Identification of expressed genes in early maturated fruit of pequi (*Caryocar brasiliense* Cambess.)

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Abstract - The pequi (*Caryocar brasiliense* Cambess.) is a species native to the Brazilian Cerrado and plays an important social and nutritional role for the population of the region. It is a species that is traditionally gathered and today is seen as a source for the production of biodiesel. The objective of this study was to identify sequences from a cDNA library that are involved in the metabolic pathways in early maturated fruit stage of pequi. To do so, a cDNA library was created and the clones were cloned and sequenced. These identified sequences were deposited in GenBank. Most of the sequences found using the Blastn and tBlastx tools are associated with protection against oxidative stress in plants, some are transcription factors and others provide structural and pathogenic resistance. The transcriptome of pequi is an effective method of identifying genes and proteins responsible for important biological characteristics of the plant, thus laying the groundwork for new studies aimed at developing a full understanding of the genomic structure of the pequi**.**

Keywords: pequi, *Caryocar brasiliense*, ESTs, molecular biology

Resumo - O pequi (*Caryocar brasiliense* Cambess.) é uma espécie nativa do cerrado brasileiro e que possui importância alimentar e social para a população que dela depende. É uma espécie que sofre ação extrativista e que, atualmente, é considerada fonte para a produção de biodiesel. Nesse trabalho o objetivo foi identificar, a partir de uma biblioteca de cDNA, seqüências que estejam envolvidas em vias metabólicas operantes no estágio inicial de maturação do fruto do pequi. Dessa forma foi construída uma biblioteca de cDNA, cujas sequências foram clonadas, isoladas e submetidas ao sequeciamento. As sequências identificadas foram depositadas no GenBank. A maior parte das sequências encontradas tanto no Blastn quanto no tBlastx estão relacionadas à proteção contra o estresse oxidativo em plantas, outras são fatores de transcrição e outros apresentam funções estruturais e de resistência a patógenos. O transcriptoma do pequi é um método efetivo na identificação de genes e proteínas responsáveis por características biológicas importantes da planta. Dessa forma, possibilita novos estudos que visem a compreensão total da estrutura genômica do pequi.

Palavras-chaves: pequi, *Caryocar brasiliense*, ESTs, biologia molecular

INTRODUCTION

The pequi (*Caryocar brasiliense* Cambess) is a medium-sized tree, native to the Brazilian Cerrado and Amazon region, which can reach 5 m in height at maturity. It requires little with regard to soil conditions, and generally grows in deep, porous and acidic soils that are poor in calcium, magnesium and organic material (Silva & Jesus, 2007).

There are reports of good adaptation to both wet and dry soils and good resistance to periods of drought (MDA/SAF, 2006). The species has economic and social value in the Cerrado region, particularly in the north of Minas Gerais (Ribeiro et al., 1994, Santos & Aoki, 1992, Silva et al., 1997, Ribeiro et al., 1997). The pulp (mesocarp) of the fruit is consumed for its high concentration of vitamin A, which plays an important role in bone, dental, capillary and skin structure as well as vision (Carvalho & Burger, 1960). It also contains B1 and B2 vitamins, fats, calcium, phosphorus, fiber, proteins and iron. This means the fruit can help curb nutritional deficiencies found in populations that live in the Cerrado, a region with few nutritional alternatives. The oil is used in popular medicinal remedies in the region for influenzas and bronchitis (Almeida & Silva, 1994, Ribeiro, 2000), as a condiment (Peixoto, 1973,

Almeida & Silva, 1994), lubricant (Lisboa, 1931, Peixoto, 1973) and in the cosmetics industry (Heringer, 1970, Peixoto, 1973, Almeida & Silva, 1994). More recently, the pulp of the fruit, after

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esterification to make it suitable for combustion, has been used to produce biodiesel. Yielding 3,200 kg of oil per hectare, the pequi is roughly 8 times more productive per hectare than soybeans (MDA/SAF 2006). The leaves of the pequi are considered medicinal, since they stimulate the secretion of bile (Brandão et al. 1992) and the ethanolic extract of the leaves has been shown to be effective against sarcoma 180, a type of skin cancer (Oliveira et al., 1970, Oliveira et al., 2005).

The advent of genome sequencing technology has generated information from DNA sequences of many plant species. With the availability of genome databases, we can predict the possible function of a gene based on its sequence information and identify candidate genes involved in a specific biochemical pathway (Ramalingam et al., 2003). Candidate genes or DNA sequences with predicted function are being used as molecular markers to associate phenotypes expressed in germplasm collections or segregating populations (Hu et al., 2003). The use of markers based on candidate genes, the characterization of germplasm constituted a useful tool in the analysis of genetic diversity, which increases the likelihood of the observed variability reflect phenotypic differences, which can not happen with the polymorphism reflected by the random markers (Benchimol, 2008).The study of the functional genomics or transcriptome of the pequi will allow the elucidation of the function of genes involved in the processes of differentiation and development and/or processes involved in responses to changes in the biotic or abiotic environment. Specific transcripts within the cDNA library create a quantitative and qualitative profile of different tissues, types of cells and stages of development, enabling the study of gene expression and genetic mapping (Boguski & Schuler, 1995, Brendel et al., 2002, Ronning et al., 2003, Rudd, 2003, Souza-Júnior et al., 2005).

The objective of this study was to identify sequences from a cDNA library that are involved in the metabolic pathways in early maturated fruit of pequi to gain a better understanding of the genetic events associated with the its maturation.

MATERIALS AND METHODS

Collection area

Pequi in early maturated fruit stage was collected in an urban area, in the São Jorge neighborhood of the city of Uberlândia in the state of Minas Gerais. The experiments were carried out in the Genetics Laboratory of the Uberlândia Federal University Institute of Genetics and Biochemistry, located in the Minas Gerais city of Uberlândia in the Brazilian central-west.

RNA Extraction

The Trizol method was used to extract RNA from the fruit. One hundred milligrams from the endocarp of the fruit was macerated, with liquid nitrogen, in a sterilized crucible with DEPC and autoclaved. One milliliter of Trizol was then added to the macerated flesh and the solution was transferred to 1.5 mL tubes and vortexed for 5 minutes. After agitation, 0.2 mL of cloroform/ml of trizol was added. This solution was again vortexed for 15 seconds and left at 30°C for 2 minutes, after which time it was centrifuged at 12,000g for 15 minutes at 4°C. The aqueous phase was transferred to a new 1.5 mL tube, and 0.5 mL of isopropanol/ml of trizol was added, mixed and incubated at room temperature for 10 minutes and then centrifuged at 12,000g for 10 minutes at 4°C. The liquid phase was discarded and the RNA precipitate was washed with ethanol 75% and centrifuged at 7,500g for 5 minutes at 4°C. The precipitate was air dried for 15 minutes and resuspended in water treated with 0.1% DEPC. The RNA was kept in an ultrafreezer at -80ºC. The amount of RNA extracted was estimated using a spectrophotometer, model GBC-UV/VIS911A (SONY), set at an absorbance reading of 260nm. This quantified and evaluated sample was diluted in milliQ water to a concentration of 2 Ug/µL, and kept in na ultrafreezer at -80ºC.

Extraction of messenger RNA

The mRNA was extracted using the *Micro-Fast TrackTM 2.0 kit (Invitrogen)* following the manufacturer's recommendations.

Construction of the cDNA library

The cDNA library was built using the *Clone MinerTM cDNA Library Construction kit (Invitrogen)* following the manufacturer's recommendations.

Plasmid Extraction

The colonies transformed after electroporation and plating in LB (Luria Bertani) medium were selected and placed to grow on plates for extraction of plasmids. To each well, 240 µL of GET solution was added, after which the plate was sealed with adhesive and vortexed for 2 minutes to resuspended the cells. The slide was centrifuged for 9 minutes at 3,700 rpm and the supernatant was discarded. Again 80 µL of GET solution was added and the slide sealed and vortexed until the cells were resuspended.

Next, 2 µL of RNase (10 mg/µL) was placed in each well of a new plate and the cell suspension was transferred to it. To this suspension, 60µL of recently prepared NaOH 0.2N/SDS1% was added. The plate was then sealed, inverted 30 times and incubated at room temperature for 10 minutes. To this suspension 60µL of recently prepared cold NaOH 3M (see attachment) was added. The microtitre plates was then sealed, inverted 30 times and incubated at room temperature for 10 minutes. The adhesive was removed after this period and the open plate incubated at 90ºC for 30 minutes. It was then sealed and cooled in crushed ice for 10 minutes and centrifuged for 9 minutes at 3,700 rpm and 20ºC.

The supernatant (100 µL) was transferred to a Millipore filter plate and centrifuged for 6 minutes at 3,700 rpm and 20ºC. The Millipore plate was removed and 100 µL of filtered isopropanol was added. The microtitre plates was again sealed with new adhesive, inverted 30 times and centrifuged for 45 minutes at 3,700 rpm and 20ºC. The adhesive was removed and the supernatant discarded. Then 150 µL of cold ethanol 70% was added to the precipitate. The slide was centrifuged for 10 minutes at 3,700 rpm and 20ºC. The supernatant was discarded and the plate inverted on absorbent paper for 15 minutes at room temperature. The plamid DNA was resuspended in 10 µL of milliQ water. The microtitre plates was covered with new adhesive and left at room temperature overnight, and then kept in a *freezer* at - 20ºC.

Reaction of sequencing in micro-tubes

Positive clones were sequenced in both directions in an automatic sequencer, *MegaBaceTM* 1000 (Molecular Dynamics, Amersham Life Sciences), using the dideoxy sequencing method with the M -13 Forward or M -13 Reverse universal primers, following the manufacturer's recommendations.

Identity analysis of and genetic databases

The sequences were initially submitted to Blastn (e value $< 10^{-5}$) (Altschul et al., 1997) against the *GenBank* database (Benson et al. 2002). The sequences were later submitted to tBlastx (e value $< 10^{-5}$) (Tatusov et al., 2003) to align the sequences obtained from the pequi with others already contained in the plant database.

RESULTS AND DISCUSSION

A total of 1,536 sequences were obtained from the pequi fruit cDNA library, and the sequences had an average length of 452 bases. Of these 1,536 sequences, 564 (36.71%) were discarded after quality analysis. Of the sequences discarded, 338 (21.09%) were rejected due to the size of the sequences and 240 (15.62%) due to the quality of the sequences.

The remaining sequences totaled 958 or 63.29%. After washing, an average number of bases with a Phred score of over 20 were found, indicating a good read, with 570 nucleotides or greater in length. The sequences were deposited in *GenBank* with the accession numbers: GR951205 to GR951217 and GT152673 to GT154478. Most of the sequences found in the Blastn and tBlastx provide some type of protection against oxidative stress of plants, some are transcription factors and others provide structural and pathogenic resistance (Tables I and II).

Table I: Analysis *in silico* (BLASTn) of sequences found in the trasncriptome of *Caryocar brasiliense.*

NSS	*NS*	*e-value*	Organismo
63	Cytochrome oxidase	$2.e^{-21}$	*Plantago lanceolata*
57	Disrupted DOF1	$2.e^{-102}$	*Zea mays*
183	Glycosyltransferase	$7.e^{-65}$	*Triticum aestivum*
45	Phosphinothricin acetyltransferase	$2.e^{-105}$	*Zea mays*
45	Superoxide dismutase 4A	$2.e^{-18}$	*Zea mays*

NSS = number of similar sequences found in the in silico analysis of Blastn
NS = name of sequence that presents similarity

Table II: Analysis *in silico* (tBLASTx) of sequences found in the trasncriptome of *Caryocar brasiliense*.

NSS	*NS*	*e-value*	Organismo
269	Cytochrome oxidase	$4.e^{-21}$	*Globa sessiliflora*
83	Disrupted DOF1	$4.e^{-99}$	*Zea mays*
71	Glycosyltransferase	$2.e^{-176}$	*Triticum aestivum*
7	Histone acetyltransferase gene	$9.e^{-8}$	*Zea mays*
68	Phosphinothricin acetyltransferase	$1.e^{-94}$	*Zea mays*
3	Putative peroxidase	$8.e^{-7}$	*Zinnia elegans*
63	Superoxide dismutase 4A	$7.e^{-65}$	*Zea mays*

NSS = number of similar sequences found in the *in silico* analysis of tBlastx
NS = name of sequence that presents similarity

The proteins related to oxidative stress or plant genetics are cytochrome oxidase,superoxide dismutase and the peroxidases. Plants in the environment are exposed to abiotic stresses such as salinity, temperature and heavy metal intoxication that affect growth and physiological processes (Levitt, 1980). Abiotic stress acts as a catalyst in the production of free radicals that result in oxidative stress in various plants, in which the superoxide (O_2), hydroxide (OH), hydrogen peroxide (H_2O_2) and alkoxy (RO) radicals are produced (Scandalios, 1993, Zhang & Kirkham, 1994, Hernandez et al., 1994, Gallego et al., 1996, Weckx & Clijsters, 1997, Loggini et al., 1999, Panda & Patra, 2000, Bakardjieva et al., 2000, Hernandez et al., 2000).

The superoxide radical has a half-life of under a second and is quickly converted into H_2O_2 by superoxide dismutase (SOD), a product that is relatively stable and can be detoxified by catalase and peroxidases (Grant & Loake, 2000). An increase in SOD enhances tolerance to oxidative stress (Bowler et al., 1992, Slooten et al., 1995, Panda & Khan, 2004). SOD is one of the first lines of defense against ROS. O_2 - is produced any place along the electron transport chain and, consequently, activates O_2 in different compartments of the cell (Elstner, 1991), including the mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts and cytosol (Alscher et al., 2002).

Cytochrome oxidase is a terminal enzyme in the electron transport chain which catalyzes the oxidation or reduction of nitric oxide (Brudvig et al., 1980). It regulates the metabolic pathway of this substance, either by interaction of nitric oxide with the enzyme, thus forming nitrous oxide (Clarkson et al., 1995, Borutaite & Brown, 1996, Palacios Callender et al., 2007) or by interaction of nitric oxide with the oxidation of the enzyme, thus forming nitrite (Zhao et al., 1995, Torres et al., 1998, Palacios-Callender et al., 2007). The primary function of cytochrome oxidase is to reduce centers that need electrons to reduce oxygen by means of four electrons, preventing formation of reactive oxygen species (Collman et al., 2007).

Peroxidases in the presence of peroxide catalyze the oxidation of some próton donating substrates, such as monophenols, diphenols, polyphenols and aminophenols (Wolf & Fatibello, 2003). The peroxidase family of proteins includes enzymes from mammals, fungi and plants. Those of plant origin contain ferriprotoporphyrin, including the prosthetic and chromophoric groups, which is responsible for the brown color of the enzymes. Peroxidase catalyzes the oxidation caused by hydrogen peroxide in substrates such as ascorbate and cytochrome C. They are related to the processes of growth and cellular differentiation and morphogenic changes in response to physical, chemical and biological stresses. An increase in activity of this enzyme in plants submitted to these conditions can be a determining factor in the capacity of adaptation, which lends itself to use as a biochemical stress marker (Piza et al., 2003).

The activity of peroxidase can be altered by external factors such as light or other types of radiation, stress (salts and temperature), senescence or growth regulators. According to Gaspar et al. (1994), peroxidase is related to the regulation or change in endogenous levels of auxin. The complexity of the responses of this enzyme has caused problems in understanding the specific of its function *in vivo* and role in the growth of the plant as well as its adaptation in the environment (Rival et al., 1997).

Peroxidases are important plant enzymes that are involved in various reactions: linking of polysaccharides, oxidation of indol-3-acetic acid, linking of monomers, lignification, healing of wounds, oxidation of phenols, defense against pathogens, regulation of cell elongation, etc. Peroxidases and polyphenol oxidases begin the oxidative degradation of the phenolic compounds near the location of cellular decompartmentalization caused by pathogens (Gaspart et al., 1982, Kao, 2003).

The pequi's ability to effectively combat free radicals formed in cells shows it is a fruit with antioxidant potential. It also prevents diseases caused by vitamin A and C deficiencies and protects against premature aging caused by the release of free radicals in cells.

The transcription factors are found in the gene DOF1 and histone acetyltransferase. The gene DOF1 codes a transcription factor, unique to plants that activate the expression of multiple genes associated with organic metabolism of acids, including phosphoenolpyruvate carboxylase (PEPC) (Yanagisawa, 1996, 1998, 2000, 2002, Yanagisawa et al., 2004). This genetic product acts as a regulatory key in the coordination of gene expression involved in the metabolism of carbon, in addition to being involved in the regulation of gene expression for luminosity (Yanagisawa, 2001). It operates, therefore, in the photosynthesis of the plant.

Histona acetiltransferase regulates the acetylation of histones and transcription factors (Legube & Trouche, 2003). An important post translation modification of histones is the acetylation of the Y- amino group in lysine residues preserved in the amino-terminal tail of the protein. The acetylation positively neutralizes modifications in the lysine and affects interactions of histones with other proteins and/or DNA (Sternglanz & Schindelin, 1999). The acetylation of histones has been associated with transcriptional activation of chromatin (Allfrey et al., 1964, Allis et al., 1985).

Glycosyltransferases (GTs) are enzymes that participate in the structural maintenance of cells, since glycosylation is one of the most important reactions that involve secondary metabolites. They participate in the maintenance of cellular homeostasis, regulation of the growth pathway of the plant and responses to environmental stress (Jones & Vogt, 2001, Lim & Bowles, 2004, Wang & Hou, 2009). They are found mainly in plants that convert products of photosynthesis into disaccharides, oligosaccharides, polysaccharides, in addition to being important molecules in the composition of cell walls (Keegstra & Raikhel, 2001).

GTs transfer simple or complex sugars from nucleotide donors to a smaller molecule receptor in the plants. Hydroxylated molecules are the most common receptors, while UDP-glycose is the most common donor in GTs catalyzing the glycosyl group transferring the reaction (Wang & Hou, 2009). Dozens of glycosyltransferase genes were identified and some were characterized functionally. The glycosylation of components of the plant by the addition of receptors usually modifies these receptors in terms of bioactivity, stability, solubility, subcellular location and links to other molecules, and this may possibly reduce the toxicity of toxic endogenous and exogenous substances (Bowles et al., 2005, Kim et al., 2006). The gene phosphinothricin acetyltransferase, with which the thorny pequi sequences presents similarity, is coded from a transferase that catalyzes the acetylation of phosphinothricin, an analog of glutamate, a herbicide that has a phytotoxic effect on plants, by inhibiting glutamine synthesis (Hérout et al., 2005, Hoagland, 1999, Gill et al., 2001), serving as a form of resistance for the plant.

Phosphinothricin is the main active ingredient of herbicides that act on the conversion of glutamic acid and ammonia into glutamine, resulting in the accumulation of ammonia ions in the plant and cell death (Wild & Manderscheid, 1984, Hoeven et al., 1994).

Although the effective role of these enzymes in the genetic constitution of the pequi (*C. brasiliense*) is still unknown, this study opens up possibilities for work on the functional genome of the species.

CONCLUSION

The transcriptome of the pequi fruit provide some type of protection against oxidative stress of plants, some are transcription factors and others provide structural and pathogenic resistance that are involved in fruit organogenesis of the species.

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