BIOTECHNOLOGICAL APPROACHES TO IMPROVE DROUGHT TOLERANCE OF *Coffea arabica*

VANESSA CRISTINA STEIN

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-graduação em Fisiologia Vegetal, para a obtenção do titulo de "Doutor"

Orientador:
Prof. Renato Paiva, PhD

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Mark Aarts	Wageningen University
Antônio Chalfun Júnior	UFLA
Luciano Vilela Paiva	UFLA
Paulo César Magalhães	EMBRAPA

Prof. PhD Renato Paiva
UFLA
(Orientador)

LAVRAS
MINAS GERAIS – BRASIL

Aos meus pais,

à minha mana Fernanda
e em especial a vó Silda
pelo incansável e constante
incentivo aos meus estudos

OFEREÇO

Ao Stenio Lasmar de Abreu
fonte de constante conhecimento,
alegria e amor em todos os momentos

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SUMMARY

GENERAL ABSTRACT

STEIN, Vanessa.Cristina. **Biotechnological approaches to improve drought tolerance of *Coffea arabica.*** 2009. 97p. Thesis (Doctor in Plant Physiology) – Federal University of Lavras, Lavras, MG. *

Overexpression of *SHINE* gene induces the expression of these stress-related genes under normal growth conditions in transgenic plants and then confers the improved tolerance to drought. In recent years rapid procedures for obtaining transgenic roots have been developed using *Agrobacterium rhizogenes*, leading to the production of so-called "composite plants" comprising a transgenic hairy root system attached to non-transformed shoots and leaves. In the chapter II was determined the effect of (over)expression of the Arabidopsis *SHN2* gene in stable and/or chimeric transgenic *Arabidopsis thaliana* and *Brassica rapa* plants subjected to drought treatment. In the chapter III was developed an efficient method for coffee genetic transformation by *A. rhizogenes* in order to create chimeric *C. arabica* (Catuaí Vermelho IAC 144) with wild-type shoots and transgenic roots for rapid testing of potentially interesting gene construct for future stable transformation using *A. tumefaciens* or *A. rhizogenes* and in the chapter IV was expressed the *SHN2* gene of *Arabidopsis thaliana* in transgenic hairy roots of *Coffea arabica* after *Agrobacterium rhizogenes*-mediated root transformation in order to improve the drought tolerance of such chimeric plants. 50% of the 35S::*SHN2* chimaric transgenic *Arabdopsis* plants exposed to 10 days withholding water and rewatered for one week recovered the drought treatment. In the coffee transformation 42% of hypocotyls infected *A. rhizogenes* contained pREDRoot plasmid had at least one transgenic roots and hypocotyls infected with *A. rhizogenes* contained pMOG22-35S::*SHN2* and showed 32% of transformation. In the coffee drought experiment the DsRED (control) chimeric plants did not recover from the 15-day dehydration treatment and completely dried out all while the 35S:*SHN2* chimeric plants recovered and become greener and stronger.

Key words: *SHINE, Agrobacterium rhizogene,* chimeric plants, drought.

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*Guidance Committee: Renato Paiva – UFLA (Advisor), Mark Aarts – WUR, Antônio Chalfun Junior – UFLA.

RESUMO GERAL

STEIN, Vanessa Cristina. **Ferramentas biotecnológicas para aumento da tolerância a seca em *Coffea arabica.*** UFLA, 2009. 97p. Tese (Doutorado em Fisiologia Vegetal) – Universidade Federal de Lavras, Lavras, MG *

A superexpressão do gene *SHINE* induz a expressão de genes relacionados ao estresse em plantas transgênicas, sobre condições normais de crescimento, e portanto confere a aumento da tolerância a seca. Atualmente, tem sido desenvolvidos protocolos para a obtenção de raízes transgênicas com *Agrobacterium rhizogenes*, liderando a produção de "plantas compostas" com sistema radicular transgênico ligado à parte aérea não transformada. No capitulo II foi determinado o efeito da superexpressão do gene *SHN2* em plantas transgênicas, estáveis e quiméricas, de *Arabidopsis thaliana* e *Brassica rapa* submetidas a tratamentos de seca. No capítulo III foi desenvolvimento de um método eficiente de transformação genética de café via *A. rhizogenes,* para a obtenção de plantas quiméricas de *C. arabica* (Catuaí Vermelho IAC 144) que podem ser submetidas a testes rápidos, com genes de interesse, que poderão ser utilizados futuramente em transformações estáveis, utilizando-se A. tumefaciens ou A. *rhizogene*; e no capítulo IV finalmente superexpressar o gene *SHN2* em raízes transgênicas de café para melhorar a tolerância à seca. 50% plantas quiméricas de Arabdopsis superexpressando o gene *SHN2* expostas submetidas a 10 dias sem irrigação e re-irrigadas por uma semana sobreviveram ao tratamento de seca. Quanto a transformação do café 42% dos hipocótilos infectados com *A. rhizogenes* contendo o plasmidio pREDRoot apresentaram pelo menos uma raiz transformada e os hipocótilos infectados com *A. rhizogenes* contendo o plasmidio pMOG22-35S::*SHN2* apresentaram 32% de transformação. No experimento de seca as plantas quiméricas de café DsRED (controle) não sobreviveram após 15 dias de desidratação enquanto que as plantas quiméricas 35S:*SHN2* sobreviveram ao tratamento.

Palavras-chave: *SHINE, Agrobacterium rhizogene,*, plantas quiméricas, tolerância a seca.

* Comite Orientador: Renato Paiva – UFLA (Orientador), Mark Aarts – WUR, Antônio Chalfun Junior – UFLA.

Chapter 1

General Introduction

1 GENERAL INTRODUCTION

Stress is usually defined as a factor that exerts a disadvantageous influence on the plants (Taiz & Zeiger, 2002), as well as the abiotic stress which is the negative impact of non-living factors such as extreme temperature, high light irradiance and water and inorganic solute supplies (Tester & Bacic, 2005; Agarwal & Zhu, 2005; Saibo, 2009) on the living organisms (Vinebrooke, 2004), that must influence the environment beyond its normal range of variation to adversely affect the population performance or individual physiology of the organism in a significant way (Vinebrooke, 2004), frequently limiting growth and productivity of major crop species (Saibo, 2009).

Over the last 25 years, from 1970 to 2005, the temperature of the planet has increased 4%, and the predictable effects of global warming include more frequent and intense extreme weather events such as heat waves, droughts, floods and hurricanes (Norcia, 2008). The Working Group of the Intergovernmental Panel on Climate Change concluded in its Third Assessment Report that Latin America is highly vulnerable to climate change and in this context it is also highly probable that crop yields will decrease significantly (Gay, 2006). In the face of a global scarcity of water resources, drought stress is already a major limiting factor in plant growth and will soon become even more severe, as desertification covers more and more of the world's terrestrial area (Vinocur, 2005).

Growth reduction has been suggested as an adaptive feature for plant survival under stress, because it allows plants to divert assimilates and energy, otherwise used for shoot growth, into protective molecules to reduce the effects of abiotic stress (Zhu, 2002) and/or to maintain root growth, improving water acquisition (Chaves et al., 2003). Adaptation to stress has been suggested to be

mediated by both preexisting and induced defenses (Pastori & Foyer, 2002). Hence, in drought stressed plants, growth and productivity are adversely affected by a series of morphological, physiological, metabolic and molecular changes (Wang et al., 2003).

At the physiological level drought and salt stress are known to induce stomatal closure (Wilkinson et al., 2001; Zhu, 2002; Chaves et al., 2003), slowing CO_2 assimilation and consequently reducing the photosynthetic rate (Flexas et al., 2002; Centritto et al., 2003) and total carbon uptake. Goodwin & Jenks (2008) suggested that cuticular water permeability also plays a role in plant adaptation to drought, especially if residual stomatal transpiration after stomatal closure is negligible. The change induced by drought is also manifested as decreased cellular osmotic potential, resulting in disruption of cellular homeostasis (Zhu, 2002). However, organic osmolytes (small solutes like amino acids and their derivatives, polyols and neutral sugars) are used by numerous water stressed organisms to maintain cell volume. Such osmolytes are often called ‘‘compatible solutes’’ and appear in high concentrations (up to 20% total dry mass) upon osmotic adjustment but, in contrast to ions, do not inhibit cytosolic enzymes. Instead, they often exert a protective effect by stabilizing biological membranes under stress conditions (Hincha & Hagemann, 2004). Consequentially, responses to abiotic stress require the production of important metabolic proteins, such as those involved in synthesis of osmoprotectants and of regulatory proteins operating in the signal transduction pathways, such as kinases or transcription factors.

The increase Ca^{2+} is a rapid response in abiotic stresses like drought and is required for the expression of some stress genes in plants (Knight et al., 1998). It is generally believed that Ca^{2+} transmits the stress signal downstream in the pathway by interacting with a protein sensor (Cheong et al., 2006), such as

calmodulin (CaM), calmodulin domain protein kinases (CDPK) and B-like proteins (CBLs). Clustering of genes based on their expression characteristics has revealed a consensus *cis* regulatory element in the promoters of Ca^{2+}-responsive genes that matches with two abscisic acid (ABA)–related *cis* elements: the ABA responsive element (ABRE) and an ABRE coupling element (ABRE-CE). ABA signaling is well established as a mediator of drought stress adaptation in several plant models (Koornneef et al., 1984; Ooms et al., 1993; Verslues & Bray, 2006). Adaptation to water stress results from an alteration in gene expression by upregulation of the major ABA- and/or stress-responsive genes, like RD (response to dehydration), COR (cold responsive), LEA/*dehydrin*-like (late embryogenesis-abundant) and aquaporin genes (Seki et al., 2001). Their products activate chaperones in order to protect cellular proteins from degradation, activate proteinases to remove damaged proteins and aquaporins are likely to control the water status of plant cells (Seki et al., 2001; Siefritz et al., 2002; Zhu et al., 2005).

The physiological and biochemical changes in plants under stress conditions are related to altered gene expression. The first step in switching on molecular responses is to perceive the abiotic signal as it occurs and to relay information about it through a signal transduction pathway. These pathways eventually lead to physiological changes or to the expression of genes and resultant modification of molecular and cellular processes. The knowledge about the signaling pathways leading from environmental stimulus to stress response in plants has increased in recent years. It is increasingly apparent that the linear pathways that were initially proposed in building hypotheses of environmental signaling responses are actually part of a more complex signaling network, in which there are multiple overlaps between branches, with, for instance, many genes induced by more than one stimulus (Knight & Knight, 2001).

Regarding gene and protein expression modulated by stress, it is well documented that abiotic stresses in general, through regulation of both gene expression and protein turnover, alter the abundance of many transcripts and proteins (Seki et al., 2002; Yan et al., 2006), indicating that transcriptional and post-transcriptional regulation play an essential role in the adaptation of cellular functions to the environmental changes. Hence, the products of genes are to function not only in stress tolerance, but also in the regulation of gene expression and signal transduction in stress responses (Shinozaki et al., 2003).

The regulation of gene expression (induction, or turning genes on, and repression, or turning gene off) has be accomplished by both positive control mechanisms and negative control mechanisms. Both mechanisms involve the participation of the regulatory gene that encodes products that regulate the expression of another gene. In positive control mechanisms, the product of the regulator gene is required to turn on the expression of one or more structural genes, whereas in negative control mechanisms, the product of the regulator gene is necessary to shut off the expression of structural genes. The product of the regulator gene acts by binding to a site called the regulator protein binding site, adjacent to the structural gene promoter. When the product of the regulator gene is bound at the regulator protein binding site, transcription of the structural gene is turned on in a positive control system or turned off in a negative control system (Snustad & Simmons, 2006) affecting down-stream expression of many enzymes and structural proteins in a more natural way than by altering single structural gene expression. The results obtained in terms of stress tolerance are often much better than when using a single gene encoding a non-regulatory protein and the observed effects on photosynthesis efficiency or photosynthetic machinery are normally positive.

The development the of new technologies for the efficient identification of genetic determinants involved in plant stress adaptation, such as molecular genetics and high throughput transcriptome, proteome, metabolome and ionome, has opened a door to the understanding of the mechanisms by which plants adapt to abiotic stress and should result in the production of new and improved stress-tolerant crops (Wood, 2008). In view of this, the genetic engineering is useful for improving the stress tolerance of plants and several different approaches have been attempted to improve the stress tolerance of plants by gene transfer (Shinozaki et al., 2003). Each stress is a multigenic trait and therefore their manipulation may result in alteration of a large number of genes as well as their products. The ability of the transcription factors (TFs) to control both multiple pathway steps and cellular processes that are necessary for metabolite accumulation, offers much promise for the manipulation of metabolic pathways (Dixit, 2004). A single transgene encoding transcription factor has been reported to confer improved drought resistance (Kasuga et al., 1999; Jeanneau et al., 2002; Yang et al., 2005; Karaba et al., 2007, Aharoni, 2004). Ten percent of the genes implicated in the drought stress response were found to encode transcription factors (Seki et al., 2002).

Given that most of these responses imply control of gene expression, transcription factors play a critical role in the abiotic stress response (Chaves & Oliveira, 2004). Transcription factors are proteins that stimulate transcription helping the RNA polymerase II to initiate transcription, some of these bind to enhance regions and others bind to cis-acting elements in the promoter and together with RNA polymerase form a preinitiation complex for transcription (Griffiths et al., 1996). By these mechanisms they induce or repress the activity of RNA polymerase, thus regulating gene expression (Riechmann et al., 2000).

Many of the transcription factor proteins appear to have at least two important chemical domains: a DNA-binding domain and a transcriptional activation domain. These domains may occupy separate parts of the molecule, or they may be overlapping. Althought the detailed mechanism of transcriptional actions is not fully understood, it appears to involve physical interactions between proteins. A transcription factor that has bound to an enhancer may make contact with one or more proteins at other enhancer, or it may interact directly with proteins that have bound in the promoter region. Through these contacts and interactions, the transcription activation domain of the factor may then induce conformational changes in the assembled proteins, paving the way for the RNA polymerase to initiate transcription. Many eukaryotic transcription factors have characteristic structural motifs that result from associations between amino acids within polypeptide chains. One of these motifs is the zinc finger, a short peptide loop that forms when two cysteines in one part of the polypeptide and two histidines in another nearby part jointly bind a zinc ion; the peptide segment between the two pairs of amino acids then juts out from the main body of the protein as a kind of finger. Mutational analysis has demonstrated that these fingers play important roles in DNA binding. A second motif in many transcription factors is the helix-turn-helix, a stretch of three short helices of amino acids separated from each other by turns. Genetic and biochemical analyses have shown that the helical segment closest to the carboxy terminus is required for DNA binding; the other helices seem to be involved in the formation of protein dimmers. In many transcription factors, the helix-turn-helix motif coincides with highly conserved region of approximately 60 amino acids called the homeodomain. A third structural motif found in transcription factors is the leucine zipper, a stretch of amino acids with a leucine at every seventh position. Polypeptides with this feature can form dimers by interactions between

the leucines in each of their zipper regions. Usually, the zipper sequence is adjacent to a positively charged stretch of amino acids. When two zippers, these charged regions splay out in opposite directions, forming a surface that can bind to negatively charged DNA. A fourth structural motif found in some transcription factors is the helix-loop-helix, a stretch of two helical regions of amino acids separated by nonhelical loop. The helical regions permit dimerization between two polypeptides. Sometimes the helix-loop helix motif is adjacent to a stretch of basic (positively charged) amino acids, so that when dimerization occurs, these amino acids can bind to negatively charged DNA. Transcription factors with dimerization motifs such as the leucine zipper or the helix-loop-helix could, in principle, combine with polypeptides like themselves to form homodimers, or they could combine with different polypeptides to form heterodimers. This second possibility suggests a way in which complex patterns of gene expression can be achieved. The transcription of a gene in a particular tissue might depend on activation by a heterodimer, which could form only if its constituent polypeptides were synthesized in that tissue. Moreover, these two polypeptides would have to be present in the correct amounts to favor the formation of the heterodimer over the corresponding homodimers. Subtle modulations in gene expression might therefore be achieved by shifting the concentrations of the two components of a heterodimer (Snustad, 2006)

The *SHINE* (*SHN*) gene encodes an AP2/EREBP transcription factor and the expression patterns, suggesting that this clade of genes acts in the regulation of lipid biosynthesis, important for deposition of the epidermal cuticula, required for protection of plants to the environment, but also for organ separation processes and wounding (Aharoni, 2004). The cuticle, mainly consisting of cutin and epicuticular waxes, plays multiple roles in plants, including the regulation of epidermal permeability and non-stomatal water loss

and protection against insects, pathogens, UV light and frost (Sieber et al., 2000). *Arabidopsis thaliana* transformed with an overexpression construct of the *SHN* gene showed enhanced drought tolerance (Aharoni, 2004).

The genetic transformation to improve drought tolerance is interesting for important crop like Coffee, which represents one of the key export and cash crops in tropical and subtropical countries with a generally favorable impact on the social and physical environment (International Coffee Organization, 2009). About 125 million people depend on coffee for their livelihoods in Latin America, Africa, and Asia. Coffee is produced in more than 68 countries between 22º N and 24º S latitudes, on a total of about 10.6 million ha. Total world production for the year 2005/06 was 6.6 million tonnes of green coffea beans, with an estimated market value of US $12.2 billion.

Coffee culture is not very easy though. Plants flower only after several years and plants require high levels of nutrients and are sensitive to drought (Da Matta et al., 2003, Cai et al., 2004). The soil water deficit during the dry season is a main environmental factor that limits the productivity of coffee (Long & Wang 1997). Recent studies indicated that because of global warming, Brazil would not be able to keep growing coffee, unless plantations could be moved farther south. A three degree increase in the average temperature in Brazil would reduce the planted coffee land in the state of São Paulo to just 38 percent of the current 97,800 square kilometers (Osava, 2002).

Moreover, there appears to be a relation between coffee production and forest preservation. In the previously cited study supported by UNEP (United Nations Environment Programme – UNEP-GRID, 2002), the expected climate change would make the areas where coffee is produced too hot to grow it any longer, and production would be expected to move to higher, cooler areas, which are currently forested. This would generate deforestation and land use change in

higher lands and probably crop or land uses changes to more profitable, but not environmental friendly, uses in areas where coffee had been grown until then (Gay, 2006).

Therefore, there is an urgent need to improve the drought tolerance of coffee. Coffee has long been bred with the view of improving important agronomic characteristics such as flowering, yield, bean size, cup quality, caffeine content and disease and drought resistance. Despite solid efforts, the progress in coffee breeding using conventional approaches has been slow due to many factors, such as the narrow genetic basis of cultivated coffee, the lack of genetic markers and efficient screening tools, as well as the long time taken for generation advancement (Vieira, 2006).

Plant genetic transformation permits gene transfer across species. It is particularly important in perennial crops and tree species in which development of new cultivars is often difficult due to their long generation time. Thus, it can be a shortcut to introduce a new trait and to develop new cultivars without losing the genetic background of the original transformed cultivar (Ribas et al., 2006)., Unfortunately, despite significant advances over the last 15 years, coffee transformation is still very laborious, with several bottlenecks in the methodology that make it far from a routine laboratory technique. Up to now, only a few genes have been transferred to coffee genotypes (Lashermes, 2008). When successful, the whole process of genetic coffee transformation, from primary inoculated explant to plantlet transfer to the greenhouse, usually takes 12-20 months (Spiral et al., 1999). Although transformation is tedious, several research teams are studying important traits for genetic engineering at the molecular level and a better utilization of genetic resources can be expected to contribute to economic and sustainable coffee production. Thus, the advances in coffee biotechnological techniques could benefit the coffee breeder and offer

new possibilities for the development of new varieties opening new perspectives in breeding for resistance to parasites as well resistance to abiotic stresses.

2 SCOPE OF THE THESIS

In Chapter II, the effect of (over)expression of the Arabidopsis *SHN* gene in stable and/or chimeric transgenic *Arabidopsis thaliana* and *Brassica rapa* plants subjected to drought treatment was determined.

In chapter III, an efficient method for coffea genetic transformation by *Agrobacterium rhizogenes* was developed, in order to create chimaric *C. arabica* (Catuaí Vermelho IAC 144) with wild-type shoots and transgenic roots for rapid testing of potentially interesting gene construct for future stable transformation using *A. tumefaciens* or *A. rhizogenes*.

In chapter IV the *SHINE* gene of *Arabidopsis thaliana* was expressed in transgenic hairy roots of *Coffea arabica* after *Agrobacterium rhizogenes*-mediated root transformation in order to improve the drought tolerance of such chimeric plants.

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Chapter 2

Transformation and selection of chimeric transgenic *Arabidopsis thaliana* and *Brassica rapa* plants and the effect of *SHINE* overexpression in stable and chimeric transgenic *Arabidopsis thaliana* plants submitted to drought

1 ABSTRACT

Overexpression of *SHINE* gene induces the expression of these stress-related genes under normal growth conditions in transgenic plants and then confers the improved tolerance to drought. In recent years rapid procedures for obtaining transgenic roots have been developed using *Agrobacterium rhizogenes*, leading to the production of so-called "composite plants" comprising a transgenic hairy root system attached to non-transformed shoots and leaves. The aim of this work was to determine the effect of (over)expression of the Arabidopsis *SHN* gene in stable and/or chimeric transgenic *Arabidopsis thaliana* and *Brassica rapa* plants subjected to drought treatment. For this the drought treatment pots with control plants and 35S::*SHN2* stable and chimeric plants were exposed to drought by withholding water for 10, 11 and 12 days, subsequently rehydrated and observed for recovery after one week. All wild-type plants subjected to drought for 10, 11 or 12 days died, while all transgenic 35S::*SHN2* stable plants subjected to the same conditions seemed to be alive. After rewatering, 40% and 30% of the 35S::*SHN2* plants submitted to drought for 10 or 11 days respectively recovered, while all plants submitted to drought for 12 days did not recovery. For 35S::*SHN2* chimaric transgenic plants, 50% of the exposed to 10 days withholding water and rewatered for one week recovered the drought treatment, while none of the 35S::GUS chimaeric control plants recovered. When water was withheld for 11 or 12 days, none of the chimeric 35S::*SHN2* and 35S::GUS plants survived.

Key-words: *SHINE*¸ composite plants, *Arabidopsis thaliana*, drought treatment.

2 RESUMO

A superexpressão do gene *SHINE* induz a expressão de genes relacionados com o estresse em plantas transgênocas sob condições normais e portanto confere tolerância a seca. Atualmente tem sido desenvolvidos processos para o abtencação de raízes transgênicas utilizando-se *Agrobacterium rhizogenes*, quepermitindo a produção de plantas "compostas" com sistema radicular transgênico e sistema aérea não transgênico. O objetivo desse trabalho foi determinar o efeito da superexpressão do gene *SHN* em plantas estáveis e quiméricas de *Arabidopsis thaliana* e *Brassica rapa* submetidas a seca. Para isso, plantas não transgênicas (controle) e plantas estáveis e quiméricas transformadas com 35S::*SHN2* foram expostas a seca por 10, 11 e 12 dias, subsequentemente reidratadas por uma semana e observada a sobrevivência. Todas as plantas não transgênicas submetidas a seca por 10, 11 ou 12 dias morreram, enquanto que todas as plantas transgênicas estáveis 35S::*SHN2* submetidas as mesmas condições pareciam estar vivas. Após a reirrigação, 40% e 30% das plantas 35S::*SHN2* submetidas a seca por 10 ou 11 dias respectivamente, sobreviveram enquantos todas as plantas submetidas a seca por 10 ou 11 dias não sobreviveram. Para as plantas transgênicas quiméricas 35S::*SHN2,* 50% das plantas submetidas a 10 dias de seca e reirrigadas por uma semana subreviveram, enquanto que nenhuma plantas controle sobreviveu. Quando a irrigação foi suspensa por 12 ou 12 dias, nenhuma das plantas quiméricas 35S::*SHN2* e 35S::GUS sobreviveram.

Palavras-chave: *SHINE*¸ plantas compostas, *Arabidopsis thaliana*, seca.

3 INTRODUCTION

Plant growth and yield are strongly influenced by abiotic stresses such as drought, flooding, salinity, metal toxicity, mineral deficiency, adverse pH, adverse temperature, and air pollution and these environmental stress presents a major challenge in our quest for sustainable food production as it reduces the potential yields by as much as 70% in crop plants (Gosal et al., 2009). The water stress imparted by drought and temperature severity is the most prevalent abiotic stress that limits plant growth and productivity, emphasizing the urgent need to develop adaptive agricultural strategies for a changing environment (Cook et al., 2007). Moreover, climate models have indicated that drought episodes will become more frequent because of the long-term effects of global warming.

The range from changes in traditional management and agronomic practices to the use of marker-assisted selection for the improvement of drought-related traits and the development of transgenic crops with enhanced tolerance of drought and improved water use efficiency would minimize drought-related losses and ensure food production for a growing population (Rivero et al., 2007). Conventional breeding approaches, involving inter-specific and inter-generic hybridizations and mutagenesis, have been used but with limited success (Carvalho, 2009). Major problems have been the complexity of drought tolerance, low genetic variance for yield components under drought conditions, and the lack of efficient selection procedures. Moreover, conventional such methods are limited with several inherent bottlenecks because trees are generally slow growing, long-lived and highly heterozygous plants (Giri, 2004). The genetic modification remains an important avenue to accelerate the domestication of trees, despite the public debate. The main advantage is that the genetic constitution of the elite clone can be maintained, and the second key argument is that transformation circumvents the long generation times that are

typical for most forest trees (Boerjan, 2005). Direct introduction of genes by genetic engineering seems a more attractive and quick solution for improving stress tolerance (Dunwell, 2000; Wang et al., 2001).

Genetic transformation has been changing plant science and agriculture around the globe. Plant transformation opens a window of opportunity to introduce traits from different species, adding characteristics which would be difficult or even impossible to acquire using traditional breeding techniques, such as tolerance to abiotic stresses like drought or frost (Ribas et al., 2006). And the role of agriculture is essential for the future of the country. A country like Brazil that currently holds about one sixth of the plant species of the world is a country that should choose to use agricultural solutions that cause less impact on natural communities. One of the alternatives to meet that objective is biotechnology. Although there are still few transgenic products approved in the country, this tool might contribute greatly to the global agricultural production by the end of this decade, with drought tolerant seeds and plants (Raven, 2007).

With the advent of innovative approaches of biotechnology, our understanding of the processes underlying plant responses to drought at the molecular and whole plant levels has rapidly progressed (Gosal, 2009). Genetic engineering of plants for tolerance to extreme abiotic stresses could be achieved by the regulated expression of stress-induced transcription factors, which in turn would regulate the expression of a large number of relevant downstream genes. Thus, modified transcription factors are powerful tools for genetic engineering as their overexpression can lead to the up-regulation of a whole array of genes under their control (Agarwal & Agarwal, 2006). With the identification of stress-inducible genes and studies on their role in stress tolerance, several approaches have been proposed to improve drought tolerance in plants. In this

way, transcriptions factors SHINE were described that, when over-expressed greatly enhance the drought tolerance of *Arabidopsis* (Aharoni, 2004).

The *SHINE* (*SHN*) gene encodes an AP2/EREBP transcription factor and the expression patterns, suggests that this clade of genes acts in the regulation of lipid biosynthesis important for deposition of the epidermal cuticula, required for protection of plants to the environment, but also for organ separation processes and wounding (Aharoni, 2004). The cuticula, mainly consisting of cutin and epicuticular waxes, plays multiple roles in plants, including the regulation of epidermal permeability and non-stomatal water loss and protection against insects, pathogens, UV light and frost (Sieber et al., 2000). *Arabdopsis thaliana* transformed with an overexpression construct of the SHN gene showed enhanced drought tolerance (Aharoni, 2004). Overexpression of *SHINE* induces the expression of these stress-related genes under normal growth conditions in transgenic plants and then confers the improved tolerance to drought.

In recent years rapid procedures for obtaining transgenic roots have been developed using *Agrobacterium rhizogenes*, a soil pathogen which elicits adventitious, genetically (Ri T-DNA) transformed roots. This leads to the production of so-called “composite plants” comprising a transgenic hairy root system attached to non-transformed shoots and leaves. *A. rhizogenes*-mediated transformation makes it possible to co-transform plant cells with more than one T-DNA at the same time. The T-DNA containing the transgene of interest in a disarmed binary vector is generally co-transformed with the resident *A. rhizogenes* Ri T-DNA containing the *root locus (rol)* genes (responsible for root proliferation) (Limpens et al., 2004). The major advantage of this approach is the rapidity and technical simplicity of *A. rhizogenes* transformation (Chabaud, 2006).

4 AIM

The aim of this work was to determine the effect of (over)expression of the Arabidopsis *SHN2* gene in stable and/or chimeric transgenic *Arabidopsis thaliana* and *Brassica rapa* plants subjected to drought treatment.

5 MATERIAL AND METHODS

5.1 Plasmids

The plasmids used at the transformation of *Arabidopsis thaliana* and *Brassica rapa* were: pMOG22-35S::*SHN2* containing the *SHINE2* cDNA, under control of the constitutive CaMV35S promoter and 35S::HPTII gene construct which confers hygromycin resistance when expressed in plants (Aharoni, 2004), the pCAMBIA1301 plasmids contained 35S::GUS marker and 35S::HPTII (Hajdukiewicz et al., 1994), and the pREDRoot plasmid contained *DsRED1* (Clontech) and 35S::NPTII gene construct which confers kanamycin resistance when expressed in plants (Limpens et al., 2004).

5.2 Preparation of *Agrobacterium* for plant transformation

The plasmids DNA pCAMBIA1301 and pMOG22-35S::*SHN2* were first introduced into *Escherichia coli* strain DH5*Af* by electroporation (Sambrook et al., 1989) and isolated using a midi-prep plasmid DNA isolation protocol (Sambrook et al., 1989).

The pMOG22-35S::*SHN2* plasmid isolated from *E.coli* was introduced into *Agrobacterium tumefaciens* strain AGL0 by electroporation (Sambrook, 1989) and selected on LB agar medium contained rifampicin (50mg.L^{-1}) and kanamycin (50 mg.L^{-1}) for 48h at 28ºC.

Both plasmids pCAMBIA1301 and pMOG22-35S::*SHN2* were subsequently introduced into *Agrobacterium rhizogenes* strain MSU440 by electroporation and selected on LB solid medium containing 50 $mg.L^{-1}$ kanamycin at 28ºC for approximately 48 hours. *A. rhizogenes* containing pRedRoot (Limpens et al., 2004) was recovered from a glycerol stock kept at -80ºC. A single colony of each was used for further experiments. To verify the proper identity of the selected colonies, plasmid DNA was isolated by midi-prep retransformed to *E. coli* by electroporation, again isolated by midi-prep and compared to the originally transformed plasmid by restriction digestion with 5U *Eco*RI and 5U *Bam*HI or 5U EcoRI and 5U *Hind*III (Figure 1). The band it is a little higher on the gel, probably because of the changing on the mobility caused by digestion solution.

To confirm the presence of the *SHN2* gene in pMOG22-35S::*SHN2* was also perform a PCR analysis using 1.5u Taq DNA polymerase (Fermentas), 1x reaction buffer, 200µM DNTP, 0.1 µM of each primer 5'ATGCTCAACACATGAGCGAAACCCTAT3', 3'CAATCCCACTATCCT TCGCAAGACCC5' and 1µg plasmid DNA on a reaction mixture of 50 µl. The amplification was performed under the following conditions: 1 cycle at 94 ◦C for 3min, 30 cycles at 94ºC for 1min, annealing at 55ºC for 1min, 72 ºC for 2 min and 1 cycle at 72ºC for 5 min. PCR was carried out using an automatic thermocycler. These primers are expected to amplify a fragment of 1369 bp when the *SHN2* gene is present (AT5G25390.1 Tair). Indeed such fragment was found for all three tested colonies, (Figure 2).

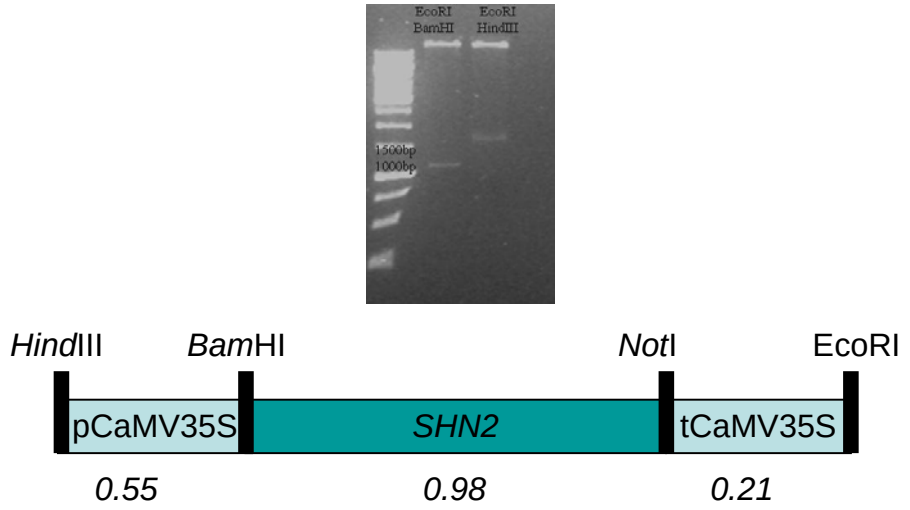

FIGURE 1 Designing of Plasmid pMOG22-35S::*SHN2* isolated by miniprep from *E. coli* colonies and digested with 5U *Eco*RI and 5U *Bam*HI (lane 1), 5U *Eco*RI and 5U *Hind*III (lane 2) and digestion solution without DNA (lane 3). 3µl of a total volume of 30 µl digested solution was separated using a 0.8% agarose gel, run in 1 x TBE buffer (Sambrook et al., 1989). Label of 1 kb.

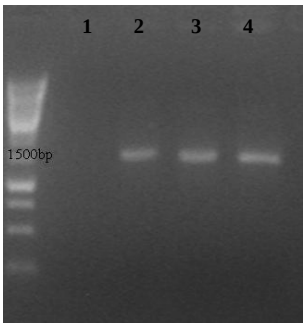

FIGURE 2 PCR analyze of pMOG22-35S::*SHN2* isolated by Midiprep from *E. coli* colonies. PCR mix control without pMOG22-35S::*SHN2* (line 1) and fragment amplified from de *SHN2* gene (line 2-4). 10µl of a total volume of 50 µl PCR solution was separated using a 0.8% agarose gel, run in 1 x TBE buffer (Sambrook et al., 1989). Label 1 kb.

5.3 *Arabidopsis thaliana* transformation with Agrobacterium tumefaciens

Arabidopsis thaliana plants were grown on rock wool weekly supplied with Hyponex (Nishimura, 1982) under long days (16h light) until they flowered. The first bolts were clipped to encourage proliferation of many secondary bolts. The pMOG22-35S::SHN2 plasmid isolated from *E.coli* was introduced into *Agrobacterium tumefaciens* strain AGL0 by electroporation (Sambrook, 1989). and select on LB agar medium contained rifampicin (50mg.$^{L-1}$) and kanamycin (50 mg.L^{-1}) for 48h at 28°C. After this one single colony was inoculated on 50 ml LB liquid medium with the same antibiotics and grown for 2 days at 28°C, 300 rpm. On the day of transformation, the *A. tumefaciens* liquid culture was centrifuged (3000 rpm, MSE table centrifuge) and the bacterial pellet was resuspended in a 5% (w/v) sucrose solution to which Silwet L-77 was added after resuspension (0.05% v/v). Above-ground parts of plant were dipped in the *A. tumefaciens* solution for 2 to 3 seconds, with gentle agitation. The dipped plants were placed under a dome or cover for 16 to 24 hours to maintain high humidity. After removal of the cover, plants were watered normally until the seeds were mature. The seeds were harvested. For selection of transformants, seeds were vapor-sterilized like described below and plated (2000 seeds/plate) on 12 cm square petridishes with ½ MS-0.8% agar medium containing 20 mg.L^{-1} hygromycin. The seeds were cold treated for 4 days at 4°C and then transferred to a climate chamber set at 25°C with 16 hrs light for 7-10 days. The putative primary transformants were transplanted to soil and used after three weeks for the drought tolerance experiment.

5.4 Hair roots transformation of *Arabidopsis thaliana* and *Brassica rapa*

A. thaliana acc. Columbia-0 (Col-0) and *B. rapa* ssp. *trilocularis* R-o-18. The seeds were sterilized by vapor-phase. For this the seeds were separated

on microcentrifuge tubes that were placed on a dessicator jar containing 250 ml beaker full with 100 ml bleach and 3 ml concentrated HCl. The jar was closed to allow sterilization by chlorine fumes and after three hours the seeds were transferred to sterile laminar flow hood. (Clough & Bent, 1998) and thereafter sowed in Petri dishes containing ½ MS medium (Murashige & Skoog, 1962) (no sugar, pH 5.8) + 0.8% agar. The dishes were incubated vertically in a climate chamber set at 20°C and 16h light. Seedlings with fully expanded hypocotyledons were used for transformation (i.e.) 5 days for *Arabidopsis thaliana* and 2 days for *Brassica rapa*).

For transformation, the *A. rhizogenes* that had the right plasmids, confirmed like described above, were recovered from a glycerol stock kept at -80ºC, for this they were placed on a plats contained LB medium with 50 $mg.L^{-1}$ of kanamycin and grown for 48h, after this just one single colony was transferred to liquid LB medium (100 ml) contained 50$mg.L^{-1}$ of kanamycin and grow again for 48h, then 200 ml of the liquid medium was transferred to new plats contained LB medium with 50 $mg.L^{-1}$ of kanamycin and grown for 48h. This plants were used to transformation.

A scalpel was dipped in the *A. rhizogenes* colonies containing either pCAMBIA1301, pMOG22-35S::SHN2 or pREDRoot. The seedlings were cut just above the hypocotyl-root boundary and arranged on one line (8 plants/plate), with the cotyledons just on the agar and the hypocotyl on the autoclaved filter paper (Figure 3 a and b). Additional *A. rhizogenes* colony material was scraped from the plate with the scalpel and put as a dot on the wound surface at the base of each seedling (Limpens et al., 2004). The dishes were sealed with parafilm, but care was taken to leave 1/3 of the side that will be the top of the plate open.

After seven days the plants were changed to new MS plates, supplemented with tricarcilin (200 $mg.L^{-1}$) to select against *A. rhizogenes* and

hygromycin (20 mg.L^{-1}) to select roots transformed with pCAMBIA1301 and pMOG22-35S::*SHN2* or kanamycin (50 mg.L^{-1}) to select roots transformed with pRedRoot. For the reason that was not possible to select the roots on kanamycin (50mg.L^{-1}) the concentrations (60 mg.L^{-1}, 75 mg.L^{-1}) were used. To avoid the contact of the non transformed leaves with the selection medium the autoclaved aluminum foil was placed between the medium and plants (Figure 3 c d). Plates were covered again for 2/3 with parafilm, leaving the top open and incubated vertically in the climate chamber. Three light conditions were tested to *Arabidopsis* and *Brassica*: 25°C and 16h light, 20°C and 12h light or 20°C and 8h light.

After 7 days on the selection medium, the growing roots were measured, to select the transformed roots. The roots that were transformed with pRedRoot were observed using Leica MZIII fuorescence stereomicroscope, wavelength 550 nm and the roots that were transformed with pCAMBIA1301 were observed after a histochemical GUS assay, for this the roots were incubated at 37ºC for overnight on the GUS solution contained 100 mM sodium phosphate buffer, pH 7.0, 0.5 mg/mL of 5-bromo-4-chloro-3-indolyl b-D-glucoronic acid (Duchefa), 0.1% Triton, and 0.5 mM each of potassium ferri/ ferrocyanide.

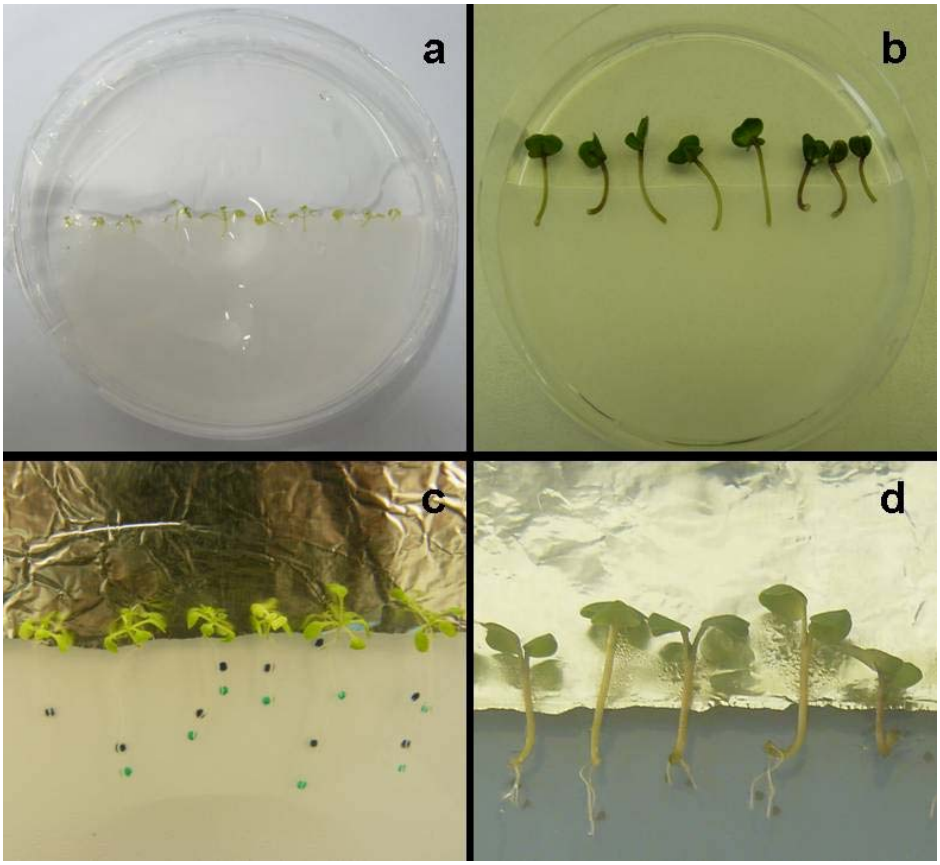

FIGURE 3 *Arabidopsis thaliana* and *Brassica rapa* seedlings infected with *Agrobacterium rhizogenes*. *A. thaliana*, 5 days old, infected with *A. rhizogenes* (a). *B. rapa*, 2 days old, infected with *A. rhizogenes* (b). *A. thaliana,* 15 days old, on MS medium contained 20mg.L^{-1} of the hygromycin (c). *B. rapa,* 10 days old, on MS medium contained 20mg.L^{-1} of the hygromycin (d).

For each transformation, 100 *Arabidopsis thaliana* and 100 *Brassica rapa* seedlings were used. The plants with roots transformed with pMOG22-35S::*SHN2* and pCAMBIA1301 as selected on ½ MS-medium containing 20 $mg.L^{-1}$ of hygromycin, were transferred to pots with ½ MS-medium containing 1.5 % (w/v) sucrose and grown for later use in drought tolerance experiments. Two light conditions were tested for the acliamtiztion, for this on the first experiment the climate chamber was set at 20°C and 8h and on second experiment the climate chamber was set 20°C and 12 h light.

5.5 Drought tolerance experiment

The control plants, transformed with pCAMBIA1301, and the 35S::*SHN2* plants (transformed with pMOG22-35S::*SHN2*) were transferred to pots containing silver sand and nutrient solutions Hyponex (Nishimura, 1982) at 85% of field capacity, and placed in a climate chamber. To know the field capacity of the sand, before the transfer of the plants, the pots were weighed and well watered and allowed to drain by gravity for 24 hours, and weighed again having the 100% field capacity (FC) and to know the water necessary to have 85% FC.

The drought treatment pots with control plants and 35S::*SHN2* plants were exposed to drought by withholding water for 10 and 11 days, subsequently rehydrated and observed for recovery after one week. The control treatment pots with control plants and 35S::*SHN2* plants were maintained at 85% FC for the rest of the experimental period, for this the pots were weight every two days and the water contend loss were addicted. The daily differences in the weight in the drought treatment pots were noted. Two pots with four plants were used per treatment. The dry weight of all plants per pot was measured at the end of the experiment.

6 RESULTS AND DISCUSSION

6.1 Hair root transformation and selection and aclimatization of *Arabidopsis thaliana* and *Brassica rapa*

For *Brassica rapa* 32% of hairy roots were transformed with pREDRoot showing red fluorescence (Figure 4a) and for *Arabidopsis thaliana* 17% of roots were transformed with pREDRoot (Figure 4b). In general, the differences in the effectiveness of transformation depend on the virulence of the *Agrobacterium* strain used (Porter, 1999) and the susceptibility of the plant species and cultivars (Lin et al., 2003). One of the most important factors determining efficient transformation is the appropriate explant selection (certain tissue or plant organ can be more suitable for transformation) in its particular developmental phase.

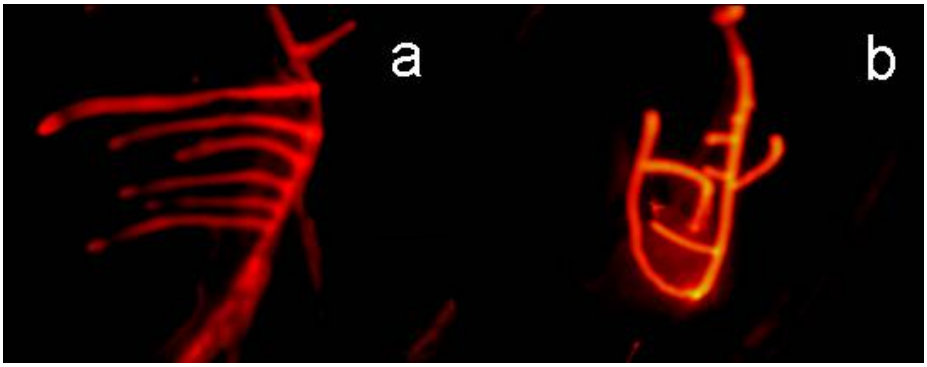

FIGURE 4 Hairy roots transformed with pREDRoot observed at Leica MZIII fuorescence stereomicroscope wavelength 550 nm. Transformed roots of *Brassica rapa* showing fluorescence in tissues expressing DsRed. (a). Transformed roots of *Arabidopsis thaliana* showing fluorescence in tissues expressing DsRed (b).

To determine the optimal concentration of antibiotics in the culture medium, since transformants for the other constructs could not be selected using fluorescence, selection using antibiotic resistance was tried. Three different kanamycin concentrations were tried, 50, 60 and 75 $mg.L^{-1}$ for transformation with pRedRoot. The medium with 50 $mg.L^{-1}$ kanamycin was not efficient to select the transformed roots, as also non-transformed roots were growing normally after 10 days on this medium. Unfortunately also kanamycin concentrations of 60 and 75 $mg.L^{-1}$ were unable to clearly inhibit untransformed root growth.

On the other hand when the plants were transformed with pCAMBIA1301 *Brassica rapa* had 52% of hairy roots transformed and *Arabidopsis thaliana* had 57% roots transformed verified by Gus assay. About the selection medium, 20 $mg.L^{-1}$ of hygromycin were sufficient, to select the transformed roots, occurring just false negative, but without false positive and this means all of the roots that were growing were transformed (Figure 5).

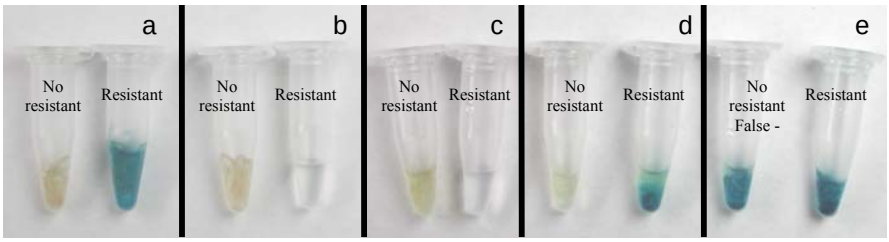

FIGURE 5 Hair roots transformation of *Brassica rapa* (a, b) and *Arabidopsis thaliana* (c,d,e) with pCAMBIA1301 selected on hygromycin and confirmed by GUS. No resitante roots GUS-negative and resistant roots GUS-positive of *Brassica rapa* (a) No resistant roots GUS-negative and anyone resistant roots of *Brassica rapa* (b) No resistant roots GUS-negative and anyone resistant roots of *Arabidopsis thaliana* (c) No resistant roots GUS-negative and resistant roots GUS-positive of *Arabidopsis thaliana* (d). No resistant roots GUS-positive – false negative and resistant roots GUS-positive (e).

Selection medium with 20 mg.L^{-1} hygromycin was used to select plants with hairy roots containing 35S::*SHN2* T-DNA (35S::*SHN2* plants) or with the pCAMBIA1301 T-DNA containing the 35S::GUS construct (control plants). In the first experiment, the plants were grown at 25ºC and 16h light. Under these conditions *A. thaliana* started to flower after three weeks, just at the time when the selection medium was applied, which was not desired. To avoid this problem plants were grown at 20ºC and the photoperiod was reduced to 8h light. Under these conditions the plants were growing slowly and after infection with *A. rhizogenes* the plants were smaller and rooting was slower compared to the long day conditions (Figure 6a and b). The environmental condition affected the development of the plants that were already stressed by the infection with *A. rhizogenes* and exposure to the selective antibiotic. Consequently the acclimatization of the plants on sand was affected and the plants did not develop well on silver sand after 3 weeks of acclimatization, even at high humidity.

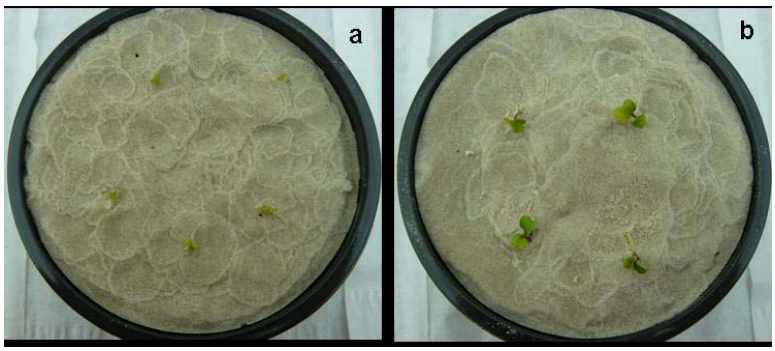

FIGURE 6 *Arabidopsis thaliana* and *Brassica rapa* chimaeric 35S::*SHN* transgenic plants after transformation with *A. rhizogenes*. *A. thaliana* seedlings, 6 weeks old, grown under short (8h) photoperiod conditions (a). *Brassica rapa* seedlings, 5 weeks old, grown under short (8h) photoperiod conditions (b).

Such deterioration is not uncommon. During acclimation to *ex vitro* conditions plants must adapt to severe changes of environment. Plants generally undergo substantial changes in leaf morphology and anatomy, including development of cuticle and effective stomatal regulation of transpiration, differentiation of leaf mesophyll and changes of chloroplast number and structure. The process of acclimation of transgenic plants is even more complicated by expression of transgenes which can lead to decreased vigor (Koperdáková et al., 2009). For example, acclimatization of *Angelonia salicariifolia* plantlets transformed with wild *A. rhizogenes* strains was problematic, with only 20% of plantlets without apparent hyperhydric appearance that could be transplanted to the greenhouse (Koike et al., 2003). To overcome the problems with acclimation, the photoperiod was increased to 12h light and after the inoculation and selection of the transformed plants, they were transferred for one week to an MS-agar medium supplemented with 15 $mg.L^{-1}$ of sucrose. On this medium, the plants developed much better and they were able to acclimatize. However, mainly for *Brassica rapa*, root formation was compromised because it was not possible to avoid completely the contact of the non-transformed hypocotyl with the selection medium. When in contact with the medium, the untransformed parts of the seedlings showed necrosis, which did not recover after transferring the plantlets to new medium without hygromycin. Therefore, only the *A. thaliana* plants transformed by *A. rhizogenes* that survived acclimatization for 2 weeks were used for the drought experiment described on item 6.3.

6.2 Drought experiment using stable transformed *Arabidopsis thaliana* plants containing 35S::*SHN2*

A drought tolerance experiment was set up with 4-week-old seedlings selected for presence of the 35S::*SHN2* and comparable wild-type (WT) plants. Plants were exposed to a period of 10, 11 or 12 days of dehydration (Figure 7) before rewatering and their recovery was monitored for a week (Figure 8). All wild-type plants subjected to drought for 10, 11 or 12 days died (Figure 7 c e), while all transgenic 35S::*SHN2* plants subjected to the same conditions seemed to be alive (Figure 7 d f). After rewatering, 40% and 30% of the 35S::*SHN2* plants submitted to drought for 10 or 11 days respectively recovered, while the 35S::*SHN2* plants submitted to drought for 12 days and all wild-type plants did not recovery (Figure 8). When comparing dry weights of all transgenic plants (dead and alive) treated with 10, 11 or 12 days drought, the transgenic plants clearly had much higher dry weights compared to the wild-type plants (Figure 9). With this results were possible to set up the time to the drought treatment used with the quimeric plants.

Aharoni et al. (2004), who initially identified the *SHN* genes, observed that wild-type plants did not recover from dehydration treatments longer than 9 days, while all seedlings derived from lines overexpressing the *SHN1* gene recovered after rewatering. Hence, on this experiment was possible easily use heterozygous primary overexpressing *SHN2* transformants showing the same phenotype observed by Aharoni et al. (2004), like including the more brilliant, shiny green color of both rosette and cauline leaves, leaf curling, reduction in trichome number (Figure 7 a b and Figure 8 a b). Contrary to Aharoni et al. (2004) experiments, in this experiment silver sand was used as a subtract instead of a peat-based soil mix (sand and perlite and two parts of compost [25% clay and 75% turf]). Soil mix holds water and yields it according to the texture and

the tension with which water is held. Sand gives up water over a much narrower range and drought can occur in sand very readily with a small withdrawal of water, moverover the sand is easy to manipulate and to control soil water content and permit recover clean roots by washing them out of the pot, while a potting mixture containing a lot of peat and other organic materials is a nuisance because these cling tightly to the roots.

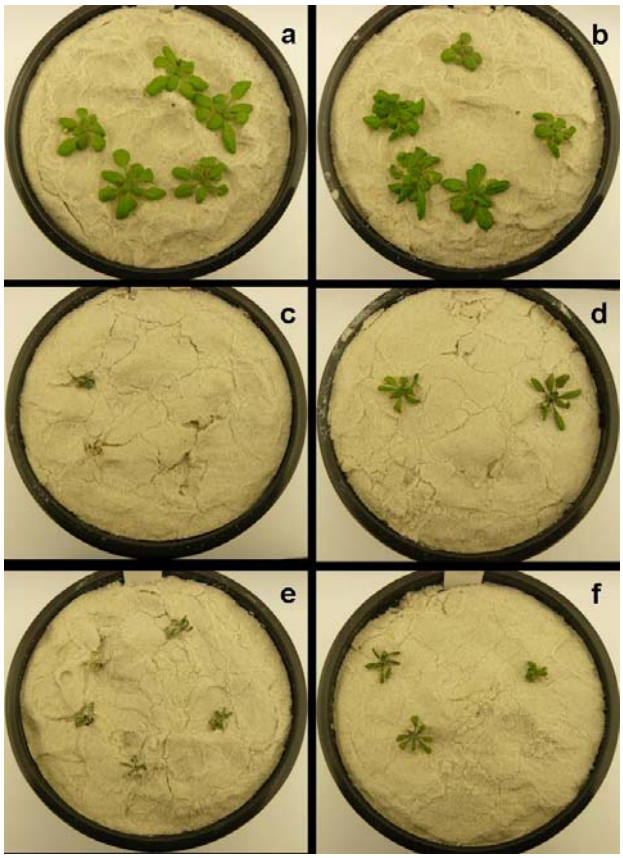

FIGURE 7 Water withholding tests of 6-week-old wild-type *Arabidopsis thaliana* plants (a, c, e) and plants transformed with the 35S::*SHN2* construct (b, d, f). Plants were watered during the experiment (a, b) or water was withheld for 10 (c, d) or 11 days (e, f).

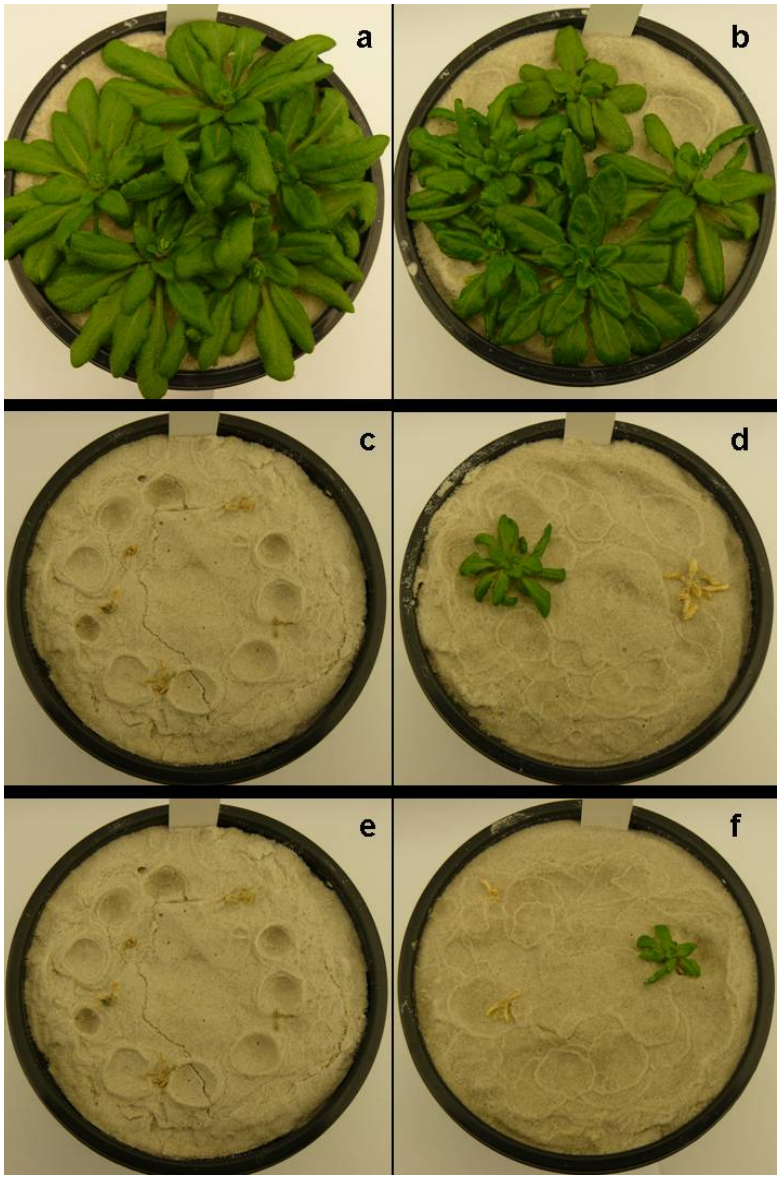

FIGURE 8 Water withholding tests of 6-week-old follow by one week of rewatering in wild-type *Arabidopsis thaliana* plants (a, c, e) and plants transformed with the 35S::*SHN2* construct (b, d, f). Plants were watered during the experiment (a, b) or water was withheld for 10 (c, d) or 11 days (e, f) and rewatered for one week.

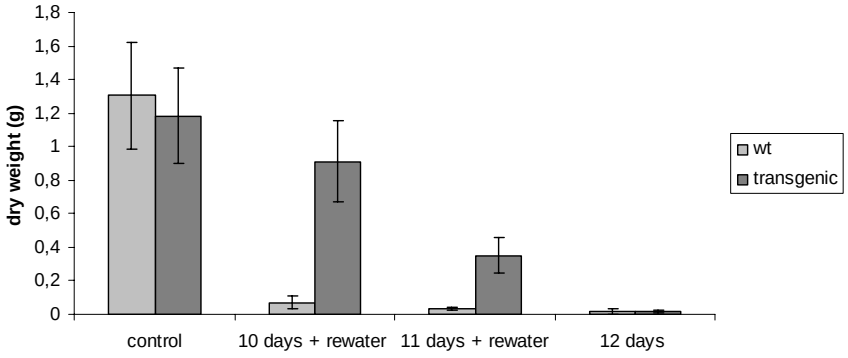

FIGURE 9 Dry weight of wild-type (WT) and transgenic 35S::*SHN2* *Arabidopsis thaliana* plants grown under control conditions, or subjected to 10, 11 or 12 days of withholding water upon which plants were rewatered for a week. Error bar indicates standard deviation.

6.3 Drought tolerance testing of chimeric *Arabidopsis thaliana* plants expressing 35S::*SHN2* in roots

It was possible to observe the 35S::GUS plants started wilting after 6 days without water, while the 35S::*SHN2* started wilting just after 8 days. As result of this about 50% of the 35S::*SHN2* chimeric transgenic plants exposed to 10 days withholding water and rewatered for one week recovered the drought treatment, while none of the 35S::GUS chimeric control plants recovered (Figure 10 c d). When water was withheld for 11 or 12 days, none of the chimaeric 35S::*SHN2* and 35S::GUS plants survived. The dry weight of the 35S::*SHN2* plants, did not differ significantly from the well-watered plants, and was significantly higher when compared with the drought-treated control plants (Figure 11).

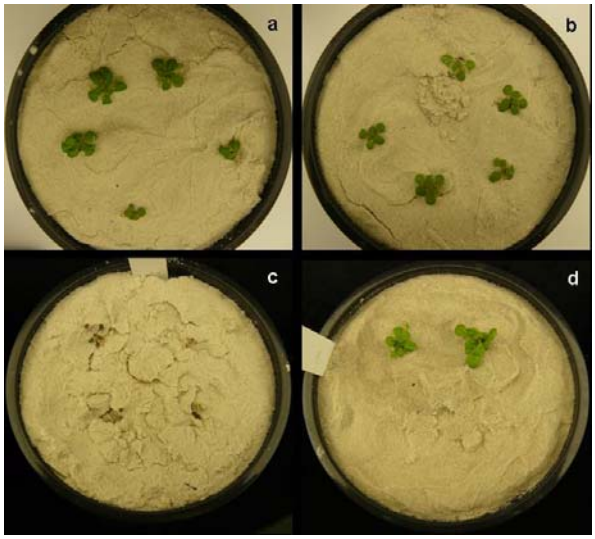

FIGURE 10 Comparison of 2-week-old *Arabidopsis thaliana* plants containing a 35S::GUS or a 35S::*SHN2* construct exposed to drought. 35S::GUS plants, well-watered for 15 days (a). 35S::*SHN2* plants, well-watered for 15 days (b). 35S::GUS plants kept without water for 10 days and rewatered for 7 days (c). 35S::*SHN2* plants kept without water for 10 days and rewatered for 5 days (d).

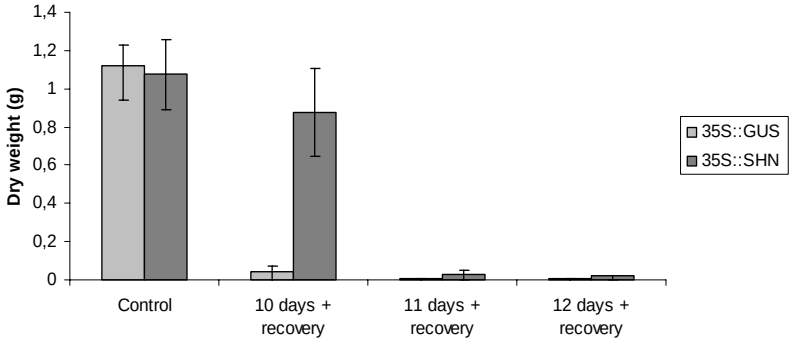

FIGURE 11 Dry weight of 35S::GUS and 35S::*SHN2 Arabidopsis thaliana* plants grown under control conditions (watered), or subjected to 10, 11 or 12 days of withholding water upon which plants were rewatered for a week. Error bar indicates standard deviation.

These results indicate that expression of *SHN2* in roots alone is already sufficient to protect plants against drought, however, the window in which root expression of *SHN2* protects the plants from drought appears to be relatively narrow. Although the shortest period of withholding water causing 35S::GUS plants to die was not determined, only a day longer withholding water was sufficient to make the difference between full recovery and complete loss of 35S::*SHN2* plants. This means the *SHN2* transcription factor possibly can induce the earliest reply to drought activating stress-responsive mechanism and this can happen in the root inducing the expression of gene involved on signal sensing and/or the transcription factor and their induced genes can be transported to the shoots where can induce the other transcription factors responsible for drought and the stress-responsive mechanisms (Figure 12).

The genes up-regulated in the *SHN* overexpressing provide supporting evidence for the actions of such mechanism in drought of the transgenic plants. Microarray analysis of drought resistant *SHN1* revealed induction of several genes involved in lipid metabolism and in cell wall synthesis, genes involved in stress signaling like kinases, a set of genes involved in calcium binding and stress induced genes such as *MYB* genes and *LEA* gene, (Trijatmiko, 2005), and Aharoni et al. (2001), reported *SHN1*, *SHN2* and *SHN3* are redundant in theis function and probably interact with the same target genes.

It is generally believed that Ca^{2+} transmits the stress signal downstream in the pathway by interacting calcium binding proteins that are possibly induced in the *SHN* overexpressing plants (Figure 12). A number of studies have shown that the calcium binding proteins like CDPKs (Ca^{2+} dependent protein kinases), Ca^{2+}/calmodulin (CaM) and calcineurin-B like proteins (CLB) are induced or activated by abiotic stress, suggesting that they may be involved in abiotic stress signaling (Urao et al., 1994; Pei et al., 1996; Tähtiharju et al., 1997; Hwang et

al., 2000). The Ca^{2+}/calmodulin (CaM) (Zik et al., 1998), stress-induced calcineurin-B like proteins (Kudla et al., 1999) and $NaCl^-$ induced biding protein (Jang et al., 2004) are highly expressed in roots and also in respond to osmotic stress the endodermis and pericycle display prolonged oscillations in cytosolic calcium, that are distinguished from the responses of the other cell types (Knight & Knight, 2001).

Cheong et al., 2003 propose that levels of some calcium sensors also may serve as a threshold (rate-limiting) factor for the responsiveness of plants to stress signals and hypothesize that the levels of both calcium and its sensor proteins are important threshold parameters in calcium-mediated signal transduction in plants. Sufficient increase in either calcium or its sensors will lead to a certain degree of activation of the downstream pathway. Kaplan et al. (2006) related that Ca^{2+}-responsive proteins, are expected to be already present at the time of the Ca^{2+} pulse and are responsible for mediating the apparent rapid gene activation. The *SHN2* transcription factor possibly increase the expression of Ca^{2+} binding proteins, leading more rapid activation of the downstream pathway.

The expression of *MYB96* genes (possibly induced by *SHN*) is significantly increased by drought. *MYB96* gene is expressed at a high level in the leaves and flowers, and it is expressed at a relatively lower level in the roots. 2B). Although the activity is low in the roots, including the primary root tips, high GUS activity is detected in lateral root primordial and *MYB96* regulates lateral root development as well as drought resistance by modulating ABA signals. This report also indicated that *MYB96*-mediated signals enhance plant resistance to drought by reducing stomatal opening (Seo, 2009)

Ca^{2+} dependent signaling lead to the activation of *LEA*-type genes (late embryogenesis abundant) (Xiong et al., 2002) and was observed that *SHN* also

induced LEA gene (Trijatmiko, 2005) (Figure 12) which are involved in protection higher plants from damage caused by environmental stress, specifically drought (Hong et al., 2005). LEAs are usually able to protect other proteins or membranes, in a fashion similar to sugars (Hoekstra et al., 2001). In addition, these proteins may have anti-oxidant properties that minimize the damaging effects of reactive oxygen species (ROS), as strongly suggested for a group 5 LEA from Arabdopsis (Mowla et al., 2006). But to induce to LEA protein the transcription factor or its products must be transported to the shoots. Chemical and protein-based factors other than ABA, cytokinins or pH could also be involved in root to shoot signaling (Alves et al, 2006). It is established clearly that xylem sap from many plant species contains proteins (Alves et al, 2006), and peptides (Neumann, 2007) and microRNAs have also been implicated as potential signal molecules that move systemically (Sunkar et al., 2007 and Zhao et al., 2007).

On this way, *SHN2* transcription factor overexpressed in Arabdopsis roots or its products perhaps is transported to the shoots inducing the LEA protein or increasing the expression of a set of downstream genes that alter cuticle properties and result in reduced transpiration.

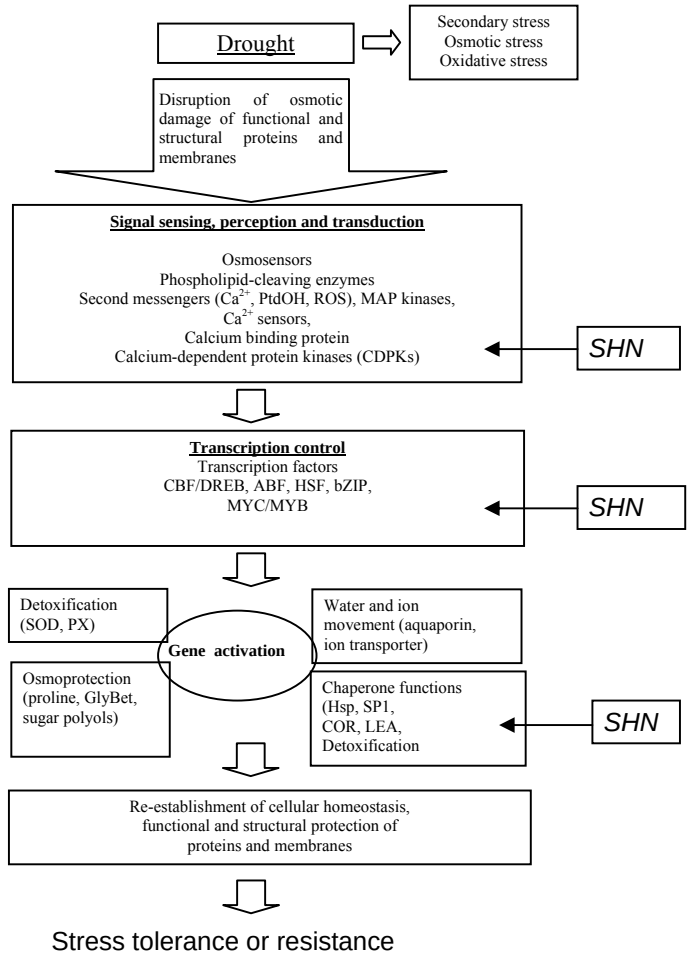

FIGURE 12 The complexity of the plant response to abiotic stress. Primary stresses, like drought is often interconnected and cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial stress signals (e.g. osmotic and ionic effects or changes in temperature or membrane fluidity) trigger the downstream signaling process and transcription controls, which activate stress-responsive mechanisms to re-establish homeostasis and to protect and repair damaged proteins and membranes. The *SHN* gene possibly induce the calcium binding proteins, *MYB* gene and LEA protein gene. Modified from (Vinocur, 2006).

7 CONCLUSIONS

Arabidopsis thaliana and *Brassica rapa* can be transformed with *Agrobacterium rhizogenes* and selected on ½ MS medium using the antibiotic hygromycin (at 20 $mg.L^{-1}$).

The acclimatization of *Arabidopsis thaliana* and *Brassica rapa* chimaeric plants after *A. rhizogenes* transformation needs to be improved.

Sand is a good substrate to be used for drought tolerance experiments.

A. rhizogenes or *A. tumefasciens* transformation of 35S::SHN does not make a difference for the phenotype.

Arabidopsis thaliana hair root transformations can be used as a very fast testing of construct and for confirmation of gene expression of at least the *SHN* gene.

Overexpression of the *SHN* gene improves the drought tolerance of chimeric and stably transformed *Arabidopsis thaliana* plants in a comparable manner.

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Chapter 3

Biotechnological approaches to improve the transformation of coffee

1 ABSTRACT

Coffee is the world's most important beverage and upon which the economy of many developing countries depends. Molecular breeding offers interesting perspectives towards the production of coffee varieties tolerant to biotic and abiotic stresses. To establish a genetic transformation system, it is necessary to have explants which are competent for the transformation process, and at least a system of genetic transference that is simple, cheap and reproducible. The main aim of this work was to develop an efficient method for coffee genetic transformation by *Agrobacterium rhizogenes* in order to create chimaric *C. arabica* (Catuaí Vermelho IAC 144) with wild-type shoots and transgenic roots for rapid testing of potentially interesting gene construct. For germination, the coffee seeds were surface-sterilized and placed in dishes containing sterile water or MS medium and incubated in darkness at 25ºC for 4 weeks. After this the germinated seeds were cut just above the hypocotyl-root boundary with a scalpel dipped in the *A. rhizogenes* colony containing either pCAMBIA1301, pMOG22-35S::SHN2 or pREDRoot and incubated at 25°C and 16h light for 4 weeks. The transformations were screened by PCR analyses (pMOG22-35S::SHN) or observed using Leica MZIII fluorescence stereomicroscope (pRedRoot) or analysed by Histochemical GUS assay (pCAMBIA1301). For the plants maintained on filter paper, 42% of *A. rhizogenes* infected hypocotyls containing pREDRoot plasmid had at least one transgenic root showing the red fluorescence, while the hypocotyls infected with *A. rhizogenes* containing pMOG22-35S::*SHN2* showed 32% of transformation. For the plants maintained on MS-medium the GUS assay showed that 30% of the plants had at least one positive-GUS and 25 $mg.L^{-1}$ of hygromycin was 67% efficient for the selection of transformed roots. 35% efficiency was found for the plants transformed with pMOG22-35S::SHN2 .

Key-words: *Agrobacterium rhizogenes*, pCAMBIA1301, pMOG22-35S::SHN2 or pREDRoot

2 RESUMO

O café um uma das bebidas mais importantes do mundo e sobre a quala a ecomonia de muitos países se desenvolveu. O melhoramento molecular oferece uma perspectiva interessante para a produção de variedades de café tolerantes ao estresse biótico e abiótico. Para o estabelecimento de um sistema de transformação genética, são necessário a obtenção de explantes competentes para o processo de transformação e um sistema simples e barato de transferência genética. O objetivo deste trabalho foi desenvolver um método para transformação genética de café via *Agrobacterium rhizogenes* criando plantas quiméricas de *C. arabica* (Catuaí Vermelho IAC 144) para aérea não transgênica e raízes. Para a germinação das sementes de café foram esterelizadas superficialmente e plaqueadas em placas de petry contendo água autoclavada ou meio MS e incubadas no escuro a 25° C e 16h de luz por 4 semanas. Depois disso as sementes germinadas foram cortadas assim do hipocotilo com o bisturi contaninado com *A. rhizogenes* contendo pCAMBIA1301, pMOG22-35S::SHN2 ou pREDRoot e inoculado a 25°C e 16h de luz por 4 semanas. A transfomações foram verificadas por PCR (pMOG22-35S::SHN) ou observadas em estereomicrocopio de fluorescentia Leica MZIII (pRedRoot) ou analisadas por métodos histoquímico GUS (pCAMBIA1301). Para as plantas mantidas em papel filtro, 42% dos hipocótilos infectados com *A. rhizogenes* contendo pREDRoot tiveram pelo menos uma raiz.fluorescente, enquanto que os hipocótilos infectados com *A. rhizogenes* contendo pMOG22-35S::*SHN2* apresentaram 32% de transfromação. Para as plantas mantidas em meio MS a analise do GUS mostrou 30% de plantas posivitas e 25 $mg.L^{-1}$ of higromicina foi 67% eficiente para a seleção das raízes transformadas. 35% de eficientcia foi encontrado para as plantas transformadas com pMOG22-35S::SHN2 .

Palavras-chave: *Agrobacterium rhizogenes*, pCAMBIA1301, pMOG22-35S::SHN2, pREDRoot.

3 INTRODUCTION

Coffee is one the most important widely traded commodities on the international markets (Dias, 2009) with a global market value of 70 to 80 billion US$/year, upon which the economy of many developing countries depends (Ribas, 2006). Coffe breeding by conventional methods is a long process involving different methods, such as selection from wild population followed by hybridisation and progeny evaluation, backcrossing and interspecific crosses. It takes more than 30 years to obtain a new cultivar from any of these methods (Carneiro, 1997). In view of this, the genetic transformation is one of the techniques that opens new perspectives for coffee improvement, allowing the fast incorporation of desirable characteristics like tolerance to biotic and abiotic stress, into elite cultivars, as well as the study of various aspects of plant sciences (Pereira, 2000) since, there are enormous amounts of DNA sequence information available in the coffee EST data set up, opening new experimental opportunities for functional genomic analysis (Vieira, 2006).

To establish a genetic transformation system it is necessary to have explants which are competent for the transformation process, and an *in vitro* culture system, which permits a high frequency of regeneration. At least a system of genetic transference that is simple, cheap and reproducible, that inserts the genetic sequence in a stable form (Birch, 1997). However, despite significant advances over the last years, coffee transformation is still very laborious, with bottlenecks in the methodology that make it far from a routine laboratory technique. Transformed somatic embryos of *Coffe arabica* were first reposted by Barton et al. (1991) using electroporation method. Regeneration of transformed embryos and plantlets resistant to kanamycin were obtained but the plantlets did not survive. Da Silva & Menéndex-Yuffá, (2003) determinated the optimal condictions for coffee somatic embryo electroporation method and obtained two

out of eight transgenic regenerated plants. Considering the biolistic transformation system, this was first used for coffee by Van Boxtel et al. (1995) but the successful regeneration of coffee plants was only published later by Ribas et al. (2001, 2005) and Cunha et al. (2004).

Ocampo & Manzanera (1991) were the fist to demonstrate that *Coffea arabica* tissues (hypocotyls of *in vitro* germinated coffee seeds) could be infected by wild strains of *Agrobacterium tumefaciens*, but no regenerated plants were reported). Another initial report on genetic *A. tumefaciens* transformation using protoplast was reported by Spiral & Peiard (1991) but regeneration was again not obtained. Following this work Spiral et al., 1993, used *A. rhizogenes* to transform torpedo-sharped somatic embryos of *C. canephora* and they regenerated plants. Kumar (2003, 2004, 2006) and Alpizar et al. (2006, 2008) reported transformation methods with different strains of *A. rhizogenes* and these studies clearly demonstrated that *A. rhizogenes* harboring binary vectors could be used for generating hairy root transgenic coffee without hairy root phenotype.

Hairy roots are adventitious roots derived from cells transformed by root inducing Ri plasmid of *A. rhizogenes.* The hairy root harbours the T-DNA segment of the Ri within its nuclear genome. Hairy root systems arising from inoculation with *A. rhizogenes* have become useful in the last decade not only because of their application in the production of valuable plant metabolites, recombinant proteins and in the detoxification of environmental pollutants, but also in their relevance as a strategy to create *de novo* metabolic pathways by introducing foreign genes or to get higher efficiency in a particular pathway or process (Guillon et al., 2006; Eapen et al., 2007) being a good strategy for studying the function of genes in roots that can be efficiently and rapidly generated on non-transformed shoots.

However, in the hair root transformation method it is important to have a good system for selection of the transformation event, since not all the roots grow after they are transformed. This can be obtained by chemical resistance or by gene reporters. Regarding the chemical resistance Spiral et al. (1993) had difficulties in screening transformation events in coffee with the kanamycin selection system. On the other hand, Hatanaka et al. (1999) obtained transformed plants of *C. canephora* using a combination of two antibiotics (hygromycin and kanamycin). Now it is well known that kanamycin selection is not as efficient as hygromycin for the selection of transgenic coffee (Kumar, 2006).

Reporter genes are useful tools to study gene expression in transformed organisms. The most widely used reporters in plants are *uidA* β-glucuronidase - GUS (Jefferson et al., 1987), luciferase (Ow et al., 1986), and gene encoding fluorescent proteins including green fluorescent protein (GFP), yellow fluorescent protein, cyan fluorescent protein, and red fluorescent protein (DsRED) (Chalfie et al., 1994; Chalfie, 1995; Cubitt et al., 1995; Matz et al., 1999). Fluorescent proteins, such as green fluorescent protein and red fluorescent protein (DsRED), have become frequently used reporters in plant biology. Compared with *GUS* and luciferase, which require the addition of specific substrates to monitor reporter activity, fluorescent proteins like DsRED, due to their intrinsic fluorescence, allow non invasive detection in living cells without the addition of substrates. This enables, for example, the real time visualization of gene expression or analysis of transformants in the first generation (Mirabella, 2004). The visual selection with DsRED also allows to avoid the selection of co-transformed roots with antibiotics or herbicides. Another advantage of the pRedRoot vector is that it can provide the possibility to select easily transgenic roots, based on fluorescence (Matz et al., 1999),

permitting to cut the non transformed roots to allow the growth of more transformed roots (Limpens, 2004).

In this work, the transformation of *C. arabica* germinated seeds using *A. rhizogenes* and the selection of the transformed roots with hygromycin, *Gus* and DsRed is reposted for the fist time.

4 AIM

The main aim of this work was to develop an efficient method for coffea genetic transformation by *Agrobacterium rhizogenes* in order to create chimaric *C. arabica* (Catuaí Vermelho IAC 144) with wild-type shoots and transgenic roots for rapid testing of potentially interesting gene construct for future stable transformation using *A. tumefaciens* or *A. rhizogenes*.

5 MATERIAL AND METHODS

5.1 Plasmids

The plasmids are described in chapter II, item 5.1.

5.2 Preparation of *Agrobacterium* for plant transformation

The *Agrobacterium* was prepared as described in chapter II, item 5.2

5.3 Preparation of coffee explants

For germination on filter paper, *Coffea arabica* L. (Catuaí Vermelho IAC 144) seeds were surface-sterilized after hand removing the parchment. The sterilization was carried out immersing the seeds in 4% HClO (w/v) bleach solution for 30 minutes and finally rinsed three times in sterile water and placed

in dishes (2 cm deep) containing sterile water. For the germination on MS medium, the coffee seeds were surface-sterilized with sodium hypochlorite 5% for 3h (Sofiatti, 2008) and the parchments were removed. After parchment removal the the seeds were immersed in 70% ethanol for 10min, sodium hypochlorite 5% for 1h and finally rinsed three times in sterile water and placed in Petri dishes (2cm deep) containing ½ MS medium (Murashige & Skoog, 1962) and solidified by phytagel (2.5 g). The seeds were placed in darkness at 25ºC for 4 weeks.

5.4 Infection of explants with *A. rhizogenes* on filter paper or culture media

A scalpel was dipped in the *A. rhizogenes* colonies containing either pCAMBIA1301, pMOG22-35S::*SHN2* or pREDRoot. The seeds germinated during the 4 weeks were cut just above the hypocotyl-root boundary and arranged in (a) dish (s) containing filter paper and water or MS-medium (Murashige & Skoog, 1962), in this case the cotyledons were placed just on the agar and the hypocotyl on the autoclaved filter paper (Fig 3 a b). Additional *A. rhizogenes* colony material was scraped from the plate with the scalpel and placed, as a dot, on the wound surface at the base of each hypocotyl **(**Limpens et al., 2004). The dishes were sealed with parafilm, but care was taken to leave 1/3 of the side that will be the top of the plate, open. Plates were incubated in the climate chamber at 25°C and 16h light for 4 weeks. After 4 weeks the plants were changed to pots containing filter paper and 1/10 MS solution (Murashige & Skoog, 1962) supplemented with tricarcilin (200 $mg.L^{-1}$) or MS-solid medium (Murashige & Skoog, 1962) supplemented with tricarcilin (200 $mg.L^{-1}$) to select against *A. rhizogenes* and hygromycin (20 $mg.L^{-1}$ and 25 $mg.L^{-1}$) to select transformed roots. Pots were incubated in the climate chamber at 25°C and 16h light for 10 days.

The plants maintained on filter paper were screened by PCR analyses (pMOG22-35S::*SHN2*) or observed using Leica MZIII fluorescence stereomicroscope (pRedRoot). The plants kept on the MS-medium containing 25mg.L^{-1} of hygromycin were screened for root growth and confirmed by PCR analyses (pMOG22-35S::*SHN2*) or by Histochemical GUS assay (pCAMBIA1301). One hundred seeds were used per transformation.

5.5 DNA analysis

For DNA isolation, the roots were harvested with a sharp pincer, transferred to eppendorf tubes, immediately frozen in liquid nitrogen and stored at -80ºC. Frozen roots from each plant were hand ground to a fine powder in a 2 mL micro centrifuge tube. Five hundred microliters of extraction buffer was added and shaken for complete homogenization and the centrifugation was done at 13000rpm for 15 minutes. The supernatant was transferred to fresh eppendorf tubes and the DNA was precipitated with 0.8 volume Isopropanol by centrifugation at 13000rpm for 15 minutes. The pellet was washed with 70% ethanol and dissolved in 400 µl of TE pH 8.0.

For the PCR analyses the following primers: 5' ATGCTCAACACATGAGCGAAACCCTAT 3' and 3´CAATCCCACTATCCTTCGCAAGACCC 5' were used for amplification of a fragment of 1369 bp when the *SHN2* gene is present (AT5G25390.1 Tair). To confirm if the amplification was not from the contamination of that bacterial DNA, the amplification of the kanamycin gene was also carried out with the primer 5' TTATTTTCTCCCAATAGGC 3' and 3' GTGGAACGGGAAAAGGACAT 5', to amplify a fragment of 1458bp. The PCR mixture consisted of 5 ng of plant DNA, 5µl of 10 x Taq buffer, 1.0 µl of 5mM dNTP, 1.25 units of Taq DNA polymerase, 1 µl from each 10pmol primer

in a final volume of 50 µl. PCR analysis was performed with a thermocycler. For DNA amplification, samples were heated to 94ºC for 5min, followed by 30 cycles at 94ºC for 1 min, at 50ºC for 1min, and at 72ºC for 2min. PCR was carried out using an automatic thermocycler. The amplified products were separated by electrophoresis on 0.8% agarose gels stained with 0.5 $mg.L^{-1}$ ethidium bromide in 0.5 x TAE (Tris-acetate/EDTA electrophoresis buffer) and visualized by fluorescence under UV light.

6 RESULTS AND DISCUSSION

6.1 Coffee hairy root transformation on filter paper

Two weeks after of infection with *A. rhizogenes* containing pREDRoot plasmid or pMOG22-35S::*SHN2*, hypocotyls swelled the root regenerated, but those roots were non transgenic (Figure 1). Hence, these roots were cut and after 4 weeks 42% of hypocotyls infected with *A. rhizogenes* containing pREDRoot plasmid (Figure 2) had at least one transgenic root showing the red fluorescence (Figure 3b), while the hypocotyls infected with *A. rhizogenes* containing pMOG22-35S::*SHN2* showed 32% of transformation (Figure 2) verified by PCR analysis (Figure 4)

FIGURE 1 Coffee hairy root transformation. Germinated coffee seeds 4 weeks old (a). Coffee germinated seeds infected with *A. rhizogenes* containing pRedRoot plasmid (b). Root regenerated after transformation with *A. rhizogenes* containing pRedRoot plasmid (c).

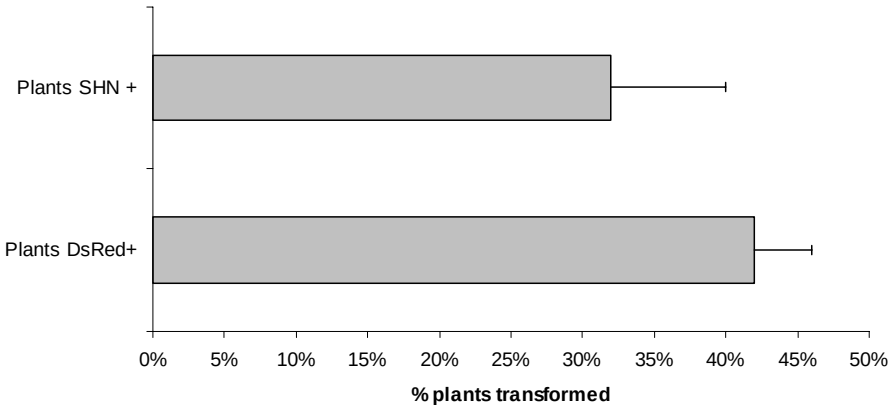

FIGURE 2 Percentage of transgenic Coffee plants transformed with DsRED and *SHN2* gene. Error bar indicates standard deviation.

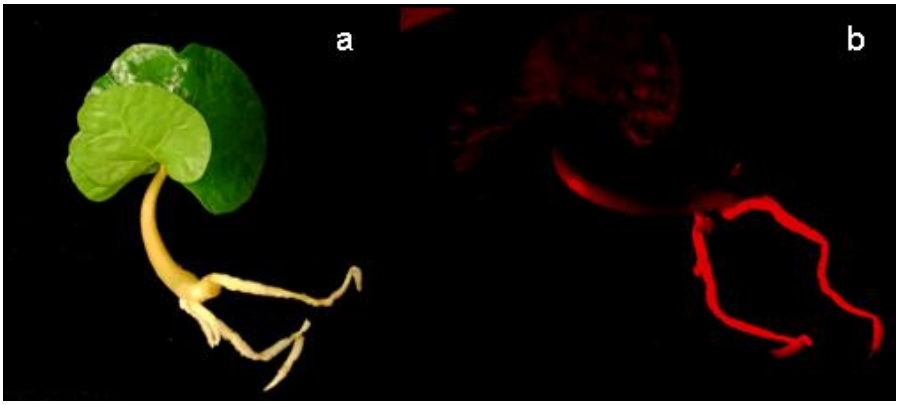

FIGURE 3 Coffee hairy root transformation. Coffee, transformed with pREDRoot 12 weeks old (a) and fluorescent red roots expressing DsRed (b).

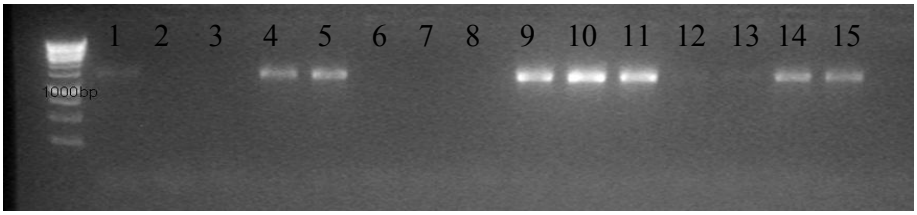

FIGURE 4 PCR analyze of DNA isolated from coffee roots transformed with pMOG22-35S::*SHN2*. Fragment amplified from de *SHN2* gene (line 1, 4, 5, 9, 10, 11, 14, 15) indicating positive transformed roots. Fragment amplified from de *SHN2* gene (line 2, 3, 6, 7, 8, 12, 13) indicating roots not transformed. PCR mix control without pMOG22-35S::*SHN2* (line 16). 10µl of a total volume of 50 µl PCR solution was separated using a 0.8% agarose gel, run in 1 x TBE buffer (Sambrook et al., 1989). Label 1 kb.

The plants that were transformed with pREDRoot and pMOG22-35S::*SHN2* were transferred to hydroponic medium (Figure 5 abc), and after 1 month the roots were analyzed again in fluorescence stereomicroscope or by PCR and some of the new root growth was transformed. The transformed plants with pREDRoot had on average 3.9 roots per plant and 2.4 transformed roots per

plant, and transformed plants with pMOG22-35S::*SHN2* had on average 4.2 roots plants and 2.3 transformed roots per plants (Figure 6). The non transgenic roots were removed to permit the growing of transgenic roots. Alpizar et al. (2006) reported the competition between the non transformed roots and transformed roots, and the removing of the non transgenic roots can permit the growing of the new transgenic roots.

The roots transformed with pRedRoot and pMOG22-35S::*SHN2* were well-branched and had few phenotype variations among roots, after 4 weeks in hydroponic medium (Figure 5 c). Thus, transformed coffee roots could not be morphologically distinguished from non transformed roots and roots growing in the air above the hydroponic medium were never observed, which means the geotropism of the transformed roots were normal. However, the term 'hairy root' is commonly used for *A. rhizogenes*-transformed roots and refers to the particular phenotype of those roots, often characterized by root thickness, highly branching root pattern and plagiotropic development attributed to increased endogenous auxin content (Nilsson & Olsson, 1997; Handa, 1991; Nguyen et al., 1997; Nin et al., 1997; Chaudhuri et al., 2005). Alpizar et al. (2006) and Kumar et al. (2005) reported that the transformed coffee roots did not display, *in vitro,* all the distinctive characteristic associated with the "hair root" phenotype, such as faster growth, high branching or plagiotropic root development.

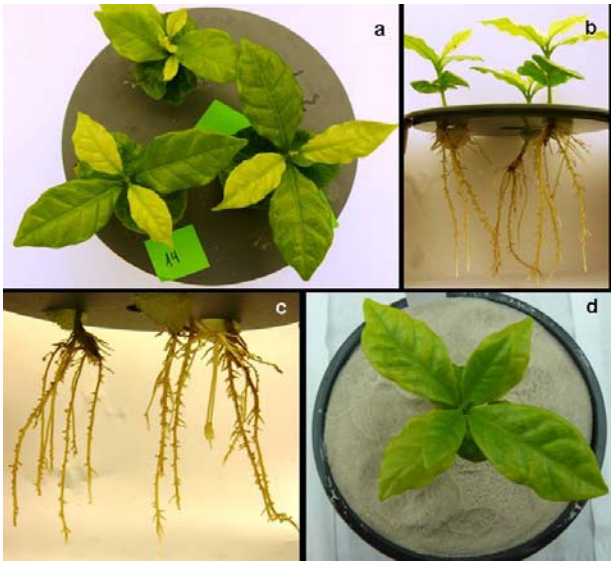

FIGURE 5 Coffee plants transformed with *A rhizogenes* containing pMOG22-35S::*SHN2* and transferred to hydroponic medium (a). Transgenic coffee roots with SHN gene growing in hydroponic medium after 5 months (b, c); Chimeric coffee transferred to sand after 6 months (d).

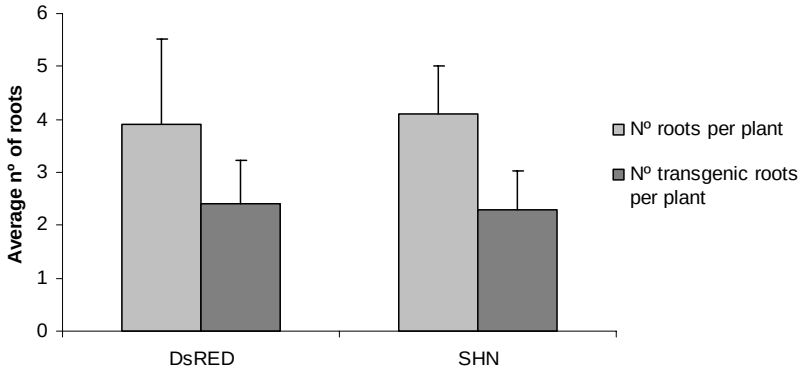

FIGURE 6 Average number of total roots per transgenic coffee plant transformed with pRedRoot and pMOG22-35S::*SHN2* compared with the average number of the transgenic roots per plant. Error bar indicates standard deviation.

6.2 Hairy root transformation of coffee germinated on MS medium

Four weeks after the transformation with pCambia1301 and pMOG22-35S::*SHN2* the plants showed the new root formation, and as reported by Alpazir (2006), and observed on plants maintained on filter paper, the transformed coffee roots did not display, *in vitro,* all the distinctive characteristics associated with the 'hairy root' phenotype.

To test the efficacy of the transformation, GUS assay was used on the roots infected with *A. rhizogenes* containing pCAMBIA1301. At this stage, 4 weeks, the hairy roots emerging from the wound site reached 3 to 6 cm in length. The GUS assay showed that 30% of the plants had at least one positive-GUS (Figure 7). Endogenous GUS activity was not observed on the assayed coffee somatic embryogenic tissues (Ribas et al., 2006) confirming that the GUS observed here is from the introduction of GUS gene on the roots. GUS activity is known as a good reporter for selecting transformed coffee plants and in this work it was also found that the DsRED fluorescent protein can be used as a marker to select transformed coffee plants.

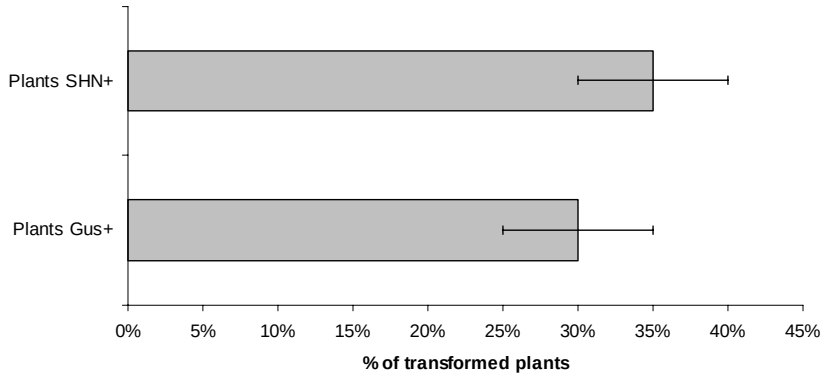

FIGURE 7 Number of coffee plants transformed with pCambia1301 and pMOG-35S::SHN compared with the total number of plants inoculated with *A. rhizogenes*. Error bar indicates standard deviation.

Gus and DsRed are shown to be a good reporter for coffee but the plasmid pMOG-35S::*SHN2* has only HPT (hygromycin gene resistance) for selection. To find the best concentration of hygromycin to select the transformed roots, the plants transformed with pCAMBIA1301 (35S-GUS marker and 35S::HPT) and pMOG-35S::*SHN2* were transferred to pots with selection medium (½ MS + 25 $mg.L^{-1}$ hygromycin), and after 10 days it was possible to observe the difference between the transformed (Figure 8b) and non transformed roots (Fig. 8c), showing the average of 1.4 and 1.2 resistant roots for plants transformed with pCAMBIA1301 and pMOG-35S::*SHN2* respectively (Figure 9). For confirmation of the selection with hygromycin, the roots transformed with pCAMBIA1301 were analyzed by GUS activity and it was confirmed that the resistant roots were GUS-positive (Figure 8a, d), and most of the non resistant roots were GUS-negative (Figure 8b, d). The non transformed roots and negative GUS were also brown and were growing slowly. Thus, 25 $mg.L^{-1}$ of hygromycin was 67% efficient for selection of transformed roots (Figure 9), only occurring a false negative but without false positive. Alpizar et al. (2006) verified that 20 $mg.L^{-1}$ allowed the growth of only the transformed plants regenerated from somatic embryos and southern analyses further confirmed the stable integration of T DNA in hygromycin resistant plantlets evidencing the concentrations and the selection used here.

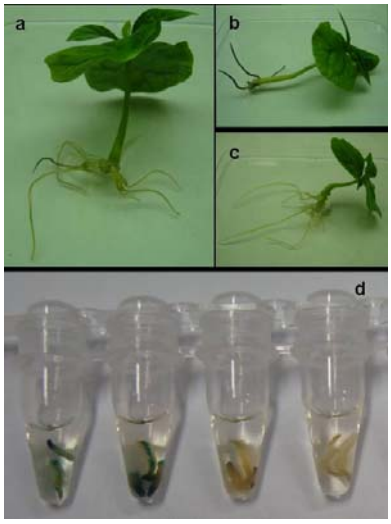

FIGURE 8 Hairy root *Coffea arabica* L. (Catuaí Vermelho IAC 44) transformation with pCAMBIA1301. Plant maintained on ½ MS medium + 25mg.L^{-1} hygromycin for 2 weeks (a,b). Plant with resistant and non resistant roots (a). Plant with non resistant roots (b). Plant maintained on ½ MS medium without hygromycin (c). Resistant roots with (Gus-positive) and non resistant roots (Gus-negative) (d).

Knowing the concentration of hygromycin to select the transformed roots it was possible to score the percentage of the transformed plants with pMOG-35S::*SHN2*. The efficiency of transformation was 35%, not differing from the percentage that was found with pCAMBIA1301 transformation (Figure 7) and also not differing from the percentage of transgenic plants cultivated on filter paper (Figure 2).

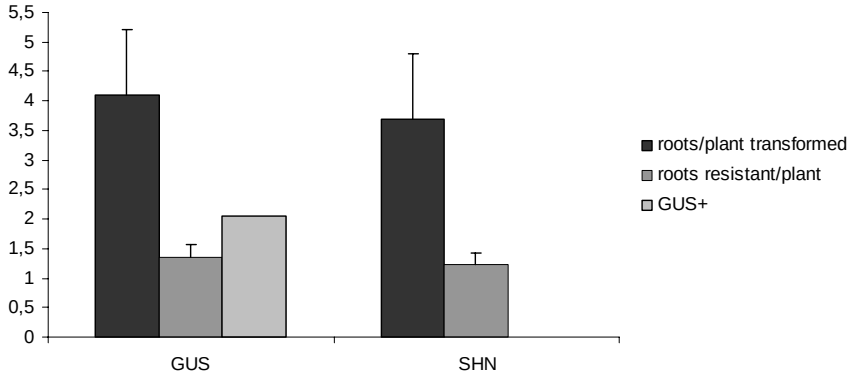

FIGURE 9 Average number of roots per transformed *Coffea arabica* L. (Catuaí Vermelho IAC 44) plants compared with average number of resistant roots and average number of GUS-positive roots. Error bar indicates standard deviation.

An alternative strategy for studying the function of genes in roots is to use 'composite' plants that can be efficiently and rapidly generated by inducing transformed roots on non-transformed shoots after inoculation with *A. rhizogenes* (Boisson-Dernier et al., 2001). Composite plants offer a major advantage over axenic hairy root cultures by generating information at the whole plant level, and give the possibility to realize functional analysis studies under non-axenic conditions (Alipizar, 2006). In coffee, protocols for *A. rhizogenes* mediated transformation and plant regeneration were first described by Spiral et al. (1993), then by Kumar et al. (2004, 2005, 2006) using somatic embryos of *C. canephora* with 3% efficiency and by Alpizar et al. (2006) using geminated embryos of *C. arabica.*

In this work the results revealed that hairy root coffee can also be obtained using germinated seeds. It was observed that the transgenic regenerated roots do not display most of particular properties as the majority of hairy root clones, exhibiting limited phenotype differences when compared with normal

non transformed root clones. These were observed even on the roots transformed on filter paper and on roots transformed on ½ MS medium. The coffee hairy roots belonging to a composite plant also exhibited a normal positive geotropism when transferred to hydroponic solution or sand soil. Alpizar et al. (2008) studing the proliferation of hairy roots revealed that these roots did not display most of the particular properties and that the majority of hairy roots clones exhibited limited phenotype differences when compared with normal non-transformed clones. In this work the low *rol* and *aux* integration variability of oncogene was also confirmed, making the hairy roots a viable and useful tool for coffee breading and offers new research perspectives. Regarding the shoots of the plants transformed with 35S::*SHN2,* they had low growth when compared with the chimeric plants transformed with DsRed, but abnormal phenotype with short internodes and stunted growth was not observed, as reported by Perthuis et al. (2005).

Thus, the method developed in this work based on infecting germinated seeds with *A. rhizogenes* conducted on filter paper or ½ MS medium is very rapid and not particularly laborious as compared with germinated embryos, which take time and ability to isolate, and also do not require a good protocol of regeneration, that it is necessary for *A. tumefaciens* mediated methods. For example, the *A. rhizogeens* transformation method, using germinated embryos, takes 5 months and *A. tumefaciens* mediated transformation with selective pressure, usually takes 14 months to produce similar well-developed transformed plantlets starting from primary explants, i.e. leaf pieces (Hatanaka et al., 1999; Leroy et al., 2000). In this work, in 2 months it was possible to produce acclimatized composite plants, bearing well-developed transformed rootstocks, directly usable for rapid validation and functional study of resistance genes. Research on coffee transformation could already benefit by this fast

transgenic approach as a research tool as long as a rapid and reproducible stable transformation system is developed, offering a new avenue for the genetic analysis of this most important crop.

7 CONCLUSIONS

Germinated seeds, 4 weeks old, of *Coffea arabica* L. (Catuaí vermelho IAC 144) can be transformed with *A. rhizogenes* reducing the time spent on the transformation processes.

It is necessary to cut the roots one more time to improve the rate of transformation.

The germination and the transformation process can be carried out on filter paper or on ½ MS medium.

The transformation on MS medium is relevant, being possible to use select medium to screen the plants transformed.

The reporters DsRED or GUS can be used to select the transformed roots, however the visilble marker is remommended instead of hygromycin selection.

On ½ MS medium 25mg.L^{-1} of hygromycin is enough to select the transformed hair roots.

The hydroponic medium can be used to acclimatize the coffee plants and to follow the development of the transgenic roots, while in sand, it is not possible to follow the root development.

A. rhizogenes mediated transformation offers a fast alternative to generate genetically transformed coffee; generating stable transgenic lines is very time-consuming.

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Chapter 4

Biotechnological approaches to improve drought tolerance of coffee

1 ABSTRACT

Drought is the most serious environmental factor limiting the worldwide productivity of agricultural crops such as coffee, with devastating economical and sociological impact. A number of genes that respond to desiccation and low temperature at the transcriptional level have been described, and their products are thought to be involved in stress response and tolerance. Overexpression of SHINE can induce the expression of these drought stress-related genes under normal growth conditions in transgenic plants and confer improved tolerance to drought. The main objective of this work was to express the *SHINE* gene of *Arabidopsis thaliana* in transgenic hairy roots of *Coffea arabica* after *Agrobacterium rhizogenes*-mediated root transformation in order to improve the drought tolerance of such chimeric plants. The chimeric plants, transformed with 35S::*SHN* or DsRED (control plants) were subjected to drought for 15 days by withholding water, and subsequently rehydrated to observe the recovery after 1 week. After only 12 days of treatment the 35S:*SHN* chimeric plants started to wither and at 15 days, the transgenic plants were completely withered but at that point the DsRED (control) chimeric plants had dried leaves. Whereas DsRED (control) chimeric plants did not recover from the 15-day dehydration treatment and had completely dried out, all the 35S:*SHN* chimeric plants recovered, becoming greener and stronger.

Key-words: Overexpression, SHINE, *Coffea arabica,* chimeric plants.

2 RESUMO

A seca é um dos fatores ambientais mais sérios que limitam a produção de culturas como o café com um desvantajoso impacto econômico e social. Tem sido descritos vários genes que respondem a dessecação e a baixas temperatuas e nível transcricional, e seus produtos são conhecidos por serem envolvidos na resposta e tolerância ao estresse. A superexpressão do gene SHINE pode induzir a espressão desses genes relacionados a seca e melhorar a tolerância a seca. O objetivo deste trabalho foi expressar o gene *SHINE* de *Arabidopsis thaliana* em plantas raizes transgênicas de *Coffea arabica* depois da transformação dom *Agrobacterium rhizogenes* para melhorar a tolerância a seca de tais plantas quiméricas. As plantas quiméricas, transformadas com 35S::*SHN* ou DsRED (plantas control) foram submetidas a seca por 15 dias por suspensão da irrigação e subsequentemente reidratadas para observar a sobrevivência depois de 1 semana. Após 12 dias de tratamento as plantas quiméricas 35S:*SHN* iniciaram murchar e ao 15 dias as plantas transgênicas completamente murchar, mas neste ponto as plantas quiméricas DsRED (controle) estavam com as folhas secas. Consequentemente as plantas quiméricas DsRED (controle) não sobreviveram aos 15 dias de desidratação enquanto que todas as plantas quiméricas 35S:*SHN* sobreviveram.

Palavras-chave: Superexpressão, *SHINE*, *Coffea arabica,* plantas quiméricas.

3 NTRODUCTION

Drought is the most serious environmental factor limiting the worldwide productivity of agricultural crops such as coffee, with devastating economical and sociological impact. (Grise, 2008). For example, in Minas Gerais, the largest coffee region in Brazil, short drought periods can result in 20 to 30% loss in yield at harvest (Rivero et al., 2007). Warmer global temperatures are expected to cause an intensification of the hydrologic cycle, with increased evaporation over both land and water. The higher evaporation rates will lead to greater drying of soils and vegetation, especially during the warm season. Climate models also project changes in the distribution and timing of rainfall. The combination of a decrease in summer rainfall and increased evaporation will lead to more severe and longer-lasting droughts in some areas (Union of Concerned Scientists, 2003).

The climatic change is an acute long-term concern: The U.N. Intergovernmental Panel on Climatic Change predicts an increase in global temperatures of 3.6 to 7.2 degrees over the next 20 years, with even greater temperature increases in the Amazon. That could mean a 10% reduction Brazil's arable land for coffee by 2020 and a one-third reduction by 2070, as the crop's suitable climate migrates into the Andean foothills of neighboring Argentina, according to a study directed by Eduardo Assad (Sibaja, 2009). The knowledge on coffee drought tolerance at the molecular, biochemical and genetic levels may permit the designing of strategies for the development of tolerant cultivars, and this would certainly help avoid damage in coffee plantations in the south and southeast regions of Brazil.

Plant genetic transformation permits gene transfer across species. It is particularly important in perennial crops and tree species in which development of new cultivars is often difficult due to their long generation time, three years

for first coffee flowering. Plant genetic transformation can be a short cut for introducing a new trait and developing new cultivars without losing the genetic background of the original transformed cultivar (Ribas et al., 2006). Dehydration-response transcription factors, which are involved in the hydric, cold and salt stress response, have been used to produce transgenic plants with better stress tolerance (Kasuga et al., 1999). A number of genes that respond to desiccation and low temperature at the transcriptional level have been described, and their gene products are thought to be involved in stress response and tolerance (Hugles & Dunn, 1996). With the identification of stress-inducible genes and studies on their role in stress tolerance, several approaches have been proposed to improve drought tolerance in plants. In this way, the transcription factor SHINE was described that, when overexpressed, greatly enhances the drought tolerance of *Arabidopsis* (Jagglo-Ottosen et al., 1998; Aharoni et al., 2004).

The *SHINE* gene encodes an AP2/EREBP transcription factor suggesting that this clade of genes acts in the regulation of lipid biosynthesis required for plant environmental protection, including organ separation processes and wounding (Aharoni, 2004). The cuticular layer, comprising cutin and waxes, plays multiple roles in plants, including the regulation of epidermal permeability and nonstomatal water loss and protection against insects, pathogens, UV light, and frost (Sieber et al., 2000). Overexpression of *SHINE* can induce the expression of these drought stress-related genes under normal growth conditions in transgenic plants and confer improved tolerance to drought.

4 AIM

The aim of this work was to express the *SHINE* gene of *Arabidopsis thaliana* in transgenic hairy roots of *Coffea arabica* after *Agrobacterium rhizogenes*-mediated root transformation in order to improve the drought tolerance of such chimeric plants.

5 MATERIAL AND METHODS

5.1 Plasmids

The plasmids are described in Chapter II, item 5.1.

5.2 Preparation of *Agrobacterium* for plant transformation

The *Agrobacterium* was prepared as described in Chapter II, item 5.2

5.3 Preparation of coffee explants, infection of explants with *A. rhizogenes* and DNA analysis

Described in Chapter III item 5.3 and 5.4 and 5.5

5.4 Drought tolerance experiments

For the drought tolerance experiments, 6-month-old plants maintained in hydroponic medium were transferred to silver sand and placed in a climate chamber (~20ºC, 12 h light). The nutrient solution Hyponex (Nishimura, 1982) was regularly supplied (every two days), keeping 85% field capacity (about 60 ml), over a period of 2 months. After this, the chimeric plants, transformed with 35S::*SHN2* or DsRED (control plants) were subjected to drought for 15 days by withholding water, and subsequently rehydrated to observe the recovery after 1 week. On the control treatment the transformants 35S::*SHN2* and DsRED

(control plants) were maintained at 85% field capacity during the experiment. Four plants per treatment were used. The survival was scored, the fresh biomass was collected and the collected plants were oven-dried at 60ºC for 5 days and the dry weight was measured.

6 RESULTS AND DISCUSSION

6.1 Drought tolerance experiment

The drought tolerance experiment was set up with 8-month-old coffee plants whose roots were transformed with either 35S::*SHN2* or DsRED (control). The chimaeric transformed plants were exposed to a dehydration period of 15 days (Figure 1 and 2). Subsequently, the plants were watered and their recovery was monitored for a week (Figure 3). DsRED (control) chimeric plants started to wither after 8 days without water (Figure 1 a b) while the 35S:*SHN2* chimeric plants still did not show strong effect related to the water deficiency (Figure 1 c d). The 35S:*SHN2* chimeric plants started to wither only after 12 days and at 15 days the transgenic plants were completely withered (Figure 2 c d) but at this point the DsRED (control) chimeric plants had dried leaves (Figure 2 a b).

Whereas DsRED (control) chimeric plants did not recover from the 15-day dehydration treatment and completely dried out (Figure 3 a, b), all the 35S:*SHN2* chimeric plants recovered, becoming greener and stronger (Figure 3 c, d). This showed that the 35S::*SHN2* chimeric plants were superior to the DsRED (control), because they maintained leaf water potential during drought, even though they were partially wilted.

During the experiment the plants overexpressing 35S::*SHN2* showed growth retardation under normal growth conditions (Figure 2 and f g h). The expression of *35S::AtDREB1A* and in transgenic Arabidopsis led to an enhanced

dehydration tolerance and also severe growth retardation under normal growth conditions (Liu et al., 1998; Kasuga et al., 1999; Dubouzet et al., 2003). Transgenic *Hipericum perforatum* plants regenerated from hairy roots transformed with *A. rhizogenes* strain A4M70GUS showed normal phenotype, but also differences in several growth parameters (Koperdakova, 2009).

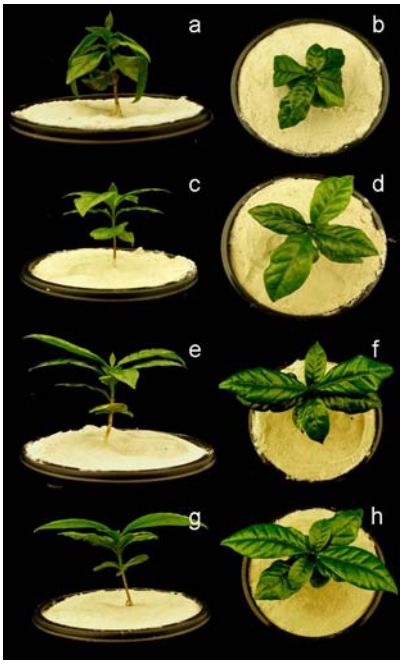

FIGURE 1 Drought tolerance of 8-month-old *A. rhizogenes* hairy root transformed chimaeric transgenic coffee. DsRed chimaeric coffee plant (control) kept without water for 8 days (a, b). 35S:*SHN2* chimeric coffee plant kept without water for 8 days (c, d). DsRed chimeric coffee plant (control) maintained at 85% field capacity (e, f). 35S:*SHN2* chimeric coffea plant maintained at 85% field capacity (g, h). In all cases representative plants are shown. For every treatment, 4 plants per genotype were used.

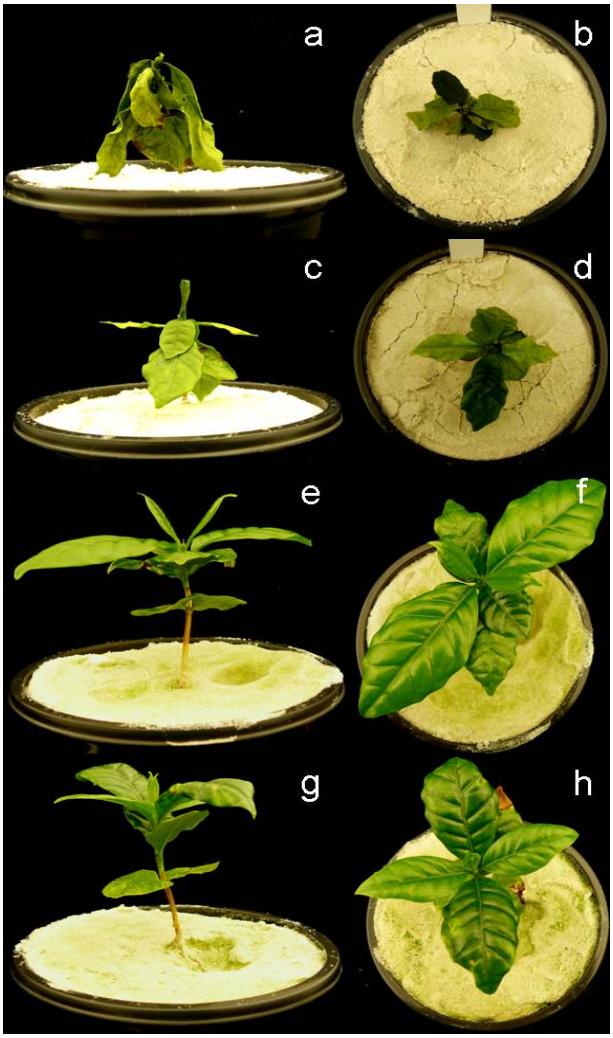

FIGURE 2 Drought tolerance of 8-month-old *A. rhizogenes* hairy root transformed chimaeric transgenic coffee. DsRed chimaeric coffee plant (control) kept without water for 15 days (a, b). 35S:*SHN2* chimeric coffee plant kept without water for 15 days (c, d). DsRed chimeric coffee plant (control) maintained at 85% field capacity (e, f). 35S:*SHN2* chimeric coffee plant maintained at 85% field capacity (g, h). In all cases representative plants are shown. For every treatment, 4 plants per genotype were used.

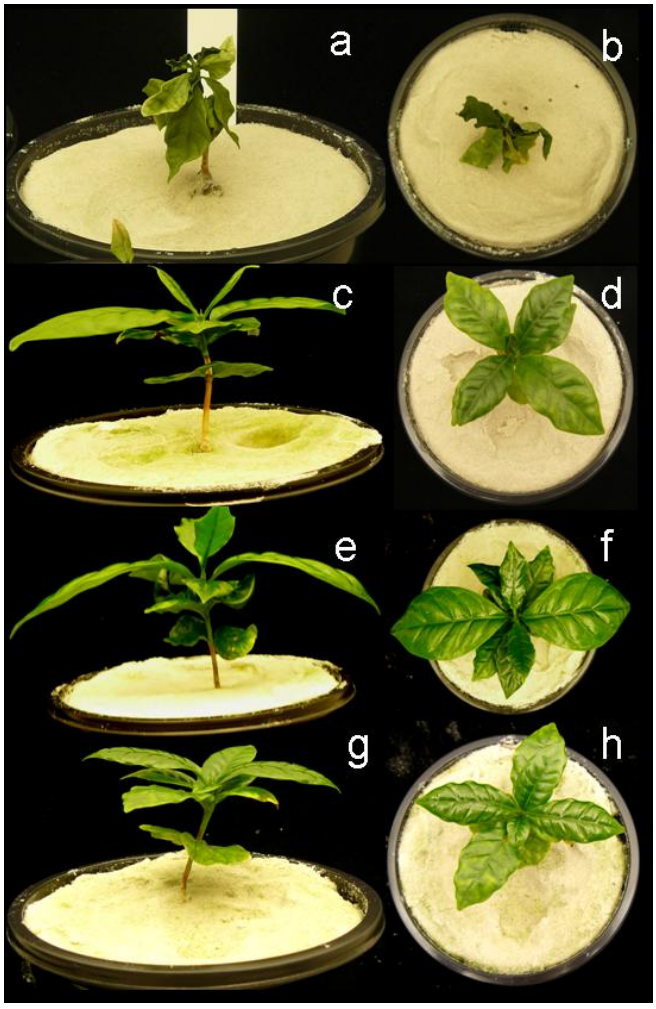

FIGURE 3 Drought tolerance of 8-month-old *A. rhizogenes* hairy root transformed chimaeric transgenic coffee. DsRed chimaeric coffee plant (control) kept without water for 15 days and rewatered for 1 week (a, b). 35S:*SHN2* chimeric coffee plant kept without water for 15 days and rewatered for 1 week (c, d). DsRed chimeric coffee plant (control) maintained at 85% field capacity (e, f). 35S:*SHN2* chimeric coffea plant maintained at 85% field capacity (g, h). In all cases representative plants are shown. For every treatment, 4 plants per genotype were used.

The shoot and root fresh and dry weight of the DsRed chimeric coffee plants (control) differed significantly (Figures 4 and 5). The plants that had been watered throughout had more fresh and dry weight when compared to the plants receiving the drought treatment. The shoot and root fresh and dry weight of the 35S::SHN plants did not significantly differ, statistically, when comparing the plants under normal and drought conditions (Figs 4 and 5). It can be observed, however, that the fresh and dry weight of non-watered 35S::*SHN2* plants did not differ from watered 35S::*SHN2* plants, while the non-watered DsRed plants differed from the watered DsRed plants, showing a decrease of the fresh and dry weight when there is no overexpression of the *SHN2* gene.

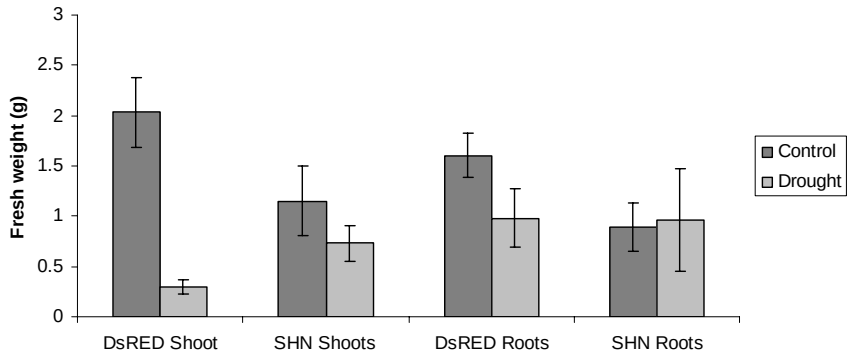

FIGURE 4 Shoot and root fresh weight of DsRed chimeric coffee plants (control) and 35S::*SHN2* chimeric coffee plants submitted to drought treatment. Statistical significance verified by Tukey test to 0.5%. Error bar indicates standard deviation.

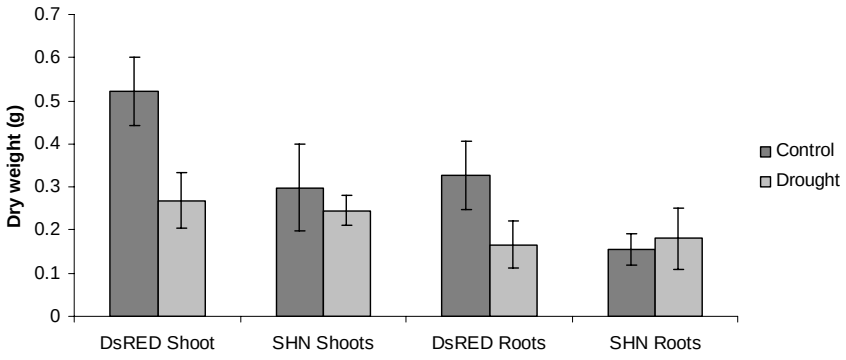

FIGURE 5 Shoot and root dry weight of DsRed chimeric coffee plants (control) and 35S::*SHN* chimeric coffee plants submitted to drought treatment. Statistical significance verified by Tukey test to 0.5%. Error bar indicates standard deviation.

The root system is the primary site of soil environment perception and the root characteristics and growth play a crucial role in maintaining the water supply to the plant. At the end of this experiment the roots of DsRed coffee plants (control) submitted to drought were dry, while the roots of the 35S::*SHN2* coffee plants were well branched and deeper. It cannot be ruled out that changes in the root system structure caused increased efficiency of the overexpression lines for water uptake under water restriction. The drought adapted coffee plants are often characterized by deep and vigorous root systems (Da Matta, 2006), Ramos & Carvalho (1997), working with 29 coffee genotypes, have associated drought tolerance with larger root dry mass, which should be related to root distribution and structure, and not quantity, in the determination of the most efficient strategy for extracting water during drought periods. Indeed, the ability to postpone drought effects in *C. canephora* clones has been associated with deeper, but not necessarily larger root systems, in terms of dry biomass (Pinheiro

et al., 2005). Aharoni (2004) observed in *Arabdopsis thaliana* 35S::*SHN2* that the root structure had longer lateral roots compared with the wild type.

It is hard to imagine that a single gene could confer full drought tolerance, as this trait is known to be determined by multiple genes. However, despite our limited knowledge of stress-associated cell metabolism (Vinocur & Altman, 2005), single transgene encoding transcription factors have been reported to confer improved drought resistance (Kasuga et al., 1999; Jeanneau et al., 2002; Yang et al., 2005; Karaba et al., 2007, Aharoni, 2004). And the results shown in chapter II also indicated that expression of *SHN2* in roots alone is already sufficient to protect plants against drought, corroborating the results with coffee.

Under drought, plant cells react defensively by initiating several signal transduction pathways that result in the accumulation of different transcripts, proteins, sugar molecules and lipophilic anti-oxidants, almost always concomitant with increased ABA levels (Cuming et al., 2007; Ramanjulu & Bartels, 2002). Different members of the AP2/EREBP family can regulate genes involved in pathogenesis, cold, drought, ethylene, and jasmonate responses (Memelink et al., 2001). Such response cascades could be executed by direct regulation of metabolic pathways, which lead to the production of metabolites essential for plant survival. The SHN transcription factor possibly permits a quicker response to hydric stress, activating the transcription of genes responsible for stress signaling and response, and this can occur in the roots where the transcription factor is being overexpressed and/or the transcription factor can be transported to the aerial part, increasing the water use efficiency (WUE) helping the plant conserve more water and delaying the drought situation.

7 CONCLUSION

1. The *SHINE2* gene of *Arabidopsis thaliana* overexpressed in transgenic hairy roots of *Coffea arabica* can improve the drought tolerance of such chimeric plants.

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