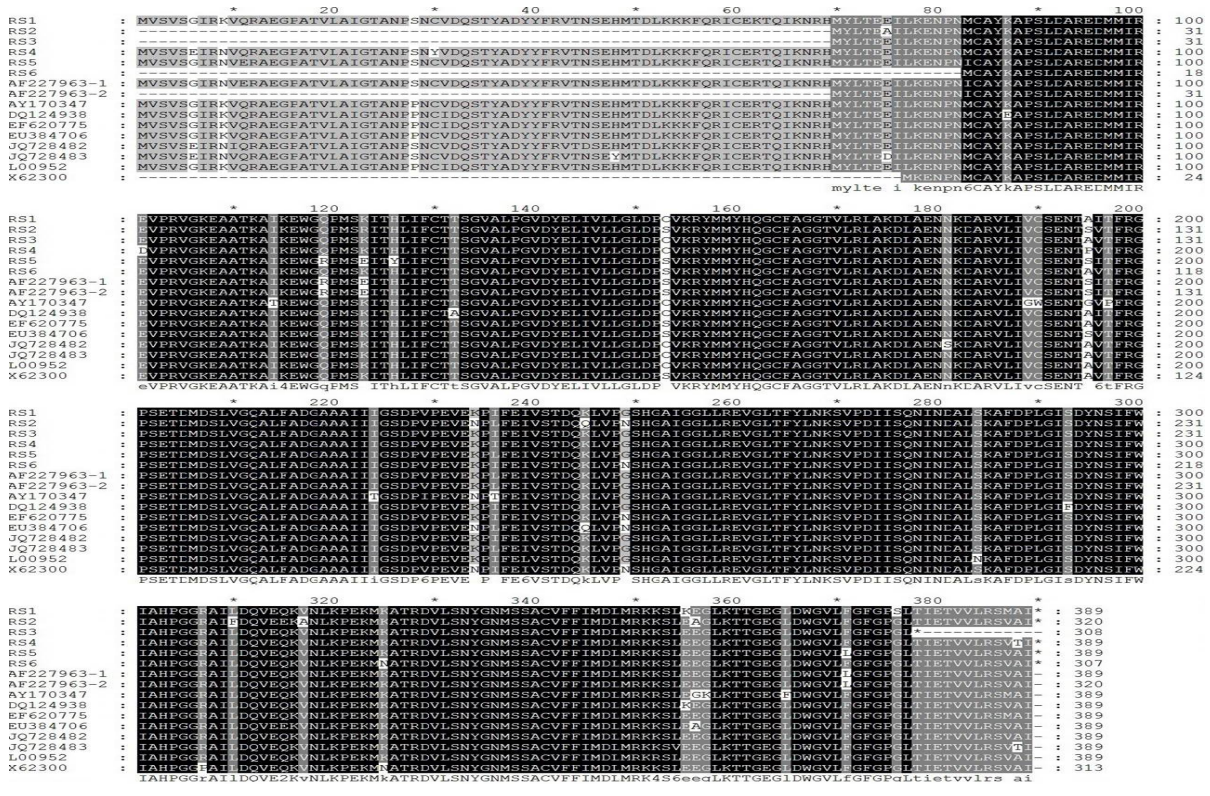


Figure 3. Amino acid alignments of RS genes in peanut. Deduced amino acid sequences for cloned RS genes from peanut "Zhenzhu Hong" variety (RS1 - RS6) and those peanut RS genes available in public database were aligned by using CLUSTALX of MEGA 4 program. Amino acid residues common to all protein are highlighted in black, the lower amino acid residues common are highlighted in light gray.

sequence of RS genes in relation to those proteins from database (**Figure 4**). The phylogenetic tree analysis revealed that all of these protein group into a branches without AY170347. The evolutionary relationship also proves the high similarity and conservation of RS gene in peanut.

BLASTP searches revealed that there were some difference between the deduced amino acid sequence of cloned RS genes and the reference sequences: the identity of RS1 and AY170347 is 95%, and RS2-EU384706 is 99%, RS3-HM130568 IS 100%, RS4-JQ728483 is 98%, RS5-L00952 is 96%, RS6-X62300 is 98%. These sequence diversity indicated that although the RS gene exhibited interspecific variation.

3.3. Expression Pattern Analysis

In order to examine the differential distributions of RS genes in peanut kernel and shell tissue (separately extracted the total RNA from them) at different developmental stages in "Zhenzhu Hong" variety (the seven samples as described in material and **Figure 1**), the relative mRNA expression levels of six RS genes were evaluated by QRT-PCR (**Figure 5**). Six RS genes preferentially expressed in peanut shells in the seven sample examined, but only lower levels of them were evident in the peanut kernel. The expression levels of six RS genes in the peanut kernel were below 0.05 fold compare with the expression level of Actin1 gene. Five of the RS genes express in shells show the same pattern, the expression level of RS genes increase gradually with the developmental stage and reach at the top point of mature stage except RS4 being higher at main leaf elongation stage. Among the six RS genes expressed in shell, RS1 is the highest expression at 16 times normalized by Actin1 gene follow by were RS3 and RS6 at about 12 fold, RS2 is about 5 times and RS5 is 3 fold, while RS4 is at the lowest express level only about 1.5 fold normalized with Actin1 gene.

3.4. Subcellular Localization in Onion Epidermal Cell

To determine the subcellular location patterns of six RS proteins, GFP fluorescence signals were examined by confocal fluorescence microscopy in onion epidermal cells. We constructed a chimeric gene for the fusion of protein encoded by the six RS genes ORF to the N-terminus of synthetic green fluorescent protein. And chimeric

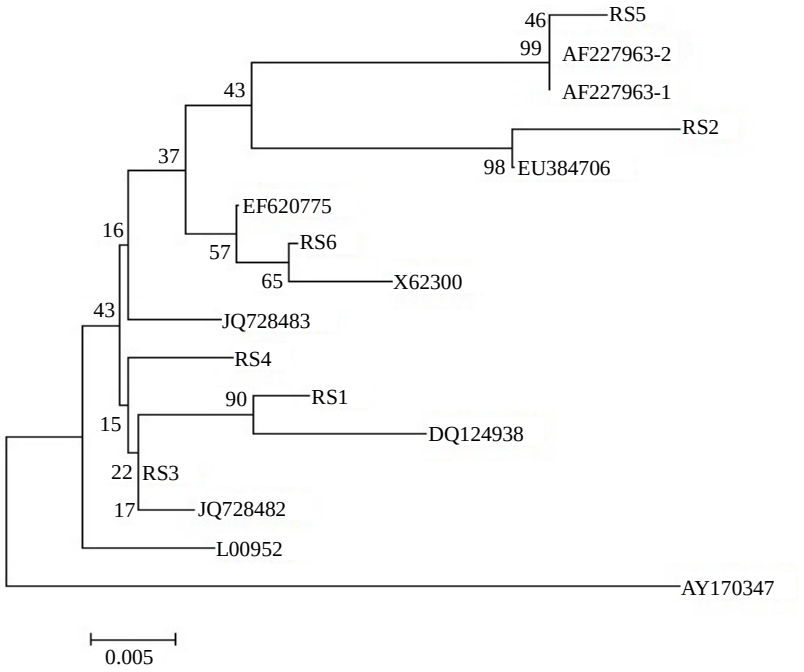


Figure 4. Phylogenetic tree analysis of RS gene in peanut by MEGA 4.0 program based on the amino acid sequences. RS1 - RS6: the deduced amino acid sequences for cloned RS genes from peanut "ZhenZhuHong" variety. AF227963, AY170347, DQ124938, EF620775, EU384706, JQ728482, JQ728483, L00952 and X62300 are the accession number of RS genes obtained from in public database.

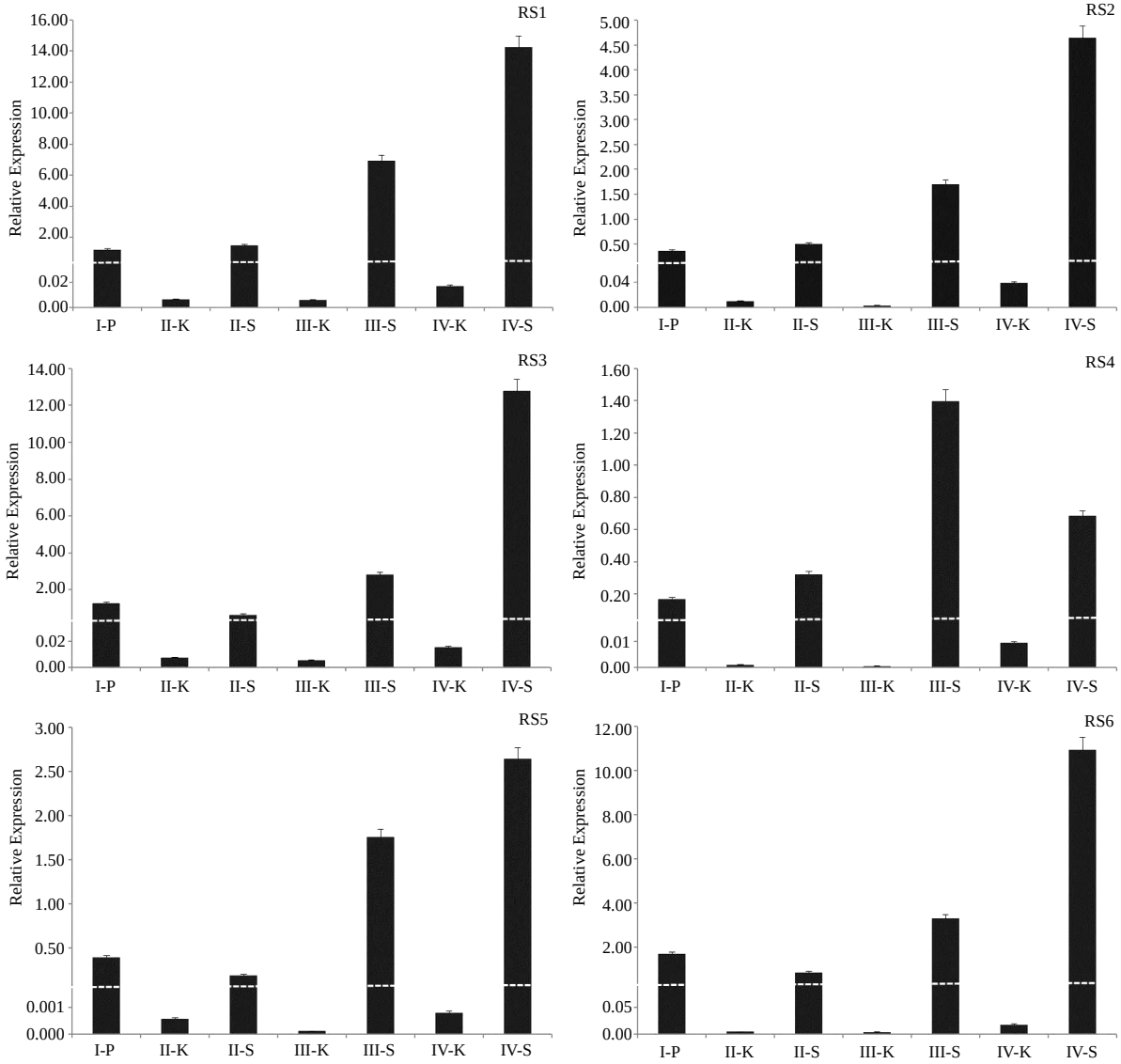
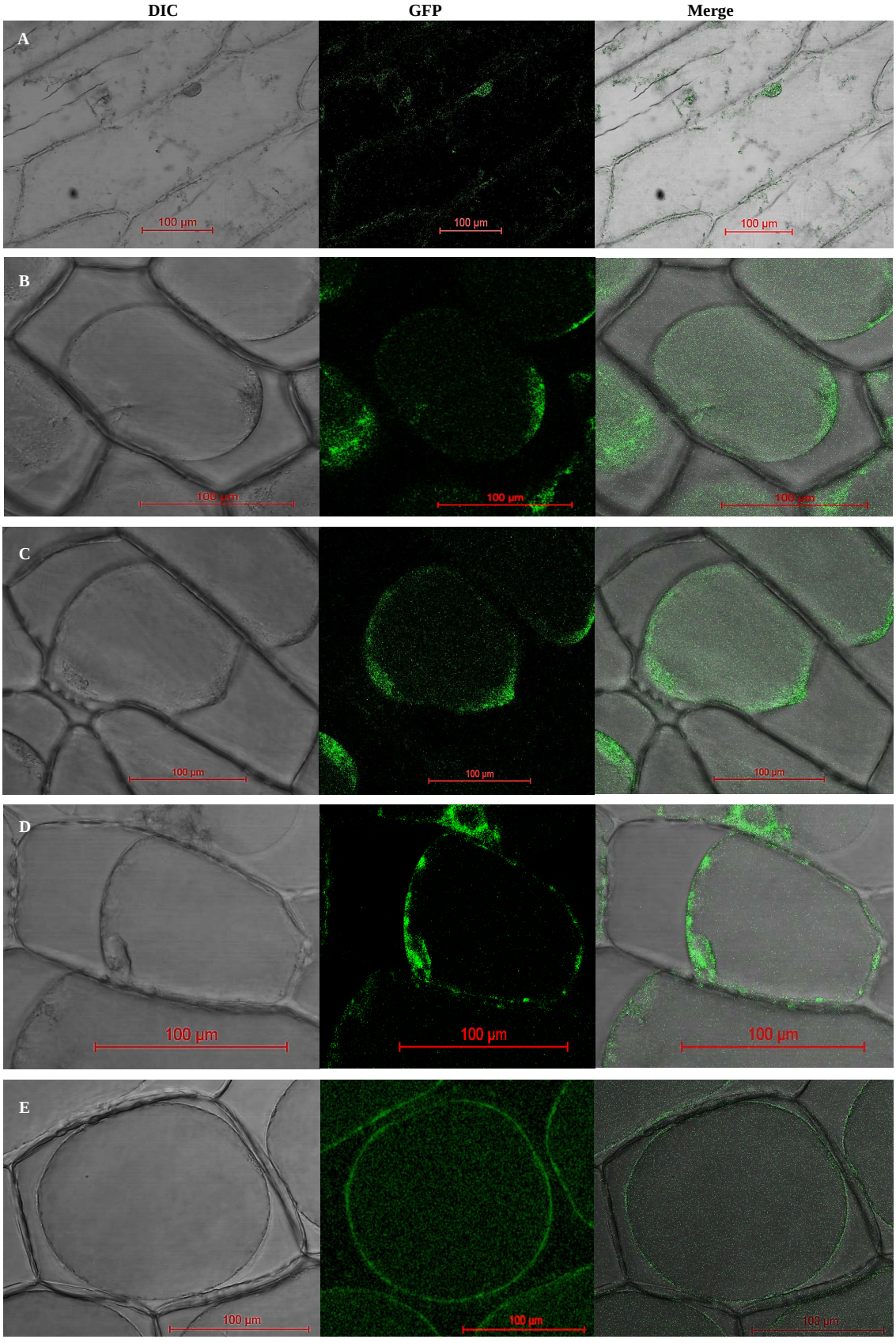


Figure 5. Expression pattern analysis of six RS genes in pods at different development stage by QRT-PCR. I: Tissue differentiation stag, II: Cotyledon elongation stage, III: Main leaf elongation stage, IV: Mature stage, K: Peanut kernel, S: Peanut shell. The values shown in this figure are the average of three independent expriment. Error bars represent the SD (n = 3) of relative mRNA expression levels of RS genes normalized to endogenous actin expression.

genes were introduced into onion epidermal cells by *Agrobacterium tumefaciens*-mediated transformation to express the fusion protein under control of the cauliflower mosaic virus (CaMV) 35S promoter. The sGFP fluorescence was imaged under a laser-scanning confocal microscope. **Figure 6**, $P_{CaMV35S}$::RS1:mGFP and $P_{CaMV35S}$::RS5:mGFP fusion protein were found to be expressed in the nucleus and plasma membrane respectively, while the $P_{CaMV35S}$::RS2:mGFP, $P_{CaMV35S}$::RS3:mGFP, $P_{CAMV35S}$::RS4:mGFP and $P_{CAMV35S}$::RS6:mGFP fusion protein were located in both nucleus inner membrane and plasma membrane.

4. Discussion

Prior investigations have provided documentary evidence indicating that resveratrol has been founded in more than 70 plant species and is mainly present in dietary plants, such as peanut, grapes and purple sweet potato It has been reported that RS genes are multiple copy in many plants [35] [36]. To learn more about the resveratrol biosynthesis in peanuts, we investigated the genes encoding RS distribution in peanut. In the present study, six


DIC
GFP
Merge
A
B
C
D
E
100 μm

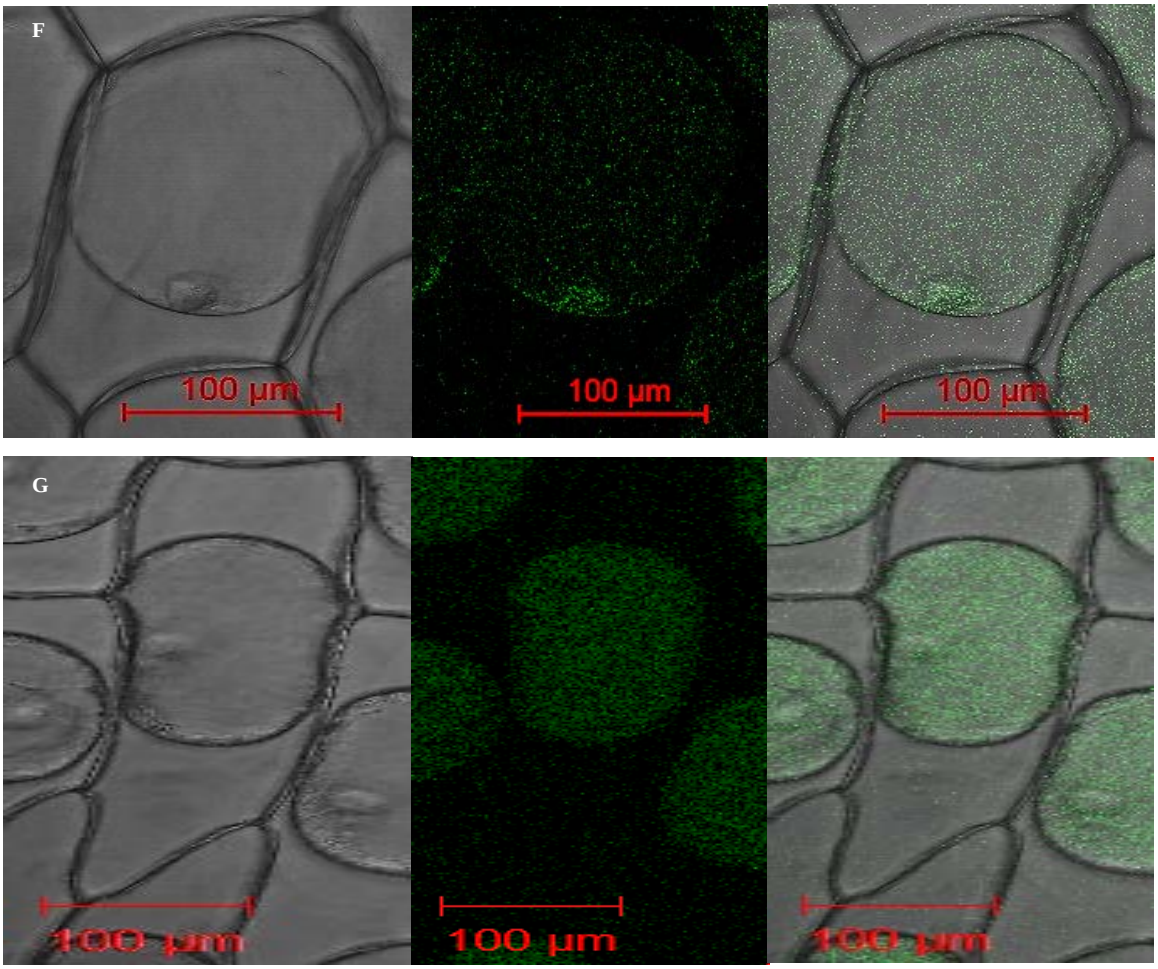


Figure 6. Subcellular localization of six RS genes. The six RS: GFP fusion protein and GFP only expressed under controlled of CaMV35S promoter in nion epidermal cells by *agrobacterium tumefaciens*-mediated transformation and monitored by scanning confocal microscope. A-F: RS1-RS6 fusion with GFP protein under control of CaMV35S promoter, G: pCAMBIA1302 Vector, the expression of GFP protein controlled by CaMV35S promoter.

RS genes were cloned and characterized from the peanut "Zhenzhu Hong" variety. The deduced amino acid sequence homologies among six RS genes were 95% - 99%, while the identity with those peanut RS genes in the public database among 92% - 98%, only contain a few of amino acid various in some varieties. All of the amino acid sequence contained Chal_Sti_Synt_N and ACP_Syn_III_C domain. This implies that RS genes were highly evolutionarily conserved in peanuts; which is also supported by the amino acid alignment (**Figure 3**) and phylogenetic analysis (**Figure 4**). These genes most likely fulfill the same function and their transcription is activated in response to different environmental conditions and developmental stages in the peanut. The concentrations of resveratrol in the plants is comparatively low under the natural cultivation, but when plants are exposed to bacterial, fungal and other serious biotic stresses or *UV* irradiation, wounding and other severe abiotic stresses, the phytoalexin resveratrol cannot be accumulated to a sufficient level if encoded by a single gene. Plants have overcome this shortcoming by evolving multiple copies of RS genes in order to counteract biotic and abiotic stress.

RS gene expression plays an important role in resveratrol synthesis and accumulation in plant tissues. The previous studies showed that, RS mRNA have detected in the peanut leaves, roots, pods, kernels, and kernel skins, and resveratrol relatively abundant in the peanut roots and shells but seeds when plants grown under the natural condition [37]-[40]. In the study, expression pattern analysis displayed the six RS genes were mainly expressed in the peanut shell at different developmental stages with different expression levels, but lower levels of them were evident in the peanut kernel. There is a correlation between resveratrol and RS mRNA accumulation,

and these results indicated tissue-specific distribution of RS gene and regulation of resveratrol synthesis in peanut. Since multiple copy RS gene present and express in peanut, On the other hand, these also indicate that peanut is an important dietary and available source of resveratrol, and resveratrol also accumulated by elicitor and abiotic stresses. And it has reported that, the expression of RS gene also can be induced by *UV* and hurt damage [20] [41] [42]. In addition, further investigation is required to determine whether all of accumulation in biotic and abiotic stress.

The particular subcellular location of RS protein was considered to be related to its physiological function in the enzymology research. The buckwheat RS protein is located in the cytosol and associates with the cytoplasmic and ER but not in nuclei plastids, mitochondria, Golgi, or tonoplasts [43]. Qiu *et al.* pointed out that RS protein in grape was mainly located on the cell wall [44]. Subcellular location of RS protein in this study showed that RS1 and RS5 were found to be expressed in the nucleus and plasma membrane respectively, while the RS2, RS3, RS4 and RS6 were located in both nucleus inner membrane and plasma membrane. It may indicate that RS2, RS3, RS4 and RS6 may play on same function and pattern, while RS1 and RS5 apart from them. The other explanation is that they function in different subcellular at the different development stage. The difference on the RS subcellular location in different organs indicated that different regulation mechanism might exist in different organs.

5. Conclusion

In summary, in this study six peanut RS genes has been isolated, characterized and analyzed the tissues expression patterns, and analyzed the localization of RS protein product. The data thus provide the molecular bases for future functional, regulatory mechanisms and enzyme kinetics studies of the peanut RS genes.

Acknowledgements

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