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Miguel Ângelo Almeida Rito

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Dissertação no âmbito do Mestrado em Biodiversidade e Biotecnologia Vegetal orientada pela Doutora Sandra Isabel Marques Correia e pelo Professor Doutor Jorge Manuel Pataca Leal Canhoto apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia
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List of Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid
4-Cl-IAA: 4-chloroindole-3-acetic acid
ABA: abscisic acid
AC: long-term callus
AFB: *AUXIN SIGNALING F-BOX*
AG15: *AGAMOUS-LIKE 15*
AGO: ARGONAUTE protein
ATP: adenosine triphosphate
AUX1: AUXIN TRANSPORTER PROTEIN 1
BAP: 6-benzylaminopurine
BBM1: *BABY BOOM 1*
cDNA: complementary DNA
CQ: gene expression values in qPCR
DCL1: DICER LIKE 1 protein
DDL: DAWDLE protein
DEPC: diethylpyrocarbonate
DNA: deoxyribonucleic acid
EtOH: ethanol
EC: embryogenic callus
FDE: denaturing loading buffer
GRF: *GROWTH RESPONSE FACTORS*
IAA: indole-3-acetic acid
IBA: indole-3-butyric acid
HCL: hydrochloric acid
HYL1: *HYPONASTIC LEAVES 1*
KOH: potassium hydroxide
LEC1: *LEAFY COTILEDON 1*
LEC2: *LEAFY COTILEDON 2*
MIR: MIRNA genes
miRNA: micro RNA
MS: Murashige and Skoog medium
NAA: 1 – Naphthaleneacetic acid
NEC: non-embryogenic callus
RNA: ribonucleic acid
RNAse: ribonuclease
RISC: RNA-induced silencing complex
SE: somatic embryogenesis
SERK: *SOMATIC EMBRYOGENESIS RECEPTOR KINASE*
PAA: 2-phenylacetic acid
PEM: pro-embryogenic cell mass
PIN: PIN-FORMED
PGR: plant growth regulator
qPCR: quantitative PCR
TF: transcription factor
TIR1: *TRANSPORT INHIBITOR RESPONSE 1*
TD: SE induction media with 2,4-D
TP: SE induction media with Picloram
UV: ultraviolet
WUS: *WUSCHEL*

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Abstract

Somatic embryogenesis (SE) is a cloning technique that allows the development of embryos from tissues other than the zygotic embryo. In order to induce embryogenic competence, the explants must be exposed to auxins or to other different stresses. In the case of *Solanum betaceum,* this induction stage will promote the formation of callus that can be embryogenic (EC) or non-embryogenic (NEC) by transferring EC to culture media devoid of auxins and with lower sucrose concentrations, embryos and plantlets develop. Thus, auxin pathway is essential in the regulation of SE, involving genes such as *TIR*, *AFB* or *GRF*'s that play a key role in auxin-mediated plant development processes.

MicroRNA's (miRNA's) are small, single-stranded RNA's known to function as post-transcriptional regulators of genes, repressing their expression through endonucleotidic cleavage. Two of those miRNA's are miR393 and miR396 that play important roles in plant growth, development and maturation. Both are crucial in the auxin pathway, by regulating *TIR* and *AFB* genes expression (miR393), and the expression of *GRF*'s genes (miR396).

This study aimed to analyse the expression of miR393 and miR396 and their target genes during tamarillo's (*Solanum betaceum* Cav.) SE from zygotic embryos and leaf explants. Total RNA and small RNA's enriched samples were extracted from embryogenic and non-embryogenic callus as well as from explants collected throughout the SE induction and embryo development processes. RNA samples were reverse transcribed into cDNA. Total RNA was used for the quantification of the expression levels of the target genes whereas the small RNA's were used for the miRNA's expression quantification by qPCR analysis.

Results show that stage and tissue-specific expressions of miR393 and miR396 and their targets suggest their possible modulation on tamarillo SE where both miRNA's share a strong inverse correlation with the corresponding target genes. MiR393 and miR396 seem to downregulate their target genes expression values during SE induction, while during somatic embryo development and germination, target genes expression values increased (miR393 - *TIR1* and *AFB2*; miR396 - *GRF1* and *GRF4*).

These results provide new insights into embryogenic competence acquisition by tamarillo tissues, as well as how that competence is maintained during callus subcultures and expressed during embryo development.

Keywords: *AFB2*, auxins, *GRF1*, *GRF4*, miR393, miR396, somatic embryogenesis, tamarillo, *TIR1*.

Resumo

A indução de embriogénese somática (ES) é uma técnica de clonagem que permite a obtenção de embriões a partir de outros tecidos além do embrião zigótico. De maneira a induzir competência embriogénica, os explantes devem ser expostos a auxinas ou outros stresses. Em *Solanum betaceum,* a etapa de indução irá promover a formação de calos que tanto podem ser embriogénicos (CE) ou não-embriogénicos (CNE) e apenas transferindo massas de CE para um meio de cultura sem auxina e com baixas concentrações de sacarose, os embriões e as plantas se desenvolvem.

A via de sinalização das auxinas é portanto essencial para a regulação da ES, e genes como *TIR, AFB* ou *GRF*'s são fulcrais em processos de desenvolvimento de plantas mediados por auxinas.

MicroRNA's (miRNA's) são pequenos RNA's de cadeia única que funcionam como reguladores pós-transcricionais, reprimindo a expressão por clivagem endonucleotídica. Dois destes miRNA's são o miR393 e o miR396 que desempenham papéis importantes no crescimento, desenvolvimento e maturação de plantas. Ambos são cruciais na via das auxinas, regulando a expressão dos genes *TIR* e *AFB* (miR393) e da família dos *GRF*'s (miR396).

Este estudo visou a analisar a expressão dos miR393 e miR396 e dos seus genes alvo durante o processo de ES em tamarilho (*Solanum betaceum* Cav.), a partir de embriões zigóticos e folhas. Amostras de RNA total e enriquecidos em smallRNAs foram extraídas de calos embriogénico e não embriogénico assim como de explantes recolhidos durante a indução de ES e durante o processo de desenvolvimento dos embriões somáticos. As amostras de RNA foram convertidas em cDNA por transcrição reversa. O RNA total foi usado para a quantificação dos níveis de expressão dos genes alvo enquanto que as amostras de smallRNAs foram usadas para quantificar a expressão dos miRNAs, por análise de PCR quantitativo.

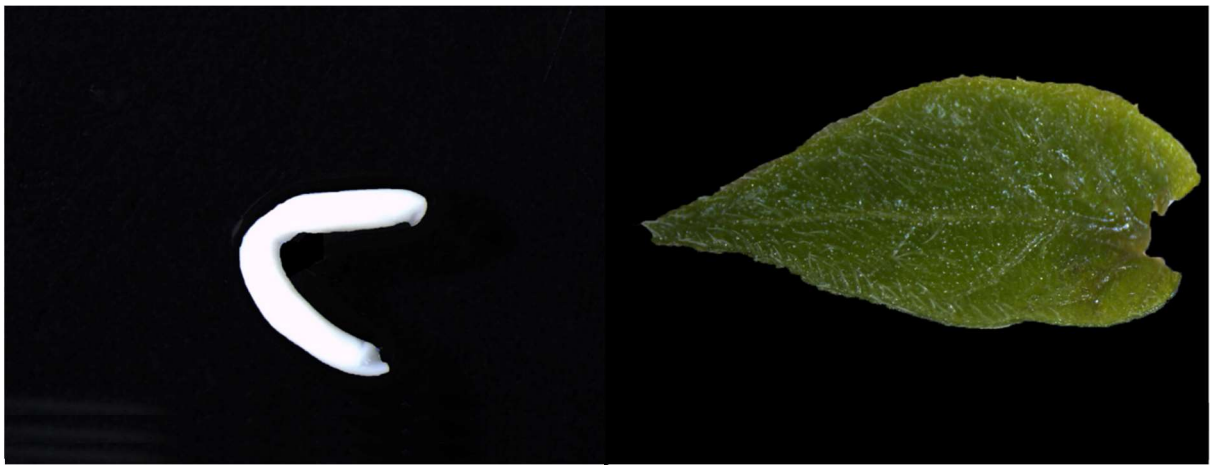
Os resultados mostram que as expressões dos miR393, miR396 e dos seus genes alvo, durante os vários estágios de ES e nos diferentes calos, sugerem a sua modulação durante a ES em tamarilho, onde ambos os miRNA's compartilham uma forte correlação inversa com os genes alvo correspondentes. Mir393 e miR396 parecem regular negativamente os seus genes alvo durante a indução de ES, sendo que durante o desenvolvimento e germinação dos embriões somáticos, os valores de expressão dos genes alvo aumentam (miR393 - *TIR1* e *AFB2*; miR396 - *GRF1* e *GRF4*).

Estes resultados fornecem novos conhecimentos sobre a aquisição de competência embriogénica a partir de diferentes explantes de tamarilho, assim como sobre o modo

como essa competência é mantida durante as subculturas de calos e expressa durante o desenvolvimento dos embriões.

Palavras-chave: *AFB2*, auxinas, *GRF1*, *GRF4*, embriogénese somática, miR393, miR396, tamarilho, *TIR1.*

1. Introduction



1.1. Work contextualization

The development of plant cell, tissue and organ culture has been crucial to biotechnology. These tools have a big impact on economically important crops that are commercialized worldwide. The applications of plant biotechnology on agronomy range from increasing crop productivity to the production of more resistant plants to biotic and abiotic stresses (Karami *et al*., 2009; Loyola-Vargas and Ochoa-Alejo, 2016). In vitro culture is an important subject in plant biotechnology; it consists in the establishment and maintenance of cells, tissues, plants or callus in controlled conditions (Chawla, 2010) allowing the study of different physiological processes (Loyola-Vargas *et al.*, 2006). At the industrial level, the first application of *in vitro* techniques was the micropropagation of plants, as this process aims at rapid multiplication, in large quantities, of plants that are genetically identical (clones) to the mother plant (Loyola-Vargas *et al*., 2006). Micropropagation can be achieved through 3 distinct processes: the proliferation of already existing meristems, in which axillar and apical shoots are used, organogenesis induction, and development of somatic embryos (Chawla, 2010).

Somatic embryogenesis is a powerful biotechnological tool with application in plant breeding, propagation, conservation strategies, genetic transformation and cryopreservation of elite genotypes (Corredoira *et al*., 2017). In recent years protocols for the cloning of economically important plants like the coffee plant, sweet orange, cotton, maize, eucalyptus and pine trees through somatic embryogenesis have been established (Fehér, 2015).

In order to induce somatic embryogenesis, the explants must be exposed to several stress conditions such as PGR's and abiotic stresses like extreme temperatures, oxidative stress, pH and others (Nic-Can *et al*., 2016). PGRs, such as auxins, play a crucial role in somatic embryogenesis induction, mainly in the dedifferentiation process where differentiated cells shift their gene expression patterns in order to become totipotent cells with embryogenic capability (Elhiti *et al*., 2016). To obtain somatic embryos from embryogenic masses the culture media must be devoid of these auxins (Fehér, 2015; Nic-Can and Loyola-Vargas, 2016).

During dedifferentiation, the cells are exposed to epigenetic mechanisms like chromatin remodelling, DNA methylation and action of small RNA's, including micro RNA's (miRNA). Epigenetics is responsible for the change in the DNA and gene expression, independent to the normal DNA sequence variation. Several authors have shown that miRNAs regulate a wide variety of genes responsible for somatic embryogenesis and are

a promising epigenetic tool for plant breeding and in biotechnological research (Mahdavi-Darvari *et al*., 2014; De-la-Peña *et al*., 2015; Blein and Laufs, 2016).

Solanum betaceum Cav. (tamarillo) has been a well-studied plant at the Laboratory of Plant Biotechnology of the Department of Life Sciences of the University of Coimbra (LBV-UC), where the first somatic embryogenesis induction protocol for this species was established (Guimarães *et al*., 1988). The reasons for the importance of this tree solanaceous species as a model do analyse somatic embryogenesis and other micropopagation techniques are based on the ability of different explant to embark upon an embryogenic pathway (Canhoto *et al*., 2005; Correia *et al*., 2011; Correia *et al*., 2012).

This work aims to analyse and compare the different expression levels of miRNA/target gene pairs throughout somatic embryogenesis.

1.2. Plant regeneration

The ability of plant to live in a wide range of diverse environments results from their ability to adapt and to transform in order to survive in an ever-changing environment. Due to their inability to run away from adverse conditions, plants have to cope with a variety of different stimuli in the exact place where they live. Thus, they have developed different strategies to adapt to an ever-changing environment, usually by modifying their pattern of development (Sultan, 2000). The unlimited growth capability of the plants enables them to optimize their resource exploitation and also their capacity to survive in unfavourable conditions (Scheres, 2007).

The meristems are tissues with undifferentiated cells called stem cells (initial cells). These cells have a high capacity to multiply indefinitely and differentiate/specialize in new tissues or organs, leading to continuous and unlimited growth (Medford, 1992; Scheres, 2007). In plants, organ differentiation is usually post-embryonic, continuous and influenced by the environment (Fehér, 2015). In order to survive to environment attacks, such as storms, fires, snow frost, and living organisms' attacks such as herbivores and pathogens, plants ensure their survivability using their regeneration capability. This regeneration capability is astonishing: it can heal small wounds but also regenerate all organs by *de novo* organogenesis (Irish, 2008) or even regenerate the whole plant from one cell by somatic embryogenesis (Loyola-Vargas and Ochoa-Alejo, 2016). Both processes are already used in *in vitro* conditions, as they are powerful tools in plant micropropagation industry for a large variety of plants used in agriculture (Loyola-Vargas and Vásquez-Flota, 2006).

In recent years, molecular analysis has been growing in the need to understand the molecular background of the different pathways of plant regeneration (Bhatia and Bera, 2015). Fascinating observations regarding the molecular mechanisms of plant regeneration have been demonstrated by the application of modern genetic, transcriptomic, epigenetic and cellular imaging approaches (Fehér, 2015).

Somatic plant cells are very flexible in terms of differentiation when compared to animal ones. It is also very well known that differentiated plant cells, under certain conditions can dedifferentiate and regain totipotency, meaning that these cells, can regress their original state and revert into an earlier developmental one. This is usually achieved when cells are exposed to hormones or PGRs (plant growth regulators) and/or environmental conditions (Sugimoto *et al*., 2011; Fehér, 2015).

Dedifferentiation is not the only way responsible for plant regeneration ability. In the published articles by Sugimoto *et al*. (2011; 2012) regeneration is also achieved by adult stem cells that situate around the veins throughout the plant body. In this case, cells differentiate, leading to a regenerated material. Nevertheless, plant regeneration may be achieved either by adult stem cells differentiation or through the dedifferentiation process (Fehér, 2015).

Although this finding might raise some questions about dedifferentiation and totipotency inheritance it cannot explain every plant regeneration pathways such as somatic embryogenesis, where embryo development induction is achieved through a differentiated plant cell, considered to be one of the most investigated and least understood types of plant regeneration capacity (Fehér, 2015). This pathway is classified as the strongest argument for the totipotency inheritance of already differentiated cells in plants (Fehér, 2015; Loyola-Vargas and Ochoa-Alejo, 2016).

1.3. Somatic embryogenesis

The first reports on successful somatic embryogenesis were presented by Steward *et al*. (1958) and Reinert (1958) in carrot tissue cultures. This technique is an asexual form of plant propagation that mimics the events of zygotic embryo development (Figueroa *et al*., 2006). As aforementioned, somatic embryogenesis is a cloning technique that allows the formation of embryos from non-zygotic origin (Loyola-Vargas *et al*., 2006). It is also used for cytological, physiological and molecular studies of plant embryogenic development patterns (Figueroa *et al*., 2006). From a mechanistic point of view, it represents a complete model of totipotency involving the action of a complex signalling

cascade chains and also gene expression patterns reprogramming (Méndez-Hernández *et al*., 2019). The genes are usually regulated to respond to external factors such as PGR's and stress conditions during culture (Nic-Can *et al*., 2016).

The mechanisms involved in SE are very complex and can be hardly reproduced in every plant although the process is similar among different species (Elhiti, 2010). Zygotic embryos are usually better explants to be used in SE induction of woody plants, followed by seedling explants, cotyledons and hypocotyls (Bonga *et al*., 2010; Corredoira *et al.*, 2019). The efficiency of SE in many woody plants is still very low and many studies have been continuously carried out to upgrade and refine SE protocols. This is due to the fact that woody species can be extremely recalcitrant in *in vitro* embryogenesis from non-zygotic material (ex: leaf tissues) (Klimaszewska *et al*., 2007; Correia *et al.*, 2011). In these group we can include *Quercus robur*, *Q. suber* and *Q. Ilex* (Corredoira *et al.*, 2014), *Eucalyptus globulus* and *Eucalyptus saligna* (Corredoira 2015), *Solanum betaceum* (syn. *Cyphomandra betacea*; Correia *et al*., 2011), and *Acca sellowiana*, considered to be good reference systems for SE in these type of plants (Guerra *et al*., 1997; Cristofolini *et al.*, 2014; Correia *et al*., 2016). In order to overcome recalcitrance and other hindrances, numerous factors thought to influence the embryogenic pathway must be analysed in to achieve suitable and efficient protocols for SE induction. SE is also an efficient tool to regenerate transgenic plants obtained *Agrobacterium spp.*, particle bombardment and chemical-mediated genetic transformation protocols (Loyola-Vargas and Ochoa-Alejo, 2016).

Economically important crops and model plants have been transformed and regenerated using SE systems (Loyola-Vargas and Ochoa-Alejo, 2016). Other studies about secondary metabolites production, using SE, have been released as documented in Loyola-Vargas and Ochoa-Alejo (2016). It was mentioned that these secondary metabolites are present in zygotic embryos and thus, they also may accumulate in somatic embryos.

Somatic embryogenesis can be divided into various stages. First, the induction phase, in which cells regain totipotency after dedifferentiation. Dedifferentiation means the regression of once differentiated cells into a less differentiated stage, considered to be a transient stem cell-like stage, regaining totipotent capacity. These cells can later redifferentiate, or more correctly, transdifferentiate into newly differentiated cells (Fehér, 2015). In order to induce this process of dedifferentiation, cells may be mechanically wounded and/or placed into cultures with appropriate conditions for induction. When induced, the original cells dedifferentiate and by cellular division start to produce an unorganized cell mass that is called callus (Ikeuchi *et al*., 2013). As mentioned before, for

some species, the callus can have both embryogenic and non-embryogenic cells. After several cell divisions, a pro-embryogenic cell mass (PEM) results from the embryogenic callus. From these PEM's somatic embryos can be develop (de Vries, *et al*., 1988) but new PEM's also form by proliferation (Steiner *et al*., 2016). In SE, these PEM's form in response to PGR's when present in the media and in order to form somatic embryos, PGR's must be removed to allow the PEM to differentiate (transdifferentiate) and form new somatic embryos (Von Arnold *et al*., 2002)

In the second stage of somatic embryogenesis, the dedifferentiated cells express the acquired embryogenic potential, resulting in a reorganization of cell physiology, metabolism and gene expression, and in which, somatic embryos are formed. In the end, these somatic embryos develop and germinate into new plantlets that are genetically identical between them and to the original mother-plant (Correia *et al*., 2011; Loyola-Vargas, 2016).

1.3.1. Somatic embryogenesis as a model to study embryogenesis

Somatic embryos are morphologically very similar to their zygotic counterparts. The fact that somatic embryos go through the same developmental phases that zygotic embryos, although with some differences, makes somatic embryogenesis a good tool to study embryo development at molecular, cellular and tissue levels (Fehér *et al*., 2003; Fehér, 2015). The biggest difference is the origin of the embryos, since the zygotic embryo results from fertilization between two gametes whereas the somatic embryos result from somatic cells that gained embryogenic capability by exposure to different stimuli (Fehér *et al*., 2003). Also, somatic embryos display a higher number of cells, are usually bigger and have a less organized surface when compared to their analogous zygotic embryos (Dodeman *et al*., 1997; Willemsen and Scheres, 2004).

These differences are related to the fact that there is no maternal constraints conditioned by the ovular tissues, but also physiologically nurtured by the maternal tissues, at least during a large period of their development (Hehenberger *et al*., 2012). Furthermore, zygotic embryos have a dormant phase since they have a seed surrounding it, but the same doesn't usually apply to somatic embryos. This makes the maturation/development phase relatively different between them. The somatic embryos develop quicker, passing the various embryo forms, globular, heart and torpedo shapes and cotyledonary stages (Méndez-Hernández *et al*., 2019) until they are ready to convert into a plantlet (Dodeman *et al*., 1997; Suhasini *et al*., 1997; Quinga *et al*., 2018). Since somatic embryos don't have a dormant stage, when they reach the cotyledonary stage

and full maturity, they are ready to initiate the shoot meristem, the seedling starts to grow and a new plantlet is formed (Yang and Zhang, 2010).

From a molecular perspective, both zygotic and somatic embryogenesis are dependent on the same key regulatory genes that have been extensively studied because of their importance in embryogenesis. They are classified as multifunctional regulators (usually transcription factors, TFs) and they are essential in the embryogenesis development (Elhiti *et al*., 2013). The most studied are *WUSCHEL* (*WUS*), the *LEAFY COTYLEDON 1* and *LEAFY COTYLEDON 2* (*LEC1* and *LEC2*), *AGAMOUS-LIKE 15* (*AG15*), BABY BOOM 1 (*BBM1*) and the *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) (Fehér, 2015). Beyond these key genes, many other genes expression is shared between both types of embryogenesis, in fact, the overall gene expression patterns in zygotic and somatic embryos are very similar (Fehér, 2015). The main difference between zygotic and somatic gene expression is in the expression of stress-related genes in somatic embryos mostly related to the effect of *in vitro* culture conditions in which PGR's responsive genes are included (Lin *et al*., 2014).

1.3.2. Stress, an essential SE inductor

Environment stress and high concentration of PGR's can trigger the dedifferentiation of differentiated cells that regain totipotency capability, resulting in a pathway for somatic embryo formation. These conditions to induce SE can be variable from species to species, and different PGRs and stress conditions are able to initiate embryo development (Zavattieri *et al*., 2010; Loyola-Vargas and Ochoa-Alejo, 2016).

Auxins and cytokinins are the most used PGRs on SE induction, working as key regulators of plant cell division (Jiménez, 2005; Singh and Sinha, 2017). The auxin/cytokinin ratio influences the morphology pathway taken by the cells. High and low ratios of cytokinins to auxin favours shoot and root *in vitro* regeneration, respectively, while a more balanced ratio promotes the formation of callus (Skoog and Miller, 1957). The most used PGR in SE induction is an auxin herbicide named 2,4-dicholophenoxyacetic acid (2,4-D; Song, 2014; Nic-Can and Loyola-Vargas, 2016). Because of its stress promotion effects, many of the transcription factors that are expressed throughout SE are stress-related (Gliwicka *et al*., 2013). It was also shown that 2,4-D also increases endogenous auxin levels in cells, namely the concentration of indole-3-acetic acid (IAA; Jiménez, 2005). 4-Amino-3,5,6-trichloropicolinic acid, commonly known as Picloram, is also considered a synthetic auxin like 2,4-D and acts in the same way, both as auxin and herbicide (Grossmann, 2007).

Other stress-related PGRs that can also play important roles in SE induction are abscisic acid (ABA), that can provoke a response in certain cases, helping in the formation of somatic embryos (Sidiqui *et al*., 1998; Nishiwaki *et al*., 2000), and ethylene that when in excess, can provoke embryo induction disruption (Bai *et al*., 2013). Gibberellic acid (GA_3) may also be used in the development of somatic embryos (Gupta and Chakrabarty, 2013). These PGRs influence the spatial and temporal expression of a panoply of genes in order to initiate the genetic shift of the somatic cells and also during its transition to somatic embryos and embryo development (Fehér, 2015)

Besides exogenous PGRs, other stress conditions can be used to initiate and/or promote embryo formation like osmotic stresses, drought, temperature, extreme pH, hypoxia, UV radiation, heavy metals, mechanical injuring and chemical treatments (Zavattieri *et al*., 2010).

Different inductive conditions must be chosen according to different species, genotypes, development stage etc. This is due to the fact that the characteristics of the explant influence its endogenous auxin levels. For different levels of endogenous auxin, different sets of conditions must be established in order to induce SE, where explants with a higher level of endogenous auxins are more responsive to the induction treatments (Jiménez, 2005). In conclusion, the plant species, genotypes, type of explant, culture media, PGRs and stress condition play a huge role in the embryogenic capability and explains the high variability of treatments used (Fehér, 2015).

1.3.3. Role of auxins in somatic embryogenesis

Auxins regulate a wide number of physiological and developmental processes such as cell division, expansion and differentiation. They are also known to be involved in tropism mechanisms, embryo seed and fruit development, and acquisition of cell totipotency (Kepinski and Leyser, 2005). Indole-3-acetic acid (IAA) is the most predominant form of endogenous auxins and was discovered by Kenneth V. Thimann in the 1920s (Friml *et al*., 2003; Grones and Friml, 2015). Discoveries of other auxins have been reported ever since, such as indole-3-butyric acid (IBA), 2-phenylacetic acid (PAA) and 4-chloroindole-3-acetic acid (4-CI-IAA). Because IAA is considered to be the most important of auxins, fewer studies are conducted on other auxins although their mechanisms may be similar to the ones in IAA (Taiz and Zeiger, 2006; Simon and Petrásek, 2011)

Auxins regulation is based on its presence in the various parts of the plant, either by local biosynthesis or directional polar transport between the cells and conjugation with

sugars or amino acids (Petrasek and Friml, 2009). Although several PGRs may be used to induce SE, auxins play the most relevant role (Altamura *et al*., 2016). Auxins are key during early and post-embryonic plant development (Elhiti *et al*., 2016). It has been theorized that SE regulation by auxins is performed by establishing auxin gradients during its induction, so dedifferentiation starts and cell division of these cells can happen before embryogenic competence is displayed (Jiménez, 2005). In a recent work by Márquez-López *et al*. (2018) it was shown that in order to initiate embryo formation, auxins must be distributed asymmetrically by polar transport.

This cell-to-cell transport is achieved by the activity of two main proteins: the AUXIN TRANSPORTER PROTEIN 1 (AUX1) an influx protein transporter (Swarup *et al*., 2004) and a group of PIN-FORMED (PIN) auxin efflux carriers (Zhou and Luo, 2018). The reason why auxins are distributed asymmetrically is due to the interaction of the influx and efflux transporters, combined with the activity of cell membrane H^+-ATPases that hydrolyse ATP while maintaining the apoplast at a pH value about 100 times lower than in the intracellular environment (Weijers *et al*., 2005).

In somatic embryogenesis little is known about endogenous levels of auxins in the initial stages of embryogenesis induction or its location in SE but some studies already enlightened some information about this case. In a work by Caeiro (2015), where endogenous levels of auxins were measured during SE induction in *Solanum betaceum*, synthetic auxins like Picloram and 2,4-D triggered specific stress responses in the inducing cells, causing endogenous auxin levels to rise, namely IAA, that is necessary for the embryogenic capability of PEM's. Is also relevant to point out that embryogenic callus had higher levels of endogenous IAA when compared to non-embryogenic callus.

In other paper by Corredoira *et al*. (2017) levels of endogenous IAA were also measured during white oak (*Quercus alba*) SE. It was observed that prior to embryogenesis induction, endogenous levels of IAA were low. After 4 weeks cells of the PEMs showed a higher IAA concentrations than the initial explants and also when compared to non-embryogenic callus IAA levels. It was also observed in this study that early somatic embryos also displayed high levels of endogenous IAA. The same also happens in zygotic embryos during their early development stages. This occurs because of the cell division and growth roles that auxins display in plants (Robert *et al*., 2013). On later stages of embryo development, namely the transition from the globular to the heart-sage embryo, auxin concentrations must decrease, in order to further develop the embryo (Suprasanna and Bapat, 2005). Yang *et al*. (2012) also observed that IAA concentrations after SE induction were 90% higher when compared to its beginning and that these concentrations would lower somatic embryo development, namely in the globular stage.

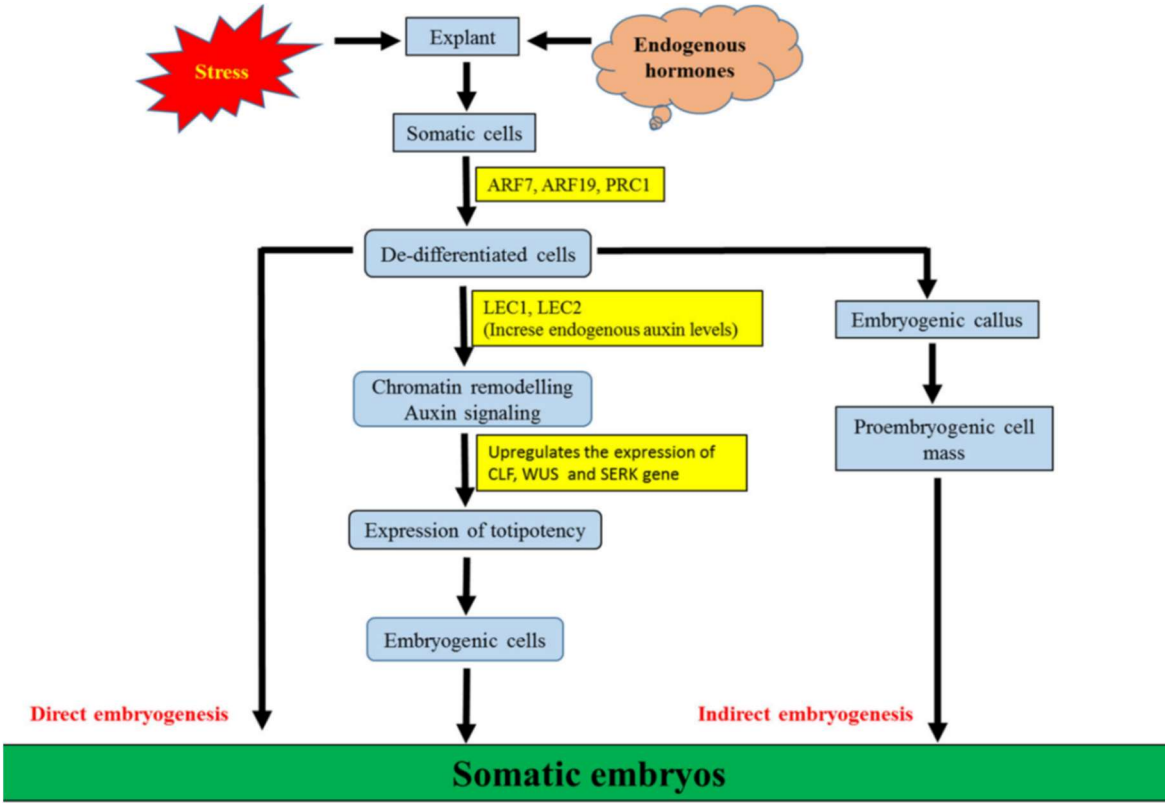


Figure 1. Different pathways of somatic embryogenesis induction. Endogenous and exogenous signals are required in order to induce SE. SE induction can be performed in two ways. By direct embryogenesis or indirect embryogenesis through the PEM formation (Kumar and Van Staden, 2017).

1.4. Epigenetic regulation of somatic embryogenesis

Epigenetics refers to changes in gene expression patterns that are independent of the normal DNA sequence variation and their mechanisms are very dynamic (Zhang and Hsieh, 2013). Epigenetics is a subject of high interest in the scientific community because it represents the link between genes and external factors, such as the environment and other stresses that induce phenotypic variation and “epiallelic” transmission (Kakutani, 2002), thus being an interesting topic in many scientific subjects including plant biotechnology.

Somatic embryogenesis is under the control of a number of metabolic, hormonal, genetic and epigenetic factors (Elhiti, 2013). Epigenetic mechanisms are critical factors in the SE signalling pathway, changing the genetic patterns of the cells embarking into an

embryogenic pathway. The differentiated cells are forced to adapt to a new environment during SE induction and are fully reprogrammed in order to dedifferentiate (Elhiti, 2013; Méndez-Hernández *et al*., 2019). Only after genetic reprogramming, those cells are ready to respond to the various stimulus and conditions in the media. In order to allow a change in the overall gene expression patterns, chromatin must be structurally reorganized (Wagner, 2003; Arnholdt-Shmitt, 2004), through involves mechanisms such as DNA methylation, or histone modifications that allow the expression or repression of genes. Gene regulation mediated by miRNA's is also an epigenetic mechanism involved in SE (Miguel and Marum, 2011; Neelakandan and Wang, 2012; Mahdavi-Dravari *et al*., 2015). These epigenetic mechanisms together with changes in hormonal pathways are pivotal to respond to stress conditions, regulating the explant development (Fehér, 2015).

1.4.1. DNA methylation

The most characterized epigenetic mechanism, DNA methylation is a chemical modification of the DNA without altering its original sequence (Neelakandan and Wang, 2012). A methyl group is added to the DNA in a specific location, usually to a cytosine nucleotide, in order to attenuate or repress the transcription of a certain gene, thus lowering its expression, leading to a transcriptionally inactive conformation and/or gene silencing. The phenomenon is considered to be a primary and heritable epigenetic mechanism that enables the suppression of specific DNA sequences (Mahdavi-Darvari *et al*., 2014). This mechanism is crucial to control development programs (Chan *et al*., 2005; Feng *et al*., 2010; Zhang, 2010). During SE, DNA methylation is also responsible for the change in the expression of stress response genes (Van Zanten *et al*., 2013).

Methylation also affects SE induction because during early embryo development, DNA methylation continues to change in response to the stress factors (De-la-Peña *et al*., 2015). Recent data have shown that DNA hypomethylation is necessary to induce SE (De-la-Peña *et al*., 2015). Global DNA methylation in the explants decreases when SE is induced, that is, when dedifferentiation proceeds. Higher methylation accumulation on the embryogenic callus affects the embryogenic competence, translating to a loss of embryogenic capacity to form somatic embryos in culture. In fact, embryogenic tissues some species, such as *Eleutercoccus senticosusand*, *Pinus nigra* and *Solanum betaceum*, have lower global DNA methylation when compared to non-embryogenic tissues (De-la-Peña *et al*., 2015; Sanches, 2017). Also in *S. betaceum*, a hypomethylation moment precedes the beginning of embryo formation although DNA methylation increases during the development phase (Sanches, 2017).

DNA methylation has proved to be involved on SE of many other species like in carrot (*Daucus carota*) (LoSchiavo *et al*., 1989), *Medicago truncatula* (Santos and Fevereiro, 2002), *Pinus nigra* (Noceda *et al*., 2009), thus, showing how important this mechanism is in SE differentiation and development.

1.4.2. Chromatin remodelling

Chromatin is a complex of DNA and histones, located in the nucleus of eukaryotic cells. Histones help in the arrangement of DNA into nucleosomes and these nucleosomes consist of DNA of around 145 base pairs covered by the histones H2A, H2B, H3 and H4 (Butler, 1983; Mahdavi-Darvari *et al.*, 2015). This structure holds the large mass of DNA present in the cell nucleus and facilitates DNA replication and gene expression as well (Mahdavi-Darvari *et al*., 2015). It reinforces the DNA macromolecule to allow replication and the expression of genes (Kumar and Van Staden, 2017). Chromatin can be conformed into two very distinct ways, either heterochromatin or euchromatin. Heterochromatin is more condensed and thus transcriptionally inactive, preventing gene expression, while euchromatin occurs in a more diffuse, transcriptionally active state, resulting in the activation of gene expression (Jarillo *et al*., 2009).

It has been demonstrated that cellular dedifferentiation is related to a global chromatin remodelling that results in alterations of gene expression patterns (Avivi *et al*., 2004). This has been studied in *Arabidopsis thaliana* (Grafi *et al*., 2007), in *Nicotiana tabacum* (Williams *et al*., 2003). *Coffea canephora* (Nic-can *et al*., 2013) and others. Chromatin remodelling is also involved in the genome stability maintenance and plant development (William *et al.*, 2003; Avivi *et al*., 2004).

The different types of histone modifications are histone acetylation, methylation and ubiquitination (Pfluger, 2007). Many studies have indicated that histone modification plays a central role on stress response in plants (Park *et al*., 2008; De-la-Peña *et al*., 2015). Among histone methylation, H3K9 methylation is one of the major epigenetic marks for gene silencing. It relates to DNA methylation, being essential in the heterochromatin formation (Saze *et al*., 2003). In a work, by Sanches (2017) the role of H3K9 in *S. betaceum* SE was studied, reporting that H3K9 methylation levels were lower in embryogenic callus when compared to non-embryogenic callus.

1.4.3. MicroRNA Regulation

MicroRNAs are small, non-protein coding regulatory RNAs that have shown to play important roles in many of developmental processes such as defence, response to environment and other factors that induce stress, hormone response and others (Bartel, 2009; Trindade *et al*., 2011; Sunkar *et al*., 2012). These miRNAs are more stable than ribosomal RNA (rRNA), small nuclear RNA (snRNA) and protein-coding messenger RNA (mRNA). For miRNA detection, microarrays, northern blot, real-time quantitative PCR (qPCR) and degradome sequencing can be used being qPCR the best tool (Zhang *et al*., 2015; Siddiqui *et al*. 2018). First discovered in *Caenorhabditis elegans* (Lee and Ambros, 2001), these small RNAs sequences of 21-24 nucleotides are derived from single-stranded RNA hairpin precursors transcribed by RNA polymerase II and are the products of *MIRNA* (*MIR*) genes (Fig. 2A).

The different members of the different *MIR* family of genes are expressed in a developmental and tissue-specific manner and in response to PGRs and/or stress stimuli (Zhao *et al*., 2007; Kruszka *et al*., 2014; Szyrajew *et al*., 2017). In order to mature in plants, the activity of the type III endoribonuclease DICER LIKE 1 (DCL1) enzyme is essential (Tang *et al*., 2003; Bartel, 2004; Jia *et al*., 2011). The pri-miRNA (primary MIR transcripts) are cleaved by DCL1, accompanied by the double-stranded RNA binding protein HYPONASTIC LEAVES 1 (HYL1), the C2H2-zinc finger protein SERRATE, and two binding proteins named CBP20 and CBP80/ABH1. In addition DAWDLE protein (DDL) stabilizes the pri-miRNA and facilitates the maturation of miRNAs (Yu *et al*., 2008; Voinnet, 2009; Szyrajew *et al*., 2017). After miRNA maturation, miRNAs are transported from the nucleus to cell cytoplasm where they bind to a protein of the ARGONAUTE family (AGO). This bound results in the formation of the RNA-Induced Silencing Complex (RISC; Fig. 2B) that is responsible for the recognition and silencing of the target genes that are complementary to the miRNA sequence (Baumberger and Baulcombe, 2005; Szyrajew *et al*., 2017). This miRNA complex directs the post-transcriptional silencing of the target gene, in the form of mRNA, by cleavage or translation repression (Brodersen *et al*., 2008; Blein *et al*., 2016; Szyrajew *et al*., 2017).

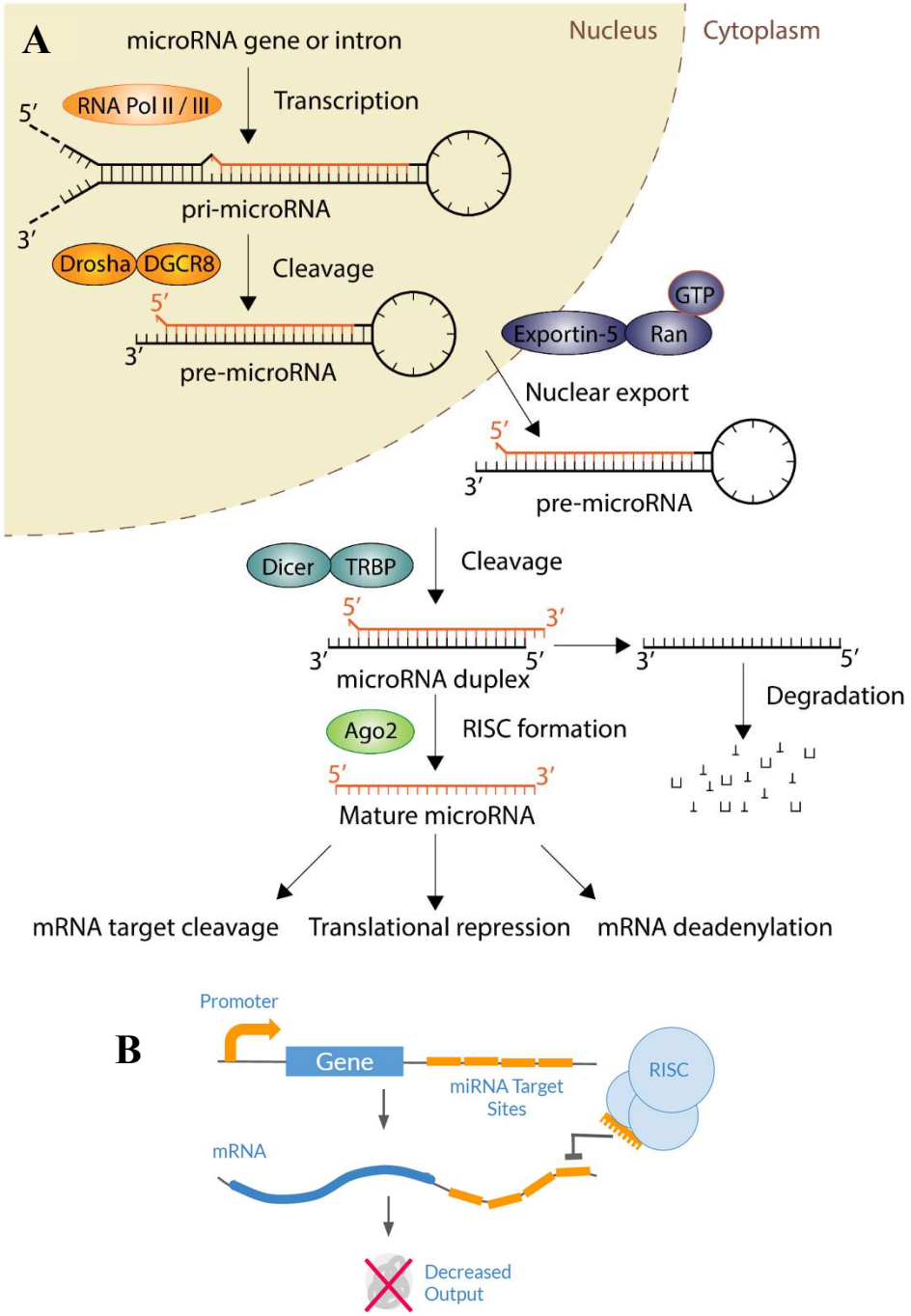


Figure 2. miRNA biogenesis and post-transcriptional silencing of target genes. A – microRNA processing pathway. RNA polymerase II or III produces the pri-miRNA, which is cleaved by the Drosha-DGCR8 complex. From here, the pre-miRNA formed is transported from the nucleus to the cytoplasm where is cleaved by the RNase Dicer forming the mature miRNA. The mature miRNA is loaded with AGO forming the RISC complex. This complex will silence the target genes by mRNA cleavage, translation repression or mRNA deadenylation (Winter *et al.*, 2009); **B –** representation of miRNA mediated target gene silencing.

The accumulation of miRNAs is directly linked to the level of proteins involved in its biogenesis. The level of proteins regulates the efficiency of pri-miRNA processing into mature miRNA (Wang *et al*., 2013; Szyrajew *et al.*, 2017).

MiRNAs can be classified as conserved miRNAs (present in most plants), less conserved miRNAs (present in a small group of plants) and species-specific miRNAs (Chen *et al*., 2018). MiRNAs bind to the 3'-untranslated region of their target gene, inhibiting their translation or facilitating mRNA degradation, resulting in the silencing of those genes (Jones-Rhoades *et al*., 2006).

Like other epigenetic mechanisms, miRNAs also play an important role in SE induction and development (Fig. 3). Low accumulation of functional miRNA products during SE was paired with the down-regulation of genes encoding enzymes in miRNA conversions like AGO and DCL1. (Wang *et al*., 2013; Szyrajew *et al.*, 2017). Various studies confirmed that miRNAs are key molecules controlling patterning and morphology of the somatic embryos (Willmann *et al*., 2011; Seefried *et al*., 2014). Kumar and van Staden (2017) pointed out that miRNAs activity is continuously being studied in order to understand their regulatory roles in SE as shown in the works by Luo *et al*. (2006), Nodine and Bartel (2010), Willman *et al*. (2011) and many others. In these works, it was visible that the patterns of miRNA changed when inducing embryogenic callus and non-embryogenic callus and also during the differentiation into a plant.

miR156 was found to be activate during SE (Nodine and Bartel, 2010). The activation of such miRNAs is key in SE induction enabling the proper pattern formation by repressing target genes and also in the transition of undifferentiated to differentiated *calli* (Chen *et al*., 2011). The expression of other miRNAs such as miR397 and miR398 also seems to be essential to maintain the totipotency of undifferentiated tissues (Chen *et al*., 2011). In *Arabidopsis thaliana* miR167 was characterized as an important regulator in somatic embryogenesis by repressing the expression of two *AUXIN RESPONSE FACTORS* (*ARF6* and *ARF8*) (Su *et al*., 2016). In maize, the increase of expression of several miRNA's expression, such as miR 156, mir159 and others, was seen in embryogenic callus (Chávez-Hernández *et al*., 2015). MiRNAs were also found to control and repress the expression of key genes that are expressed during SE like *LEC2* (Willmann *et al*., 2011).

In *Arabidopsis thaliana*, a report by Szyrajew *et al*. (2017), found that around 85% of the miRNA's analysed had a difference in expression when SE was induced. miRNA's like miR156, miR390, miR393 and miR396 were analysed and considered to be very relevant in SE. miR390 has an important role in auxin response, in the production of transacting small-interfering RNAs (tasiARFs; Marin *et al*., 2010) that repress the activity

of *ARFs* namely ARF2, ARF3 and ARF4. It was shown that this particular miRNA was significantly accumulated in SE induction. In another study in *A*. *thaliana,* the expression of *GROWTH RESPONSE FACTORS* (*GRF*) family genes were shown to be differentially expressed during SE, and it was confirmed that they were targets of the miR396 that by itself is also differentially expressed during SE (Hewezi and Baum, 2012).

In *Citrus sinensis* (sweet orange), 50 known miRNA's alongside with 45 novel miRNA's were studied and the conclusion was that miRNA's in embryogenic callus have their expression reduced when compared to non-embryogenic callus (Guo *et al.,* 2007; Pan *et al*., 2009).

Auxins are key elements in the SE process, which means that auxin-related miRNAs also play an important role on it. One miRNA that was identified and confirmed to be linked with auxin-related genes expression is miR393. MiR393 not only regulates auxin signalling but also a response to biotic and abiotic stress as well as root and leaf development. (Etemandi *et al*., 2014; Iglesias *et al.*, 2014; Zhang *et al*., 2015; Wang *et al*., 2018). A study by Xu *et al.* (2017) also concluded that *TIR1* homologs and miR393 silencing are necessary for fruit/seed set development and leaf morphogenesis. Many other studies also concluded that *TIR1* and *AFB2* were negatively regulated by miR393 during plant development (Fig. 4) and when these were exposed to abiotic stresses (Vidal *et al*., 2010; Chen *et al.*, 2011; Wang *et al*., 2018). In *Arabidopsis thaliana,* miR393 inhibits shoot regeneration by repressing *TIR1* (Wang *et al*., 2018). It is also involved in shoot regeneration in *in vitro* cell cultures, where it is highly expressed in non-totipotent callus comparing to totipotent callus (Qiao *et al*., 2012). In SE this miRNA contributes to embryogenic transition by silencing the auxin receptors *TIR1* and *AFB2* which results in tissue sensitivity modulation to the auxin treatments (Wójcik and Gaj, 2016; Chen *et al*., 2018)

miR396 is involved in leaf development (Fig. 4), namely morphogenesis and growth polarity by regulating its target genes, especially the GRF family-like *GRF1* and *GRF4* (Chen *et al*., 2018). A study by Ercoli *et al.* (2016) showed that miR396 has a key role in the control of cell proliferation and elongation. The post-transcriptional repression of *GRF*s by this miRNA results in the co-expression of the TFs with proliferating cells (Debernardi *et al*., 2012; Ercoli *et al*., 2016). miR396 overexpression causes a reduction of *GRF* expression.

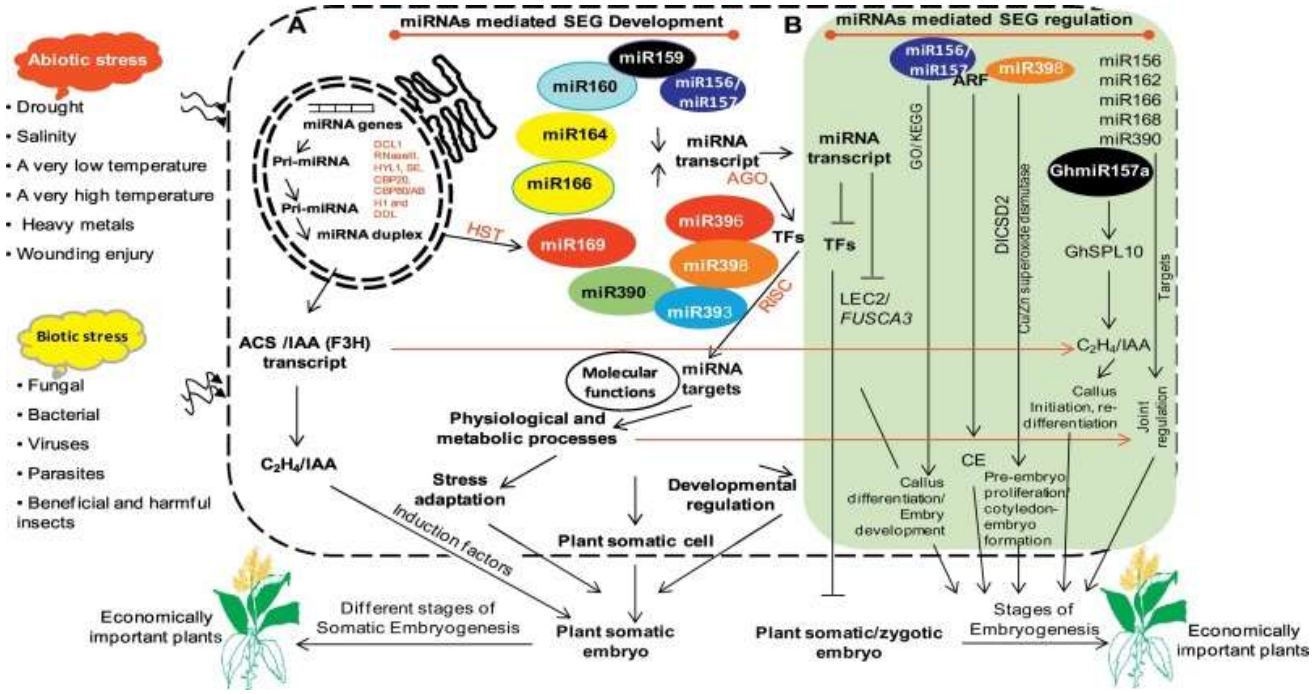


Figure 3. Role of miRNAs in the development and regulatory mechanism of somatic embryogenesis. Expression of miRNA transcript during the stages of SE and their influence in molecular, physiological and development processes involving plant hormones under stress, leading to a cellular response which results in the improvement of crops. **SEG**, somatic embryogenesis; **ACS**, ACC synthase; **IAA**, Indole-3, acetic acid; **C2H4**, ethylene; **HST**, hasty; **AGO**, Argonaut protein; **RICS**, induced silencing complex; **TFs**, transcription factors; **LEC 2**, leafy cotyledon 2; **FUSCA 3**, B3-domain transcription factor; **ARF**, auxin regulatory factor; **CE**, cotyledon embryo; **DICSD2**, superoxide dismutase; **GhmiR157a**,*Gossypium hirsutum*; **GhSPL10**,*Gossypium hirsutum* GhmiR157a target (Siddiqui *et al*., 2018).

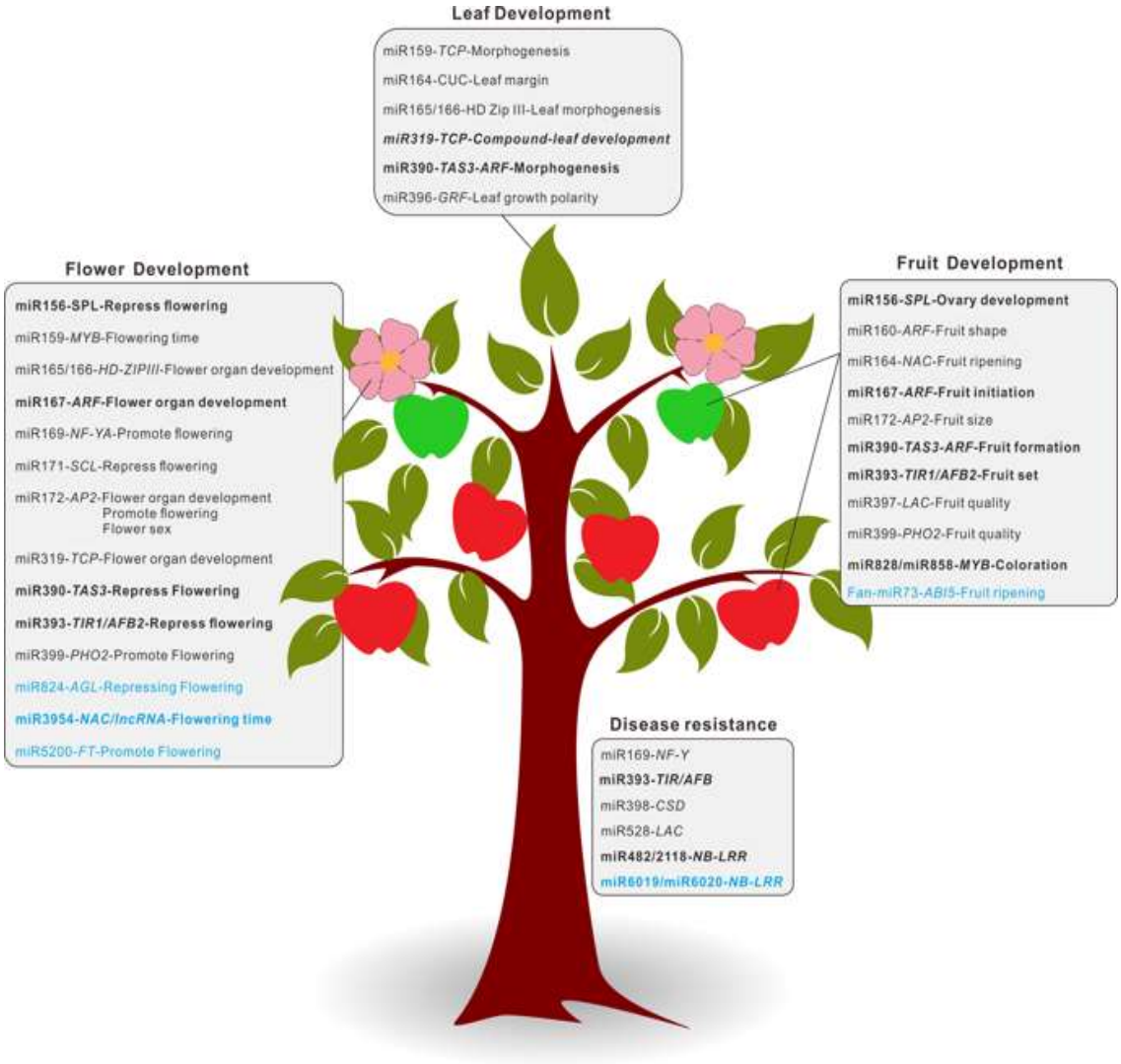


Figure 4. Main miRNA pathways involved in the development of horticultural traits. (Chen *et al*., 2018).

1.5. Tamarillo

The Solanaceae, known as the nightshade family, comprises about 2700 species of plants from which around 50% belong to genus *Solanum* (Olmstead *et al.*, 2007). The plants associated with this family are much diversified since there are various types of trees, shrubs, epiphytes, annual herbs, lianas and creepers. Species with high economic interest that belong to this family are: potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), pepper (*Capsicum spp*.) and tobacco plant (*Nicotiana tabacum*) (Mueller *et al.*, 2005)

Tamarillo (*Solanum betaceum* Cav.) belongs to the Solanaceae family and is also known as tree tomato or *tomate de árbol*. Native to South America, more exactly, Bolivia, Chile, Ecuador and Peru, it spread to Central America, Southern of Europe, Australia, New Zealand and also in the Portuguese archipelagos of Madeira and Azores (Canhoto *et al.*, 2005).

Its natural habitat is the sub-tropical region and is usually found in altitudes between 700 and 2000 metres. In colder climates, where the temperatures usually do not reach above 10ºC it prefers lower altitudes. As other solanaceous, it is very resistant to diseases and other plagues but it can be attacked by aphids, fruit fly, nematodes, viruses and mildew (Eagles *et al*., 1994).

This plant is a small perennial tree (Fig. 5A), with deciduous leaves with variable sizes of 10 to 30 centimetres of length. The flowers have 5 petals, white-pink colour, and usually, are assembled in small groups on the branches. Generally, the flowers blossom during summer to autumn transition, but can also appear during other times of the year. Pollination is mostly autogamic, what can explain the low genetic variability observed in wild varieties of tamarillo (Barghchi, 1986). The fruits have long peduncles and are usually isolated in groups of 3 to 12 (Fig. 5B), have an oval shape and reach maturity between the months of October to April. Size-wise they can reach 5-10 centimetres of length and be 3-5 centimetres wide. The epicarp of the fruit can have 3 colours, dark red, orange and yellow, whereas the pulp can have a colour in the yellow/orange spectrum. Not all the fruit is edible, as the epicarp is very stiff and has an unpleasant flavour and so it is advised its removal before eating. The pulp is very juicy and has a bittersweet flavour. The seeds are flat and round and can be consumed with the fruit (Fig. 5C).

There is an increasing economic interest in tamarillo fruits (Fig. 5D) that can be eaten fresh or processed as juices and jam. These fruits are highly nutritious as they have high amounts of protein, vitamins C, E and pro-vitamin A, minerals like potassium and iron and have a lower concentration of carbon hydrates and caloric value (McCane and WIddowson, 1992). Although being very nutritious, these fruits also have in its constitution anthocyanins and carotenoids that have biological, therapeutic and preventive importance, making them a valuable asset, appealing to its exploration (Kou *et al*., 2008; Hurtado *et al.*, 2009).

Figure 5. ***Solanum betaceum*** **(Tamarillo). A –** tamarillo tree; **B –** grouped tamarillo oval fruits; **C –** tamarillo fruit is very pulpous; **D –** tamarillo fruits for sale in a Madeira island's market.

Tamarillo propagation can be achieved through seedlings, staking (Prohens and Nuez, 2001) or grafting, and using laboratory techniques like micropropagation from axillary meristems (Barghchi, 1998), somatic embryogenesis (Guimarães *et al.*, 1996; Canhoto *et al.*, 2005; Correia *et al.*, 2009) and organogenesis (Obando *et al.*, 2001). The seeds are an easy way to produce tamarillo plants but they don't assure genetic uniformity and are useless when the objective is the propagation of genotypes of interest. To achieve this asexual propagation methods must be used. A problem in tamarillo is that traditional techniques for cloning proved to be ineffective (Pringle *et al.*, 1991) so, biotechnological methods like in vitro cloning and genetic transformation proved themselves as viable alternatives for the reproduction and improvement in tamarillo (Barghchi, 1998).

1.5.1. Somatic embryogenesis in tamarillo

Somatic embryogenesis induction in tamarillo was first reported in 1988 from zygotic embryos and hypocotyls explants (Guimarães *et al.*, 1988). Eight years later (Guimarães *et al.*, 1996), the same group described the obtention of tamarillo plants through organogenesis and somatic embryogenesis in different explants like hypocotyls, cotyledons, roots and zygotic embryos. Mature zygotic embryos were the first explants used for somatic embryogenesis induction being observed that, somatic embryo differentiation may proceed through two different embryogenic pathways and using two different types of auxins: NAA and 2,4 – D. In the presence of NAA, somatic embryos arise in the original medium following small callus formation. When 2,4-D is used, zygotic embryos produced an embryogenic callus formed by proliferating PEMs that could be maintained by subsequent subcultures in media with the same auxin. In the case of young leaves, the best auxin to use in order to obtain PEM is Picloram. These embryogenic *calli*, can be transferred to media without auxin, allowing the development of somatic embryos (Canhoto *et al*., 2005; Correia *et al*., 2011).

The embryogenic callus obtained from the various explants can be subcultured several times in order to increase its fresh mass since embryogenic callus can maintain their capability to form somatic embryos, for a long period of time (Lopes *et al*., 2006; Correia *et al*., 2011). In order to obtain embryogenic masses, the right explant must be used. In the case of tamarillo and other, the explant reflects its capacity to induce SE. In tamarillo explants from mature embryos and young leaves must be used (Canhoto *et al*., 2005; Correia *et al*., 2011). In old leaves, it was shown by Canhoto *et al*. (2005) that only non-embryogenic masses would rise from them. Another factor to take into account is that red cultivars have a better response to SE induction when compared to the orange ones and they also are more stable in culture (Canhoto *et al*., 2005)

Other biotechnological techniques were already used in tamarillo plant propagation namely axillary shoot proliferation (Cohen and Elliot, 1979; Barghchi, 1986), organogenesis by Guimarães *et al*. (1996) and genetic transformation techniques (Cohen *et al.*, 2000; Correia *et al.*, 2019). Nevertheless SE is the more studied technique in this plant, either from a micropropagation point of view, or from a functional molecular biology approach, including, proteomics and transcriptomics analysis (Guimarães *et al*., 1998; Canhoto *et al*., 2005; Correia *et al*., 2011; Correia *et al.*, 2012; Caeiro, 2015; Correia *et al.*, 2016; Sanches, 2016; Correia *et al*., 2019). The fact that tamarillo belongs to the nightshade family can be very impactful in the search for information and knowledge about

Solanaceae plants, notable plants like tomato and pepper that have a huge economic relevance crop production

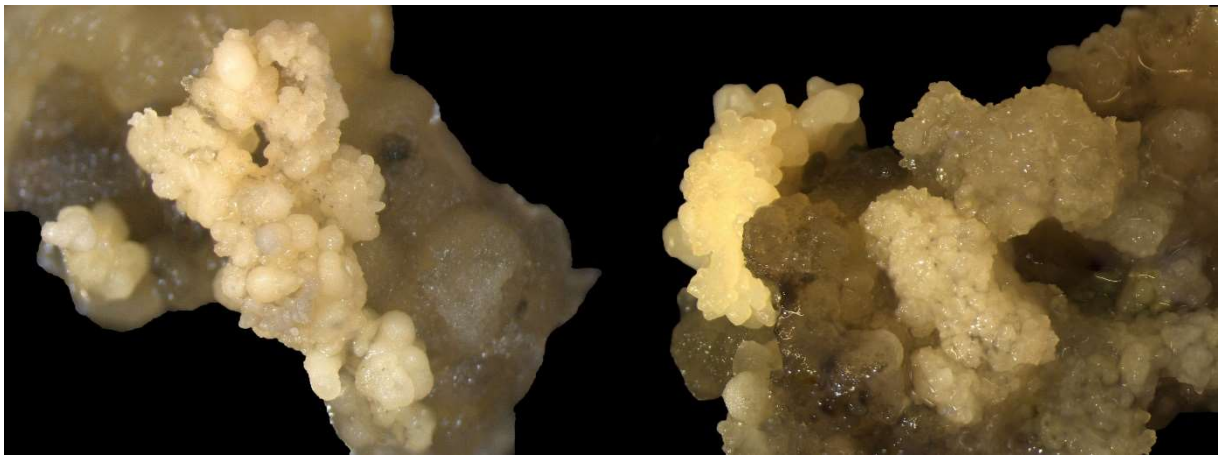
1.6. Objectives

In the follow-up of several molecular analysis made in the last years at LBV-UC, including proteomic (Correia *et al*., 2012; 2018) and transcriptomic (data not published) data analysis of somatic embryogenesis samples from tamarillo, this work aims to contribute with the analysis of new molecular actors involved in the process, namely specific miRNA's and their putative target genes.

Following a functional biology approach in the analysis of tamarillo SE the general aim of the present work was the analysis of miRNA/target gene pairs' expression at different phases of the somatic embryogenesis process, including early and late induction times, the proliferation of callus and development and germination of somatic embryos.
The specific objectives for this work were:

1. to induce SE in mature zygotic embryos and young leaves in order to obtain embryogenic and non-embryogenic cell lines;
2. to address the morphological changes during SE induction, development and germination of embryos;
3. to extract good qualityRNA samples from several samples in each SE stage;
4. to determine the expression of miR393, its targets *TIR1* and *AFB2*, miR396 and its targets *GRF1* and *GRF4* by quantitative PCR (qPCR);
5. to analyse, and correlate if possible, the different miRNA's and targets' expression in the SE process, as well in embryogenic and non-embryogenic calli, in order to show evidence in their interactions.

2. Materials and Methods



2.1. Seed germination

Plant lines were established using seeds from various red tamarillo trees located in the Botanical Garden of the University of Coimbra. The trees chosen were tagged as C7 and C8. The seeds were removed from mature fruits. Then they were sterilized with 5% (w/v) calcium hypochlorite for containing 2-3 drops of the detergent Tween20, 10-15 minutes under stirring. The seeds were washed afterward with sterile distilled water at least three times and then placed in Petri dishes with wet cotton and paper in an incubator at dark conditions and 25 ºC. After a few days, some seeds germinated. After 1 to 2 weeks, the germinated seeds were transferred to tubes (15 cm x Ø 22 mm) containing MS medium (Murashige and Skoog, 1962), supplemented with 3% w/v of sucrose and 0,7% w/v of agar (Duchefa Biochemie, Netherlands), with a pH between 5.6 and 5.8, adjusted with KOH (base) and HCl (acid). Each tube contained 12 ml of this medium. Cultures were kept in a growth chamber, at 25 ºC, in a 16h light/ 8h dark photoperiod with a light intensity of 15-20 µmol $m^{-2}s^{-1}$ (cool-white fluorescent lamps) for 2-3 weeks.

2.2. Plant multiplication by axillary meristem proliferation

After germination, young plants were selected. In sterilized conditions, using a scalpel and forceps the leaves were removed from the plant and the stem was cut (diagonal cuts, to ensure a better contact with the culture medium) in various segments with 1-2 centimetres of length, each one possessing at least one axillar or apical meristem. Those segments were inoculated in test tubes or plastic containers (Combiness Microbox, 11 x 8 cm, 565 mL, XXL filters) with MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, 0.2 mg/l of 6-benzilaminopurine (BAP) (Duchefa Biochemié, Netherlands) and 0.7% (w/v) agar, with a pH between 5.6 and 5.8. The shoots were maintained in a growth chamber at 25ºC in a 16h light/ 8h dark photoperiod and a light intensity of 15-20 µmol $m^{-2}s^{-1}$ (cool-white fluorescent lamps) and subcultured into fresh medium every 1 to 2 months.

2.3. Somatic embryogenesis induction in leaves and zygotic embryos

The procedures used in this work for somatic embryogenesis induction and development of the somatic embryos and plants were already described in details in Canhoto *et al*. (2005) and also in Correia and Canhoto (2018).

Shortly, young leaves, from the shoots previously established, were partitioned in 4 or 2 pieces, about the same size (1-2 cm) and were mechanically wounded, on the abaxial side, using a scalpel. Each leaf segment was transferred to a test tube, with their abaxial side face down, containing 12 ml of MS medium, supplemented with 9% w/v of sucrose, 5 mg/l of Picloram (© Sigma-Aldrich, Missouri, USA) and 0.25% (w/v) Phytagel™ (© Sigma-Aldrich, Missouri, USA) as gelling agent, and a pH between 5.6 and 5.8. This medium was used for the induction of SE in leaves and named as "TP" medium. The cultures were kept in the dark, at 25 ºC. After 12 weeks the explants were examined to check for somatic embryogenesis induction, and the simultaneous presence of embryogenic and non-embryogenic callus.

To induce SE from zygotic embryos, the embryos were carefully removed from the seeds (collected from fruits of the red tamarillo trees in the Botanical Garden and disinfected as mentioned above), using a scalpel and a needle, and transferred to test tubes containing MS medium, supplemented with 9% w/v of sucrose, 2 mg/l of 2,4-D (© Merck KGaA, Darmstadt, Germany) and 0.25% w/v of Phytagel™, with a pH between 5.6 and 5.8. This medium was used for the induction of SE in zygotic embryos and named as "TD" medium. The tubes were kept in the dark, at 25 ºC. After 12 weeks the explants were examined to check if somatic embryogenesis was induced. New lines of embryogenic (E_C81) and non-embryogenic lines (NE_C81) were then established, isolated and proliferated in tubes containing TD induction medium.

Samples were collected from both leaves and zygotic embryos induced explants during the induction phase at the times 0 (t0), 2 (It2), 4 (It4), 6 (It6), 8 (It8), 10 (It10) and 12 (It12) weeks for subsequent RNA extraction. For each time 3 biological samples were taken in order to analyse gene expression during SE induction. To analyse miRNA expression 2 biological samples were taken from t0, It2 and It8 as they were considered the most crucial points in the expression of the target genes.

2.4. Maintenance and proliferation of embryogenic, non-embryogenic and long-term established *calli*

Part of the analysis was made with lines of embryogenic and non-embryogenic *calli* previously established. Those lines were originated from leaves, following a similar procedure as described above, and designated as: EC (embryogenic callus, established and subcultured for 2 years), AC (long-term callus, previously embryogenic, established and subcultured for 4 years) and NEC (non-embryogenic callus, established and subcultured for 6 years). All these lines (EC, AC and NEC) and lines C81 (embryogenic and non-embryogenic *calli*, originated from a zygotic embryo) were routinely maintained following the following procedure: 100 mg of callus were transferred to a tube with MS medium, supplemented with 9% (w/v) of sucrose, 5 mg/l of Picloram (in the case of *calli* with leaf origin) or 2 mg/l of 2,4-D (in the case of *calli* with zygotic embryo origin) and 0.25% (w/v) of Phytagel™, with a pH between 5.6 and 5.8. The test tubes were kept in the dark, at 25 ºC. After 6 weeks the procedure was repeated, and *calli* subcultured on the same fresh culture medium, in order to achieve a time coincident with the cell's maximum proliferation rate. EC and E_C81 lines, AC line NEC and NE_C81 lines were collected in order to perform RNA extractions (3 samples each) and miRNA/gene expression analysis.

2.5. Development and germination of somatic embryos

To promote the formation of somatic embryos, embryogenic masses from either the leaves or zygotic embryo inductions were transferred to a medium without auxins and lower sucrose concentrations. Briefly, 100 mg of embryogenic callus from both EC and E_C81 lines were transferred into flasks (20 x 13 cm) containing 20 ml of MS medium, supplemented with 3% (w/v) of sucrose, devoid of auxins and 0.7% (w/v) of agar, with a pH between 5.6 and 5.8. The flasks were kept in the dark, at 25 ºC, for 4 weeks. Somatic embryo samples after 1 (Dt1), 2 (Dt2), 3 (Dt3) and 4 (Dt4) weeks in development medium were also collected (2 samples each) in order to perform RNA extractions and gene expression analysis. Dt1 was the time chosen to analyse miRNA expression.

After 4 weeks in the development conditions, the somatic embryos were transferred to a fresh new medium and 16h light/8h dark conditions to germinate. In this new medium, the embryos that formed were during the development phase were exposed to a lower sucrose concentration and also to the presence of light, in order to differentiate into newly cloned plantlets. This medium that we address as "germination medium", was composed of MS medium components with 2.5% (w/v) of sucrose and 0.7% (w/v) of agar,

with a pH between 5.6 and 5.8. The flasks were then maintained in a growth chamber, at 25 ºC, in 16h light/ 8h dark photoperiod with a light intensity of 15-20 µmol.$m^{-2}s^{-1}$ (cool-white fluorescent lamps) for 4 weeks. At the first week of the germination (G), embryos that start to change their colour to green (2 samples each) were collected to perform RNA extractions and miRNA/gene expression analysis.

Figure 6 summarizes the micropropagation techniques used in the present work.

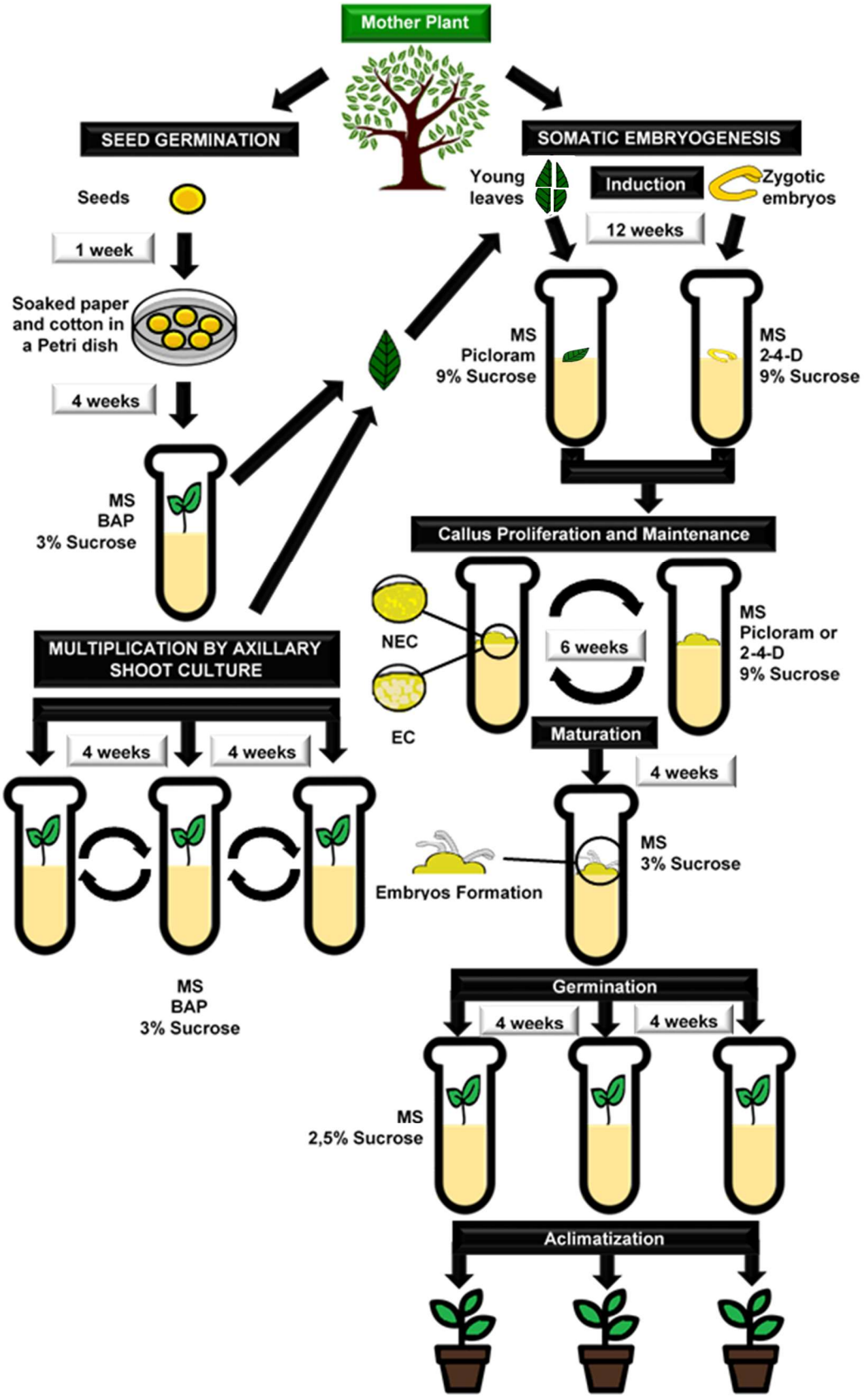


Figure 6. Schematic representation of seed germination, multiplication by axillary shoot culture and SE and plant regeneration in tamarillo.

2.6. Total RNA and small RNAs enriched extractions

As said above RNA was extracted from multiple samples, of both leaves and embryos explants, during the various phases of the somatic embryogenesis process: induction, proliferation and maintenance, development and germination. Before the extraction of total RNA, samples were frozen using liquid nitrogen and stored at -80 ºC until their use. The masses of these samples were approximately 100 mg.

To perform the extractions, the samples were grinded in liquid nitrogen in a cooled, DEPC (diethylpyrocarbonate)-treated mortar. DEPC overnight treatment (0.1% v/v DEPC water), followed by autoclaving at 121 ºC for 20 minutes twice, was used to remove potential RNAses from all the material used in RNA extractions (mortars, pestles and spatulas). Samples were then turned into a fine powder using a pestle and kept in a frozen state using liquid nitrogen, to avoid RNA degradation and reduce the RNAses activity. The powdered samples were transferred into new cold RNAse free 1.5 ml Eppendorf tubes using a frozen spatula. After this initial stage, RNA extractions proceeded as recommended by the Total RNA extraction kits' manufacturers.

Two different types of RNA extraction were performed. The first one to extract total RNA samples and the other to extract small RNA's enriched samples. For total RNA extraction, NucleoSpin® RNA Plant (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany) was used following the manufacturer's instructions. For small RNA enriched extractions, 2 different approaches were used: one with Direct-zol™ RNA MicroPrep (ZYMO RESEARCH, California, USA), according to the manufacturer's instructions, and the other with an adapted protocol used for *Medicago truncatula* (Trindade, 2010). Following this second protocol, 700 µl of RLT (lysis of cells and tissues) extraction buffer (Qiagen, Hilden, Germany) with 2% v/v β-mercaptoethanol was added to each sample. Each tube was mixed by inverting the tube and vortex. Then, 750 µl of phenol pH 4.3 (SIGMA) was added, mixed and vortexed. The samples were centrifuged for 10 to 15 min. at 12000 rpm. The upper phase was carefully collected and divided into 2 RNase-free tubes. About 650 µl of phenol:chloroform:isoamyl (25:24:1) were added to each tube and then centrifuged for 3 min. at 12000 rpm. This step was repeated when necessary. The upper phase was removed and an equal amount of chloroform:isoamyl (24:1) (+/- 600 µl) was added. The samples were centrifuged once again for 3 min. at 12000 rpm. The resultant upper phases were pooled in only 1 RNAse-free tube. 5 µl of 4M Na-acetate (pH 5.2) per 100 µl of the sample volume was added, and two volumes of chilled EtOH (96%) were pipetted into each tube. The tubes were mixed by gentle inversion and placed overnight at -20 ºC. The next day the tubes were centrifuged for 10-15 minutes at 12000

rpm. The pellet that formed was washed with 70% ethanol and left inside the *hotte* to dry all the ethanol. This step was repeated once or twice.

RNA extracts from both total RNA and small RNA enriched samples were diluted in RNase free H_20. Total RNA was diluted in 50 µl, small RNA was diluted in 15 µl when using the Direct-zol™ RNA MicroPrep kit and 50 µl when using the *M. truncatula* adapted protocol. The final concentration of RNA of each sample was measured using a spectrophotometer (NanoDrop™, Thermo Scientific, Massachusetts, USA). The concentration values were read at an absorbance peak of 260 nm. RNA purity was confirmed with the A260/A280 ratio between 1.9 and 2.2 and A260/A230 ratio between 1.8 and 2.3.

To ensure the good quality of the RNA obtained, the samples were evaluated in an agarose and acrylamide gel electrophoresis. For those, a denaturing loading buffer (FDE) was assembled with the following: 10 ml deionised formamide, 200 µl of 0.5 M EDTA (pH 8.0) and 10 mg of bromophenol blue. To 2 µl of RNA of each sample in an RNase free tube, 3 µl of FDE was added and placed in a heat block denaturing at 65 ºC for 15 min. To check the quality of Total RNA a 1.5% agarose gel (1xTBE) was used and ran at about 80 V for 1 to 1.5 hours. In order to check the small RNA, a polyacrylamide gel was used and ran following the instructions in Rio *et al*. (2010). The reason that the small RNAs samples were run in a polyacrylamide gel electrophoresis is that these samples contain RNAs with a very low number of nucleotide pair bases, such as microRNAs.

2.7. cDNA synthesis from total RNA and small RNA enriched samples

To produce cDNA for further analysis 1µg of RNA from each sample was used, so that cDNA concentration was equal across all samples. For total RNA samples, cDNA synthesis was performed using NZY First-Strand cDNA Synthesis Kit (NZYTech, Lda. – Genes and Enzymes, Lisbon, Portugal) according to the manufacturer's instructions. For Small RNA samples, the kit used was Mir-X™ miRNA First-Strand Synthesis Kit (Takara Bio USA, California, USA) according to the manufacturer's instructions (Fig. 7)

2.8. Quantification of miRNAs and target gene expression by quantitative PCR

In this experiment, the expression of miRNA's, miR393 and miR396, and their putative target genes, *TIR1*, *AFB3*, *GRF1* and *GRF4*, was quantified. For the analysis of the miRNA's the kit TB GreenTM Advantage® qPCR Premix (Takara Bio USA, California, USA) was used according to the manufacturers manual. To analyze the expression of target genes the kit used was NZYSpeedy qPCR Green Master Mix (2x) (NZYTech, Lda. – Genes and Enzymes, Lisbon, Portugal), following the instructions provided and the samples were diluted 50 times. Samples with the mix were pooled in a 96-well qPCR plate and measured in C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Lda., Amadora, Portugal) (Fig. 7). For reliable quantitative PCR's, reference genes were also chosen in order to normalize the data (Udvardi *et al*., 2008). U6 gene (provided in TB GreenTM Advantage® qPCR Premix) was chosen when quantifying the miRNA's expression and *Ef1α* gene when quantifying the target genes expression (Correia *et al*., 2012; Correia *et al*., 2019).

Expression analysis of miRNA's in zygotic embryos SE process was not possible to perform. This was the result of a lack of material to perform qPCR's that were personalized in order to analyze miRNA expression.

All the primers (Table 1), with the exception of *TIR1* gene primers (designed for *Solanum lycopersicum*), were designed for *Solanum betaceum* transcript sequences obtained from embryogenic cells RNAseq and smalRNAseq libraries (data not published). These primers were designed using the NCBI primer design tool. Before their use in quantitative PCR all primers were tested using samples of cDNA, from RNA extracts belonging to the proliferation stage of the somatic embryogenesis, by Reverse Transcriptase PCR RT-PCR) using NZYTaq II 2x Green Master Mix (NZYTech, Lda. – Genes and Enzymes, Lisbon, Portugal), following the provided protocol, proceeded by an agarose gel electrophoresis to check if only one band of the correct size was present in each sample.

Table 1 – Primers sequences used in qPCR

Gene	Forward Primer	Reverse Primer
AFB3	CTGTACGGAAATGGGGTGCT	GCAGAGTACGGGGAACCAAA
GRF1	ATCCAGAGTTCCTGAGCTGC	GCAACAGCAACAAGGTGTCG
GRF4	GCATGCTCCAATTTCACCCTC	CTGTGCCACCGGACCTAGTA
TIR1	AGATGGCTGTCCAAAGCTCC	GAGCCTTGTCTCCAAACGGA
EF1α	ACAAGCGTGTCATCGAGAGG	TGTGTCCAGGGGCATCAATC
miR393	CACGCAATCATGCGATCT	Provided in TB GreenTM Advantage® qPCR Premix
miR396	CACGCATTCCACAGCTTT	Provided in TB GreenTM Advantage® qPCR Premix
U6	Provided in TB GreenTM Advantage® qPCR Premix	Provided in TB GreenTM Advantage® qPCR Premix

2.9. Statistical analysis

The expression values (Cq) obtained for target genes, miRNA's and reference genes were assembled in order to be analyzed. Data were first normalized using the adequate reference gene to each target gene or miRNAs. The method used to analyze the qPCR data was the relative quantification method, or $2^{-\Delta\Delta CT}$ method, where the ΔΔCT value = (CQ Target – CQ Reference) (Livak and Schmittgen, 2001).

The normalized data were statistically analyzed using Graph Pad Prism. To analyze the variation of each miRNA/gene' expression values obtained during each instant of the SE process and between different callus, one-way ANOVA and Tukey test were performed.

To measure the correlation degree between miRNA and their targets a Pearson correlation test was performed where values vary between -1 and 1. 1 corresponds to a perfect positive correlation, -1 to a perfect negative correlation and 0 indicates there is no association between the two variables. Positive values of correlation coefficient indicate a tendency of one variable to increase or decrease together with another variable while negative values of correlation indicate that the increase of values of one variable is

associated with the decrease of values of the other variable and vice versa. The closer to -1 or 1, the stronger the variables are correlated and if close to 0 it indicated that there is a weak association between variables.

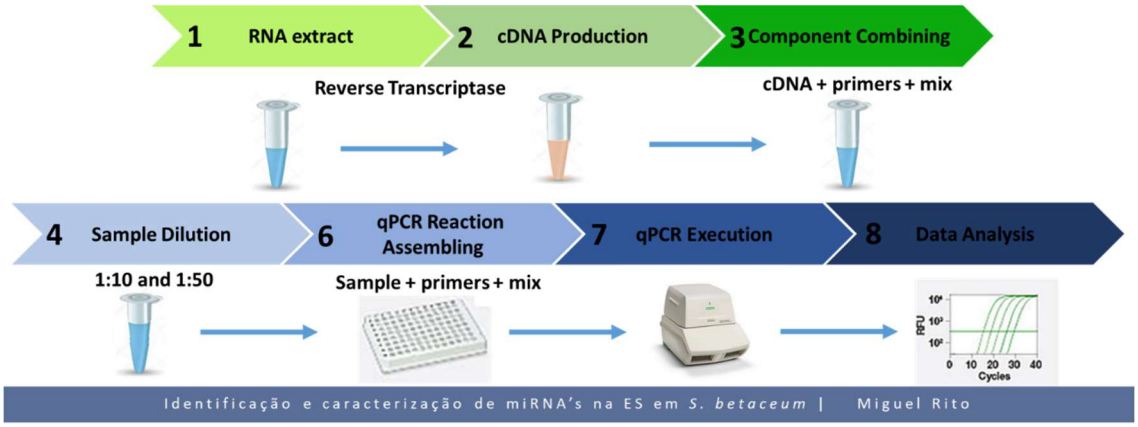
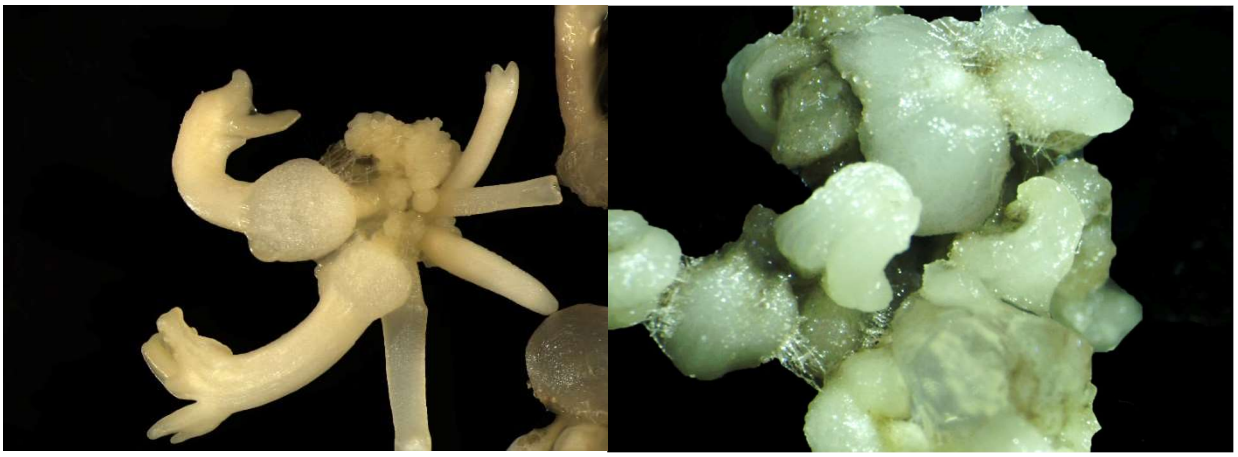


Figure 7. From RNA extraction to qPCR execution.

3. Results



3.1. Generating new embryogenic lines of tamarillo from leaves and embryos, callus maintenance and somatic embryo development and germination

3.1.1. Seed germination and multiplication by axillary shoots

Seeds from tamarillo trees were germinated in order to obtain recently established genotypes of red tamarillo plants. Two different trees (C7 and C8) located at the Botanic Garden of the University of Coimbra were tested. After germination, the seeds were placed into fresh medium, and after 4 weeks the seedlings were micropropagated in MS medium with 0.2 mg/l BAP, thus obtaining various shoots of the same genotype (Fig. 8). The establishment of this micropropagated material was the basis for the somatic embryogenesis induction from leaf explants in “TP” induction medium.

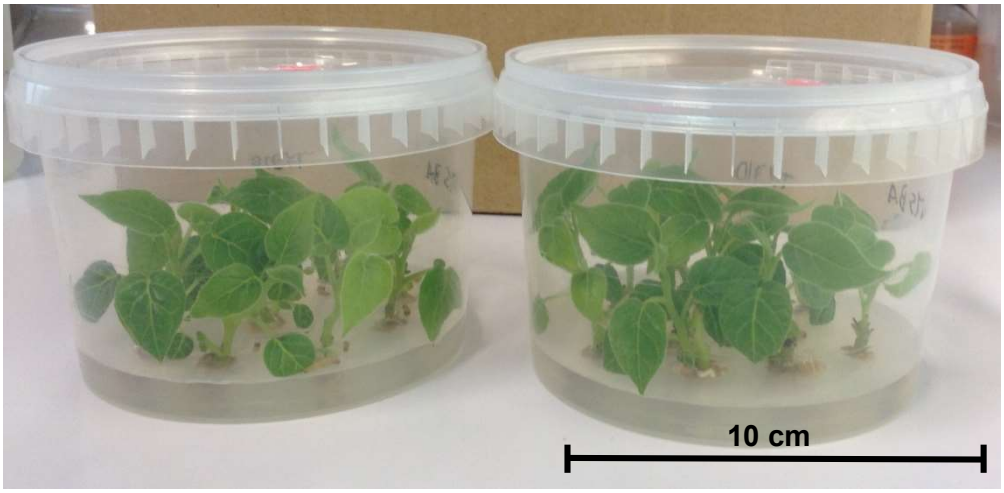


Figure 8. In vitro propagation by axillary shoot proliferation. The figure shows two boxes containing shoots that were used as source of leaves for somatic embryogenesis induction.

3.1.2. Somatic embryogenesis induction

From more than 150 explants collected from young leaves of C7 and C8 lines and induced for SE, no embryogenic masses were obtained and only non-embryogenic proliferating callus was established (Fig. 9). A high percentage (≈90%) of explants

responded to the culture medium conditions by dedifferentiating between the 2nd and 4th week of culture (Figs. 9B and 9C) and by the 4th week, a homogenous mass had emerged (Fig. 9C). After the 6th week (Fig. 9D) the entire explant was dedifferentiated into a friable translucent non-embryogenic callus, sometimes with a yellow or grey color. These masses proliferated fast in the medium. Afterward, this tissue was subcultured in "TP" medium. During the 8th week (Fig. 9E) the same type of callus was still present, and no embryogenic callus was formed. No embryogenic callus formed between the 10th and 12th weeks (Figs. 9F and 9G). Only non-embryogenic callus was able to proliferate.

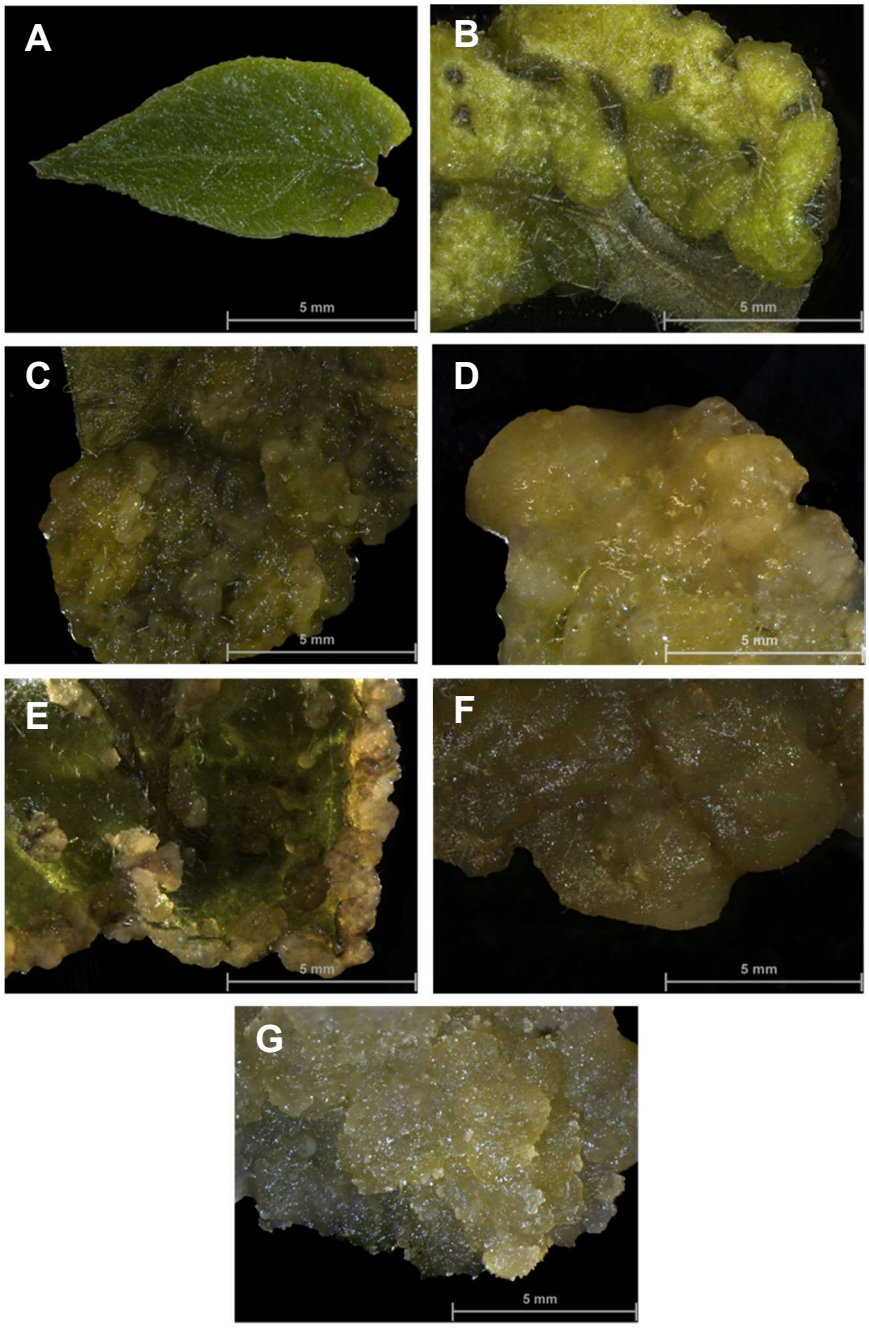


Figure 9. Timeline of callus formation in induction in young leaves. A – A small young leave collected from an in vitro plantlet; **B –** 2nd week of induction; **C –** 4th week of induction

;D – 6th week of induction; **E –** 8th week of induction: In this picture is interesting to see that the callus only formed from the mechanically injured parts of the plant; **F –** 10th week of induction; **G –** 12th week of induction.

Zygotic embryos were placed in “TD” media in order to induce SE. From 320 embryos inoculated, SE induction by the induction of embryogenic callus was achieved for ≈4% of the explants. As for leaf explants, the non-embryogenic callus formed showed high proliferation rates, friable texture and translucent with a yellow/grey color. On the other hand, embryogenic callus had a low rate of proliferation, being a slow-growing mass. The embryogenic masses formed were more compact than the non-embryogenic callus and also with a granular structure and white opaque color. These differences make it easier to identify both types of callus. After twelve weeks on induction medium, both embryogenic and non-embryogenic callus were separated and cultured separately in “TD” media.

In the 2nd week of induction (Fig. 10B) a yellowish mass formed around the embryo, indicating that dedifferentiation was induced. After 4 weeks (Fig. 10C), yellow and translucent masses have already been formed, indicating that non-embryogenic callus started to form and proliferate. On the 6th (Fig. 10D) week this non-embryogenic callus continued to grow and proliferate. The first signs of embryogenic callus formation (Fig. 10E 1) began after eight weeks (Fig. 10E). Somatic embryos formed by the 10th week (Figs. 10F and 10G), which is an evidence of the presence of newly formed PEM although most of the callus present is non-embryogenic as in Fig. 10G. In the end of SE induction (Figs. 10H and 10I) non-embryogenic, embryogenic and somatic embryos formed.

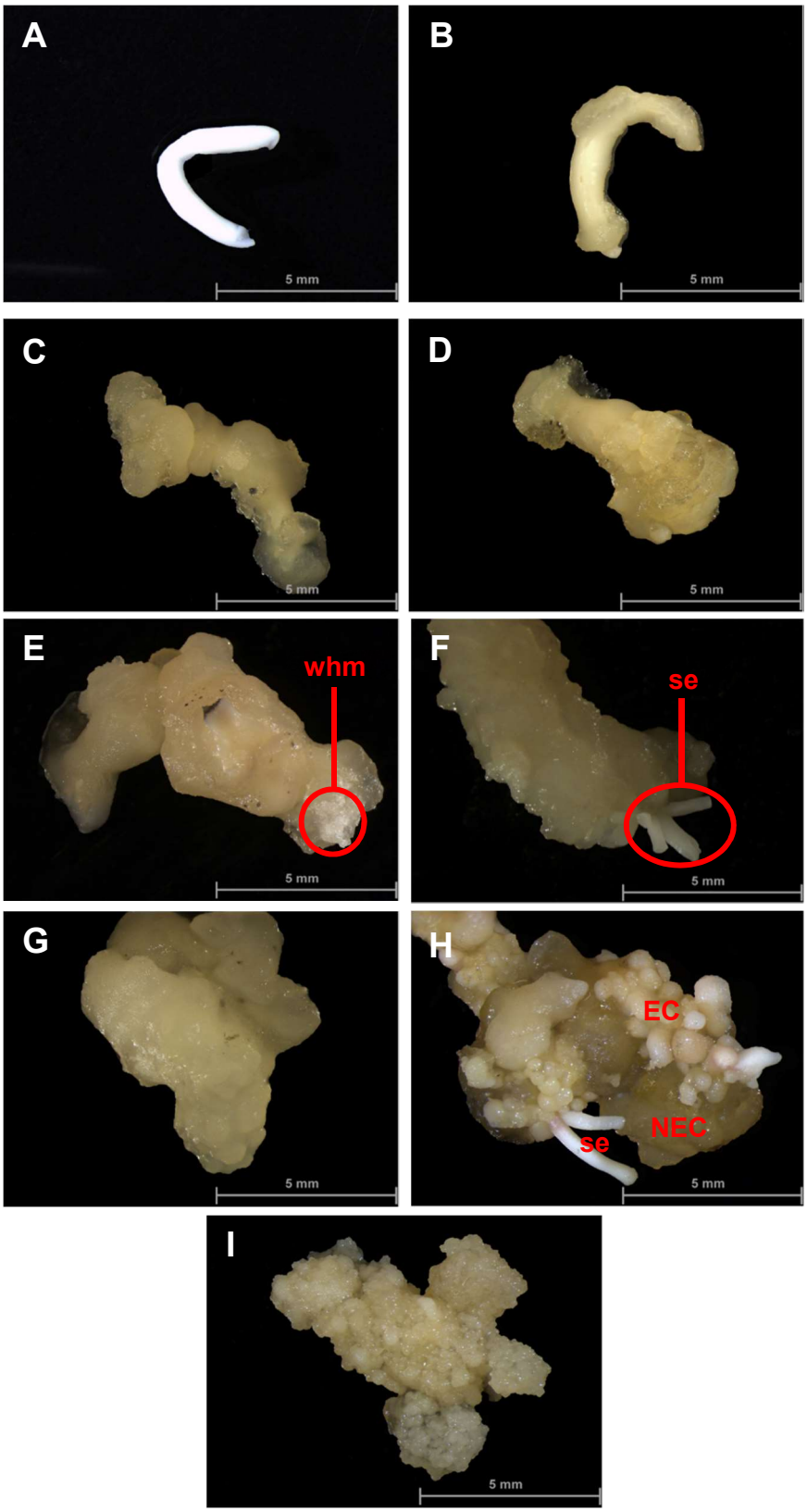


Figure 10. Timeline of SE induction in embryos from the initial mature zygotic embryo till the 12th week of culture. A – Embryo removed from tamarillo seed in order to induce SE; **B –** 2nd week of induction; **B –** 2nd week of induction; **C –** 4th week of induction:; **D –** 6th week of induction; **E –** 8th week of induction: whm – white

heterogeneous mass; **F, G –** 10th week of induction: se – somatic embryos; **H, I –** 12th week of induction: NEC – non-embryogenic callus, EC – embryogenic callus.

3.1.3. Callus proliferation and maintenance

As mentioned in the section Materials and Methods, 3 *calli* lines from leaf SE were chosen from previously established *calli* lines in *in vitro* culture. All lines were successfully maintained during this work. EC (Fig. 11A), which was the embryogenic line, had the slower rate of proliferation and this type of callus had a grainy heterogeneous texture with white opaque structures. AC (Fig. 11B), the long term callus line, proliferated rather quickly, indicating loss of embryogenic capability loss. It was characterized by their yellow grainy texture, although being rather homogenous when compared to the embryogenic line. NEC line (Fig.11C) corresponds to the non-embryogenic callus and proliferates very quickly and in large amounts. It has a frail grey and translucent texture and showed no embryogenic capability.

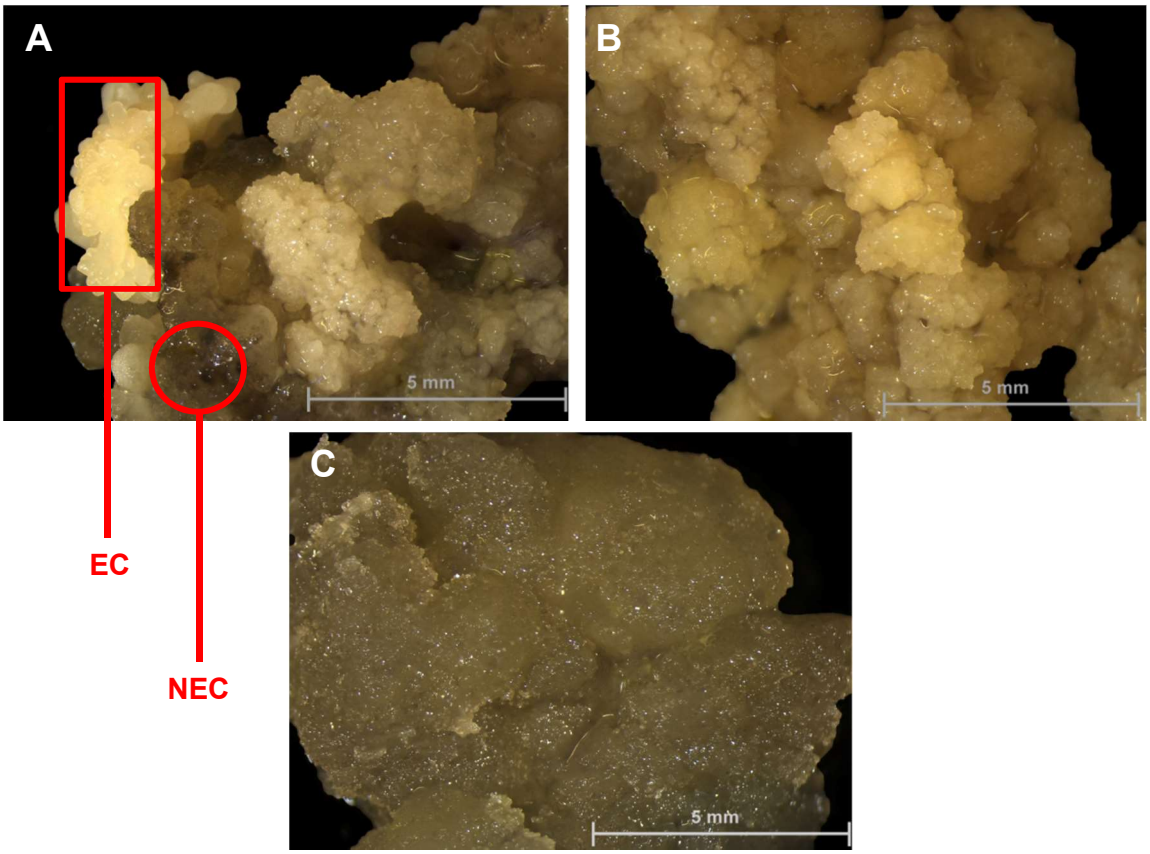


Figure 11. Leaf origin callus proliferation in TP media. A – Embryogenic callus, with embryogenic (EC) and non-embryogenic masses (NEC); **B –** Long term callus; **C –** Non-embryogenic callus.

The callus obtained by the culture of zygotic embryos was also cultured in “TD” media to obtain more material for RNA extraction. Both embryogenic and non-embryogenic masses were obtained from the same line, C81. As already referred embryogenic (Fig. 12A) callus presents a more granular and compact structure, with a slow proliferation rate while non-embryogenic masses (Fig. 12B) are more friable and translucent with a high proliferation rate.

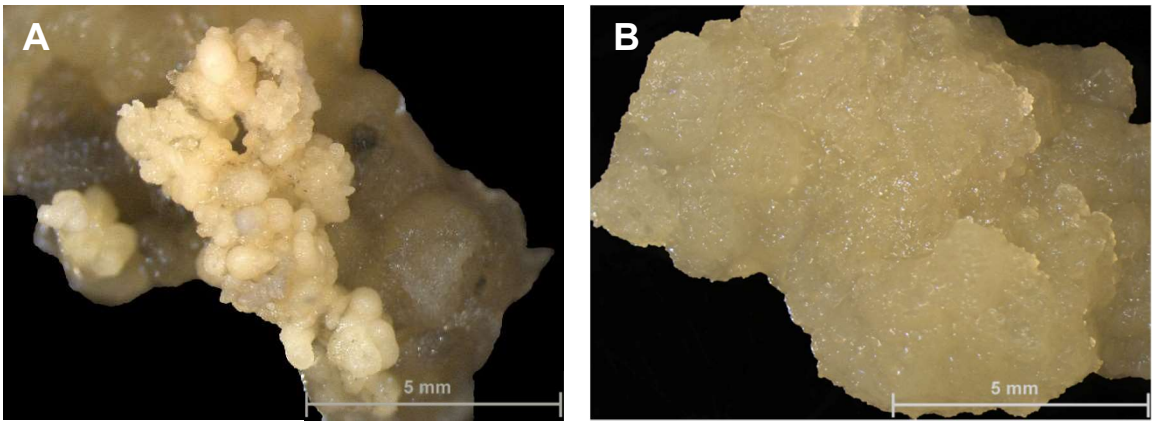


Figure 12. Zygotic embryo origin callus proliferation in TD media. A – Embryogenic callus; **B –** Non-embryogenic callus.

3.1.4. Somatic embryo development and germination

Embryogenic callus from the EC line was collected and inoculated in development medium for 4 weeks. In the first 3 weeks not particular features were noticed. Only on the last week, the development of somatic embryos was visible (Fig. 13A) although they were very small and seemed to not be developed enough. After 4 weeks, the somatic embryos that formed were reallocated to the germination medium where the formation of plantlets was expected. Only after 4 weeks the embryos started to germinate and became green (Fig. 13B), forming a complete plantlet afterward, although some plantlets had deformations and were unable to fully develop.

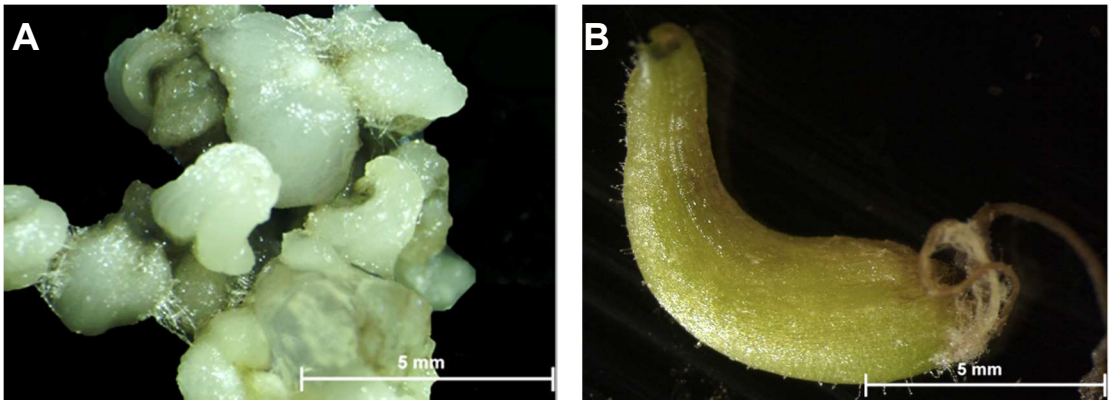


Figure 13 – Development and germination of somatic embryos from EC callus (leaf origin). A – Somatic embryos formed after 4 weeks in maturation media; **B –** Germinating somatic embryo.

Embryogenic callus from E_C81 line (induced from zygotic embryos) was also placed in maturation medium for 4 weeks. On the first week of the development minor changes were seen. Somatic embryos started to develop into embryos in a globular stage (Fig. 14A). By the second week embryos were developing and presenting a globular form (Fig. 14B). They were only formed from the white masses of embryogenic callus. The homogeneous masses of non-embryogenic callus, didn't show the capacity to form somatic embryos. By the third week, almost fully developed elongated embryos were clearly seen (Fig. 14C). On the fourth and final week, the embryos were fully developed, mostly at the cotyledonary stage as seen in Fig. 14D, although some embryos might not fully develop (Fig. 14E). At the end of the development stage, the embryos were transferred to germination medium. In the case of the somatic embryos from C81 callus, one week was enough for them to start germinating and becoming green as seen in Fig. 14F, G and H. Some transferred embryos did not germinate as seen in Fig. 14F and 14H.

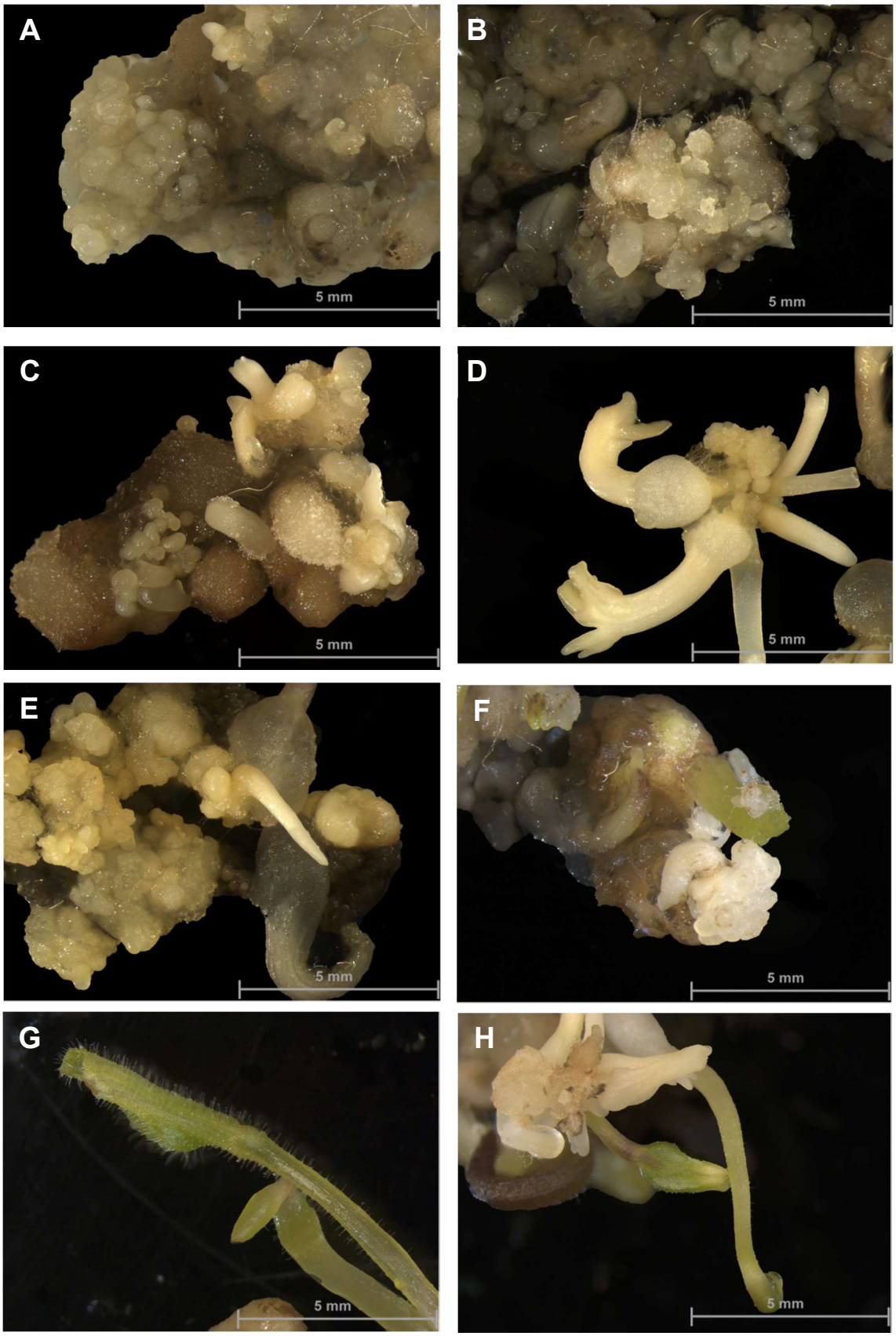


Figure 14. Development and germination of somatic embryos from E_C81 callus (embryo origin). A – First week of embryogenic callus in the maturation media; **B –** 2nd week of somatic embryo development; **C –** 3rd week of development; **D, E –** 4th week of

embryo development; **F, G, H –** somatic embryo germination.

The development and germination of somatic embryos were very much alike zygotic embryogenesis. The induced somatic embryos passed through the several stages of embryo development. The germination was also very similar, although some embryos presented some aberration, such as abnormal growth and leaf development.

3.2. miRNA and predicted target genes expression evaluation

3.2.1. RNA extraction

Before cDNA synthesis, the RNA was tested to evaluate its quality. In every extract RNA content was quantified using the A260/A280 ratio that should be between 1.9 and 2.2, and the A260/A230 ratio also that should be between 1.8 and 2.3. The results obtained are indicated in table 2. Extracted samples with NucleoSpin® RNA Plant and Direct-zolTM RNA MicroPrep had high yields of RNA concentration and the absorbance values were suitable, corresponding to values between the ones aforementioned. The adapted protocol from RNA extraction in Medicago truncatula wasn't suitable and therefore only Direct-zolTM RNA MicroPrep was used. Although RNA extractions following the adapted protocol produced samples with high RNA content, the values of A260/280 were very low (Table 2), meaning that the samples were contaminated by phenol or other contaminants.

Table 2 – Evaluation of RNA quantification and purity (example). EC – embryogenic callus; AC – long-term callus; NEC – non-embryogenic callus.

Extraction method	Samples	RNA quantification (ng/µl)	A260/A280 ratio	A260/A280 ratio
Total RNA - NucleoSpin® RNA Plant	EC	910.0	2.12	2.27
		443.4	2.10	2.19
	AC	355.2	2.12	2.26
		461.8	2.11	2.13
	NEC	617.9	2.10	2.25
		1610.9	2.14	2.31
Small RNA enriched samples - *Medicago truncatula* protocol	EC	9897.7	1.62	0.66
		8017.4	1.58	0.63
	AC	5151.3	1.52	0.44
		5820.7	1.96	0.82
	NEC	9752.5	1.61	0.67
		6466.2	2.02	0.86
Small RNA enriched samples - Direct-zol™ RNA MicroPrep	EC	288.8	2.14	2.24
		224.5	1.91	1.93
	AC	320.6	2.10	2.22
		534.7	2.07	2.11
	NEC	425.5	2.05	1.75
		486.2	2.09	1.96

3.2.2. Testing for RNA quality

After testing for the quality of the RNA, it was still necessary to check in an agarose gel, in the case of Total RNA, or in an acrylamide gel, in case of SmallRNA enriched samples (Fig. 15). SmallRNA enriched samples were analyzed on an agarose gel when necessary to check if the 28S ribosomal RNA and 18S ribosomal RNA bands did not show degradation signs.

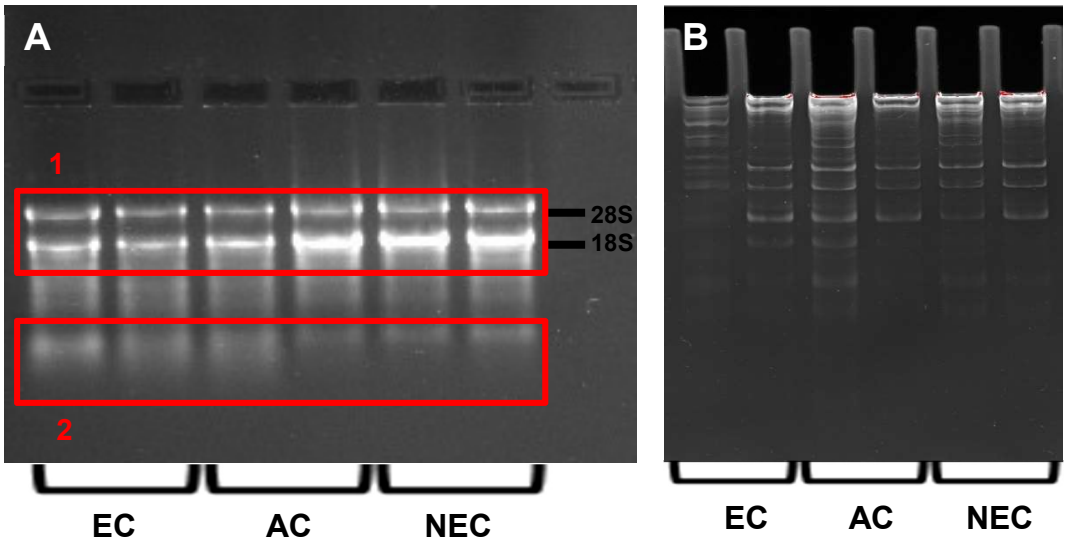


Figure 15. Testing RNA quality in agarose and acrylamide gels. A – Agarose gel. As highlighted (1) the intensity of both bands of 28S and 18S gives a clue on the quality of the RNA. 2 represents diffused bands of SmallRNAs present in the sample. **B –** Acrylamide gel used to analyse SmallRNA samples. **EC –** Embryogenic callus; **AC –** Long-term callus; **NEC –** Non-embryogenic callus.

3.2.3. Primer specificity evaluation

To proceed to the gene expression analyses, the designed primers were tested (Fig. 16)

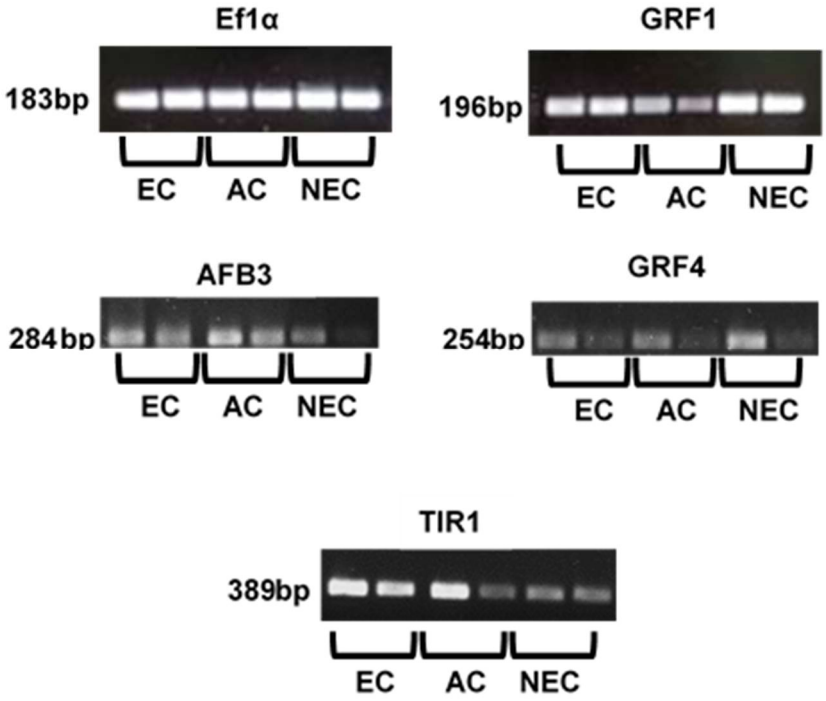


Figure 16. Primers testing. As observed all the primers only amplified a certain region of the DNA. ***EF1α –*** 183 bp; ***GRF1 –*** 196 bp; ***GRF4 –*** 284 bp; ***AFB3 –*** 254 bp; ***TIR1 –*** 389 bp;. **EC –** Embryogenic callus; **AC –** Long-term callus; **NEC –** Non-embryogenic callus.

3.2.4. Quantification of miRNAs and predicted target gene expression during the SE in leaves and zygotic embryos

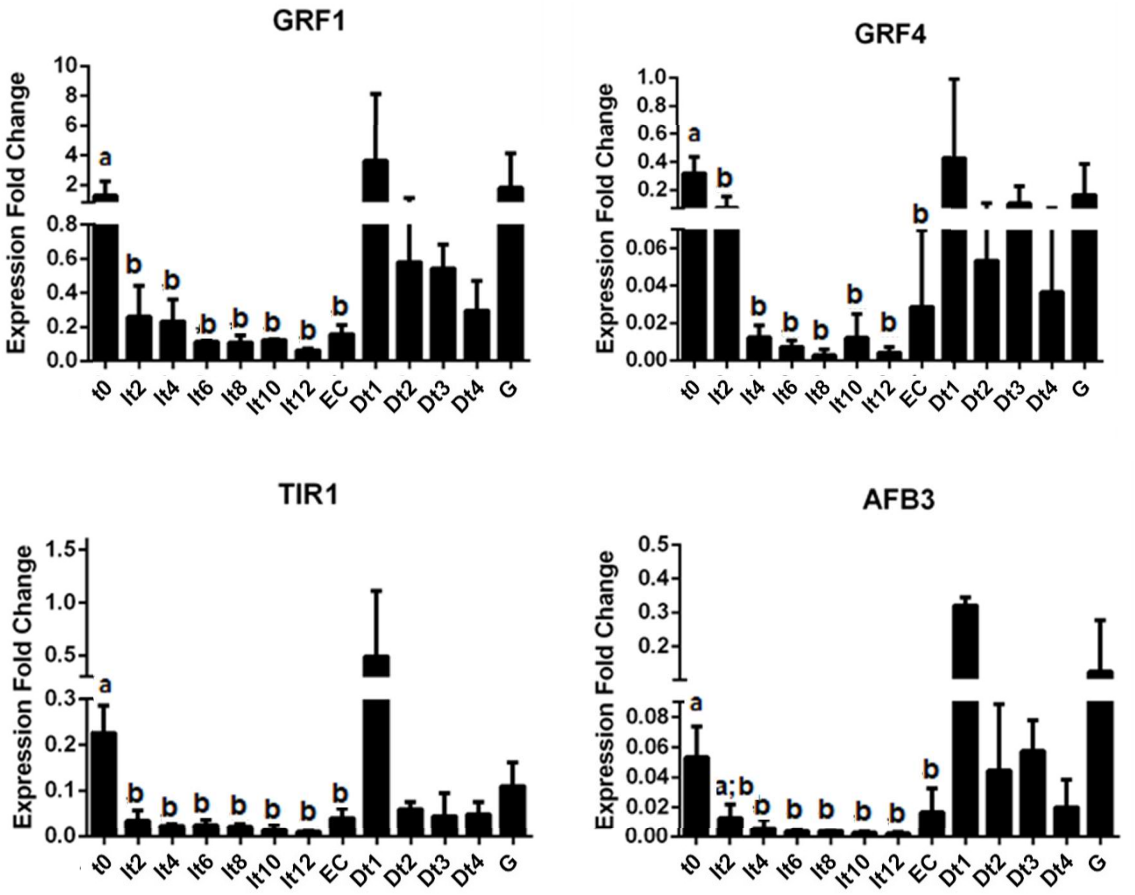


Figure 17. Target genes expression during SE process (leaf). Error bars correspond to standard deviation values. Values indicated with the same letter were not statistically different at p ≤ 0,05, using Tukey test. **t0 –** Young leaf; **It2 –** 2nd week of induction; **It4 –** 4th week of induction; **It6 –** 6th week of induction; **It8 –** 8th week of induction; **It10 –** 10th week of induction; **It12 –** 12th week of induction; **EC –** Embryogenic callus; **Dt1 –** 1st week of development; **Dt2 –** 2nd week of development; **Dt3 –** 3rd week of development; **Dt4 –** 4th week of development; **G –** Germination. **Note:** Because only two samples were analysed during development and germination, a statistical analysis was not performed.

For the four genes (Fig. 17), there was a tendency to lower expression levels during SE induction and in the embryogenic callus (EC) when compared to the initially differentiated explant (t0). From It2 to It12 the expression of all the genes was rather similar along time. The only statistically significant difference was between t0 and the other times of induction when the explants were placed in a medium with auxin (Picloram) and dedifferentiation was occurring.

During the development stage (Dt1 - Dt4), the expression of genes rose significantly, when compared to the levels registered during induction, although it decreased during this stage as the explants were placed into a medium devoid of auxins, and embryos start to form and develop. In the germination stage (G1), the expression of genes was very similar to the initial explant (t0).

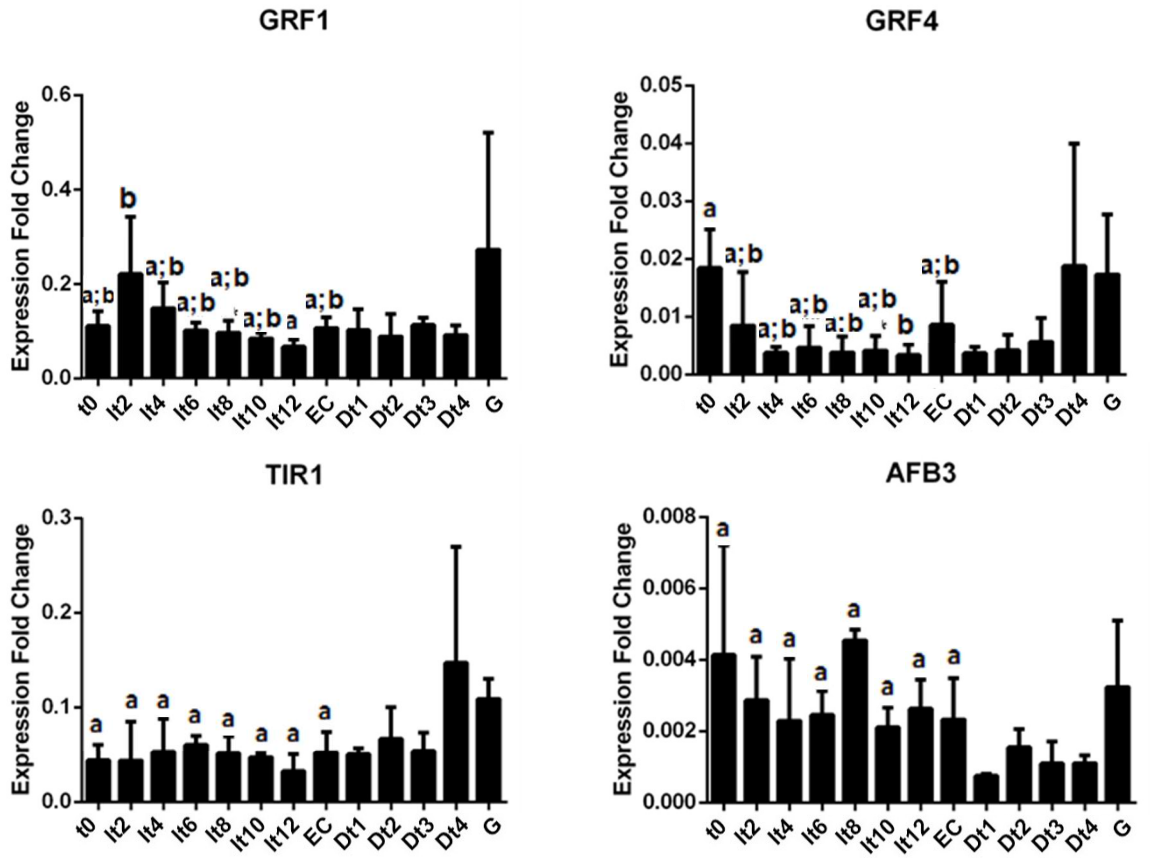


Figure 18. Target genes expression during SE process (embryo). Error bars correspond to standard deviation values. Values indicated with the same letter were not statistically different at p ≤ 0,05, using Tukey test. **t0 –** Young leaf; **It2 –** 2nd week of induction; **It4 –** 4th week of induction; **It6 –** 6th week of induction; **It8 –** 8th week of induction; **It10 –** 10th week of induction; **It12 –** 12th week of induction; **EC –** Embryogenic callus; **Dt1 –** 1st week of development; **Dt2 –** 2nd week of development; **Dt3 –** 3rd week of development; **Dt4 –** 4th week of development; **G –** Germination. **Note:** Because only two

samples were analysed during development and germination, a statistical analysis was not performed.

Like it was found for the results observed for induced zygotic embryos, the expression levels of all four genes were very similar throughout the SE process and there are almost no statistically significant differences. When comparing *GRF1* expression during the different times the biggest differences were found in It2 and G times although with big variations meaning that this gene is expressed almost in a regular way. For *GRF4* the biggest differences were in t0, Dt4, and G when comparing to the induction times. *TIR1* also showed regular levels of gene expression during the SE process although with a slight variation at the end of the development stage and during germination. *AFB3* had higher expression during SE induction and germination and lower during the development phase.

In leaves, miRNA was extracted from a young leaf, from the 2nd and 8th weeks of SE induction, from embryogenic callus and from the 1st week of the development stage and germination. These times were selected according to the stages where gene expression differentiated the most.

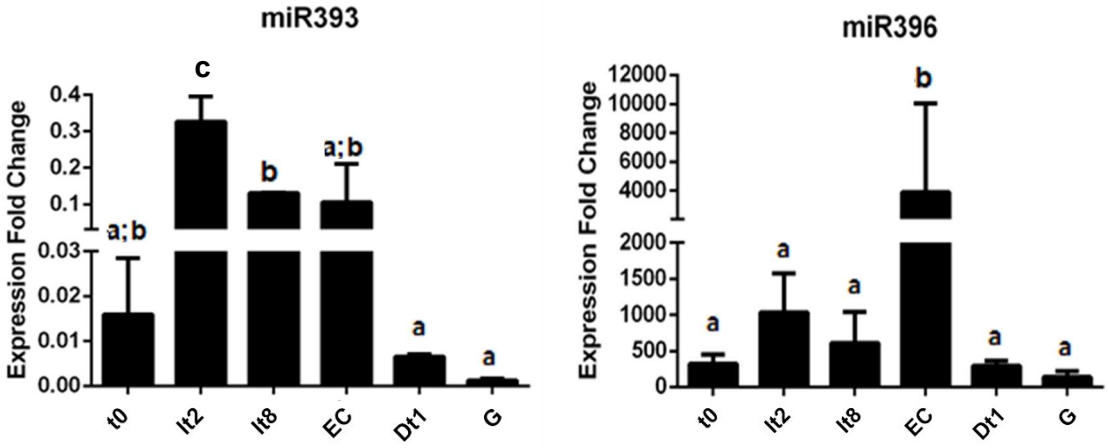


Figure 19. miRNAs expression in SE process (leaves). Values indicated with the same letter were not statistically different at p ≤ 0,05, using Tukey test. **t0 –** Young leaf; **It2 –** 2nd week of induction; **It8 –** 8th week of induction; **EC –** Embryogenic callus; **Dt1 –** 1st week of development; **G –** Germination.

For miRNA393 the statistical differences observed were between It2, the beginning of SE induction, and the remaining samples, and between It8 and Dt1 and G. According to the data of in figure 19, miRNA expression increases when SE is induced and high expression values are registered during the induction times and in embryogenic callus.

During the development phase and germination, the miRNA expression values plummeted being lower than those from the original explant.

The miRNA396 expression levels are only statically significant higher when comparing embryogenic callus (EC) with the other samples. Nevertheless, there was an increase of miRNA396 expression during SE induction and it quickly rose in embryogenic callus. Once again during the development and germination phases, the expression values plummeted, similarly to miRNA393.

3.2.5. Correlation between miRNA/target genes during the SE process in leaves

When SE was induced on leaves, a pattern was observed in the target genes and miRNAs expression. During SE induction phase expression of target genes decreases while the expression of the miRNA's increases. During the development and germination stages, the expression of target genes increases significantly while the expression levels of the miRNA's plummeted in both cases. Following this results, a negative correlation between predicted target genes and miRNAs was observed since as expression values of miRNA increases, the expression of target genes decreases and vice versa (Fig. 17 and 19). In the case of miR393, *TIR1* and *AFB3* are downregulated (Fig. 20) and in the case of miR396, both *GRF1* and *GRF4* are downregulated (Fig. 21).

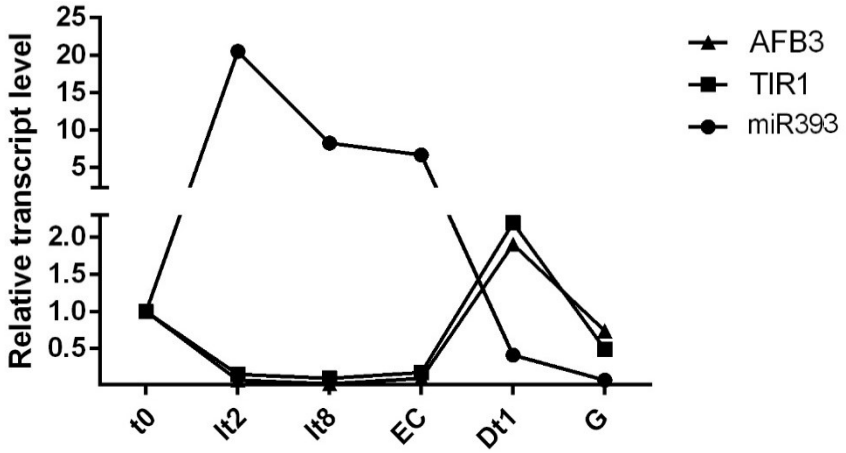


Figure 20. Expression levels of miR393 and its target genes. The relative transcript level was calculated based on the expression of t0. The expression levels during SE induction, development and germination stages were compared to the ones in t0. Pearson correlation test showed that miR393 shares a strong inverse correlation for both target genes (-0,669 for AFB3 and -0,572 for TIR1). Pearson correlation also showed that both target genes share a very strong correlation (0,979). **t0 –** Young leaf; **It2 –** 2nd week of induction; **It8 –** 8th week of induction; **EC –** Embryogenic callus; **Dt1 –** 1st week of

of development; **G –** Germination.

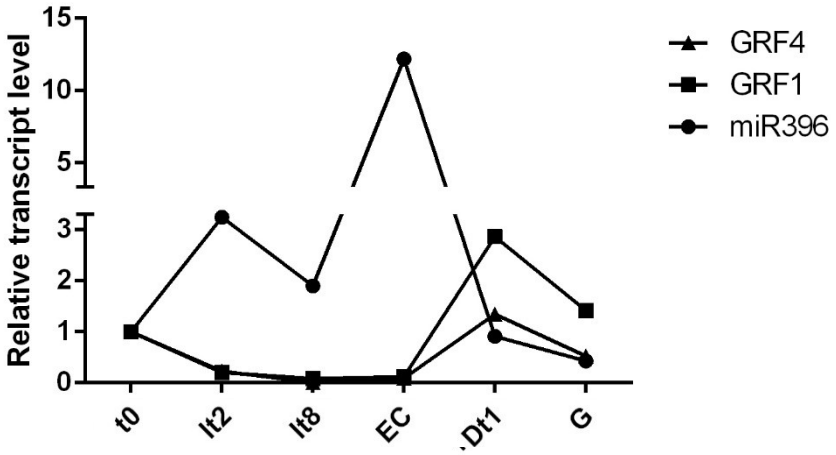


Figure 21. Expression levels of miR396 and its target genes. The relative transcript level was calculated based on the expression of t0. The expression levels during SE induction, development and germination stages were compared to the ones in t0. Pearson correlation test showed that miR396 shares a strong inverse correlation for both target genes (-0,500 for GRF1 and -0,515 for GRF4). Pearson correlation also showed that both target genes share a very strong correlation (0,902). **t0 –** Young leaf; **It2 –** 2nd week of induction; **It8 –** 8th week of induction; **EC –** Embryogenic callus; **Dt1 –** 1st week of development; **G –** Germination.

3.2.6. Quantification of miRNAs and predicted target genes expression in established calli lines

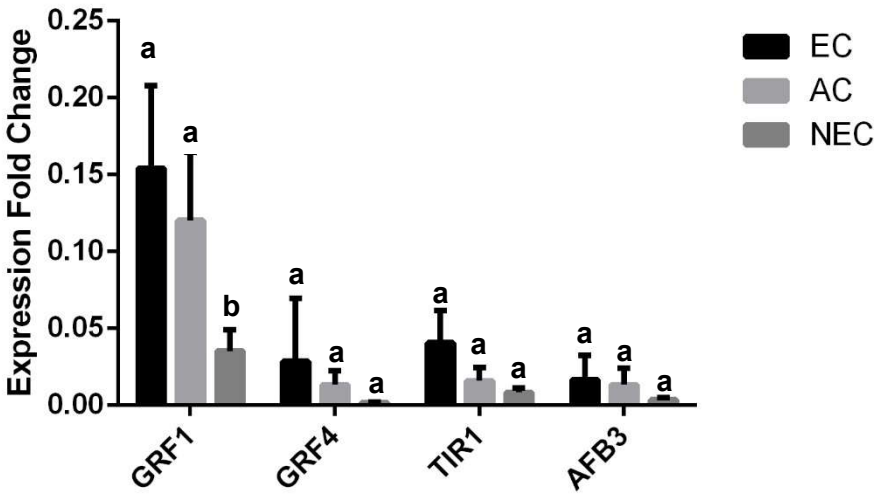


Figure 22. Target genes expression in embryogenic, long-term and non-embryogenic callus (leaf origin). Error bars correspond to standard deviation values. Values indicated with the same letter were not statistically different at $p \leq 0,05$, using Tukey test. **EC –** Embryogenic callus; **AC –** Long-term callus; **NEC –** Non-embryogenic callus.

GRF1 gene expression is significantly higher in the embryogenic when compared to non-embryogenic callus (Fig. 22) but not when compared to long term callus. The values variation from both embryogenic and long-term callus is high. The expression of this gene in non-embryogenic callus is very low.

For the other genes, *GRF4*, *TIR1*, and *AFB3* no statistical differences between the expression of genes were observed in the different lines (Fig. 22) although a pattern appears. Embryogenic callus usually displays a higher gene expression, although with strong variations, whereas and non-embryogenic callus displayed lower gene expression. In the case of long term callus the values were intermediate between embryogenic and non-embryogenic.

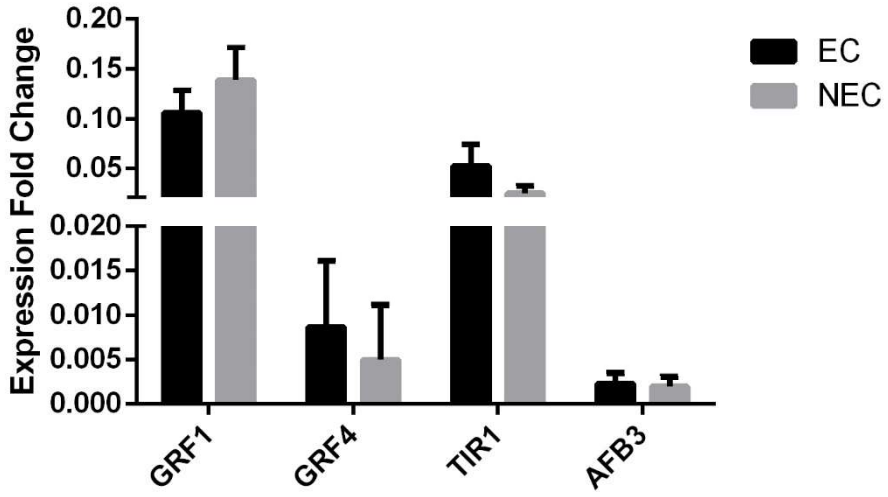


Figure 23. Target genes expression in embryogenic and non-embryogenic callus (zygotic embryo origin). No significantly differences were registered, at p ≤ 0,05, using Tukey test.. **EC –** Embryogenic callus; **NEC –** Non-embryogenic callus.

Gene expression was rather similar in both embryogenic and non-embryogenic callus for all four genes. The same type of pattern was observed in the Figure 23. Callus resulting from zygotic embryo SE induction shoed no significant differences between them.

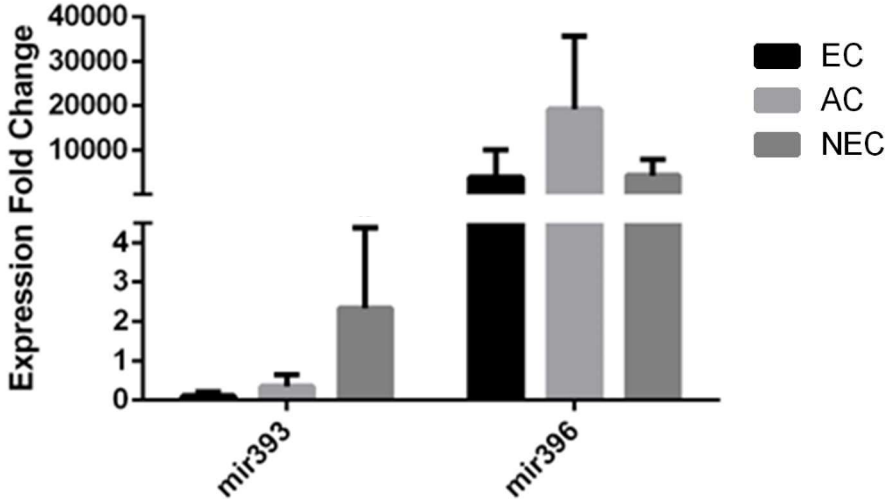


Figure 24. miRNA expression in embryogenic, long-term and non-embryogenic callus (leaf origin). No significantly differences were registered on both miRNA gene expression in the three types of callus, at p ≤ 0,05, using Tukey test. **EC –** Embryogenic callus; **AC –** Long-term callus; **NEC –** Non-embryogenic callus.

In the case of miR393, there was an increase in miRNA expression in non-embryogenic callus when compared to both embryogenic and long-term callus. Once again the embryogenic capability of each callus might be the differentiator factor. In the case of the miR396, no differences were registered, where expression values were very close between each other, although long-term callus had a higher expression of this miRNA.

4. Discussion

4.1. Generating new embryogenic lines of tamarillo and development and germination of somatic embryos

4.1.1. Somatic embryogenesis induction in leaves and embryos

Somatic embryogenesis in tamarillo can be achieved in different tissues such as leaves and embryos (Guimarães *et al*., 1996; Canhoto *et al*., 2005; Correia, 2011). In the case of SE induced in young leaves, embryogenic callus was not obtained and only non-embryogenic callus formed. This was a mishap since a new cell line was not established. What happened was due to the fact that the cell line, EC, that was already in culture was relatively old and their totipotent capability might not be at its peak as explained by Benson (2000), although embryogenic callus can keep their embryogenic capability for a long time (Lopes *et al*., 2006).

The reasons behind the unsuccessful SE induction in leaves might be due to several factors and the most prominent one is the genotype. Many reasons can influence SE induction such as the type of auxin used, the media, the age of the explant and also the genotype (Fehér, 2015). In the case of tamarillo, it was also described that the genotype is important for SE induction as shown in Correia (2011). So, the genotypes used for leaf SE induction might not have been the most suitable, resulting in the inability of producing embryogenic masses. The non-embryogenic callus obtained had a friable and translucent structure with a grey/yellow color, as already observed by Correia (2011).

In the study by Canhoto *et al.* (2005), it was concluded that the use of zygotic embryos of tamarillo to induce SE could be achieved either by using immature or mature embryos. In the present work SE induction in zygotic embryos was successfully achieved and an important cell line was obtained for the study. The fact that this is a new cell line is important because it's in its peak of totipotency capability. Embryogenic callus obtained was characterized by its white opaque granular compacted structure with a slow rate of proliferation while non-embryogenic callus was characterized by its friable and translucent structure with a high rate of proliferation. Both *calli* looked the same as the ones characterized in Canhoto *et al.* (2005) and Correia (2011). Direct embryogenesis (Quiroz-Figueroa *et al*., 2006) also happened during zygotic embryo SE, where somatic embryos started to appear on the edge of the explant. Such was already described in Canhoto *et al*. (2005).

4.1.2. Development and germination of somatic embryos

Embryogenic (EC) and non-embryogenic (NEC) callus from C81 line, embryogenic callus (EC), long-term callus (AC) and non-embryogenic callus (NEC) of the older cell lines were successfully proliferated *in vitro*. In order to induce the formation of somatic embryos only the embryogenic lines were used because they are the only ones with embryogenic capability, whereas AC has a low or no embryogenic capability, because of the callus longevity and NEC has no embryogenic capability. In several works, it was reported a decrease in the ability to develop somatic embryos in long-term embryogenic callus, which is often due to somaclonal effects accumulating during prolonged subcultures (Correia *et* al., 2011; Park *et al*., 2011; Currais *et al*., 2013). Somaclonal variation is produced during tissue culture and implies genotypic and phenotypic variations wherein the genotype, and these changes may be genetic or epigenetic like gene methylation and post-transcriptional gene expression regulation by means of miRNA action. These mechanisms will result in gene expression changes in the callus (Jaligot *et al*., 2011; Miguel and Marum, 2011). As aforementioned in the work by Benson (2000) in somehow recalcitrant plants like tamarillo, we may observe a decrease in their morphogenetic and totipotent capacity.

Somatic embryos formed from both embryogenic lines as mentioned in the results section. In newly established lines of zygotic embryo originated embryogenic callus, usually a high number of embryos formed associated with a strong development while in leaf originated embryogenic callus the same did not apply. Leaf originated EC' embryos were not as developed as those originated from zygotic embryos, what can be explained by the fact that this cellular line already had 3 years of *in vitro* culture. As explained above this might compromise the quality and quantity of the embryos. It also explains the reason behind the amount of time needed for these somatic embryos to germinate. In the case of leaf originated EC' somatic embryos, it took about 4 weeks until they germinate and acquire a green color whereas, for the ones of zygotic embryo EC, only one week was all it took for them. Another aspect to point out is that some embryos of leaf originated EC presented some deformations and sometimes would not form a fully developed plantlet. The same results were also already registered in works such as in Canhoto *et al*. (2005), Correia (2011) and Sanches (2016).

4.2. Target genes and miRNA expression evaluation and correlation

4.2.1. Target genes expression

In the case of SE induction in leaves, a decrease of gene expression in all four tested genes (*GRF1*, *GRF4*, *TIR1* and *AFB2*) was found, mainly during the first 2 weeks of induction. For the remaining of the induction time, gene expression showed to be very stable. It is also relevant to underline that gene expression in embryogenic callus was a little higher when compared to the last week of SE induction. As previously mentioned, the type of callus that resulted in leaf SE induction was only non-embryogenic callus and the gene expression values were lower in non-embryogenic callus when compared to embryogenic callus. This might explain the observations that expression levels are higher in EC when compared to the latter times of somatic embryogenesis. The decrease of these genes expression values could be due to the presence of the auxin Picloram (Grossmann, 2007).

This auxin acts as a stress factor stimulating the dedifferentiation process. During SE induction, when the dedifferentiation process begins, a shift in gene expression patterns occurs (Fehér, 2015; Nic-Can and Loyola Vargas, 2016). This means that for SE to occur, some genes will have their expression up or downregulated. In the case of the four studied genes, their expression was downregulated.

When comparing EC, AC and NEC the decrease of gene expression seems to be correlated to the lack of embryogenic capability. Embryogenic callus had the highest gene expression values, non-embryogenic callus the lowest and the long-term callus gene expression was in-between. During the development of somatic embryos an increase of expression of all four genes was registered, followed by a constant decrease until the fourth week of development. The development of the embryos only happened once the auxin was removed, and as a result, it was registered a big increase in the genes' expression in the first week. When the somatic embryos were germinated gene expression levels of the target genes were very close to the ones measured in the explants before SE induction.

Zygotic embryos are formed by cells in a lesse differentiated state than those of a leaf, which means that totipotency is easier to express in the embryos (Verdeil *et al*., 2007; Fehér, 2015). In Merkle *et al*. (1995) it was referred that the further the explant is from a zygotic embryo stage, the more reprogramming is required to convert the explant. This means that a zygotic embryo does not need a bigger reprogramming in SE induction when

compared with more differentiated tissues, like tissues from leaves. This results in a lower dedifferentiation of the zygotic embryo tissues thus reducing the impact of a bigger shift of gene expression patterns that is usually correlated with dedifferentiation (Fehér, 2015; Nic-Can and Loyola Vargas, 2016) and also the impact of epigenetic mechanisms like DNA methylation and miRNA regulation (Mahdavi-Darvari *et al*., 2014; De-la-Peña *et al*., 2015; Blein and Laufs, 2016).

Considering this framework it is permissible to admit a correlation between the lower dedifferentiation of the embryo during SE induction with its impact on the stable expression of genes during SE induction registered for zygotic embryos. In conclusion, the most undifferentiated explant, the zygotic embryo, does not suffer a huge impact during dedifferentiation that may translate into stable gene expression during SE induction. As aforementioned, the expression of the four genes was higher on the germination stage, when the first differentiated tissues appeared during the formation of a plantlet. This result may lead to speculate that dedifferentiating and undifferentiated tissues can be correlated to lower gene expression values, whereas differentiated tissues usually present higher expression values of those genes.

When comparing leaf and zygotic embryo SE there were several differences that can be compared. First the difference in how gene expression levels were altered during the whole process and second the fact that gene expression values during zygotic embryo SE were usually lower when compared to the leaf SE. On the first point, was found that in leaf SE, gene expression considerably varies through the several stages. Gene expression lowers during the induction stage, increases in the development stage and also in the germination, while on embryos the expression never changes much, where it only increases in the germination phase. On the second point, gene expression levels registered in zygotic embryo SE were very similar to the values registered in the SE induction times in leaf SE. Which means that undifferentiated tissues share similar gene expression values. What it can be concluded from this comparison is that differentiated tissues have a higher expression of the analysed genes when compared to the ones in undifferentiated tissues or the fact that these genes already have a low expression on the embryo and it is maintained until the embryo germinates and the differentiated tissues forms. The lack of bibliography means that the comparison between the zygotic embryo and leaf SE should be more studied in order to better understand and evaluate this association.

TIR1 and *AFB2* are auxin receptors that regulate the auxin response (Dharmasiri *et al*., 2005b) and of the two, *TIR1* is the most studied. *TIR1* is the auxin receptor that mediates rapid degradation of Aux/IAA proteins, controlling the expression of auxin-

regulated genes (Dharmasiri *et al*., 2005a). *TIR1* and *AFB2* expression levels plummeted during leave SE induction when the auxin was introduced in the media. The same happened in the works by Chen *et al*. (2018) and Wójcik and Gaj (2016) where both *TIR1* and *AFB2* expression lowered in SE induction. On the other hand, the results obtained in zygotic embryo SE don't correspond to the bibliography. The lower expression of these genes results in the tissue sensitivity modulation to the auxin treatments, meaning that this regulation is of the utmost importance in order to induce SE (Wójcik and Gaj, 2016). *TIR1* is also important in regulating root growth, leaf inclination and tillering, meaning that in order for normal development, their expression must be controlled (Chen *et al*., 2011). During embryo development and germination, *TIR1* levels increased, which means that this gene is necessary for the embryos to develop and germinate. The same can be applied to *AFB2* since these share common tasks.

GRF's are transcription factors that control cell proliferation during leaf development and also in the development and growth of plant organs and structures (Kim *et al*., 2003; Rodriguez Gonzalez *et al*., 2010; *et al*., 2016). In the present work, *GRF1* and *GRF4* gene expression levels, during leaf SE induction, dropped on the second week and then maintained until the of the induction stage. Hewezi and Baum (2012) also reported that *GRF* family genes were differentially expressed during SE. During the development and germination stages their levels rose which means that the *GRF*'s might also play a role during somatic embryo development as documented by Vestman *et al*. (2011).

4.2.2. MicroRNAs expression

As referred in the Results section, miRNA expression levels quantification were analysed in leaves SE but it wasn't possible in the case of zygotic embryo SE.

During SE both miR393 and miR396 had low expression levels in the original explants, were upregulated during the induction phase and in embryogenic callus and down-regulated during the development and germination of embryos. When comparing miR393 expression in EC, AC and NEC, its expression was higher in NEC and lower in both EC and AC while in miR396 the expression values were almost the same in all three, although a bit higher in AC.

miR393 and miR396 are considered to be two of the most important miRNA involved on the control of SE (Szyrajew *et al*., 2017) as they were highly expressed during the induction phase of SE. Other authors have shown that the expression of miR393 was also regulated during the SE process, where it was especially upregulated at the beginning of SE (Wójcik and Gaj, 2016). Similar results were obtained by Hewezi and Baum (2012)

working with *A. thaliana* in which expression of miRNA396 was also considered to be differentially expressed during the SE process.

A study by Qiao *et al*. (2012) also correlated the expression values of miR393 in both totipotent and non-totipotent callus, where this miRNA expressed highly in non-totipotent callus and lower in totipotent callus. The same results were obtained in this work. Evidence about miR396 expression in different types of callus are non-existent and the same applies to the measure of miR393 expression levels during somatic embryo development and germination. In a work by Vestman *et al*. (2011) miR396 was found to be downregulated during early to late embryo development much alike to what happens in this work.

Not much information about these miRNA in SE induction and development and germination of somatic embryos is available. Most of the information found about these miRNA during these stages is recent and much is still left to be investigated.

4.2.3. miR393, *TIR1* and *AFB2* correlation

Many studies reported that miR393 controlled the expression of both *TIR1* and *AFB2* genes during response to biotic and abiotic stresses and plant development (Vidal *et al*., 2010; Chen *et al.*, 2011; Etemandi *et al*., 2014; Iglesias *et al.*, 2014; Zhang *et al*., 2015; Xu *et al*., 2017; Wang *et al*., 2018) and in somatic embryogenesis (Yang *et al*., 2012; Wójcik and Gaj, 2016; Chen *et al*., 2018).

miR393 shares a strong inverse correlation with the genes *TIR1* and *AFB2*, meaning that when expression levels of miR393 increases, the levels of *TIR1* and *AFB2* decreases and *vice-versa*. This shows evidence that miR393 may regulate the expression of both genes during the SE process, although by this process one can't prove that miR393 does regulate both target genes. Nonetheless, referring to the bibliography we may assume that this correlation is indeed true and not just a coincidence.

As mentioned before expression levels of miR393 rose during SE induction and declined in the stages of development and germination of the somatic embryo while the target genes had an inverse behavior. The genes behaved very similarly, presenting a strong correlation. Firstly they were downregulated during SE induction and upregulated during the development and germination stages. This means that when the auxin was introduced, miR393 expression rose and the expression of the target genes lowered and when the auxin was removed the inverse was registered, where miR393 expression plummeted and target genes' expression ascended. As reported by Szyrajew *et al*. (2017) this miRNA is fundamental for the induction of SE since it regulates auxin-related genes expression and is also relevant in the response of biotic and abiotic stresses like the

introduction of a PGR in the media, like Picloram that induces stress and auxin response (Grossmann, 2007), or mechanical injuring (Etemandi *et al*., 2014; Iglesias *et al.*, 2014; Zhang *et al*., 2015; Wang *et al*., 2018). By controlling these mechanisms, this miRNA contributes to embryogenic transition by actively modulating the tissue sensitivity to the auxin treatments (Wójcik and Gaj, 2016; Chen *et al*., 2018).

This modulation of the sensitivity of the tissues is only achieved by silencing the *TIR1* and *AFB2* genes that function as auxin receptors, regulating the auxin response, this means that by upregulating miR393, *TIR1* and *AFB2* genes are downregulated (Wójcik and Gaj, 2016; Chen *et al*., 2018). The same happens during plant development and when exposed to abiotic stresses (Vidal *et al*., 2010; Chen *et al.*, 2011; Wang *et al*., 2018). This also means that miR393 is responsible for proper homeostasis of auxin signalling in plant development (Windels *et al*., 2014; Wójcik and Gaj, 2016).

In the present work, a similar behaviour was reported where miR393 expression increased in the beginning and *TIR1* and *AFB2* expression decreased. Based on the previous studies we may assume that during SE induction in tamarillo leaves, miR393 controls the expression of both *TIR1* and *AFB2* genes in order to respond to the auxin presence and stress factors, resulting in the modulation of the sensitivity of the tissues to the auxin treatment, thus resulting in the dedifferentiation process which allows SE to initiate.

TIR1 and *AFB2* play a role in the development of the plant by regulating the auxin response, with *TIR1* mediating the rapid degradation of Aux/IAA proteins, controlling the expression of auxin-regulated-genes (Dharmasiri *et al*., 2005a; Dharmasiri *et al*., 2005b; Vidal *et al*., 2010; Chen *et al*., 2011; Si-Ammour *et* al., 2011; Wang *et al*., 2018). This auxin receptor will control the endogenous auxin content in order to somatic embryos develop and germinate. As documented in this work, *TIR1* and *AFB2* expression levels rose once the media was devoided of auxins, while miR393 expression lowered. The downregulation of miR393, the regulator of the target genes, allows the upregulation of *TIR1* and *AFB2* genes. This means that by upregulating both genes, they will be necessary in the development and germination in order to obtain a new fully developed plant.

When comparing the expression values of miR393 and target genes in the different callus, an inverse correlation can also be made. In EC target gene expression is higher and miRNA expression is lower, while in NEC the reverse happens. This is another evidence that miR393 controls the expression of *TIR1* and *AFB2*. Although different, the expression values between EC and NEC are not very far. This may be due to the fact that the embryogenic line used is already old as it may be losing its embryogenic capability (Correia *et al*., 2011), being this the reason why it was so important to establish a new

embryogenic line from leaf explants. Nevertheless, the reason why both miRNA and target genes expression is different in the different callus remains to be studied more deeply, although the miRNA/target gene pair may be important in the embryogenic capability of the callus.

In sum, it may be assumed that miR393 plays a big role during the process of SE in tamarillo. miR393 responds to the presence of auxin and stress by downregulating *TIR1* and *AFB2* genes in order to start SE induction, allowing the transition of somatic cells. When the auxin is no longer present, its expression levels lower, allowing *TIR1* and *AFB2* gene expression to rise in order to participate in the somatic embryo development.

4.2.4. miR396 and *GRF*'s correlation

miR396 has been a subject of study in different aspects of plant development but mainly mechanisms other than SE. Szyrajew *et al*. (2017) referred that this miRNA was strongly expressed during SE induction phase and that it controlled genes belonging to the *GRF* family genes. Hewezi and Baum (2012) also showed similar results where *GRF* family genes were differentially expressed during SE and were confirmed targets of miR396 that also expressed differentially during the same process. The regulation of *GRF*'s family genes by miR396 was also confirmed during cell proliferation, plant development and growth of organs and structures (Debernardi *et al*., 2012; Ercoli *et al*., 2016; Rodriguez *et al*., 2016; Chen *et al*., 2018).

miR396 shares a strong inverse correlation with the genes *GRF1* and *GRF4*. When expression levels of miR396 increases, the levels of *GRF1* and *GRF4* decreases and *vice-versa*. Based on this data, miR396 may regulate the expression of both genes during the SE process. This data is not enough to prove that they do share a relation but by resorting to previous studies we may assume that this correlation is indeed true.

Very similarly to the case of miR393, miR396 and its target genes behaved in a very similar way. While miR396 expression levels increased in SE induction and decreased during germination and germination of somatic embryos, both *GRF*'s expressed in an inverse way, very similar to each other (strong correlation). Once again the presence of the auxin seems to be key in the miR396 regulation of *GRF1* and *GRF4* genes.

In order to initiate SE, miR396 should be highly expressed (Szyrajew *et al*., 2017) and during the development stage, its expression should lower, allowing the *GRF*'s expression to rise in order to promote the development of the somatic embryos (Vestman *et al*., 2011). Although its function is not well described in SE, miR396 is involved in many processes of plant development by controlling *GRF*'s gene expression (Ercoli *et al*., 2016; Chen *et al*.,

2018), differentiation (Rodriguez *et al*., 2010) and stress responses (Omidbakhshfard *et al*., 2015; Liu *et al*., 2017). Because of its function, we may associate that this miRNA plays a role in SE induction by responding to the stress factors, downregulating *GRF1* and *GRF4* expression while during the development stage, miR396 is downregulated and both *GRF*'s are upregulated in order to help in the development and differentiation of the somatic embryos.

Attending to the results, miR396 is highly expressed during SE induction in order to respond to both auxin and mechanically injuring induced stress by downregulating both *GRF1* and *GRF4* genes. This confirms miR396 as an important regulator during SE induction. Afterward, miR396 is downregulated in response to the stress agent removal, the auxin, regulating upwards the expression of *GRF1* and *GRF4*. This upregulation means that both genes will be performing a task during the development and germination stages. *GRF1* and *GRF4* will be helping in the development and differentiation of the somatic embryos.

When comparing miRNA/target genes expression levels between the different *calli*, its visible a higher expression of the target genes in EC when compared to NEC but the same difference is not seen in the correlated miRNA. When comparing miR396 expression in EC, AC and NEC, almost no differences are visible, which is contrary to the expected, since miR396 expression influences the expression of both *GRF1* and *GRF4*. Once again the age of the embryogenic callus may take a toll on these results. Because the EC is a bit old, it may influence the expression of the miRNA, resulting in a higher expression, when probably the opposite should have been registered. More information is needed to be gathered in order to fully understand how miRNA expression differs in the different types of callus.

Overall, it seems that miR396 plays an important role in SE induction but also in the development of the somatic embryos. When stress factors are present at the beginning of SE, miR396 responds by downregulating *GRF1* and *GRF4*, allowing SE to begin. When auxin is removed, miR396 responds by being downregulated, resulting in the upregulation of GRF1 and GRF4 genes that will play a role during the development of the somatic embryos.

5. Concluding remarks

Embryogenic callus was successfully obtained from zygotic embryos explants but not from leaf explants. This may be due to the fact that the genotype chosen might have been incorrect.

From embryogenic *calli* of two different origins (leaf and zygotic embryo), somatic embryo formation and development occurred. In the case of somatic embryos from leaf EC, the embryos developed slower and/or didn't develop normally, which indicates that the age of the cell line could have an impact on the quality of the newly formed somatic embryos, thus meaning that it is important to establish and analyse a new cell line.

TIR1, *AFB2*, *GRF1* and *GRF4* are genes that participate during several processes of plant growth and development and also during SE, where their expression values fluctuate during the SE. In the case of leaf SE, low expression levels were registered in SE induction and in the different callus, while during the development and germination stages the expression levels increased and in some cases the values registered in the germination stage were closely related to the original explant. In the case of zygotic embryo SE the expression values didn't vary much during the SE process and also when comparing EC and NEC callus. Although the level of differentiation of the zygotic embryo may be a reason for these results, more information needs to be gathered in order to reach a further conclusion.

Post-transcriptional gene expression regulation by the action of miRNAs has proven to be an important epigenetic mechanism during somatic embryogenesis, where miR393 and miR396 expression oscillates during the full SE process. High expression values were registered during SE induction and in the different types of callus, embryogenic, long-term and non-embryogenic callus.

miR393 seems to regulate *TIR1* and *AFB2* genes during SE. At the beginning of SE induction, miRNA393 downregulates both target genes, in response to the auxin and stress, so the tissues start to dedifferentiate. When the media is devoid of auxins, miRNA393 expression lowers allowing that both genes participate in the embryo development.

In the case of miR396, strong evidence shows that *GRF1* and *GRF4* gene expression is controlled by this miRNA during SE. During SE induction, miR396 has a stress-related response, silencing both *GRF1* and *GRF4* expression. During embryo development, miRNA393 expression lowers and in contrast both GRF's expression increases. *GRF1* and *GRF4* will actively participate in the development and growth of the somatic embryo.

Much more information needs to be gathered in order to further explore and complement relevant matters of this study such as the comparison between target genes

expression in both leaf and zygotic embryo SE, and their function throughout the process, the differences between miRNA/target genes expression in the different callus and other addressed issues. Still, many questions are to be answered in order to fully understand the role of these pairs in SE and more studies are required consequently. More data should also be gathered in order to reinforce the present data and other protocols, such as protoplast transformation, are required to prove that miR393 controls the expression values of *TIR1* and *AFB2* and that miR396 controls *GRF1* and *GRF4* expression values.

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