PROSPECTING OF TRANSCRIPTS EXPRESSED DIFFERENTIALLY USING ISSR MARKERS IN PEANUT SUBMITTED TO WATER STRESS

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ABSTRACT: Abiotic stresses affect many processes related to yield in several crops. The ability to response to these events depends on the defense mechanism and stress level that each crop is submitted. In peanut, such events have been studied via physiological and biochemical assays, associated to molecular tools, generating the best understanding of associated factors to resistance. In this paper, a prospective study of differentially expressed genes was performed in a drought-tolerant peanut variety submitted to water stress, using ISSR primers as molecular tool. RNA was collected when plants reached 50% of stomatal closure (measured through porometry) from youngest fully expanded leaf at 9:00 am. Reverse transcription was performed using ISSR primers that amplified 45 transcripts from different expression levels. All activated transcripts obtained from stressed plant were sequenced. The obtained sequences were analyzed in Arabidopsis thaliana and Arachis databanks and just four ones showed homology with known genes that are involved in metabolic pathways in response to abiotic and biotic factors.

Index terms: molecular markers, *Arachis*, abiotic stress.

PROSPECÇÃO DE TRANSCRITOS DIFERENCIALMENTE EXPRESSOS POR MEIO DE MARCADORES ISSR EM AMENDOIM SUBMETIDO A ESTRESSE HÍDRICO

RESUMO: Estresses abióticos afetam muitos processos relacionados à produtividade de várias culturas. A habilidade de responder a estes eventos depende do mecanismo de defesa e do nível de estresse a que cada cultura é submetida. No amendoim, tais eventos têm sido estudados por meio de ensaios fisiológicos e bioquímicos associados a ferramentas moleculares, gerando uma melhor compreensão quanto aos fatores associados à resistência. Neste trabalho, uma prospecção de genes diferencialmente expressos foi realizada utilizando-se primers ISSR em uma variedade de amendoim resistente à seca submetida ao estresse hídrico. O RNA foi coletado de folhas jovens completamente expandidas, às 9 horas, quando as plantas atingiram o fechamento dos estômatos em 50% (medido por meio de porometria). Reações de transcriptase reversa foram realizadas utilizando primers ISSR. Quarenta e cinco fragmentos foram amplificados com diferentes níveis de expressão. Todos os transcritos ativos, obtidos das plantas estressadas, foram sequenciados. As sequências foram analisadas em bancos de dados de *Arachis* e *Arabidopsis thaliana* e apenas quatro apresentaram homologia com genes conhecidos, envolvidos em vias metabólicas em resposta a fatores abióticos e bióticos.

Termos para indexação: marcadores moleculares, *Arachis*, estresse abiótico.

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INTRODUCTION

Peanut is an economic expressive crop worldwide. It is cultivated in a number of countries under the most diverse environmental conditions. The plant exhibits considerable plasticity in its physiological mechanisms, which enables it to adapt itself to environments with significant climatic variations, especially in semiarid regions, where a large area of production is concentrated (BEGHIN et al., 2006; GRACIANO et al., 2011).

According to Azevedo Neto et al. (2010), under conditions of water scarcity, the peanut triggers a genetic command to deepen its roots and extract water from a depth in order to prevent desiccation during periods of low precipitation. Production, however, is reduced because the water deficit decreases the metabolism of the plant and reduces the synthesis of essential secondary compounds for the proper structural development.

Despite the ability of peanut to tolerate semiarid environments, water stress reduces its growth. However, depending on the development stage, vegetative and reproductive growths may not be influenced in the same way. For example, early drought in the growing season of upright and runner genotypes has a relatively small effect on pod productions than if it takes place since the phases of flowering until grain filling (NOGUEIRA; TÁVORA, 2005). In addition, water deficit alters several physiological processes in plants, including an increase in diffusive resistance to water vapor due to stomatal closure, what reduces transpiration and, consequently, the CO_2 supply for photosynthesis. Many of these effects reflect plant adaptation mechanisms to the arid environment. Other associated mechanisms to drought tolerance have been reported, such as the root growth or a number of linked attributes to the plant phenology. Genotypes that have high partition coefficients (greater efficiency in the translocation of photoassimilates to the reproductive organs), for example, exhibit less tolerance to water stress (AZEVEDO NETO et al., 2010; NOGUEIRA; TÁVORA, 2005).

In Brazil, the greatest area of peanut production is concentrated in the Southeastern region. However, farmer's number in the Northeastern region have increased due to peanut short cycle, adaptation abilities and attractive market (SANTOS et al., 2010). Climatic adversity is constant in this region, especially regarding the distribution and amount of rainfalls. Thus, the selection of tolerant genotypes to the semiarid environment is a constant concern in peanut breeding programs.

According to Miyasaka and Medina (1981), the abiotic component affects the efficiency of the photosynthesis process on the molecular level through the desiccation of the cytoplasm, as well as on the physiological level due to stomatal closure. As mechanisms of tolerance to biotic and abiotic factors are complex on the molecular level, studies in this area are essential to the understanding of physiological and biochemical responses when plants are submitted to them, because genes may be associated to a combination of both.

The literature describes several studies that have identified cultivars that are resistant to biotic and abiotic stresses (MISHRA et al., 2005; RENSINK et al., 2005) by molecular tools. In peanut crops, Jain et al. (2001) identified down and up-regulated transcripts from resistant and sensitive genotypes to water stress. Bertioli et al. (2003) identified genes involved in the mechanism of disease resistance in Arachis varieties. The identification of such genes can significantly contribute to the understanding of mechanisms associated to water stress and serves as reliable molecular markers in studies involving assisted selection for genetic breeding and thereby minimizes the often incurred operational costs under field conditions.

A number of PCR-based methods has been adapted for the identification and characterization of differentially expressed genes, since Differential Display (DD), cDNA-

AFLP and subtractive libraries to more sophisticated methods, such as Microarray and SAGE. All of them have their own peculiarities, limitations, advantages and operational costs (MISHRA et al., 2005).

The aim of this study was prospecting differentially displayed genes through the PCR methods in peanut plants submitted to water stress.

MATERIAL AND METHODS

The African peanut cultivar 55 437 was selected to the present study, because it is highly tolerant to semiarid conditions, has short cycle (75 to 85 days) and earliness (SANTOS et al., 2010). Seeds were obtained from Cotton Brazilian Agricultural Research Company (Embrapa Cotton) (access CNPA 76 AM) and were sown in pots containing soil previously corrected and fertilized under greenhouse conditions. Plants were daily watered for 25 days as recommended. Afterward, they were divided in two treatment: control (plants were normally watered) and stressed plants (plants were hold without water until reach 50% of closed stomata for five days). Transpiration and diffusive resistance were daily monitored with a LICOR dynamic equilibrium porometer (model LI/ 1600). By previous tests, all the analyzes of the expanded leaves on the upper third of the main axis were daily performed at 9:00 am. On the fifth day following the water reduction, when the stressed plants reached 49.7% of stomatal closure in comparison to the control ones, some leaves were collected and conditioned in liquid nitrogen for the immediate extraction of total RNA.

RNA extraction and RT-ISSR reactions

Total RNA was extracted from fresh expanded young leaves (1g) using Trizol reagent (Invitrogen). RNA purity and concentration were analyzed in denatured agarose gel (1.2%) and in a spectrophotometer (FEMTO, model 700S).

The reverse transcription reaction (RT-PCR) was performed using the M-MLV kit (Invitrogen), according manufacturer's recommendations: 25 pmol of each primer (Table 1), 12.5 mmol L^{-1} of dNTP and 100 ng of RNA were added to a final volume of 20 μL. PCR amplifications were performed in 50 μL of reaction mixture with the following reagents: 5 μL of cDNA, 10 μmol L^{-1} of primers, 1 unit of Taq DNA polymerase, 0.25 mmol L^{-1} of each dNTP, 25 mmol L^{-1} of $MgCl_2$ and 1x reaction buffer (Invitrogen). Ten ISSR primers from the UBC series (University of British Columbia) were used. The PCR program was performed in a termocycler (Mastercycler gradient, Eppendorf) programmed as follows: an initial step at 95 ºC/ 5 minutes, 30 cycles of denaturing at 94 ºC/ 30 seconds, annealing at 48 ºC/ 45 seconds, extension at 72 ºC/ 2 minutes and a final extension cycle at 72 ºC/7 minutes. The ISSR amplification products were mixed with equal volumes of loading dye (98% formamide, 10 mM EDTA and 0.05% xylene cyanol) and SYBR Gold (Invitrogen) and loaded into a agarose gel (0.8%). A 1 Kb marker (Ladder plus, Invitrogen) was used to estimate band sizes. Further, gel was analyzed under UV light and digitally photo-documented (Bio-Imaging System, model MiniBis Pro).

Purification of amplified fragments and cloning

All molecular procedures were carried out as manufacturers' recommendations. The obtained activated transcripts from stressed plant were purified using SNAP kit (Invitrogen) and cloned in pGEM®-T Easy Vector System (Promega). Transformants were obtained by eletroporation using a MicroPulser Electroporator (Bio-Rad Laboratories) using *Escherichia coli*, INVF cell (Invitrogen) and plasmidial DNAs (minipreps) were performed using Wizzard kit (Promega). The quality and concentration of plasmidial DNAs were verified in agarose gel (0.8%).

TABLE 1. ISSR primers used in differential display and number of transcript identified in peanut plants submitted to water stress.

Primers (UBC)	Sequence	Down-regulated	Up-regulated	Activated
1- 827	5'- ACACACACACACACACG -3'	04	01	01
2- 884	5'- H^1B^2HAGAGAGAGAGAGAG -3'	01	-	01
3- 813	5'- CTCTCTCTCTCTCTCTT -3'	03	-	-
4- 820	5'- GTGTGTGTGTGTGTGTC -3'	01	-	01
5- 808	5'- AGAGAGAGAGAGAGAGC -3'	02	-	02
6- 874	5'- CCCTCCCTCCCTCCCT -3'	01	-	-
7- 812	5'- GAGAGAGAGAGAGAGAA -3'	01	01	02
8- 846	5'- CACACACACACACACAR^{3}T -3'	02	-	03
9- 858	5'- TGTGTGTGTGTGTGTGTGR^{3}T -3'	02	-	-
10- 838	5'- TATATATATATATATAR^{3}C -3'	–	–	–
Total	10	17	02	10

Degenerated bases: H^1: A, C or T; B^2: C, G or T; R^3: A or G.

Sequencing of samples

One aliquot of each miniprep (20 ng/μL) was sequenced at the Human Genome Research Center, of the Universidade de São Paulo, Brazil. The obtained nucleotide sequences from the active transcripts were analyzed using the BLAST program (Basic Local Alignment Search Tool) from the National Center for Biotechnology Information-NCBI (http://www.ncbi.nlm.nih.gov), focusing on the available *Arabidopsis thaliana* and *Arachis* databanks.

RESULTS AND DISCUSSION

Forty four fragments were obtained, of which twenty nine were down regulated (C1, C2, C3, C4, C5, C6, C7, C8 and C9; Figures 1A, 1B and 1C), three were up regulated (E1 and E7; Figures 1A and 1C, respectively); and thirteen were only activated in stressed plants (E1, E2, E4, E5, E7 and E8; Figures 1A, 1B and 1C). Primer 812 generated the greatest number of transcripts – eight low expressed, one high expressed and two activated. However, the greatest number of activated transcripts was obtained with primer 846 (Table 1). Both primers are rich in AC and AG repetitions.

Among the sequenced activated transcripts, just four genes are involved in stress mechanism pathways when compared to the *A. thaliana* and *Arachis* databanks. Table 2 shows a summary of the main results from the sequenced transcripts.

The primer 812 transcript showed homology with a class of TIR-NBS-LRR proteins from *A. thaliana* (NM123889), with lysine-rich motifs, and is involved in plant defense processes against disease, as transcript 884 (NM128455). According Meyers et al. (1999) and Tameling et al (2002), these genes are associated to resistance to biotic stress in superior plants, as well as the programmed cell death in hypersensitivity response. This last event may be related to the stress effect to which the peanut plants were submitted.

The transcript 808 showed homology with a gene that codifies for arabinogalactan proteins (AGPs) from glycoprotein family. These proteins belong to a structural class present in the leaves, stems and roots of all

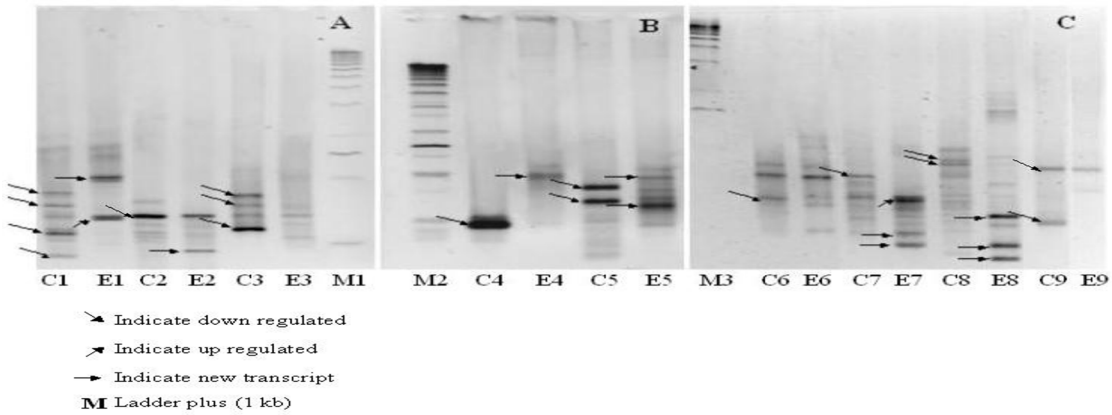

FIGURE 1. Differentially displayed transcripts in stressed (E) and control (C) peanut plants. A- primer 812, B- primer 808, C- primer 846. M1, 2 and 3- DNA marker, 1 Kb (Invitrogen).

TABLE 2. Result of BLAST sequence alignment from four clones obtained from active transcripts in stressed plants.

Clone (bp)	Access number	Description	Identity (%)
812 (400)	NM123889.1	*Arabidopsis thaliana* disease resistance protein (TIR-NBS-LRR class)	82
808 (700)	NM116972.4	*Arabidopsis thaliana* AGP10 (Arabinogalactan protein 10)	80
884 (400)	NM128455.2	*Arabidopsis thaliana* leucine-rich repeat protein kinase	84
	AY747418.1	*Arachis hypogaea* isolate PLTRP2G09 resistance protein PLTR gene	76
846 (300)	NM001085124.1	*Arabidopsis thaliana* peptidyl-tRNA hydrolase family protein	84
	AY157777.1	*Arachis cardenasii* clone C8_Y_57 resistance protein gene	80

superior plants (SHOWALTER, 2001) and constitute a class of proteoglycans that are essential to the regulation of expansion and cell division (SCHINDLER et al., 1995).

According to Bohnert et al. (1995) and Shinozaki et al. (1999), the functions of AGPs may be associated to the regulatory processes that are initiated during the loss of water from the cell, when the cellular metabolism is adjusted and the genes are induced to promote an increase in the protection of the cytoplasm, organelles and cell membranes, as well as alterations in cell osmotic potential and a greater regulation in other genes expression. Thus, the homology of transcript 808 with directly involved proteins in cell adjustment processes suggests a possible relationship with water stress response pathway associated to the genes.

The transcript 846 showed homology with proteins from the hydrolase family

(peptidyl-tRNA), both from *A. thaliana*. When it was compared in the *Arachis* databanks, however, only transcripts 884 and 846 showed homology with biotic stress pathways genes, both of them which are linked to disease resistance factors of the type PLTR (AY747418) and NBS (AY157777), respectively.

The exposure of plants to a particular stressing condition triggers a specific response which is related to the current state of the plant, which generates biochemical and physiological processes that are interrelated to the stress. Regarding the molecular aspect, Mittler (2006) reports that, for each stressing condition to which the plant is subjected, a unique response is generated and little overlapping in the display of transcripts is found when stressing conditions are from abiotic origin, as the ones caused by heat, drought, cold, salinity, high temperature or mechanical damage. There are cases, however, in which gene pathways involved in events of a biotic origin coincide with abiotic events, which promote common pathways for both types of stress. This is the case, for example, of the *A. thaliana* ROS genes (Reactive Oxygen Species), which act in association under both kinds of stress conditions. According to Pereira (2010), water stressed plants also under high temperatures generate high production of ROS due to these conditions what limits the availability of CO_2 in the dark reaction and leaves oxygen as one of the main reactive products of the photosynthesis.

CONCLUSIONS

It was verified that most of the transcripts were associated to biotic stress, particularly resistance to disease, by the sequencing results. Differences in the genetic expression level were observed in some transcripts that exhibited more intense band patterns in the control samples than the ones in the stressed samples.

Differentially displayed products were obtained by ISSR primers cloning when plants were exposed to water stress. However, to achieve a better understanding of the interrelations of biotic and abiotic events on peanut plants, further studies must be performed and associates physiological and molecular aspects.

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