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IDENTIFICATION AND VALIDATION OF DIFFERENTIALY EXPRESSED GENES RELATED TO DROUGHT RESPONSE IN SIRIEMA *Coffea arabica* PLANTS

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Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Curso de Mestrado em Biotecnologia Vegetal, para a obtenção do título de "Mestre".

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A Deus, aos meus pais José Antônio e Vanilce, às minhas irmãs Juliana e Amanda e ao meu noivo Thiago,

dedico

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ABSTRACT

Brazil is the main world producer of coffee, which after oil is the most commercialized commodity in the world. In coffee plants flowering is induced by rainfall or irrigation after a period of water deficit, however, prolonged periods of drought can affect yield and quality of fruits. Drought affects plants impairing growth and development of them. Identification and the understanding of drought tolerance mechanisms, as well as the development of technologies that allow plants to tolerate longer periods of drought are presented as important alternatives to preserve or even increase the brazilian and worldwide agricultural production. The SSH technique (Supression Subtractive Hybridization) allows the obtention of small cDNA libraries enriched with differentially expressed transcripts present in one of the compared sample. This technique has been used efficiently to obtain information about many biological processes, such as identifying genes that are induced under biotic and abiotic stresses. In this study, three subtractive cDNA libraries of young leaves from 6 months old drought-stressed Siriema coffee plants, 24RI48-24NI (24 days after 48hours of re-irigation – 24 days non-irrigated), 24RI48-24RI24 (24 days after 48hours of re-irigation – 24 days after 24 hours of re-irrigation) and 12NI-12I (12 days non-irrigates – 12 days irrigated) were prepared, cloned, sequenced and analyzed. Hypothetical and unknown proteins, putative novel proteins, and some protein-encoding genes were identified. The protein-encoding genes found have relation to stress signaling, stress response and direct or indirect relation to recovery of re-irrigated plants. One gene from each library was selected for quantitative expression analysis by qRT-PCR and validation of the subtraction. For library 1, the selected gene (INV) was differentially expressed between the samples and validated the results. For library 2, differential expression was also detected for the selected gene (CAB11) in quantitative analysis, validating the library results. For library 3, the primers designed for expression analysis showed low efficiency to conduct the quantitative analysis, and validation was not possible. These results contribute to the study of drought tolerance in Siriema progeny and may provide information for further studies and the development of technologies that allow coffee plants to tolerate longer periods of drought.

Keywords: Abiotic stress. Drought. SSH. qRT-PCR.

RESUMO

O Brasil é o principal produtor mundial de café, que depois do petróleo é a mercadoria mais comercializada no mundo. Em plantas de café, a floração é induzida pela chuva ou irrigação após um período de déficit hídrico, no entanto, períodos prolongados de seca podem afetar o rendimento e a qualidade dos frutos. A seca afeta plantas, prejudicando o seu crescimento e desenvolvimento. A identificação e compreensão dos mecanismos de tolerância à seca, bem como o desenvolvimento de tecnologias que permitam que as plantas tolerem longos períodos de seca são apresentados como alternativas importantes para preservar ou mesmo aumentar a produção agrícola brasileira e mundial. A técnica de SSH (Hibridização Subtrativa Supressiva) permite a obtenção de pequenas bibliotecas de cDNA enriquecidas com transcritos diferencialmente expressos presentes em uma das amostras comparadas. Esta técnica tem sido usada de forma eficiente para obter informações sobre muitos processos biológicos, tais como a identificação de genes que são induzidos sob estresse biótico e abiótico. Neste estudo, três bibliotecas subtrativas de cDNA de folhas de plantas de café Siriema sob déficit hídrico, denominadas 24RI48-24NI (24 dias 48 horas após re-irrigação – 24 dias não irrigadas), 24RI48-24RI24 (24 dias 48 horas após re-irrigadas – 24 dias 24 horas após re-irrigação) e 12NI-12I (12 dias não irrigada – 12 dias irrigada) foram preparadas, clonadas, seqüenciadas e analisadas. Proteínas hipotéticas e desconhecidas, putativas novas proteínas, e alguns genes foram identificados. Os genes encontrados têm relação com a sinalização e resposta ao estresse, e relação direta ou indireta com a recuperação de plantas re-irrigadas. Um gene de cada biblioteca foi selecionado para análise de expressão quantitativa por qRT-PCR e validação da subtração. Para uma biblioteca, o gene selecionado (INV) foi diferencialmente expresso entre as amostras, validando os resultados. Para a biblioteca 2, a expressão diferencial também foi detectada para o gene selecionado (CAB11) na análise quantitativa, validando os resultados da biblioteca. Para a biblioteca 3, os primers desenhados para análise de expressão mostraram baixa eficiência para conduzir a análise quantitativa e a validação não foi possível. Estes resultados contribuem para o estudo da tolerância à seca na progênie Siriema e podem fornecer informações para estudos e desenvolvimento de tecnologias que permitam que plantas de café tolerem períodos prolongados de seca.

Palavras chave: Estresse abiótico. Déficit hídrico. SSH. qRT-PCR.

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SUMMARY

CHAPTER 1
GENERAL INTRODUCTION AND LITERATURE REVIEW

ABSTRACT

Brazil is the main world producer of coffee, which after oil is the most commercialized commodity in the world. Since the beginning of coffee production in Brazil, it has been directly related to economic development and industrialization, therefore is one of the most important crops of the country. In 2011 the production is estimated in 43,5 million bags of 60 kg. In coffee plants, flowering is induced by rainfall or irrigation after a period of water deficit, however, prolonged periods of drought can affect yield and quality of fruits. Drought affects plants impairing its growth and development. Due to prognostic for climate changes in the coming years, drought has been pointed out as one of the most important subjects of study, aiming at searches for mechanisms to mitigate its impact over plants and, consequently, to the production. Accordingly, the identification and the understanding of drought tolerance mechanisms, as well as the development of technologies that allow plants to tolerate longer periods of drought are presented as important alternatives to preserve or even increase the brazilian and worldwide agricultural production.

Key words: Coffee. Drought. Tolerance. Abiotic Stress

RESUMO

O Brasil é o principal produtor mundial de café, que depois do petróleo é a mercadoria mais comercializada no mundo. Desde o início da produção de café no Brasil, esta tem sido diretamente relacionada ao desenvolvimento econômico e à industrialização, portanto, é uma das culturas mais importantes para o país. Em 2011, a produção é estimada em 43,5 milhões de sacas de 60 kg. Em plantas de café, a floração é induzida pela chuva ou irrigação após um período de déficit hídrico, no entanto, períodos prolongados de seca podem afetar o rendimento e a qualidade dos frutos. A seca prejudica as plantas afetando o crescimento e desenvolvimento das mesmas, e devido aos efeitos deste e de outros estresses abióticos, a produção mundial de alimentos está em declínio nos últimos anos. Devido ao prognóstico para as mudanças climáticas nos próximos anos, a seca tem sido apontada como um dos temas mais importantes para o estudo, na busca de mecanismos para mitigar o seu impacto sobre as plantas e, conseqüentemente, na produção. Assim, a identificação e compreensão dos mecanismos de tolerância à seca, bem como o desenvolvimento de tecnologias que permitam que as plantas tolerem longos períodos de seca são apresentados como alternativas importantes para preservar ou mesmo aumentar a produção agrícola brasileira e mundial.

Palavras-chave: Café. Seca. Tolerância. Estresse Abiótico

1. General Introduction

The coffee tree belongs to the Rubiaceae family, which consists of more than 10.000 species grouped in 630 genera, among them, the *Coffea* genus, comprising about 90 species. The species of *Coffea* genus are distributed in a wide region through the African continent, Madagascar and neighboring regions (BERTHAUD; CHARRIER, 1988). Among *Coffea* species only *Coffea arabica* and *Coffea canephora* are economically important for worldwide coffee production.

Brazil is the main world producer of coffee, which after oil is the most commercialized commodity in the world. Since the beginning of coffee production in Brazil, it has been directly related to economic development and industrialization, therefore is one of the most important cultures of the country. In 2011, the production is estimated in 43,5 million bags of 60 kg (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2011).

In coffee plants, flowering is induced by rainfall or irrigation after a period of water deficit (CARR, 2001), however, prolonged periods of drought can affect yield and quality of fruits. Some cultivars are more tolerant than others, and several studies have been carried out to understand tolerance mechanisms in coffee plants (DAMATTA; MAESTRI; BARROS, 1997; DIAS et al., 2007; GRISI, 2006).

Siriema is a progeny from Blue Mountain (*C. arabica*) and *Coffea racemosa* crossing, backcrossed with Mundo Novo and then with Catimor, to incorporate resistance to the disease rust, low stature and productivity. This progeny was created and evaluated for multiple resistance to rust and leaf miner, performed in 2002/2003, along with cultivars Catucaí, Acauã, Palma and lineages of Catuaí and Acaiá (MATIELLO; ALMEIDA; SILVA, 2004). During these experiments it was noticed that Siriema plants showed characteristics of drought tolerance and was then indicated as a candidate for breeding to develop a tolerant cultivar. Recent studies with Siriema plants have confirmed tolerance to drought (GRISI, 2006; MELO, 2008) but molecular studies associated with physiological experiments were not carried out.

The understanding of tolerance mechanism and the genes involved in response pathways through expression profiling studies will be useful in biotechnological approaches to improve stress tolerance (HAZEN; WU; KREPS, 2003), becoming an important step towards the development of stress tolerant crops (MAHAJAN; TUTEJA, 2005). In order to understand the molecular regulation of stress responses,

the most relevant subset of differentially expressed genes must be identified, cloned and studied in detail (HAZEN; WU; KREPS, 2003). Some techniques have been applied in order to identify differentially expressed genes, such as microarrays, suppressive subtractive hybridization (SSH) and RNA-seq.

The SSH method has been an important tool in identifying and isolating cDNA of differentially expressed genes. In a general manner, the method involves the hybridization of a cDNA population (tester) to an excess of cDNA from another population (driver) and the isolation of the non-hybridized (target) from the common hybridized sequences. The method allows efficient and fast results from a low concentration of Poly (A) RNA, which makes the method advantageous in differential expression studies.

Therefore, the importance of studies that allow a better comprehension of the response to environmental stresses that directly affect coffee production, especially drought, is highlighted. Understanding these processes will contribute to the development of biotechnological tools that allow manipulating coffee plants in order to optimize tolerance to extreme drought conditions. The study of differentially expressed genes related to stress response becomes essential to elucidate the routes involved in this process.

2. Literature Review

2.10 Responses of plants to drought and gene expression

Stress is usually defined as an external factor that exerts a disadvantageous influence on the plant (TAIZ; ZAIGER, 2009). The plants are constantly exposed to a variety of environmental stresses, some of them biotic, like pathogen infection and insect herbivory, and others abiotic, such as high or low temperatures, salinity and drought (XIONG; YANG, 2003). Drought affects growth due to photosynthetic decline, metabolic constrains imposed by osmotic stress and interference with nutrient availability on the soil as it dries (CHINNUSAMY; SCHUMAKER; ZHU, 2004). Physiological responses to drought include stomatal closure, decreased photosynthetic activity, altered cell wall elasticity, among others (AHUJA et al., 2010).

Plants respond to drought using different strategies depending on the genotype. There are plants that escape from drought by completing its life cycle (early flowering

and early maturity) before serious soil and plant water deficits develop. On the other hand, some species are able to maintain cellular hydration while the soil is dry, delaying the effects of the stress. Furthermore, there are plants that can keep the metabolism even under water privation, because of osmotic adjustment (BURSSENS et al., 2000). Nonetheless, the adaptations cited above have some disadvantages. A genotype of short life cycle usually presents lower yields, compared to normal conditions. Also, the mechanisms that confer drought resistance by reducing water loss (such as stomatal closure and reduced leaf area) usually result in reduced assimilation of carbon dioxide. Furthermore, osmotic adjustment increases drought resistance by maintaining plant turgor, but the growth of the plants as also the yield may be affected (TRIJATMIKO, 2005). Consequently, crop adaptation must reflect a balance among escape, avoidance and tolerance while maintaining adequate productivity.

Plant adaptive strategies to stress, such as adjustment of growth, development, cellular and molecular activities are coordinated and fine-tuned by a rapid activation of the intracellular signaling pathway against unfavorable environmental changes (LUDWIG et al., 2005). The environment signals are primary perceived by membrane receptors that, once activated trigger a cascade to transmit the intracellular signal. After the signal perception by the membrane receptors, the transduction is possible due to second messengers formation, including calcium, reactive-oxygen species (ROS) and inositol phosphate. The second messengers are responsible for the increase of intracellular concentration of calcium and the alteration is sensed by calcium binding proteins. These sensor proteins trigger a phosphorylation cascade that reach most of the stress response genes or transcription factors that control these genes (MAHAJAN; TUTEJA, 2005).

In several species, a large number of genes that respond to drought stress are early induced genes. These genes are induced within minutes of stress signal perception and are often expressed transiently. This group is consisted of regulatory proteins (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2007), which are protein factors involved in signal transduction and stress-responsive gene expression. These include various transcription factors, kinases protein, phosphatases protein, proteins involved in phospholipid metabolism, and other signaling molecules.

Drought triggers the formation of the phytohormone abcisic acid (ABA), which induces expression of stress-related genes (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2007), and therefore are part of the ABA-dependent pathway, one of the

routes for signaling and regulation of many drought-responsive genes. These genes contain the ABA-responsive element (ABRE) in the promoter, which is regulated by transcription factors triggered by ABA, such as many basic leucine zipper factors (bZIP) (KAUR; GUPTA, 2005; UMEZAWA et al., 2010).

DRE (dehydration-responsive element)/CRT (C-RepeaT) is a *cis*-acting element that functions in ABA-independent expression pathway in response to abiotic stresses (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2007). Transcription factors belonging to the ERF/AP2 family bind to the DRE/CRT elements, for instance DREB2 genes, which are induced by dehydration stress and may activate other genes involved in the drought stress tolerance (YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2009). Furthermore, the SHINE clade of ERF/AP2 transcription factors is also thought to be involved in the induction of drought tolerance (TRIJATMIKO, 2005), among others.

The molecular re-programming triggered by stress causes extensive changes in gene expression, leading to alterations in the biochemical and proteomic machinery (AHUJA et al., 2010). Genes induced during stress conditions are not only thought to function in the regulation of genes for signal transduction but also in the stress response, protecting cells by the production of important metabolic proteins. The products of these genes, which are late induced by drought stress, are denominated functional proteins (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2007). This group includes proteins that function in abiotic stress tolerance, molecules such as chaperones, LEA proteins, osmotin, mRNA binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, various proteases and others. In contrast with the early and transiently induced genes, most of functional proteins encoding genes are induced after hours of stress perception and its expression are often sustained (MAHAJAN; TUTEJA, 2005).

Many genes induced by abiotic stresses have been already identified, using mainly microarray systems (YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2009). The results of such studies demonstrate the complexity of the response mechanism regulated by the different regulatory systems cited above. Nevertheless, many genes remain unknown or their function and processes in which they are involved were not described yet, and further studies are needed.

2.11 Drought and coffee production

Drought affects plants impairing its growth and development. Due to prognostic of climate changes in the next years, drought has been pointed out as one of the most important subjects of study, in a search for mechanisms to mitigate its impact over plants and, consequently, in production.

According to recent data, the temperature raised 1,4ºC in the last century in Minas Gerais, and the forecast for this century is that the temperature will raise 3ºC, and that changes in patterns and distribution of rainfall will also occur (BARBOSA, 2008). Based on prognostics of climate changes from Intergovernmental Panel on Climate Change - IPCC (2001), global temperatures will increase 1 to 5,8 ºC, and Assad et al. (2004) estimated the impact in agroclimatic zoning of coffee in Brazil if the temperature rises 1ºC, 3ºC or 5,8ºC. The authors concluded that a temperature rising of 3ºC will reduce the area of production in Minas Gerais to 6,1% of the state, and irrigation will be needed to supply water. To date, the state of Minas Gerais is the main producer of coffee in Brazil, responsible for 50% of the national production (CONAB, 2011) and therefore, changes in temperatures and rainfall distribution will severely affect national production and consequently, the economy.

Water deficit and high temperatures are the main abiotic constraints to coffee production (DAMATTA; RAMALHO, 2006), despite coffee plants can stand moderate drought conditions. Coffee plants present a more uniform flowering after a certain period of water deficit followed by irrigation or rainfall (NASCIMENTO et al., 2008). In general, under field conditions the water potential rarely declines to values lower than -1,5 MPa and the water deficit is slowly established, allowing the plants to adjust to the conditions (RENA; MAESTRI, 2000). However, when the water potential declines to values around -3,0 MPa the plants hardly or do not recover after re-irrigation (MELO, 2008; PINHEIRO et al., 2004). Values lower than -2,0 MPa are considered dangerous to the coffee plants, due to reduction of stomatal conductance and, consequently, of the photosynthetic rate (LIMA et al., 2002).

The coffee plants under drought conditions generally present an increase in cellular osmolarity, which results from the accumulation of compatible osmotically active solutes, and is associated with turgor maintenance and osmotic equilibrium without disturbing macromolecular-solvent interactions. Furthermore, the physiological mechanisms underlying coffee tolerance to drought are also related to the

strong sensitivity of coffee stomata to both soil and atmospheric water deficits (DAMATTA; RAMALHO, 2006).

Dias et al. (2007) reproduced drought conditions experimentally on coffee plants and observed a decrease of the water potential and transpiration. These authors evaluated Catucaí and Siriema progenies and considered that the latter had the best water use efficiency. Grisi (2006) compared Siriema and Catuaí in response to drought exposure and Siriema showed a greater tolerance than Catuaí.

Melo (2008) developed a work aiming at evaluating tolerance and recovery ability of Siriema coffee plants under drought conditions. The experiment was carried out in a greenhouse where plants were subjected to watering withholding up to 30 days, while several parameters were evaluated, such as starch, total soluble and reducing sugars content, activity of SuSy, Neutral and Acid Invertases, Nitrate Reductase enzymes, stomatal resistance and density, water potential, transpiration, and others. During the experiment period, the measurements were made on a group of plants under daily irrigation (Control), a second group maintained non-irrigated and, within this group, subgroups were re-irrigated after 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days without watering. In the re-irrigated plants, the parameters were measured 24h and 48h after re-irrigation. The conclusions of this work were that Siriema plants presented characteristics of tolerance, standing long periods of drought and showing values of recovery near to control plants after re-irrigation up to 24 days without watering (Figure 1). At 12 days of experiment, stomatal resistance started increasing on stressed plants compared to control plants, while transpiration started decreasing. Enzymes activity and sugars content differed from control approximately at 12 days of experiment as well.

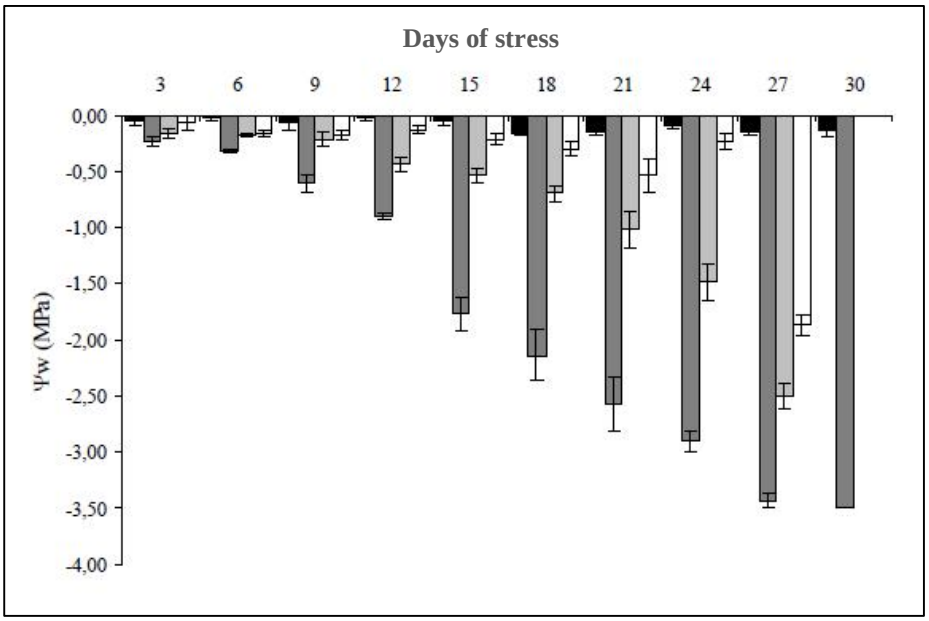

Figure 1. Water potential of coffee plantlets Siriema progeny under drought stress. Irrigated plants (■), non-irrigated (■), 24 hours after re-irrigated (■) and 48 hours after re-irrigated (■). Bars represent standard error of four samples mean. Extracted from Melo (2008)

Therefore, the identification and the understanding of the molecular mechanisms of drought tolerance in Siriema progeny may contribute to further development of tolerant coffee cultivars that can cope with extreme environmental conditions. The development of technologies that allow plants to tolerate longer periods of drought are presented as important alternatives to preserve or even increase the Brazilian and worldwide agricultural production, considering the negative prognostics for the incoming years.

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CHAPTER 2

CONSTRUCTION, ANALYSIS AND VALIDATION OF THREE SUPRESSIVE SUBTRACTIVE HYBRIDIZED (SSH) cDNA LIBRARIES FROM LEAVES OF SIRIEMA COFFEE PLANTS SUBJECTED TO DROUGHT

ABSTRACT

The SSH technique (Supression Subtractive Hybridization) allows the obtention of small cDNA libraries enriched with differentially expressed transcripts present in one of the compared sample. This technique has been used efficiently to obtain information about many biological processes, such as identifying genes that are induced under biotic and abiotic stresses. In this study, three subtractive cDNA libraries of leaves of drought-stressed Siriema coffee plants, 24RI48-24NI, 24RI48-24RI24 and 12NI-12I were prepared, cloned, sequenced and analyzed. Hypothetical and unknown proteins, putative novel proteins, and some protein- encoding genes were identified. The protein- encoding genes found have relation to stress signaling, stress response and direct or indirect relation to recovery of re-irrigated plants. One gene from each library was selected for quantitative expression analysis by qRT-PCR and validation of the subtraction. For library 1, the selected protein- encoding gene (INV) was differentially expressed between the samples and validated the results. For library 2, differential expression was also detected for the selected protein- encoding gene (CAB11) in quantitative analysis, validating the library results. For library 3, the primers designed for expression analysis showed low efficiency to conduce quantitative analysis, and validation was not possible.

Key words: SSH. qRT-PCR. Drought. Siriema

RESUMO

A técnica da hibridização subtrativa supressiva permite a obtenção de pequenas bibliotecas de cDNA enriquecidas com transcritos diferencialmente expressos presente em uma das amostras comparadas. Esta técnica tem sido usada de forma eficiente para obter informações sobre muitos processos biológicos, tais como a identificação de genes que são induzidos sob estresse biótico e abiótico. Neste estudo, três bibliotecas subtrativas de cDNA de folhas de plantas de café Siriema sob déficit hídrico, denominadas 24RI48-24NI, 24RI48-24RI24 e 12NI-12I foram preparadas, clonadas, seqüenciadas e analisadas. Proteínas hipotéticas e desconhecidas, putativas novas proteínas e alguns genes codantes foram identificados. Os genes encontrados têm relação com a sinalização e resposta ao estresse, e relação direta ou indireta com a recuperação de plantas re-irrigadas. Um gene de cada biblioteca foi selecionado para análise de expressão quantitativa por qRT-PCR e validação da subtração. Para uma biblioteca, o gene selecionado (INV) foi diferencialmente expressos entre as amostras validando os resultados. Para biblioteca 2, expressão diferencial também foi detectada para o gene selecionado (CAB11) na análise quantitativa, validando os resultados da biblioteca. Para a biblioteca 3, os primers desenhados para análise de expressão mostraram baixa eficiência para conduzir a análise quantitativa e validação não foi possível.

Palavras chave: SSH. qRT-PCR. Seca. Siriema

1. Introduction

The SSH technique (Supression Subtractive Hybridization) allows the achievement of small cDNA libraries enriched with differentially expressed transcripts present in one of the compared sample (DIATCHENKO et al., 1996). This technique has been efficiently used to obtain information about many biological processes, such as identifying genes that are induced under biotic and abiotic stresses.

At first, two populations of mRNA are converted to cDNA, and designated *tester* or *driver*. *Tester* is the cDNA which contains specific transcripts (differentially expressed); *driver* is the reference cDNA. Tester and Driver are hybridized and, consequently, the hybridized sequences are removed. The non-hybridized cDNA contains the genes that are expressed in tester but not in driver, which are the target sequences to be studied. After cloning and sequencing the fragments, part of the sequences of the differentially expressed genes present in the *tester* population are obtained. The different steps and principle of the SSH are shown in a scheme in the Figure 2.

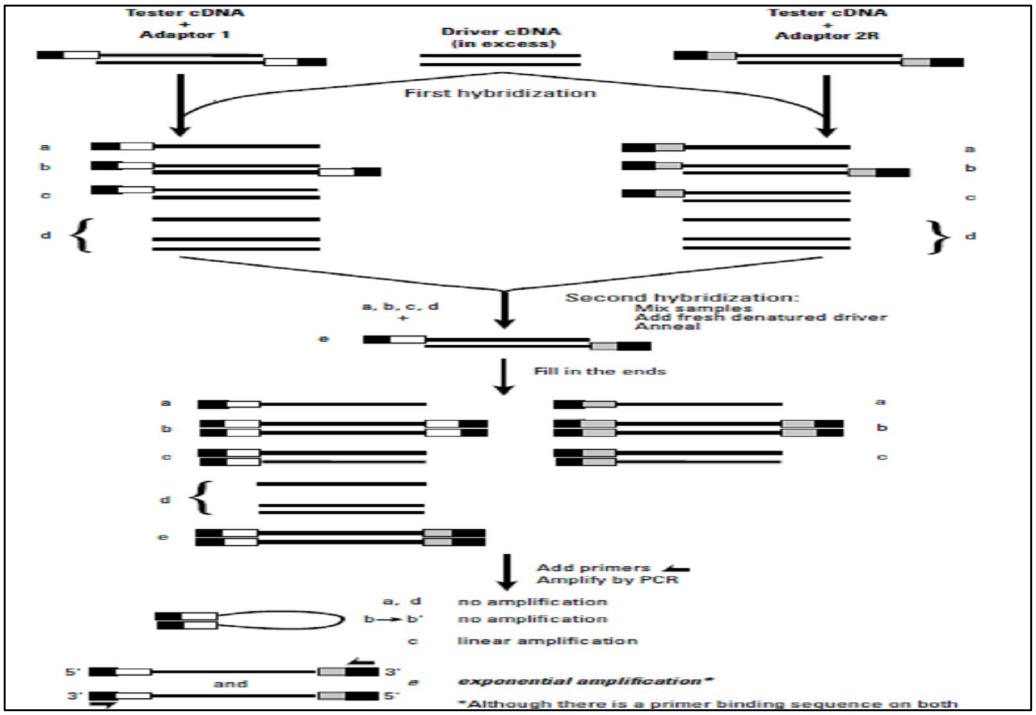

Figure 2. Schematic representation of the SSH method. Two tester populations are created with different adaptors, and Driver cDNA has no adaptors. Two hybridizations are performed and the product is amplified by PCR. The lines represents *tester* or *driver* cDNA; the black boxes represents the external region of 1 and 2R Adaptors which have the sequence of the first primer used in the PCR (PCR primer 1); The white boxes represents the internal region of the adaptor1,

which correspond to the Nested PCR primer 1; the gray boxes represents the internal region of the 2R Adaptors and corresponds to the *Nested* PCR *primer* 2R.

Recently, the differential expression analysis by the SSH method became more common, mainly in works that aimed at identifying genes related to biotic and abiotic stresses responses mechanism, including drought. Fu et al. (2007) obtained from drought stressed rice plants many cDNA sequences of genes related to signaling, signal transduction and response to stress. Sequences were further confirmed by quantitative reverse transcriptase PCR (qRT-PCR). The study showed consistent results when compared to other methods of differential expression study, such as microarray.

Ouyang et al. (2007) identified genes related to salt stress by using the SSH method in roots of tolerant and sensible tomato plants. Many genes responsible for signal transduction, protein synthesis, transcription factors, cellular transport and others were found. Several salt-induced ESTs were also identified by the SSH method in *S. maritima* (SAHU; SHAW, 2009) and in *Vitis vinifera* (DALDOUL et al., 2010), and in the former work few genes were selected to confirm the results by qRT-PCR.

Differentially expressed genes associated with a disease named as *citrus decline* were identified by the SSH method (ABREU, 2007). Many genes that are already described as related to stress conditions, such as LEA (Late Embryogenesis Abundant) proteins, metalothioneins, hydrolases, ubiquitin, and other genes were identified as being differentially expressed.

In sugarcane plants under drought conditions, the SSH method was used to identify several genes, which were involved in a wide range of processes in the plant, for instance, carbohydrate metabolism, protein metabolism, photosynthesis, ethylene biosynthesis, signaling and others. Also, hypothetical proteins and genes of unknown function were identified (PRABU et al., 2010). The results were validated by semi-quantitative RT-PCR

These and other works show the efficiency of the SSH method to identify differentially expressed genes under stress conditions. In this work three subtractive cDNA libraries were made from leaves of Siriema progeny coffee plants subjected to drought conditions and re-irrigated after a certain period, in order to identify genes related to stress tolerance and recovery in these plants.

2. Aims

The aim of this work was identifying differentially expressed genes in Siriema coffee plants related to drought tolerance and recovery by using Supressive Subtractive Hybridization (SSH) in leaves of plants subjected to drought and of re-irrigated plants, and additionally, to validate differential expression of selected genes by qRT-PCR.

3. Material e Methods

3.1 Experiment set up

The plant material was provided by the experiment carried out by Melo (2008). The experiment was carried out in a greenhouse with 50% of solar radiation interception, located at the experimental area of the Plant Physiology Sector from Universidade Federal de Lavras. Plants of *Coffea arabica*, Siriema progeny, 6 months old, were cultivated in 3 liter plastic bags and subjected to water withholding up to 30 days, and a control group was normally irrigated during the experiment period. A group of plants was re-irrigated after 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days, and leaves from the third pair were harvested 24 and 48 hours after re-irrigation. Furthermore, non-irrigated plants and control plants were similarly harvested each three days during the 30 days of experiment. The samples were rinsed with distilled water, immediately transferred to liquid N_2 and stored under -80 ºC. Plants of 12 days, irrigated and non-irrigated, and plants of 24 days, non-irrigated, re-irrigated 24h and re-irrigated 48h were selected for RNA extraction and construction of the SSH library. The samples were selected based on the results obtained by Melo (2008), summarized in the item 2.2 of chapter 1.

3.2 RNA Extraction

The total RNA from plants of 12 days, irrigated (12I) and non-irrigated (12NI), along with plants of 24 days, non irrigated (24NI), re-irrigated 24h (24RI24) and re-irrigated 48h (24RI48) was extracted following the *Concert*™ (Invitrogen) method. The tissue was grinded in liquid N_2 and 0,5 mL of *Concert*™ was added to 100 mg of grinded tissue. The samples were homogenized and centrifuged for 2 minutes at 12.000 rpm at room temperature. Afterwards, 100 µL of 5M NaCl and 300µL of chloroform

were added to the supernatant, and the solution was exhaustively homogenized by inversion.

The samples were centrifuged again for 10 minutes at 12.000 rpm in +4ºC and the upper aqueous phase was transferred to a new tube. An equal volume of isopropanol was added and the tubes were centrifuged as described before; the supernatant was discarded. 1 mL of 75% ethanol was added to wash the pellet, the tubes were centrifuged for 1 minute at 12000 rpm in room temperature, and the supernatant was discarded. The tubes containing the precipitated RNA were left at room temperature to dry completely. The pellet was diluted with RNAse free water.

3.3 mRNA Purification

Before starting the purification process, in order to reduce the salt excess and increase the *MicroPoly(A)Purist kit* efficiency, 100 µL of 5M Ammonium Acetate, 1 µL of glycogen and 250 µL of 100% ethanol were added to the total RNA samples. The solutions were mixed and left for 30 minutes at -70ºC to precipitate. Afterwards, the tubes were centrifuged at 13.400 rpm for 25 minutes at 4ºC, and the supernatant was discarded. 1 mL of 70% ethanol was added to the pellet, mixed by vortex and centrifuged for 10 minutes. The supernatant was removed with a fine pipette.

After removing the salt excess, the pellet was diluted in 250 µL of nuclease free water (provided by the kit) and vigorously mixed. Next, 250 µL of binding solution (2X) was added and mixed again.

Each sample was transferred to a tube containing Oligo(dT) Cellulose, and mixed by inversion and slow pipetting to dilute the resin. Afterwards, the tubes were incubated for 5 minutes at 70 ºC to denature the secondary structure and maximize the hybridization between the poli(A) sequences present in the great part of mRNAs, and the poli(T) sequences contained in the Oligo(dT) Cellulose. The tubes were incubated for 60 minutes at room temperature and slow agitation, and afterwards were centrifuged at 6.600 rpm for 3 minutes at room temperature. The supernatant was removed and reserved on ice until confirming the purification.

The precipitate containing Oligo (dT) was diluted in 500 µL of Wash Solution 1 to remove non specific material and ribosomal RNA. One column corresponding to each sample was placed in a tube and the Oligo (dT) Cellulose suspension was transferred to the columns. The tubes were centrifuged at 6.600 rpm for 3 minutes at

room temperature. The collected material was discarded and a second round was performed by adding 500 µL of Wash Solution 1 to the column.

After discarding the collected material again, 500 µL of Wash Solution 2 was added to the columns and mixed by vortex. The mixture was centrifuged at 6.600 rpm for 3 minutes at room temperature, and a second round was performed following the same steps.

The column was transferred to a new tube and 200 µL of RNA Storage Solution pre-warmed at 70 ºC was added. The suspension was thoroughly mixed and centrifuged at 7.300 rpm for 2 minutes. The solution removes the bound RNA poli(A) from the Oligo(dT) Cellulose. The poli(A) RNA is collected at the bottom of the tube and the columns were discarded.

For mRNA precipitation, the tubes were left for 30 minutes in a -70 ultrafreezer. Afterwards, the RNA recovery was performed by centrifugation at 11.400 rpm for 25 minutes at 4ºC. The supernatant was carefully removed with a fine pipette and 1 mL of 70% ethanol was added to the pellet, mixing by vortex. The mixture was centrifuged for 10 minutes at 4 ºC and the supernatant removed as described before. The pellet was diluted in 20 µL of RNA Storage Solution and stored in -80ºC ultrafreezer until the cDNA synthesis.

3.4 Suppressive Subtractive Library

Three subtracted cDNA Libraries were made from the extracted mRNA. The libraries were: 24 days re-irrigated 48h – 24 days non-irrigated (Library 1: 24RI48 – 24NI), 24 days re-irrigated 48h – 24 days re-irrigated 24h (Library 2: 24RI48 – 24RI24) and 12 days non-irrigated – 12 days irrigated (Library 3: 12NI – 12I). Afterwards, reverse libraries, i.e., composed of the same samples but hybridized inversely were made in order to avoid false-positives.

To prepare a SSH library, it is necessary to synthesize cDNA from purified mRNA. For that, the S*MART PCR cDNA Synthesis Kit* (CLONTECH) was used, which allows the synthesis of high quality cDNA from small quantities (nanograms) of poli A^+ RNA. Afterwards, the hybridization and subtraction were performed by using the PCR SELECT cDNA Subtraction kit (CLONTECH), following the manual instructions from the Adaptors ligation step. The followed steps from cDNA synthesis to the PCR amplification of the hybridization products are described in this section.

3.4.1. Synthesis of the first strand of cDNA

For each sample of 3µL of RNA PoliA$^+$, 1 µL of 3' SMART CDS Primer II A (12 µM) e 1 µL SMART II A Oligonucleotide (12 µM) was added in a 0,5 mL tube. After homogenizing and briefly centrifuging, the mixture was incubated at 72^{o}C for 2 minutes and cooled on ice for 2 minutes. To bind the content to the bottom, the tube was centrifuged again.

A mixture containing 2 µL of the 5X First Strand Buffer, 1 µL of DTT (20 mM), 1 µL of dNTP Mix (10 mM of each dNTP) and 1 µL of AMV Reverse Transcriptase was prepared and added to each tube, mixed gently with a pipette and centrifuged. The tubes were incubated for 1h at 42 ^{o}C in a thermal cycler. The samples were diluted in 90 µL of TE Buffer (10 mM Tris [pH 7.6], 1 mM EDTA).

3.4.2. Synthesis of the second cDNA strand

For amplification of the second strand of cDNA by PCR, two 0,5 mL tubes with 4 µL of the diluted cDNA were prepared. A PCR Mix was prepared with the following aliquots for each reaction: 74 µL deionized water, 10 µL of 10X *Advantage* 2 PCR Buffer, 2 µL 50X dNTP (10 mM each dNTP), 2 µL 5´ PCR Primer II A (12 µM) and 2 µL 50X *Advantage* 2 Polimerase Mix. The components were homogenized in vortex and briefly centrifuged. Afterwards, 90 µL of Master Mix were added to the tubes containing the single stranded cDNA.

The following amplification program was initiated: 95°C 1 min and 30 cycles of 95°C 15 sec, 65°C 30 sec and 68°C 6 min. After the program was finished, 2 µL of 0,5 M EDTA was added to terminate the reaction. The final product contains amplified double stranded cDNA.

3.4.3. Column Chromatography

The two tubes containing the amplified cDNA product were mixed in a 1,5 mL tube. An equal volume of phenol: choloroform:isoamyl alcohol (25:24:1) was added and vigorously mixed by vortex. After centrifuging at 14.000 rpm for 10 minutes at room temperature to separate the phases, the upper phase (aqueous) was removed and transferred to a new tube. Afterwards, 700 µL of n-butanol was added and vortexed thoroughly, to concentrate the PCR product to a volume of 40-70 µL. The tubes were

centrifuged again at 14.000 rpm for 1 minute and the upper phase (n-butanol) was removed and discarded.

For each sample a *CHROMA SPIN* 1000 Column was inverted several times to completely resuspend the gel matrix. The upper cap was removed, then the bottom cap was removed and the column was transferred to a tube, and 1,5 mL of 1X TNE buffer was added to the column. The buffer drained through the column until it reached the same level of the gel matrix in the column. The collected buffer in the tube was discarded.

The samples were carefully applied to the center of the gel surface, avoiding the samples to flow along the inner wall of the column. Next, 25 µL of 1X TNE buffer was applied and flowed through the column completely. A new aliquot of 150 µL of the buffer was applied to the column and flowed again through the column. Afterwards, the column was transferred to a new tube.

To obtain the purified fraction of cDNA, a new aliquot of 320 µL of 1X TNE buffer was applied to the column and collected in the tube. 10 µL of this fraction was collected and transferred to a clean tube labeled as Sample B for agarose gel/EtBr analysis. The column was transferred to a new tube, 75 µL of the buffer was applied and aliquot was taken to gel analysis (Sample C).

The samples A and B were analyzed in 1,5% agarose gel to confirm the presence of purified cDNA in the sample. After analysis the samples were mixed, following the manual recommendations, and collected to proceed with the digestion with *RsaI* restriction enzyme.

3.4.4. Restriction Enzyme Digestion (*RsaI*)

In order to generate shorter and blunt-ended double-stranded cDNA, which are necessary for both adaptor ligation and subtraction, a digestion with an endonuclease (*RsaI*) was performed. To each sample of purified cDNA, 36 µL of 10X RsaI restriction buffer and 1,5 µL of RsaI (10 units) were added and mixed by vortex. The mixture was incubated for 3 hours at 37 ºC and afterwards 8 µL of 0,5 M EDTA was added to terminate the reaction.

3.4.5. Purification of digested cDNA

The purification of digested cDNA was performed by using the DNAclear® Kit (Ambion®) following the manual instructions. Before proceeding with the

purification, one *Micro Filter Cartridge* for each sample was assembled in a Collection Tube and equilibrated with 30 µL of cDNA Binding Buffer incubated for 5 minutes at room temperature.

Aliquots of 100 µL of each sample were prepared and 250 µL of cDNA Binding Buffer was added to each sample and mixed carefully by pipetting. The mixture was applied to the *Micro Filter Cartridge* and centrifuged at 10.400 rpm for 1 minute to flow the mixture through the filter. The collected material was discarded and the filter was placed in a new tube. Afterwards, 500 µL of cDNA Wash Buffer to each filter and centrifuged for 10.400 rpm for 1 minute. The collected material was discarded again and the tubes were briefly centrifuged to collect any ethanol residue present in the Wash Solution.

The filter was placed in a new tube and, to elute the precipitate, an aliquot of 10 µL of pre-heated (55ºC) nuclease free water was added to the filter and centrifuged for 1 minute at 10.400 rpm. The step was repeated and then the filter was discarded. The content of the tube was the purified digested cDNA.

3.4.6. Adaptors Ligation

Tester samples were submitted to adaptors ligation to the ends before proceeding with the hybridization with the Driver samples in excess. 1 µL of the Tester sample (digested cDNA with *RsaI* and purified) was diluted in 5 µL of Sterile Water to initiate the adaptors ligation protocol.

In a 0,5 mL tube a Mix was prepared containing 3 µL of Sterile Water, 2 µL of 5X Ligation Buffer and 1 µL of T4 DNA Ligase (400 units/µL) per reaction, and two reactions were prepared for each Tester DNA following the manual instructions.

The tubes were incubated at 16ºC for 14 hours. After incubation, 1 µL of EDTA/Glycogen Mix was added to stop the reaction, and the samples were warmed at 75ºC for 5 minutes to inactivate the ligase. The tubes were briefly centrifuged and stored at -20ºC.

3.4.7. First Hybridization

In the hybridization process, the sample Driver in excess is added to the sample Tester, and the samples are denaturated by heating and the single strand cDNA sequences present in tester and driver that are similar bind to each other. The first hybridization reactions were prepared in two different tubes, denominated Tester 1-1

and Tester 2-2 (components are described in the Table 1), in the same order they were added to the reaction.

Table 1. First Hybridization Reaction set up.

	Sample for Hybridization	
	1	**2**
Component	**Tester 1-1**	**Tester 1-2**
Driver cDNA digested whith Rsa I	1.5 µL	1.5 µL
Adaptor 1-ligated Tester 1-1	1.5 µL	—
Adaptor 2R-ligated Tester 1-2	—	1.5 µL
4X Hybridization Buffer	1.0 µL	1.0 µL
Final Volume	4.0 µL	4.0 µL

The samples were incubated at 98ºC for 1,5 minutes to denature the tester and driver cDNA. Afterwards, the firs hybridization reaction was performed in thermal cycler Eppendorf Mastercycler at 68°C for eight hours. After this period, the products of the reaction were immediately used for the second hybridization reaction.

3.4.8. Second Hybridization

The two samples obtained from the first hybridization were mixed in this step, and fresh denaturated driver DNA was added to further enrich for differentially expressed sequences. New hybrid molecules were formed, consisting of differentially expressed cDNAs with different adaptors on each end.

For the second hybridization the following steps were followed:

a) Denaturation of driver cDNA: 1 µL of cDNA driver not diluted was mixed with 1 µL of 4X Hybridization Buffer and 2 µL of sterile water. An aliquot of 1 µL from this mixture was incubated in thermal cycler at 98°C for 1,5 minutes;

b) Mix of the second hybridization reaction: the hybridization samples 1 and 2 from the first hybridization reaction were mixed without being removed from the thermal cycler and the fresh denaturated driver cDNA was added to the tube.

Afterwards, the second hybridization reaction was performed in water bath at 68ºC for 14 hours. After the incubation, the samples were diluted in 200 µL of Dilution Buffer (HEPES-HCl 20 mmol/L pH 8,3, NaCl 50 mmol/L e EDTA 0,2 mmol/L pH

8,0). The mixture was incubated in thermal cycler Eppendorf Mastercycler at 68°C for 7 min and used for the PCR.

3.4.9. First and Second PCR Amplification

The differentially expressed cDNAs present in the product of the second hybridization reaction were selected during two PCR rounds (Advantage PCR kit – Clontech). In the first amplification reaction, only the double stranded cDNAs with different adaptor sequences on each end are exponentially amplified. In the second amplification, nested PCR was used to reduce background and enrich for differentially expressed sequences.

The following mix was prepared: 1 µL of tester cDNA from the second hybridization, 19,5 µL of Sterile Water, 2,5 µL of 10X PCR Buffer, 0,5 µL of dNTPs Mix (10 mmol/L each), 1 µL of PCR primer 1 (10 µmol/L) and 0,5 µL of Advantage cDNA Polymerase Mix 50X.

The mix was incubated in thermal cycler Eppendorf Mastercycler at 75°C for 5 min for adaptors extension. Immediately after incubation, the program used was as follows: 94°C for 25 s, followed by 27 cycles of 94°C for 10 s, 66°C for 30 s and 72°C for 1,5 min.

Afterwards, 3 µL of each PCR product were diluted in 27 µL of water and 1 µL from the diluted samples were used for the second amplification reaction. The aliquot from the diluted product of the first PCR was mixed with 18,5 µL of Sterile Water, 2,5 µL of PCR Buffer 10X, 1 µL of Nested PCR primer 1 (10 µmol/L), 1 µL of Nested PCR primer 2R (10 µmol/L), 0,5 µL of dNTP mix (10 mmol/L) and 0,5 µL of Advantage cDNA Polymerase Mix 50X.

The amplification reaction followed the program: 21 cycles of 94°C for 10 s, 68°C for 30 s and 72°C for 1,5 min in Eppendorf Mastercycler thermal cycles. The products from the second PCR were visualized in a 2% agarose/EtBr gel run in TAE [1X].

3.4.10. Cloning the final product of the subtractive hybridization in a vector

The final product of the hybridization was cloned into TOPO® (Invitrogen™ TOPO TA Cloning® kit) vector. For cloning, 4 µL of PCR was used, and 1µL of salt solution and 1 µL of vector were added. A tube was prepared for each cDNA library.

The mixture was incubated at room temperature for 20 minutes, and bacterial transformation was performed.

3.5 Bacterial Transformation

After cloning, *E. coli* cells were transformed, by adding 2 µL of cloning reaction was added to tubes containing 50 µL of One Shot® Chemically Competent DH5α *E. coli*, mixing gently. The mixture was incubated on ice for 30 seconds and transferred to water bath at 42ºC for heat shock and immediately transferred to ice again. Afterwards, 250 µL of S.O.C. media (2%Tryptone, 0.5% Yeast Extract, NaCl 10 mM, KCl 2.5 mM, $MgCl_2$ 10 mM, MgSO4 10 mM, glucose 20 mM). The tubes were incubated shaking at 200 rpm for 1 hour at 37ºC.

The transformed *E. coli* cells were selected in LB (Lysogeny Broth) + Agar media, Ampicilin (50 mg/L) and X-gal (20mg/L). Two plates for each transformation were prepared, one with 50 µL and other with 200 µL of transformation. The plates were incubated at 37ºC overnight.

3.6 Miniprep

All the white colonies from the plates were picked and cultured in 96 well plates, containing 1 mL of *CircleGrow* with 1 µL/mL of Ampicilin (100mg/µL) in each well. The plates were sealed and two small holes were made to allow air circulation. Afterwards the plates were incubated at 37 ºC shaking overnight. After incubation, Miniprep for plasmidial DNA extraction was performed.

The plates were centrifuged for 6 minutes at 3700 rpm. The seal was removed and the supernatant was discarded, by inverting the plates and then the plates were left inverted for 5 minutes. It was added 250 µL of GET (Glucose 20%, EDTA 0,5M pH 8,0, Tris-Hcl 1M pH 7,4, MilliQ H_2O) to each well, mixing by vortex for 2 minutes to resuspend the cells, and centrifuged for 10 minutes at 3700 rpm. The supernatant was removed as described before and 80 µL of GET were added to the wells, mixing by vortex to resuspend the cells.

Polypropylene microplates with 96 wells were prepared with 2,5 µL of RNAse (10mg/mL) in each well and the bacterial suspension was transferred to these plates. A solution containing 0,2M NaOH/1% SDS and 80 µL of this solution were added to each well. The plates were sealed and inverted 30 times, briefly centrifuged and incubated for 10 minutes at room temperature. Afterwards, 80µL de 3M KOAc (at

4ºC) were added, repeating the same steps described for agitation and incubation. A white precipitate was formed at this point. The Seal was removed and the plates incubated for 30 minutes at 90ºC. After incubation, the plates were placed on ice for 10 minutes and centrifuged for 10 minutes at 3700 rpm.

After centrifuging, 100 µL of the supernatant was transferred (avoiding to touch the white precipitate) to Millipore (filter) plates added to a polypropylene plate and centrifuged for 6 minutes at 3.700 rpm in order to flow the supernatant through the filter. Afterwards, the Millipore plates were discarded and 100 µL of isopropanol was added to the filtered supernatant. The plates were sealed, inverted 30 times to mix the content and centrifuged at 20ºC for 45 minutes at 3700 rpm. The supernatant was discarded and 200 µL of cold 70% ethanol was added to each well. The plates were centrifuged again for 5 minutes at 20ºC at 3700 rpm and the supernatant removed. Afterwards, the plated were incubated for 15 minutes at 37ºC to evaporate the ethanol and the pellet was resuspended in 60 µL of milliQ water. The plates were sealed, left at room temperature for 14 hours, agitated, briefly centrifuged and stored at -20ºC.

3.7 Sequencing

After plasmidial DNA extraction, 1 µL was used for sequencing. A Mix was prepared with: 1 µL of *M13 foward Primer* (5 µM), 4 µL of Pre-mix and 4 µL of milliQ water autoclaved. The reaction was run in *Eppendorf Mastercycler* thermo cycler following the program: 30 cycles at 95°C for 20 seconds, 50°C for 15 seconds and 60°C for 1 minute.

The amplified products from the sequencing reaction were precipitated, by adding 1 µl of Ammonium Acetate (7,5 mol/L) to the samples. Afterwards, 95% ethanol, two times the volume of the samples, was added and the plates were sealed, inverted and incubated at room temperature for 15 minutes. After centrifuging for 45 minutes at 6.600 rpm at 20 °C, the supernatant was discarded and the pellet was washed with 100 µl of 70% ethanol. The plates were centrifuged for 15 min at 6.600 rpm, 20°C. The supernatant was discarded; the plates were briefly centrifuged (3.300 rpm, 1 min), inverted to remove the ethanol excess and incubated at 37°C for 15 min. After applying 10 µl of *loading solution* to each well, the plate was injected in Mega Bace 1000 sequencer. The parameters of sequencing were: voltage of injection: 2 Kv; time of injection: 80 seconds; running voltage: 6 Kv; running time: 240 minutes. The sequences were given by the Sequence Analyzer software.

3.8 Analysis of the cDNA sequences in Database

The sequences from the libraries were compared to sequences available in the database "*National Center for Biotechnology Information*" (NCBI), (http://www.ncbi.nlm.nih.gov).The sequences were screened for the presence of vector in the sequences, by the tool *VecScreen* and compared to similar sequences by using the BLAST*n* program. The most similar sequence found compared by the BLAST software, the attributed function and the species from which the sequence referred were used to identify the genes corresponding to each EST analyzed.

3.9 qRT-PCR Analysis

3.9.1. Treatment of RNA samples with DNAse

The RNA samples were treated with DNAse (TURBO DNAse – AMBION), following the manual instructions to eliminate DNA contamination before cDNA synthesis. A 25 µL reaction was prepared in 0,5 mL tubes, with 2,5 µL (0,1 vol.) of 10X DNAse buffer, 0,5 µL of DNAseI enzyme and 5 µg of RNA. The amount of RNA was calculated for each sample to reach 5 µg, and the volume was completed to 25 µL with sterile water.

First, the samples were incubated for 30 minutes at 37 ºC. Next, 2,5 µL (0,1 vol.) of DNAse inactivation was added and the samples were incubated for 2 minutes at room temperature. Afterwards, the tubes were centrifuged at 10000 g for 1,5 minutes and the supernatant was transferred to a new tube. The same reaction was performed to the three biological replicates for each treatment.

The RNA quantity and quality were measured in Nanodrop®, and integrity and DNA contamination were verified in agarose gel electrophoresis (1%, TAE 10X, stained with GEL RED).

3.9.2. cDNA synthesis

Synthesis of cDNA for qRT-PCR analysis was performed afterwards DNAse treatment, using the High Capacity (INVITROGEN) cDNA synthesis kit, following the manual instructions. A total of 1 µg of treated RNA was used as template for cDNA synthesis. The reaction was prepared to a final volume of 20 µL, with: 2,0 µL of

Buffer, 0,8 µL of dNTP, 2,0 µL of Primer, 1,0 µL of Reverse Transcriptase enzyme and 4,2 µL of water. The program followed in thermocycler was: 25 ºC for 10 minutes, 37 ºC for 120 minutes, 85 ºC for 5 minutes, hold at 4 ºC.

3.9.3. qRT-PCR assay

Some protein- encoding genes were selected from the libraries for expression analysis by qRT-PCR and primers were designed to the target genes. The detection system was the Rotor-Gene (QIAGEN), using SYBR Green, which intercalates double stranded cDNA and emits fluorescence. The cDNA was obtained from the treated RNA of 12NI, 12I, 24NI, 24RI24, 24RI48 samples, in biological triplicates.

A 10 µL reaction was prepared for each sample, with 5 µL of Rotor-Gene SYBR GREEN (QUIAGEN), 0,4 µL of each primer (0,5 µM) and 1 µL of cDNA. Besides biological triplicates, the samples were analyzed in technical triplicates. The PCR program was: 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds, 65 °C for 30 seconds and 72 °C for 30 seconds. At the end of the cycles, temperature was gradually raised from 65 °C to 95 °C in order to obtain the melting curve to identify possible primer dimers or unspecific amplification.

The method used to obtain the expression was the comparative C_T (Threshold Cycle). The normalization was performed using the equation $\Delta C_T = C_T$ (target gene) - C_T (reference gene). The calibration was determined by the formula $\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (calibrator). The calibrator is the sample used as baseline to the comparative expression analysis. The relative quantification was obtained by the formula $2^{-\Delta\Delta C_T}$.

The transcripts abundance was normalized by the expression of the reference genes *Ubiquitin* (*UBI*) and GAPDH in the same samples.

4. Results and Discussion

4.1 Libraries

The total RNA isolated from 24NI, 24RI48, 24RI24, 12NI and 12I samples were visualized in EtBr agarose gel (1%, TAE 10X), showed in figure 3. For each sample 1 µL of RNA was applied, with 8 µL of water and 1 µL of DYE.

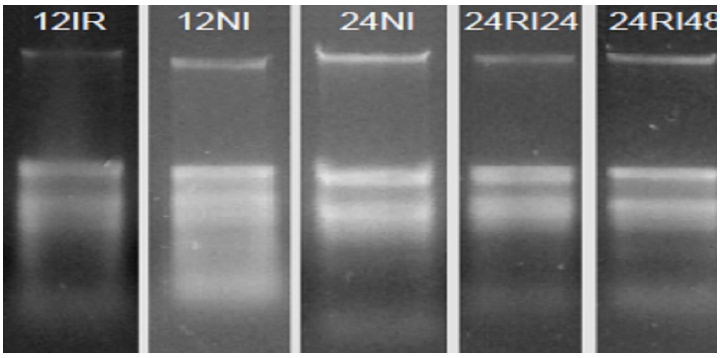

Figure 3. Agarose gel (EtBR; 1%; TAE 10X) picture of RNA samples isolated from coffee leaves under stress conditions. Samples in the order they were applied to the gel: 12I: 12 days irrigated; 12NI: 12 days non-irrigated; 24NI: 24 days non-irrigated; 24RI24: 24 days 24 hours after re-irrigated; 24RI48: 24 days, 48 hours after re-irrigated.

After all the steps for hybridization were completed for library 1 (24RI48-24NI), library 2 (24RI48 – 24RI24) and library 3 (12NI – 12I), two rounds of PCR were performed in order to amplify the differentially expressed genes. The second PCR products were visualized in EtBr agarose gel (2%), along with an amplification of a control sample from the kit (Figure 4).

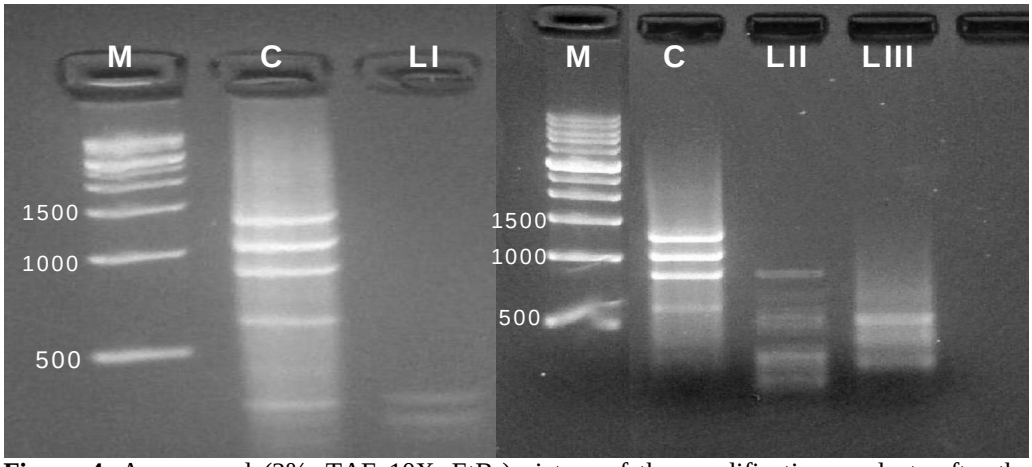

Figure 4. Agarose gel (2%, TAE 10X, EtBr) picture of the amplification products after the second PCR amplification of Library 1 (LI), Library 2 (LII) and Library 3 (LIII) along with a subtracted control sample from kit. M: 1kb marker.

The samples were then cloned and all white colonies were picked for miniprep and sequencing of the transcripts. The number of colonies obtained for each library and the subsequent number of ESTs generated by sequencing are listed in table 2.

Table 2. Number of white *E.coli* colonies picked for sequencing and the number of ESTs generated by sequencing for each Library.

Library	Number of colonies	Number of ESTs
1	187	146
2	190	139
3	120	101

Furthermore, the protein- encoding genes, putative proteins, unknown proteins and hypothetical proteins found by BLASTn to which each EST was similar were listed. No hits with the database were found for 26, 11 and 12 ESTs in Library 1, 2 and 3, respectively. Additionally, similarity with no-plant organisms was found for 40, 87 and 61 ESTs and these sequences were not listed. The libraries in reverse direction were similarly analyzed (data not showed) in order to identify sequences present in both directions, what is considered a false positive result, but no false positive was identified.

4.1.1. Library 1

In Library 1, the differential expression between plants 48 hours after re-watered of 24 days under drought and plants under drought for 24 days, with no re-watering, was analyzed, and protein- encoding genes related to tolerance and recovery were expected in this library. Many hypothetical proteins and unknown proteins were identified, but many of them were identified in differential expression analysis of biotic or abiotic stressed plants and deposited in the database, what suggests that these proteins may play key roles in stress response and further studies are needed to describe these proteins and characterize their function.

Moreover, no hits with the database were found for 26 ESTs, indicating that these ESTs can correspond to novel proteins. Further analysis such as protein prediction along with other bioinformatics analysis can provide important information in order to determine whether these ESTs correspond to putative proteins or not.

Few protein- encoding genes were identified, only six, what partially impairs a wide comprehension of the stress response mechanism occurring in the tested plants. However, for some of them there are some information in the literature, allowing a comprehension of what is differentially expressed in re-irrigated plants and may relate to their recovery, as observed in physiological analysis. All sequences found in library 1 are listed in table 3 and the known protein- encoding genes are discussed below.

Table 3. List of the ESTs from Library 1 which had similarity with sequences deposited in the NCBI. The accession number of the sequences found by BLAST, the quantity of ESTs similar to the same sequence, the best e-value and the name of the sequence are listed.

ACCESSION NUMBER	QNT	BEST E-VALUE	NAME
AM748496.1	18	2,00E-15	*Vigna unguiculata* partial mRNA for putative 23S rRNA pseudouridine synthase
CT028787.1	22	5,00E-14	Poplar cDNA sequences
CT033829.1	5	2,00E-11	Poplar cDNA sequences
CT029475.1	2	3,00E-15	Poplar cDNA sequences
AM748435.1	2	2,00E-14	*V. unguiculata* partial mRNA for putative plant disease resistance response protein family
AK226691.1	1	4,00E-44	*A. thaliana* mRNA for ubiquitin-specific protease 8
EU164537.1	2	6,00E-06	*Coffea canephora* BAC genomic sequence
AJ635225.1	1	0.035	*T. aestivum* mRNA for acid beta-fructofuranosidase precursor (inv1 gene)
GU123895.1	1	2,00E-15	*Coffea arabica* clone BAC 140-17D
NM_001196699.1	1	0.54	*Z. mays* hypothetical protein
AC235257.1	1	0.84	*G. max* strain Williams (hypothetical protein)
AM479703.1	1	0.024	*V. vinifera* whole genome shotgun sequence (hypothetical protein)
DQ465754.1	1	0.007	*S. drummondii* clone mRNA
EF107674.1	10	8,00E-04	*Coffea pseudozanguebariae* ethylene receptor (ETR1) gene
CU137658.1	1	0.010	*M. truncatula* chromosome 5 clone mth2-54e16
AM748484.1	1	0.022	*V. unguiculata* partial mRNA for putative ATP synthase CF1 epsilon subunit (atpE gene)
AM490238.1	1	0.024	*Z. mays* mRNA for putative wuschel homeobox protein (wox5A gene)
CT834194.1	1	8,00E-09	*O. sativa* (indica cultivar-group) cDNA

23rRNA pseudouridine synthase

Pseudouridine synthases are enzymes responsible for a post-transcriptional modification in rRNA, and is the most frequent single base modification in ribosomal RNA. The function of pseudouridines remains unclear, but it seems to play a critical role in ribosome functioning, and therefore, in protein synthesis. Depletion of one or more pseudouridines in yeast caused reduced growth rate of the cells, decreased rate of translation and other phenotypic effects (KIPPER et al., 2011).

Protein- encoding genes directly or indirectly associated with protein synthesis are generally identified in subtractive cDNA libraries made from stressed plants. For instance, gene ontology analysis of ESTs generated from SSH of severely drought stressed chickpea (*Cicer arietinum* L) identified ESTs related to translational regulation activity (DEOKAR et al., 2011). A pseudouridine synthase was identified in a subtractive cDNA libraries with Mn toxicity response in *Vigna unguiculata* (FÜHRS et al., 2008), demonstrating that this gene is correlated to stress response, although its function is not yet clear.

Ubiquitin-specific protease 8 (USP8)

Ubiquitin specific proteases (USP) are deubiquitinating enzymes, and plays regulatory role in ubiquitin pathways. USP8 proteins are thought to be related to growth (NIENDORF et al., 2007). Posttranslational modification of proteins by mono or polyubiquitination represents a central mechanism for modulating a wide range of cellular functions, like protein stability, intracellular transport, protein interactions, and transcriptional activity.

Proteins related to ubiquitin pathway are also commonly found in differential expression analysis, for instance Prabu et al. (2010) found ubiquitin-conjugating families of enzymes. This gene was not found in literature as being differentially expressed in similar conditions to this work, but the presence of this gene may be related to resuming of growth after the stress period.

Acid beta-fructofuranosidase precursor (inv1)

Acid invertases catalyze the irreversible hydrolysis of sucrose into the constituent monosaccharides fructose and glucose (HUANG et al., 2011). Vacuolar acid invertases play an important role in sugar storage, cell enlargement and osmoregulation (ROITSCH; GONZALEZ, 2004), therefore contributing to osmotic adjustment and tolerance.

Upregulation of acid invertases genes were already detected for many stresses, such as cold and water deficiency (GREINER et al., 1999; KIM et al., 2000). More recently, differential expression of acid invertase genes in roots of metallicolous (tolerant) and non-metallicolous (sensitive) populations of *Rumex japonicus* under copper stress was tested and the metallicolous population showed significant upregulation of vacuolar acid invertases, while for non-metallicolous population the

expression was not affected (HUANG et al., 2011). The enhanced transcript levels of invertases genes may correlate to the tolerance, and this can be also the case for Siriema coffee. Although invertases would be expected to be in higher level in stressed plants because of its important role in osmotic adjustment, the presence of this gene in library 1 indicates that invertases remain playing an important role during recovery period. In the physiological analysis, activity of the vacuolar acid invertase showed constant increasing during all experiment (MELO, 2008), for both stressed and re-irrigated plants.

ETR1

Ethylene is involved in regulation of several processes in plants, such as seed germination, seedling growth, leaf and petal abscission, organ senescence, ripening, stress responses, and pathogen responses (ABELES; MORGAN; SALTVEIT JUNIOR, 1992). Ethylene receptors act as negative regulators of the ethylene signal transduction pathway (ZHAO; SCHALLER, 2004). Thus, a decrease in receptor levels is predicted to sensitize the plant such that it responds to lower levels of ethylene than usual.

The ETR1 protein level decreased as an effect of osmotic stress in *Arabidopsis thaliana* (ZHAO; SCHALLER, 2004). Decrease in ETR1 expression during stress may indicate that ethylene pathway is active while plants are stressed, and upregulation of this gene during recovery after a period of stress, as indicated in library 1, can be due to repression of ethylene pathway. These results are an indicative of its function in this condition and further analysis must be carried out in order to elucidate ETR1 expression profile and ethylene function in drought-stressed coffee plants.

ATP synthase CF1 epsilon subunit

CF1 is a subunit of chloroplast ATP synthase, and is comprised of many subunits, among them the epsilon (Ɛ) subunit. A chloroplast ATP synthase subunit (CF0) was identified in drought stressed sugarcane leaves (PRABU et al., 2010). In tolerant wild watermelon, the amount of the Ɛ subunit in CF0CF1 ATP synthase was decreased by drought/excess light stresses, while other subunits of the same enzyme did not change during the stress. Moreover, the amount of the Ɛ subunit was recovered by resuming irrigation (KOHZUMA et al., 2008). The authors suggested that the

decomposition of the Ɛ subunit contributes to the protection of the thylakoid against over-acidification under drought conditions. Therefore, the presence of this gene is also an indicative of recovery in re-irrigated plants.

WOX5A

The wuschel homeobox 5A is a transcription factor which plays a key role in the determination of cell fate and cell differentiation in plants and animals. These transcription factors were found to be down-regulated in drought stressed amaranth roots (HUERTA-OCAMPO et al., 2010).

When re-irrigated, coffee plants may up-regulate expression of these transcription factors in order to stimulate cell differentiation and consequently, growth, which is inhibited during the stress period. Further analysis, for instance expression analysis of this gene in different tissues and periods of stress compared to re-irrigated plants will allow a better comprehension of WOX5A involvement in the process of drought stress and recovery in coffee plants.

4.1.2. Library 2

In Library 2, differential expression of plants 24 days under drought conditions, 48 hours after re-irrigation compared to plants 24 hours after re-irrigation was tested. In this library, genes activated during plant recovery period are identified. During the physiological experiments, the period between 24 and 48 hours after re-irrigation showed to be essential to plant recovery (MELO, 2008). As in the case of library 1, many of the ESTs showed similarity with hypothetical proteins, many of them identified in studies with biotic and abiotic stresses, including drought. No hits with the database were found for 11 ESTs, and further analyses are needed to these sequences in order to identify whether these are putative proteins. The sequences found for library 2 are listed below. Furthermore, four protein-encoding genes were identified and are discussed below.

Table 4. List of the ESTs from Library 2 which had similarity with sequences deposited in the NCBI. The accession number of the sequences found by BLAST, the quantity of ESTs similar to the same sequence, the best e-value and the name of the sequence are listed.

ACCESSION NUMBER	QNT	BEST E-VALUE	NAME
AM748466.1	3	7,00E-06	*Vigna unguiculata* partial mRNA for putative single-stranded nucleic acid binding R3H
XM_002278335.1	2	6,00E-21	PREDICTED: *V. vinifera* hypothetical protein
DQ123928.1	2	0.015	*Coffea arabica x Coffea canephora* mRNA sequence
XM_002264008.1	1	1,00E-04	PREDICTED: *V. vinifera* hypothetical protein
FJ809741.1	1	0.84	*Solanum pennellii* chromosome 11
XM_002440024.1	1	0.12	*Sorghum bicolor* hypothetical protein
AC007188.7	1	0.71	*A. thaliana* chromosome 2 (unknown protein)
AC067753.4	1	0.68	*A. thaliana* hypothetical protein
NM_001074595.1	1	0.021	*O. sativa* (japonica cultivar-group) mRNA (hypothetical protein)
NM_121896.2	1	6,00E-04	*A. thaliana* protein kinase family protein mRNA
DQ139800.1	1	3,00E-46	*V. vinifera* MSA gene
AM472467.1	1	0.16	*V. vinifera* whole genome shotgun sequence
XM_002285850.1	1	4,00E-13	PREDICTED: *V. vinifera* hypothetical protein mRNA
XM_002464466.1	1	0.71	*Sorghum bicolor* hypothetical protein
AM431307.2	1	0.96	*Vitis vinifera* hypothetical protein
AC124967.38	1	0.49	*Medicago truncatula* chromosome 8
AM465476.2	1	0.27	*V. vinifera* whole genome shotgun sequence
CT029989.1	1	2,00E-05	Poplar cDNA sequences
AJ579711.2	1	7,00E-29	*Beta vulgaris* mRNA for chloroplast chlorophyll a/b binding protein
BT052406.1	1	3,00E-28	*Medicago truncatula* unknown mRNA
XM_002524334.1	1	5,00E-15	*R.communis* conserved hypothetical protein, mRNA
CU228715.1	1	0.25	Populus EST from severe drought-stressed leaves
NM_001175885.1	1	0.070	*Zea mays* hypothetical protein
XM_002314860.1	1	0.67	*P. trichocarpa* predicted protein, mRNA

Putative single-stranded nucleic acid binding R3H

According to the InterPro database, R3H is a domain that binds single-stranded nucleic acids and the sequences that contain R3H motif are mostly hypothetical proteins predicted from sequencing projects. This motif was also found in a sequence obtained from a subtractive cDNA library Mn-toxicity response in *Vigna unguiculata*

(FÜHRS et al., 2008), but no further information about this motif function was provided.

MSA gene

Little information available was found about MSA gene. A note at the GenBank page about the sequence found by BLAST, which is from *Vitis vinifera*, describes the gene as being associated with maturation and stress, and that it is an abcisic acid-induced protein. Also, the note refers to the protein as part of the regulatory proteins, which are protein factors involved in signal transduction and stress-responsive gene expression (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2007). These types of proteins are generally early induced after stress, and are transiently expressed. In Library 2 it was not expected to find early induced genes, but since this library represents genes being induced after a sudden change in environmental conditions, re-watering after a 24 days period of drought stress, this gene may be involved in this process of rapid change in gene expression activation for response.

Protein kinase family protein

Kinases are enzymes that phosphorylate proteins, and are involved in several pathways in the cell. According to the TAIR database (Arabidopsis Information Resource), this protein kinase family found in library 2 is located in peroxisome and was expressed in different stages of pollen maturation in *Arabidopsis thaliana*. Protein kinases are regulatory proteins and several types of kinases are generally found in SSH works with stress response in plants (DALDOUL et al., 2010; DEOKAR et al., 2011; GUAN et al., 2010; PRABU et al., 2010), although this protein kinase family was not found in similar works.

Chloroplast chlorophyll a/b binding protein

The chloroplast light harvesting a/b protein (cab11) gene is involved in photosynthesis, and according to InterPro database, its functioning is believed to be mediate the distribution of excitation energy between photosystems I and II. A SSH work showed that the transcript levels of the chlorophyll a/b binding protein were

downregulated during drought stress in cheakpea (DEOKAR et al., 2011). In case of Library 2, it would be expected to find genes related to photosynthesis, since the photosynthetic rate usually decreases during stress conditions, and this library was created intending to identify differentially expressed genes related to recovery of plants after re-irrigation.

4.1.3 Library 3

The library 3 represents differentially expressed genes between irrigated plants during 12 days and non-irrigated plants in the same period. For this library, genes involved in tolerance were expected, since from the physiological results the main differences between irrigated and non-irrigated plants started at 12 days of experiment (MELO, 2008). Similar to the other libraries, many hypothetical and unknown proteins were found. For 12 ESTs no hits with the database were found, and these sequences may contain non described proteins that may be involved in tolerance mechanism. Analysis of these sequences is needed in order to identify whether they are putative proteins and provide more information about them. Furthermore, only three protein-encoding genes already identified were found in Library 3, what makes more difficult to understand the processes, but some information in the literature were found for these genes, which are discussed bellow.

Table 5. List of the ESTs from Library 3 which had similarity with sequences deposited in the NCBI. The accession number of the sequences found by BLAST, the quantity of ESTs similar to the same sequence, the best e-value and the name of the sequence are listed.

ACCESSION NUMBER	QNT	BEST E-VALUE	NAME
CT033829.1	3	3,00E-09	Poplar cDNA sequences
AM748496.1	2	0.004	*Vigna unguiculata* partial mRNA for putative 23S rRNA pseudouridine synthase (yibC gene)
CT028555.1	1	8,00E-08	Poplar cDNA sequences
XM_002265554.1	1	2,00E-44	PREDICTED: *Vitis vinifera* hypothetical protein
DQ228355.1	1	3,00E-11	*Solanum tuberosum* clone 153A08 ribosomal protein S7-like protein mRNA
AC139354.7	1	4,00E-06	Medicago truncatula clone mth1-64n13
JI333903.1	1	0.23	TSA: *Petunia axillaris* PETAX002274:Contig1 mRNA sequence
AC125411.1	1	0.68	*Oryza sativa*, Nipponbare strain, clone OSJNAb0079B22

AK319568.1	1	4,00E-09	*Solanum lycopersicum* cDNA, clone: HTC in leaf
CT029673.1	1	1,00E-05	Poplar cDNA sequences
AM085801.1	1	0.33	*Anacardium occidentale* microsatellite DNA
AM435492.2	1	0.5	*Vitis vinifera* contig VV78X205074.50, whole genome shotgun sequence
AC182709.2	1	0.43	*Populus trichocarpa* clone Pop1-85D11
XM_002264008.1	1	0.012	PREDICTED: *Vitis vinifera* hypothetical protein
EU965668.1	1	0.78	*Zea mays* clone 287825 glucan endo-1,3-beta-glucosidase 4 precursor
AM748484.1	1	0.003	*Vigna unguiculata* partial mRNA for putative ATP synthase CF1 epsilon subunit (atpE gene)

23S rRNA pseudouridine synthase

The pseudouridine synthase gene was similarly found in Libraries 1 and 3. As cited before, this gene was also identified in a subtractive cDNA library with Mn toxicity response in *Vigna unguiculata* (FÜHRS et al., 2008), but its function in stress response is not clear. As the periods tested in library 1 and 3 are the periods in which the main changes in metabolism occurs (MELO, 2008), this gene up-regulation may be correlated to the increasing in protein production, since it is directly related to ribosome functioning.

Ribosomal protein S7-like protein mRNA

The ribosomal S7-like was identified in library 3. The S7 ribosomal protein is required for head assembly of the ribosomal subunit 30S (FREDRICK; DUNNY; NOLLER, 2000). The ribosomal S7 gene was identified in wheat mitochondria and showed similarity with the S7 ribosomal protein in bacteria and chloroplasts (ZHUO; BONEN, 1993), but homologue sequences were not found to soybean, what shows that this gene is not present in all plants. In *Solanum tuberosum*, similarity with wheat S7 protein was identified and the sequence was deposited in the *GenBank* as S7-like protein, which is the sequence retrieved by BLASTn of one EST in Library 3, but further information was not available.

Glucan endo-1,3-beta-glucosidase 4 precursor

The glucan endo-1,3-beta-glucosidase 4 gene plays an important role in pathogen defense in plants. In addition to pathogen attack, the expression of b-1, 3-glucanases has been shown to be induced by abiotic elicitors such as ethylene, salicylic acid, and methyl jasmonate. Additionally, b-1,3-glucanases have been involved in several physiological and developmental processes such as microsporogenesis, seed germination, pollen development, and fruit development (JUNG; HWANG, 2000). In Library 3 this gene may indicate response to stress, since from 12 days of experiment, non-irrigated plants started being more affected by drought in many aspects (MELO, 2008).

4.2 qRT-PCR analysis

The use of complementary methods, such as quantitative and semi-quantitative PCR methods, to validate SSH results is a common practice in recent studies (DEOKAR et al., 2011; PRABU et al., 2010; SAHU; SHAW, 2009), by selecting one or more genes. In order to validate the results of the libraries and test whether differential expression was detected by the SSH technique in Siriema coffee plants, qRT-PCR was performed for selected protein-encoding genes from each library. The selected protein-encoding genes, the respective library, the primer sequence and the amplicon size are listed in table 6.

Table 6. List of the protein-encoding genes selected for gene expression analysis. The respective libraries to which the genes belong, forward and reverse primers sequence and amplicon size are presented.

Library	Gene	Forward Primer (5´- 3´)	Reverse Primer (5´- 3´)	Amplicon size (bp)
1	acid beta-fructofuranosidase precursor (INV)	GCCGCCAATCGT TCTCTTAC	GACCGAGAAAC CTCTGCTTCA	102
2	chlorophyll a/b binding protein (CAB11)	TACAATGTCACT GGAGAAGGAC	TTGAACGATTGT GTTGTGCCATG	78
3	glucan endo-1,3-beta-glucosidase 4 (GLU)	CCGACCTTTCAA ACCTACCA	ATCTCCTCGTTT GTGACGC	157

The RNA samples were treated with DNAse to eliminate DNA contamination from RNA samples. RNA integrity and absence of DNA contamination were tested in an agarose gel (1%, TAE 10X GEL RED), as showed in figure 5. Afterwards, cDNA was synthesized and primers efficiency test was performed for further qRT-PCR

analysis. Three independent samples for each treatment (12I, 12NI, 24NI, 24RI24 and 24RI48) were used.

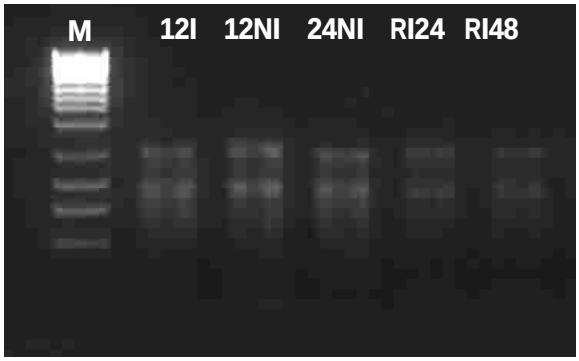

Figure 5. Agarose gel (1%, TAE 10X, GELRED) picture of RNA samples from leave of plants under the treatments: 12 days irrigared (12I), 12 days non-irrigated (12NI), 24 hours after re-irrigated (RI24) and 48 hours after re-irrigated (RI48) treated with DNAse. M: 1kb marker.

Furthermore, a primer efficiency test was performed for each pair of primers. From the synthesized cDNA of three independent samples for each treatment, a pool containing 1 μL of each sample (15 μL) was diluted in 50 μL of sterile water, forming a 1:5 dilution. Afterwards, serial dilution was performed to 1:25, 1:125, 1:625 and 1:3125. A real time expression analysis was performed using the serial dilutions as a template for each pair of primers, as also for the reference genes UBI and GAPDH.

The threshold baseline was defined to determine de Threshold Cycle (Ct), which corresponds to the number of cycles required to reach the determined fluorescence intensity. Afterwards, Ct values of each sample dilution were plotted against the logarithm of its corresponding dilution, and in some cases, the last dilution point was omitted (1:3125). The efficiency was calculated by the Rotor-Gene Q Series software. The efficiencies of UBI, GAPDH, INV, CAB11 and GLU were, respectively: 1.0, 1.0, 0.92, 0.90 and 0.57. The GLU primers presented low efficiency and were not used for gene expression analysis.

Furthermore, real time gene expression analysis was performed. All samples were analyzed with the reference genes (GAPDH and UBI) to further normalization of the results. The samples corresponding to Library 1, 24RI48 and 24NI were tested with INV primers, and the samples from Library 2, 24RI48 and 24RI24 were tested with CAB11 primers. The mean values of three technical replicates for each biological

replicate was normalized with the mean values of the reference genes in the same biological replicates (ΔC_T). The calibrator for INV were 24NI samples, and for CAB11, 24RI48 samples. After $\Delta\Delta C_T$ calculation, the expression level was calculated ($2^{\Delta\Delta CT}$). The results are shown in figure 6.

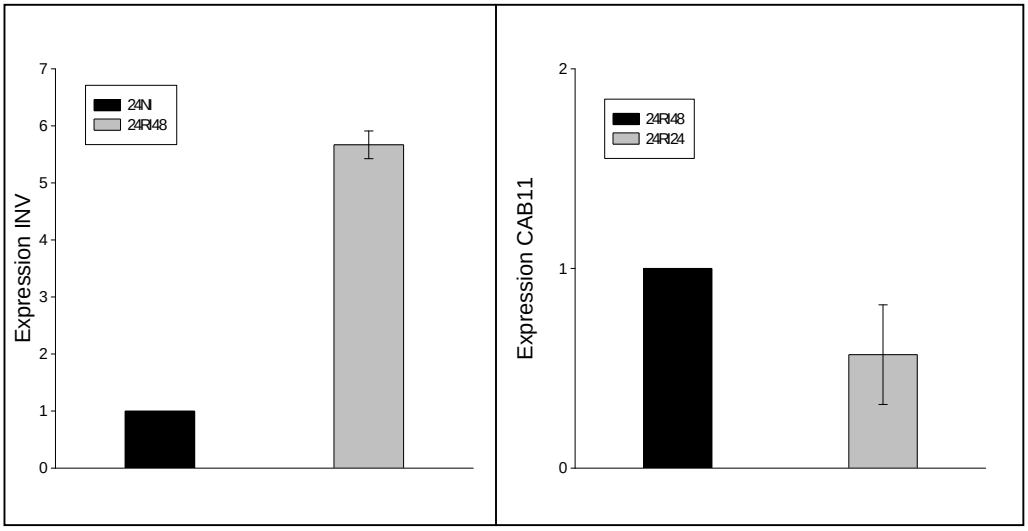

Figure 6. Expression analysis of INV in 24NI and 24RI48 plants (Library 1) and CAB11 in 24RI48 and 24RI24 (Library 2). Columns are mean values of three plants and bars represent standard error.

The expression of INV gene was about 5,6 fold higher in 24RI48 samples than in 24NI samples. This shows differential expression between the samples, as indicated by the subtracted cDNA library, thus library 1 can be considered validated. Further analysis of the expression profile of this gene in all periods and treatments will allow better understanding of how this gene is regulated along the experiment and its contribution to tolerance and ability to recover of Siriema plants.

In case of CAB11 gene, the expression in 24RI24 samples was about 0,57 of 24RI48 expression, what indicates the same pattern of differential expression presented by SSH method and therefore validate the results. Analysis of the expression profile of this gene in all conditions will allow a better comprehension of its relation to drought stress and recovery in Siriema coffee plants.

5. Conclusion

In this study, three subtractive cDNA libraries of leaves of drought-stressed Siriema coffee plants, 24RI48-24NI, 24RI48-24RI24 and 12NI-12I were prepared, cloned, sequenced and analyzed. Hypothetical and unknown proteins, putative novel proteins and some protein-encoding genes were identified. The protein-encoding genes found have relation to stress signaling, stress response and direct or indirect relation to recovery of re-irrigated plants.

One gene from each library was selected for quantitative expression analysis by qRT-PCR and validation of the subtraction. For library 1, the selected gene (INV) was differentially expressed between the samples and validated the results. For library 2, differential expression was also detected for the selected gene (CAB11) in quantitative analysis, validating the library results. For library 3, the primers designed for expression analysis showed low efficiency to conduce quantitative analysis, and validation was not possible.

These results contribute to the study of drought tolerance in Siriema progeny and may provide information to further studies and the development of technologies that allow coffee plants to tolerate longer periods of drought.

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