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Expressão diferencial dos genes *VuUCP1a* e *VuUCP1b* em caupi sob estresse salino[1]

Differential expression of *VuUCP1a* and *VuUCP1b* in caupi under salt stress

Francisco Edson Alves Garantizado[2]*, José Hélio Costa[2], Ivan de Godoy Maia[3] e Dirce Fernandes de Melo[2]

Resumo - O estresse salino afeta o crescimento e o desenvolvimento das plantas, induzindo respostas bioquímicas e fisiológicas como mecanismo de adaptação para sua sobrevivência. As proteínas desacopladoras de planta (pUCPs) dissipam o gradiente eletroquímico mitocondrial de prótons como calor e são codificadas por famílias multigênicas. Elas atuam como sistema de defesa evitando a formação de espécies reativas de oxigênio geradas em resposta a estresses ambientais. O objetivo do trabalho foi estudar a expressão de genes das pUCPs (*VuUCP1a* e *VuUCP1b*) em raízes e folhas de plântulas de *Vigna unguiculata* submetidas ao estresse salino (NaCl 100 mM). Sementes foram germinadas no escuro e após 3 dias, as plântulas foram transferidas para solução de Hoagland, permanecendo 3 dias antes da aplicação do estresse. As raízes e folhas foram coletadas com 0; 6; 12 e 24 horas, após adição de NaCl, para avaliar o perfil de expressão dos genes da pUCP por RT- PCR. A análise de transcritos mostrou que *VuUCP1a* é expresso em raízes e folhas revelando um perfil constitutivo enquanto que *VuUCP1b* é dependente do tecido, isto é, expresso nas folhas, sem alteração em resposta ao estresse e induzido pelo estresse salino nas raízes. A peculiaridade da duplicação gênica de *pUCP1* em caupi com perfil diferencial de expressão, sugere tanto um papel dessa enzima nos mecanismos de ajustamento ao estresse salino quanto promove essa espécie como um modelo atrativo para compreensão do papel da família multigênica da pUCP em plantas.

Palavras-chaves - Estresse salino. pUCP. *Vigna unguiculata.*

Abstract - Salt stress affects growth and development of plants, inducing a variety of physiological and biochemical responses as an adaptation mechanism for survival. The plant uncoupling mitochondrial proteins (pUCPs) are able to dissipate the proton electrochemical gradient as heat and are encoded by a multigene family. They works as defence systems avoiding the formation of reactive oxygen species promoted by environmental stress. The aim of this work was to study gene expression of pUCPs (*VuUCP1a* and *VuUCP1b*) in roots and leaves from *Vigna unguiculata* seedlings under salt stress (100 mM NaCl). Seeds were germinated in the dark and after 3 days, the seedlings were transferred to Hoagland's medium and grown for 3 additional days before being submitted to the stress condition. Roots and leaves were harvested at 0; 6; 12 and 24 hours after addition of NaCl for total RNA isolation and RT-PCR assays. Expression analysis by RT-PCR showed that *VuUCP1a* is constitutive in leaves and roots while *VuUCP1b* is expressed as tissue-dependent presenting a constitutive profile in leaves and a differential one in roots from seedlings under salt stress. The uniqueness of *pUCP1* gene duplication in cowpea with differential expression suggest a role of this enzyme in the adjustment of salt stress as well as promotes this species as an attractive model to understand the role of pUCP gene members in plants.

Key word - Salt stress. pUCP. *Vigna unguiculata.*

* Autor para correspondência
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[2]Programa de Pós-Graduação em Bioquímica, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências/UFC, Av. Eng. Humberto Monte, Bloco 907, Campus do Pici, Fortaleza-CE, Brasil, 60.451-970, edsongarantizado@yahoo.com.br, costajhe@yahoo.com.br, fernandesdemelod@gmail.com
[3]Programa de Pós-Graduação em Genética, Departamento de Genética, Instituto de Biociências, Universidade Estadual Paulista-Unesp, São Paulo-SP, Brasil, igmaia@ibb.unesp.br

Introduction

Salinity is a major abiotic stress limiting the productivity of many plant species and consequently affects plant survival, biomass, plant height and the capacity of plants to collect water and nutrients (MANIVANNAN et al., 2008; ZUSHI et al, 2009). These damages are especially revealed pronounced in arid and semi-arid regions as Brazilian Northeast, where cowpea (*Vigna unguiculata*) is a widely cultivated species (CAVALCANTI et al., 2007). In fact, the salinity imposes two stresses on plant tissue: a water-deficit that results from the relatively high solute concentrations in the soil, and ion toxicity due altered K/Na rations and Na^+ and Cl^- concentrations that are harmful to plants (ALVES et al., 2008; CHEN et al., 2008). Salt stress affects almost every aspect of the physiology and biochemistry of plants, promoting ionic, osmotic, nutritional and hormonal imbalances, leading to a decrease in metabolic efficiency and even to injurious secondary effects such as oxidative stress promoted by reactive oxygen species (ROS) (MUNNS; TESTER, 2008; PANG; WANG, 2008; PARIDA; DAS, 2005). ROS are produced predominantly in chloroplasts, mitochondria, and peroxisomes causing oxidative damage to membrane lipids, proteins, and nucleic acids (ASHRAF, 2009; BANU et al., 2009; JEZEK; HLAVATÁ, 2005) and can induce responses such as new gene expression (GILL; TUTEJA, 2010). However, in response to the salinity deleterious effects, plants can suffer metabolic changes as a mechanism of adjustment to adverse conditions, ensuring its survival (MUNNS, 2002; SILVA et al., 2009). Under steady state conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms (FOYER; NOCTOR, 2005).

Plant mitochondria shows besides the enzymes involved in a classical electron transport chain, coupled to oxidative phosphorylation, another non-phosphorilating electron transport pathway mediated by alternative oxidase (AOX). Indeed, plant mitochondria possess another uncoupling enzyme named plant uncoupling protein (pUCP) (BORECKÝ et al., 2006). The pUCPs are integral membrane proteins that are present in the inner mitochondrial membrane and are able to dissipate the proton electrochemical gradient as heat (ITO-INABA et al., 2009; VERCESI et al., 2006). AOX and pUCP function are not fully understood, but it seems involved in protecting oxidative damage by ROS (BLOKHINA; FAGERSTEDT, 2010). Both of them can be considered as a tool for ROS level regulation/elimination (GRABEL'NYKH et al., 2009; PASTORE et al., 2007).

It is well known that AOX expression is regulated by salt stress (COSTA et al., 2007; JACOBY et al., 2010; SMITH et al., 2009), but little is known for pUCP expression (DLASKOVÁ et al., 2006; PASTORE et al., 2007; TRONO et al., 2006). Up to date, apparently, there is no study with cowpea pUCPs in response to salt stress. These proteins are encoded by a multigene family and are related to plant adjustment mechanisms under environmental stresses (BORECKÝ et al., 2006; CHEONG et al., 2002; NANTES et al., 1999; NOGUEIRA et al., 2003; SEKI et al., 2002; SWIDZINSKI et al., 2002). Two pUCP genes were identified in cowpea: *VuUCP1a* and *VuUCP1b* (GARANTIZADO, 2007). Therefore, the aim of this work was to study roots and leaves pUCPs expression from cowpea seedlings submitted to salt stress.

Materials and methods

Plant material and stress conditions

Vita 5 cowpea seeds (*V. unguiculata* (L.) Walp) were obtained from the seed bank of the Departamento de Fitotecnia, Universidade Federal do Ceará, Fortaleza, Ceará, Brasil. Seeds were surface sterilised for 5 min in 0.5% (w/v) CaOCl, rinsed with water, and germinated in the dark, at 25 °C, on filter paper soaked with distilled water. After 3 days, the seedlings were transferred to hydroponics systems, containing Hoagland solution (HOAGLAND; ARNON, 1950) in a greenhouse, staying three days before the application of salt stress. After six days of germination, seedlings were separated into two groups: control and salt stress. The salt stress group was induced by adding to 100 mM NaCl in the nutrient solution and the control group submitted only Hoagland solution. Leaves and roots were harvested at 0; 6; 12 and 24 hours after addition of 100 mM NaCl, immediately frozen in liquid nitrogen and stored at -80 °C before extraction of total RNA.

Extraction of total RNA and semi-quantitative RT-PCR

Two hundred milligrams of roots were powdered with liquid nitrogen in a mortar with a pestle. Total RNA was then extracted using the RNeasy Plant Mini Kit according to the manufacturer protocol (Qiagen, Hilden, Germany). Total RNA (1.5 mg) from each sample was heated to 75 °C in a water bath for 10 min, cooled 2 min on ice and used for reverse transcription. Semi-quantitative RT-PCR was performed using the Ready To Go RT-PCR beads kit (Pharmacia) with specific primers for each pUCP gene and it was concomitantly controlled by amplification of *V. unguiculata* actin cDNA. Specific actin primers were obtained according COSTA et al. (2004). The VuUCP1a and VuUCP1b primers were obtained from both gene sequences retrieved from genespace sequence (GSS) database of Cowpea Genomics Initiative (CHEN

et al., 2007). The PCR parameters for a semi-quantitative estimation were according COSTA et al., 2010).

PCR assays were carried out to establish the optimal annealing temperature of primers at 55 °C for *actin*, *VuUCP1a* and *VuUCP1b*. Preliminary experiments with various PCR cycle numbers indicated that in the RT-PCR conditions of this study, amplifications were not in the plateau phase, and therefore allowed for semi-quantitative estimations of transcript levels. The cycle number used for each gene was 27 for *Actin*, *VuUCP1a* and *VuUCP1b*.

The primer sequences were: VuUCP1a sense: 5′ GTGTGTACTATTCCGTTG 3′ and antisense: 5′ TGCTATATTGGGGCCAAG 3′; VuUCP1b sense: 5′ GTGTGTACTATTCCTCTC 3′ and antisense: 5′ GTTATGTTGGGTCCAATC 3′; Actin sense: 5′ GCGTGATCTCACTGATGCC 3′ and antisense: 5′ TCGCAATCCACATCTGTTGG 3′. The RT-PCR products were analyzed by electrophoresis in a 1.5% (w/v) agarose gel, stained with ethidium bromide and photographed with a gel imaging system (itf labortechnik-Germany).

Results and discussion

Salinity, as several others biotic and abiotic stresses, alters the equilibrium between the production and the scavenging of ROS (GILL; TUTEJA, 2010; MUNNS; TESTER, 2008; PANG; WANG, 2008; PARIDA; DAS, 2005). ROS are produced continuously in different cellular compartments such as chloroplast, mitochondria and peroxisomes and their imbalance cause oxidative damage to membrane lipids, proteins, and nucleic acids (ASHRAF, 2009; BANU et al., 2009; JEZEK; HLAVATÁ, 2005). Plant mitochondria possess two uncoupling systems mediated by alternative oxidase (AOX) and plant uncoupling protein (pUCP) that have been linked to systems involved in the prevention of the ROS formation (BLOKHINA; FAGERSTEDT, 2010; GRABEL'NYKH et al., 2009; PASTORE et al., 2007).

Since the UCP was found for the first time in plants (VERCESI et al., 1995), several groups have asked why plant mitochondria have another energy-dissipating system in addition to alternative pathway mediated by alternative oxidase (AOX). Similar to AOX the pUCP is encoded by a multigene family, however, in spite of the great progress in the plant genome sequencing in the recent years only pUCP gene families of two plants have been well characterized, sugarcane and *Arabidopsis thaliana* (BORECKÝ et al., 2006) with five (*SsUCP1-5*) and six (*AtUCP1-6*) gene members, respectively. The physiological role of pUCP gene members in plants have been focused on their gene regulation (BORECKÝ et al., 2006; VERCESI et al., 2006). Concerning salt stress response *AtPUMP1* (*AtUCP1*) in Arabidopsis is induced by salinity (VERCESI et al., 2006).

Two pUCP genes were previously identified in cowpea (*VuUCP1a* and *VuUCP1b*) presenting at least 83% of identity between deduced amino acid sequences. These genes are orthologous to *GmUCP1a* and *GmUCP1b* from soybean, a leguminous as it is the case of cowpea (GARANTIZADO, 2007). Considering the apparent importance of Arabidopsis pUCP1 in response to salinity became curious to study the expression of these duplicated UCP1 in cowpea.

Plant uncoupling proteins (pUCPs) gene expression in roots (FIG. 1) and leaves (FIG. 2) from cowpea seedlings submitted to saline stress (100 mM NaCl) were carried out at 0; 6; 12 and 24 h after stress submission. Figure 1 shows the *VuUCP1a* and *VuUCP1b* expression profile in roots from cowpea seedlings in control and stress conditions. In addition, Actin cDNA was used as constitutive gene to control the semi-quantitative measure. It can be seen that *VuUCP1a* revealed the same profile expression in both conditions as a constitutive gene. On the other hand, *VuUCP1b* was differentially expressed revealing an increase in the transcript level mainly observed at 12 and 24 h.

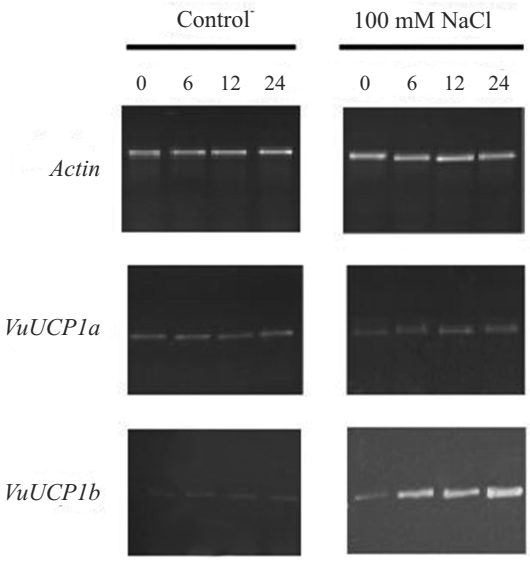

Figure 1 - Expression analysis of *VuUCP1a* and *VuUCP1b* genes in cowpea roots at 0, 6, 12 and 24 hours of stress with 100 mM NaCl and control condition. The amplification of *Actin* was used as a constitutive control. The results are representative of three repetitions of RT-PCR assays

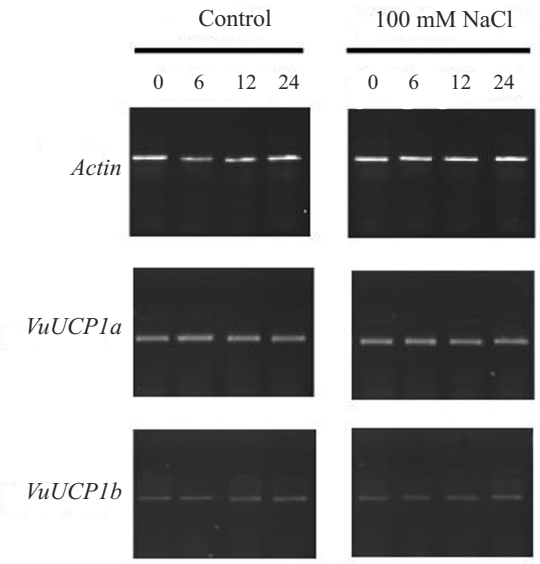

Figure 2 - Expression analysis *VuUCP1a* and *VuUCP1b* genes in cowpea leaves at 0, 6, 12 and 24 hours of stress with 100 mM NaCl and control condition. The amplification of *Actin* was used as a constitutive control. The results are representative of three repetitions of RT-PCR assays

In order to investigate if the differential expression of both genes is tissue-dependent an expression analysis was performed in leaves collected simultaneously with roots. As observed in Figure 2, there was no change in *VuUCP1a* and *VuUCP1b* expression profile in leaves.

There are few studies of gene expression in response to salt stress and there are controversial results. Transcript levels of two distinct pUCP-related genes in durum wheat seedlings grown under control and salt stress conditions were not enhanced (TRONO et al., 2006). In fact, it has been characterized two pUCP1 genes in wheat i.e., *WhUCP1a* and *WhUCP1b* but there is no date related to stress condition (VERCESI et al., 2006). Contrarily, an increase in mRNA for *ZmpUCP1* was found in maize shoots and roots after 24h treatment with 250 mM NaCl (DLASKOVÁ et al., 2006). In the case of Arabidopsis, a dicot model, it was revealed a down-regulated expression pattern promoted by high-salinity stress for *AtUCP4* and *AtUCP5* (SEKI et al., 2002). However, other abiotic stresses as low and high temperatures (NANTES et al., 1999; NOGUEIRA et al., 2003; SWIDZINSKI et al., 2002) drought, wound (CHEONG et al., 2002) induced up-regulation in *AtUCP* genes. These results are puzzling but it could be suggested a differential regulation at cell or tissue/organ level among different species. Our data contributed to put in evidence cowpea as an interesting model since it presented gene duplication of UCP1 (*VuUCP1a and VuUCP1b*) showing a tissue and gene-dependent expression under salt stress. These results are promisors concerning biotechnological application since this knowledge could be useful to produce plants more tolerant to salt and oxidative stresses promoting enhancement of productivity of many plant species.

Conclusion

The results suggest a tissue and gene-dependent pUCP1 (*VuUCP1a* and *VuUCP1b*) expression under salt stress in cowpea, with a constitutive expression of *VuUCP1a* in roots and leaves and induction of *VuUCP1b* in roots.

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