

Studies toward the identification of transcription factors in cassava storage root

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Transcription factors play important roles in several physiological processes. In recent years many transcription factors have been isolated from plants and they are emerging as powerful tools in the manipulation of plant traits. In this work we initiated studies in order to isolate transcription factors from cassava (*Manihot esculenta* Crantz), an important tropical and subtropical crop. Our results show three kinds of proteins expressed differentially in cassava storage root and immunologically related to the opaque-2 transcription factor from maize. Southwestern experiments showed two proteins capable of interacting *in vitro* with the DNA sequence of the *be2S1* gene from the Brazil nut tree.

Key words: bZIP, cis-acting elements, expression, gene regulation, opaque-2, Southwestern.

Estudos para a identificação de fatores de transcrição em raiz de reserva de mandioca: Fatores de transcrição desempenham importantes funções em vários processos fisiológicos. Nos últimos anos, muitos fatores de transcrição têm sido isolados de plantas, emergindo como poderosas ferramentas na manipulação de características agrônômicas. No presente trabalho, iniciamos estudos para isolar fatores de transcrição de mandioca (*Manihot esculenta* Crantz), importante cultura tropical e subtropical. Nossos resultados revelaram três tipos de proteínas diferencialmente expressas na raiz de reserva de mandioca (*Manihot esculenta* Crantz): e imunologicamente relacionadas com o fator de transcrição opaco-2 de milho. Experimentos de Southwestern mostraram duas proteínas capazes de interagir *in vitro* com uma seqüência de DNA do gene *be2S1* de castanha-do-brasil.

Palavras-chave: bZIP, elementos em cis-atuantes, expressão, regulação gênica, opaco-2, Southwestern.

Plant transcription factor studies have contributed substantially in the elucidation of gene expression mechanisms in several physiological processes, such as development of seeds (Schmidt et al., 1990; Yunes et al., 1994) and vascular tissues (Yin et al., 1997), ABA and light responses (Foster et al., 1994), plant defense and stress tolerance (Singh et al., 2002). Transcription factors are sequence-specific DNA-binding proteins that are capable of activating and/or repressing transcription. They are largely responsible for the selectivity of gene regulation and are often expressed in a tissue-specific, developmental-stage-specific, or stimulus-dependent manner. The utilization of transcription factors in the ma-

nipulation of plant traits and in the regulation of transgene expression has been described (Martin, 1996; Ordiz et al., 2002; Segal et al., 2003).

A well described transcription factor of the bZIP class, essential for the expression of endosperm-specific storage protein genes, is opaque-2 (O2) from maize, involved in the regulation of expression of the b-32 albumin, pyruvate orthophosphate dikinase-1 and the 22- and 14-kDa zein gene families (Schmidt et al., 1990; Madalloni et al., 1996). The maize relatives coix and sorghum contain homologous O2 genes that regulate the expression of α -coixin and α -kafirin storage protein genes, respectively (Ottoboni et al., 1993; Vettore et al., 1998).

We have found in the promoter of a brazil nut 2S albumin gene (*be2S1*) sequences related to *cis*-acting elements found in the α -zein and α -coixin promoters. These elements were located around an ACGT or ACCT core and were able to interact with maize O2 (Vincentz *et al.*, 1997). This finding in particular led us to speculate whether the nucleotides coding for the rather conserved basic region of this factor might be of use in prospecting for potential components of regulatory networks in crops of socio-economical importance. For these investigations we chose cassava (*Manihot esculenta* Crantz) because nowadays cassava is one of the most important food crops in the tropics and is ranked in fourth place, after rice, maize and sugarcane with respect to furnished calories (Puonti-Kaerlas, 1998).

The cassava storage root results from the swelling of adventitious roots through secondary growth. According to anatomical studies initiated by Cabral *et al.* (2000), the cassava storage root contains five different tissue layers: L1-composed of phellogen and phelloderm, L2-composed of phloem and vascular cambium, L3-composed of parenchyma cells at an early developmental stage, L4-composed of secondary xylem and developed parenchyma cells packed with starch granules and L5-composed of primary xylem and mature parenchyma cells. Studies on the isolation and characterization of cassava storage root protein genes were recently initiated by de Souza (2001). In these studies an 18 kDa protein was identified with high similarity to the small heat-shock protein class, as well as an alcohol-soluble protein similar to allergenic Hev b5 from rubber-tree (de Souza, 2001; de Souza *et al.*, 2002).

In the present work our aim was to identify in cassava potentially transactivating bZIP-like coding regions and proteins through genomic hybridization with homologous probes, immunological homologies and DNA-protein interaction experiments. Southern blot analysis showed that genomic sequences with homologies to maize O2 are present in the cassava genome. Strong hybridization signals, as intense as the signals obtained with maize DNA itself (figure 1, lane 1), were observed only with cassava DNA (lanes 10 and 11). Weak signals were observed in Brazil nut and taro DNA samples (lanes 4, 8 and 9).

Proteins reacting with antibodies raised against maize O2 and interacting with the DNA sequence of the *be2S1* gene from the Brazil nut tree are expressed differentially in the tissue compartmentalization model of cassava storage root described by Cabral *et al.* (2000). Our results show that polyclonal antibodies raised against the bZIP domain of O2

recognized a protein of about 25 kDa and detected only in layer 2 (composed of phloem and vascular cambium), while a 33 kDa protein was present in all layers of cassava root (figure 2B). Antibodies against O2 also reacted with a 45 kDa protein detected in layers 2 and 4 (figure 2B). No reaction was seen with these proteins and the pre-immune serum (figure 2A).

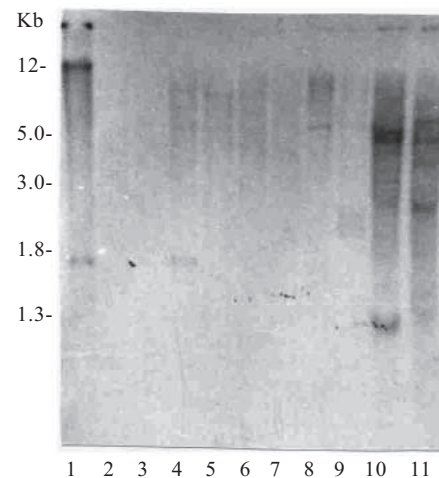


Figure 1. Identification of homologue sequences to the regulatory O2 gene from maize in the genome of various plant species by Southern blot analysis. Restricted DNA samples were separated on 0.8% agarose gel and probed with a radiolabeled probe from the basic region of O2 of maize. Lanes 1: Genomic DNA from maize digested with *Hind III*; 2 and 3: DNA samples from potato digested with *EcoRI* and *Hind III*, respectively; 4 and 5: DNA samples from Brazil nut digested with *EcoRI* and *Hind III*, respectively; 6 and 7: DNA samples from bean digested with *EcoRI* and *Hind III*, respectively; 8 and 9: DNA samples from taro digested with *EcoRI* and *Hind III*, respectively; 10 and 11: DNA samples from cassava digested with *EcoRI* and *Hind III*, respectively.

In the present work we used four DNA fragments (**A**-the 1.0 kb *PstI/NcoI* fragment from the α -coixin gene promoter of coix, **B**-the 285 bp *Sau 3AI* fragment from the *PstI/NcoI* from the α -coixin gene promoter of coix; **C**-the 1.4 kb and **D**- the 265 bp fragments *EcoRI/HindIII/NcoI* from the pTJ2 plasmid containing the *be2S1* gene promoter of Brazil nut) containing *cis*-elements to O2 to test the *in vitro* DNA-protein interaction with cassava root proteins. Two fragments contain entire promoter sequences of α -coixin (**A**) and almost entire promoter sequence of *Be2S1* (**C**) genes, while the other fragments contain the specific *cis*-elements to opaque-2. Fragment B (285 bp α -coixin) contains the *cis*-element AGACATGTCAT described by Yunes *et al.* (1994). The DNA

fragment with 265 bp from *Be2S1* gene (D) contains the F1 (TCCACGTCGA), F2 (GCCACCTCAT) and F3 (TCCACGTACT) elements described by Vincentz et al. (1997). Our results show that only fragment C, containing the almost entire promoter sequence from *Be2S1* gene, was capable of interacting with proteins from cassava root (figure 3). Interestingly, the 45 kDa protein immunologically related to opaque-2 showed interaction with this fragment in samples from tissue layers 2, 3 and 4. A protein with molecular mass above 45 kDa and present only in the tissue layer 3 also interacted with this element (figure 3A). No reaction was observed for proteins from leaves.

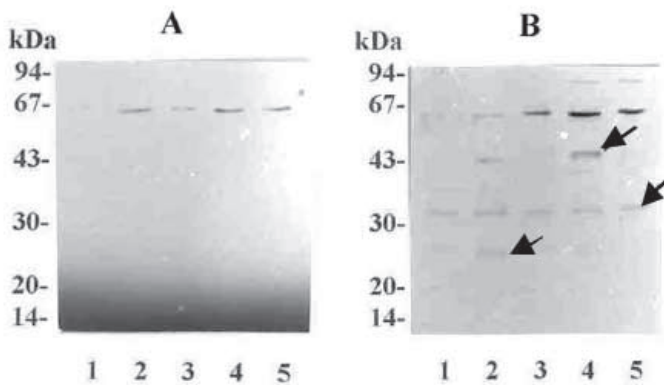


Figure 2. Identification of O2-like proteins in cassava storage root extracts by Western blot analysis. Samples containing 40 μ g of total protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with polyclonal antibodies raised against the O2 bZIP domain (B) and pre-immune serum (A). Lanes: 1 to 5- crude extracts from cassava root layers L1 to L5, respectively. The arrows indicate the proteins recognized by anti-O2 antibodies.

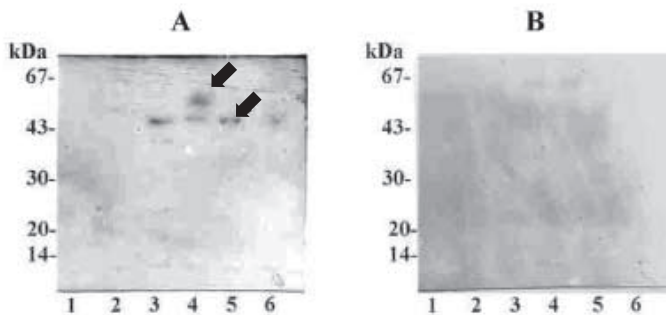


Figure 3. Southwestern analysis of cassava proteins with 1.4 kb promoter fragment of *be2S1* gene from Brazil nut trees (A) and 1.0 kb promoter fragment from the α -coixin gene (B). Lanes: 1- crude extract from leaves; 2 to 6- crude extract from storage root layers 1 to 5, respectively. The arrow indicate the proteins interacting with the *be2S1* promoter.

The prospecting for genes with socio-economically interesting features is a priority for genetic resources and biotechnology programs. Within this context we initiated a search for regulatory genes in an important tropical and subtropical crop, the cassava. O2 is a regulatory protein identified in three cereal species (maize, coix and sorghum) that regulates the gene expression in seeds. In the present work we attempted to test the hypothesis of the presence of O2 like proteins in cassava storage root. Our results show the presence of sequences homologous to the basic region of opaque-2 from maize in the cassava genome. Since the basic region is a conserved domain among bZIP proteins, the maize opaque-2 gene is more closely related to cassava than other species analyzed, under the experimental conditions used. Studies of homology at the antigenic relations level indicated the presence of potential bZIP regulatory proteins in cassava root proteins. Interestingly, the 45 kDa protein immunologically is related to opaque-2 and also showed interaction with the DNA fragment from the *Be2S1* gene promoter from Brazil nut. Since our results show no interaction between the DNA fragment (D) containing F1 and F2 *cis*-acting elements to opaque-2 from *Be2S1* and this 45 kDa protein (data not shown), it is probable that the interaction observed occurred despite the presence of others *cis* elements in this promoter sequence. The protein with molecular mass above 45 kDa was not recognized by opaque-2 antibodies (figure 2B), indicating that this protein detected only in parenchyma cells at early developmental stage (figure 3A) can belong to another transcription factor class. Future experiments will focus on the isolation of genomic sequences coding for these potential regulatory proteins and functional analysis.

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