Evaluation of a cassava translationally controlled tumor protein (MeTCTP) reveals its function in thermotolerance of Escherichia coli and in vitro chaperone-like activity

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Genet. Mol. Res. 16 (4): gmr16039835
Received September 11, 2017
Accepted November 08, 2017
Published November 29, 2017

DOI http://dx.doi.org/10.4238/gmr16039835

ABSTRACT. The TCTP (Translationally controlled tumor protein) is a highly conserved protein family found in eukaryotic organisms, which has been associated to the performance of several functions at the cellular level, as those related to growth, development, and responses against environmental stresses. However, studies on TCTP from plants are still incipient. Cassava (Manihot esculenta Crantz) is a crop of great socioeconomic importance for millions of people in the world, especially because of its high energy content. Previous studies reported the identification of a cassava TCTP (MeTCTP) with potential roles in storage root formation and salt stress response. Here, our main goal was to increase the understanding about the roles of MeTCTP in response to abiotic stresses. We verified that the overexpression of recombinant MeTCTP in Escherichia coli increased tolerance of bacterial cells to heat stress. In addition, the recombinant MeTCTP was purified by nickel affinity and evaluated by chaperone activity assays, which showed its ability to preserve the function of NdeI restriction enzyme under thermal denaturation.
Plants are subjected to a wide variety of abiotic stresses, such as drought, salinity, high and low temperatures, which have been responsible for huge yield losses worldwide. In order to overcome such adverse conditions, new insights about physiological components that contribute to the endogenous defense mechanisms of plants are extremely relevant (Marques et al., 2017). Thus, the identification and prospection of gene products that contribute to abiotic stress response constitute an essential step for generating of plants with tolerance against such stress.

The translationally controlled tumor protein (TCTP) was firstly identified by its translational regulation in mammalian tumor cells (Gross et al., 1989); however, it is not a tumor-specific protein, being also found in normal cells and widely expressed in eukaryotes (Sanchez et al., 1997; Woo and Hawes, 1997; Thiele et al., 2000; Gnanasekar et al., 2002; Bonmer and Thiele, 2004). Also, it is known that the regulation of TCTP expression occurs at both transcriptional and translational levels (Xu et al., 1999; Bommer and Thiele, 2004). TCTP comprises a family of small hydrophilic proteins of 18 to 20 kDa conserved in eukaryotic organisms. In animals, the role of TCTP in the performance of important functions at cellular level is well-established, as those related to growth, development and endogenous stress, as well as its Ca$^{2+}$ and microtubule-binding properties (Bommer and Thiele, 2004). TCTP is a key component of the TOR signaling pathway (target of rapamycin), an important growth regulator (Deprost et al., 2007; Berkowitz et al., 2008; Brioudes et al., 2010). In addition, TCTP was described as a novel heat shock protein with chaperone-like activity (Gnanasekar et al., 2009).

Compared to the TCTPs from animals, the studies focused on plant TCTPs are still incipient, especially considering the vast genomic complexity and the high variety of molecular defense strategies adopted by plants. As example of TCTP effect in plant growth and development, Cao et al. (2010) reported that the silencing of BoTCTP gene decreased the vegetative growth in cabbage plants. On the other hand, studies have reported the TCTP roles in response to abiotic stresses by its increased transcript levels under salt stress (Qin et al., 2011; Guo et al., 2012; Li et al., 2013; Santa Brigida et al., 2014), extremes of temperature (Cao et al., 2010), drought (Kim et al., 2012), and mercury (Wang et al., 2015) stress conditions. In addition, the TCTP relationship with abiotic stress has been also evidenced by silencing (Cao et al., 2010) and/or over-expression (Kim et al., 2012; Wang et al., 2015; de Carvalho et al., 2017) of TCTP genes in transgenic plants.

As mentioned above, besides the TCTP roles in growth and development of plants, this protein has a pivotal participation in stress response, justifying studies based on identification and functional evaluation of TCTP genes and their products for use in molecular breeding to the generation of well-adapted plants, thereby overcoming abiotic stresses at the field level. Among plants with socio-economic importance, cassava (*Manihot esculenta* Crantz) is a major tropical food crop for millions of people in the world. This crop is especially important to smallholder farmers and low-income people because of its high energy potential (high starchy carbohydrate content in storage roots) and high yield and productivity potential in areas which presents poor or acid soils and where rainfall is low (El-Sharkawy, 2004). In cassava, TCTP was firstly reported as a gene possibly involved in storage root formation (de Souza et al., 2004). Later, Santa Brigida et al. (2014) performed the cloning and characterization of cassava TCTP gene coding for a 19 kDa protein, named MeTCTP, with increased transcript levels in cassava leaves submitted to sodium chloride treatment. In addition, these authors verified that the overexpression of recombinant MeTCTP in *Escherichia coli* leads to increased tolerance to salt stress in bacteria cells (Santa Brigida et al., 2014). In order to increase our understanding about the role of MeTCTP against abiotic stresses, here we evaluated if the overexpression of the recombinant MeTCTP contributes to the tolerance of *E. coli* against heat stress, as well as the function of this protein as a molecular chaperone.

**MATERIALS AND METHODS**

**MeTCTP production and purification**

Bacteria cells of *E. coli* strain Rosetta (Novagen, USA) transformed with pET29a-MeTCTP construct with recombinant protein containing a C-terminal 6xHis tag were previously obtained by Santa Brigida et al. (2014). To analyze the MeTCTP production levels from cell crude extracts, heterologous expression of MeTCTP was induced by Isopropyl-b-D-thiogalactoside (IPTG) 1 mM and incubation at 37°C with constant agitation of 180 rpm. The total protein samples were taken at different incubation times (0, 1, 2, 3, 4, 5 and 6 hours) after induction.
with IPTG. For total protein extraction, bacteria cells were collected by centrifugation at 10,000 rpm for 10 minutes, followed by pellet washing on 10 mM Tris pH:8.0 and resuspension in phosphate buffered saline (PBS: NaCl 137 mM, Na$_2$HPO$_4$ 10 mM, KH$_2$PO$_4$ 1.8 mM, KCl 2.7 mM, pH: 7.4). Bacteria cells were disrupted by repeated cycles of freezing and thawing. After centrifugation at 12,000 rpm for 10 minutes at 4°C, the supernatant containing soluble recombinant protein was collected and analyzed on SDS-PAGE according to Laemmli (1970). The normalisation of volume of supernatant (µl) applied on electrophoresis was performed according to procedure described by Machado et al. (2013).

MeTCTP His-tagged recombinant protein was affinity purified by nickel column (NiTA-agarose, Novagen, USA) using elution buffer with 250 mM imidazole, according to manufacturer’s instructions. To verify the efficiency of purification process, total protein from bacteria cells transformed with pET29a-MeTCTP, flow through fraction and purified MeTCTP samples were analysed by SDS-PAGE. As negative control, we used total protein sample from bacteria cells transformed with empty pET29a vector. Purified MeTCTP sample was quantified using a Qubit fluorometer (Invitrogen, USA) and used in assays of chaperone activity.

Heat tolerance assay

For assays of tolerance to heat stress, bacterial cells containing the pET29a-MeTCTP construct were cultured in LB medium containing IPTG 1 mM at different temperatures according to procedure described by Reddy et al. (2012). After incubation at different temperatures (37°C, 45°C and 50°C), bacteria cells growth was monitored by optical density (OD) at 600 nm. Assays were repeated at least five times. For each treatment were performed six repetitions. As negative control we used bacteria cells transformed with an empty pET29a vector. Assays were carried out in a completely randomized design and factorial scheme (bacteria cells transformed with empty pET29a or pET29a-MeTCTP vs temperatures conditions). Data were subjected to analysis of variance (ANOVA, p ≤ 0.01) and the means of the treatments were compared by the Tukey’s test (α ≤ 0.01), using GENES software (Cruz, 2011).

Assay for the chaperone activity of recombinant MeTCTP

The procedures described by Hess and FitzGerald (1998) and Barros et al. (2015) were used in assays of in vitro chaperone activity for MeTCTP. Enzymatic activity of NdeI was assayed by cleavage of DNA pGEM-3Z vector (Promega, USA) containing only one site for this restriction enzyme. Plasmid DNA was prepared by alkaline lysis with SDS according to Birnboim and Doly (1979). Samples containing plasmid DNA were digested with NdeI at 50°C for 30 minutes in the presence and absence of purified MeTCTP (2.2 µg). The plasmid DNA digested with NdeI at 37°C for 30 minutes was used as control sample. All samples were analyzed by agarose gel electrophoresis stained with ethidium bromide, including a sample of plasmid DNA uncut. Assays were repeated at least five times, and for each treatment were performed three repetitions.

RESULTS

Heterologous expression and purification of recombinant MeTCTP

As first step, for obtaining large amounts of MeTCTP for functional analysis, it was performed its heterologous expression in bacterial system aiming at evaluating of the effect of induction time on recombinant protein yield. Then, we used a bacterial clone transformed with pET29a-MeTCTP construct and able to express the recombinant MeTCTP with a C-terminal 6×His obtained by Santa Brigida et al. (2014). In Figure 1 is shown a SDS-PAGE of total protein samples from bacteria cells with pET29a-MeTCTP construct after expression induction by IPTG 1 mM and incubation at 37°C during different times (0h, 1h, 2h, 3h, 4h, 5h and 6h). Except the negative control (0h), the successfully normalized pET29a-MeTCTP samples showed a band with about 20 kDa, which is in accordance with the molecular weight reported by Santa Brigida et al. (2014). In addition, the highest level of expression was detected in sample induced for 6 hours (Figure 1), that was the time of induction used to produce total protein sample for purification of recombinant MeTCTP by a nickel column. As shown in Figure 2, the recombinant MeTCTP was purified with success, according to SDS-PAGE of flow through fraction and purified MeTCTP samples obtained from purification process. As negative control, we used total protein sample from bacterial cells transformed with an empty pET29a vector.
Figure 1. SDS-PAGE of total protein samples from bacteria cells transformed with the pET29a-MeTCTP construct. It is shown the expression level of the recombinant MeTCTP for each time after induction with IPTG (0, 1, 2, 3, 4, 5 and 6 hours). The molecular weight corresponding to the 20 kDa recombinant protein is indicated by arrow. M: Protein marker.

Figure 2. SDS-PAGE of the MeTCTP purification. It is shown the protein fractions from purification of recombinant MeTCTP using a NiTA-agarose column, as well the total protein samples from bacteria cells expressing the pET29a-MeTCTP construct and an empty pET29a vector as control. The molecular weight corresponding to the 20 kDa recombinant protein is indicated by arrow.

Bacteria cells protection against heat stress by overexpression of MeTCTP

In order to investigate if MeTCTP could protect in vivo bacteria cells against heat treatment, we analyzed the protection potential of recombinant MeTCTP in bacterial growth during such stress, according to procedure described in the Material and methods. Under high temperatures (45°C and 50°C), in comparison to bacterial growth at optimum temperature (37°C), the bacterial growth rates were considerably reduced in both bacteria cells expressing the pET29-MeTCTP construct and an empty pET29a vector. However, such decrease of growth was significantly higher in the negative control, indicating the effect of MeTCTP overexpression in the advantage of bacterial growth (Figure 3). Thus, the MeTCTP favored tolerance of bacterial cells under thermal stress.
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**Figure 3.** Evaluation of effect of the recombinant MeTCTP on bacterial growth under heat stress. Bacteria cells transformed with pET29a-MeTCTP construct were submitted to different temperatures (37°C, 45°C and 50°C) with 1 mM IPTG for 12 hours, followed by evaluation of optical density at 600 nm. Bacteria cells transformed with an empty pET29a vector were used as control. Distinct uppercase letters denote different means by Tukey’s test (α ≤ 0.01) for comparisons between bacteria cells transformed (with pET29a-MeTCTP or control) growing in a same temperature condition. Distinct lowercase letters denote different means by Tukey’s test (α ≤ 0.01) for comparisons between bacteria cells transformed with a same gene construct growing in different temperature conditions.

**In vitro chaperone activity of MeTCTP**

Taking into account the results obtained from thermotolerance assays of bacterial cells expressing MeTCTP, and since it is known that some proteins contribute for plant defense response against heat stress acting as molecular chaperones, we seek to verify the potential of MeTCP as a molecular chaperone. For this purpose, we evaluated the ability of the recombinant MeTCTP on the maintenance of digestion activity of NdeI restriction enzyme under conditions of protein denaturation. According to our results, the sample containing MeTCTP showed a DNA intense band of linearized pGEM-3Z vector, indicating complete digestion of plasmid DNA by NdeI under high temperature (50°C), in comparison to the DNA sample digested at 37°C. On the other hand, the activity of NdeI was partially inactivated by high temperature in the sample without MeTCTP (Figure 4). Therefore, our results revealed that MeTCTP acted as a molecular chaperone by preserving the activity of NdeI under the conditions of protein denaturation here adopted.
In cassava, previous studies reported the potential roles of MeTCTP in salt stress response, since transcript levels were increased in leaves treated with sodium chloride. In addition, functional studies in *E. coli* showed that the overexpression of recombinant MeTCTP favored the bacterial growth in salt stress conditions (Santa Brígida et al., 2014). Here, our results with heterologous expression of MeTCTP showed a growth advantage of bacterial cells under thermal stress. Heterologous expression in bacterial system has been used with success in several functional studies of plant proteins with roles in tolerance to heat stress, such as LEA proteins (Reddy et al., 2009; He et al., 2012; Barros et al., 2015); however, such studies for TCTP are still rare. As example, our results are in agreement with those obtained by Gnanasekar et al. (2009) for TCTPs from human and *Schistosoma mansoni* that conferred protection against heat stress in bacterial cells. Likewise, the TCTP from *Stichopus monotuberculatus* favored bacterial growth under thermal stress (Ren et al., 2014). In contrast, studies reported by Parmar (2016) revealed that the expression of a mammalian TCTP rendered the bacterial cells more sensitive to temperature, pH and oxidative stresses. Since TCTP is found in eukaryotes and there is no information of similar proteins in prokaryotic organisms (Hinojoza-Moy et al., 2008), the molecular function by which the recombinant MeTCTP conferred tolerance against thermal stress in bacterial cells remains to be elucidated. However, our results contribute to functional characterization of this cassava protein, indicating that the MeTCTP can also participate on adaptation of cassava plants against heat stress.

Regarding the possible molecular function of MeTCTP in conferring tolerance against thermal stress, our results revealed that this protein has ability of chaperone by stabilizing and preserving the activity of *NdeI* enzyme under heat denaturation conditions. As far we known , it is the first report of a plant TCTP with chaperone activity; however, our results are consistent with those obtained by Gnanasekar et al. (2009) that identified TCTPs from human and *S. mansoni* able to interact with denatured proteins and protect them from the harmful effects of thermal shock. Thus, these authors suggested that TCTP is a novel small molecular weight heat shock protein (14-33 kDa) with chaperone-like activity (Gnanasekar et al., 2009). It is known that chaperones are proteins with properties to provide heat tolerance by avoiding the denaturation and favoring protein refolding of target proteins (Hartl, 1996). It is also known that the most of the chaperones are heat shock proteins (HSPs), with expression increased by heat shock, being classified into families according to their molecular weight and amino acid sequence homology (Perez et al., 2009). Thus, based on its 19 kDa, the MeTCTP can be considered a small HSP; however, this protein has no homology with known HSPs. Therefore, our results with MeTCTP are in concordance with the findings of Gnanasekar et al. (2009) that the TCTP can be considered a novel small HSP with chaperone activity. Also regarding the MeTCTP function as a molecular chaperone, the presence of putative binding domain to Rab GTPase within MeTCTP sequence (Santa Brígida et al., 2014) can be a feature supporting this function, since studies reported by Thaw et al. (2001) verified that TCTPs contain structural similarity to a family of guanine nucleotide-free chaperones that binds to the GDP/GTP free form of Rab proteins. However, the region in amino...
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acid sequence of proteins from TCTP family related to their chaperone activity has not yet been experimentally validated.

In conclusion, our results showed that recombinant MeTCTP was able to act as a molecular chaperone in experimental conditions tested here. Since the MeTCTP was functionally active to protect bacterial cells against heat stress, then it is possible that in cassava plants this protein plays similar function.

Here, we reported for the first time the participation of MeTCTP in thermotolerance by overexpression in bacterial cells and its chaperone property. The results presented here and those previously obtained by Santa Brígida et al. (2014) regarding the MeTCTP roles in salt stress response confirm the participation of plant TCTP as a multifunctional protein in tolerance against abiotic stresses.

ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Pará (FAPESPA), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Universidade Federal do Pará (UFPA), Brazil.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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