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Coupled transcript and metabolite identification: insights on induction and synthesis of resveratrol in peanut, wild relatives and synthetic allotetraploid

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ABSTRACT. Resveratrol is an antioxidant that is a promising antitumoral, cardioprotective and neuroprotective agent. It has been found in a restricted number of plants including peanut (*Arachis hypogaea* L.) and its wild relatives. The objective of this study was to understand the relationship between resveratrol content and the expression of putative resveratrol synthase genes in four *Arachis* genotypes. Two diploids and two tetraploid were analyzed. Contents of resveratrol on non- and UV-treated leaves were estimated using HPLC. Resveratrol synthase (RS) was analyzed using RT-qPCR with primers developed in this study. Sequences of six *Arachis* species were amplified using two degenerated primer pairs that were designed based on *Arachis* and general RS available at GenBank. Those sequences were used to qPCR primers design. Test and control leaves were collected

from plants cultivated in greenhouse and three biological replicates were evaluated for each genotype. The synthesis of resveratrol in leaves was induced by treatment with UV for 2.5 h. All genotypes studied synthesized resveratrol. Concentrations ranged from 193.66 µg/g in synthetic allotetraploid to 371.97 µg/g in *A. duranensis*. Natural and induced allotetraoploids showed lower levels of resveratrol than their diploid parents. Untreated samples did not produce significant amounts of resveratrol. The analysis of resveratrol content and levels of RS mRNA allowed the identification of one gene induced by the UV treatment. The data showed different amounts of RS in the different genotypes suggesting early and late response to the UV induction in the different species. The understanding of the variation found among species will help to identify species that have high resveratrol content and their ideal pos-induction times. This also will allow analysis of other tissues where high levels resveratrol would be very important, such as in seeds.

Key words: Resveratrol; *Arachis*; qPCR; HPLC; Resveratrol synthase

INTRODUCTION

Peanut (*Arachis hypogaea* L.) has high nutritional value due to its high content of proteins, oils, carbohydrates, minerals, and vitamins (Wu et al., 2009). Besides, it also synthesizes resveratrol (trans-3,5,4'-trihydroxystilbene) (Jeandet et al., 2012), that is a phytoalexin that is part of defense system to biotic and abiotic stresses, such as UV irradiation (Chung et al., 2003). It is also a powerful antioxidant that has been considered therapeutic for humans (Baur and Sinclair, 2006).

Resveratrol was first reported in peanut grains by Ingham (1976). Recently, it was also found in UV-treated leaves of ten wild species of the section *Arachis* (Lopes et al., 2013), including *A. duranensis* and *A. ipaënsis*, the progenitors of the cultivated peanut (Kochert et al., 1996).

Section *Arachis* is one of the nine taxonomic sections of genus *Arachis* and it comprises 31 species (Krapovickas and Gregory, 2007; Valls et al., 2013) that are phylogenetically very close to peanut (Bechara et al., 2010). Section *Arachis* species are found in different environments and because of that they may harbor resistant genes related to abiotic and biotic stress that are not found in cultivated peanut (Pande and Rao, 2001). The variation in many important traits have been evaluated in *Arachis* species to verify their value in peanut breeding programs, mainly in Brazil, which possesses the largest gene bank of wild species of *Arachis* (Fávero et al., 2009).

Resveratrol is synthesized by a one-step reaction (Hanhineva et al., 2009) of resveratrol synthase gene (RS) that is differentially regulated under different stimuli and in different tissues (Chung et al., 2003) and belongs to a large multigene family (Vannozzi et al., 2012). *Arachis hypogaea* RS genes were already cloned (Schröder et al., 1988; Huang et al., 2012). However, little is known about the RS in wild species of *Arachis*.

The study of RS expression in wild plants will shed light on resveratrol production in wild species, helping identifying their real potential as sources of resveratrol and alleles to improve cultivated peanut production.

The objective of this study was to evaluate the variation in resveratrol content and RS gene among five *Arachis* genotypes to get information that will help the better understanding of resveratrol synthesis among different *Arachis* genotypes.

MATERIAL AND METHODS

Material

Seeds of five *Arachis* genotypes (Table 1) were germinated in germitest paper. Plantlets were transferred to pots containing 500 g soil and kept regularly watered in a greenhouse for three months.

Table 1. Species, sample identifications, genome type, and ploidy level.

Species	Sample identification	Genome	Ploidy level
A. cardenasii	GKP10017	AA	2N
A. hypogaea	cv. IAC-Tatu	AABB	4N
	cv-IAC Runner	AABB	4N
A. duranensis	V14167	AA	2N
	K7988	AA	2N
A. ipaënsis	K30076	BB	2N
A. magna	KG30097	BB	2N
A. stenosperma	V10309	AA	2N
Synthetic Allotetraploid	*A. ipaënsis* K30076 X *A. duranensis* V14167	BBAA	4N

Resveratrol induction by UV

Fifty leaves from 15 plants of each genotype were collected in greenhouses few hours before the UV treatment. They were equally divided in two groups (treated and control) and placed in trays (45 cm x 30 cm) containing a layer of germitest paper under a layer of approximately 1 cm of cotton moistened with 200 mL water. UV induction in leaves was performed as described by Lopes et al. (2013). Leaves were placed at a laminar flow chamber (Trox Model FLV series: 235-81) under UV lamps (Philips TUV 30W/ G30 T8 Longlife) for 2 h 30 min at room temperature and control samples were maintained in the same conditions but under the dark. After that, both samples were maintained in the dark at room temperature for 15 h.

Leaves of each genotype had the leaflets detached from the petiole and homogenized within each treated and control group. One gram of leaflets was then transferred to two 50-mL tubes, immediately frozen in liquid nitrogen and stored at -80°C.

Samples of each genotype were divided so that half of the material was used to analyze the relative expression of RS and the other half to analysis of resveratrol content.

RS expressions analysis

Development of primers for RT-qPCR analysis

RS sequences of wild *Arachis* species were obtained using two pairs of degenerated primers (RsAra and RsGb) that were designed using two sets of sequences. The first set comprised only *Arachis* RS sequences identified using BLASTn, that were aligned using the Clustal X and primers were designed to conserved regions using PriFi (Fredslund et al.,

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2005). The selected primers were RsAra-F 5'CGCCACGAAGGCCATGMARGARTGGGG3' and RsAra-F 5'GGGCCGGGRTGNGCDAT3'. The second set comprised all RS and stilbene synthase of all species available at GenBank at that time. Those sequences were also aligned using Clustal X - jalview. The sequences were submitted to the Consensus-Degenerate Hybrid Oligonucleotide Primers (http://bioinformatics.weizmann.ac.il/blocks-bin/codehop.pl) to design primers to conserved regions of proteins. The selected primers of this second search were RsGb-F 5'CACATGACYGAYCTCAAGAARAAATTTCAGCGCAT3' and RsGb-R 5'GTTGCAGCCTCTTTTCCAACYCTTGGTACC T3'. Both pairs of primers were designed to amplicons to have about 600 bp length.

RsAra and RsGb primers were used to amplify genomic regions of *A. hypogaea* cv. IAC-Tatu, *A. hypogaea* cv. IAC-Runner, *A. cardenasii* (accession GKP10017), *A. magna* (KG30097), *A. stenosperma* (V10309), *A. duranensis* (K7988, V14167), and *A. ipaënsis* (KG30076). PCRs were carried out using 25 ng genomic DNA. The cycling conditions were 5 min at 92°C, and 35 times of the following cycle: 1 min at 92°C, 1 min at 55°C, 1 min at 72°C, followed by a final extension of 5 min at 72°C. The fragments obtained from each pair of primers and DNA of the seven genotypes were excised from the purified gel using DNA Geneclean® II kit. The fragments obtained for each species were cloned into the pGEM®-T Easy Vector. Twelve clones of each genotype were sequenced for each genotype.

The fragment sequences were aligned and a phylogenetic tree was obtained using the Similarity tree - Staden program. RT-qPCR primer pairs were designed using sequences from different clusters of the tree.

Alignments were fed into the Primer3-Plus software (Untergasser et al., 2012). (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) using the parameters defined by Morgante et al. (2011) and the Primique software was used (http://cgi-www.daimi.au.dk/cgi-chili/primique/front.py) when fragment specific pair of primer was not found. The primers selected are listed on Table 2.

Table 2. Sequences of primers selected based on the phylogenetic tree. Primer pairs identified as RSGbAhT or as RSArAhT were designed using resveratrol synthase gene sequences of *Arachis hypogaea* (Ah) and primer pairs identified as RSArAs or as RSGbAsA were based on sequences from *A. stenosperma* (V10309 accession) resveratrol synthase (As).

Primer designations	Forward	Reverse
RSArAs01	TCC TAA CAT GTG CGC ATA CAA	TTG CAG CCT CTT TTC CAA CT
RSArAs02	TCC TAA CAT GTG CGC ATA CAA	CAG CCT CTT TTC CAA CTC TT
RSArAs06	AGG AGA ATC CTA ACA TGT GC	GTT GCA GCC TCT TTT CCA AC
RSArAhT05	TCC TAA CAT GTG CGC ATA CAA	GTT GCA GCC TCT TTT CCA AC
RSArAhT06	CAA GGG TAT TGA TGG ATG TGA A	AAC GAC GGT GCC TTA TAT GC
RSGbAsA6b	CCT GGC GTT GAT TAC GAA CT	AGC AGC CTT GGT GGT ACA TC
RSGbAsA12	CCG TCC GTG AAG AGG TAC ATG	AAC CAC CAG CAC ACG AGC AC
RSGbAhG8	TCC GTC CGT GAA GAG GTA CAT G	AAC CAC CAG CAC ACG AGC AC
RSGbAhT14	GGC GTT GCA TTG CCT GGT GTT G	CAG CCA AGT CCT TAG CCA AGC G

Relative expression of RS genes

Leaves from treated and control samples were immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from 250 mg plant material using the method of Chang et al. (1993) followed by purification on Invisorb Spin Plant RNA Mini columns (Invitek, Berlin, Germany) to eliminate impurities. RNA integrity was checked at 1% agarose gel stained with ethidium bromide. Total RNA was quantified at 260 nm using the NanoDrop®

ND - 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and its purity confirmed as a 260/280 nm ratio above 1.8.

DNAse treatment and cDNA synthesis were carried out in subsequent steps, in the same tube. Genomic DNA contaminants were removed from total RNA by treatment with DNase (TURBO DNAfree™, Ambion, USA), according to the manufacturer instruction, followed by first strand cDNA synthesis performed at 42°C for 60 min on a Master Cycler thermocycler (Eppendorf AG, Hamburg, Germany) using SuperScript™ III RT and anchored Oligo(dT) (Invitrogen, Carlsbad, CA, USA), according to the manufacturer instruction. DNase and reverse transcriptase were heat inactivated in the tube and the resulted cDNA was directly used in RT-qPCR assays.

DNA contamination in cDNA samples was checked by RT-PCR using a pair of conserved primers that flank an intron region in *Arachis* (Actin C24 Fwd-5' GAGCTGAAAGAT TCCGATGC 3' and Actin C24 6Rev-5' GCAATGCCTGGGAACATAGTC3'), as previously described (Morgante et al., 2011), which allows the distinction between PCR products amplified from genomic DNA and cDNA templates.

Real-time PCR was performed according Morgante et al. (2011), with adaptations. qRT-PCR assays were performed in a F7300 Real Time PCR (Applied Biosystems, Foster City, CA, USA) using 5 mL Platinum SYBR Green qPCR Super Mix-UDG w/ROX kit (Invitrogen) in a 10-mL final volume reaction containing 2 mL diluted cDNA and 0.2 mM of each primer. The following two reference genes were used: *Arachis* Ubiquitin (Fwd-5'AAGCCGAAGAAGATCAAGCAC3', Rev-5'GGTTAGCCATGAAGGTTCCAG3') (Luo et al., 2005) and Ribosomal 60S (Fwd-5'GAGCTGAAAGATTCCGATGC3', Rev-5'GCAATGCCTGGGAACATAGTC3') (Jain et al., 2006). Those two pairs for primers were designed in intron flanking regions which also allows the distinction between PCR products amplified from genomic DNA and cDNA templates. For each sample, three independent analytical replicates were performed. The amplification conditions were: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, and a dissociation curve at 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. The result of the reaction was expressed in TC value (value for the number of cycles required for the fluorescent signal reaches detection threshold) and calculated by Real Time PCR Miner 2.2 program (Zhao and Fernald, 2005) as fluorescence values of the reactions. Individual results expressed as CT values were grouped according to species analyzed and treatment (treated and untreated) for statistical analysis.

The threshold cycle (Ct) was calculated by the RT-qPCR software automatically (threshold value at 0.2). Raw fluorescent data (normalized reporter values, Rn values) were also exported. Amplification efficiencies were calculated from raw fluorescent data (Rn values), using the Real-time PCR Miner program (Zhao and Fernald, 2005). Calculation of relative gene expressions detected using eight primer pairs were performed using REST-MSC (Relative Expression Software Tool-Multiple Condition Solver) software version 2 (Pfaffl et al., 2002). REST uses the Pairwise Fixed Reallocation Randomization Test© to calculate result significance and will indicate if the reference gene used is suitable for normalization.

Determination of resveratrol content in leaves

Resveratrol was extracted using a method modified from Potrebko and Resurreccion (2009). The internal standard (0.840 mL of phenolphthalein) was added to each sample immediately after maceration and solution phase were separated suing centrifugation at

10,000 rpm, at 25°C for ten minutes. The liquid phase was transferred to another tube and 5 mL 80% EtOH were added to the tube containing solid leaf residues, mixed and centrifuged again. This procedure was repeated three times. The same amount of hexane was added to the supernatant and the residue was diluted in 6.8 mL 15% ethanol prior to analysis by high performance liquid chromatography (HPLC). Acetonitrile gradient and 0.02% aqueous phosphoric acid was used as mobile phase: acetonitrile 0 min, 13%; 6-9 min, 15%; 17 min, 17%; 28-33 min, 28%; 40 min, 50%; 45 min, 60%; 46-48 min, 80%, 49-54 min; 13%; flow 1.0 mL/min. The UV absorption was monitored at 308 nm, 280 nm and also at the length of maximum absorption (PDA). The injection volume was 10 μL. Resveratrol was quantified by the addition of phenolphthalein to the extracts and standards, as internal standard (Lopes et al., 2013). Each sample was subjected to ultrasound bath for 4 min twice. The analysis of data was done according to Potrebko and Resurreccion (2009).

Correlation between the concentration of resveratrol and the relative expression of RS

Each treatment included three biological replicates. For data analysis, it was adopted the program for statistical language R - version 2.13.2, freely download at http://www.r-project.org (R 2012). A model of mixed regression was used to relate the results of resveratrol and RS relative expression.

RESULTS AND DISCUSSION

The amplicons obtained using both degenerated primers pairs had the expected size (around 500 bp). The 124 high-quality sequences from the two different sets of primers did not align, suggesting that the primers used allowed the amplification of different regions of the same gene or different genes (data not shown).

Phylogenetic trees were built based on sequences obtained using the two degenerated primer pairs. However, only the tree obtained using nucleotide sequences obtained using the primer pair that was selected based in *Arachis* sequences is presented (Figure 1) here because they show very similar results. Variation was found among sequences of each sample. In general, no 100% similarity was observed between sequences in both trees and sequences of each species were spread into different clusters having higher similarity to other species sequences than to those of the same species. The intra-specific variation might be due to the fact that RS gene family also happens in the wild species, as reported for the cultivated peanuts (Lanz et al., 1990). The amplification in all samples using primers designed using sequences from two different species suggested that RS gene family originated before speciation, at least in section *Arachis* that comprises the samples analyzed. RS gene family has not been studied in wild *Arachis*, but evidences show peanuts and their wild relatives have many similarities on gene content (Bertioli et al., 2016). Thus, we assume wild *Arachis* also have a RS gene family. The variation observed also is because the alignment used to get Figure 1 was based on nucleotide sequences instead of amino acid which probably would increase the similarity among the sequences since the conserved domains would show up.

Nine nucleotide sequences from different clusters of both trees were selected to analyze different RS genes and identify those that were induced by UV light. Sequences RSArAs01, RSArAs02, RSArAs06, RSArAhT05, RSArAhT06 were placed in different clusters as it can be seen in Figure 1. *A. hypogaea* and *A. stenosperma* sequences were chosen to primer design

among the species that had amplicons sequenced since the first is the cultivated and previous data on RS expression is available (Chung et al., 2003; Zhu et al., 2014) and the second is a source of genes for *A. hypogaea*, since some of its accessions had more resistance to some diseases that affect the cultivated one (Fávero et al., 2009).
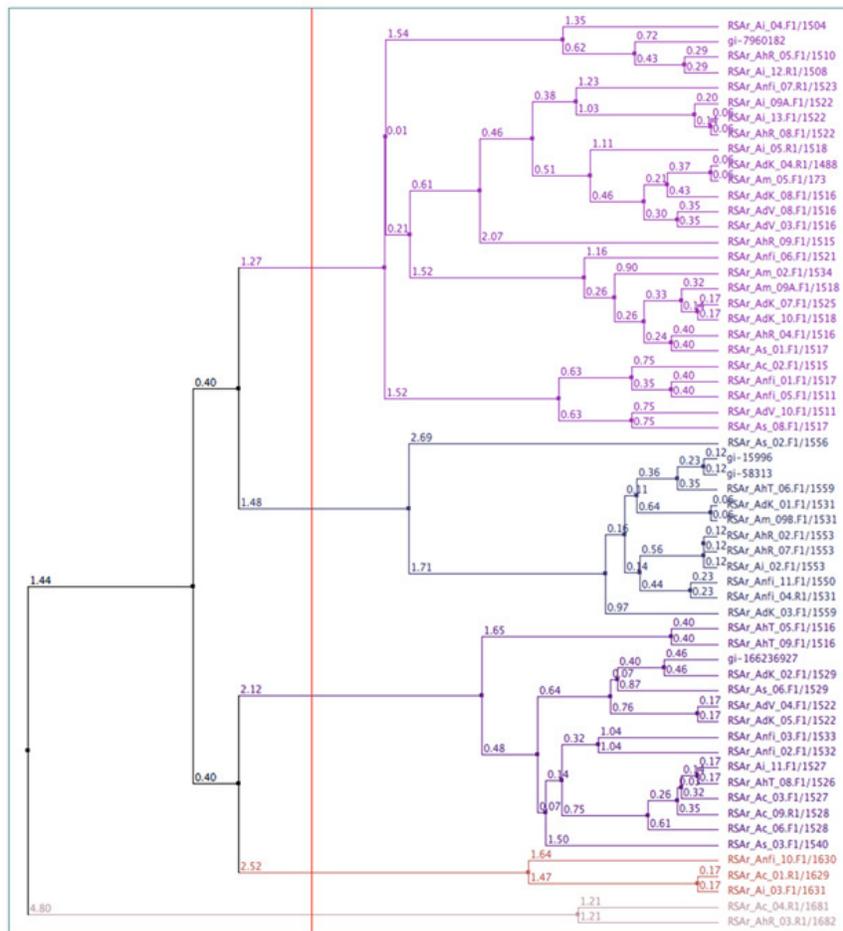

Figure 1. Phylogenetic tree obtained using DNA sequences of the fragments amplified through degenerate primers for *Arachis* RS (RSAR) and DNAs of six species of *Arachis* and a synthetic amphidiploid (*A. hypogaea*, *A. cardenasii*, *A. duranensis*, *A. magna*, *A. stenosperma*, *A. ipaënsis*, *A. duranensis*, *A.ipaënsis*) and RS sequences from GenBank (gi).

Eight of the nine pairs allowed the amplification of one specific sequence that had the expected size (150-200 bp) in all samples. The primer pair RSGbAsA12 resulted in two peaks (data not shown) indicating no specificity and it was not used for further analysis. The sequences of RS used for primer designing are probably in coding regions with important biological functions and therefore these sequences have been maintained in all species over time, before the emergence of different genomes in *Arachis* section.

RSGbAhT14, RSArAhT5, RSArAs2 and RSArAs6 allowed the identification of genes that were upregulated in samples treated with UV and RSArAs01, RSArAhT06, RSGensteA06b, and RSGenhypTG08 showed no differential expression between test and control in all four genotypes analyzed (Table 2).

Resveratrol content

The resveratrol content in each genotype samples was also assessed. Only traces of resveratrol were detected in all control samples, not allowing quantification. On the other hand, resveratrol was detected in all UV-treated leaves (Table 3).

Table 3. Relative frequencies of four transcripts and resveratrol content in UV-treated leaves in four *Arachis* genotypes. The superscript letters (a, b and c) indicate significant differences among resveratrol contents.

Sample	RT-qPCR (Normalized values)				HPLC (µg/g)
	RSArAs02	RSArAhT05	RSArAhT06	RSGenAhT14	
A. hypogaea	2.58	2.6	1.97	7.73	211.73[a]
Synthetic allotetraploid	6.4	7.72	14.96	14.93	193.66[b]
A. duranensis	30.17	15.21	15.29	30.13	371.97[c]
A. ipaënsis	58.84	116.71	29.47	58.84	225.85[a]

The superscript letters (a, b and c) indicate significant differences among resveratrol contents.

Data showed that accessions of *A. ipaënsis* and *A. duranensis* have higher concentrations of resveratrol than the two allotetraploids, which is a surprising result since many other studies showed differences between diploids and polyploids, the latter having better characteristics than the former (Fávero et al., 2009; Xu et al., 2014; Michelotto et al., 2015; Leal-Bertioli et al., 2015, 2017). The lack of polyploidy superiority has been previously observed for *Arabidopsis thaliana* and *Pyrus communis,* in which only a few metabolites differ between diploids and synthetic autotetraploids. The authors suggest that the higher amount of metabolites observed in naturally occurring polyploids may be due to secondary natural selection (Tsukaya et al., 2015). In the current case, this explains the lower amount of resveratrol in the synthetic allotetaploid, but not in the naturally occurring peanut. The lack of polyploidy superiority may be attributed to the time required for adjustment, adaptation, and evolution after the genomic shock induced by polyploidization (Caruso et al., 2013). Furthermore, since there is evidence on variation to UV stimulus (Correia et al., 1999) and if that variation to biotic stress response is reflected on gene expression pattern, the smaller production found in polyploids may be due to different reasons such as the variation on sensibility among the samples analyzed to UV stimulus, resulting in early and delayed responses. Therefore, a large set of *Arachis* diploids and allopolyploids must be analyzed to have a better view of the impact of polyploidization on resveratrol content variation.

RS gene expression and resveratrol content

The relationships between relative frequencies of transcripts identified using primers RSArAs2, RSGbAhT14, RSArAhT5, and RSArAs6 and the amount of resveratrol in each biological replicate of each genotype analyzed are shown in Figure 2. The samples were more scattered when resveratrol content was plotted against relative frequencies of transcripts

detected using primer pairs RSArAhT5 and RSArAs6. Grape has a RS gene family and the alleles have different behaviors under different treatments (Vannozzi et al., 2012). Thus, primers may have detected other RS genes that not have relationship with RS induction by UV, may being induced by other stimuli or they may be similar to a RS gene, like chalcone synthase genes.
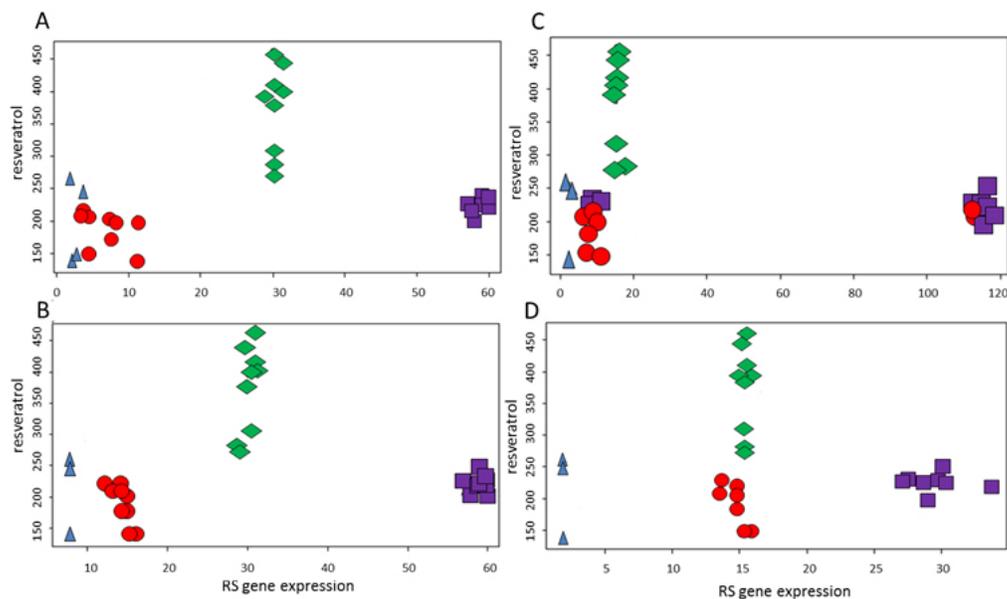

Figure 2. Relationship between the concentration of resveratrol and the relative expression levels of resveratrol synthase identified by the primers pairs RSArAs2 (**A**), RSGbAhT14 (**B**), RSArAhT5 (**C**), and RSArAhT06 (**D**) to *A. hypogaea* (blue triangle), synthetic allotetraploid (red circle), *A. duranensis* (green diamond), *A. ipaënsis* (purple square).

Biological replicates of each genotype had similar expression patterns for RSArAs2 and RSGbAhT14, suggesting those are part of a RS gene that were induced by UV treatment and they may amplify parts of the same genes. The function of these genes was previously validated by transgenic studies with *Nicotiana benthamiana* (Condori et al., 2009).

Previous analysis of RS in peanuts has been done using Southern blot (Lanz et al., 1990), but wild *Arachis* species RS had never been analyzed, so this is the first report of the use of RT-qPCR to analyze RS in cultivated and wild *Arachis* species. The use of sequences for primer design from different clusters in the tree (Figure 1) and the establishment of relation with resveratrol content was a successful approach to the identification of primers that allowed the analysis of the mRNA of genes involved in resveratrol synthesis under UV induction.

The behavior of the synthetic allotetraploid was similar to natural tetraploid (*A. hypogaea*) and when compared to the two species both showed low relative expression and production of resveratrol (Figure 2). *A. duranensis* showed higher concentrations of resveratrol, but the value of the relative expression was lower when compared to other samples analyzed. *Arachis ipaënsis* had intermediate concentrations of resveratrol despite it had the highest values of relative expression. Positive correlation between resveratrol content and level of a

RS mRNA was observed in *A. hypogaea* and that was explained by transcriptional control of RS activity (Chung et al., 2003). Based on the assumption diploids and synthetic allotetraploid have similar behavior to *A. hypogaea*, the higher expression of RS on tetraploids may have happened 15 h after treatment, since low relative expression and production of resveratrol. *A. duranensis* had the highest level of resveratrol in relation the other analyzed species when compared with the others three genotypes analyzed the values of genetic expression was lower than in *A. ipaënsis*. If *A. duranensis* has similar behavior to the cultivated peanut, the data suggested that larger amounts of resveratrol may be found before 15 h, because the transcript levels compared to found in the other species were not the highest observed, suggesting the beginning of a reduction in its expression. The results suggested a late induction in *A. ipaënsis* since the transcript levels were the highest and the resveratrol level was the lowest at the collection point. If *A. ipaënsis* has behavior similar to *A. hypogaea*, the best point for analysis of resveratrol in this species can be reached 15 h after treatment. This was also observed in species of grapevine (wild and cultivated) one sequence of RS had different expression levels in various hours post inoculation with *Uncinula necator* for each genotype, showing a species-specific expression profile (Xu et al., 2011).

This is the study trial of RS using RT-qPCR in *Arachis* and the first one in wild *Arachis* species. The successful results here obtained will allow future studies including more periods after induction to understand better the kinetics of resveratrol production and its relationship with resveratrol synthase control.

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