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Levels of MeLEA3, a cDNA Sequence Coding for an Atypical Late Embryogenesis Abundant Protein in Cassava, Increase Under In Vitro Salt Stress Treatment

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Abstract Cassava (*Manihot esculenta* Crantz) is a tropical food crop that is well adapted to critical climate and soil conditions, including drought, one of the most serious abiotic stresses that limit crop production in agriculture. Expression of late embryogenesis abundant (LEA) proteins is involved in acquisition of tolerance to drought, cold and high salinity in many different organisms. Here, we report on the characterization of a cassava MeLEA3 cDNA sequence and its deduced amino acid sequence. MeLEA3 protein was deduced from a full-length cDNA sequence of 664 bp with a 285-bp open reading frame, consisting of 94 amino acid residues, with a calculated molecular weight of 10 kDa and a theoretical isoelectric point of 9.66. The most abundant amino acid found in MeLEA3 protein was alanine (18.09%), followed by lysine (9.57%) and serine (9.57%). A search for conserved domain revealed that MeLEA3 belongs to Pfam family LEA3, PF03242. Furthermore, Kyte–Doolittle algorithm and MitoProt II software analyses showed that MeLEA3 is a hydrophobic atypical LEA protein predicted to be exported to mitochondria. In addition, semi-quantitative RT-PCR assays showed that accumulation of MeLEA3 transcripts is increased in leaves treated with sodium chloride, indicating a potential role in salt stress response. Our results also revealed no introns within the MeLEA3 genomic sequence. This is the first study on characterization of a LEA sequence in cassava that can contribute to understanding molecular mechanisms involved in resistance of this crop to abiotic stresses.

Keywords Abiotic stress · Bioinformatics analyses · Cassava · MeLEA3 protein · Salt stress response · Semi-quantitative RT-PCR

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Introduction

Late embryogenesis abundant (LEA) proteins are involved in acquisition of tolerance to drought, cold, high salinity, and osmotic stress of exogenous abscisic acid (Hong-Bo et al. 2005). These proteins were first identified during the late stage of seed development accompanied by dehydration in cotton (Dure et al. 1981; Galau et al. 1986; Baker et al. 1988). Subsequently, these proteins were also found in seedlings, roots, and other organs (Hou and Guo 1998; Zhang et al. 2002; Federspiel 2000). As indicated by their name, LEA proteins are expressed in high quantity during late embryo development and can represent up to 4% of the total cellular proteins (Roberts et al. 1993). Nowadays, it is known that LEA-related proteins represent a widespread

adaptation to water deficit (Garay-Arroyo et al. 2000) distributed across archeal, eubacterial, and eukaryotic domains (Battaglia et al. 2008).

LEA proteins are mainly small molecular weight proteins ranging from 10 to 30 kDa (He and Fu 1996); they have been grouped into various LEA families based on occurrence of amino acid motifs (Dure et al. 1989; Wise 2003) that confer protection against different stress conditions. For example, group 1 LEA protein of wheat confers tolerance to osmotic stress in yeast (Swire-Clark and Marcotte 1999), and rice OsLEA3 belonging to group 3 LEA induces resistance to drought (Xiao et al. 2007).

LEA proteins can also be classified as typical and atypical based on their physicochemical properties. Typical LEA proteins are also called hydrophilins since they are very hydrophilic, with hydrophilicity index greater than 1, and glycine is their most abundant amino acid (Wise and Tunnacliffe 2004; Battaglia et al. 2008), while those showing hydrophobic characteristics are known as atypical LEA proteins (Galau et al. 1993; Naot et al. 1995; Zegzouti et al. 1997; George et al. 2009).

Although LEA proteins play crucial roles in cellular dehydration tolerance, their physiological and biochemical functions are unknown. However, studies indicate that they can participate in protecting cellular structures by retaining water, sequestration of ions, and acting as molecular chaperones in protection of other proteins (Bray 1993; Ingram and Bartels 1996; Kovacs et al. 2008). Other studies revealed that LEA proteins have the ability to stabilize labile enzymes under freezing conditions (Hara et al. 2001; Sanchez-Ballesta et al. 2004).

In the last 10 years, many LEA genes have been isolated and characterized from different crops (Krüger et al. 2002; Grelet et al. 2005; Kim et al. 2005; Nakayama et al. 2008; Boucher et al. 2010). Research on LEA genes is necessary to understand the molecular mechanisms involved in plant response to abiotic stresses. Furthermore, considerable advances have been made in developing transgenic crops resistant to abiotic stresses using such genes (Xiao et al. 2007; Babu et al. 2004; Wang et al. 2009).

Although significant advances have been made towards the identification and characterization of LEA sequences in many different crops, little is known about genes involved in mechanisms of tolerance to abiotic stress in cassava (*Manihot esculenta* Crantz). Cassava is one of the most important tropical food crops, cultivated for its starchy storage roots as a valuable source of calories; around 600 million people in Africa, Asia, and Latin America depend on this plant for their survival. In sub-Saharan Africa, an average consumption of cassava exceeds 300 kg per person/year (FAO 2003). In developing countries, cassava is often cultivated by subsistence farmers since the crop presents easy propagation systems, high drought tolerance, and low demand for nutrients, producing reasonably well under critical climate and soil conditions.

The identification of LEA sequences in cassava was firstly reported by our group, which isolated a cDNA sequence coding for a putative LEA protein possibly related to tuberization of the storage root since it was preferentially expressed in these roots in comparison to adventitious roots (de Souza et al. 2004). Recently, Lokko et al. (2007) and Sakurai et al. (2007) reported the identification of some expressed sequence tags induced in cassava under stress conditions, including drought. Here, we aimed to characterize this previously isolated cDNA sequence and its deduced amino acid sequence, designated MeLEA3, by bioinformatics analyses. In addition, we evaluated MeLEA3 gene expression in leaves under in vitro salt stress and investigated the presence of introns within the MeLEA3 genomic sequence.

Materials and Methods

Plant Material and Salt Stress Treatment

Cassava leaves and roots harvested in field conditions were kindly provided by Dr. Eloisa Cardoso from EMBRAPA Amazonia Oriental (EMBRAPA-CPATU, Belém-PA, Brazil) and Prof. Geraldo Rodrigues Coqueiro from the Universidade Federal Rural da Amazônia (Belém-PA, Brazil). Detached leaves were treated with 200 mM sodium chloride (NaCl) solution at 0, 2, 4, 8, and 12 h and immediately frozen in liquid nitrogen until RNA isolation. Sample at 0 h of NaCl treatment corresponds to leaves collected from cassava plants and immediately frozen in liquid nitrogen.

Cassava MeLEA3 cDNA Sequence

The MeLEA3 cDNA sequence used here has been previously identified as MALC04-C04 accession deposited in Embrapa-Cenargen GeneBank (Brasilia-DF, Brazil) and possibly related to storage root formation of cassava (de Souza et al. 2004). This sequence was registered in the National Center for Biotechnology Information (NCBI) GenBank under accession no. GR421259.1.

RNA Extraction and Semi-Quantitative RT-PCR Assays

Total RNA of roots was isolated according to methodology described by Jones et al. (1985). To isolate total RNA of leaves, we used methodology reported by Chang et al. (1993). RNA samples were quantified using a Qubit fluoremeter (Invitrogen, USA) and analyzed on formaldehyde agarose gel. RNA samples were treated with DNase I

following the manufacturer's guidelines (Invitrogen, USA) in order to eliminate genomic DNA contamination. The first strand cDNA was then synthesized with RevertAid H Minus Reverse Transcriptase (Fermentas, USA) and an oligo dT primer. cDNAs were quantified using the Qubit fluoremeter (Invitrogen, USA) and used in polymerase chain reaction (PCR) amplifications. MeLEA3 primers (MeLEA-F: 5′atggctcgctctttctcagacg3′ and MeLEA-R: 5′ ttaatgcttcttcaacagcatagccct 3′) flanking the open reading frame (ORF) of MeLEA3 were based on the cDNA sequence shown in Fig. 1. The Tubulin gene was used as internal control to confirm the initial amount of cDNA in PCR assays. Tubulin primer sequences (MeTub-F: 5′ atccttctcaagggcagcaagat3′ and MeTub-R: 5′acatggaaagta catggcctgctg3′) were based on cassava accession BM260004 available in GenBank. PCR assays with 15, 20, 25, 30, 35, 40, and 45 cycles were tested in order to obtain DNA fragments during exponential phase of amplification. PCR conditions used were: initial denaturation at 94°C for 5 min and 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1.5 min, followed by 20 min at 72°C. Each PCR assay was repeated three times. Relative intensities of ethidium-bromide-stained reverse transcriptase PCR (RT-PCR) products were measured by densitometry using the ChemiImager 4400 software (Alpha-Innotech Corporation, CA) and normalized against Tubulin values. The DNA fragments were cloned into pGEM-T vector (Promega, USA) and sequenced in order to confirm amplification of correct gene.

DNA Extraction and MeLEA3 Genomic Sequence Amplification

Genomic DNA was extracted from cassava leaves using the Purelink plant total DNA purification kit, following the manufacturer's guidelines (Invitrogen, USA). Purified genomic DNA was used as template in PCR assays, with MeLEA-F and MeLEA-R primers flanking the MeLEA3 ORF. PCR products were cloned into pGEM-T vector (Promega, USA) and sequenced.

Nucleotide Sequencing and Bioinformatics Analyses

Nucleotide sequences of clones containing cDNA and genomic fragments were obtained using the Big Dye Terminator kit (Applied Systems, USA) and aligned with MeLEA3 sequence (Fig. 1) using ClustalW multiple alignment program (Thompson et al. 1994). MeLEA3 cDNA sequence was evaluated by ORF Finder tool from NCBI to analyze by similarity with the non-redundant GenBank protein database using the BLASTX algorithm (Altschul et al. 1997). Molecular weight and isoelectric point of MeLEA3 deduced amino acid sequence were predicted by ExPASy Proteomics Server (http://ca.expasy.org/tools/pi_tool.html). For conserved domain analysis, MeLEA3 protein was evaluated using Pfam database (Bateman et al. 2000). Hydropathy plot was obtained according to the Kyte–Doolittle algorithm (Kyte and Doolittle 1982). Phylogenetic analysis was performed using the software PAUP 4.0b10, with maximum parsimony method and heuristic search strategy with random addition taxa. The confidence level for each node was tested using bootstrap method with 2,000 pseudoreplicates (Felsenstein 1985). TMHMM (http://www.cbs.dtu.dk/services/TMHMM) was used to predict transmembrane domains according to the method described by Krogh et al. (2001). SignalP v.2.0 (http://www.cbs.dtu.dk/services/SignalP) was used to predict signal peptides according to Nielsen and Krogh (1998). PSORT was used to predict signals for subcellular localization (Nakai and Kanehisa 1992). Mitochondrial localization was predicted using MitoProt II software (Claros and Vincens 1996). For secondary structure prediction of MeLEA3 protein, we used PSIPRED protein structure prediction server (Jones 1999). A search for splicing sites in the MeLEA3 genomic sequence was performed using SplicePort (Dogan et al. 2007).

Results and Discussion

In Silico Analyses of MeLEA3 cDNA and Deduced Amino Acid Sequences

Cassava adapts well to a variety of soil and climatic conditions, such as drought, one of the most serious abiotic stresses that limits plant growth and crop production in agriculture. Here, we aimed to increase our knowledge of the molecular basis of cassava abiotic stress resistance by characterization of the MeLEA3 cDNA sequence previously isolated by our group.

The MeLEA3 full-length cDNA shown in Fig. 1 has a total of 664 bp with an initiation codon ATG at position 197 nt and a stop codon TAA at position 479 nt, comprising a 285-bp ORF. This cDNA contains a 196-bp 5′ untranslated region and a 183-bp 3′-noncoding region containing a usual polyadenylation AATAA at a position 79 nt downstream of stop codon. MeLEA3 protein consists of 94 residues, accounting for a predicted molecular weight of 10 kDa and isoelectric point of 9.66.

A search for conserved domain in Pfam database revealed that MeLEA3 belongs to LEA3 family, PF03242, e-value 6e-24. In addition, a BLAST search of MeLEA3 against the non-redundant GenBank protein database showed maximum identity of 62% with the late embryogenesis protein -5 (ACV50424.1) isolated from roots of *Jatropa curcas* challenged with sodium chloride and also predicted to be a member of pfam03242 family. Members

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1   gcccgttggactaaatcaggtcacaccgggggtccaagtgaccgaggccgattcaagga  60

61  tttgacgcccccggcagtggagagaccgggtacaagagcaagttcttaccgctgggaagg 120

121 ttcttccataagagataaagttttccaacagttagcttcatcgctgttcctccaaggttt 180

181 ctcaattcgaatcgcaatggctcgctctttctcagacgcaaagtttctttctgctttcat 240
                    M  A  R  S  F  S  D  A  K  F  L  S  A  F
241 caccaaagcaatcaacgggagaggactttcagcatctgctcctgctgcaccacaaggagg 300
     T  K  A  I  N  G  R  G  L  S  A  S  A  P  A  A  P  Q  G  G
301 tgtgcgcagcctccccaaaggaggagcttccgcggtcaagaaaattgcggaagagaagat 360
     V  R  S  L  P  K  G  G  A  S  A  V  K  K  I  A  E  E  K  I
361 tgcatccgcacagaaggtcgcatggattccagatccccataccggatgctatagaccaga 420
     A  S  A  Q  K  V  A  W  I  P  D  P  H  T  G  C  Y  R  P  E
421 gaatgtaaccgaggagattgatgctgccgagtcaagggctatgctgttgaagaagcatta 480
     N  V  T  E  E  I  D  A  A  E  S  R  A  M  L  L  K  K  H  *
481 aacatagaaacagataaaagtctttcttattgattcattgatgtggtcgtataaagaaac 540

541 aagaaaggatggatatataaataatcggccggtgatctatgaatgtcttatgctttatat 600

601 ctttgctgtagcttctctaccctccctgtttaggagaaagaagaatttttcaaaaaaaa 660

661 aaaa
```

Fig. 1 The full-length cDNA sequence and deduced amino acid sequence of cassava MeLEA3. Start codon ATG and stop codon TAA are *boxed*. Potential polyadenylation signal is *underlined*. *Underlined* amino acids correspond to the N-terminal presequence which can direct MeLEA3 protein to mitochondria. This nucleotide sequence appears in the NCBI GenBank under accession no. GR421259.1

of LEA3 group are involved in response to dehydration and salt stresses (Xiao et al. 2007; George et al. 2009).

To predict whether signal peptide or signals for subcellular localization are present in the MeLEA3 protein, we analyzed its sequence using different computational tools, among them the MitoProt II software, which showed that this protein is predicted to be exported to mitochondria at 96.84% probability. MeLEA3 protein is predicted to be synthesized as a precursor containing an N-terminal presequence of 46 amino acids (Fig. 1) that is probably cleaved after protein to be imported into the mitochondria, resulting in a 5 kDa mature protein. Although most of LEA proteins are hydrophilic and localized in the cytoplasm (Swire-Clark and Marcotte 1999; Soulages et al. 2002), studies show that LEA proteins can also be found in nucleus, mitochondria, and chloroplast, where these proteins probably play different functional roles. For example, a genome-wide analysis of *Arabidopsis thaliana* reported by Hundertmark and Hincha (2008) identified 51 genes coding for LEA proteins; of them, 13 genes encoded were mainly members of LEA3 and LEA4 groups predicted to be located in chloroplast/mitochondria. Recently George et al. (2009) reported the identification of the 10 kDa atypical Pj LEA3 protein from *Prosopis juliflora* that also belongs to LEA3 Pf03242 family and is predicted to be located in chloroplast/mitochondria. In addition, alignment between MeLEA3 and Pj LEA3 proteins revealed that these proteins are 46% identical (data not shown). Although Pj LEA3 function is still unknown, Northern blot analysis showed that its expression was upregulated in *P. juliflora* leaves under 90 mM H_2O_2 stress, indicating a potential role in dehydration response (George et al. 2009).

Kyte–Doolittle algorithm analysis revealed that MeLEA3 protein shows a slightly hydropathic pattern, and the amino-terminal sequence is more hydrophobic than the carboxyl-terminal (Fig. 2a), as found in atypical LEA proteins from *Gossypium hirsutum* (Galau et al. 1993), *Citrus sinensis* (Naot et al. 1995), and *P. juliflora* (George et al. 2009). In addition, amino acid composition analyses of MeLEA3 showed a high content of alanine (18.09%), followed by lysine (9.57%) and serine (9.57%), unlike hydrophilins, typical LEA proteins which have glycine as their most abundant amino acid (Wise and Tunnacliffe 2004; Battaglia et al. 2008).

Our results of phylogenetic analysis also classified MeLEA3 as an atypical LEA. For this analysis, we used the first six hits of BlastX search for MeLEA3 sequence: late abundant protein- 5 from *J. curcas* (ACV50424.1), e-value 5e-22; indole-3-acetic acid-induced protein ARG2 from *Ricinus communis* (XP_002523101), e-value 2e-22; predicted protein from *Populus trichocarpa* (XP_002301483), e-value 1e-21; late embryogenesis abundant protein-5 from *Populus suaveolens* (ABF29697.1), e-value 4e-21; predicted protein from *P. trichocarpa* (XP_002321000.1), e-value 3e-17; and an unknown protein from *P. trichocarpa*× *Populus deltoids* (ABK96509), e-value 6e-17. We also included typical and atypical LEA protein sequences available in GenBank. An unrooted phylogenetic tree of LEA proteins is depicted in Fig. 2b, where MeLEA3 and its homologues, including the atypical PjLEA3 (ABG66530.1), form a distinct group (7) from typical LEA proteins (1, 2, 3, 4, and 6); however, they showed a phylogenetic relationship with known atypical LEA5A and LEA5D proteins from *G. hirsutum* (P46521.1, P46522.1) and LEA5 protein from *C. sinensis* (Q39644.1) (5).

Fig. 2 **a** Hydropathic plot of MeLEA3 protein according to the Kyte–Doolittle algorithm. *Regions above a score of zero* are hydrophobic. **b** Unrooted phylogenetic tree of LEA proteins based on maximum parsimony method. *Numbers above each node* are bootstrap values in%. The different LEA groups are shown by *different colors*. Typical LEA proteins (groups *1*, *2*, *3*, *4*, and *6*). Known atypical LEA proteins (group *5*) and MeLEA3 and its homologues (group *7*)

Fig. 3 Secondary structure of MeLEA3 protein of cassava using the PSIPRED protein structure prediction server

Based on confidence values for secondary structure prediction of MeLEA3 (Fig. 3) using the PSIPRED Server, we found that the majority of amino acids were predicted to be α-helices that can be important for the functional role of this protein. This result can be explained by high content of amino acid propensities for α-helices, such as alanine and lysine (Costantini et al. 2006), within MeLEA3 sequence. Furthermore, according to a report by Wise (2003), a common characteristic of members of group 3 LEA is a high content of α-helices. Studies indicate that atypical LEA proteins can require a globular conformation since they remain in an insoluble state after boiling (Baker et al. 1988; Zegzouti et al. 1997). On the other hand, many LEA proteins were found unfolded in solution (Soulages et al. 2002; Goyal et al. 2003), and some were able to change to a more folded state under dehydration stress (Goyal et al. 2003; Shih et al. 2004). In addition, recent studies have evidenced that hydrophilic and hydrophobic LEA proteins can protect from dehydration stress by different mechanisms. For example, Grelet et al. (2005) identified a hydrophilic mitochondrial LEA protein expressed in seeds of *Pisum sativum* predicted to form amphipathic helices and able to protect enzymes from drying. Later, Tolleter et al.

Fig. 4 Expression analysis of MeLEA3 in cassava by semi-quantitative RT-PCR. DNA bands amplified from non-stressed storage roots (*R*) and leaves (*L*) (**a**) and detached leaves under 200 mM NaCl at 0, 2, 4, 8, and 12 h (**b**) were visualized on agarose gel stained with ethidium bromide. Tubulin gene was used as internal control. **c** Relative intensities of RT-PCR products from leaves under salt stress were quantified by densitometry using the ChemiImager 4400 software and normalized against Tubulin values. The data represent the mean ± SD of the three experiments

(2007) confirmed that this LEA protein is natively unfolded and adopts a reversible α-helical structure upon desiccation; it was also able to interact with membranes in a dry state and protect liposomes subjected to drying. On the other hand, MtPM25, a hydrophobic atypical protein from *Medicago truncatula* seeds, was unable to protect membranes but able to prevent aggregation of proteins during stress (Boucher et al. 2010).

Introns Within *MeLEA3* Genomic Sequence and Expression Analyses in Detached Leaves Under In Vitro Salt Stress

Here, we also investigated the presence of introns within coding sequence of the MeLEA3 gene, taking into account the importance of such elements in gene expression regulation of plants (Nelsen and Marcotte 2000; Rose 2008; Samadder et al. 2008). A 295-bp fragment corresponding to the MeLEA3 ORF was amplified from genomic DNA of cassava leaves. Its nucleotide sequence was aligned with MeLEA3 cDNA, showing no introns within this genomic sequence. This result was confirmed by a search of potential sites for splicing using the SplicePort program. Few studies of identification and functional analysis of introns from LEA genes have been reported. For example, Nelsen and Marcotte (2000) identified a wheat group 1 Lea intron which was able to enhance *β-glucuronidase* gene expression in cereal cells. Wang et al. (2007) reported the identification of 34 LEA genes in genome of rice: 64.7% and 20.6% of them contain only one intron and two introns, respectively, while 14.7% have no intron.

According to studies reported by de Souza et al. (2004), MeLEA3 and Mec1 transcripts were found in storage roots but not in adventitious roots of cassava, suggesting a potential role of these genes in the tuberization process. In addition, Northern blot analysis revealed that *Mec1* gene coding for the Pt2L4 glutamic acid-rich protein is strongly expressed in storage roots in comparison to other organs, including leaves, where no signal hybridization was detected (de Souza et al. 2006). We then performed semi-quantitative RT-PCR assays, a methodology which has been employed successfully in gene expression studies of different crops (Meng et al. 2009; Li et al. 2010; Wang et al. 2010; Zhang et al., 2010; Zhuang et al. 2010) in order to evaluate whether the *MeLEA3* gene could also be strongly expressed in storage roots. Leaf and root samples used in RT-PCR assays were obtained from plants grown under field conditions in the Amazon region, where humidity rates can reach up to 90%. Our results showed that unlike the *Mec1* gene, the *MeLEA3* gene is expressed in both organs, with a higher expression in leaves than storage roots (Fig. 4a). Studies show that LEA gene expression has no tissue specificity since it can be found in different parts of the plant, such as seeds, stems, leaves, and roots (Hou and Guo 1998; Zhang et al. 2002; Federspiel 2000; Hundertmark and Hincha 2008). Although most LEA genes can be induced or enhanced by various stress conditions, they can also be downregulated or expressed constitutively (Wang et al. 2007; Oztur et al. 2002). Dehydration-related stress conditions used to evaluate LEA gene expression have included NaCl, polyethylene glycol, and abscisic acid treatments (Xiao et al. 2007; Kim et al. 2005). Therefore, to evaluate the response of MeLEA3 under dehydration conditions, we examined changes in its expression in detached leaves treated with 200 mM NaCl by RT-PCR assays. Fig. 4b, c shows the RT-PCR products analyzed on agarose gel and quantification of the band intensity expressed as MeLEA3/Tubulin relative densities, respectively. Our results revealed that levels of MeLEA3 increase under in vitro salt stress treatment, with the highest expression level at 8 h, suggesting a potential role in stress response.

This is the first study on characterization of a LEA sequence in cassava, a drought tolerant crop that can be grown on depleted and marginal soil. Although elucidation of the exact function of MeLEA3 was not the goal of the present study, our results suggest that this hydrophobic atypical LEA protein predicted to be exported to mitochondria can be involved in acquisition of tolerance to dehydration stress in cassava since its transcript accumulation was upregulated in leaves under salt stress conditions. The results obtained here can contribute to understanding molecular mechanisms involved in resistance of cassava to abiotic stresses. In addition, the MeLEA3 sequence could be a potential candidate gene for generation of drought-resistant crops; however, further studies, including elucidation of its functional roles, are necessary.

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