BÁRBARA CASTANHEIRA FERRARA BARBOSA

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Supervisor

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Dedicated to my beloved mom,
Eunice Leite Castanheira,
the reason of my life.

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RESUMO GERAL

O zinco foi identificado como um nutriente essencial para as plantas nos anos 1970. Em plantas, ele é absorvido na forma Zn^{2+}, e as suas funções estão relacionadas com as propriedades de um cátion bivalente, com uma forte tendência para formar complexos tetraédricos. O zinco age quer como componente metálico de enzimas ou como cofatores. Por exemplo a álcool desidrogenase, superóxido dismutase, anidrase carbónica e RNA polimerase são enzimas que contêm Zn. O zinco também exibe um papel na ativação de enzimas envolvidas no metabolismo de carboidratos, proteínas, triptofano e ácido indol-acético (AIA). Acredita-se que as metalotioneínas (MTs), proteínas conhecidas por participar no processo de homeostase de metais, são parcialmente responsáveis pela absorção de zinco em plantas. Metalotioneínas são proteínas de baixo peso molecular, ricas em resíduos de cisteína que podem se ligar a metais, formando compostos do tipo metal-tiolato. Em contraste com os numerosos relatórios sobre a estrutura gênica e padrões de expressão, as funções das MTs não foram precisamente determinadas. Dessa forma, este estudo objetivou caracterizar in silico os prováveis genes que codificam metalotioneínas e analisar a expressão de alguns destes genes em resposta ao fornecimento de zinco em café. As sequências selecionadas obtidas a partir das pesquisas realizadas no banco de dados CAFEST tiveram a sua filogenia avaliada por árvores filogenéticas e seus perfis de expressão in silico foram analisados por Northerns eletrônicos. O banco de dados CAFEST mostrou um elevado número de sequências relacionadas com metalotioneínas. As árvores filogenéticas demonstraram uma elevada similaridade entre as sequências encontradas no CAFEST e as sequencias de outras espécies, e os Northerns eletrônicos detectaram a sua expressão em diferentes tecidos, fases de desenvolvimento e condições de stress. As análises quantitativas de expressão

dos genes que codificam MTs mostraram que estes genes são expressos diferencialmente em resposta às diferentes doses de zinco fornecidas.

GENERAL ABSTRACT

Zinc was identified as an essential nutrient for plants in the 1970s. In plants, it is absorbed as Zn^{2+}, and its functions are related to the properties of a divalent cation, with a strong tendency to form tetrahedral complexes. The zinc acts either as enzyme metallic components or as cofactors. For example, alcohol dehydrogenase, superoxide dismutase, carbonic anhydrase and RNA polymerase are Zn containing enzymes. Zinc also displays a role in activating enzymes involved in the metabolism of carbohydrates, protein, tryptophan and indole acetic acid (IAA). It is hypothesized that metallothioneins, which are proteins known to participate in the process of metal homeostasis, are partially responsible for the zinc up-take in plants. Metallothioneins (MTs) are low molecular weight, cysteine-rich proteins that can bind metals forming metal-thiolate clusters. In contrast to the numerous reports about MTs gene structure and expression patterns, the functions of MTs are still elusive. Thus, this study aimed to *in silico* characterize the putative metallothionein-like genes and to analyze the expression of some of these genes in response to zinc supply in coffee. The selected sequences obtained from the searches performed in the CAFEST database had their phylogeny assessed by phylogenetic trees and the in silico expression profiles were analyzed by electronic Northerns. The CAFEST database showed a high number of sequences related to metallothionein-like genes. The phylogenetic trees demonstrated a high similarity between the sequences found in the CAFEST and those form other species, and the electronic Northerns detected their expression in different tissues, development stages and stress conditions. The expression analyses of the genes encoding putative MTs showed that these genes are differentially expressed in response to zinc supply.

SUMMARY

CHAPTER 1 – GENERAL INTRODUCTION

1 INTRODUCTION

Mineral nutrients are essential to plant development and have specific functions in plant metabolism. As they are divided in Micro and Macronutrients, mineral nutrients are required in different rates. Macronutrients are needed in large amounts and include: nitrogen (N), phosphorus (P), and potassium (K) calcium (Ca), magnesium (Mg), and sulfur (S). Micronutrients are needed in small quantities although indispensible for plant growth. They are: boron (B), copper (Cu), iron (Fe), chloride (Cl), manganese (Mn), molybdenum (Mo) and zinc (Zn). Thus, when the present amount is not satisfactory, nutrient deficiency in cells promote severe metabolic alterations. These disorders often result in visible symptoms such as chlorosis, necrosis and reduced growth. Symptoms of mineral deficiencies are to some extent characteristic for each nutrient, depending upon the severity of deficiency, species, variety and environmental factors (MARSCHNER, 1995).

Zinc, a micronutrient, is especially involved in numerous processes related to plant growth and development. Identified as an essential nutrient for plants in the 1970s, it is known to take part in enzymes synthesis, and also acting as cofactors, which are necessary for enzyme activation. For example, alcohol dehydrogenase, superoxide dismutase, carbonic anhydrase and RNA polymerase are enzymes containing Zn. In addition, it is related to the activation of enzymes involved in the metabolism of carbohydrates, protein, tryptophan and indole acetic acid (IAA) (MARSCHNER, 1995).

Metallothioneins (MTs) are low molecular weight, cysteine-rich proteins that can bind metals forming a metal-thiolate clusters. It is, thus, hypothesized that metallothioneins, which are proteins known to participate in the process of metal homeostasis, are partially responsible for the zinc up-take in plants.

2 BACKGROUNG

2.1 Metallothioneins

Metallothioneins (MTs) constitutes a super-family of low molecular weight proteins (5-10 kDa) ubiquitously distributed in nature (FREISINGER, 2008). The dominant feature of metallothioneins is the high content of cysteine residues, what makes it possible for divalent metal ions (Zn^{2+}, Cu^{2+}, Cd^{2+}) to bind to thiol groups of cysteine residues (FREISINGER, 2008). Metal-MT ligation is mainly accomplished by formation of metal-thiolate clusters, but the participation of nonproteic ligands has also been reported. The remarkable combination of high thermodynamic but low kinetic stability is one of the main features of the metal-MT complexes that allow them to bind metals very tightly, and promptly exchange them to other proteins (DOMENECH et al., 2007).

The spatial structures of MTs have been uncovered as structure with separate domains, α and β. The different metal reactivity and metal affinity of two domains suggests different functional roles, that is, N-terminal β domain is involved in the homeostasis of essential metal ions (KAGI; SCHAFFER, 1988; WILLNER; VASAK; KAGI, 1987), and C-terminal α domain, is involved in the sequestration of excess and/or toxic metal ions (WRIGHT et al., 1987).

The first description of MTs occurred more than 50 years ago, when Margoshes and Vallee (1957) identified a chelator of cadmium in the equine renal cortex, responsible for the accumulation of this element in the kidneys. Since then, its presence has been widely detected in animal species, plants, algae and prokaryotic organisms (COBBETT; GOLDSBROUGH, 2002). The physiological functions of MTs include the alleged transfer of essential metal ions, zinc and copper to various metalloenzymes and transcription factors (VALLEE, 1991), as well as storage and detoxification of metal ions including cadmium and mercury (GIORDANI et al., 2000; ZIMERI et al., 2005), and increasingly, roles in protection against reactive oxygen species

(CHUBATSU; MENEGHINI, 1993; AKASHI et al., 2004). The high conservation of certain domains in this group of proteins indicates its importance throughout the evolutionary pathway of many species.

According to the actual system of classification, the MT superfamily is subdivided into 15 families, and the plant MTs are part of the fifteenth (15) family. This classification system also includes subfamilies, subgroups, isolated isoforms and alleles (BINZ; KAGI, 2001).

Most MTs plant contains two cysteine-rich domains separated by a central area free of cysteines, but with hydrophobic and aromatic amino acids (exclusive of plants). All plants MTs can be subdivided based on the sequence of amino acids into four types according to the distribution of cysteine residues in two domains (ROBINSON et al., 1993; COBBETT; GOLDSBROUGH, 2002). Type 1 metallothioneins have six cysteine residues in a grouped arrangement Cys-X-Cys, distributed equally in the amino and carboxy terminal domain. Type 2 proteins contain four pairs of cysteines at the amino terminal end, starting from a configuration Cys-Cys and ending at the first domain with Cys-X-X-Cys and the typical distribution at the carboxy terminal end (ROBINSON et al., 1993; COBBETT; GOLDSBROUGH, 2002). The Type 3 MTs exhibit a distinctive arrangement of four cysteines in the amino terminus (Cys-X-X-Cys-X- Cys-X-X-X-X-Cys). The MTs related to wheat Ec gene expressed during development of embryos are classified as Type 4 and have cysteine residues in all three domains (ROBINSON et al., 1993; COBBETT; GOLDSBROUGH, 2002).

Plants contain genes that are homologous in structure and function to genes of MT animals and fungi (RAUSER, 1999). The first plant MT protein was isolated from wheat embryos (Ec), with three cysteine domains and demonstrated ability to bind zinc (LANE; KAJIOKA; KENNEDY, 1987). Thereafter, MT genes have been reported in other monocots and dicots species, including maize, wheat and soybean (KAWASHIMA et al., 1991; FOLEY; SINGH, 1994). The MTs of various types are encoded by small

gene families with two or more genes, even in the small genome of Arabidopsis (ZIMERI et al., 2005) where nine members of the four types have been reported. Rice has also been identified to contain nine MT genes distributed in several chromosomes (WONG et al., 2004). Futhermore, three new genes were found in rice making a total of eleven MT genes in this species (ZHOU et al., 2006). In most species, one or more isoforms of any of the four types of MTs were reported, but not all. In *Populus trichocarpa x deltoides* six MTs genes were found, representatives of types 1, 2 and 3 (two of each type) (KOHLER et al., 2004). Six MTs genes have been identified in various studies in cotton: three of type 1, one of type 2 and two of type 3 (HUDSPETH et al., 1996; JORDAN et al., 2005; XUE et al., 2009). In tomato three MTs genes have been reported, all belonging to type 2 (GIRITCH et al., 1998).

Each gene has a specific temporal and spatial expression pattern (COBBETT; GOLDSBROUGH, 2002). Generally, type 1 MTs genes are constitutively expressed for most tissues examined. Type 2 MTs transcripts are abundantly detected in leaves at various development stages, flowers and fruits, and to a lesser extent in roots, whereas the expression of type 3 MTs is almost exclusive to fruit and leaves of plants without fruits. Transcripts or Ec Type 4 MTs, are exclusive to seeds (COBBETT; GOLDSBROUGH, 2002; FREISINGER, 2008). In Arabidopsis, the type 1 MTs were more highly expressed in roots than in leaves, while the MTs transcripts of types 2 and 3 were detected in higher levels in leaves than in roots (ZHOU; GOLDSBROUGH, 1995; MURPHY et al., 1997). Most of the predicted proteins of plants were not isolated, except for wheat (LANE; KAJIOKA; KENNEDY, 1987) and Arabidopsis (MURPHY et al., 1997), and the ability of each protein to bind metals has not been established, except for wheat Ec. Therefore, the expression of metallothioneins has been mainly inferred from sequences of DNA /mRNA.

The function of metallothioneins is still elusive, but evidence indicates they have an important role in the homeostasis of metals, probably

associated with transportation. The synthesis of MTs can also be induced by various agents such as hormones, growth factors, tumor promoters, physical-chemical stresses and under many other chemicals conditions, in vivo and in cell cultures (KAGI, 1991; FORDHAM-SKELTON et al., 1997; COBBETT; GOLDSBROUGH, 2002). In mammals it is well established that MTs maintains the homeostasis of zinc and copper and protect cells from the toxicity of cadmium and oxidative stress (COYLE et al., 2002). Since the plants MTs efficiently bind metals (EVANS et al., 1992; MURPHY et al., 1997) and some MTs genes are transcriptionally regulated by metals (MURPHY; TAIZ, 1995; COBBETT; GOLDSBROUGH, 2002), it was suggested that metallothioneins might also have an important role in homeostasis and metal tolerance in plants.

Evidence suggests the importance of MTs to copper tolerant Arabidopsis, which may be associated with metal homeostasis in plants, regulating the concentration of certain ions (ROBINSON et al., 1993; ZHOU; GOLDSBROUGH, 1995). Transcripts MT1 accumulate in leaves of Arabidopsis exposed to Cu^{2+}, and to a lesser extent for Zn^{2+} and Cd^{2+}, while those of MT2 increased in the presence of Ag^{+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, or heat shock (ZHOU; GOLDSBROUGH, 1995). Arabidopsis genes MTs (MT1a and MT2a) when expressed in mutant yeast lacking the metallothionein gene (CUP1), reconstituted and increased the tolerance to elevated contents of Cu^{2+} and Cd^{2+} and MT2a was capable of restoring the growth if there were high levels of Cd (ZHOU; GOLDSBROUGH, 1995). The same gene from Arabidopsis (MT2a) partially restored the growth of a mutant cyanobacteria (smTA gene deletion) in the presence of Zn (FORDHAM-SKELTON et al., 1997). Turchi et al. (2012) demonstrated that the overexpression of a type 2 MT from *Pisum sativum* in white poplar resulted in an enhanced tolerance and increased Zn and Cu sequestration. Furthermore, the ability of the 4 types of MTs from Arabidopsis to bind to metals was investigated (GUO; MEETAM; GOLDSBROUGH, 2008). With this propose, six isoforms (MT1a, MT2a, MT2b, MT3, MT4a and MT4b) were introduced and

expressed in mutant yeast sensitive to copper and zinc, Δcup1, Δzrc1Δcot1 respectively. The four types of MTs conferred similar levels of tolerance and copper accumulation in mutant Δcup1. However, the MTs type 4a and 4b conferred greater Zn tolerance and accumulation in the mutant Δzrc1Δcot1t, evidencing the preference of these proteins for different metals (GUO; MEETAM; GOLDSBROUGH, 2008). Zhao et al. (2009) verified that transgenic rice plants, super expressing the OsMT1a (a type 1 MT-like gene), accumulated 53% more zinc in grains than wild type plants.

The large number of Cys residues in MTs suggests that they might be involved in the detoxification of reactive oxygen species (ROS) or in the maintenance of redox level (CHUBATSU; MENEGHINI, 1993; KANG, 2006). With a structure rich in sulphydryl groups, MTs provide a "sink" to trap free radicals, that is, the multiple cysteine residues can react with superoxide ($\bullet O_2^-$) and hydroxyl radicals (•OH) leading to their degradation (KLASSEN; CAGEN, 1981; SATO; BREMNER, 1993).

All the amino acid sequences of MTs from plants derived from conceptual translation of mRNA sequences/cDNA identified in studies of gene expression or in sequencing projects. Currently, about 25% of all the entries in the protein sequences database Swiss-Prot are listed under the name 'metallothioneins' (FREISINGER, 2008). MTs proteins have no recognized enzymatic activity, and the purification of this protein from plant extracts is difficult, possibly due to instability and susceptibility to degradation in the presence of oxygen (MURPHY et al., 1997). Moreover, only nine MTs proteins were characterized, and eight of them were exclusively expressed as recombinant proteins in Escherichia coli (FREISINGER, 2008).

2.2 Zinc

Zinc was identified as an essential nutrient for plants in the 1970s. In plants, it is absorbed as Zn^{2+}, and its functions are related to the properties of

a divalent cation, with a strong tendency to form tetrahedral complexes. The zinc acts either as enzyme metallic components or as cofactors. For example, alcohol dehydrogenase, superoxide dismutase, carbonic anhydrase and RNA polymerase are enzymes containing Zn. Zinc also displays a role in activating enzymes involved in the metabolism of carbohydrates, protein, tryptophan and indole acetic acid (IAA) (MARSCHNER, 1995).

Research shows us that zinc is required for the synthesis of Tryptophan, which in turn is the precursor for the synthesis of IAA. Thus, Zinc deficient plants exhibit low levels of auxins. In the absence of IAA plant growth is inhibited, particularly internode growth and leaf size. A more direct influence of zinc deficiency is that of grain or seed yield which are reduced to a greater extent. Zinc also plays a major role in plant fertilization, as pollen grains contain a very high concentration of zinc. At flowering time during fertilization most of the zinc taken up is incorporated into the developed seed.

2.2.1 Protein synthesis and function

Zinc is an element well known to participate in protein synthesis and to contribute to the structural integrity of a number of proteins. According to Malavolta (2006), zinc deficiency causes a disruption in protein biosynthesis and nitrate reduction, promoting a decrease in the level of RNA. This is due to impairments in the structural integrity of ribosomes and activities of enzymes involved in protein synthesis (e.g. RNA polymerase) (MARSCHNER, 1995). Consequently, the activity of enzymes and zinc finger proteins (DNA-binding proteins) are seriously affected under Zn deficient conditions. Zinc finger proteins contribute to innumerous cellular processes and are required for gene expression and regulation.

It is estimated that up to 10% of the proteins in biological systems need Zn for the maintenance of structural integrity and consequently for the proper protein function (ANDREINI et al., 2006). Therefore, zinc has an

important role in energy metabolism of the plant, including the production of dry matter. For these reasons it is not surprising that plants are highly susceptible to low concentrations of Zn in tissues.

2.2.2 Structural and functional integrity of cell membranes

The structural integrity of proteins and proper functioning of biological membranes are under the control of Zn, which greatly affects protein architecture and controls the permeability of biological membranes, which are needed at very high concentrations. When Zn is deficient, the structural integrity of membranes is compromised resulting in degenerative changes in membrane function. Consequently, there is an alteration in uptake and transport of mineral nutrients (CAKMAK, 2000)

Zinc deficiency is an ordinary problem in semi-arid regions where salt accumulation is also a common problem in soil. The loss of structural integrity of root cell membranes in low zinc conditions may induce a high root uptake of Na, leading to salt damage. Improving the zinc nutritional status of plants grown under saline soil conditions is therefore important to minimize excess salt accumulation in plants (CAKMAK, 2000).

2.2.3 Detoxification of reactive oxygen species (ROS)

Reactive oxygen species (ROS), such as superoxide radical (O_2^-) and hydroxyl radical (OH), are the main cause of cell damage under various stress factors, such as drought, heat and freezing, which are responsible for oxidative damage of chlorophyll, proteins, biological membranes and DNA. Among ROS, O_2^- is very important because it generates other toxic O_2 species. Furthermore, a number of enzyme systems or metabolic processes in plant cells generate ROS by transferring electrons onto oxygen, particularly during photosynthetic or mitochondrial electron transport (ASADA, 1999).

Superoxide dismutase (SOD) is a Zn-dependent enzyme that detoxifies superoxide radicals and plays a key role in the defense systems

against ROS. There are three types of SOD enzymes and the Zn-containing one is the predominant form in both plant and mammalian cells. In plants with zinc deficiency, SOD activity is significantly depressed leading to a higher susceptibility of cells to an oxidative attack by ROS. Due to the reduced biosynthesis of proteins, activity of other antioxidant enzymes is also reduced in Zn deficiency, which potentiates production of more ROS.

Superoxide generating NADPH-oxidase is a universal enzyme system, very sensitive to Zn supply, which produces superoxide radicals in biological systems and represents an important source of ROS in different stress conditions. Evidences suggest that the activity of superoxide generating NADPH-oxidase is increased under Zn deficiency (CAKMAK; MARSCHNER, 1988). Also, there is a very close relationship between cell damage (lipid peroxidation) and the enhanced activity of superoxide generating NADPH-oxidase in different cell systems, another reason why plants are highly susceptible to environmental stress factors under zinc deficiency.

2.2.4 Photo-oxidation and light damage

Chloroplasts are the major organelles producing ROS, especially during the photosynthesis process, in which large amounts of electrons are produced for CO_2 fixation, particularly under high light intensity. If photosynthetic CO_2 fixation is reduced due to any stress factor, the electrons released and the light energy absorbed by pigments during photosynthesis is used intensively for the activation of oxygen instead of CO_2 reduction. This transfer of electrons and light energy to O_2 produces ROS leading to so-called “photooxidative damage” to chloroplasts (CAKMAK; MARSCHNER, 1988).

Due to the low activity of the protective enzymes against ROS (such as superoxide dismutase) in zinc deficient plants, ROS generation and photooxidative damage is additionally intensified. Zinc deficient plants are,

therefore, highly sensitive to high light conditions, especially in combination with an environmental stress factor, quickly developing leaf chlorosis and necrosis when exposed to high light intensity.

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CHAPTER 2 - CHARACTERIZATION, CLONING AND SEQUENCING OF A PUTATIVE GENE ENCODING A METALLOTHIONEIN-LIKE PROTEIN IN *COFFEA ARABICA*.

ABSTRACT

Metallothioneins (MTs) are low molecular weight, cysteine-rich proteins that can bind metals forming metal-thiolate clusters. In contrast to the numerous reports about MTs gene structure and expression patterns, the functions of MTs are still elusive. In our search in the database of coffee Expressed Sequence Tags (CAFEST) using keywords and sequences of known metallothionein-like proteins, we obtained 27 EST-contigs. These sequences, that showed a reliable similarity, were clustered and annotated. Multiple alignments were performed, comprising the sequences found in this work and sequences of metallothioneins from other species. Phylogenetic trees were designed and the expression profile was assessed by *in silico* Northerns. The expression of the ESTs occurred more often in libraries of seedling and leaves treated with arachidonic acid. However, the MTs showed a wide expression profile, being detected in many tissues. These results indicate the probable involvement of coffee MTs in different phases of the plant development.

RESUMO

Metalotioneínas (MTs) são proteínas de baixo peso molecular, ricas em resíduos de cisteína, capazes de se ligar a metais formando compostos do tipo metal-tiolato. Embora hajam inúmeras pesquisas sobre estruturas gênicas e padrões de expressão, as funções das metalotioneínas permanecem indefinidas. Na nossa busca no banco de dados CAFEST, usando palavras-chave e sequências de metalotioneínas já descritas, obtivemos 27 EST-contigs. Essas sequências, que apresentaram uma alta similaridade, foram clusterizadas e anotadas. Foram realizados múltiplos alinhamentos compreendendo as sequências encontradas neste trabalho e sequências de metalotioneínas de outras espécies. Árvores filogenéticas foram desenhadas e o perfil de expressão foi avaliado por Northerns in silico. A expressão dos ESTs ocorreu mais frequentemente em bibliotecas de plântulas e folhas tratadas com ácido araquidônico. No entanto, as MTs apresentaram um perfil de expressão de amplo, tendo sido detectadas em muitos tecidos. Estes resultados indicam o provável envolvimento das MTs de café em diferentes fases do desenvolvimento da planta.

1 INTRODUCTION

Metallothioneins (MTs) are proteins of low molecular weight, part of a cysteine-rich superfamily. The cysteine domains have great affinity to metal ions, and have shown to associate more commonly to Zn^{2+} and Cu^{+}. Specifically when referring to plants MTs, it is preferred to use the term *MT-like* since most of them are inferred from DNA sequences derived from different species. Based on the number and arrangement of cysteine residues, all the plant MTs belong to class II and can be further subdivided into four types (COBBETT; GOLDSBROUGH, 2002). The analysis of the expression pattern of these four types of MTs revealed that type 1 MTs are mainly found in roots (HUDSPETH et al., 1996), whereas types 2 and 3 MTs are found in the leaves (ZHOU; GOLDSBROUGH, 1995; HSIEH et al., 1996) and expression of type 4 MTs was only found in developing seeds.

The effort to sequence coffee genome resulted in an EST (*Expressed Sequence Tags*) database – CAFEST (VIEIRA et al., 2006). This allowed the search of putative genes of interest, related to numerous physiological traits. Also, it made possible to determine the tissues where the genes of interest are expressed and the levels of this expression.

Thus, this study aimed to characterize in silico the putative genes that encode metallothionein-like proteins in coffee (Coffea arabica).

2 MATERIALS AND METHODS

2.1 In Silico essay

The computational essays consisted of a series of analyses carried out with the purpose to characterize the putative MT-like proteins in coffee.

2.1.1 Search for EST-contigs

First, a search for MTs sequences was performed by keyword criteria at NCBI database and obtained sequences were annotated. Then, a search using BLAST (*Basic Local Alignment Search Tool*) in the CAFEST (http://bioinfo04.ibi.unicamp.br) database was performed using as queries the sequences previously obtained, as well as keywords. Sequences showing reliable similarity (e-value <4.10) were deposited in the system for sequence management and manipulation − the GeneProject − and clustered using the CAP3 program (HUANG; MADAN, 1999). After clustering, the selected contigs and singlets were annotated and compared against public databases of protein to obtain more information about the probable proteins encoded by these sequences and elimination of false sequences. Only sequences involving the conserved domain were selected for subsequent analysis. Later, chosen sequences were also used as templates for a new search by CAFEST, in a process called saturation, with the intention to extend or supplement the resulting sequences, or even find new ones. This process was repeated until no significant *read* returned in the search.

2.1.2 Phylogenetic Analysis

Multiple alignments were performed comprising the protein coding sequence from the selected *contigs* and homologous sequences published in NCBI.

The ORFs (Open Reading Frames) were obtained by ORFinder tool, found at the NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov). The protein encoding sequences were acquired by the translation tool found in protein database ExPASy (http://www.expasy.ch). Multiple alignments were performed in ClustalW software (THOMPSON; HIGGINS; GIBSON, 1994) using default parameters. Then a phylogenetic tree was constructed by MEGA5 software, version 5.0 (TAMURA et al., 2007) with default parameters, *neighbor-joining* comparison model (SAITOU; NEI, 1987), distance-P method and *pair-wise* deletion. The validity of the trees and the phylogenetic distance of the clusters could be measured by probabilistic bootstraps test with 1000 replications (SITNIKOVA; RZHETSKY; NEI, 1995).

2.1.3 Electronic Northern

For the *in silico* analysis of the spatial expression patterns, Eletronic Northerns were designed. Through this, we calculated the appearance frequencies of the *contigs* forming *reads* in each library. Subsequently, the data were normalized to give an accurate picture of the expression degree of candidate genes for each treatment and locus of the plant. The procedure for standardization was to multiply each read by the ratio between the total number of reads in all libraries and library number of reads in which it was expressed. The sequences were rearranged according to the sites of expression and their data were fed into a matrix, linking genes and libraries. The EST-contigs and libraries were grouped by hierarchical clustering using the programs Cluster and Treeview (EISEN et al., 1999). The expression results were presented in a gray scale, where zero or negative expression is represented by lighter color and is gradually increased up to black, which represents the maximum degree of expression.

2.1.4 Primer Design

Based on the computational essays, two *contigs* were selected for further investigation.

In order to isolate the putative coffee MTs genes, primers (table 1) were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/).

Table 1– Primers designed for fragment isolation

Gene	Primer Sequence	Amplicon (pb)
CaMT19 F	5'CACCTTCTCACCATGTCGGACA	209
CaMT19 R	5'GCGTCAATTGTCACAGGTGCAG	
CaMT21 F	5'CACCAGAATGTCGTGCTGCG	248
CaMT21 R	5' CCTCATTTGCAGTTGCAGGGA	

2.2 Cloning and Sequencing

2.2.1 Plant Material

The plant material was collected in the experimental field of Universidade Federal de Lavras (UFLA) in Lavras-MG (21 ° 14'42" S 45 ° 00'00" W) and the experiment was developed at the Laboratory of Molecular Physiology of Plants (LFMP -UFLA). For RNA extraction of coffee, we used completely expanded leaves of Coffea arabica cv. Ruby. The samples were preserved immediately in liquid nitrogen and subsequently stored at -80 ° C.

2.2.2 DNA extraction

The DNA extraction from leaves followed the CTAB protocol with slight modifications. The samples were quantified in a spectrophotometer (Nanodrop ® ND-1000 Espectrophotometer) at the A260 and checked on 0,8% agarose gel, satined with 2 mL of GelRed (Biotium).

2.2.3 RNA extraction and cDNA synthesis

The RNA extraction followed the manual's recommendations given for the ConcertTM Kit Plant RNA Reagent (Invitrogen) with minor changes. Subsequently the samples were treated with the inhibitor Turbo DNA-free kit (Ambion), for removing residual DNA contamination. The RNA integrity was checked on 0.8% agarose gel. The samples were quantified in a spectrophotometer (Nanodrop ® ND-1000 Espectrophotometer) at the A260. The samples that exhibited a high degree of integrity and purity were used for cDNA synthesis, the cDNA Kit High-Capacity Reverse Transcription (Applied Biosystems). After cDNA synthesis, the samples were stored in a freezer at -20 ° C until use.

2.2.4 Isolation, cloning and transformation of amplification products

The isolation of the fragments of interest was performed through PCR with GoTaq polymerase (Promega) with both cDNA and DNA templates. Denaturation, annealing and extension temperatures and mastermix concentrations for PCR were optimized for each pair of primers. The products of the amplifications were subjected to electrophoresis on agarose gel 1.2% (w / v) stained with 2mL of GelRed (Biotium), under an electric current of 110 V in TAE buffer (1mM EDTA pH 8.0, 40 mM TRIS pH 8.0, 20mM acetic acid) for 40 min. The fragments obtained were eluted from the agarose gel by MinElute Kit (Qiagen ®) and quantified using Nanodrop 8000 (Thermo Scientific).

Isolated fragments were then ligated to the pCR II vector, using TOPO-TA kit (Invitrogen®) following manufacturer instructions.

Recombinant vectors were inserted in *E. coli*, TOP10 strain chemically competent (Invitrogen®) in Kan50 supplemented medium. 2μL of ligation products were mixed to 40μL *E. coli*, incubated on ice for 30 minutes, followed by heat shock of 42oC for 45 seconds, and incubated again on ice for 2 minutes. 250μL of SOC medium (Invitrogen®) was added

to the recombinant cells, incubated in a shaker (300 rpm) at 37°C for 45 minutes. After this, cells were plated on solid LB medium enriched with 50μL/mL of kanamycin (Kan50), and left for 16 hours at 37 °C.

2.2.5 PCR analysis of transformed colonies

Transformed colonies were submitted to a PCR, using an inoculum of each colony resistant to the selective medium added to a mastermix containing the specific primers and the GoTaq polymerase enzyme (Promega®). PCR products were submitted to an electrophoresis gel in 1% agarose.

2.2.6 Extraction of plasmid DNA

Plasmid DNA was extracted from transformed colonies using Plasmid Miniprep Kit (Qiagen). One colony containing the gene was added to 10 mL of LB medium supplemented with Kan50 and kept in an orbital shaker at 200 rpm, 37°C for 16 hours. After that, cultures were centrifuged to pellet the cells, and used for plasmid DNA extraction. DNA was diluted in DEPC water and stored at -80°C.

2.2.7 Sequencing of circular DNA

The clones selected were submitted to the automated sequencer MegaBACE 1000 (Amersham Biosciences), through the enzymatic method based on DNA synthesis in vitro in the presence of nucleoside triphosphates chain terminators (SANGER et al., 1977), by using the kit "Big Dye Terminator" (Applied Biosystems). M13 reverse and M13 (-20) forward primers were used to generate sequences.

2.2.8 Sequences similarity search

The resulting sequences from the above step were compared by BLASTX (ALTSCHUL et al., 1997) with those obtained previously in the in silico analysis as well as the ones deposited in the GenBank.

3 RESULTS AND DISCUSSION

3.1 In Silico assay

The search for putative MT-like genes in the CAFEST database resulted in more than 1000 reads. After clustering, we obtained 27 contigs and 78 singlets. In the annotation process, this number decreased to 6 contigs and 1 singlet (represented by the letter S in the name). The ORFinder tool was used to predict the coded proteins (table 2).

Table 2 – Protein Prediction

Gene	Reading Frame	ORF size	Predicted protein size
CaMT3	+1	243pb	80aa
CaMT5	+1	243pb	80aa
CaMT8	+1	198pb	65aa
CaMT19	+2	198pb	65aa
CaMT21	+2	243pb	80aa
CaMT25	+2	198pb	65aa
CaMTS2	+2	198pb	65aa

When submitted to a similarity search by blastp, the predicted proteins showed high identity levels (table 3) with metallothioneins from coffee and from other species. One *contig* (CaMT21) in particular presented a complete identity, corroborating it stands for a coffee metallothionein already presented in the GenBank. Hence, based on the alignments performed and in sequence size, the *contigs* CaMT21 and CaMT19 were selected for further analyses.

Table 3 – Comparison of the sequences related to metallothioneins found in the CAFEST database with the best alignment generated from blastp of these sequences in the NCBI database.

Type	Contigs	blastp	e-value	Identity	Positives
2	CaMT3	P43396.1 Metallothionein-like protein 1 [*Coffea Arabica*] 80aa.	3e-45	79/80 (99%)	79/80 (99%)
	CaMT5	P43396.1 Metallothionein-like protein 1 [*Coffea Arabica*] 80aa.	1e-20	56/57 (98%)	56/57 (98%)
	CaMT21	P43396.1 Metallothionein-like protein 1 [*Coffea Arabica*] 80aa.	6e-46	80/80 (100%)	80/80 (100%)
3	CaMT8	AAK08208.1 metallothionein-like protein [*Citrus unshiu*] 68aa	6e-23	46/65 (71%)	52/65 (80%)
	CaMT19	AAK08208.1 metallothionein-like protein [*Citrus unshiu*] 68aa	6e-23	46/65 (71%)	52/65 (80%)
	CaMT25	AAK08208.1 metallothionein-like protein [*Citrus unshiu*] 68aa	1e-23	47/65 (72%)	53/65 (82%)
	CaMTS2	AAK08208.1 metallothionein-like protein [*Citrus unshiu*] 68aa	6e-23	46/65 (71%)	52/65 (80%)

According to the classification of plants metallothioneins in four types proposed by Cobbett and Goldsbrough (2002), type 2 proteins contain four pairs of cysteine at amino terminal end, starting from a configuration Cys-Cys and ending with Cys-X-X-Cys (X being any amino acid but Cysteine). Type 3 MTs exhibit a distinctive arrangement of four cysteines in the amino terminus (Cys-X-X-Cys-X-Cys-X-X-X-X-X-Cys). Thus, our proteins could be divided into the types 2 and 3 (Figures 1 and 2). Turchi et al. (2012) states that, the occurrence of multiple MTs genes accounts for their different roles in heavy metal sequestration and in other mechanisms essential for plant growth.

Although categorized as Type 1, the metallothionein deposited in GenBank (and our analogous contig) indeed correspond to Type 2, according to Cobbett and Goldsbrough (2002) classification. This becomes clear when we analyze the cysteine residues position in the alignment of

these proteins with known Type 2 MTs (Figure 1). The phylogenetic tree (Figure 3) constructed comprising rice MTs from all classes and our selected contigs corroborates with the classification as Type 2.

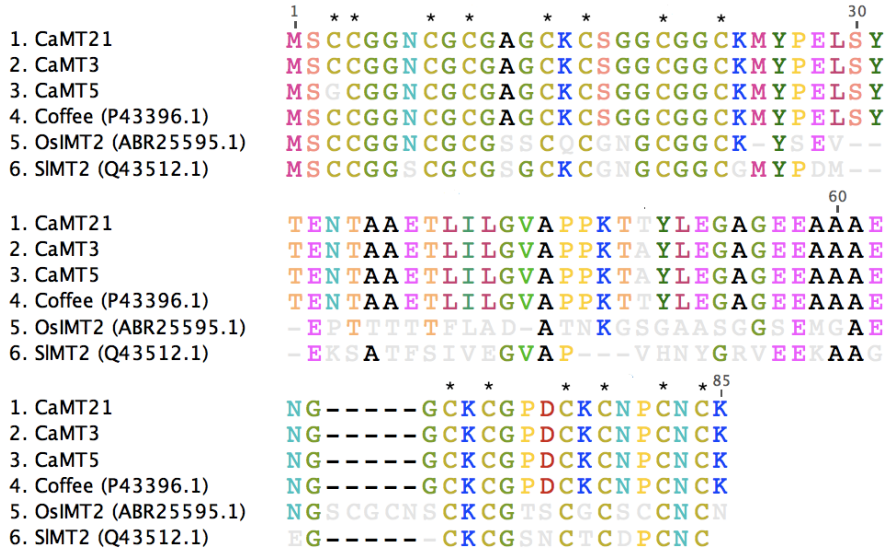

Figure 1- Alignment of the select contigs and published type 2 metallothioneins.

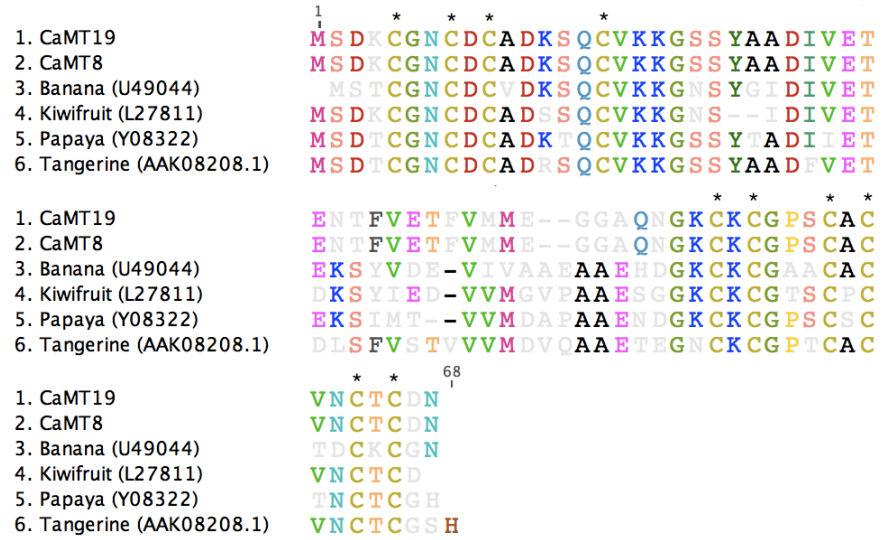

Figure 2- Alignment of the select contigs and published type 3 metallothioneins.

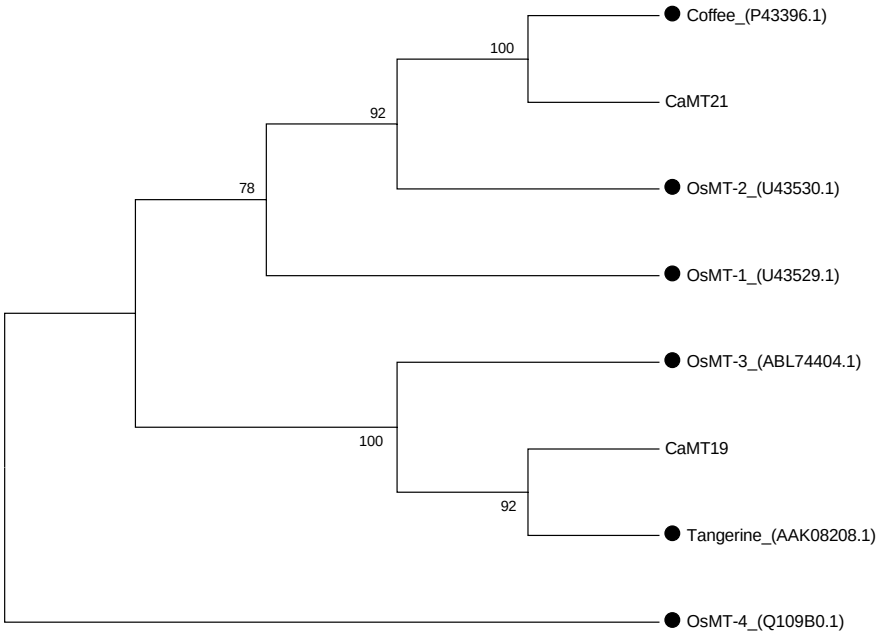

Figure 3 Phylogenetic analysis involving the putative metallothioneins found in the CAFEST database and homologous sequences obtained in the NCBI database.

The expression pattern accessed by the e-Northern (Figure 4) evidenced that the expression of the ESTs occurred more often in libraries of seedling and leaves treated with arachidonic acid. Plants treated with exogenous Arachidonic Acid presented an increased expression of general stress-responsive genes (SAVCHENKO et al., 2010), coherent with the proposed alternative function for plant MTs as protectants from oxidative damages (AKASHI et al., 2004; WONG et al., 2004).

Nevertheless, the MTs showed a wide expression profile, being detected in many tissues, which indicates the probable involvement of coffee MTs in different pathways and phases of plant development. These results are consistent with the evidences of the involvement of MTs in the process of seed development (BRKLJACIC et al., 2004) and leaf senescence (NAVABPOUR et al., 2003; BHALERAO et al., 2003), in addition to its function in metal homeostasis and detoxification (ROBINSON et al., 1993).

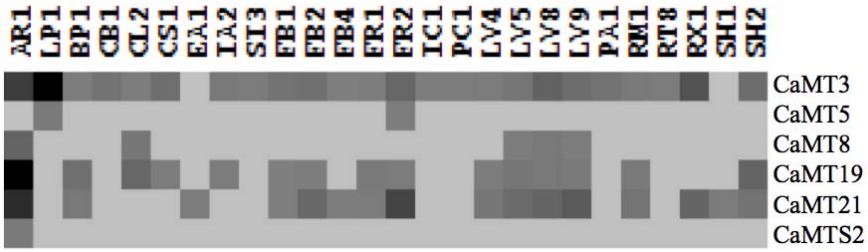

Figure 4- In silico expression profile of putative Metallothionein-like genes found in the database CAFEST. The normalized numbers of reads are represented in a gray scale, where zero or negative expression is represented by lighter colored and is gradually increased up to black, which represents the maximum degree of expression. Contigs and singlets are represented in rows and columns as libraries. The libraries as defined by Vieira (2006) are: plantlets and leaves treated with arachidonic acid (AR1 and LP1); suspension cells treated with acibenzolar-S-methyl (BP1); suspension cells treated with acibenzolar-S-methyl and brassinoesteroids (CB1); hypocotyls treated with acibenzolar-S-methyl (CL2); suspension cells treated with NaCl (CS1), embryogenic calli (EA1 and IA2); germinating seeds (SI3); flower buds in different developmental stages (FB1, FB2 and FB4); flower buds + pinhead fruits + fruits at different stages (FR1, FR2); non embryogenic calli with and without 2,4 D (IC1, PC1); young leaves from orthotropic branch (LV4, LV5); mature leaves from plagiotropic branches (LV8, LV9); primary embryogenic calli (PA1); leaves infected with leaf miner and coffee leaf rust (RM1); suspension cells with stress from aluminum (RT8); stems infected with *Xylella spp*. (RX1); Water deficit stresses on field plants (pool of tissues) (SH2).

3.2 In Vitro assay

The amplification products obtained in the polymerase chain reaction resulted in fragments with the following estimated sizes: 200pb for CaMT19 with cDNA template, 700pb for CaMT19 with DNA template, 250bp for CaMT21 with cDNA template and 750pb for CaMT21 with DNA template (Figure 5), suggesting the existence of introns in the coding sequences.

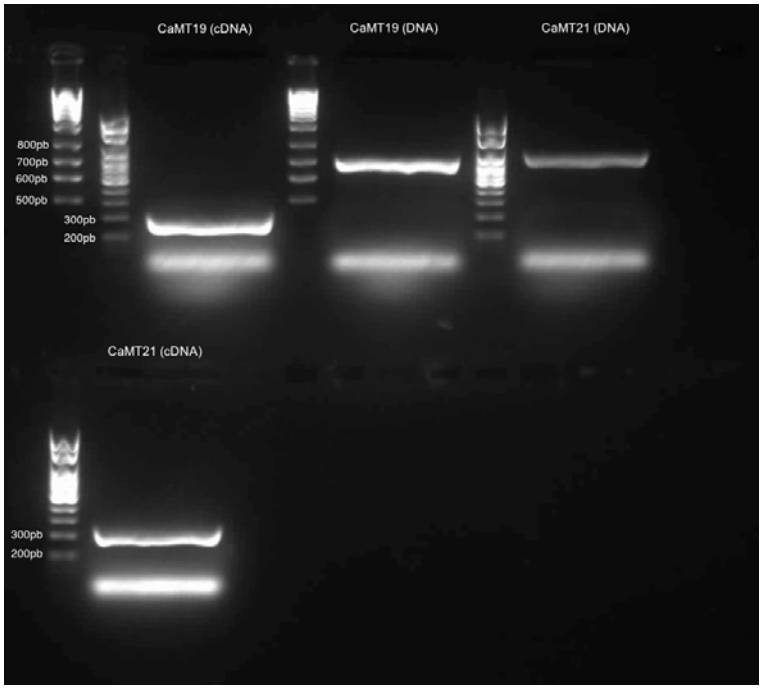

Figure 5 – Agarose gel with the isolated fragments.

An intron can be defined as any nucleotide sequence within a gene that is removed by RNA splicing. The term refers to both the DNA sequence and the corresponding sequence in RNA transcripts. Nowadays it is recognized that introns can play important roles in gene regulation via alternative splicing and nonsense-mediated decay. A less well-known role of introns is that they can provide a boost to gene expression (PARRA et al., 2011).

The alignment of the sequences obtained in sequencing for both DNA and cDNA templates of CaMT21 (Figure 6) shows the possible localization of this intron, corroborating with the results visualized in the electrophoresis gel. However, the sequence obtained by the DNA clone is smaller than expected (700pb), which could be explained by the low accuracy in sequencing borders of long fragments. Unfortunately, the sequencing of CaMT19 did not work, thus making it impossible to predict the gene structure.

Zhou et al. (2006) reported the existence of one or more introns in the gene structure of eleven rice metallothionein-like genes. However, in rice, all type 2 metallothioneins presented two introns. On the other hand, five MTs genes identified from the Arabidopsis genome also contain two exons interrupted by a single intron (ZHOU; GOLDSBROUGH, 1995), which is the same pattern presented in our results.

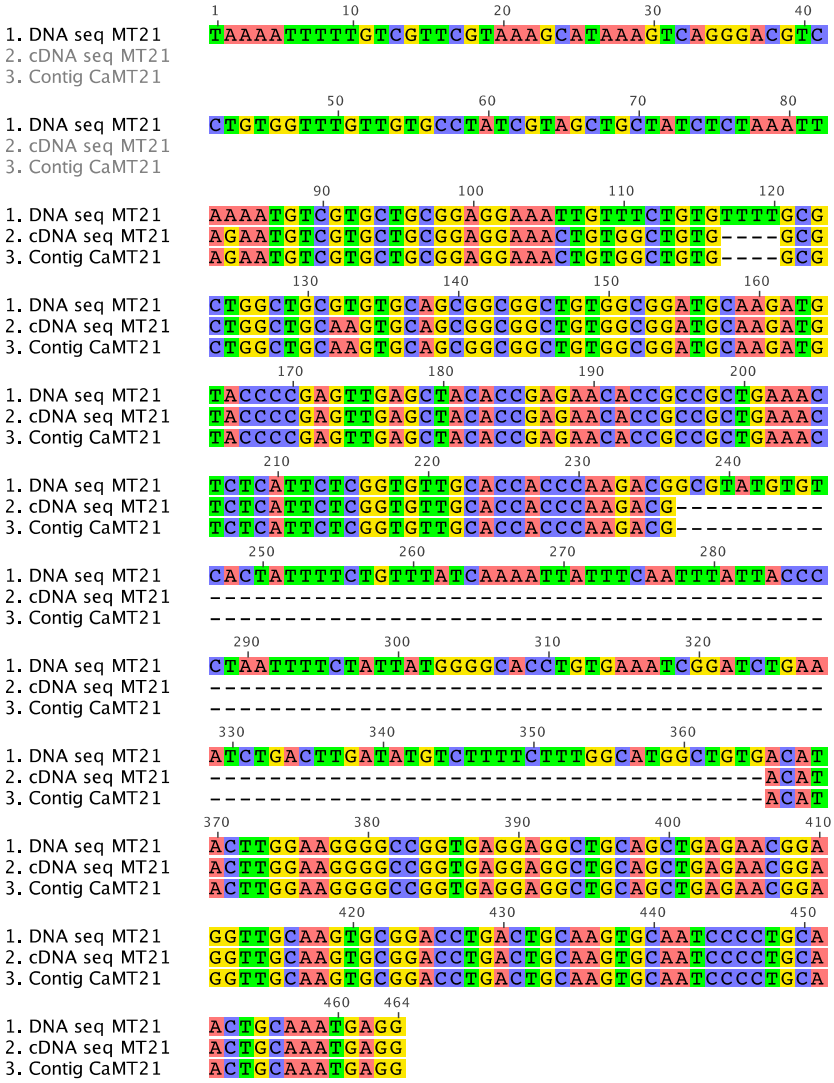

Figure 6 – Alignment of the sequences obtained by DNA and cDNA clones and the contig selected in the *in silico* analysis.

4 CONCLUSION

The CAFEST database proved to be quite representative with respect to potential members of the Metallothionein superfamily in coffee.

The conserved motifs and phylogenetic analysis showed a high degree of conservation between the coffee sequences and those sequences of other species.

The results of the in silico expression analysis corroborate with the many conditions and stages of development in which MTs are known to play important roles in diverse physiological processes.

The amplification reaction and gene sequencing results indicates the probable existence of an intron that can possibly be related to MTs gene regulation.

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CHAPTER 3 – QUANTITATIVE EXPRESSION ANALYSIS OF PUTATIVE METALLOTHIONEIN-LIKE GENES IN RESPONSE TO ZINC SUPLY

ABSTRACT

Zinc is an essential micronutrient to physiological and metabolic processes as a component of various enzymes and its deficiency leads to a reduction in internode length, the formation of small, narrow leaves and rosettes at branch tips and lower dry matter production. Additionally, it has been demonstrated that the supply of Zn positively influences the production and quality of coffee beans, a characteristic that increases the value of the commercial product. Moreover, metallothioneins (MTs) are proteins known to participate in the process of metal homeostasis, hypothesized as being partially responsible for the zinc up-take in plants. Thus, we aimed to estimate the metallothionein differential gene expression in coffee in response to zinc supply and correlate this expression to an increase in zinc contents in leaves. The 42 coffee plantlets (Catuaí 144) were grown in modified half strength Hoagland's nutrient solution, as the hydroponics system was chosen in order to minimize the variation of zinc and other nutrients supply. The quantification of zinc contents in leaves before application resulted in an average of 15ppm in the plants submitted to zinc deficiency and 25ppm in the positive control plants, in which the zinc supply was constant, indicating a scenario of zinc deficiency. Furthermore, in the qPCR assay, both CaMT19 and CaMT21 putative metallothionein-like genes showed a higher expression level in the 0.6% $ZnSO_4$ dose corroborating with the results obtained in the zinc quantification, in which the greatest assimilation also occurred in the 0.6% $ZnSO_4$ dose. Therefore, our results indicate a differential expression of MT genes in response to zinc supply.

RESUMO

O zinco é um micronutriente essencial para os processos fisiológicos e metabólicos uma vez que atua como componente de várias enzimas. Sua deficiência leva a uma redução do comprimento de entrenós, à formação de folhas pequenas e estreitas, rosetas em pontas de ramificação e menor produção de matéria seca. Além disso, foi demonstrado que o fornecimento de Zn influencia positivamente a produção e a qualidade dos grãos de café, uma característica que aumenta o valor do produto comercial. Adicionalmente, metalotioneínas (MTs) são proteínas que se sabe participarem no processo da homeostase de metal, por isso acredita-se que estas estejam envolvidas na absorção de zinco. Assim, procurou-se estimar a expressão diferencial de genes que codificam metalotioneína em café em resposta ao fornecimento de zinco e correlacionar esta expressão com um aumento do teor de zinco em folhas. As 42 plântulas de café (Catuaí 144) foram cultivadas em solução nutritiva de Hoagland, meia força, modificada. O como o sistema hidropônico de cultivo foi escolhido de forma a minimizar a variação no fornecimento de zinco e outros nutrientes. A quantificação dos teores de zinco em folhas antes da aplicação resultou numa média de 15 ppm nas plantas submetidas à deficiência e 25 ppm nas plantas do controle positivo, no qual o fornecimento de zinco foi constante, indicando um cenário de deficiência de zinco. Ainda, no ensaio de qPCR, tanto CaMT19 e CaMT21 mostraram um maior nível de expressão na dose de 0,6% $ZnSO_4$, corroborando com os resultados obtidos na quantificação de zinco pós aplicação, em que a maior assimilação também ocorreu na dose de 0,6% $ZnSO_4$. Portanto, os nossos resultados indicam que há uma expressão diferencial dos genes de MTs em resposta ao fornecimento zinco.

1 INTRODUCTION

In higher plants, Zn is essential for physiological and metabolic processes as a component of various enzymes (DECHEN; HAAG; CARMELLO, 1991) such as carbonic anhydrase and alcohol dehydrogenase CuZn-superoxide dismutase. It is also an activator of several types of enzymes, including dehydrogenases, aldolases, isomerases and transphosphorilases (MARSCHNER, 1995).

Zn deficiency causes a reduction in internode length, the formation of small, narrow and lanceolate leaves and rosettes at branch tips, causing death of branches, younger leaves becoming coriaceous and brittle, interveinal chlorosis and lower dry matter production, particularly of aerial parts (CAKMAK; MARSCHNER.; BANGERTH, 1989; CAKMAK; MARSCHNER, 1993). Surveys have shown that most coffee plantations analyzed had a zinc deficiency (MARTINEZ et al., 2004). This nutrient is essential in important physiological processes and its absence affects the growth and yield of coffee (MALAVOLTA; CARVALHO; GUIMARÃES, 1983; FÁVARO, 1992; MELLO et al., 1999).

Poltronieri, Martinez and Cecon (2011) have demonstrated that the supply of Zn positively influences the production and quality of coffee beans, a characteristic that increases the value of the coffee product.

Additionally, metallothioneins (MTs) are proteins known to participate in the process of metal homeostasis. The capacity of MTs to bind metals is due to the cysteine conserved domains that interact with metals forming metal-thiolate clusters. It is therefore hypothesized that metallothioneins are partially responsible for the zinc up-take in plants.

Thus, we aimed to estimate the metallothionein differential gene expression in coffee in response to zinc supply and correlate this expression to an increase in zinc contents in leaves.

2 MATERIALS AND METHODS

2.1 Plant Material

Experiments were carried out at the greenhouse of the Plant Physiology Sector, of the Universidade Federal de Lavras. Coffee plantlets (6 months old) from the Catucaí variety, cultivar 144, collected at the experimental field of the Empresa de Pesquisa Agropecuária de Minas Gerais, at Três Pontas, Minas Gerais, Brazil, were used.

2.2 Plant growth condition

The hydroponics system was chosen in order to minimize the variation of zinc and other nutrients supply. The 42 coffee plantlets were grown in modified half strength Hoagland's nutrient solution (SCHAT; VOOIJS; HUIPER, 1996) prepared with no Zn, except for the positive control in which the Zinc supply (2μM $ZnSO_4$) was kept throughout the whole experiment period. The plastic buckets with 6 liters capacity, were washed in 0,1N HCl solution, to eliminate zinc contamination. Two plantlets were placed in each bucket. For the first month the solution was refreshed twice and thereafter once a week. The plantlets were kept in the hydroponic system during 4 months, when zinc deficiency symptoms were already visible. The pH was measured at least two times a week and adjusted to 6,0 if necessary.

2.3 Quantification of Zinc content in leaves

Two leaves from each plant were collected in order to measure the zinc content. The quantitation experiment was conduced by the Laboratory of Foliar Analysis, of the Chemistry Department of the Universidade Federal de Lavras, following the laboratory's standards procedures.

Two quantification events were performed, one before the zinc application and another one after.

2.4 Zinc Application

Zinc was supplied in four different concentrations, each concentration being applied to three buckets with two plantlets (6 plants in total). The chosen concentrations were: 0,3; 0,6; 0,9 and 1,2% $ZnSO_4$. To each liter of zinc solution, 1ml of Nonylphenol detergent was added, in order to guarantee homogenous zinc dissemination. 20ml of solution was given to each plant, except for the positive and negative controls, in which it was given 20ml of distilled water.

After 8.6 hours, which is the time needed for the plant to absorb 80% of the supplied zinc (FÁVARO, 1992); leaves were collected. The leaves destined for in vitro experiments were immediately frozen in liquid nitrogen. The others, destined for determination of zinc levels, were thoroughly washed in running water and then dried.

2.5 RNA extraction and cDNA synthesis

Leaves were harvested and immediately frozen in liquid nitrogen. Then the tissues were ground by a mortar and pestle and samples were conditioned in 1.5ml eppendorf tubes.

The RNA extraction followed the manual's recommendations of the ConcertTM Kit Plant RNA Reagent (Invitrogen) with minor changes. For 100mg of frozen tissue, 600µl of cold Plant RNA Reagent was added. The samples were then incubated horizontally at room temperature for 10 min and subsequently centrifuged for 2 min at 12000 xg. The supernatant standardized to 500µl were transferred to new tubes and 100µl of 5M NaCl was added. After mixing, 300µl of chloroform was added and mixed thoroughly by inversion. The samples were then centrifuged for 10 min at 12000 xg at 4°C. The supernatants (450µl) were transferred to a new tube and the same volume of isopropanol was added. The samples were incubated at -20°C for 30 minutes and again taken to centrifugation for 20 min at 12000 xg at 4°C. The supernatants were discarded. To wash the pallet, 1ml of ethanol was added and the tubes were again centrifuged for 1min at 12000

xg. The aqueous phase was discarded and the pallet suspended again in 30µl of Mili-Q sterilized water.

All samples were treated with Turbo DNase free (Ambion) in a reaction containing 0.1 volumes of DNase 10X turbo buffer, 1µl of turbo DNase enzyme and water free of DNase/RNase to arrive at a total volume of 25 ml. This reaction was incubated at 37 °C for 30 min. Subsequently 0.1 volume of the DNase inactivation reagent was added and the samples were incubated for 5 min at room temperature. Then the tubes were centrifuged for 1.5 min at 10,000 g and the supernatant transferred to new ones. The samples were stored at -20 °C.

To evaluate the integrity of the samples, the RNA was submitted to electrophoresis on agarose gel 1.2% (w / v) stained with GelRed Nucleic Acid Gel Stain and viewed on a transilluminator UVITEC FireReader XS-D-77Ls 20.M. The samples were quantified in spectrophotometer (Nanodrop ® ND-1000 Espectrophotometer) at A_{260} nm in order to determine the quantity and quality of RNA. To guarantee that the samples were free of DNA contamination, a PCR was performed with RNA as a template and the primers designed to qPCR. The GoTaq (Promega) standard protocol was followed.

The cDNA synthesis was performed using the cDNA Kit High-Capacity Reverse Transcription (Applied Biosystems). First, RNA was prepared at a concentration of 1µg in a final volume of 10µl. After this, a mix was prepared containing 2µl of 10X buffer enzyme, 2µl of 10X RT Random primers, 0.8µl of dNTP mix (100 mM), 1µl Reverse Transcriptase MultiScribeTM, and water for a total volume of 10µl/sample. To each solution prepared in 10µl of 1µg RNA 10µl of this mix were added. The tubes were taken to MultiGene Gradient thermocycler Labnet, programmed with three steps: 10 min at 25°C for primer annealing, 2h at 37°C for the enzyme polymerization and 5 min at 85°C to inactivate it. The samples were stored at -20 ° C.

2.6 qPCR

Based on the MTs sequences obtained from the in silico study, primers were designed for the qPCR experiment using the program Primer3 (version 0.4.0; http://frodo.wi.Mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 4).

Table 4 Primers designed for qPCR

Gene	Primer Sequence	Eficiency (%)
CaMT19 F	5'TGTGGAGACCTTCGTGATGA	97%
CaMT19 R	5'TCACAGGTGCAGTTGACACA	
CaMT21 F	5'TCTCATTCTCGGTGTTGCAC	99%
CaMT21 R	5' AGTTGCAGGGATTGCACTTG	
GAPDH F	5'TTGAAGGGCGGTGCAAA	92%
GAPDH R	5'AACATGGGTGCATCCTTGCT	
UBQ F	5'AAGACAGCTTCAACAGAGTACAGCAT	99%
UBQ R	5'GGCAGGACCTTGGCTGACTATA	

For the quantitative gene expression analysis the real-time PCR cycler Rotor Gene-Q (Qiagen) was employed, using the SYBR Green detection system and cDNA derived from RNA extracted from coffee leaves. The thermal conditions of the reaction were: 5 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and 10 seconds at 60°C. Data were collected and stored in the Rotor-Gene Q software. For each reaction, we used 2.5µl of cDNA, 2.5µl of each primer and 12.5µl of 2x Rotor-Gene SYBR Green PCR Master Mix (Qiagen) for a final volume 25.0µl/sample. Negative controls and melting curves were included in all analyzes. The qPCR experiment for each gene under study was conducted on different cDNAs obtained from two biological replicates with three technique replicates for each, and the results were normalized using C_Ts (Cycle

Threshold) obtained by the expression of the housekeeping genes ubiquitin (UBQ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (BARSALOBRES-CAVALLARI et al., 2009). The C_T was determined by the cycle number in which the fluorescence generated in a reaction crosses the baseline (threshold). The method used was the comparative C_T. As one of the requirements for the use of this method, a validation experiment was performed to show that the amplification efficiencies of target and reference genes are similar (Table 4). With this in mind, the standard curves were performed for the genes under study at the following dilutions: 1:5, 1:25, 1:125, 1:625 and 1:3125. This procedure also defined the cDNA concentration used in each reaction, which was 10 ηg/μl. The concentration of each primer was 1.5μM.

2.7 Statistical analysis

The statistical analysis of the relative gene expression was performed by the T-test in which significant differences between means were determined at the 5% significance level ($P \leq 0,05$).

3 RESULTS AND DISCUSSION

3.1 Zinc quantification

The quantification of zinc contents in leaves (Figure 8) before application resulted in an average of 15ppm in the plants submitted to zinc deficiency and 25ppm in the positive control plants, in which the zinc supply was constant. There is some disagreement among authors when it comes to the optimal Zn concentration. According to Willson (1985) and Reuter and Robinson (1988) the critical concentration of Zn in the leaves of coffee varies from 15 to 30 mg kg^{-1}. For Malavolta, Vitti e Oliveira (1997) these levels range from 15 to 20 mg kg^{-1} and Martinez et al. (2004) found levels ranging from 6 to 13 mg kg^{-1} and 12-30 mg kg^{-1}. In any case, the verified levels are close to the critical lower level, indicating a zinc deficiency in these plants.

As the quantification method demands large amounts of tissue (0.5g of dry matter) it was necessary to collect leaves from different plants in order to form pools of samples of each treatment. This is also why a single quantitation was done before application and another one after, thus not allowing any statistical analysis.

Furthermore, the quantification of Zinc after the application showed that the greatest assimilation occurred in the 0.6% $ZnSO_4$ dose, in which the zinc content almost doubled. Malavolta, Vitti e Oliveira (1997) claimed that the nutrient supplementation has a critical physiological-economical level, defined as the optimal concentration of nutrients in leaves: below it the production decreases and above it fertilization is no longer economically viable. Pozza et al. (2009) determined that for coffee the optimal concentration for zinc supplementation is 0.5% $ZnSO_4$, in accordance with the observed results. Even though the 0.9% $ZnSO_4$ dose also presented a great improvement in the zinc contents of leaves, it was lower than the one presented by 0.6% $ZnSO_4$ dose.

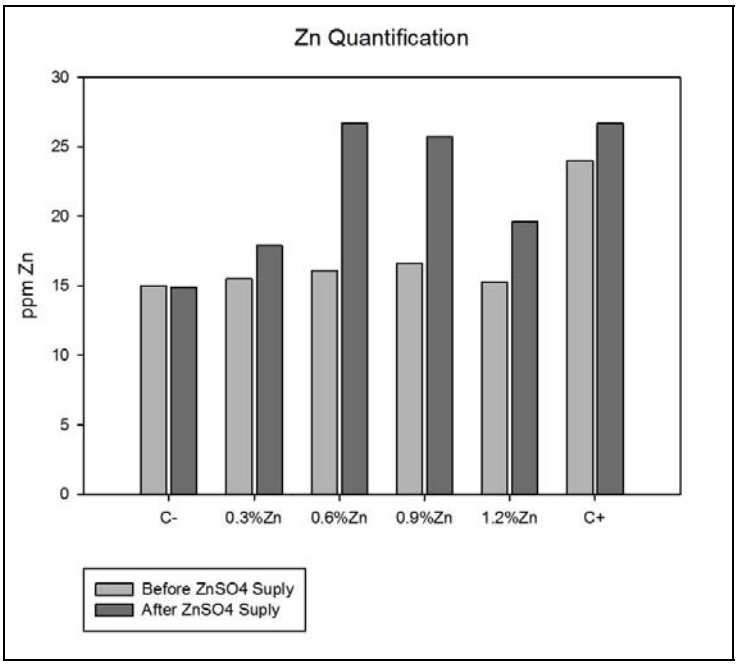

Figure 8 Zinc concentrations in leaves before and after $ZnSO_4$ supply.

Additionally, the zinc supplementation showed to be efficient in restoring the zinc contents to its ideal concentration, achieving levels similar to those of plants kept under sufficient zinc supply.

According to Cakmak and Marschner (1993), one symptom of Zn deficiency is the reduction in internode length which leads to the formation of rosette leaves at branch tips. Our plants showed this rosette formation (Figure 9), reassuring the Zn deficiency diagnosis.

Figure 9 Coffee plants with rosette formation in branch tips.

3.2 qPCR assay

All the genes of this study (reference and target) had primers efficiency above 90% (Table 4). The reference genes used in the work, were described in different experimental conditions for growing coffee (BARSALOBRES-CAVALLARI et al., 2009).

One of the most striking features of metallothioneins is the fact that the expression of certain MTs might be induced by a variety of agents and conditions; hormones, metals, senescence (COBBETT; GOLDSBROUGH, 2002; ZHOU; GOLDSBROUGH, 1995; HSIEH; LIU; HUANG, 1995), although only a few MTs expression studies have been conducted. We tested the induced expression of the putative metallothionein-like genes CaMT19 and CaMT21 transcript upon Zn exposure.

The CaMT19 gene presented a high expression only in the 0.6% $ZnSO_4$ dose (Figure 10), the other doses presented no statistical differences to the negative control. In 0.6% $ZnSO_4$ the expression was approximately 65% higher than that of the negative control and around 5% higher than the positive control, although there was no statically difference between the 0.6% $ZnSO_4$ dose and the positive control. This result corroborates with the

ones obtained in the zinc quantification, in which the greatest assimilation occurred in the 0.6% $ZnSO_4$ dose. Similarly, the CaMT21 gene also presented an enhanced expression in the plants of the 0,6% $ZnSO_4$ dose (Figure 11), being around 40% higher than the negative control. Unlike CaMT19, CaMT21 presented a smaller expression level in all doses when compared to the positive control.

Jack et al. (2007) tested the induced expression of the *SvMT2b* (a type 2 MT) transcript in *Silene vulgaris* upon Zn and Cd exposure and were unable to detect any significant change in levels. Additionally, a study performed on hybrid aspen grown on contaminated soil evidenced that the MT2b mRNA levels correlated with foliar Cd and Zn concentrations and that the foliar MT2b expression is metal-responsive (HASSINEN et al., 2009). Turchi et al. (2012) demonstrated that by over-expressing type 2 MTs in plantlets, the sequestration of Zn could be promoted. All these reports support our findings, but how much each gene studied accounts for zinc sequestration remains to be ascertained.

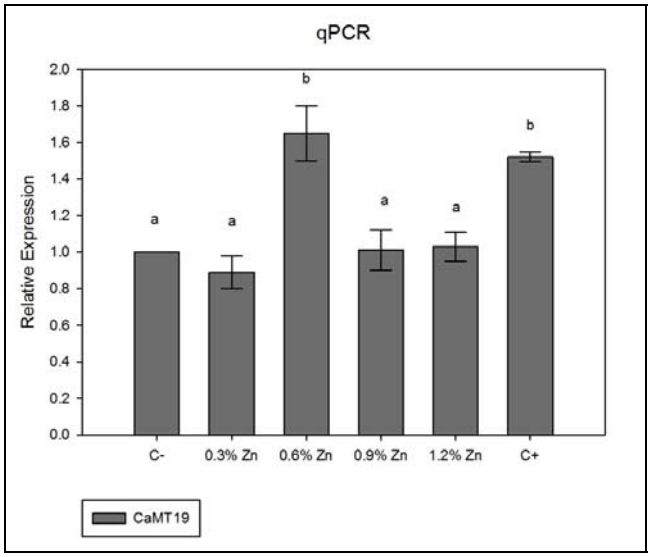

Figure 11 – Relative CaMT19 gene expression in response to Zinc supply. The same letter indicates no significant differences between average values.

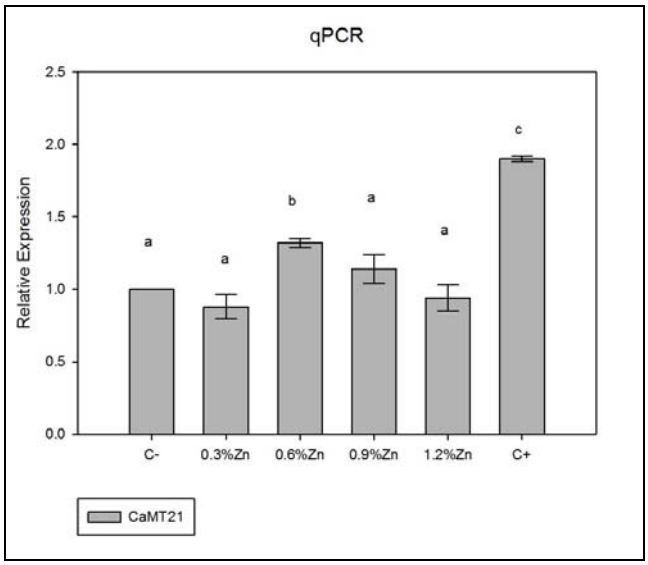

Figure 12 – Relative CaMT21 gene expression in response to Zinc supply. The same letter indicates no significant differences between average values.

4 CONCLUSIONS

The zinc supply resulted in a differential expression of the studied metallothionein-like genes under diverse $ZnSO_4$ doses. The greatest expression level occurred in the 0,6% treatment thereafter decreasing in the subsequent higher doses.

5 FINAL REMARKS

Our findings support the hypothesis that there is a positive correlation between the expression of metallothionein-like genes and zinc assimilation in coffee plants, but further studies are needed in order to elucidate the role of each metallothionein-like gene in the response to zinc supply. Also, the possible conserved function of these genes needs to be investigated in transformation events and mutant analyses to corroborate if the putative CaMT19 and CaMT21 act as metallothionein-like genes in coffee.

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