Somatic embryogenesis in *Cyphomandra betacea* (Cav.) Sendt (tamarillo): optimization and molecular analysis

Sandra Isabel Marques Correia

Universidade de Coimbra

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Sandra Isabel Marques Correia

Orientador:

Prof. Doutor Jorge Manuel Pataca Leal Canhoto

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Picture on the cover is a Elizabeth Hawkes painting.

Tamarillo Tree

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2-DE – Two dimensional electrophoresis
2,4-D – 2,4-dichlorophenoxyacetic acid
A. thaliana – Aranidopsis thaliana
ABA – Abscisic acid
B5 – Gamborg culture medium (Gamborg, 1968)
BA – Benzyladenine
BSA – Bovine serum albumin
bp – Base pairs
CAPS - N-cyclohexyl-3-aminopropanesulfonic acid
cDNA – Complimentary deoxyribonucleic acid
CHAPS - 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid
DNA - Deoxyribonucleic acid
dNTP's - Term referring to the 4 deoxyribonucleotides: dATP, dCTP, dGTP and dTTP
DTT – Dithiothreitol
E. coli – Escherichia coli
EC – Embryogenic callus
EDTA – 2-[2-(bis(carboxymethyl) amino) ethyl-carboxylmethyl) amino] acetic acid
EST – Expressed sequence tags
EtBr – Ethidium bromide
FW – Fresh weight
GA3 – Gibberellic acid
hRNA – Hairpin ribonucleic acid
IBA - Indole-3-butyric acid
IEF – Isoelectric focusing
Kin – Kinetin
LB – Luria broth medium
LC MS/MS - Liquid chromatography combined with tandem mass spectrometry
LEC – Leaf embryogenic callus
LNEC – Leaf non-embryogenic callus
mRNA – Messenger ribonucleic acid
$MgCl_2$ - Magnesium chlorid
MS - Murashige and Skoog culture medium (Murashige and Skoog, 1962)
MTase - Methyltransferase
NAA - 1-Naphthalene acetic acid
NEC – Non-embryogenic callus
NEP – Non-embryogenic protein
OPC - Operon Technologies Kit C, sequences of the arbitrary primers
OT – Orange tamarillo
PEMs – Proembryogenic masses
pic – Picloram
PCR - Polymerase chain reaction
PGR(s) - Plant growth regulator(s)
PTGS – post-transcriptional gene silencing
RAPD - Random amplified polymorphic DNA
RNA - Ribonucleic acid
RT – Reverse transcriptase

RTA – Red tamarillo plant A
RTC – Red tamarillo from Coimbra
RTL – Red tamarillo from Leiria
SAM - Shoot apical meristem
SDS – Sodium-dodecyl sulfate
SE – Somatic embryogenesis
SE - Standard error
SEM – Scanning electron microscopy
TBS-T - Tris-HCl Buffered Saline-Ttween
T-DNA – Transfer DNA
tRNA – Transfer ribonucleic acid
Taq polymerase - Enzyme originally isolated from the bacteria *Thermus aquaticus;*
TBE – Tris-borate-EDTA
TDZ – Thidiazuron
Tris – Tris-hydroxymethylaminomethane (2-amino-2-(hydroxymethyl) propane-1,3-diol
YT – Yellow tamarillo
Zt – Zeatin
ZEEC – Zygotic embryo embryogenic callus
ZENEC – Zygotic embryo non-embryogenic callus

GENERAL SUMMARY

Cyphomandra betacea (Cav.) Sendt (tamarillo) is a small solanaceous tree which produces edible high nutritional fruits. Several lines of research have shown the interest of this species to understand particular aspects of *in vitro* morphogenesis, in particular somatic embryogenesis (SE). SE is an important biotechnological tool with great potential for rapid large-scale clone propagation. In addition, genetic transformation and cryopreservation procedures in many plant species rely on efficient SE protocols. One of the pathways to induce SE in tamarillo is a two-step process in which embryogenic tissues and non-embryogenic callus are first produced (induction phase) in an auxin-rich medium and then developed into embryos, following the transfer to an auxin-free medium (development phase). Explants as zygotic embryos, hypocotyls and leaves can participate in this type of somatic embryo formation. To uncover some of the limiting steps of SE induction and to identify markers directly related to this process, which would allow achieving more efficient results, not only with tamarillo but also with other woody species, three strategies were followed: the improvement of induction and somatic embryo development protocols, the investigation of a SE inhibitory protein, previously identified, and a comparison between proteomic profiles of embryogenic and non-embryogenic *calli*.

Previous works have shown that SE in tamarillo can be easily induced from juvenile plant material. In this work we were able to clone an adult tamarillo tree through SE. To overcome the lack of potential of adult tissues for SE, an indirect approach was attempted, in which shoots from an adult tree were first established *in vitro* and then wounded leaves were used for SE induction. These results open the way for large-scale cloning of elite tamarillo trees through SE. The optimization of the somatic embryo development protocol was also achieved by increasing the number of somatic embryos that can be converted into plantlets. The manipulation of sucrose concentration showed that 0.11M sucrose and dark conditions before conversion increase the number of morphologically normal somatic embryos produced, a critical step to enhance the yield of converted embryos. The comparison between mature zygotic embryos and normal somatic embryos, after the maturation period, reflects an inefficient accumulation of storage compounds in tamarillo somatic embryos, namely in what concerns lipids

storage. Poor storage accumulation may be responsible for the abnormal development displayed by most of these embryos.

In tamarillo, embryogenic and non-embryogenic cells arise side by side from the same cultured explants, a condition ideal to evaluate molecular and biochemical changes occurring in the different types of *calli*. Moreover, different types of explants and auxins led to the same morphological responses, indicating that different tissues can perceive the same stimulus in a similar way, pointing out to a general behavior of the cells under certain stimulus, which can be extended to understand embryogenesis in a more integrated perspective.

Previous comparative proteomic analyses identified a protein (NEP25, 26.5 kDa) consistently present in non-embryogenic *calli*. In this work, we evaluated the role of NEP25 on somatic embryogenesis induction, following two experimental strategies: 1) SE induction on knock-out lines of *Arabidopsis thaliana* and 2) post-transcriptional *NEP25* gene down-regulation in tamarillo. Tamarillo *NEP25* gene down-regulation was achieved by an improved *Agrobacterium*-mediated transformation protocol that allowed obtaining more than 80 self-rooted kanamycin resistant plants from a single embryogenic cell line. The data obtained associate (for the first time) a putative member of the SpoU methylase super family with the inhibition of SE. This is a protein family that promotes tRNA/rRNA stabilization through its methylation. The absence of particular phenotypes resulting from gene silencing in *Arabidopsis thaliana* or from NEP25 down-regulation in tamarillo suggests that other proteins of this family may have a redundant role during the SE induction process.

Trying to find embryogenic-specific proteins, a comparative analysis of the proteome of tamarillo's embryogenic and non-embryogenic *calli* was performed. Through two-dimensional electrophoresis (2-DE) and LC-MS/MS, proteins exclusively or predominantly expressed in embryogenic tissues were identified, such as enolases, adenylate kinases, fumarate hydratases, treonine synthases (metabolic proteins). On the other hand, pathogenesis-related proteins and fructokinases (stress and defense related proteins) occur mainly in non-embryogenic callus. An interesting differential expression of heat-shock proteins in embryogenic cells was also detected. The data so far obtained indicate that, for the same type of *calli* (embryogenic tissue or non-embryogenic callus), a strong resemblance occurs between protein profiles, in spite of the auxin (2,4-D or picloram) or explant (young leaves or zygotic embryos) tested.

The information gathered with this work may help to answer to some of the limiting steps of SE in tamarillo, to which fundamental knowledge from the classical model plants has been insufficient. Finding proteins directly involved in the acquisition of embryogenic competence may help to understand the regulatory mechanisms of this process.

RESUMO GERAL

Cyphomandra betacea (Cav.) Sendt (tamarillo) é uma pequena árvore da família *Solanaceae* que produz frutos comestíveis bastante nutritivos. Várias linhas de investigação têm demonstrado o interesse desta espécie para a compreensão de aspectos específicos da morfogénese *in vitro*, em particular da embriogénese somática (ES). A ES é uma ferramenta biotecnológica importante com um enorme potencial para a rápida propagação clonal em grande escala. Além disso, os procedimentos de transformação genética e criopreservação em muitas espécies vegetais baseiam-se em protocolos eficazes de ES. Uma das muitas formas de induzir ES no tamarilho consiste num processo em duas fases no qual os tecidos embriogénicos e os calos não embriogénicos são inicialmente produzidos (fase de indução) num meio rico em auxina e, após a sua transferência para um meio isento de auxina, formam embriões (fase de desenvolvimento). Vários tipos de explantes, como embriões zigóticos, hipocótilos e folhas têm capacidade para sofrer embriogénese somática. De modo a ultrapassar alguns dos passos restritivos da indução de ES e identificar marcadores directamente relacionados com este processo, que permitiriam obter resultados mais eficazes, não só no tamarilho mas também com outras espécies lenhosas, foram seguidas três estratégias: a melhoria dos protocolos de indução e desenvolvimento de embriões somáticos, a investigação de uma proteína inibidora de ES, previamente identificada, e a comparação dos perfis proteicos de calos embriogénicos e não embriogénicos.

Trabalhos anteriores têm demonstrado que a indução de ES no tamarilho pode ser facilmente obtida a partir de material vegetal juvenil. Com este trabalho, foi-nos possível clonar uma árvore de tamarilho adulta por ES. Para superar o reduzido potencial de ES nos tecidos adultos, foi experimentada uma abordagem indirecta, na qual os rebentos de uma árvore adulta foram, primeiramente, estabelecidos *in vitro* e, depois, as folhas foram utilizadas na indução de ES. Estes resultados abrem o caminho para uma clonagem em larga escala de árvores seleccionadas de tamarilho por ES. A optimização do protocolo de desenvolvimento de embriões somáticos também foi alcançada aumentando o número de embriões somáticos que podem ser convertidos em plântulas. A manipulação da concentração de sacarose para 0,11M e a realização das culturas no escuro, antes da conversão, aumentam o número de embriões somáticos morfologicamente normais produzidos, um passo essencial para aumentar a produção de

embriões convertidos. A comparação entre embriões zigóticos maduros e embriões somáticos normais, após o período de maturação, reflecte uma acumulação ineficaz de compostos de armazenamento nos embriões somáticos de tamarilho, nomeadamente em relação ao armazenamento de lípidos. A fraca acumulação de reservas poderá ser responsável pelo desenvolvimento anormal registado na maioria destes embriões.

No tamarilho, as células embriogénicas e não embriogénicas surgem lado a lado nos mesmos explantes, uma condição ideal para avaliar as alterações moleculares e bioquímicas observadas nos diferentes tipos de calos. Além disso, os diferentes tipos de explantes e auxinas causam as mesmas respostas morfológicas, o que indica que diferentes tecidos podem responder ao mesmo estímulo de forma semelhante, apontando para um comportamento geral das células sob a acção de determinados sinais que podem ser utilizados para compreender a embriogénese numa perspectiva mais integradora.

Análises proteómicas comparativas anteriores identificaram uma proteína (NEP25, 26.5 kDa) presente, de forma consistente, em calos não embriogénicos. Neste trabalho, avaliámos a função da NEP25 na indução de embriogénese somática, seguindo duas estratégias experimentais: 1) indução de ES em linhas *knock-out* de *Arabidopsis thaliana* e 2) *down-regulation* pós-transcripcional do gene *NEP25* no tamarilho. A *down-regulation* do gene *NEP25* em tamarilho foi conseguida através de um protocolo optimizado de transformação via *Agrobacterium* que permitiu obter mais de 80 plantas resistentes à canamicina a partir de uma única linha celular embriogénica. Os dados obtidos associam (pela primeira vez) um membro putativo de uma super-família de metilases de RNA (SpoU) à inibição de ES. Esta é uma família de proteínas que promove a estabilização de tRNA/rRNA através da sua metilação. A ausência de fenótipos específicos resultantes do silenciamento de genes em *Arabidopsis thaliana* ou da *down-regulation* de *NEP25* no tamarilho sugere que outras proteínas desta família poderão ter um papel redundante no processo de indução de ES.

Na tentativa de encontrar proteínas específicas de embriogénese somática, foi realizada uma análise comparativa dos perfis proteicos de calos embriogénicos e não embriogénicos de tamarilho. Através de electroforese bidimensional (2-DE) e LC-MS/MS, foram identificadas proteínas expressas, exclusiva ou predominantemente, em tecidos embriogénicos, como, por exemplo, enolase, adenilato-cinase, fumarato-hidratase, treonina-sintase (proteínas metabólicas). Por outro lado, as proteínas associadas à patogénese, como a frutocinase (proteínas de stress e defesa), ocorrem

principalmente em calos não embriogénicos. Também foi detectada uma interessante expressão diferencial de proteínas de choque térmico nas células embriogénicas. Os dados até agora obtidos indicam que, no mesmo tipo de calos (tecido embriogénico ou calo não embriogénico) verifica-se uma grande semelhança entre os perfis proteicos, independentemente da auxina (2,4-D ou picloram) ou explante (folhas jovens ou embriões zigóticos) testados.

A informação recolhida com este trabalho poderá ajudar a responder a alguns dos passos restritivos da ES no tamarilho, para a qual os conhecimentos fundamentais sobre as plantas modelo tradicionais têm sido insuficientes. Encontrar as proteínas directamente envolvidas na aquisição de competência embriogénica poderá ajudar a compreender os mecanismos reguladores deste processo.

CHAPTER 1

GENERAL INTRODUCTION

Part of this chapter is submitted to be published as a book chapter:

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1. INTRODUCTION

1.1. TAMARILLO (CYPHOMANDRA BETACEA (CAV.) SENDT.)

1.1.1. CHARACTERIZATION AND DISTRIBUTION

Tamarillo, *Cyphomandra betacea* (Cav.) Sendt. (Fig. 1A), is a Solanaceae also know by other names such as tree tomato or "tomate de la paz" (Bois, 1927). The tamarillo designation appeared in 1967, in New Zealand, after a rapid increase in the production and consumption of this fruit, which led to the need of having a commercially appealing trade name which could not be mistaken with the well known tomato (*Lycopersycon esculentum*, now *Solanum lycopersicum*) a relative of tamarillo (Morton, 1987). Originated from South America, more specifically from the Andean region of Peru, Chile, Ecuador and Bolivia (Dawes and Pringle, 1983; Meadows, 2002), it spread to Central America and West Indies and, later on, to the Portuguese islands of Azores and Madeira and to southern Europe. By the end of the 19th century, it had reached Australia and New Zealand. Today, although currently grown in California, Argentina, Colombia and Venezuela, New Zealand is the main producer and exporter (Meadows, 2002).

Tamarillo is a small (2 to 4 meters high) perennial tree, with deciduous leaves of 10-30 cm of length. Flowers (Fig. 1B), which are rose-shaped and scent usually develop in small groups at the top of the branches. They blossom mostly during the transition from summer to fall but can, however, appear in other times of the year. Pollination is mainly autogamic, what may explain the low variability observed in natural populations of this species (Barghchi, 1986). Fruits (Fig. 1C), with long peduncles, can appear isolated or in groups of 3-12 units and are egg-shaped, sharp at both ends. Their size range from 5 to 10 cm long, and 3 to 5 cm wide, reaching maturity from October to April. The epicarp can be dark red, orange or yellowish or even a mixture of these three colors. Pulp color can also range from orange-red to yellow. Fruits are edible but the skin is thick and has an unpleasant taste, thereby it should be removed before eating. Pulp is consistent and juicy, with a sweet-sour taste. Seeds which are flat and round shaped, can be eaten together with the endocarp, but they are bigger and harder than those of tomato fruits.

Figure 1 - *Cyphomandra betacea*. **(A)** Tamarillo tree growing at the Botanical Garden of the University of Coimbra. **(B)** Flowers at different developmental stages. **(C)** Fruits.

1.1.2. ECONOMIC RELEVANCE AND PRODUCTION

This species is usually cultivated because of its fruits which, when ripe, can have several uses, from salads to industrial processing products, like juices or jams. In terms of nutritional value, tamarillo fruits have a relatively high content of proteins (1.5-2g/100g), vitamin C (30-45mg/100g), vitamin E (1.86mg/100g), provitamin A and some minerals like potassium and iron. Furthermore, the carbohydrate content is low (7.7g/100g), as well as the caloric value (around 28cal/100g) (McCane and Widdowson, 1992).

In recent years several studies aiming to evaluate the content and type of anthocyanins and carotenoids in tamarillo fruits have been carried out (de Rosso and

Mercadante, 2007; Kou *et al.*, 2008; Hurtado *et al.*, 2009). The results have shown that some of these compounds may have important biological, therapeutic, and preventive antioxidant properties making them a valuable food resource which deserves to be better explored both as a fruit and as a source of compounds that can eventually improve human health.

Until recently, among the several varieties of tamarillo known, only the red (most widely grown) and yellow varieties were usually commercialized. Fruits of the yellow variety have a strong, acid taste (citric acid content between 2.1 and 2.4%), with low sugar levels (McCane and Widdowson, 1992). Fruits of this variety are mostly used in canned food preparation, while the red ones, given their high anthocyanin content and acidity, are less appropriate for this purpose. The red varieties are the consumers favourite, being more attractive, with a superior flavour and, in some aspects, a higher nutritional value. Recent developments may, however, boost the interest for this fruit since some countries, such as New Zealand and USA (California), are implementing intensive plant breeding programmes to obtain new cultivars more attractive for consumers (Meadows, 2002).

Propagation of tamarillo cultivars can be achieved through seeds or cuttings (Prohens and Nuez, 2001), or by grafting onto wild trees of *Solanum mauritiantum*. Seeds produce upright trees with higher branches, while cuttings usually develop into smaller plants with hanging branches, which need an artificial support. Seed germination can be faster if the seeds, after being washed and dried, are stored in the cold for 24 hours before sowing. Young plants may develop in pots until they reach, approximately, 40 cm high (more or less a period of 4 months). To achieve a successful propagation, the cuttings must be collected from branches with 1-2 years old, 10-25 mm thick and 30-40 cm long.

Although seeds germinate easily, they do not assure genetic uniformity and are useless if the objective is to propagate selected genotypes. In this case, asexual methods of propagation have to be used. The low success rate of cross-pollination and the high incidence of incompatibly phenomena within this species, plus its phytosanitary problems (Mossop, 1977) have made the use of traditional techniques inadequate for improving tamarillo cultivars. Thus, biotechnological methods such as *in vitro* cloning and genetic transformation appear as useful tools for *C. betacea* plant breeding (Barghchi, 1998), hence complementing the more conventional methods of breeding.

1.1.3. *IN VITRO* REGENERATION AND TRANSFORMATION SYSTEMS

An increasing number of assays describing tamarillo micropropagation through axillary shoot proliferation (Figs. 2A-C) (Cohen and Elliot, 1979; Barghchi, 1998; Obando *et al.*, 1992), organogenesis (Figs. 2D-E) (Guimarães *et al.*, 1996; Obando and Jordan, 2001) and somatic embryogenesis (Figs. 3 and 4) (Guimarães *et al.*, 1988, 1996; Lopes *et al.*, 2000; Canhoto *et al.*, 2005; Correia *et al.*, 2009) have been published. Attempts to obtain genetically modified plants of tamarillo have also been reported (Atkinson and Gardner, 1993; Cohen *et al.*, 2000; Cruz and Tomé, 2007). Micropropagation of tamarillo through axillary shoot proliferation was the first method to be applied (Barghchi, 1986; Cohen and Elliot, 1979). Then, regeneration by organogenesis on leaf explants (Obando *et al.*, 1992) and from protoplasts (Tomé *et al.*, 1992) was achieved.

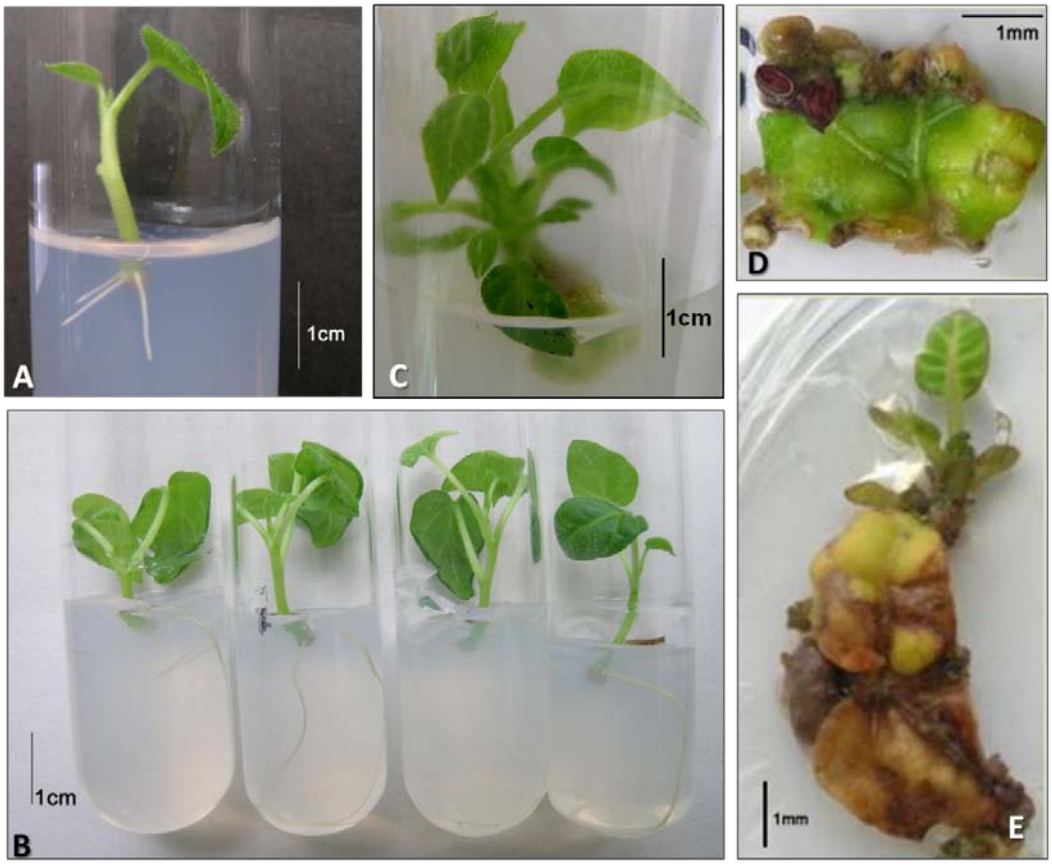

Figure 2 - Propagation of tamarillo by axillary shoot proliferation and organogenesis induction. **(A)** Apical shoot rooted on MS medium, after 2 weeks in culture. **(B)** Plantlets of different clones established *in vitro*. **(C)** Axillary shoot proliferation on MS medium supplemented with a cytokinin, after 2 weeks in culture. **(D)** and **(E)** Adventitious bud formation **(D)** and shoot developement **(E).**

In 1988, our laboratory reported, for the first time, success on somatic embryogenesis (SE) induction in tamarillo through the culture of mature zygotic embryos and hypocotyls (Guimarães *et al.*, 1988). In a follow-up study (Guimarães *et al.*, 1996), the same group described the obtention of tamarillo plants by organogenesis and SE from different kinds of tamarillo explants (hypocotyls, cotyledons, roots and mature zygotic embryos) and from protoplasts. In this set of works cultures were initiated directly from zygotic embryos, from different parts of 3-week-old aseptically grown seedlings, and from leaf disks or petiole segments. In tamarillo somatic embryo formation can follow two quite distinct pathways. In the presence of the auxin NAA (1-naphthaleneacetic acid) zygotic embryos differentiate in somatic embryos following the formation of a reduced callus in a one-step process. On the other hand, when the stronger auxins 2,4-D (2,4-dichlorophenoxyacetic) or Picloram are used zygotic embryos or young leaf segments produce an embryogenic tissue that can be maintained by successive subcultures in the same auxin-containing medium. Following the transfer to auxin-free conditions, the embryogenic *calli*, formed by proembryogenic masses, develop into embryos and later into plantlets. More details about plant regeneration through SE are given in section 2.3.1. Shoot regeneration was also observed from hypocotyls, cotyledon and root explants in MS medium (Murashige and Skoog, 1962) containing benzyladenine (BA), as well as from the cut edges of leaf disks and petiole segments of rapidly growing leaves. Shoot rooting is currently achieved by treatments with IBA (indole-3-butyric acid). Chromosome counts in root tip cells of plants regenerated either by organogenesis or SE, showed a normal diploid set of chromosomes (2n=24). However, in long-term (more than 2 years) embryogenic callus cultures, plants displaying abnormal chromosome numbers were often found (Canhoto *et al.*, 2005).

Trying to eliminate virus in contaminated tamarillo plants, Barghchi (1998) carried out several assays in which shoot tips were submitted to thermotherapy for several periods of time (32-36ºC). Immunosorbent electron microscopy and serological tests confirmed that the plants thus obtained were virus-free.

Previous works have already reported the application of the *Agrobacterium*-mediated transformation method to obtain genetically modified tamarillo plants regenerated via organogenesis (Atkinson and Gardner, 1993). In that work the avirulent LBA4404 *Agrobacterium tumefaciens* strain was used to introduce the pKIWI110

binary vector into leaf disks. All regenerated plants were kanamycin-resistant, and some of them also expressed the β- D-glucuronidase (*gus*A) reporter gene and chlorsulfuron resistance. More recently, *Agrobacterium*-mediated transformation was also used to obtain tamarillo plants resistant to TaMV (tamarillo mosaic virus) that were regenerated by axillary shoot proliferation (Cohen *et al.,* 2000). Genetic transformation studies associated with efficient protocols of *in vitro* regeneration may play a decisive role in developing new clones resistant to abiotic stress, virus, pests and diseases that, by conventional breeding, are difficult to obtain.

More recently, micropropagation of tamarillo plants through the formation of adventitious buds on the abaxial surface of leaf explants and their multiplication by axillary bud proliferation have also been carried out (Obando and Jordan, 2001; Gatita and Almeida, 2003). Anther culture has also been tried by Barghchi (1998), that reported the induction of pollen *calli* on media supplemented with BA but further haploid plant production could not be achieved. Preliminary assays made by our group led to the formation of multicellular microspores possessing up to 16 cells but which did not develop further.

1.1.4. SOMATIC EMBRYOGENESIS IN TAMARILLO

Since 1988, different aspects related with SE induction and somatic embryo development of tamarillo have been studied at our laboratory (Guimarães *et al.*, 1988, 1996; Lopes *et al.*, 2000; Canhoto *et al.*, 2005; Correia *et al.*, 2009) making it a suitable model to understand the cytological and molecular mechanisms involved in somatic embryo formation and development, a morphogenic process with important applications both for plant cloning and genetic transformation and also to better understand embryo formation and development.

Several explants of tamarillo have the potential to initiate embryogenic cultures including mature zygotic embryos (Figs. 3 and 4A), young leaves (Figs. 3 and 4B-E), cotyledons and hypocotyls. The recalcitrance of adult material for SE induction has been often reported for many species, in particular trees (Thorpe and Stasolla, 2001), hence limiting the cloning of selected plants. An alternative way to clone adult tamarillo plants by SE can be the *in vitro* establishment of the plants by axillary shoot proliferation followed by induction of embryogenesis in leaf segments of these plants (Correia *et al.*, 2011). As previously stated, SE induction is routinely achieved with an

auxin-rich culture medium in which the inclusion of higher sucrose levels (9%) strongly increases somatic embryo formation, raising the efficiency of SE induction to values around 85% (Fig. 3; Canhoto *et al.*, 2005).

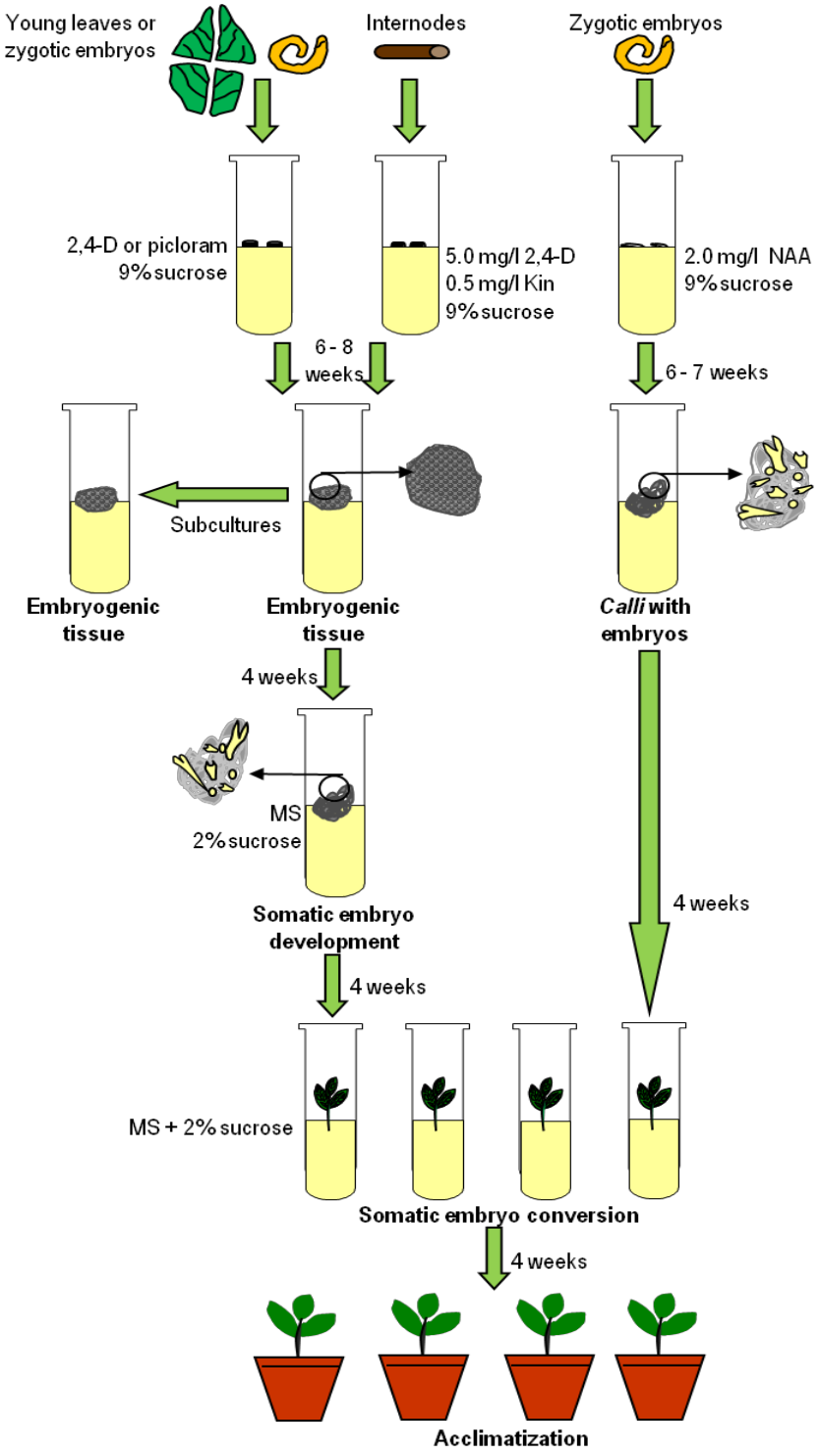

Figure 3 – Schematic representation of the protocols for SE induction in tamarillo (adapted from Canhoto *et al.*, 2005).

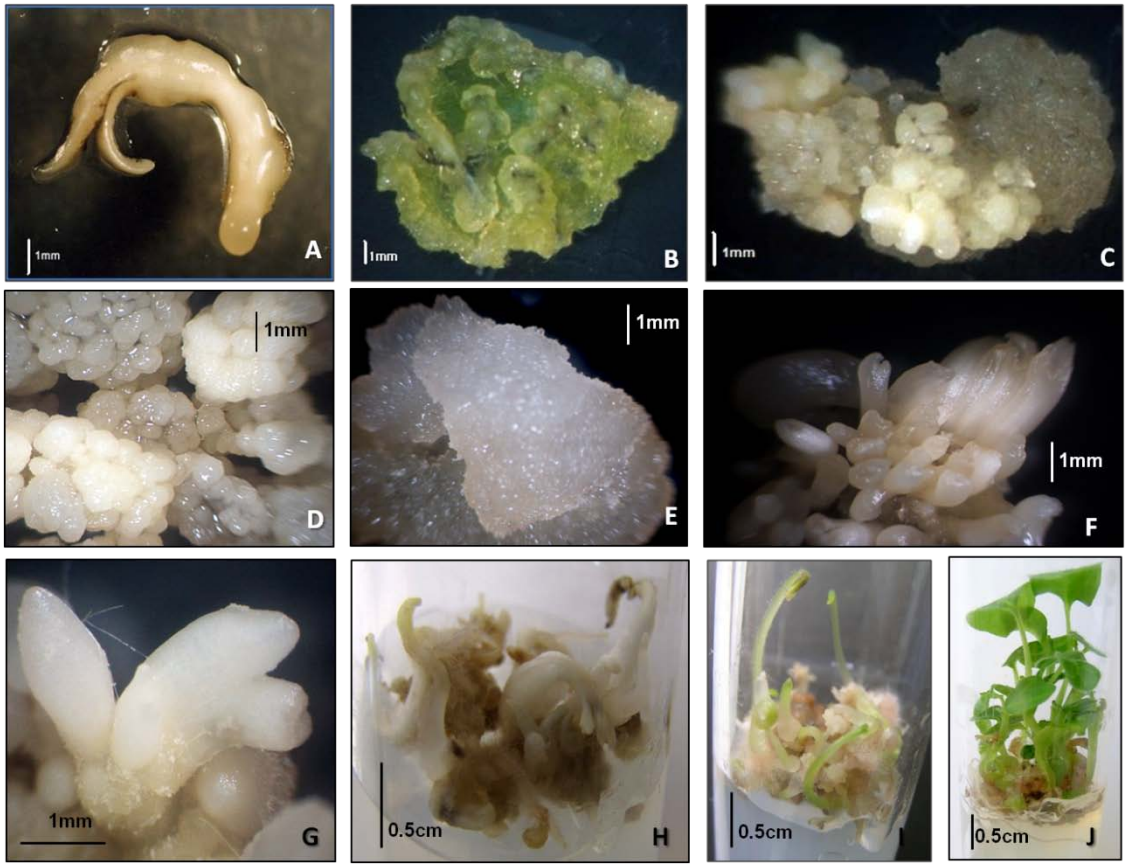

Figure 4 - Somatic embryogenesis in tamarillo. **(A)** Zygotic embryo after 2 weeks on a 2,4-D containing medium. **(B)** Leaf explant after 1 month in the induction medium - MS supplemented with picloram. **(C)** Embryogenic and non-embryogenic (darker) zones formed on a leaf explant after 10-12 weeks of culture. **(D)** Embryogenic tissue. **(E)** Non-embryogenic callus. **(F)** and **(G)** Somatic embryos at different developmental stages after 3 weeks in a MS basal medium without auxins. Note the abnormal morphology of the embryos in **(G)**, showing fused or undifferentiated cotyledons. **(H-J)** Somatic embryo conversion and plantlet development, after 6 weeks on MS medium.

Tamarillo mature zygotic embryos were one of the first explants tested for SE induction, revealing that two types of somatic embryo differentiation can be induced depending on the kind of auxin used: NAA, Picloram or 2,4-D as already stated. Although the somatic embryos or the embryogenic tissues developed mainly from the hypocotyl region of the zygotic embryos, when NAA was used, the hypocotyl of zygotic embryos formed a small callus from which somatic embryos differentiated. These somatic embryos matured in the original NAA containing medium after 4 – 6 weeks of culture (Guimarães *et al.*, 1996). Since somatic embryos are induced and matured in the same culture medium, this process of embryogenesis has been called "one-step" or direct SE (DSE) and has been reported in other woody species studied at

our laboratory, such as *Feijoa sellowiana* (Canhoto and Cruz, 1996; Correia and Canhoto, 2010) *Myrtus communis* (Canhoto *et al.*, 1999) and carob (Canhoto *et al.*, 2006) When several concentrations of NAA were tested, the best results (42.3% induction) were obtained with 2.0 mg/l NAA, even though a wide range of concentrations (0.1 – 10 mg/l) of this auxin were able to induce SE (Guimarães *et al.*, 1996). In contrast with the results obtained with NAA, when zygotic embryos or leaf sections from six-week-old seedlings or from one-month-old shoots were cultured in the presence of the auxins 2,4-D or picloram, a slow growing callus was induced after 4 to 6 weeks of culture (Lopes *et al.*, 2000; Maia, 2002). By the 8^{th} to the 10^{th} week of culture whitish clusters of embryogenic cells were formed in some areas of the callus (Figs. 4C-D), which continued to proliferate. Subculture of the embryogenic tissue on media devoid of auxins or containing 0.1 mg/l gibberellic acid (GA_3) allowed the differentiation of the embryogenic masses in somatic embryos that progressed through stages resembling zygotic embryo development (Fig. 4F). In this case somatic embryos are obtained through a "two-step" process, indirect SE (ISE), since two different media are necessary to achieve full somatic embryo differentiation (Williams and Maheswaran, 1986).

Light and electron microscopy (SEM and TEM) analysis following SE assays revealed that on hypocotyl cultures, in the presence of NAA, somatic embryos arose from a meristematic layer, probably of multicellular origin, in a process resembling somatic embryo differentiation in *Feijoa sellowiana* (Canhoto and Cruz, 1996). On the other hand, in the presence of 2,4-D, cell divisions begin next to the vascular bundles, leading to the formation of a callus tissue (Guimarães *et al.*, 1996). Sub-epidermal and epidermal cells also acquire meristematic characteristics forming a continuous layer of densely cytoplasmatic cells at the periphery of this callus. Some of these cells have the potential to organize themselves into proembryogenic masses that continue to proliferate, giving origin to new masses of embryogenic cells through division of the most peripheral cells (Guimarães *et al.*, 1996).

In leaves, the first cell divisions occurred mainly in the one-cell thick palisade layer, then spread throughout the mesophyll cells with the consequent formation of a callus tissue. Later on, within this callus, some densely cytoplasmatic cells originated globular proembryogenic masses which, after subculture on a hormone free medium further differentiated into somatic embryos. Histochemical studies revealed that starch

is very abundant in almost every leaf cell at the beginning of the culture and its presence within the cells decreases during the callus formation phase. On the other hand, in zygotic embryo cultures, lipids and proteins are the explant's main cell reserves, which gradually disappear during the callus formation phase (Lopes *et al.*, 2000).

The formation of embryogenic callus offers a great potential for large-scale production (Merkle *et al.*, 1995) and the possibility of being used as a source of cell lines for assays of plant genetic transformation. Tamarillo embryogenic tissue can be maintained *in vitro* in an auxin containing medium for several years without the loss of the embryogenic potential. However, some lines of these long-term embryogenic tissue are quite unstable in culture and variations in the chromosome number and amount of DNA have been detected. RAPD analysis showed polymorphisms among embryogenic tissue of the same origin (Lopes *et al.*, 2000). Chromosome abnormalities, including the occurrence of tetraploid plantlets, were detected through chromosome counting (Lopes *et al.*, 2000). Studies at our lab have also shown that plantlets obtained from five-year-old embryogenic tissue displayed more abnormalities than those regenerated from more recent embryogenic tissue (one year or younger). The data obtained also seems to indicate that chromosome aberrations occur during culture and do not pre-exist in the cultured tissues. Differences were also observed in the multiplication rate among several callus lines and among cultivars (Canhoto *et al.*, 2005).

Independently of the tested conditions and explant sources, embryogenic tissue did not develop into embryos in an auxin-rich medium. Subsequent embryo development only occurs when masses of embryogenic *calli* originated from leaves or zygotic embryos, were transferred to an auxin-free medium containing 2% sucrose. The early phases of somatic embryo development could be observed on the callus surface within 3 weeks. Somatic embryo development is an asynchronous process (Fig. 4F) during which most somatic embryos went through different morphological phases similar to those occurring during zygotic embryogenesis (globular, heart-shaped, torpedo and cotyledonar). Also, a suspensor-like structure is often present in somatic embryos morphologically similar to those described forother species studied at our laboratory (Canhoto *et al.*, 2009; Correia and Canhoto, 2010).

The asynchronous embryo development, the continuous *calli* proliferation and the presence of anomalous embryos (Fig. 4G) make difficult to establish an efficient

quantification method to evaluate plant regeneration through SE. Nevertheless, more assays on somatic embryo conversion and germination are being conducted at our lab to reduce some of these problems and increase the rates of plant regeneration. Recent data has shown that the manipulation of light conditions and the inclusion of abscisic acid (ABA) in the medium before germination increases the number of morphologically normal somatic embryos produced (Chapter 3).

Following conversion into plantlets, (Figs. 4H-J) these are submitted to an acclimatization stage in the greenhouse before transference to field conditions Previous work reported the development of plantlets with morphological abnormalities during the initial phases of plant development which, after further growth, recovered the normal phenotype. The high levels of plant mortality observed during the acclimatization phase in those assays seem to be related to abnormal plant development (Canhoto *et al.*, 2005).

Since the conditions for SE induction and plant regeneration are well characterised in tamarillo, biochemical and molecular studies can be performed to better understand this morphogenic process. Some of the advantages of tamarillo for this purpose are: 1) the possibility to induce SE in several organs such as zygotic embryos, hypocotyls and leaves, 2) long-term embryogenic tissue can be maintained in culture without the loss of embryogenic potential, 3) somatic embryo conversion into plantlets is easy and several plants can be obtained from a single callus, 4) SE can be induced in explants derived from adult trees, and 5) embryogenic and non-embryogenic cell lines can be obtained from the same explants, hence possessing the same genotypic background. This last aspect is particularly important since we can compare the two types of cell lines at the molecular level and try to find proteins or mRNA specific to each situation that can further lead to the identification of genes promoting or inhibiting the embryogenic process.

During the assays of SE induction on different explants of tamarillo (Lopes *et al.*, 2000) the protein electrophoretic patterns of embryogenic and non-embryogenic *calli* was compared trying to identify protein markers associated with these two types of *calli* (Ferreira *et al.*, 1998). A 26.5 kDa protein (NEP25) was consistently found in non-embryogenic *calli* derived from several explants, such as zygotic embryos and isolated hypocotyls, cultured in the same conditions, suggesting that this protein can be considered a good marker for the non-embryogenic *calli* of tamarillo.

1.2. MOLECULAR ASPECTS OF SOMATIC EMBRYOGENESIS INDUCTION

SE is one form of asexual reproduction by which somatic cells, under suitable induction conditions, undergo a complete genome shift and embark into a new developmental pathway ending in the formation of asexual embryos morphologically identical to their zygotic counterparts (Zimmerman, 1993; Schmidt *et al.*, 1997; Komamine *et al.*, 2005). During this unique developmental process cells have to dedifferentiate, activate cell division, and reprogram their physiology, metabolism, and gene expression patterns (Yang and Zhang, 2010). Thus, SE is the most clear demonstration of totipotency, showing that somatic cells contain the essential genetic blueprint for complete plant development, and that embryogenesis is not an exclusive of the zygote and can proceed in the absence fertilization (Zimmerman, 1993).

Since the first observations of somatic embryo formation in carrot cell suspension cultures by Steward *et al.* (1958) and Reinert (1959), the potential for SE has been shown to be characteristic of a wide range of tissue culture systems from both gymnosperms and angiosperms plants (von Arnold *et al.*, 2002; Quiroz-Figueroa *et al.*, 2006; Yang and Zhang, 2010). However, zygotic embryos and zygotic embryos derived organs such as cotyledons, or hypocotyls have proved to be the most responsive organs. In general, the SE process can be divided into two phases: induction and expression (Namasivayam, 2007). During the induction phase, differentiated somatic cells acquire embryogenic competence and proliferate as embryogenic cells. In the expression phase, the embryogenic cells display their embryogenic competence and differentiate to form somatic embryos (Jimenez, 2001).

SE induction involves differentiated somatic cells acquiring embryogenic competence and proliferating as embryogenic cells (Yang and Zhang, 2010). Initiation of the embryogenic pathway seems to be restricted to certain responsive cells that have the potential to activate genes involved in generating embryogenic cells (Nomura and Komamine, 1985; Quiroz-Figueroa *et al.*, 2002). Once these yet unknown genes are activated, an embryogenic gene expression program replaces the established gene expression pattern in the explant tissue (Quiroz-Figueroa *et al.*, 2006).

As SE induction in cultured tissues is a multi-factorial event, determining specific physical and chemical factors that switch on the embryogenic pathway of development is an important but also difficult step (Karami *et al.*, 2009). It has been

proposed that plant growth regulators (PGRs) and stresses play a central role in mediating the signal transduction cascade leading to the reprogramming of gene expression, followed by a series of cell divisions that induce either unorganized callus growth or more organized growth leading to SE (Dudits *et al.*, 1991; de Jong *et al.*, 1993). In fact, induction by plant hormones, particularly auxin, is generally considered to be the most important factor (Rose, 2004) and is by far the most widely studied. Other chemical signals, such as the nutritional components of culture medium, and physical factors, such as lighting and the liquid or solid form of culture medium, have also been shown to play a role in SE induction in some species.

Auxins are considered to be positional and pattering signal molecules that play a major role in zygotic embryogenesis (Souter and Lindsey, 2000) as well as in SE (Chugh and Khurana, 2002; Pasternak *et al.*, 2002; Feher *et al.*, 2003). Activation of auxin responses may be a key event in cellular adaptation and genetic, metabolic, and physiological reprogramming, leading to the embryogenic competence of somatic plant cells (Feher *et al.*, 2003). Once embryogenic cells have been formed, they continue to proliferate and form proembryogenic masses (PEMs). Auxin is required for proliferation of PEMs but is in general inhibitory of somatic embryo development (Nomura and Komamine, 1995; Filonova *et al.*, 2000). The degree of embryo differentiation taking place in the presence of auxin varies in different species. Embryogenic cultures of some species and some genotypes can be subcultured for an extended period in a medium containing PGRs and still retain their full embryogenic potential whereas in most crops the embryogenic potential decreases with prolonged culture and is eventually lost (von Arnold *et al.*, 2002).

Among different auxins, 2,4-D has been the most commonly used for SE induction. Some reports have shown that the formation of an embryogenic cell is related to nuclear DNA hypermethylation in the presence of 2,4-D (Leljak-Levanic *et al.*, 2004). The role of DNA methylation, performed by DNA methyltransferase 1 (DNMT1), in modifying gene expression during *Arabidopsis* embryogenesis, was also demonstrated by Xiao *et al.* (2006). Therefore, dynamic changes in chromatin structure induced by DNA methylation under the influence of 2,4-D lead to genomic reprogramming in somatic cells. These modifications might affect hormone-responsive genes, specifically those required for the acquisition of embryogenic competence (Fig. 5). Data suggest that 2,4-D may acts mainly as a stress substance rather than a

phytohormone, hence triggering the acquisition of embryogenic competence by plant cells (Karami and Saidi, 2010).

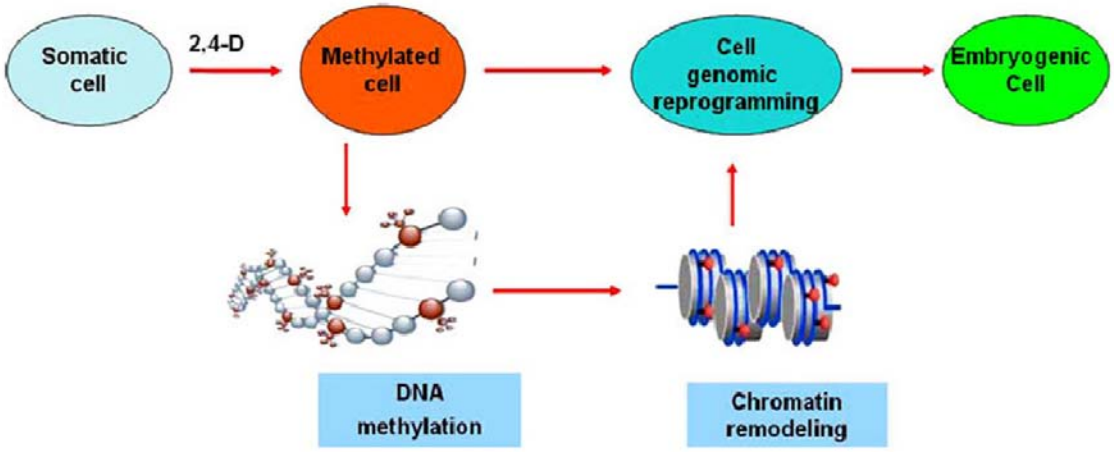

Figure 5 – Model representation of embryogenic competence acquisition, by DNA methylation, in the presence of 2,4-D (Karami and Saidi, 2010).

In fact, in recent years, an increasing number of works have indicated that the stress-response of cultured tissues, stimulated by heavy metal ions, high osmotic pressure, dehydration, explant wounding or high temperature, plays a major role in somatic embryo induction (Karami and Saidi, 2010; Zavattieri *et al.*, 2010). Plants respond to abiotic stresses by altering the expression of many of their genes. This altered expression is a major mechanism of adaptation and survival during the stress periods (Hasegawa *et al.*, 2000). Early phases of SE are characterized by the induction of many stress-related genes, leading to the hypothesis that SE is an extreme stress response of cultured plant cells (Dudits *et al.*, 1995). Stress-related genes were also induced in these embryogenic cultures, a reason for SE to be interpreted as the outcome of an *in vitro* adaptation process to a new environment (Feher *et al.*, 2003).

MtSK1 gene and *GLUTATHIONE-S-TRANSFERASE* (*GST*) genes are among the most common stress-related genes expressed during SE induction. *MtSK1* gene is a member of the class of plant kinases called the SnRK group, which is thought to play a role in stress responses of plants. Nolan *et al.* (2006) have cloned and investigated the stress kinase gene *MtSK1* in relation to SE in *Medicago truncatula*, concluding that its expression was stimulated by wounding and not by PGRs levels. This may indicate a sustained stress–response of cultured tissue throughout the culture process. The

transcripts of *GST* genes were detected in abundance during auxin induction and in somatic embryos. Members of the *GST* gene family are up-regulated during auxin-induced SE and appear to be major regulators of the interacting genes expressed in response to auxin. On the other hand, *GST* genes are also induced by ABA under various biotic and abiotic stresses, and may have a potential role in detoxifying excessive amounts of auxin, thus regulating its intracellular concentration (Thibaud-Niessen *et al.*, 2003).

Increased levels of the hormone ethylene in response to biotic and abiotic stresses, including wounding have also been reported (Jiménez, 2005). Although reports on the effects of ethylene on SE are contradictory, Mantiri *et al.* (2008) showed that ethylene was necessary for SE in *M. truncatula*, by inducing the expression of *SOMATIC EMBRYO RELATED FACTOR1* (*MtSERF1*) in embryogenic *calli*.

Explant cells can be induced to go through a direct or indirect SE by modulating tissue culture conditions (Sharp *et al.*, 1980). In direct somatic embryogenesis (DSE), proembryogenic competent cells are already present, thus a minimal proliferation or reprogramming of unorganized tissue precedes embryo formation, which occurs directly on the surface of organized tissues such as a leaf, stem segment, zygotic embryo, young inflorescence, protoplasts or microspores (Williams and Maheswaran, 1986). In alternative, indirect somatic embryogenesis (ISE) requires a major reprogramming in order to obtain proliferating *calli* with embryogenic ability before embryo formation (Williams and Maheswaran, 1986; Yeung, 1995). In these cases, more complex media, with additional factors, are required to induce dedifferentiation and initiation of cell division in the explant before they can express embryogenic competence (Williams and Maheswaran, 1986). The main factors involved in each case depend on the source and physiological state of the explant employed and the type and concentration of the growth regulators, as is the case for tamarillo. During ISE, both embryogenic tissue and nonembryogenic *calli* are present. These two types of cell proliferations are easily distinguishible on the basis of their morphology, color and growth rate. Embryogenic tissues present a nodular appearence and a smooth surface, while embryogenic cells that form somatic embryos are characterized generally as small and isodiametric in shape. These cells have large and densely staining nuclei and nucleoli and are densely cytoplasmic, with small vacuoles, thin cell walls, and a higher metabolic activity. In

contrast nonembryogenic *calli* are rough, friable, and translucent, with highly vacuolated cells.

The switching of somatic cells into embryogenic cells involves a series of events associated with the molecular recognition of internal signals and external stimuli (Chugh and Khurana, 2002; Karami *et al.*, 2009). The perception and response to these events triggers various signal cascades, and the downstream pathways followed during the transition of single cells to somatic embryos eventually result in specific gene expression and SE (Dudits *et al.*, 1991; Zimmerman, 1993; Chugh and Khurana, 2002; Feher *et al.*, 2003). It is considered that analyses of gene expression during SE can provide information for better understanding the overall process (Chugh and Khurana, 2002; Feher *et al.*, 2003; Stasolla *et al.*, 2004; Yang and Zhang, 2010).

Since the early preliminary experiments on differential gene expression (Sung and Okimoto, 1981; Wilde *et al.*, 1988), studies increasingly focused on characterizing genes specifically expressed during SE in many plant species, such as carrot (Aleith and Richter, 1991), grapevine (Gianazza *et al.*, 1992), peanut (Rani *et al.*, 2005), alfalfa (Domoki *et al.*, 2006), cotton (Zeng *et al.*, 2006), conifers (Cairney and Pullman, 2007) and potato (Sharma *et al.*, 2008). This set of works has resulted in the identification of several genes that are specifically activated or that exhibit differential expression during SE (Chugh and Khurana, 2002; Rojas-Herrera *et al.*, 2002; Feher *et al.*, 2003), such as *SERK* (Schmidt *et al.*, 1997), *BABY BOOM* (*BBM*, Boutilier *et al.*, 2002), *WUSCHEL* (*WUS*, Zuo *et al.*, 2002) and *LEAFY COTYLEDON* (*LEC*, Curaba *et al.*, 2004; Gazzarrini *et al.*, 2004; Gaj *et al.*, 2005).

Of all the genes that have been isolated during SE, only one, *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*), has successfully been shown to be a specific marker distinguishing individual embryo-forming cells in carrot suspension cultures (Schmidt *et al.*, 1997). The *SERK* gene was found to be expressed during proembryogenic mass formation and up to the globular stage during carrot SE (Schmidt *et al,*. 1997). It was also detected in zygotic embryos up to the early globular stage, but not in unpollinated flowers or any other tissue. Later, an *Arabidopsis thaliana* homologue (*AtSERK1*) of the carrot *SERK* cDNA was cloned as one of the five members of a small gene family (Hecht *et al.*, 2001). The *AtSERK1* was shown to be expressed in several tissues, including the shoot apical meristem (SAM) and cotyledons

of *Arabidopsis* seedlings treated with auxin. These are the sites where embryogenic callus emerges in *Arabidopsis* (Mordhorst *et al.,* 1998; von Recklinghausen *et al.*, 2000), which indicates that *AtSERK1* expression was not restricted to embryogenic cells, but was characteristic of those cells capable of responding to hormone signals and competent to form somatic embryos or embryogenic tissues. Overexpression of *SERK1* does not result in any obvious plant phenotypes but gives a 3- to 4-fold increase in embryogenic competence, which indicates that *SERK1* not only marks embryogenic competence but also promotes the transition of somatic cells to an embryogenic state (Hecht *et al.,* 2001).

The acquisition of embryogenic potential followed by the transition from somatic cells to somatic embryos, coupled with the establishment of body plan and embryo maturation, involves molecular events encompassing not only differential gene expression, but various signal transduction pathways for activating or repressing numerous gene sets (Zimmerman, 1993). Along with *SERKs*, calmodulin-mediated signal transduction has been also implicated in SE induction. Calmodulin (CaM), calcium-dependent protein kinase (CDPK), and calcineurin B-like proteins (CBL family), detect cellular calcium signals that enhance SE thus confirming the role of Ca^{2+} as an important mediator in the process (Anil and Rao, 2000).

Induction of embryo development can occur on leaves of *Arabidopsis* plants ectopically expressing *LEAFY COTYLEDON 1* (*LEC1*; Lotan *et al.,* 1998) or *LEC2* (Stone *et al.,* 2001). *LEC1* encodes a transcription factor homologue to the CCAAT box binding factor HAP3 subunit (Lotan *et al.*, 1998), and is required for the specification of cotyledon identity and the completion of embryo maturation in *Arabidopsis* (Meinke 1992; West *et al.*, 1994). The pattern of *LEC1* gene expression strongly suggests that *LEC1* functions specifically during embryogenesis, including the earliest embryonic period (Lotan *et al.*, 1998).

Molecular techniques, such as differential display analysis, subtractive hybridization, and construction of cDNA libraries, have been crucial to identify genes that exhibit differential activity. Differential display was very effective for isolating genes involved in the very early stages of SE (Yoshida *et al.*, 1994; Momiyama *et al.*, 1995; Linkiewicz *et al.*, 2004). Alexandrova and Conger (2002) identified two SE related genes with possible nuclear regulatory functions, *DGE1* and *DGE2*, that were expressed in embryogenic but not in non-embryogenic leaf cultures of *D. glomerata*.

Charbit *et al.* (2004) isolated five cDNAs that could be used to distinguish between *calli* prior to induction, thus enabling an early estimation of callus embryogenic potential.

Using subtractive hybridization, during *Brassica napus* microspore embryogenesis, a differentially expressed cDNA was identified and named *BABY BOOM* (*BBM*, Boutilier *et al.*, 2002). The *BBM* gene also encodes a transcription factor belonging to the AP2/ERF family. Ectopic expression of *BBM* in *Arabidopsis* primarily induces spontaneous somatic embryo formation from seedlings, although ectopic shoots and callus also develop at a lower frequency (Boutilier *et al.*, 2002). In tobacco, heterologous *BBM* expression induces spontaneous shoot and callus formation, while a cytokinin pulse is required for somatic embryo formation (Souter and Lindsey, 2000). The ability of *BBM* to promote organogenesis and embryogenesis in the absence of exogenously applied growth regulators suggested that *BBM* may act by stimulating the production of plant hormones and/or increasing the cell sensitivity to these substances. Zuo *et al.* (2002) used a chemically inducible activation tagging system to identify genes which over-expression induces the formation of somatic embryos in *Arabidopsis* tissues without the need for external hormonal treatments. The identified gene, *PLANT GROWTH ACTIVATOR 6* (*PGA6*), was found to be identical to *WUSCHEL* (*WUS*), a homeobox gene which encodes a transcription factor that regulates the pool of stem cells in the shoot meristem and is regulated by a feedback loop involving the *CLAVATA* (*CLV*) genes (Laux *et al.*, 1996). *WUS/PGA6* was presumably involved in promoting vegetative to embryogenic transition and/or maintaining the identity of the embryonic stem cells. In *Coffea canephora WUSCHEL (WUS*) has been shown to cause dedifferentiation when expressed in somatic cells followed by the production of new stem cells that can lead to SE or organogenesis (Arroyo-Herrera, 2008).

Moreover, transcriptomic studies were applied to investigate global gene expression in SE (Thibaud-Nissen *et al.*, 2003; Stasolla *et al.*, 2004). ESTs uniques to embryogenic cell clusters in carrot (Yasuda *et al.*, 2001), *Coffea arabica* (Rojas-Herrera *et al.*, 2002) and embryogenic *calli* of *Lycium barbarum* (Cui *et al.*, 1999) have been detected. By means of subtractive hybridization and macroarrays, Zeng *et al.* (2006) isolated ESTs involved in SE, and then produced a draft molecular interaction network representing complex gene expression during SE (Zeng *et al.*, 2007).

In recent years there has been a growing interest in proteomic approaches to better understand SE. Since proteins directly influence cellular biochemistry and

provide a more accurate analysis of cellular changes during growth and development (Chen and Harmon, 2006), the identification of proteins associated with somatic embryo development may provide insights onto SE.

Two embryo-specific proteins (77 and 43 kDa) were earlier reported in carrot by Sung and Okimoto (1981). Similar studies performed on rice revealed the presence of several polypeptides in the range of 40 to 44 kDa, which were more abundant in embryogenic tissues than in nonembryogenic *calli* (Chen and Luthe, 1987). Detection of embryogenesis-related proteins from total protein extracts has also been reported for *Cichorium intybus* (Hilbert *et al.*, 1992), *Dactylis glomerata* (Hahne *et al.*,1988), and *Cupressus sempervirens* (Sallandrouze *et al.*, 1999). Pedroso *et al.* (1995) detected two polypeptides, E1 and E2, specifically related to the process of proembryo induction and globular embryo development of *Camelia japonica*. Fellers *et al.* (1997) identified two proteins with 43 kDa/pI 7.6 and 27 kDa/pI 8.2 that can be used as markers for embryogenic potential in wheat *calli*. Blanco *et al.* (1997) found a marker protein for the regeneration potential of sugarcane embryogenic tissue. Hvoslef-Eide and Corke (1997) detected proteins specific to birch embryogenic cultures. An investigation of total protein expression using two-dimensional gel electrophoresis during ontogeny of carrot SE allowed Dodeman and Ducreux (1996) to identify markers for the induction phase and different development stages.

Recent improvements in high resolution two-dimensional gel electrophoresis and mass spectrometry (Hurkman and Tanaka, 2007) have made the large-scale protein profiling and identification a dynamic area of research in plant biology (Hochholdinger *et al.*, 2006). These approaches were applied to the study of SE in several plant species including cassava (*Manihot esculenta*, Baba *et al.*, 2008; Li *et al.*, 2010), cowpea (*Vigna unguiculata*, Nogueira *et al.*, 2007), cyclamen (*Cyclamen persicum*, Winkelmann *et al.*, 2006; Lyngved *et al.*, 2008; Bian *et al.*, 2010; Rode *et al.*, 2011), date palm (*Phoenix dactylifera*, Sghaier-Hammami *et al.*, 2009), feijoa (*Acca sellowiana*, Cangahuala-Inocente *et al.*, 2009), grapevine (*Vitis vinifera*, Marsoni *et al.*, 2008; Zhang *et al.*, 2009), *Medicago truncatula* (Imin *et al.*, 2004, 2005), oak (*Quercus suber*, Gómez *et al.*, 2009), rice (*Oryza sativa, Yin et al.*, 2008), Valencia sweet orange (*Citrus sinensis*, Pan *et al.*, 2009) and white spruce (*Picea glauca*, Lippert *et al.*, 2005).

The majority of these reports included comparative studies between embryogenic and non-embryogenic *calli*, as well as between gametic and SE and/or

differential time-course proteomic studies in order to detect protein markers during distinct embryonic developmental stages.

An analysis of the changes found in the referred proteomic profiles shows marked differences in carbohydrate, energy/metabolism and stress/defense-related proteins. Among stress-related proteins, some are involved in oxidative stress, such as ascorbate peroxidase and manganese superoxide dismutase in grapevine (Marsoni *et al.*, 2008; Zhang *et al.*, 2009) as well as glutathione S-transferase, phospholipid hydroperoxide and glutathione peroxidase in Valencia sweet orange (Pan *et al.*, 2009). SE also requires strong induction of protein biosynthesis. The dynamic protein turnover during SE of cyclamen is indicated by the upregulation of putative 26S proteasome regulatory particle triple-A ATPase subunit 5a (Bian *et al.*, 2010).

Comparison of somatic and zygotic embryos revealed that their proteomes reflected mainly the different environmental conditions, which resulted in differential expression of proteins involved in metabolic pathways and stress response (Winkelmann *et al.*, 2006).

Proteomic studies also described several extracellular proteins as markers for SE, which could offer the possibility of determining embryogenic potential of plant cells in culture long before any morphological changes (Schimdt *et al.*, 1997; Chugh and Khurana, 2002; Alexandrova and Conger, 2002). Arabinogalactan proteins, nonspecific lipid transfer proteins and germin/germin-like proteins are important groups of extracellular proteins that help triggering embryogenic potential in plant cells (Tchorbadjieva, 2005).

1.3. THE SIGNIFICANCE OF MODEL SYSTEMS AND ORGANISMS

The availability of plant SE model systems has created effective tools for examining the details of plant embryogenesis. Somatic embryo models have been useful for studying cell differentiation processes in plants and for increasing our understanding about the functional aspects of genes already implicated in SE (Mordhorst *et al.*, 2005; Quiroz-Figueroa *et al.*, 2006).

The development of somatic embryos closely resembles, both morphologically and physiologically, the development of zygotic embryos, probably due to the conservation of the underpinning cellular and molecular mechanisms in both processes (Karami and Saidi, 2010). Therefore, SE has been extensively used as an experimental

system to investigate the morphological, biochemical and physiological events of embryogenesis (Zimmerman, 1993). Especially in carrot, a large quantity of somatic embryos of the same development phase can be easily obtained using a tissue culture system, making carrot a widely used model plant for researches on SE (Feher *et al.*, 2003; Ikeda *et al.*, 2006).

Species that easily form somatic embryos such as *Arabidopsis*, carrot, white spruce, alfalfa and soybean, have been used for identifying and characterizing signal molecules that are important for the induction and maintenance of embryonic development (Mordhorst *et al.*, 1997; Ikeda-Iwai *et al.*, 2002; Yazawa *et al.*, 2004; Mordhorst *et al.*, 2005; Quiroz-Figueroa *et al.*, 2006; Raghavan, 2006).

Arabidopsis thaliana has been extensively used as a model plant for genetic and molecular studies of various events in plants, including embryogenesis. Thus, SE induction in *A. thaliana* has been reported by several authors (Pillon *et al.*, 1996; Gaj, 2001; Ikeda-Iwai *et al.*, 2002; Gaj *et al.*, 2005), who used immature zygotic embryos as explants in a B5 (Gamborg *et al.*, 1968) induction medium supplemented with an auxin (2,4-D). In addition to a well characterized system for SE induction *A. thaliana* offers the advantage of a wide range of information available for all researchers. Since the sequencing of the *A. thaliana* genome was completed, in 2000 (Arabidopsis Genome Initiative, 2000), a variety of new genomics resources has been created. Some examples include large numbers of genetic polymorphisms (Jander *et al.*, 2002; Borevitz *et al.*, 2007), homozygous T-DNA populations from the Salk Institute (Alonso and Ecker, 2006) and genome-wide expression data from researchers around the world (Schmid *et al.*, 2005). Different types of data, such as DNA, RNA, protein and phenotype have been deposited in resources of different scales, including large-scale public repositories (for example, GenBank), community-specific databases (for example, TAIR) and project specific databases.

There is no doubt that model organisms have many advantages and without them our understanding of the mechanisms underlying many developmental and physiological processes would be much more limited. However, the discoveries based on model organisms such as *Arabidopsis* must be tested in other organisms or systems to verify their effectiveness. Moreover, new model systems that can bring new approaches to broad our scientific knowledge are welcomed. In this context, and in our point of view, somatic embryogenesis induction in tamarillo seems to be an interesting system to better understand particular aspects of somatic embryo formation. One of the

main advantages of this system is that embryogenic tissue and non embryogenic *calli* can be formed in the same explants under the same induction conditions. Moreover the two types of *calli* are very distinct and easy to separate, hence allowing the establishment of embryogenic and non-embryogenic lines of the same origin. It must also be pointed out that somatic embryogenesis in tamarillo can be achieved from different tissues and in the presence of different auxins such as 2,4-D, NAA and picloram. Finally, tamarillo is a tree, a non-taxonomic group of plants in which somatic embryogenesis is difficult to achieve, particularly in explants of adult or adult-derived origin. The economic importance of the tree is also relevant since the interest in tamarillo fruits is increasing in several markets, Portugal included. Based on this background we decided to go further on our research on tamarillo somatic embryogenesis hoping that the outputs of this research may contribute to better understand this developmental process.

1.4. OBJECTIVES

Somatic embryogenesis induction in woody species is often a recalcitrant process, strongly dependent upon culture conditions and of the genotype of the donor plants.

Tamarillo has shown to be a very useful plant to better understand particular aspects of *in vitro* morphogenesis, in particular SE. Since embryogenic and non-embryogenic *calli* can be obtained from the same explant, molecular and biochemical studies can compare the two different types of *calli* in different conditions trying to identify molecular markers related with the embryogenic (or non-embryogenic) process. Other problems affecting SE in tamarillo are: 1) the difficulties in establishing efficient protocols of SE induction from adult material of selected lines, and 2) the premature germination of poorly developed embryos that affects the levels of normal plant regeneration. Most of the work with SE induction in woody species refers to protocols that are initiated from juvenile explants, such as zygotic embryos.

In order to uncover some of the limiting steps of SE induction and to identify markers directly related to this process, which would allow achieving more efficient results, not only with tamarillo but also with other woody species, three strategies were followed: the improvement of induction and somatic embryo development protocols,

the investigation of a SE inhibitory protein and a comparison between proteomic profiles of embryogenic and non-embryogenic *calli*.

Therefore, the first set of experiments (Chapter 2) aimed to develop a reliable methodology for the propagation of adult tamarillo trees through SE. To achieve that we followed two strategies: 1) a direct approach in which explants from an adult tree were tested for their embryogenic potential, and 2) an indirect approach in which an adult tree was first established *in vitro* through axillary shoot proliferation and then leaves from this material were tested for SE induction. Scanning electron microscopy (SEM) and cytological observations were performed to better monitor the steps of the induction process. RAPD analysis was used to verify the genetic identity of the obtained clones.

The second set of experiments (Chapter 3) was designed to analyze the effect of several different treatments on the development of tamarillo somatic embryos in terms of their maturation and to evaluate the histochemical differences between mature/cotyledonar somatic and zygotic embryos. Sucrose and ABA concentrations, as well light conditions were the variables tested.

The goal of the third set of experiments (Chapter 4) was to characterize a previously identified protein (NEP25, 26.5 kDa) as a possible negative regulator of somatic embryogeneis induction. Expression assays (RT-PCR and immunoblots) were conducted to follow NEP25 expression in tamarillo.

To evaluate the putative role of NEP25 in SE induction two experimental approaches were followed: 1) SE induction from NEP25 knock-out lines of *Arabidopsis thaliana*, and 2) post-transcriptional down-regulation of *NEP25* gene in transgenic tamarillo plants.

The purpose of the last set of experiments (Chapter 5) was to identify embryogenic-specific proteins directly involved in the induction of SE. In order to achieve it, a comparative analysis of the proteome of embryogenic and non-embryogenic *calli* of tamarillo, obtained under different culture conditions, was performed. 2-DE analysis followed by LC-MS/MS allowed the identification of several potential markers for SE.

Part of this research was already published in international journals and presented in national and international meetings while some data wa submitted for publication as indicated at the beginning of the chapters.

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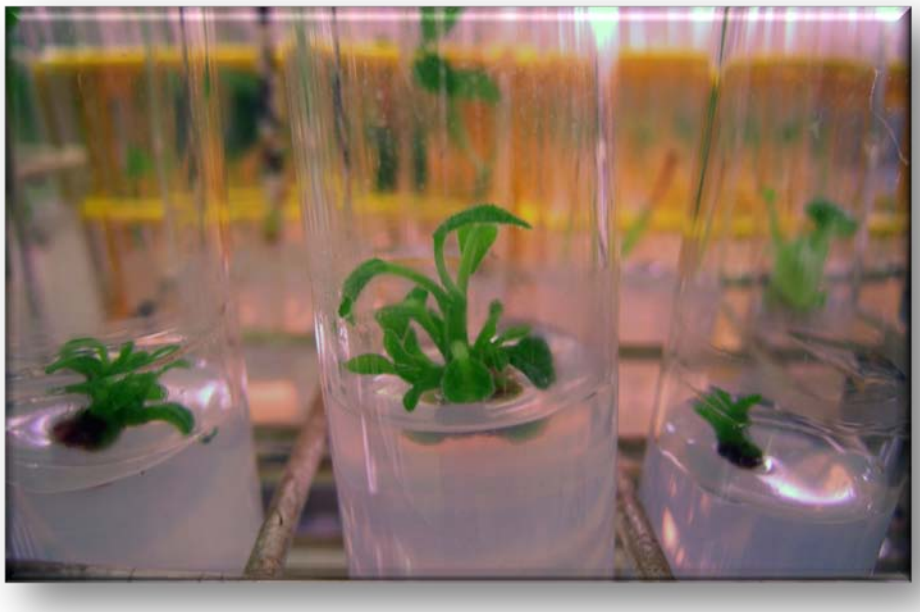

CHAPTER 2

SOMATIC EMBRYOGENESIS INDUCTION SYSTEM FOR CLONING AN ADULT *Cyphomandra betacea* (Cav.) Sendt. (TAMARILLO)

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2. SOMATIC EMBRYOGENESIS INDUCTION SYSTEM FOR CLONING AN ADULT *Cyphomandra betacea* (Cav.) Sendt. (TAMARILLO)

2.1. ABSTRACT

Somatic embryogenesis is a valuable tool for plant breeding. In recent years, different aspects related with somatic embryogenesis (SE) induction in tamarillo have been studied at our lab. In this work, results concerning the establishment of a protocol for cloning an adult tamarillo tree through SE are presented. Attempts to induce SE in tamarillo from various explants directly taken from an adult tree were unsuccessful and only *calli* with no embryogenic potential were initiated. To overcome the lack of potential of adult tissues for SE an indirect approach was attempted in which shoots from an adult tree were first established *in vitro* and then wounded leaves were used for SE induction. A low rate of embryogenic tissue formation was obtained (19.4%) but it was in the range of initiation rates from leaf explants of *in vitro* cloned plantlets of different tamarillo cultivars (red, orange and yellow) that originated from a single seedling (13.3-54.4%). High variation in SE initiation among juvenile controls could not be explained by different organogenic potential as no significant differences in shoot proliferation or rooting ability during micropropagation could be detected. Subcultures of embryogenic lines from the adult tree allowed us to obtain a large amount of embryogenic tissue that, after 8 weeks on a PGR free medium gave an average of 111 plants per g of fresh mass of embryogenic tissue. A RAPD comparative analysis of somatic embryo-derived plantlets and the donor tree confirmed that the plantlets had no variation in the DNA regions amplified by twelve primers. These results open the way for large-scale cloning of elite tamarillo trees through SE.

Keywords: embryogenic tissue; genotype; shoot culture; picloram; RAPD analysis; somatic plant.

2.2. INTRODUCTION

Tamarillo, *Cyphomandra betacea* (Cav.) Sendt., is a solanaceous tree also known by other common names such as tree tomato or 'tomate de la paz' (Bois, 1927) depending on the region where it grows. Originating from the Andean region of Peru, Chile, Ecuador and Bolivia it has spread to several other world areas (Dawes and Pringle, 1983; Meadows, 2002) and nowadays New Zealand is the main producer and exporter of this fruit crop. The main economic interest in this species is related to the high nutritional value of its fruits, which can be eaten fresh or processed, and which have a relatively high content of proteins, vitamin C, vitamin E, provitamin A and minerals, such as potassium and iron (McCane and Widdowson, 1992). More recent studies have shown that tamarillo fruits are also rich in anthocyanins and carotenoids and may be used as a source of antioxidants (Hurtado *et al.*, 2009; Kou *et al.*, 2008).
In vitro propagation by axillary shoot proliferation (Barghchi, 1986; Cohen and Elliot, 1979), induction of organogenesis (Guimarães *et al.*, 1996; Obando and Jordan, 2001) or somatic embryogenesis (Correia *et al.*, 2009) and genetic transformation techniques (Atkinson and Gardner, 1993; Cohen *et al.*, 2000) are valuable biotechnological tools that have been applied to tamarillo breeding. SE is of particular interest because it has a great potential for the improvement of tree species and for rapid large-scale clonal propagation, genetic transformation, and cryopreservation of desirable selected lines (Park, 2002; von Arnold, 2008). Moreover, somatic embryo induction and development serve as model to understand genetic regulation of embryo development (Rose *et al.*, 2010; Yang and Zhang, 2010).

SE induction and somatic embryo development of tamarillo have been studied at our laboratory for several years using mature zygotic embryos or explants such as cotyledons, hypocotyls and roots obtained from seedlings (Canhoto *et al.*, 2005; Lopes *et al.*, 2000). As in most other woody species (Bonga *et al.*, 2010) immature or mature zygotic embryos were found to be the most suitable and successful explants, followed by seedling explants, such as cotyledons and hypocotyls. Regeneration of trees from juvenile explants usually implies the implementation of strategies that can allow the storage of embryogenic material, in culture or by cryopreservation, until the evaluation of the juvenile propagated material can be made in the field (Dunstan *et al.*, 1995). Since most trees are characterized by long juvenile phases this approach is only practical for species of high economic interest due to the high costs of long field tests.

The recalcitrance of adult material to SE induction is well known and is one of the major problems preventing wider use of SE technology in both angiosperms and conifers (Thorpe and Stasolla, 2001; Klimaszewska *et al.,* 2007). Effective protocols of SE induction from adult trees permit a faster propagation of the elite trees, hence increasing genetic gains in each breeding generation.

In some species, flowers (Gambino *et al.,* 2007) or floral organs such as petals, anthers or ovaries isolated from flower buds (Stefanello *et al.,* 2005; Vidal *et al.,* 2009) have been successfully used to propagate adult selected trees. However, in most of tree species, this type of explants proved to be recalcitrant. In spite of the difficulties in cloning adult trees through SE there have been recent reports on the induction of SE and plant development from explants of adult trees of both angiosperms (Mauri and Manzanera, 2003; Sriskandarajah and Lundquist, 2009; San-José *et al.,* 2010) and conifers (Klimaszewska *et al.,* 2011).

The main goal of the present work was to develop a reliable methodology for the propagation of adult tamarillo trees through SE. We followed two strategies: 1) a direct approach in which explants from an adult tree were tested for their embryogenic potential, and 2) an indirect approach in which an adult tree was first established *in vitro* through axillary shoot proliferation and then leaves from this material were tested for SE induction. The results of these experiments showed the superiority of an indirect approach.

2.3. MATERIAL AND METHODS

2.3.1. Establishment of shoot cultures from adult tamarillo

Shoot cultures of *Cyphomandra betacea* were initiated from branches (1–2 cm thick) collected from a 13-year old plant (named A) growing at the Botanical Garden of the University of Coimbra (Fig. 1A). Branch segments (20 to 25 cm) were washed in running water, sprayed with a fungicide solution (Benlate, 6% w/v) and set upright in jars containing water to allow the development of axillary shoots in a growth cabinet at 20ºC and 80–90% relative humidity, under a 16h photoperiod at 15-20 µmol $m^{-2}s^{-1}$ (cool white fluorescent lamps). After 2 weeks, the developed axillary shoots (circa 1.5 - 1cm) were used as explants source. Nodal segments, 1.5-2 cm long, possessing only one axillary bud were stripped of leaves, washed with detergent (2-3 drops of Tween 20) in

a volume of 100 ml of water, and surface sterilized by a brief immersion (30 s) in 70% (v/v) ethanol followed by disinfection (15 min) in 5% (w/v) calcium hypochlorite solution containing 2–3 drops of Tween 20 under stirring. Nodal segments were then washed three times in sterile water. Thirty axillary shoot buds, with small amount of stem tissues, were excised from each nodal segment and inoculated individually in test tubes (15 x 2.2 cm) with 15 ml/tube MS medium (Murashige and Skoog, 1962) supplemented with 0.8 µM benzyladenine (BA) and 0.07 M sucrose. Agar (Panreac, Spain) 0.6% (w/v) was added to the medium and the pH adjusted to 5.7 before autoclaving at 121ºC for 20 min (800-1100 g/cm^2 gel strength after autoclaving). The clonal shoots of this red tamarillo tree are depicted as RTA.

2.3.2. Establishment of shoot cultures from seedlings of tamarillo

Shoot cultures were also established and propagated from seedlings of *in vitro* germinated seeds of four tamarillo genotypes: yellow tamarillo (YT), orange tamarillo (OT) and two genotypes of red tamarillo cultivar, named RTC and RTL. Each of these four genotypes was obtained from a single seedling through the successive culture of shoot tips (1.5 cm) in test tubes containing MS medium but without plant growth regulators (PGRs).

Shoot cultures were subcultured once per month onto fresh medium of the same composition and kept in a growth chamber at 25ºC, under 14 h photoperiod at 15-20 µmol m^{-2}s^{-1} (cool white fluorescent lamps).

2.3.3. Rooting of juvenile clones

Shoots (1.5 – 2.0 cm in length) of the four seedling-derived genotypes (YT, OT, RTC and RTL), were rooted in MS medium with different indole-3-butyric acid (IBA) concentrations: 0 µM (control, MS0), 1.2 µM (MS1) and 2.4 µM (MS2). In all cases 0.07 M sucrose and 0.6% (w/v) agar was added. Cultures were kept in a growth chamber under 16 h photoperiod at 15-20 µmol m^{-2}s^{-1} (cool-white fluorescent lamps) and a constant temperature of 25ºC. The number of rooted shoots was recorded after 10 and 30 days, as well as the number and length of formed roots. Three replicates of 8 shoots each were tested.

2.3.4. Somatic embryogenesis induction from leaf explants of seedling-derived shoots

Leaf explants from cloned shoots of YT, OT, RTC and RTL genotypes were used for induction of SE. Axillary shoots were cultured for 2 months in the establishment medium (MS medium without PGRs) before SE induction experiments began. All the procedures used in these assays, from SE induction to somatic embryo maturation and germination, have been previously described in details (Canhoto *et al.*, 2005). Briefly, the most apical expanding leaves were excised from proliferating cultures and used as initial explants for SE induction. Fifteen leaf explants per line, in 7 replicated treatments, were randomly wounded with the tip of a scalpel (see Fig. 2d) and placed (abaxial side down) in test tubes containing 15 ml of MS medium plus 0.25 M sucrose and 20 µM picloram. Gellan gum (Phytagel, Sigma) 0.25% (w/v) was added to the medium and the pH adjusted to 5.7. This medium was named TP (Table 1). Cultures were incubated at 25 ± 1ºC in the dark and the percentage of explants forming embryogenic tissue was evaluated after 12 weeks of incubation. After this period embryogenic cell clumps were separated from the non-embryogenic callus and subcultured is test tubes (each 4 – 5 weeks) on the same medium.

Mature zygotic embryos (30 explants per assay, in 10 replicated treatments) were isolated from seeds of the A tree previously disinfected in a 7% (w/v) calcium hypochlorite solution, with a few drops of Tween 20 for 20 minutes rinsed in sterilized water, and placed in test tubes containing 15 ml of MS medium plus 0.25 M sucrose and 9 µM 2,4-Dichlorophenoxyacetic acid (2,4-D) -,TD induction medium (Table 1). Results (% of explants forming embryogenic tissue) were also recorded after 12 weeks of culture.

2.3.5. Somatic embryogenesis induction directly from explants of adult tree

Different types of explants from the field-growing adult tree A were used for SE induction namely, pith segments, floral pieces (ovaries, stamens, petals and sepals), peduncles, and leaf segments.

Pith segments (2 mm thick, 0.5 x 0.5cm) were isolated from soft-woody branches that were cut in 2-3 cm segments. These segments were disinfected by immersion in water containing a few drops of a commercial detergent (5 min), rinsed

with 70% ethanol (1 min) and placed in a 7% (w/v) calcium hypochlorite solution containing a few drops of Tween 20, for 15 minutes. After three washes in sterile distilled water the pith segments were inoculated on MS medium with 0.25% w/v gellan gum (Phytagel, Sigma) containing 20 µM picloram, 9 µM 2,4-D or 27 µM 1-naphthaleneacetic acid (NAA), or a combination of auxin and cytokinin (9 µM kinetin, zeatin, or thidiazuron) as indicated in Table 1. Each treatment consisted of three replicates of 15 explants.

Leaves (5 – 10 cm length) were sterilized according to the same procedure. However, a more diluted calcium hypochlorite solution (5% w/v) was used. Following disinfection the midrib was removed and 0.5 x 0.5 cm segments were placed (abaxial side down) on the culture medium.

Floral explants and peduncles were isolated from non-pollinated tamarillo flowers, collected between May and July, surface disinfected with a solution of 7% (w/v) calcium hypochlorite containing a few drops of Tween 20, for 15 minutes, followed by 3 rinses in sterile water. The different explants were inoculated in 60 mm Petri dishes containing the same media and cultured in the same conditions as above (Table 1).

Table 1 – Composition of the culture media used in the experiments of somatic embryogenesis induction in different explants of tamarillo.

Explant	Explant origin	Somatic embryogenesis induction medium (MS basal medium, 0.26M sucrose)	
		Medium	Plant Growth Regulators (µM)
Leaf segments	Micropropagated shoots of YT, OT, RTC, RTL and RTA;	TP	Picloram (20)
Zygotic embryos	Seeds of plant A	TD	2,4-D (9)
Pith segments, leaf segments and floral pieces (petals, sepals, ovaries, stamens and petioles)	Plant A	TP	Picloram (20)
		TPK	Picloram (20) + Kinetin (9)
		TPZ	Picloram (20) + Zeatin (9)
		TPT	Picloram (20) + Thidiazuron (9)
		TD	2,4-D (9)
		TDK	2,4-D (9) + Kinetin (9)
		TDZ	2,4-D (9) + Zeatin (9)
		TDT	2,4-D (9) + Thidiazuron (9)
		TN	NAA (27)
		TNK	NAA (27) + Kinetin (9)
		TNZ	NAA (27) + Zeatin (9)
		TNT	NAA (27) + Thidiazuron (9)

2.3.6. Indirect somatic embryogenesis induction from explants of the adult tree

Leaf explants from *in vitro* grown shoots derived from the A tree were also used for SE induction. The conditions tested in this type of cultures were identical to those used for juvenile controls. Three replicates of twelve explants were tested. The results were taken after 12 weeks of culture in the dark, at 25±1ºC.

2.3.7. Somatic embryo conversion and plant acclimatization

Pieces of 80 mg of embryogenic tissue from the RTA genotype obtained in the induction medium (TP) were transferred to test tubes containing 15 ml of MS medium, (pH 5.7) without PGRs and with sucrose concentration lowered to 0.07 M (conversion medium). The cultures were kept under a 16 h photoperiod at 15-20 µmol $m^{-2}s^{-1}$ (cool-white fluorescent lamps at 25ºC) and the number of plantlets obtained per mass of embryogenic tissue (3 replicates of 80 mg each) was registered after 8 weeks.

Rooted plantlets were transferred to pots containing a mixture of sand, loam and peat (1:1:1) (covered with plastic) and placed in a growth cabinet chamber at 20ºC and 80–90% RH, under a 16 h photoperiod at 25-35 µmol $m^{-2}s^{-1}$ (cool-white fluorescent lamps). After 12 weeks of acclimatization plants were grown under standard greenhouse conditions.

2.3.8. RAPD analysis

Total genomic DNA was isolated from young leaf tissue samples of tree A and of two somatic embryo-derived plantlets of the RTA clone. Leaf tissues (100 mg) were grounded into a fine powder in liquid nitrogen using a mortar and pestle. Genomic DNA was extracted using the DNeasy® Plant Mini kit (Qiagen). The concentration and quality of the genomic DNA was determined by using a ND-1000 Spectrophotometer (NanoDrop Technologies). A total of 12 arbitrary decamer primers (Operon Technologies) was screened for RAPD amplification. The DNA amplification was carried out on a Thermal Cycler (BioRad) using the following profile: one cycle of 1.5 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 35 °C and 2 min at 72 °C, followed by one cycle of 5 min at 72 °C. Twenty five ng of DNA were run using the selected primers following the same PCR profile. The final volume for the PCR reaction was 25

µl containing 5 µl of 5X GoTaq® buffer (Promega), 1.5 mM of $MgCl_2$, 0.3 mM of each dNTP, 1U of GoTaq® DNA polymerase (Promega) and 0.4 µM of the primer. The PCR reaction products were separated by electrophoresis in an agarose gel (1.5% w/v) in 1X TBE buffer, stained with ethidium bromide, and visualized and documented under UV light. Six hundred nanograms of a DNA size ladder (HyperLadder™ II, Bioline) was loaded in the gel along with the PCR products.

2.3.9. Scanning electron microscopy (SEM) and cytological observations

For SEM observations embryogenic tissue and non-embryogenic *calli* from the RTA clone cultured on TP medium, as well as embryogenic tissue cultured for 3, 6, 9 and 15 days on plant conversion medium were fixed for 2h in a 2.5% (w/w) glutaraldehyde and 0.2 M sucrose solution prepared with 0.1 M phosphate buffer, pH 7.0, and post-fixed at room temperature for 1h in 1% (w/v) osmium tetroxide solution prepared with the same buffer. Samples were thoroughly dehydrated in an ethanol series (20, 40, 60, 80, 95 and 100% v/v) and critical point dried with carbon dioxide as the transition fluid and coated with gold. The specimens were mounted in aluminium stubs and examined in a JEOL JSM-T330 scanning electron microscope operating at 20 kV.

For cytological observations, small pieces (1 mm) of embryogenic tissue and non-embryogenic callus of the RTA clone cultured in the TP medium were placed on a microscope slide, squashed in acetocarmine and observed with a Nikon Eclipse E400 microscope equipped with a Nikon digital camera (model Sight DS-U1) using the Act-2U software.

2.3.10. Statistical analysis

Statistical analysis (SPSS Statistics 17.0) was performed by analysis of variance (ANOVA) and the significantly different means were identified by using the Tukey test ($p<0.05$).

2.4. RESULTS

2.4.1. Establishment of shoot cultures and rooting

Nodal segments obtained from a 13-year old tree (Fig. 1A) showed a high degree of contamination when cultured *in vitro* with about 78% (from a total of 30) of the explants displaying fungal growth. At the end of the culture period (six weeks) all the non-contaminated explants developed at least one shoot with a length between 3 – 4 cm and possessed at least 2-3 nodes (Figs. 1B and 1C). When necessary, nodal segments from these shoots were used to obtain new ones in the same culture conditions, thus increasing the stock of plant material used in SE initiation experiments. Clonal material of four genotypes (YT, OT, RTC and RTL), each one derived from a single seedling was also obtained and genetically uniform explants (leaves) of each clone were tested for SE induction (see below).

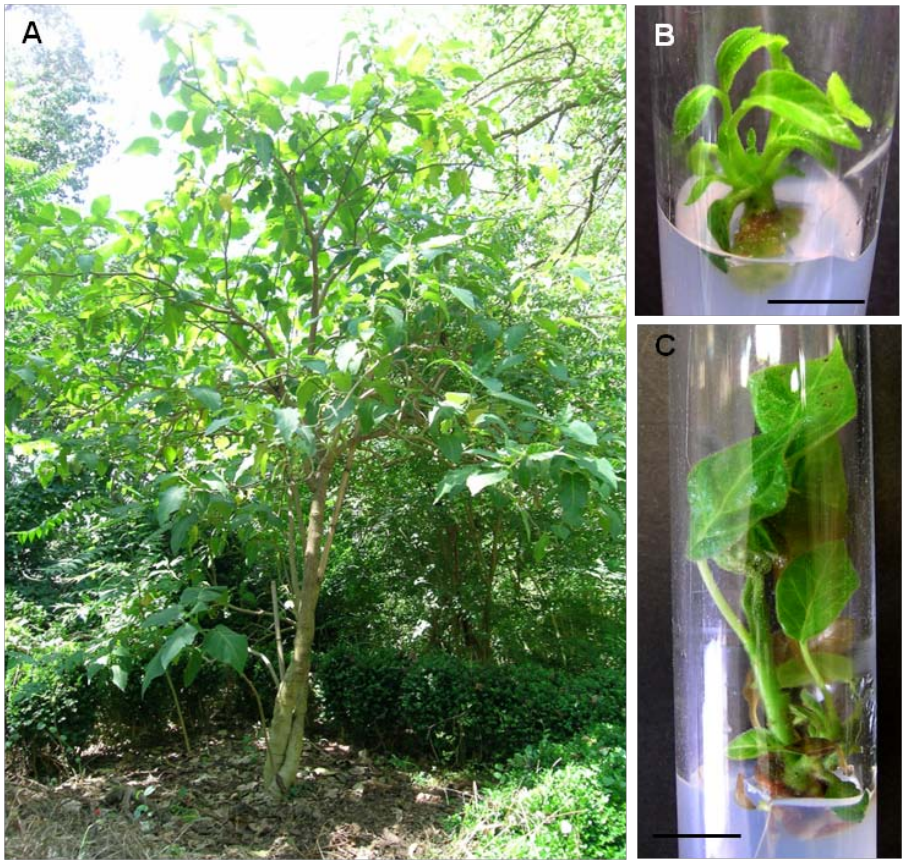

Figure 1 - *In vitro* cloning of an adult tamarillo tree ("A") using axillary bud micropropagation. **(A)** Tamarillo tree growing at the Botanical Garden of the University of Coimbra; **(B-C)** Different stages of shoot development resulting from the proliferation of axillary buds, after 2 weeks **(B)** and 6 weeks **(C)** of culture on full MS medium supplemented with 0.8 µM BAP and 0.07 M sucrose. Bars: 0.5 cm.

Table 2 – Root induction in shoots of tamarillo juvenile clones. Results were taken after 10 and 30 days of culture.

Genotype	Culture medium	Rooted shoots after 10 days (%)	Rooted shoots after 30 days (%)	Number of roots per shoot (after 30 days)	Shortest root (cm)	Longest root (cm)
YT	MS0	38	100	2.38±0.49[a]	3.00±0.75[a]	5.24±0.44[a]
	MS1	63	87.5	2.43±0.22[a]	3.84±0.67[a]	5.69±0.33[a]
	MS2	25	75	2.17±0.31[a]	3.30±0.72[a]	4.44±0.77[a]
OT	MS0	75	87.5	2.57±0.48[a]	4.04±0.49[a]	5.64±0.51[a]
	MS1	0	100	2.88±0.40[a]	3.14±0.53[a]	5.19±0.51[a]
	MS2	0	100	3.25±0.73[a]	3.21±0.69[a]	4.39±0.61[a]
RTC	MS0	50	100	2.63±0.32[a]	4.95±0.43[a]	6.14±0.29[a]
	MS1	25	100	2.63±0.50[a]	3.72±1.18[a]	4.32±1.09[a]
	MS2	25	100	2.50±0.37[a]	2.75±0.69[a]	3.65±0.43[b]
RTL	MS0	13	100	2.63±0.26[a]	3.24±0.59[a]	5.11±0.29[a]
	MS1	13	100	2.25±0.50[a]	3.84±0.50[a]	5.30±0.82[a]
	MS2	63	100	2.25±0.68[a]	4.28±0.54[a]	5.62±0.51[a]

Root length values are the mean (±SE) of three replicates (8 explants each). In the same column, numbers followed by the same letter are not significantly different (Tukey test, $p<0.05$)

The rooting of the cloned seedlings from shoots indicated that IBA was not required for root formation and development (Table 2). Generally no significant differences were detected in rooting frequency on media with or without IBA (Table 2). Root formation was a fast process with roots usually appearing as soon as by the 10th day of culture (one exception: genotype OT). Near 100% rooting was obtained after 30 days of culture even on the IBA free-medium, in particular when the red genotypes were tested (RTC and RTL). At the end of the experiment (30 days) there were no significant differences in average number of roots per plant (2.17-3.25) and on the average shoot length (2.75-6.14 cm). RTC genotype was the only one in which a significant difference between the different media was observed, with the shortest roots observed on MS2 medium.

2.4.2. Embryogenic tissue formation

From all the explants tested, embryogenic tissue was obtained from mature zygotic embryos on TD medium (Fig. 2A) or from leaf segments of *in vitro* proliferated shoots on TP medium. Other explants showed the infection rate higher than 50% for and in some cases (leaves) it reached almost 100%. The produced *calli* were variable in form, shape, structure, color, and growth rate depending on the type of explant and culture medium (Figs. 2B and 2C; Table 1), and none of them had an organogenic or embryogenic potential. Petals and stamens (Figs. 2B and 2C, respectively) were the only explants that formed *calli* in all the tested media.

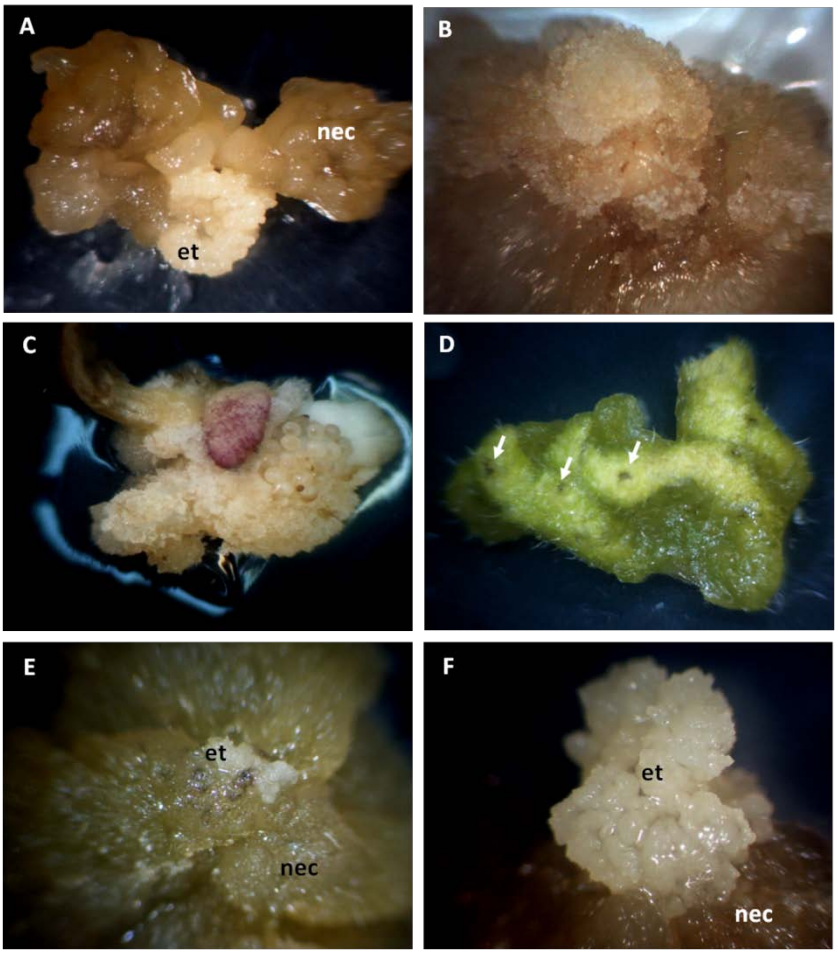

Figure 2 - Callus and embryogenic tissue formation in different explants. **(A)** Embryogenic tissue (**et**) formation on a mature zygotic embryo after 8 weeks on TD medium. Note the surrounding non-embryogenic callus (**nec**); **(B, C)** non-embryogenic callus from a petal on TNT medium (**B**), and from a stamen in TDZ medium (**C**) after 8 weeks; **(D, F)** embryogenic tissue induction on leaves of micropropagated shoots from the tree A cultured on TP medium; **(D)** wounded leaf explant (arrows point to the wounds) after 3 weeks of culture; **(E)** early development of embryogenic tissue (**et**) after 6 weeks of culture on TP medium; **(F)** well developed embryogenic tissue (**et**) after 10 weeks on TP medium. Note the non-embryogenic callus (**nec**) in figures **A**, **E** and **F** surrounding the embryogenic tissue (**et**).

Callus from wounded leaves (Fig. 2D) of RTA started to appear by the 6th week of culture and 2-3 weeks later, areas of whitish, compact embryogenic cell masses could be distinguished among the more friable, darker and fast growing callus (Fig. 2E). These small embryogenic areas continued growing and could be easily separated from the surrounding non-embryogenic callus (Fig. 2F). Embryogenic tissues separated from the non-embryogenic callus and subcultured on the same TP medium were slow growing, and formed compact whitish globular masses of cells of about 50-200 µm in size (Figs. 3A and 3C) whereas non-embryogenic *calli*, when subcultured onto the same medium were soft, fast growing and translucent (Figs. 3B and 3D). Cytological observations of both types of tissues showed the presence of globular cell masses formed by isodiametric cytoplasmic-rich cells in embryogenic tissues (Fig. 3E) and of enlarged and highly vacuolated cells (Fig. 3F) in non-embryogenic callus. A similar phenomenon occurred when mature zygotic embryos and juvenile clones were tested. Thus, in either of both types of the explants the embryogenic tissue could be separated from non-embryogenic *calli* and subcultured on media of the same composition (TD or TP), at 4-5 week intervals, without changes in their morphological appearance or loss of its embryogenic potential. Through this process, a large amount of embryogenic tissue was obtained from the different genotypes. Regarding the potential of embryogenic tissue formation from explants of the A plant (19.4% ± 2.8), the frequency of embryogenic tissue formation was similar to that obtained with mature zygotic embryos (24% ± 5.0). However, it must be stressed out that the culture medium used as well as the type of explant and the genotype were all different.

Beside the explant type, the data also indicated that the genotype significantly affected embryogenic cell masses formation (Table 3). The best results were obtained with leaf segments of the orange cultivar genotype (OT) in which over 54% of the explants produced embryogenic tissue (Table 3). High frequencies of embryogenic tissue formation (39.7%), but not significantly different from those obtained with OT, were also achieved with one of the red clones (RTL). In all other clones (RTL, YT, RTA) initiation rates were significantly lower and below 20% (Table 3). Interestingly results obtained from the adult red clone (RTA, 19.4%) were similar to juvenile clones, including one clone of the same red cultivar as RTA (RTC, 13.3%).

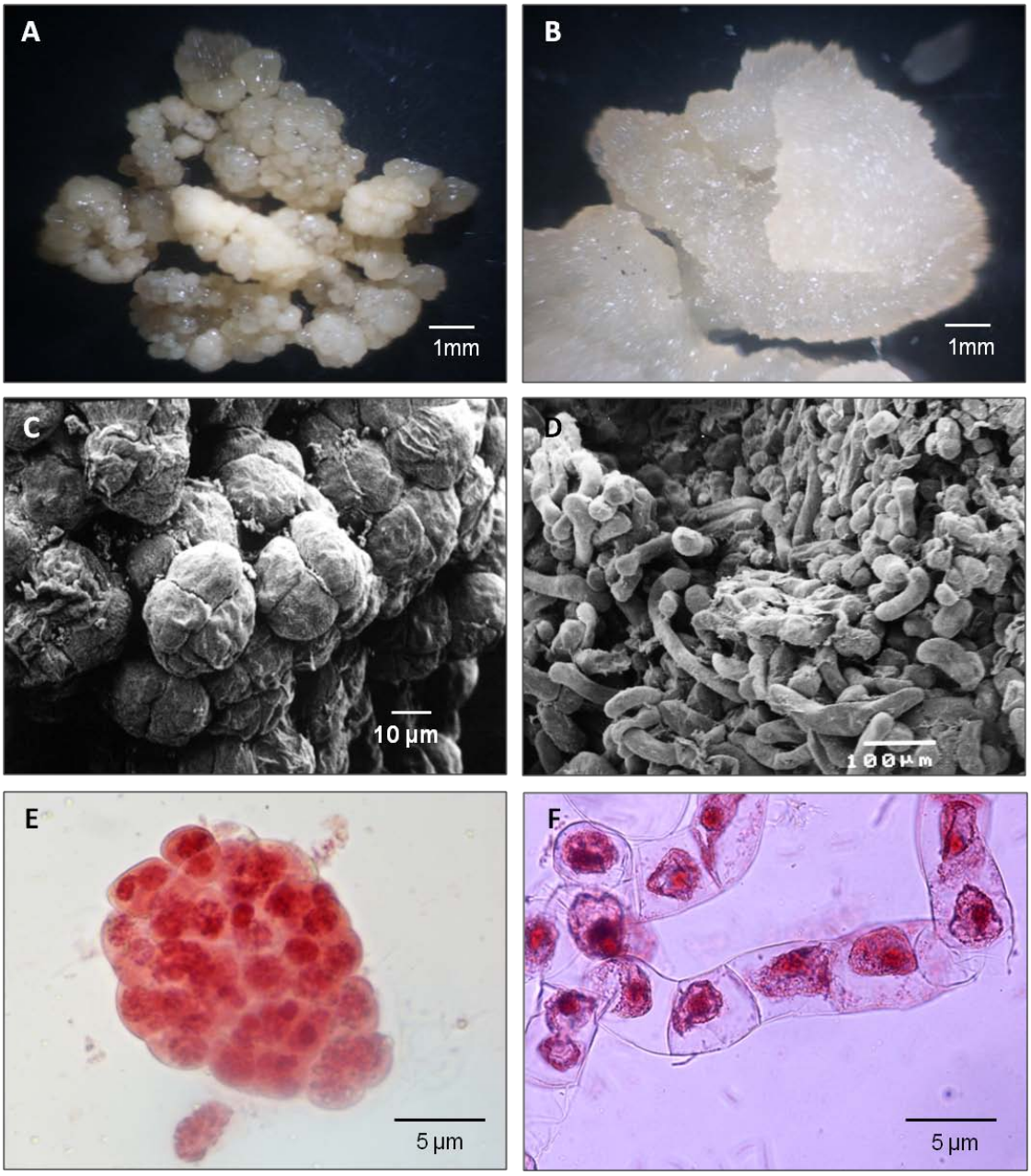

Figure 3 - Comparison between embryogenic tissue (**A**, **C** and **E**) and a non-embryogenic callus (**B**, **D** and **F**) derived from leaves of micropropagated shoots from the tree A subcultured for 1 month on TP medium. **(A)** Embryogenic tissue; **(B)** non-embryogenic callus; **(C)** SEM image of the surface of the embryogenic tissue; **(D)** SEM image of the surface of a non-embryogenic callus; **(E)** squash of an embryogenic tissue stained with acetocarmine showing a cell mass; **(F)** cells of a non-embryogenic callus stained with acetocarmine.

Table 3 – Role of the explant origin on the formation of embryogenic tissue. Media containing picloram (20µM) were used and the results taken after 12 weeks of culture.

Explant	Genotype	Embryogenic tissue formation (%)
Leaves from shoots of cloned seedlings	OT	54.4±9.06[a]
	YT	15.5±4.97[b]
	RTL	13.3±4.20[b]
	RTC	39.7±6.45[a]
Leaves from shoots of an adult plant (A)	RTA	19.4±2.76[b]

Values in percentage are the mean (±SE) of at least three replicates. Values followed by the same letter are not significantly different (Tukey test, $p<0.05$)

2.4.3. Somatic embryo development, conversion and acclimatization

To promote somatic embryo development and conversion into plantlets embryogenic cell masses from the adult material (RTA line) were transferred to a medium without auxin. In this condition, the embryogenic masses started to develop (Figs. 4A-D) and went through the classical stages of globular, heart-shaped, torpedo and cotyledonary embryos resembling their zygotic counterparts (Figs. 4A-D). Somatic embryo development was not synchronized and abnormal embryo phenotypes were often seen (Fig. 4D). By the 8th week of culture embryos converted into plantlets (Figs. 4E and 4F). Further plantlet development was achieved by isolating the growing emblings followed by their transfer to the same culture medium, until they reached a length of 5-8 cm (Fig. 4G) and were able to be acclimatized (Fig. 4H).

A RAPD analysis comparing two of these plantlets, obtained by SE from A tree showed similar band profiles when 12 arbitrary primers were used, indicating no variation. The results for three of these primers are shown on Fig. 5. Due to the high number of developing somatic embryos it was difficult to determine the number of embryos formed per embryogenic tissue. Instead, the number of plantlets per mass of embryogenic callus was evaluated. After 8 weeks of culture, an average of 1 plant was formed per each 9 mg of embryogenic tissue (111 plants/g).

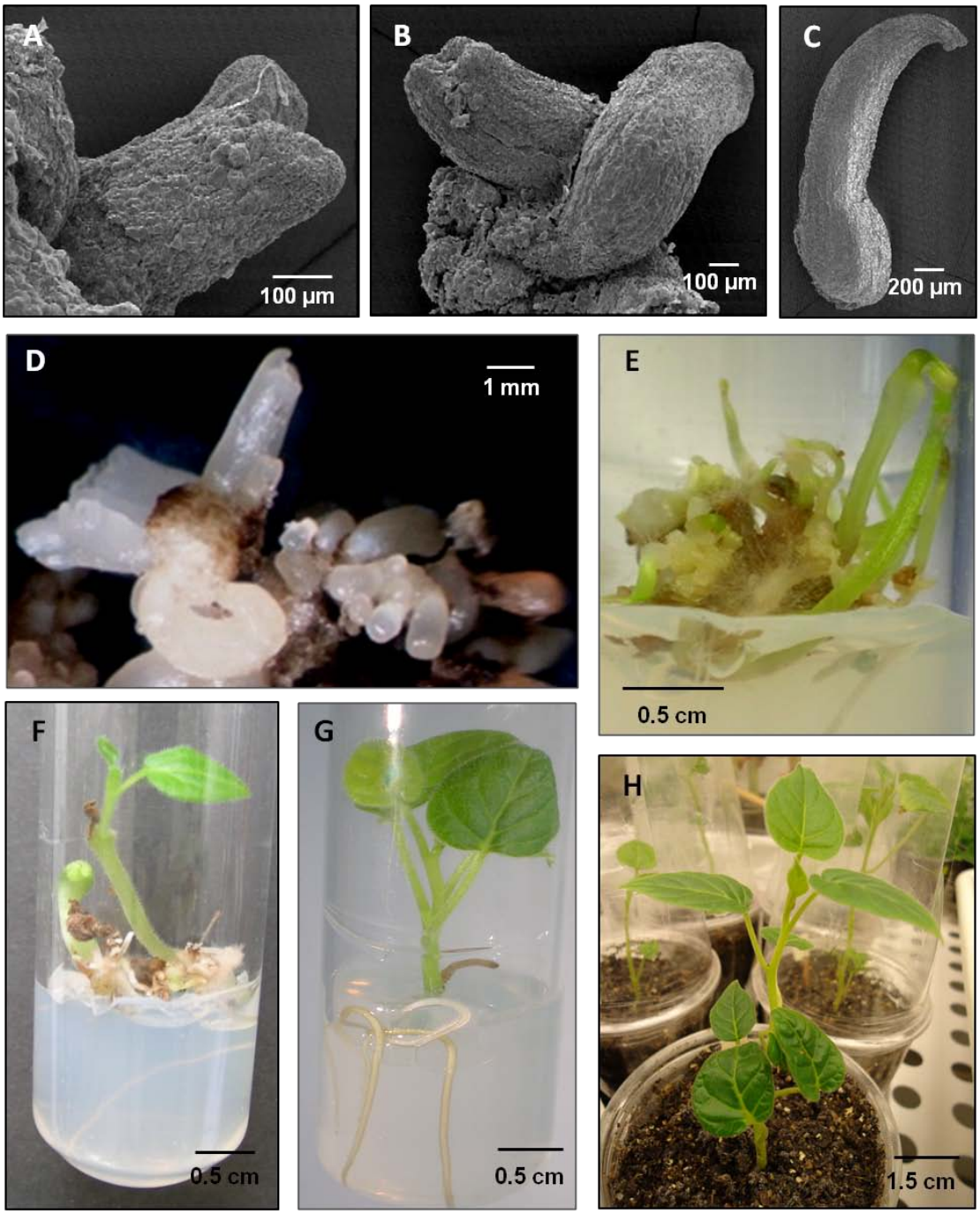

Figure 4 - Development, and conversion of somatic embryos and acclimatization of somatic plants derived from the adult tree A. **(A-C)** SEM aspects of somatic embryo development after 2 weeks on MS medium; (**D)** cluster of developing somatic embryos in the same conditions as indicated above; **(E)** somatic seedlings after 8 weeks of development; **(F)** plantlets resulting from the conversion of somatic embryos after 8 weeks; **(G)** morphologically normal somatic embryo-derived plant just before acclimatization (10 weeks); **(H)** Young tamarillo plants during the acclimatization process (4 weeks) after transferring from the test tubes.

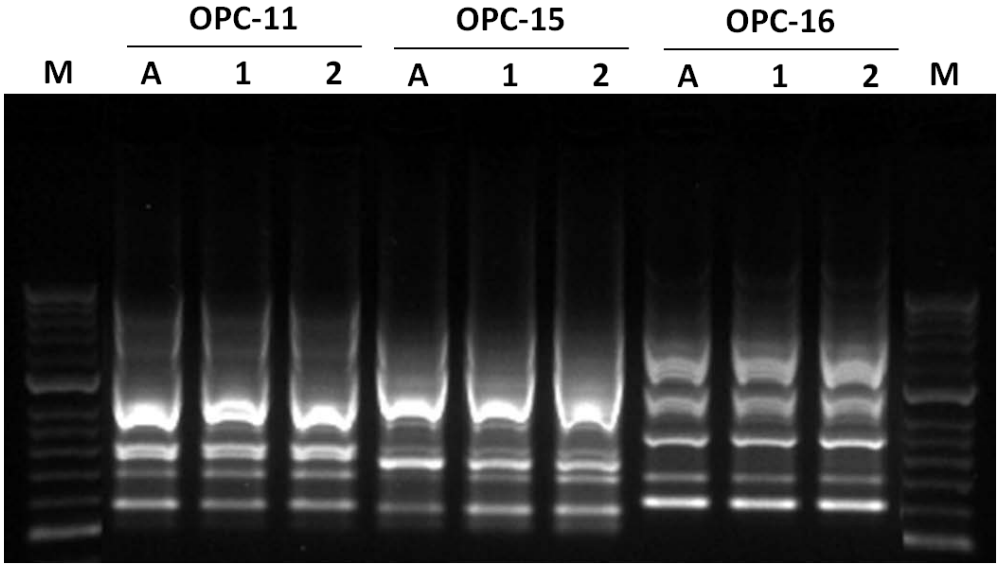

Figure 5 - RAPD analysis of the tree A and two somatic embryo-derived plants obtained from leaves of micropropagated shoots of the same tree (1 and 2). Results of the amplification with three primers (OPC-11, OPC-15 and OPC-16). M – 0.05-2kb HyperLadder^{TM} II (Bioline) marker.

2.5. DISCUSSION

Somatic embryogenesis has a great potential in crop improvement because it allows efficient cloning, rapid large-scale multiplication and genetic transformation of selected lines. One of the major drawbacks that has hindered the wider use of SE technology in trees has been the difficulty in using explants originated from mature trees (von Arnold, 2008; Bonga, 2010). The ability of trees to be vegetatively propagated decreases with increasing age, and mature trees have a low capacity to be cloned by SE. In spite of this impediment, SE has been induced from tissues of several adult tree species including avocado (Sánchez-Romero *et al.*, 2006), coffee (Etienne, 2005), cork oak (Toribio *et al.*, 2005), litchi (Raharjo and Litz, 2007) and more recently 10-year-old spruce (Klimaszewska *et al.*, 2011).

2.5.1. The low embryogenic potential of various explants from mature trees

Our results with tamarillo support the classical observation that tissues directly collected from adult field-grown trees have low ability to undergo SE. Previous works

with this species have shown that embryogenic tissue and somatic embryos can be obtained from embryonal (mature zygotic embryos) or post-embryonal tissue such as young leaves derived from seedlings (Canhoto *et al.,* 2005; Lopes *et al.,* 2000). Attempts to induce the same kind of response with similar protocols in different types of explants from one mature tree have failed. In all explants tested only friable, fast growing non embryogenic *calli* were obtained in contrast to the slow-growing embryogenic tissue that has been obtained from mature zygotic embryos or young leaves (Canhoto *et al.,* 2005). The embryogenic tissue is formed by proembryogenic masses that can proliferate in the presence of an auxin (picloram or 2,4-D). When transferred to an auxin-free medium proembryogenic masses developed to form somatic embryos through successive morphological phases characteristic of zygotic embryo development (Lopes *et al.,* 2000). We were also unable to achieve SE induction from developed leaves of mature plants as occurs in other species such as coffee (Etienne, 2005). In our case, the tamarillo leaf explants, taken directly from an adult tree, could not be established in culture due to the high level of contamination, which might be due to the mucilaginous trichomes covering leaf surface and making an efficient disinfection procedure difficult. Nevertheless, the few leaf explants (less than 5) that were not contaminated produced only non-embryogenic callus. More effective means of disinfection must be applied for estimating embryogenic ability of leaf explants, although contamination by endophytic organisms could be also a problem. The reason(s) why cells from adult tissues are less prone to dedifferentiate and to be reprogrammed into truly rejuvenated cells remains unclear. According to some authors (Litz and Gray, 1995; von Arnold, 2008) the differences between tissues of mature and juvenile plants in terms of regeneration potential may be epigenetic resulting in the inhibition of the regenerative potential of more differentiated cells. Recently, Bonga *et al.* (2010) have discussed some options that can be explored to overcome the difficulties encountered in the regeneration of woody plants from tissues of adult trees.

2.5.2. Embryogenic ability of *in vitro* propagated mature tree compared with control seedlings

Since direct approaches to induce SE in tamarillo from adult plants proved to be unsuccessful, we developed a strategy based on *in vitro* establishment of adult trees (micropropagation) to produce explants. Leaf explants were found to express

embryogenic potential with quite high initiation rate (19.4%) and regeneration of somatic plants. A similar protocol has been developed by Corredoira *et al.* (2006) for chestnut and by Gomes *et al.* (2009) for strawberry tree. An alternative way to explore would be leaves from epicormic shoots as was done in cork oak by Toribio *et al.* (2005).

When we tested leaf segments of the tamarillo juvenile controls for SE induction the orange cultivar showed the highest ability (54% of explants) for embryogenic tissue formation. The morphological aspect of the embryogenic tissue was identical in all the explants and genotypes tested. For embryogenic tissue originated from either mature zygotic embryos or leaves from seedlings this was described in previous works (Canhoto *et al.,* 2005; Lopes *et al.,* 2000). About one fifth (19.4%) of the leaves from adult derived explants produced embryogenic tissue, a result that was not significantly different from that obtained with the low responsive genotypes of seedling origin and within the range observed for control seedlings. It is well known that SE and rooting are two auxin and genotype-dependent processes. However, the interaction between these two factors in tamarillo seems to be stronger in the case of embryogenic tissue formation than in the case of rooting. In fact, whereas rooting rates of nearly 100% were obtained even without auxin (IBA) in all the juvenile genotypes, embryogenic tissue formation was highly genotype-dependent with the orange tamarillo clone displaying the highest embryogenic potential. However, the data about the role of the genotype should be interpreted carefully. The observation that a particular genotype under specific conditions of culture gives lower rates of SE induction than other does not necessarily mean that it is a low responsive genotype. Culture conditions can be improved and in different conditions the genotypes can behave differently.

The relatively low frequency of embryogenic tissue formation from leaves derived from shoots of an adult tree is not an obstacle for tamarillo cloning because the tissue can be subcultured in the same medium and increase its fresh mass from which somatic embryos can be obtained. Previous work on tamarillo has shown that the embryogenic cell masses could be maintained in culture for long periods of time without loss of the embryogenic potential (Lopes *et al.,* 2000). Morphological and SEM observations showed that callus derived from the adult A tree behaved in the same way. Presently we have healthy embryogenic tissue maintained for almost two years on an auxin containing medium from which somatic embryos can be obtained upon transfer to PGR-free medium. In tamarillo both embryogenic tissue and non-embryogenic callus

were produced from the same explant regardless the origin: zygotic embryos, leaves from seedlings, or leaves from shoots of a micropropagated adult tree. In general, for the same explant, the amount of non-embryogenic callus formed was higher than the embryogenic tissue. However, through separation of the embryogenic tissue and its subculture on the same medium a pure embryogenic culture was obtained, unless left in culture for over 4 months. It is possible that when the embryogenic tissue is separated from the non-embryogenic callus some vacuolated non-embryogenic cells could also be transferred. However, in the fresh medium, these cells do not proliferate suggesting that a certain cell density might be necessary for non-embryogenic cell proliferation. The reason why some cells of a particular explant, embark into an embryogenic pathway whereas others form non-embryogenic callus remains unclear. Did these two types of *calli* originate from different cell types or are particular microenvironments inside the callus responsible for triggering different morphogenic responses? Time course histological studies and the comparison between embryogenic cell masses and non-embryogenic *calli* in terms of protein and nucleic acid profiles may help to clarify these different types of cell fate.

2.5.3. Somatic embryo yield and quality

Previous works on SE in tamarillo showed that somatic embryos often display morphological abnormalities (Canhoto *et al.*, 2005; Lopes *et al.*, 2000). In this work we have also observed abnormalities and although a quantitative evaluation was not carried out, morphological observations under a stereoscope indicated that abnormal embryos were always present regardless the origin of embryogenic tissues. For some species, as feijoa, the presence of abnormal embryos impairs the success of plant regeneration through SE (Correia and Canhoto, 2010). In tamarillo it was observed that, in most of the cases, these abnormalities do not block embryo conversion to plantlets. The number of somatic embryos formed per unit of fresh mass of embryogenic tissue was highly variable. On average, 1 plant could be produced per 9 mg of fresh mass embryogenic tissue. This amounts to about 111 plants per g fresh mass of embryogenic cell masses.

In conclusion, the present study reports, for the first time, a reliable protocol for SE induction from an adult tree of tamarillo. A RAPD analysis indicated that, at the least for the two regenerated plants there were no variations in the analyzed regions of DNA, but a more detailed study with a higher number of plants must be carried out to

ascertain genetic fidelity. This is particularly important for long-term callus cultures of tamarillo.

We are now applying this protocol to cloning the orange tamarillo cultivar. However, differences may appear in trees of different cultivars as has been observed with the material of seedling origin. Further studies should be directed towards increasing the frequency of embryogenic tissue formation and the number of regenerated plantlets per mass of embryogenic tissue. Genomic and proteomic approaches are under way to compare the embryogenic tissue and non-embryogenic callus.

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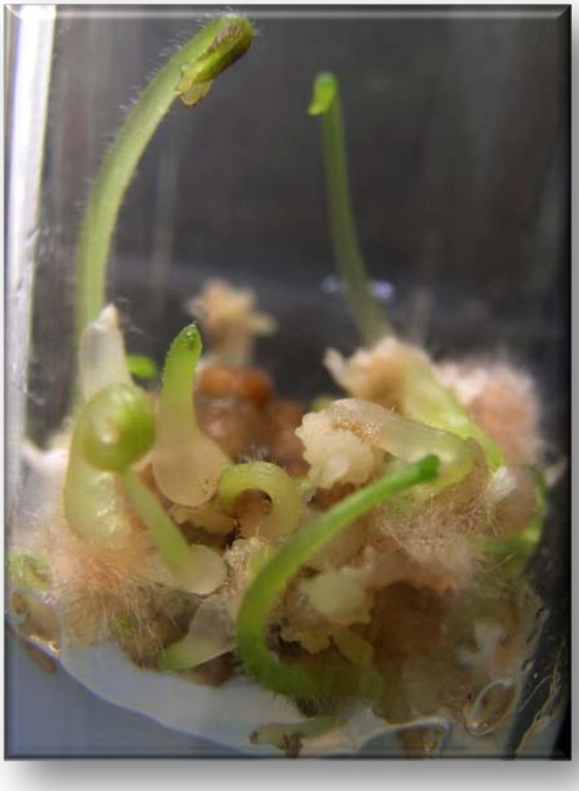

CHAPTER 3

SOMATIC EMBRYOGENESIS IN TAMARILLO: APPROACHES TO INCREASE THE EFFICIENCY OF EMBRYO DEVELOPMENT

Chapter submitted to an SCI journal (Plant Cell Tissue & Organ Culture)

3. SOMATIC EMBRYOGENESIS IN TAMARILLO: APPROACHES TO INCREASE THE EFFICIENCY OF EMBRYO DEVELOPMENT

3.1. ABSTRACT

Somatic embryogenesis induction and somatic embryo development of the Solanaceous tamarillo tree have been studied at our lab, and successfully used on the regeneration of these plants from several types of explants. Somatic embryogenesis is induced in MS medium containing the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram and high sucrose concentrations (0.25M), and the embryogenic tissues obtained must be transferred to an auxin-free medium, with reduced sucrose levels, in order to obtain somatic embryos. This regeneration protocol, through a two-step system, is often impaired by an effective transition from the proembryogenic masses that form the embryogenic tissue to embryo development. In this work, we intended to optimize the somatic embryogenesis system of tamarillo by increasing the number of somatic embryos that can be converted into plantlets. Our results have shown that the presence of a high number of anomalous embryos did not significantly affect plant conversion, hence indicating that SAM development is not affected in anomalous somatic embryos. It was also shown that the manipulation of sucrose concentration in the development medium (0.11M) and dark conditions before conversion increases the number of morphologically normal somatic embryos produced. The comparison between mature zygotic embryos and normal somatic embryos, at the cotyledonary stage, shows an inefficient accumulation of storage compounds in tamarillo somatic embryos, namely in what concerns lipid storage, which could explain the abnormal development of most of these embryos.

Keywords: ABA; dark conditions; embryo maturation; histochemical analysis; storage compounds; sucrose;

3.2. INTRODUCTION

Cyphomandra betacea (Cav.) Sendt. is a solanaceous softwood tree native of South America (Bois, 1927) and commonly known as tamarillo (Barghchi, 1998) . Used mainly because of its high nutritional edible fruits, this species has spread to several world regions, such as Central America, Southern Europe and New Zealand, which is nowadays the main producer and exporter of this fruit crop (Dawes and Pringle, 1983; Morton, 1987; Meadows, 2002). Tamarillo can also be cultivated in temperate regions of the Northern hemisphere, where it blossoms mostly during the transition from summer to fall, although flowering can also occur during other times of the year depending on the climatic conditions.

Somatic embryogenesis induction and somatic embryo development of tamarillo have been studied at our lab for several years and have been successfully used for the regeneration of tamarillo plants from zygotic embryos, seedling-derived explants such as cotyledons, hypocotyls and roots, and from leaves of *in vitro* proliferating shoots (Guimarães, *et al.* 1988; Guimarães, *et al.* 1996; Lopes, *et al.* 2000; Canhoto, *et al.* 2005). More recently an induction system to clone adult trees was also developed (Correia *et al.*, 2011). The assays performed with these different explants have given important insights about the conditions for somatic embryogenesis induction. Thus, it was found that somatic embryogenesis can be induced in MS (Murashige and Skoog, 1962) medium containing the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram and 0.25M sucrose (induction medium). The embryogenic tissues, subcultured in the same media, continued to proliferate, giving origin to new embryogenic clumps of cells. To promote embryo development, the embryogenic tissues must be transferred to a MS basal medium or to a medium containing gibberellic acid (GA_3) and reduced levels of sucrose (Guimarães *et al.*, 1996, Canhoto *et al.*, 2005).

For tamarillo, as for many other woody species, the development of efficient somatic embryogenesis protocols of plant regeneration may represent a useful method for clonal mass propagation of selected material, genetic transformation and germplasm cryopreservation (Park, 2002; von Arnold, 2008). Somatic embryogenesis has been induced in hundred of plants including angiosperms and gymnosperms (Namasivayam, 2007; Yang and Zhang, 2010). However, in most of the cases, the main problem is not somatic embryogeneis induction but rather further embryo development, maturation and conversion. Thus, effective protocols of somatic embryogenesis are often impaired by

several bottle-necks such as (1) difficulties in the *in vitro* maintenance of embryogenic tissues (2) loss of embryogenic potential after successive subcultures, (3) high levels of abnormal embryos produced and (4) the low maturation and subsequent conversion rates of somatic embryos into plantlets (Corredoira *et al.*, 2003; Stasolla and Yeung, 2003; Perán-Quesada *et al.*, 2004; Hussain *et al.*, 2009). In tamarillo, as in other species in which plant regeneration through somatic embryogenesis occurs through a two-step system, one of the major problems is an effective transition from the proembryogenic masses that form the embryogenic tissue to embryo development, which is often impaired by the formation of abnormal embryos and precocious germination of many others. This situation may be caused by an inadequate maturation of the embryos, an important phase of somatic and zygotic embryo development following the classic morphogenic phases from globular to cotyledonary embryos.

During maturation, embryo cells undergo various physiological changes, which become evident by the deposition of storage materials, repression of germination and acquisition of desiccation tolerance (Jiménez *et al.*, 2005; Vahdati *et al.*, 2008). In some species, in particular among gymnosperms, such as Norway spruce (Bozhkov *et al.*, 2002; von Arnold, 2008), effective protocols for somatic embryo development and maturation have been implemented and, as a consequence, the yield of plant conversion could be increased. Also, in some angiosperms (Mauri and Manzanera, 2003; Pinto *et al.*, 2008), the manipulation of the culture conditions gave a major contribute to boost the rate of plant conversion.

Trying to increase the number of somatic embryos that can be formed from tamarillo embryogenic tissues following transfer of the embryogenic tissue to an auxin-free medium, it was decided to investigate the role of some modifications in the composition of the culture media as well in the culture conditions on somatic embryo development. The more general goal of this work is to optimize the somatic embryogenesis system of tamarillo by increasing the number of somatic embryos that can be converted into plantlets. To better understand the process of somatic embryo development, the storage of reserves was analyzed by histochemical and biochemical techniques and compared with the levels of storage compounds present in mature zygotic embryos. The results of these approaches are now presented.

3.3. MATERIALS AND METHODS

3.3.1. Establishment and maintenance of embryogenic tissues

Embryogenic tissues were obtained and maintained following the methodology described by Lopes *et al.* (2000) and Correia *et al.* (2011). Briefly, the most apical expanding leaves were excised from *in vitro*-cloned shoots of tamarillo (red cultivar) and cultured in the induction medium containing the nutrients of the MS formulation (Murashige and Skoog, 1962), 0.07M sucrose, 0.6% (w/v) agar and the pH adjusted (with KOH) to 5.7. The leaf explants were randomly punctured and placed (abaxial side down) in test tubes (15 x 2.2 cm) containing 15 ml induction medium plus 0.25M sucrose and 20 µM of the auxin picloram. Phytagel 0.25% (w/v) was added to the medium and the pH adjusted to 5.7. Cultures were incubated at 24±1ºC in the dark for 12 weeks. After this incubation period, embryogenic areas were isolated and subcultured at 4 weeks intervals in the same medium. All the embryogenic tissues used for proliferation, germination and conversion studies were taken from a single embryogenic line (Fig. 1A) that was multiplied and maintained for 1 year before the subsequent assays.

3.3.2. Yield of the embryogenic process

First, in order to determine the yield of the embryogenic system in tamarillo, the number of somatic embryos produced per embryogenic tissue was evaluated. Somatic embryos were divided into normal and anomalous. Normal somatic embryos were considered those that reached the cotyledonary stage of development and showed a normal phenotype: two well formed cotyledons and an embryo axis. Embryos displaying some kind of abnormality, such as an altered number of cotyledons, enlarged embryos, fused embryos or any other bizarre appearance were considered abnormal. The assay was performed transferring an initial mass (an average of 0.043±0.004g) of embryogenic tissue to test tubes containing the development medium (MS plus 0.07M sucrose, 0.6% agar, pH5.7). The cultures were placed under a 16h daily illumination regime of 15-20 µmol $m^{-2}s^{-1}$ of photosynthetically active radiation provided by cool-white fluorescent lamps at 24ºC±1ºC. After one month under these conditions, the fresh weight of *calli* and somatic embryos was registered and the number of normal and

abnormal embryos recorded (Table 1). These *calli* were then transferred to new tubes containing the same culture medium for another 2-month period under the same conditions. By the end of the assay, after a total of 3 months of culture, total fresh weight and the number of plantlets per explant were registered.

3.3.3. Effect of the development media and culture conditions on somatic embryo development

Trying to increase the number of somatic embryos reaching the cotyledonary stage of development, embryogenic tissue was transferred to different development media and placed under various culture conditions. White opaque clusters, consisting, in average, of 100 mg of embryogenic tissue each, were removed from the induction medium and transferred to 100 ml flasks containing 25 ml of solidified medium, for 4 weeks, under different conditions. In total, 5 replicates per treatment were used, and the experiments repeated twice.

The influence of the medium on somatic embryo development was tested using full strength MS basal medium supplemented with different sucrose concentrations: 0.07M (S1), 0.11M (S2) and 0.16M (S3), and either free of growth regulators or supplemented with 8µM abscisic acid (ABA, S1A and S2A). S1 medium (0.07M and no ABA supplement) was the control situation.

To evaluate light influence, the above treatments (S1, S1A, S2, S2A) were performed under two light conditions (Table 2): one group was maintained in the dark, at 24±1ºC (S1D, S1AD, S2D, S2AD), and the other group was incubated under a 16h daily illumination regime of 15-20 µmol $m^{-2}s^{-1}$ photosynthetically active radiation provided by cool-white fluorescent lamps at 24ºC±1ºC (S1L, S1AL, S2L, S2AL).

The efficiency of the development conditions was evaluated by counting the number of mature cotyledonary and anomalous somatic embryos after 3 weeks of culture. Fresh weight at the end of this period was also registered and statistical analysis (SPSS Statistics 17.0) was performed through an analysis of variance (oneway ANOVA) with the significantly different means determined by the Tukey test (p=0.05).

Following somatic embryo development, mature cotyledonary somatic embryos were transferred to test tubes containing MS basal medium, 0.07 M sucrose, 0.6% (w/v) and agar, pH5.7 and cultured for one more month under a 16h daily illumination regime

of 15-20 µmol $m^{-2}s^{-1}$ photosynthetically active radiation provided by cool-white fluorescent lamps at 24ºC±1ºC., to achieve plantlet formation.

3.3.4. Extraction and quantitative analyses of storage compounds

To calculate the amount of storage compounds present in cotyledonary zygotic and somatic embryos biochemical assays were carried out. Intact cotyledonary zygotic embryos were carefully removed from sterilized seeds (7% w/v calcium hypoclorite, for 20 minutes) whereas cotyledonary zygotic embryos were isolated from the embryogenic cultures after a 3-week period in the development medium. Both zygotic and somatic embryos were first rinsed with sterilized water, dried on filter paper, weighted and fast-frozen in liquid nitrogen before being kept at -80ºC. Three replicates of each sample were analysed per assay. Statistical analysis (SPSS Statistics 17.0) was performed using the Levene test for homogeneity of variance and the T-test for independent samples (p=0.001, α=0.05). The procedures adopted for each type of compound are indicated in the following sections.

Total lipid extraction and quantification: previously frozen samples (2.5 g) were lyophilised and all the subsequent treatments were made in terms of dry weight. The extraction was made using a modified Folch procedure (Folch *et al.*, 1957) with chloroform:methanol (2:1) as the extraction solvent. The lyophilised samples were homogenized in 7 ml of extraction solvent, followed by centrifugation (10 min.) at 3.000 rpm. The supernatant phase was removed and the pellet washed two times with 1 ml aliquots of chloroform:methanol:water (3:48:47), which was then evaporated under vacuum conditions, on a rotary evaporator, at 45ºC. The residuum was collected at the bottom of the flask, transferred to a glass vial, dissolved in 2 ml of chloroform and again evaporated on the rotary evaporator at 45ºC. This last residuum was redissolved in 1 ml of cloroform and kept in a freezer (-20ºC) until quantitative analyses were performed

Total lipid quantification was performed based on the reaction of lipid degradation products with aromatic aldehydes, which results in a red coloration that can be quantified at 528 nm (Zöllner and Kirsch, 1962).

Total protein extraction and quantification: frozen samples (100 mg) were grounded to a fine powder in a pre-cooled mortar using liquid nitrogen. Tissue powder was transferred to sterile tubes and re-suspended with 200µl of an ice-cold extraction buffer [50 mM sodium citrate pH 5.5, 5% w/v sodium dodecyl sulfate (SDS), 0.01% w/v bovine serum albumin (BSA), 150mM calcium chloride, 2% β-mercaptoethanol; protease inhibitors as indicated by the manufacturer (SIGMA FAST^{TM} Protease Inhibitor)] and centrifuged (30 min., 13,200 rpm). After centrifugation, the supernatant was transferred to a new tube and kept on ice until protein quantification.

The protein levels were determined by the colorimetric method of Bradford (1976) using coomassie brilliant blue G-250 (Sigma). The absorbance was read at 595nm. The determination of total proteins was performed using BSA as standard.

Starch extraction and quantification: the extraction and determination of starch levels were based on the procedures of McCready *et al.* (1950). For total soluble sugars extraction, samples were macerated using 2 ml of methanol:chloroform:water (MCW) (12:5:3), and centrifuged for 10 min at 2.000 rpm. The supernatant was recovered and the pellet was re-extracted using 2 ml of MCW. One part chloroform and 1.5 part water were added for each four parts of the supernatant, followed by a new centrifugation for 10 min at 2,000 rpm, from which two phases were obtained. The upper aqueous phase (soluble sugars) was removed and the pellets were ground with 1 ml of 30% (v/v) perchloric acid and centrifuged for 15 min at 10,000 rpm. The supernatant containing starch was transferred to a new tube and the pellets were re-extracted twice. The supernatants were then combined and the pellets eliminated (McCready *et al.*, 1950). The sugar and starch concentrations were calculated using anthrone at 0.2%, in accordance with Umbreit *et al.* (1964) and using glucose as standard. The absorbance was read at 620 nm.

3.3.5. Histochemical analysis of cotyledonar somatic and zygotic embryos

Storage compounds were also analysed by histochemical studies. Cotyledons of zygotic and somatic embryos were both fixed for 2h in a 2.5% (w/w) glutaraldehyde and 0.2 M sucrose solution prepared with 0.1 M sodium cacodylate buffer, pH 7.2, and post-fixed, at room temperature, for 1h, in 1% (w/v) osmium tetroxide solution prepared with the same buffer. Samples were thoroughly dehydrated in an ethanol series (20, 40,

60, 80, 95 and 100% v/v) and embedded in Spurr's resin (Spurr, 1967). The blocks were polymerised at 65ºC overnight. For light microscopy, semi-thin (0.5-1µm) cross sections of the cotyledons were cut with a LKB ultra-microtome using glass knifes and stained by Sudan Black B (Bronner, 1975) for lipid detection, by mercury bromophenol blue (Mazia, 1953) for protein detection, and periodic acid-Schiff (PAS) reaction (McManus, 1948) for starch detection.

3.4. RESULTS

3.4.1. Evaluation of somatic embryo and embling formation

Embryogenic masses of tamarillo (Fig. 1A) are formed by clumps of isodiametric meristematic cells that keep on growing without somatic embryo formation whether maintained in an auxin containing medium. When this tissue was transferred to an auxin-free medium, with reduced sucrose levels, the embryogenic masses start to organize into somatic embryos (Fig. 1B) which go through the typical phases of somatic embryo development in dicotyledons (Fig. 1C). However, sometimes it was difficult to find heart-shaped or torpedo somatic embryos. After one month in this medium, fresh weight has increased as much as 30 times due mainly to the developing embryos (Table 1, Fig. 1D). At this moment, an evaluation of the number of somatic embryos per initial embryogenic tissue showed that the average number of cotyledonary somatic embryos formed was 143.9 per g of initial tissue, whereas the total number of abnormal somatic embryos was about three times higher, showing a value of 482.6 embryos per g of initial embryogenic tissue (Table 1). At the end of the experiment, fresh weight was much higher that at the end of the first subculture (1 month) and the number of developing plantlets (494.2 per g of initial embryogenic tissue) clearly outnumbered the number of normal somatic embryos initially developed, reaching a value that was approximately the same as the total of normal and abnormal somatic embryos (Table 1). This indicates that at least some of the abnormalities displayed by the somatic embryos do not interfere with embryo germination. Abnormal somatic embryos displayed different phenotypes such as fused, "cup-like", or absent/non-developed cotyledons, and fused or/and large-sized embryos (Fig. 1E). When the experiment was finished, many somatic embryos at different morphological stages were still present in the cultures (Fig. 1F and

1G) indicating that many more plants could have be obtained if other subcultures had been made.

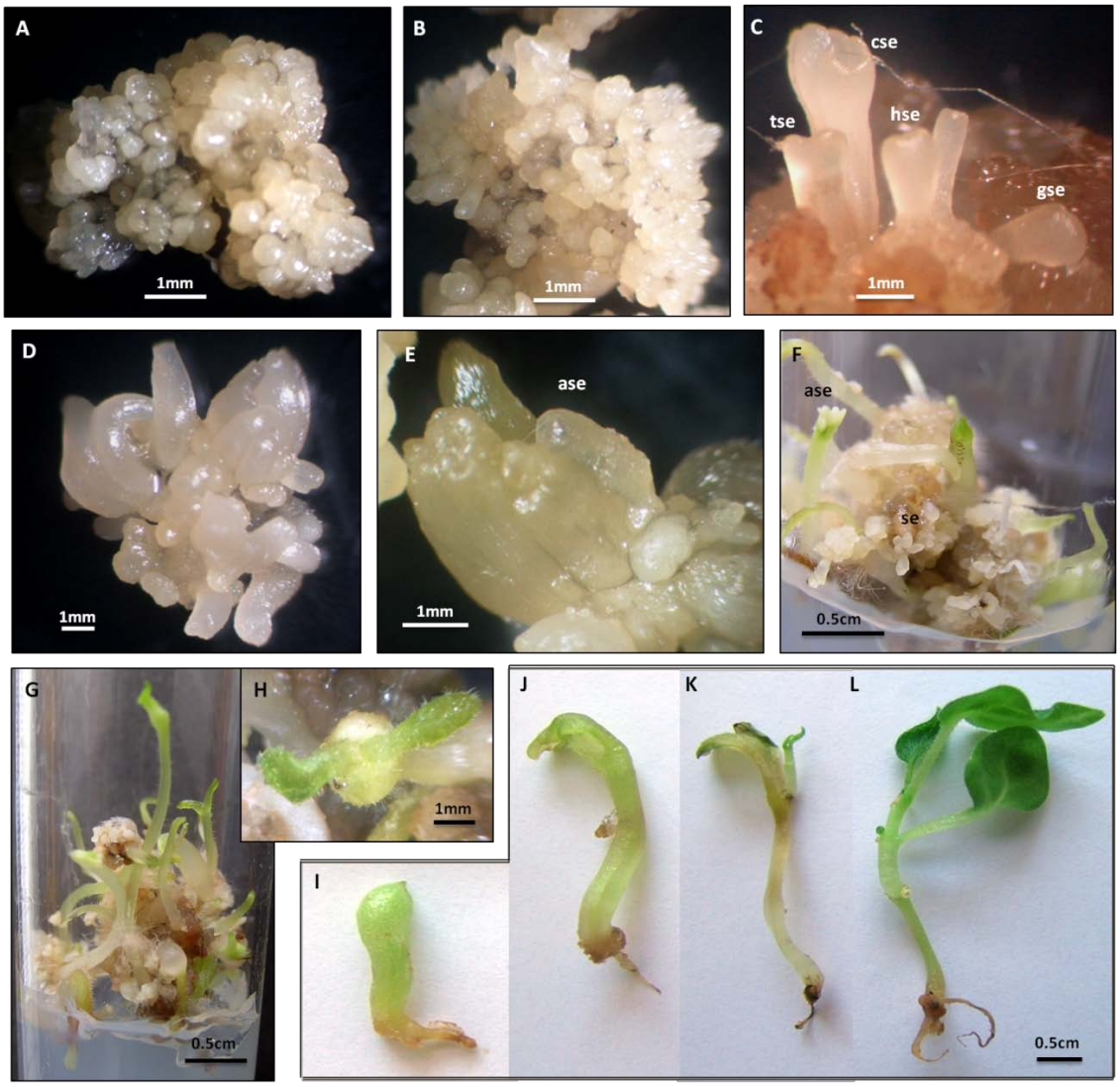

Figure 1 – Tamarillo somatic embryo development and conversion in MS medium supplemented with 0.07M sucrose and photoperiod conditions. **(A)** Embryogenic tissue induced from leaves; **(B)** Somatic embryos start to develop after 1 week, through an asynchronous process, as observed in **(C)**, where, after 2 weeks, several stages of somatic embryo development are present (**gse**, globular, **hse**, heart-shaped, **tse**, torpedo and **cse**, cotyledonar somatic embryos); **(D - E)** Somatic embryos, after a 4-week development period. Note the presence of large-sized anomalous somatic embryos (**ase**); **(F)** Somatic embryo conversion into plants after 8 weeks in culture - masses of embryogenic tissue and developing somatic embryos (**se**), and anomalous somatic embryos (**ase**) are still present; **(G-L)** Plantlet conversion after 10 weeks in culture showing apical and root development. Note the characteristic development of the shoot apical region from inside a sheath-like structure and the adventitious roots formation.

A common feature observed was the presence of a sheath-like structure involving the embryo apical meristem, which developed from the inside of this structure during conversion (Figs. 1G and 1H). In spite of the conspicuous morphological abnormalities, most of these embryos were able to germinate and be converted into viable plantlets (Fig. 1I-L). It was also observed that in some of the embryos formed, a root apical meristem seems not to exist since the roots are adventitious, formed after conversion in the development medium (Figs. I-L).

Table 1 - Mass proliferation (measured in fresh weight – FW) and total number of somatic embryos (SE) and emblings obtained after 1 and 3 months on the embryo development medium (MS medium, 0.07M sucrose) and under photoperiod conditions.

Explant	Initial embryogenic tissue FW (g)	Callus + SE FW after 1 month (g)	Number of SE per explant (after 1 month of culture)		Number of emblings per explant (after 3 months)
			Normal / cotyledonary SE	Abnormal SE	
1	0.049	1.06	8	8	2
2	0.052	1.32	2	13	7
3	0.026	1.16	3	33	23
4	0,041	0.96	10	28	41
5	0.040	1.57	8	15	23
6	0.050	1.06	6	28	32
Mean±SE*	0.043±0.004	1.19,±0.1	6.2±1.3	20.8±4,1	21.3±6.0
Number of SE / emblings per g of initial embryogenic tissue FW*			143.9±30.2	482.6±95.3	494.2±139.5

*****Mean number ± standard error.

3.4.2. Effect of the development media and culture conditions on somatic embryo development

In all media and conditions tested, embryogenic masses developed into somatic embryos similar to those obtained in the initial experiments, including phenotypically normal somatic embryos and different types of abnormalities (Figs. 2A and 2B). Somatic embryo development was not synchronized and different stages could be found

in the same callus (Fig. 2A). The presence of a large number of anomalous somatic embryos (Fig. 2C and 2D), large-sized, fused or with fused “cup-like” cotyledons, was a constant for all treatments. The results clearly show that sucrose has a crucial role in the development of the embryogenic tissue into somatic embryos, with the best results being obtained when 0.11M sucrose (S2) was used both for normal and abnormal somatic embryo formation (Fig. 3). Besides, the number of anomalous embryos continued to exceed the number of normal somatic embryos formed. When higher sucrose concentrations (0.16M) were tested, the number of somatic embryos sharply dropped and was not significantly different from the data recorded when the lowest sucrose concentration (S1, 0.07M) was tested (Fig. 3). Also morphologically, these somatic embryos were very similar to those obtained with the lowest sucrose concentration (Fig. 2E). The level of sucrose in the medium affects not only somatic embryo development but also tissue proliferation. However, when this last parameter was analysed the results showed that lower sucrose levels (0.07M) presented the best results either in light or dark conditions (Table 2). Since most embryos are formed in media containing 0.11M sucrose, the strong increase in fresh weight (Table 2) could not result exclusively from somatic embryo formation and development but also from the proliferation of the embryogenic cells in an auxin free medium.

The results also indicate that ABA is an important factor for somatic embryo development. Thus, it was found that, in the presence of ABA, the number of normal cotyledonary embryos, was significantly higher than when it was not present, but only when 0.11M of sucrose was used and the explants were kept under dark conditions (Fig. 4A). Nevertheless, there were no significant differences between the number of normal embryos obtained in the dark, when MS 0.11M sucrose was supplemented with ABA (S2A), and the number of embryos obtained with MS 0.11M sucrose not supplemented with ABA (S2), either in light or in dark conditions (Fig. 4A). As found for the number of anomalous somatic embryos, normal embryo formation was significantly promoted in S2 under photoperiod conditions. In all the other conditions tested, even though not at a significant level, the number of anomalous embryos was consistently higher under light conditions (Fig. 4B).

Morphological observation of the somatic embryos at the more developed stages showed that embryos formed under dark conditions, in MS medium with 0.11M sucrose and ABA (Fig. 2F), were more whitish and opaque than the more translucent ones formed under light conditions (Fig. 2G).

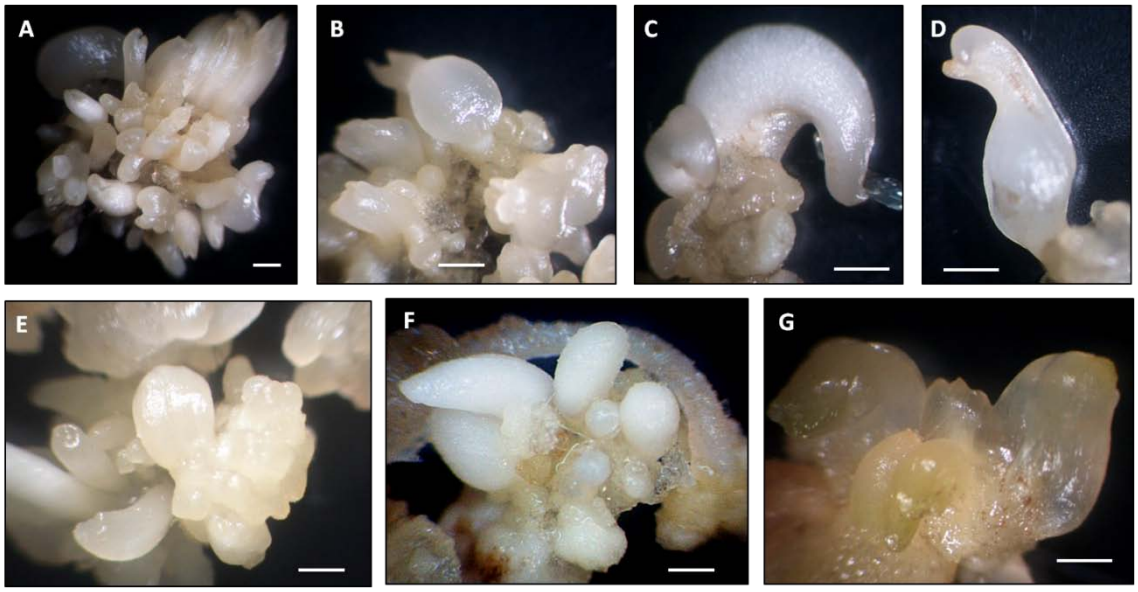

Figure 2 – Tamarillo somatic embryo development, after 4 weeks, under different culture conditions **(A-D)** Somatic embryos obtained in MS medium supplemented with 0.11M sucrose and dark conditions; note the somatic embryo asynchronous development and the presence of large-sized anomalous embryos, with fused or "cup-shaped" cotyledons; **(E)** Somatic embryos developed in MS medium supplemented with 0.16M sucrose in the dark; **(F-G)** Comparison between embryo development in MS medium supplemented with 0.11M sucrose and 8µM ABA, in the dark **(F)** and MS medium supplemented with 0.07M sucrose, in light conditions **(G)**. Note the white compact color of the somatic embryos in **(F)** when compared to the translucent somatic embryos in **(G)**.

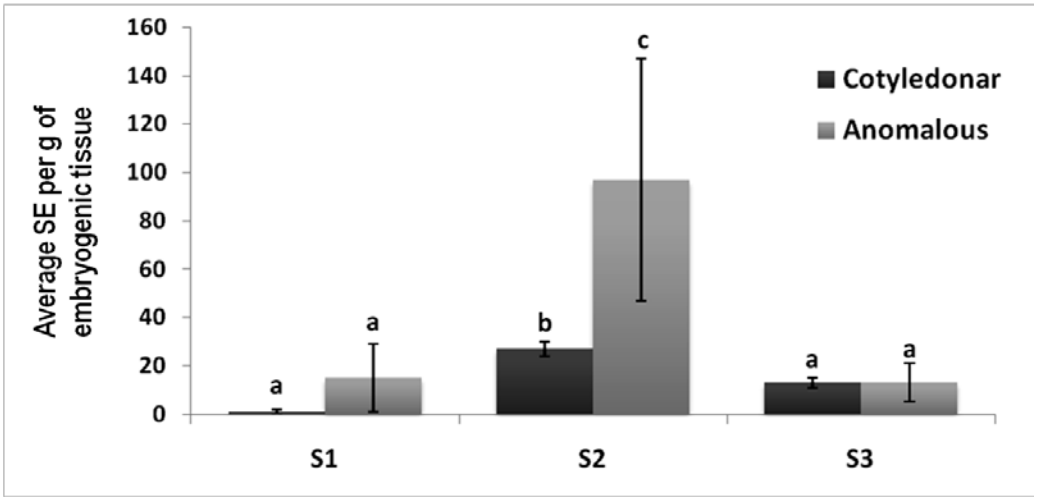

Figure 3 - Effect of the sucrose concentration on the development medium on somatic embryo development under dark conditions. The values are means ± standard error of 5 replicates from two experiments. Different superscript letters indicate significant differences at $p<0.05$, according to the Tukey test.

Table 2 - Tamarillo embryogenic masses proliferation under different developmental conditions.

	Treatment conditions*			Embryogenic tissue fresh weight (g)**	
	Sucrose concentration	ABA 8µM (Yes/No)	Light (L) /Dark (D)	Initial	After maturation
S1L	0.07 M	No	L	0.107	0.812±0.120[a]
S1D		No	D	0.113	0.813±0.155[a]
S1AL		Yes	L	0.106	0.406±0.630[b]
S1AD		Yes	D	0.118	0.546±0.221[c]
S2L	0.11 M	No	L	0.119	0.275±0.302[b]
S2D		No	D	0.105	0.553±0.188[c]
S2AL		Yes	L	0.111	0.496±0.883[c]
S2AD		Yes	D	0.104	0.423±0.280[b]
S3D	0.16 M	No	D	0.116	0.395±0.408[b]

* MS medium was used as basal medium, solidified with 0.6% (w/v) agar, pH 5.7;
** Values are means ± standard error of 5 replicates from two experiments. Values followed by the same superscript letter are not significantly different at $p<0.05$, according to the Tukey test.

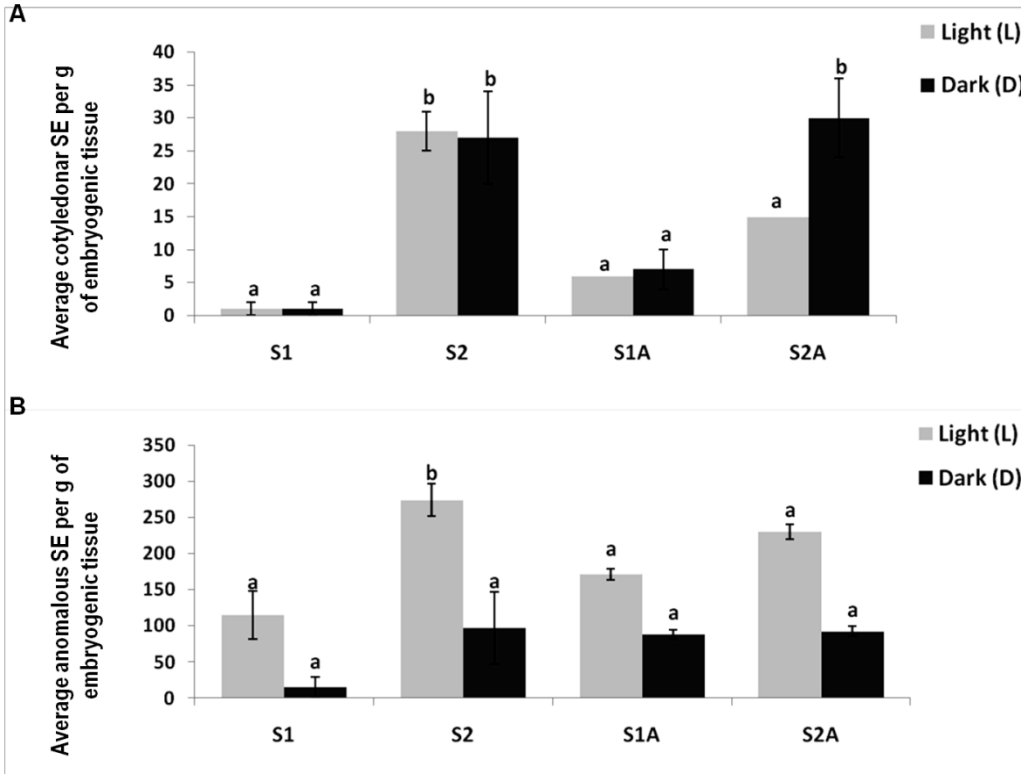

Figure 4 - Effect of medium composition (sucrose concentration and presense/absence of 8µM ABA) and light on somatic embryo development. The values are means ± standard error of 5 replicates from two experiments. Different superscript letters indicate significant differences at $p<0.05$, according to the Tukey test.

3.4.3. Biochemical and histochemical analysis of storage compounds

In order to compare the accumulation of storage compounds in somatic and zygotic embryos of tamarillo and to understand whether somatic embryos conversion could be affected by poor deposition of reserves, histological and biochemical analyses (Table 3) were performed. A general staining with toluidine blue indicated that cells of the cotyledonary zygotic embryos were large, more or less isodiametric, and completely packed with reserves (Fig. 5A). Histochemical analysis showed that the reserves were mainly lipids and proteins, respectively stained with Sudan Black B (Fig. 5B) and blue bromophenol (Fig. 5C). In somatic embryos, reserves were more scarce and cells were more vacuolated (Figs. 5D and 5E). Another important difference was found in starch content. Starch grains could not be found in cells of zygotic embryos, but their presence was common in somatic embryo cells (Fig. 5B). When the reserves were analysed by biochemical assays, the results were not completely consistent with those perceived by the histochemical data. Thus, while the total lipids were much higher in zygotic than in somatic embryos, as in the case of the histological studies, the levels of total proteins were not significantly different in the two types of embryos, although slightly higher in the zygotic ones (Table 3). The same is true for the starch content which was found to be similar in both types of embryos (Table 3) but that could not be demonstrated in the histochemical studies in which, as above-mentioned, starch could not be detected in zygotic embryos. Like starch, soluble sugars were present in similar amounts in the two types of embryos. The shortage in reserve compounds observed in somatic embryos can be one of the reasons affecting normal somatic embryo development and further conversion into plantlets.

Table 3 - Levels of lipids, proteins, soluble sugars and starch (mg/g fresh weight – FW) of tamarillo cotyledonary-staged somatic and zygotic embryos. The values presented are the mean ± SE of three replicated treatments. Values followed by different letters inside parameters indicate significant differences according to Tukey test ($p<0.05$).

Cotyledonary embryos (100mg FW)	Total lipids (mg/g FW)	Total proteins (mg/g FW)	Soluble sugars (mg/g FW)	Starch (mg/g FW)
Somatic	2.3±0.04 [a]	2.4±0.76 [a]	7.70±0.03 [a]	6.93±0.01 [a]
Zygotic	46.6±0.72 [b]	2.9±0.06 [a]	6.58±0.05 [a]	5.92±0.05 [a]

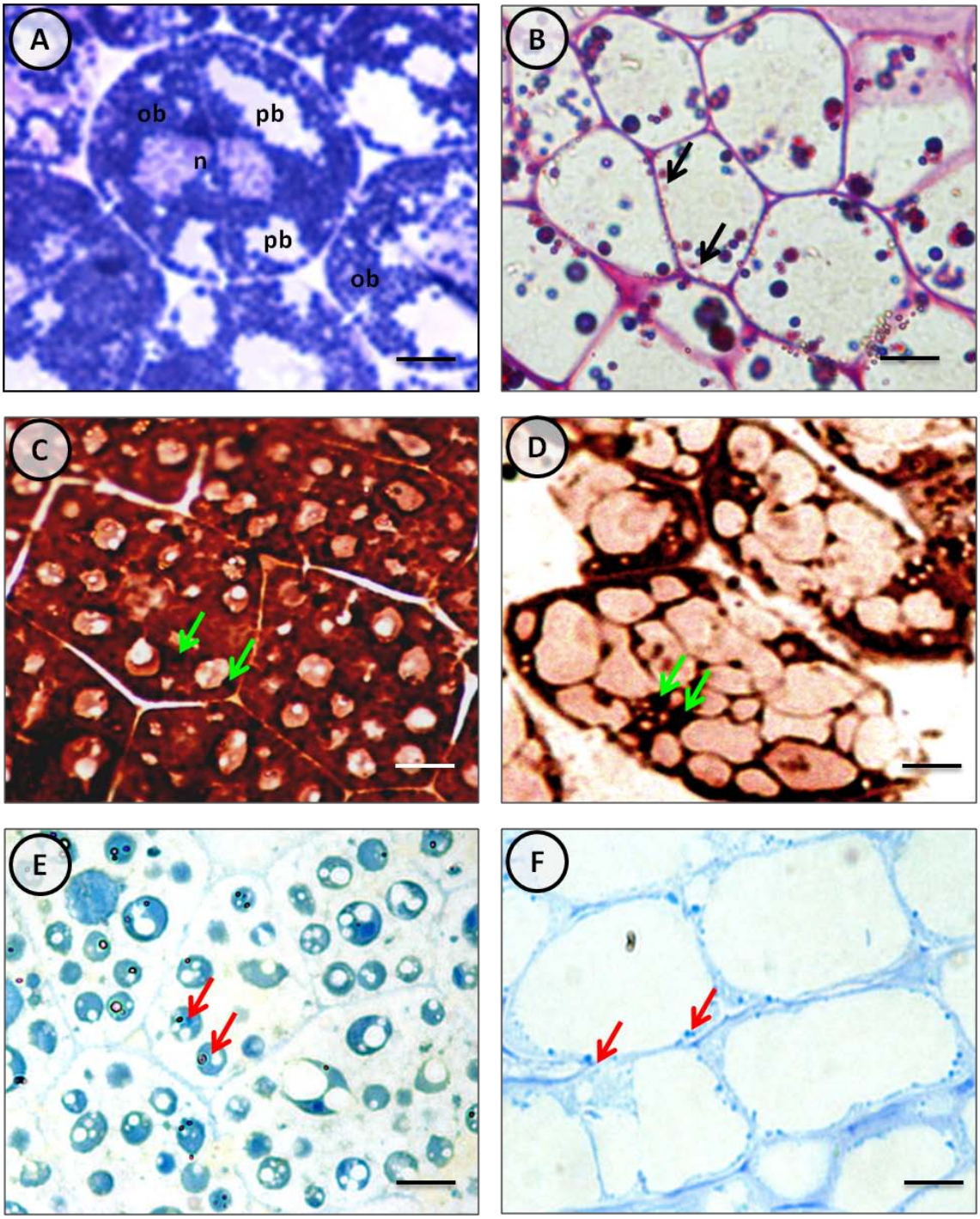

Fig. 5 Histochemical analysis of somatic and zygotic mature embryos cotyledons. **(A)** General staining, with toluidine blue, of a mature zygotic embryo cotyledon (**n**, nucleous; **ob**, oil bodies; **pb**, protein bodies); **(B)** Total starch detection, in somatic embryos cotyledons, with the PAS procedure; **(C-D)** Sudan Black B staining of total lipids from the cotyledons of a mature zygotic embryo **(C)** and of a cotyledonar somatic embryo **(D)**; **(E-F)** Total protein analysis, by bromophenol blue staining, of a mature zygotic embryo **(E)** and of a cotyledonar somatic embryo **(F)**. Arrows indicate the presence of the identified compound. Bars 100 µm.

3.5. DISCUSSION

Somatic embryogenesis is a method of *in vitro* plant multiplication that allows the clone propagation of selected genotypes (Thorpe and Stasolla, 2001). Nevertheless, an efficient regeneration protocol by somatic embryogenesis depends on the efficiency of somatic embryo germination and conversion into plantlets (Yang and Zhang, 2010). Thus, somatic embryo development must mimic, as much as possible, zygotic embryo development, which means that somatic embryos should display morphological or physiological abnormalities that could impair the embryogenic process (Ikeda and Kamada, 2005). However, this ideal situation is not always observable and, for many species, the number of somatic embryos obtained is much higher than the final number of plants, due to embryo abnormalities and/or deficiencies in germination and conversion (Canhoto *et al.*, 1999; Yang and Zhang, 2010).

All the previous work with tamarillo at our laboratory has contributed to a well established somatic embryogenesis induction protocol that allows obtaining a large number of somatic embryos. However, it has been shown that embryo quality is often poor, leading to the occurrence of morphological abnormalities, germination difficulties and precociously germinated embryos. In the literature, those abnormalities have been frequently related to apical meristems malformations or to constraints occurring during somatic embryo development (Corredoira *et al.*, 2003; Stasolla and Yeung, 2003; Perán-Quesada *et al.*, 2004; Hussain *et al.*, 2009). Preliminary results with tamarillo (Lopes *et al.*, 2010), and also with other species (Correia and Canhoto, 2010), have demonstrated that somatic embryo cells are often vacuolated, hence suggesting a weak ability to accumulate storage compounds during maturation with further negative impacts on embling production .

The data obtained in our work showed that plantlet formation is not strongly conditioned by the high number of anomalous somatic embryos formed, since the number of plantlets was 3 times higher than the number of normal somatic embryos obtained after the maturation period. This is probably an indication that the meristematic zones are well formed and not negatively affected by the culture conditions, at least in what concerns the shoot apical meristem. In fact, during conversion it was found that primary root development was sometimes affected and that, in these situations, root development occurred from adventitious root formation. Further studies concerning the characterization of shoot and root apical meristems in

somatic and zygotic embryos must bring some light in these aspects. An interesting feature observed during tamarillho somatic embryo conversion was the formation of a sheath-like structure completely surrounding the shoot apex and roughly resembling the coleoptiles of gramineous plants. Attempts to find similar structures described in the literature were unsuccessful. By now, we do not have a quantitative analysis about the percentage of somatic embryos forming this structure but they are quite common. The structural/functional role of this sheath must be analysed in detail in further studies. Since the high number of anomalous somatic embryos did not difficult the conversion into plantlets, it seems that the transition from embryogenic tissue to plant development is more critical for the process yield than the conversion. Thus, we have tried to improve plant conversion phase through the establishment of more appropriate culture conditions for somatic embryo development and maturation.

The obtained results indicated that sucrose concentrations of 0.11M are better for embryo development than the lower concentrations (0.07M) previous used. The type and concentration of carbohydrates as osmoticum in the culture media have been pointed as factors that strongly affect somatic embryo maturation and conversion (Corredoira *et al.*, 2003, Troch *et al.*, 2009). Sucrose, as a low weight osmoticum, has been proven to improve maturation when present in high concentrations, has seen in hoalm oak (Mauri and Manzanera, 2003), but higher sucrose levels can also be negative for some species as was the case in camphor tree (Shi *et al.*, 2009). These results are in accordance to the ones observed for tamarillo, suggesting that intermediate sucrose levels (0.11M) could reduce the drastic change from the induction medium (0.25M sucrose) to the development medium conditions. Another explanation can be related to the fact that a higher sucrose availability in the culture medium could be mobilized to compensate the low levels of storage compounds in the developing somatic embryos.

Although in some woody plants maturation and germination of somatic embryos have been obtained in media lacking phytohormones (Canhoto *et al.*, 2005, Shi *et al.*, 2009) in most cases the application of a specific treatment is necessary. Abscisic acid (ABA) and osmotic stress are known to be important factors for seed maturation in many angiosperms (Vahdati *et al.*, 2008). Inclusion of ABA into the culture medium during the final phases of somatic embryo development, simulating the natural increase in endogenous hormones observed in several zygotic embryos (Jiménez, 2005), has been recognized as a factor for promotion of normal development and maturation of somatic embryos and their uniformity in several species, like horse chestnut (Capuana

and Debergh, 1997), Norway spruce (Bozhkov *et al.*, 2002), avocado (Perán-Quesada *et al.*, 2004) or persian walnut (Vahdati *et al.,* 2008). For tamarillo, the presence of ABA did not improve the development process, since the number of cotyledonary somatic embryos obtained in media containing this hormone was not significantly different from the results obtained when it was not present. Instead, 0.11M sucrose and dark conditions showed a positive role on embryo development. Thus, sucrose levels and dark conditions appear to be more relevant for the improvement of development / maturation conditions than culture in a medium supplement with ABA. Nevertheless, it could be important to perform more assays with other ABA concentration and osmotica that could be more suitable for tamarillo. An interesting aspect that should be referred is that the embryo quality in tamarillo could be easily perceived by their color, with milky embryos possessing more reserves than the more translucent ones (data not presented). Based on this observation, image analysis methods can be developed for automated evaluation of embryo quality.

With respect to light conditions, according to Gaj (2004), most authors use photoperiod or darkness during somatic embryogenesis. Nevertheless, systematic studies on the effects of light on *in vitro* response of cultured explants (and in SE in particular) are limited. Assays with *Eucalyptus globulus* showed that light may influence the quality of the maturation process, suggesting that darkness should be maintained until the cotyledonary stage is reached (Pinto *et al.*, 2008). This observation correlates to what was observed for tamarillo somatic embryos.

Although the protocol of somatic embryo conversion in tamarillo enables the production of a great number of emblings per g of initial embryogenic tissue, it must be highlighted that the full potential of the protocol was not explored since embryogenic tissue continued to proliferate even in the auxin free media used for embryo development. Embryogenic tissue proliferation is an uncommon feature in embryogenic systems of this type and may be an indication of auxin habituation (Kevers *et al.*, 1996). In alternative, it could means that embryogenic cell proliferation is dependent on other conditions rather than auxin. Tissue proliferation in an auxin free medium deserves further analysis because embryo formation and plant conversion in these conditions can overcome the problems often caused by high auxin and/or strong auxins present in the induction medium (Jiménez, 2005).

Since the maturation and development process of a somatic embryo should be as similar as possible to the one of a zygotic embryo (Ikeda and Kamada, 2005), the next

stage of this work focused in the biochemical and histochemical differences between cotyledonary somatic embryos and mature zygotic embryos. The obtained results show that somatic embryos have significant reduced levels (about 20 times) of total lipids when compared to zygotic embryos. Histological analysis revealed that most of the cell volume is filled with lipid bodies, which indicates that the quantified lipids are in fact storage lipids. Lipid storage in embryo cells during the maturation stage of zygotic embryo development is a common feature for many species (Bewley and Black, 1994). Those lipids are mobilized during the early stages of germination. The correlation of lipid accumulation with a hormonal effect triggered by ABA (Thomas and Jiménez, 2005) suggests that the culture conditions used for tamarillo somatic embryos maturation can still be improved, namely by the use of better suited ABA concentrations.

When storage proteins were analysed, histological analysis showed that protein accumulation is more reduced in somatic embryos, however biochemical quantification showed equivalent values for both types of embryos. One explanation could be related to the fact that storage proteins are highly glycosylated, which contributes to the appearance of large protein vacuoles with an effective lower protein content than expected. Supporting this observation is the fact that the PAS technique, presumably specific for carbohydrates, also stain protein vacuoles. Other possibility is that, in somatic embryos, the protein could not accumulate so intensively in protein bodies and may have a more diffuse distribution in the cytoplasm. Ultrastructural studies comparing the subcellular organization of cells in both types of embryo cotyledons may help to clarify these contradictory observations

When starch accumulation was analysed, the histological data showed that zygotic embryos do not accumulate this storage compound, but somatic embryos do. The accumulation of starch in somatic embryos is probably the result of a modification in cellular metabolism that might be a consequence of the high sucrose levels used in the culture medium. Some studies (Merkle *et al.,* 1995; Yeung, 1995; Thorpe and Stasolla, 2001) have focused on the association between synthesis and accumulation of starch and the development of somatic embryos. The results showed an increase in starch levels in the late development stages, indicating that starch is accumulated in higher levels in somatic embryos than in the zygotic ones. These studies also suggested metabolic differences between zygotic and somatic embryos, the later being less efficient in converting carbohydrates in lipids and storage proteins.

The set of results obtained allowed verifying that the inefficient accumulation of storage compounds in tamarillo somatic embryos could explain the low rates of normal embryos obtained. Further analysis should focus on the analysis of the effect of different maturation treatments tested in relation to the storage compounds accumulated in the embryo cells, since the improvement of maturation conditions could be very important to the yield of the somatic embryogenesis process in this species.

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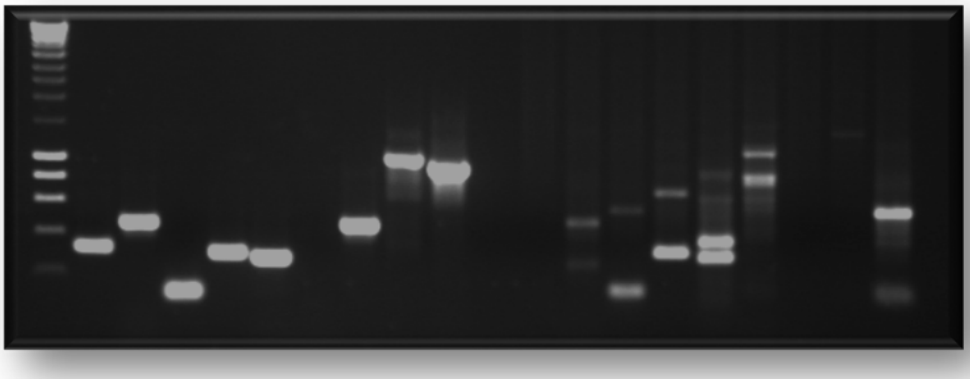

CHAPTER 4

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF A PUTATIVE NEGATIVE REGULATOR OF SOMATIC EMBRYOGENESIS INDUCTION IN A SOLANACEOUS TREE (C*yphomandra betacea*)

4. MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF A PUTATIVE NEGATIVE REGULATOR OF SOMATIC EMBRYOGENESIS INDUCTION IN A SOLANACEOUS TREE (*Cyphomandra betacea*)

4.1. ABSTRACT

Somatic embryogenesis (SE) is an important biotechnological tool with great potential for rapid large-scale clone propagation. In addition, genetic transformation and cryopreservation procedures in many plant species rely on efficient SE protocols. Different aspects related to SE induction and somatic embryo development in tamarillo, a woody plant of the Solanaceae family, have been studied in our laboratory. The experiments performed led to the development of a protocol for the *in vitro* establishment of embryogenic and non-embryogenic *calli* of tamarillo. Previous comparative proteomic analyses identified a protein (NEP, 26.5 kDa) consistently present in non-embryogenic *calli*. A cDNA corresponding to the gene which encodes this protein was isolated from a cDNA library, prepared from non-embryogenic *calli*, and sequenced (Faro *et al.*, 2003). The isolated sequence encodes a 222 amino acid long protein showing a high degree of identity with the *Arabidopsis thaliana* tRNA/rRNA methyltransferase (SpoU) family protein, and also significant alignments (>75%) with predicted proteins found in *Vitis vinifera*, *Populus trichocarpa*, *Ricinus communis* and *Glycine max*. In this work, we evaluated the role of NEP25 in somatic embryogenesis induction, following two experimental strategies: 1) SE induction on knock-out lines of *Arabidopsis thaliana*, and 2) post-transcriptional down-regulation of the *NEP*25 gene in tamarillo. Homozygous plants were evaluated for their ability to undergo SE, when compared to the wild type controls. Morphogenetic aspects, such as rooting and rosette and inflorescence development, were also compared. Although no significant differences were found in terms of development or SE induction rates, RT-PCR analysis of embryogenic and non-embryogenic explants showed a higher expression of this gene in the latter, in conformity to what has been observed in tamarillo. Tamarillo's *NEP25* gene down-regulation was achieved using the GATEWAY™ cloning technology to prepare a construct encoding a hairpin RNA targeting the *NEP25* gene. In approximately 6 months, an improved *Agrobacterium*-mediated transformation protocol for tamarillo leaf explants, allowed obtaining more than 80 self-rooted kanamycin resistant plants from a single embryogenic cell line. After confirmation of *NEP25* gene

down-regulation, SE was induced from tamarillo transgenic plants in parallel with a control wild-type line. As observed for *Arabidopsis*, no significant differences were found between induction rates in transformed and wild-type lines, yet the NEP25 expression continued to be more evident in non-embryogenic callus. The data obtained so far associates (for the first time) a putative member of the SpoU methylase super family with the somatic embryogenesis induction process. The absence of phenotypes resulting from gene silencing suggests that other proteins of this family may have a redundant role during the SE induction process.

Keywords: non-embryogenic protein; SpoU RNA methylases; post-transcriptional gene silencing; hairpin RNA, knock-out.

4.2. INTRODUCTION

Embryogenesis is one of the most important steps in the life cycle of plants. In Angiosperms, it involves several crucial processes that begin with double fertilization, followed by determination of the embryo axes and its morphologic changes throughout globular, heart-shaped and torpedo-shaped stages. Subsequently, storage proteins accumulate in the embryo, and finally, the embryos become desiccated and dormant. These processes are regulated by numerous factors, including phytohormones, enzymes, and other substances related to embryogenesis. Many studies have been conducted, using diverse experimental techniques, aiming to understand the mechanisms that control plant embryogenesis. Somatic embryogenesis is one of the most useful experimental tools to investigate the morphological, biochemical and physiological events of embryogenesis (Meinke, 1991; Zimmerman, 1993; Ikeda *et al.*, 2006).

Besides being an important tool for large-scale plant propagation, somatic embryogenesis offers an attractive model system for studies on embryo development since somatic embryos follow a development pathway very similar to that of their zygotic counterparts (Dodeman *et al.*, 1997) and are more accessible than zygotic embryos. Furthermore, they can be produced in high numbers in a single test tube.

The search for markers of plant embryogenesis has been an important area of research in plant development. Several physiological, biochemical and molecular markers associated with the embryogenic competence of cells have been reported and

reviewed (Yang and Zhang, 2010), including genes related to cell differentiation, morphogenesis, desiccation tolerance, and signal transduction (Ikeda *et al.*, 2006). Nevertheless, and despite all the efforts, the molecular mechanisms that control the transition of somatic cells, with specific functions into cells capable of forming an embryo, hence expressing totipotency, are one of the least understood areas of the somatic embryogenesis process.

However, the switching from a plant genomics research to an expansive functional genomics research has allowed researchers to choose from a multitude of different methods that are contributing to the characterization of several plant genes related to somatic embryogenesis. Such methods allow analyzing the different constituents of the cell that help to deduce gene function, namely the transcripts, proteins and metabolites. Similarly, the phenotypic variations of entire mutant collections can now be analyzed in a fast and efficient way. The different methodologies have developed to form their own fields within the functional genomics technological platform and are termed transcriptomics, proteomics, metabolomics and phenomics. Gene function, however, cannot be exclusively inferred by using only one of such approaches (Holtorf *et al.*, 2002).

The complete genome information now available for some key species such as *Arabidopsis thaliana* is of crucial importance for an effective functional plant gene analysis. Many embryo-defective mutants have been isolated and analyzed in *Arabidopsis*, allowing a rapid analysis of gene function (Krysan *et al.* 1999; Speulman *et al.* 1999). However, the majority of the genes causing the embryo-defective mutants were related to housekeeping phenomena (e.g., cell division, cell differentiation, phytohormone response and other indispensable survival processes), and only few embryogenesis-specific genes have been found (Ikeda *et al.*, 2006). Thus, many aspects of the embryogenesis program remain to be elucidated.

More recently, gene silencing could be achieved by the transformation of plants with constructs that express self-complementary (termed hairpin) RNA containing sequences, homologous to the target genes (Fig. 1). The DNA sequences encoding the self-complementary regions of hairpin RNA (hpRNA) constructs form an inverted repeat (Smith *et al.*, 2000) that triggers the suppression of gene expression. Double-stranded RNA (dsRNA) is perceived as foreign and triggers the degradation of itself and homologous RNA within the cell, in a process of post-transcriptional gene silencing (PTGS). In recent years, this methodology has been successfully employed to determine

gene function in many organisms (Waterhouse *et al.,* 1998; Chuang and Meyerowitz, 2000; Schawb *et al.,* 2006;). The efficiency and effectiveness of hpRNA as a tool for silencing plant genes makes this the method of choice over antisense or cosuppression methods. The application of hpRNA silencing depends only on the ability to deliver the hpRNA construct efficiently into the plant cell and on the availability of gene sequences, making it a functional genomics tool that can be applied to understand the biology of virtually any plant species (Heliwell and Waterhouse, 2003).

In tamarillo, the search for molecular markers of somatic embryogenesis induction started with a study of somatic embryogenesis induction from different explants of tamarillo, followed by the analysis of protein electrophoretic patterns of embryogenic and non-embryogenic *calli* . This comparative analysis intended to identify protein markers associated with these two types of *calli* (Ferreira *et al.,* 1998). A 26.5 kDa protein (NEP25) was consistently found in non-embryogenic *calli* derived from several explants, such as zygotic embryos and isolated hypocotyls, cultured in the same conditions, suggesting that this protein could be considered a good marker for the non-embryogenic *calli* of tamarillo. In a further work, this protein was isolated and sequenced (Faro *et al.,* 2003).

Cyphomandra betacea is a small perennial Solanaceous tree intensively studied in our laboratory, with well established protocols for somatic embryogenesis induction (Lopes *et al.,* 2000; Canhoto *et al.,* 2005; Correia *et al.,* 2011). Also, several other groups reported the use of biotechnological methods, such as *in vitro* cloning and genetic transformation (Atkinson and Gardner, 1993; Cohen *et al.,* 2000), as useful alternatives for tamarillo plant breeding (Barghchi, 1998). In all the reports, tamarillo has shown to be a very useful plant to better understand particular aspects of *in vitro* morphogenesis, in particular somatic embryogenesis. Allowing to associate genetic transformation studies with efficient protocols of *in vitro* regeneration trough somatic embryogenesis, tamarillo appears to be a plant that could have an important role in the study of tree development processes.

In the present work, we aimed to analyze the expression of the previously identified NEP25 in order to find some clues that could bring insights into the role of NEP25 during somatic embryogenesis induction in tamarillo. To accomplish that objective, several functional genomics tools were applied, from proteomics analysis to gene suppression assays. Thus, two strategies were followed: 1) somatic embryogenesis induction on knock-out lines of *Arabidopsis thaliana,* and 2) post-transcriptional gene

down-regulation of the *NEP25* gene in tamarillo. The combination of the two methodologies allowed us to discuss the role and possible use of NEP25as a marker of non-embryogenic potential.

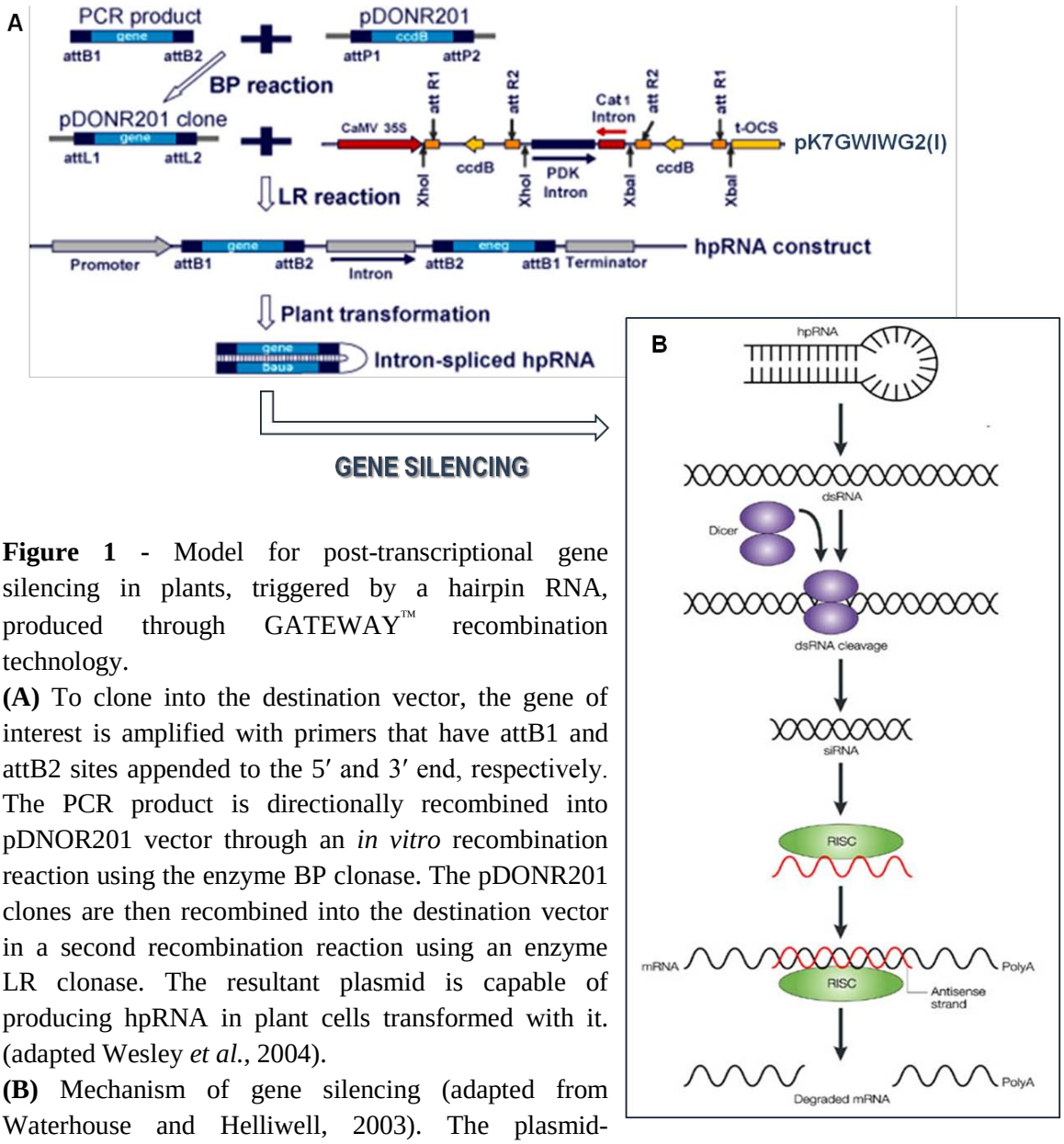

Figure 1 - Model for post-transcriptional gene silencing in plants, triggered by a hairpin RNA, produced through GATEWAY™ recombination technology.

(A) To clone into the destination vector, the gene of interest is amplified with primers that have attB1 and attB2 sites appended to the 5′ and 3′ end, respectively. The PCR product is directionally recombined into pDNOR201 vector through an *in vitro* recombination reaction using the enzyme BP clonase. The pDONR201 clones are then recombined into the destination vector in a second recombination reaction using an enzyme LR clonase. The resultant plasmid is capable of producing hpRNA in plant cells transformed with it. (adapted Wesley *et al.*, 2004).

(B) Mechanism of gene silencing (adapted from Waterhouse and Helliwell, 2003). The plasmid-expressed hairpin RNA (hpRNA) is exported from the nucleus. In cytoplasm, the dsRNA is cleaved by Dicer generating siRNA, in which the "guide strand" is identified and released. The "guide strand" is then integrated in the active RNA Interference Specificity Complex (RISC). The siRNA guides RISC to the target mRNA. RISC delivers the mRNA to cytoplasmic processing bodies (P-bodies), wherein mRNA decay factors are concentrated, and the target mRNA is cleaved and degraded.

4.3. MATERIAL AND METHODS

4.3.1. Plant materials and growth conditions

Induction of embryogenic cultures of tamarillo was conducted using the methodology described by Lopes *et al.* (2000) and elsewhere in this thesis (see Chapters 2 and 3). Leaves excised from *in vitro*-cloned shoots of tamarillo (red cultivar) and mature zygotic embryos were used as explants. The most apical expanding leaves were collected from 6 weeks developed shoots, randomly punctured and placed (abaxial side down) in test tubes containing induction medium. Zygotic embryos were isolated from seeds of an adult red tamarillo tree. Seeds were first disinfected in a 7% (w/v) calcium hypochlorite solution, with a few drops of Tween 20 for 20 minutes, rinsed in sterilized water and placed in test tubes containing the induction medium. For both types of explants, the induction medium was MS basal medium (Murashige and Skoog, 1962) plus 0.25 M sucrose, 0.25% (w/v) gelrite and pH 5.7, supplemented with 20µM Picloram, in the case of leaves, or 9µM 2,4-D, in the case of zygotic embryos. Cultures were incubated at 24±1ºC under dark conditions during 12 weeks. After this incubation period, embryogenic and non-embryogenic areas were isolated and subcultured at 4 weeks intervals in the same medium. Through all the induction period, samples for proteomic (Table 1) and RT-PCR analysis were collected and fast-frozen in liquid nitrogen before being kept at -80ºC.

Somatic embryo-derived plants were obtained by transferring masses of embryogenic tissue to MS basal medium plus 0.07 M sucrose, 0.6% (w/v) agar and pH 5.7, under a 16h daily illumination regime of 15-20 µmol $m^{-2}s^{-1}$ photosynthetically active radiation, provided by cool-white fluorescent lamps, at 24ºC±1ºC, for 8 weeks.

Arabidopsis thaliana (Col-0) and the SALK T-DNA insertion line (SALK_027418), also of the Columbia ecotype (Col-0), were obtained from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/cgi-bin/tdnaexpress;v Alonso *et al.*, 2003) and provided by the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk/). Seeds were surface-disinfected (1 bleach : 5 ethanol, 15 min.) and placed on solid half-strength MS medium, supplemented with 0.03M sucrose, 0.8% (w/v) phytagar (Gibco BRL) and kanamycin (50 mg/mL, only for SALK line), followed by further cultivation in soil, under short day conditions (8 h light/16 h dark), at 20°C.

4.3.2. Synthesis of a cDNA library and NEP25 cDNA cloning

In a previous work, Faro *et al.* (2003) identified, by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), a 26.5 kDa protein systematically expressed in non-embryogenic *calli* of tamarillo obtained in the conditions described in the previous section. This protein was named NEP25 (Non-Embryogenic Protein 25) and the corresponding cDNA was cloned from a non-embryogenic *calli* cDNA library. The cDNA library was synthesized using a ZAP-cDNA synthesis kit (Stratagene) and directionally cloned into the EcoRI and XhoI sites of Uni-ZAP®XR phage λgt11 (Stratagene), as described by the manufacturer. Total RNA was isolated from established non-embryogenic *calli*, obtained from leaf explants, and the mRNA poli A+ was purified in a S-Sepharose oligo dT column. Recombinant plasmids were excised from the cDNA library and transformed into *E. coli* DH10B (Invitrogen). The cDNA expression library was screened by hybridization with a polyclonal antibody raised against the 26.5 kDa protein. Positive clones were detected and the DNA purified. The sequences obtained for the different clones encoded the same protein and the amino acid sequence that was first described (Faro *et al.*, 2003).

The fragments of the expected size were cloned in a pCR®2.1-TOPO® vector (Invitrogen, Paisley, U.K.), and transformed into competent *E. coli* cells TOP10F'. The *E. coli* cells were grown in Luria broth medium with 100µg/ml of ampicilin to select positive clones. The isolated clones were used for plasmid extraction and DNA sequencing.

4.3.3. NEP25 sequence bioinformatics analysis

Database searches were performed using the BLAST network service (Altschul *et al.*, 1997) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) or at the UniProtKB (http://www.uniprot.org/) and also at the SOL Genomics Network (http://solgenomics.net/). Sequence alignments and comparisons were performed using ClustalX 2.0.11 (Larkin *et al.*, 2007) and the MultiAlin tool (available online, Corpet, 1988). Phylogenetic and molecular evolutionary analyses of the full-length amino acid sequences of NEP25 and similar proteins were conducted using *MEGA* version 5 (Tamura *et al.*, 2011).

Arabidopsis thaliana databases, such as TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org/) and *Arabidopsis* eFP Browser (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), with data from the gene expression map of *Arabidopsis*, were used to analyze *Arabidopsis* sequences similar to NEP25.

Protein predictions were performed using the ProtParam tool (http://www.expasy.ch/cgi-bin/protparam, Gasteiger *et al.*, 2005) and WoLF PSORT prediction (http://wolfpsort.org/; Horton *et al.*, 2007). Predicted post-translational NEP25 modifications were analyzed with NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/, Blom *et al.*, 1999) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/, Gupta *et al.*, 2004). NEP25 sequence exposed regions were detected by three-dimensional modulation of the protein conformation, using the software Swiss-Model, accessible via the ExPASy web server (http://swissmodel.expasy.org/, Arnold *et al.*, 2006; Larsen *et al.*, 2006).

4.3.4. Protein extraction, separation and immunoblot analysis

Leaf explants, zygotic embryos, induced explants and embryogenic (EC) and non-embryogenic callus (NEC) samples (Table 1) were ground to a fine powder in liquid nitrogen. While still frozen, the powder was suspended in 4ml of cold acetone (containing 0.2% (w/v) DTT, 10% (w/v) TCA). The suspension was then transferred to clean centrifuge tubes, incubated overnight at -20ºC and centrifuged for 30 min at 20,000 ×g at 4ºC. The supernatant was carefully decanted, and the remaining protein pellet was washed twice in cold acetone (containing 0.2% (w/v) DTT), incubated for 30 min at -20ºC, centrifuged 30 min at 20,000 ×g at 4ºC and vacuum-dried. The resulting pellet was re-suspended in 500µl per tube of an IEF solubilization buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid) and 1% (w/v) DTT]. The suspension was sonicated and incubated at room temperature for 2 h in a rotary shaker, and then centrifuged at 20,000 ×g for 1 h to remove the insoluble material. Total protein concentration (Table 1) was assessed using a 2-D Quant Kit (GE Healthcare; Amersham Biosciences) following manufacturer's guidelines, and using BSA as the standard. Samples were aliquoted and stored at -20ºC until being used.

Proteins were resolved by 10% SDS-PAGE. To each aliquot of 20µg of the solubilized proteins, 2% SDS and bromophenol blue were added to each aliquot of 20µg of the solubilized proteins before separation. Some of the gels were stained with Coomassie Blue R-250 (Sigma-Aldrich, Madrid, Spain) and protein patterns compared between samples. Prestained Precision Protein Standards (Bio-Rad, Amadora, Portugal) were used as molecular weight markers (250kDa-10kDa).

Proteins resolved by SDS-PAGE (gels not stained) were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore), in transferring buffer (CAPS, N-cyclohexyl-3-aminopropanesulfonic acid 10% methanol), overnight, at 4ºC, 40V. The membrane was incubated in TBS-T (Tris-HCl Buffered Saline-Ttween) buffer with 5% skim milk (blocking solution) for 45 minutes at room temperature, and then with anti-NEP25 polyclonal antibody diluted to 1:1500 a blocking solution, for 1 h at room temperature (Jacek *et al.*, 1992). This antibody was raised against the peptide antigen IPQYGCGTASLN, corresponding to residues 123-134 of an exposed region of NEP25 (Genosphere Biotechnologies, Paris, France). Membranes were then placed in the SNAP i.d. Protein Detection System (Millipore), washed 4 times with TBS-T, with vacuum running continuously, and incubated with an alkaline phosphatase-conjugated anti-rabbit IgG monoclonal antibody (Sigma-Aldrich, Tokyo, Japan; diluted to 1:5000 with a blocking solution) for 10 minutes at room temperature. Detection was performed by enhanced chemifluorescence technique (GE Healthcare, Buckinghamshire, UK).

4.3.5. Quantitative reverse transcription-PCR analysis of tamarillo NEP25

Abundance of NEP25 transcripts in the several tissues was determined using total RNA extracted from leaves, zygotic embryos, induced explants and embryogenic and non-embryogenic callus 100mg samples, isolated with RNeasy Plant mini kit (Qiagen, Hilden, Germany). The concentration and quality of the RNA was determined by using a ND-1000 Spectrophotometer (NanoDrop Technologies). For the qRT-PCR, first-strand cDNA synthesis was performed using 900ng of total RNA treated with DNase (RNase-Free DNase Set, Qiagen, Hilden, Germany) and the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) form Roche (Mannheim, Germany). The reaction mix contained 1x reaction buffer, 5mM $MgCl_2$, 1mM deoxynucleotide mix, 1µM oligo-p$(dT)_{15}$ primer, 2.5U/µl of RNase inhibitor and 1U/µl of AMV Reverse Transcriptase, in a final volume of 20µl.

The resulting single–stranded cDNAs were amplified by polymerase chain reaction utilizing sequence-specific primers, designed using Primer Premier 5.0 (Premier Biosoft). Primer pair 5'-ACATAGCAAAGAGACACAACGTCGGAA-3' and 5'-TTGAGGAAGGTTTTAGCATCGGCAA-3' was used to amplify a 175bp fragment of the cDNA sequence. The *ELONGATION FACTOR 1-α* (*EF1α*) gene (Nicot *et al.*, 2005) was used as an internal control for the qRT-PCR, with a 380bp fragment amplified by the primer pair: 5'-ACCCGTGAACATGCATTGCTTGCT-3' and 5'-ACACCAGTCTCAACACGACCAACA-3'. Amplification reactions were carried out with 1µl of the synthesized cDNA, in a final reaction volume of 20 µl containing 1X GoTaq® buffer (Promega), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 1U/µl of GoTaq® DNA polymerase (Promega) and 0.2 µM of each primer. The annealing temperatures were 54ºC, for NEP25 primers, and 60ºC for EF1α primers, and 35 cycles of amplification were necessary to observe the expected bands. PCR reaction products were separated by electrophoresis in an agarose gel (1.5% w/v) in 1X TBE buffer, stained with ethidium bromide, and visualized and documented under UV light. Six hundred nanograms of a DNA size ladder (HyperLadderTM II, Bioline) was loaded in the gel along with the PCR products. All reactions were carried out in triplicate and the results were processed statistically, after gel analysis with ImageJ 1.43 software (http://rsb.info.nih.gov/ij).

4.3.6. Somatic embryogenesis induction in *Arabidopsis thaliana* NEP25 knock-out lines

Screening for homozygous T-DNA insertion lines: genomic DNA was isolated from rosette leaves of 4-week-old plants and used for PCR-based screening to identify homozygous T-DNA insertion mutants. PCR primers used for genotyping were two gene-specific primers: SALK_027418LP (5'-TGACTTGACAAAAGCCCTCAC-3') and SALK_027418RP (5'-GGCAGAGACCAACAATCATTG-3') and a T-DNA left border primer Lba1 (5'-TGGTTCACGTAGTGGGCCATCG-3') as described by Stepanova and Alonso (2003).

Primary somatic embryo induction: Arabidopsis thaliana (Col-0) wild-type and homozygous T-DNA insertion mutants were grown for 8 weeks (section 4.3.1.) before the somatic embryogenesis induction procedure. Somatic embryo induction was

performed using a modified method based on that described by Ikeda-Iwai *et al.* (2002). Immature green siliques were collected and surface-disinfected in a 5% (w/v) calcium hypochlorite solution, with Tween 20, for 20 minutes. Disinfected siliques were dissected, under sterile conditions, with very fine needles, using a dissecting microscope. The immature seeds containing torpedo zygotic embryos were placed on somatic embryogenesis induction medium consisting of B5 basal medium (Gamborg, 1968) plus 0.06 M sucrose and 4.5 µM 2,4-D). The medium was solidified by the addition of 2,5 g/l phytagel, in 60 mm Petri dishes (10 seeds/dish, 15 dishes per line in 4 replicated treatments). Cultures were incubated at 24±1ºC in photoperiod (16h light / 8h dark) conditions for 4 weeks. After the induction period, the results were observed and registered. Observations focused on those aspects referred in literature, such as callus formation, precociously germinated embryos, non-responsive explants and primary somatic embryo formation. Statistical analysis (SPSS Statistics 17.0) was performed by analysis of variance (ANOVA) and the significantly different means were identified by using the Tukey test ($p = 0.05$).

mRNA expression analyses: Total RNA was isolated from rosette leaves, primary somatic embryos and callus explants (after induction) using the RNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) form Roche (Mannheim, Germany) and 0.9 µg of total RNA as starting material (section 4.3.5). PCR primers, used to amplify a 610 bp fragment of *AT4G15520* gene transcripts, were: 5'-AAAGCGTCACAACGTCGGAACTTTA-3' and 5'-ACCGTCCAAAAGGTCGGATGATGA-3'. *Arabidopsis ACTIN 1* gene was used as an internal control for the qRT-PCR, with a 391 bp fragment amplified by the primer pair: 5'- GGCGATGAAGCTCAATCCAAACG-3' and 5'-GGTCACGACCAGCAAGATCAAGACG-3'. PCR reaction products separation was carried as described in section 4.3.5. The separation of PCR reaction products was carried as described in section 4.3.5. All reactions were performed in triplicate and the results were processed statistically, after gel analysis with ImageJ 1.43 (http://rsb.info.nih.gov/ij).

4.3.7. Down-regulation of NEP25 gene in transgenic tamarillo plants

Cloning of NEP25 sequences: GATEWAY™ cloning technology (Invitrogen) was used to directionally insert a 264 bp specific fragment of NEP25 into a destination vector. The specific *NEP25* gene fragment was made GATEWAY-compatible with template-specific primers (with attB1 and attB2 sequences) and amplified by PCR from the plasmid pCR ®2.1-TOPO® (Invitrogen, Paisley, U.K.). Primer Premier 5.0 (Premier Biosoft) was used to design the specific primer pair: 5'-TGCCGATGCTAAAACCTTCC-3' and 5'-CCAGCCCAAACTCCAAAT-3'. After the recombination of the PCR-product with pDONR221 (BP recombination reaction), a pENTRY vector was obtained which was then recombined with the destination vector (LR recombination reaction). The selected destination vector was the binary plasmid pK7GWIG2(I) obtained from UGent – VIB Research (Belgium). The T-DNA region of this vector contains a negative selectable marker (ccdB gene) to select against non-recombinant clones, promoter and terminator of *35S*, the GATEWAY cassette (two inverted AttR regions separated by an intron) and the *neomycin phosphotransferase II* gene (*nptII*) as the plant selectable marker. PCR and *in vitro* BP and LR Clonase™ (Invitrogen) recombination reactions were carried out according to the manufacturer instructions. The recombination reaction product was confirmed by PCR and restriction analysis with HindIII (New England BioLabs®). In transformed plants, the T-DNA construct will produce double-stranded RNA (hairpin RNA) from the NEP25 sequence, triggering post-transcriptional gene silencing.

Bacterial strain: The obtained recombinant plasmid containing the NEP25 sequence was incorporated, by electroproration, into LBA4404 *Agrobacterium tumefaciens* competent cells. *Agrobacterium* was grown on Luria broth (LB) medium with appropriate antibiotics (rifampicin 50 µg/ml and spectinomycin 50 µg/ml). For cocultivation, isolated colonies of bacteria were picked from selection plates and grown overnight in 10 ml of LB liquid medium, at 28ºC, until an optical density of 0.6-0.8 at 600 nm was reached. Prior to plant inoculation, 20 µM of acetosyringone was added to the bacterial suspension.

Plant tissue culture and transformation: One-month-induced leaf explants of tamarillo (section 4.3.1.) plantlets were used as explants for transformation. Transformation medium was composed of liquid somatic embryogenesis induction medium and the bacterial suspension supplemented with acetosyringone, in a proportion of 1:50. The leaves (at the beginning of the dedifferentiation process) were removed from the induction medium and wounded with a scalpel before immersion in the transformation medium for 5 minutes. After this pretreatment, explants were rinsed thoroughly in sterilized water, blotted dry on sterile filter paper and placed on induction medium regeneration plates with 20 µM acetosyringone. After a 2-day cocultivation period in dark conditions, explants were rinsed in a MS salt solution with carbenecillin (250 µg/ml) and cefotaxime (250 µg/ml), sterilized water and blotted dry on sterile filter paper. Explants were then transferred to induction medium plates with carbenecilin (250 mg/l), cefotaxime (250 µg/ml) and kanamycin (100 mg/l), and maintained in the dark, at 24±1ºC, being transferred to fresh induction medium selective plates at 14 days intervals. After 2-3 months, embryogenic cells, that proliferated under kanamycin (50 µg/ml) selection, were transferred to embryo development and conversion selective medium - MS basal medium containing 50 µg/ml kanamycin, solidified with 0.8% (w/v) phytagar (Gibco BRL). Plants obtained from kanamycin-resistant somatic embryos were established in the growth chamber as described in section 4.3.1. All the experiments were followed by controls that were treated as described above except for the presence of antibiotics in the culture media.

DNA extraction and PCR analysis: For PCR analysis, total genomic DNA was isolated from young leaves of kanamycin-resistant somatic embryo-derived plants and non-transformed controls. Leaf tissues (100 mg) were grounded into a fine powder in liquid nitrogen using a mortar and pestle. Genomic DNA was extracted using the DNeasy® Plant Mini kit (Qiagen). The concentration and quality of the genomic DNA was determined by using a ND-1000 Spectrophotometer (NanoDrop Technologies). The DNA amplification was carried out on a Thermal Cycler (BioRad). The primers used for amplification of a 700bp fragment of the *nptII* gene were 5'-GAGGCTATTCGGCTATGACTG-3' and 5'-ATCGGGAGCGGCGATACCGTA-3', and those used for amplification of a 1537bp fragment between the T-DNA intron and promoter P35S were 5'-TGCCACCTTATTCAACCAT-3' and 5'-TCTCATCAAGACGATCTACC-3'. For each PCR, the amount of DNA was 15-20 ng

of the appropriate plasmid as positive control and 75-100 ng of plant DNA, in a final reaction volume of 25 µl containing 5 µl of 5X GoTaq® buffer (Promega), 1.5 mM of $MgCl_2$, 0.3 mM of each dNTP, 1U of GoTaq® DNA polymerase (Promega) and 0.4 µM of each of the specific primers. DNA amplification used the following profile: one cycle of 1.5 min at 94°C, 35 cycles of 30 s at 94°C, 45s at 65ºC°C (*nptII* primers) or 45s at 60ºC (*intron* and *p35s* primers) and 2 min at 72°C, followed by one cycle of 5 min at 72°C. The PCR reaction products were separated by electrophoresis in an agarose gel (1.5% w/v) in 1X TBE buffer, stained with ethidium bromide, and visualized and documented under UV light. Six hundred nanograms of a DNA size ladder (HyperLadderTM II, Bioline) was loaded in the gel along with the PCR products.

Evaluation of NEP25 down-regulation in transgenic lines: For determining the level of down-regulation of NEP25, RT-PCR and immunoblot assays were performed with 3 transformed lines and 1 control line of selected tamarillo plants. Tissue expression was examined for NEP25 transcripts using total RNA extracted from leaves and induced explants, as described in section 4.3.5. Protein extraction, separation and immunoblot analysis were conducted according to the procedure described in section 4.3.4.

Leaf explants from the transformed plants underwent somatic embryogenesis induction (see section 4.3.1.) to comfirm if their embryogenic potential was modified, when compared to the control line. Four replicated treatments, with an average of 25 explants per line were performed.

4.4. RESULTS

4.4.1. Somatic embryogenesis induction in leaf explants and zygotic embryos

Both types of explants used, young leaves from cloned seedlings and cotyledonary zygotic embryos, underwent a dedifferentiation process, after which two types of callus (EC and NEC) were formed. Even though two different auxins induce SE in the explants tested, a similar behavior of the cultures was observed, as shown in Figs. 6A and 7A. Callus from wounded leaves (Fig. 6A) or from zygotic embryos (Fig. 7A) started to appear by the 6th week of culture and, 2-3 weeks later, areas of whitish, compact embryogenic cell masses could be distinguished among the more friable and

fast growing non-embryogenic callus. These small embryogenic areas could be easily separated from the surrounding non-embryogenic callus and subcultured in the same culture medium, without losing their embryogenic potential..

4.4.2. NEP25 sequence analysis

Bioinformatics analysis of NEP25 cDNA (666 bp) and amino acid (221 aa) sequences (Fig. 2) allowed to collect a set of data for the interpretation of the obtained results.

The BLASTP (Altschul *et al.,* 1997) search through databases revealed 192 hits in the Viridiplantae group. In the present analysis, the 12 sequences with the highest scores (>294) and lowest E-values (> 7e-79) were selected for phylogenetic analysis (Fig. 2). These 12 sequences include representative eudicot species (*Arabidopsis lyrata, Arabidopsis thaliana, Glycine max, Populus trichocarpa, Ricinus communis, Vitis vinifera*), monocot species (*Oryza sativa, Sorghum bicolor, Zea mays*) and a conifer (*Picea sitchensis*). A BLASTN (Altschul *et al.*, 1997) search through All SGN Unigene sequences database allowed including in our analysis three predicted proteins from three Solanaceae species (*Nicothiana benthamiana, Solanum lycopersicum, Solanum tuberosum*).

Multiple sequence alignment, performed with ClustalX, showed a high percentage of conserved domains between residues 10 and 150 (Fig. 3A), which conforms with the BLAST identification of a putative conserved domain of the SpoU rRNA Methylase family (E-value 2.38e-31), a family of proteins with RNA methyltransferase activity and RNA binding and processing functions, that probably use S-AdoMet (S-adenosyl-L-methionine).

In *Arabidopsis thaliana*, this protein family includes at least five different proteins (Fig. 4), expressed by different genes, that share the conserved domain SpoU_MeTrfase (IPR001537), found in RNA methyltransferases TrmH, and also in NEP25. Multialignment of the RNA methyltransferases sequences clearly showed the presence of those conserved domains (Fig. 4A). Databases search allowed verifying that the molecular weights of these proteins range from ~24 to ~200 kDa and that they are differentially expressed in *Arabidopsis* tissues (Fig. 4B). Interestingly, their higher levels of expression are associated with embryo development and functioning of the shoot apical meristem development.

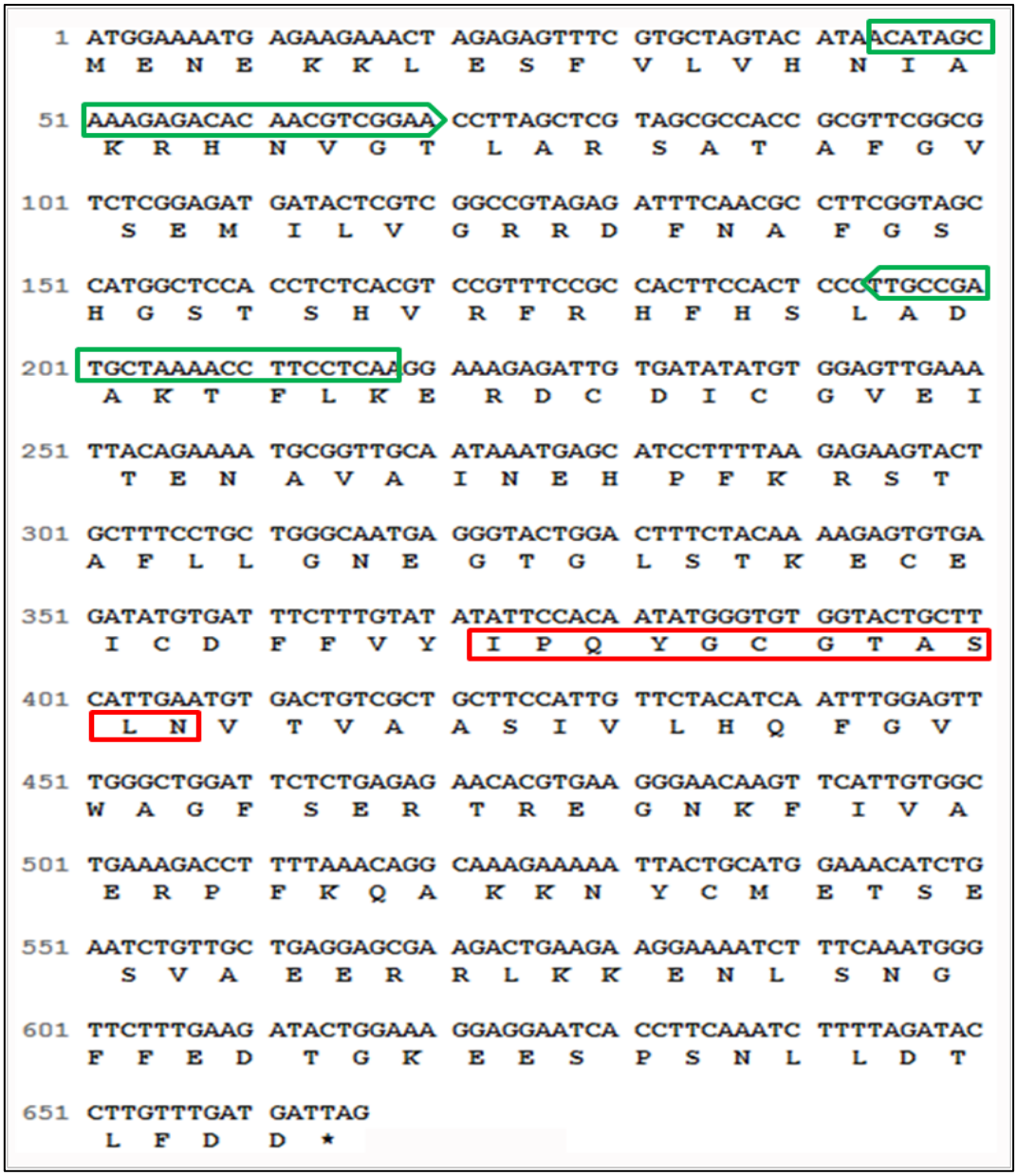

Figure 2 - NEP25 cDNA sequence and the corresponding deduced amino acid sequence (Faro *et al.*, 2003). The green boxes correspond to primer sequences. Red box highlights the peptide sequence utilized to produce the polyclonal antibody against NEP25.

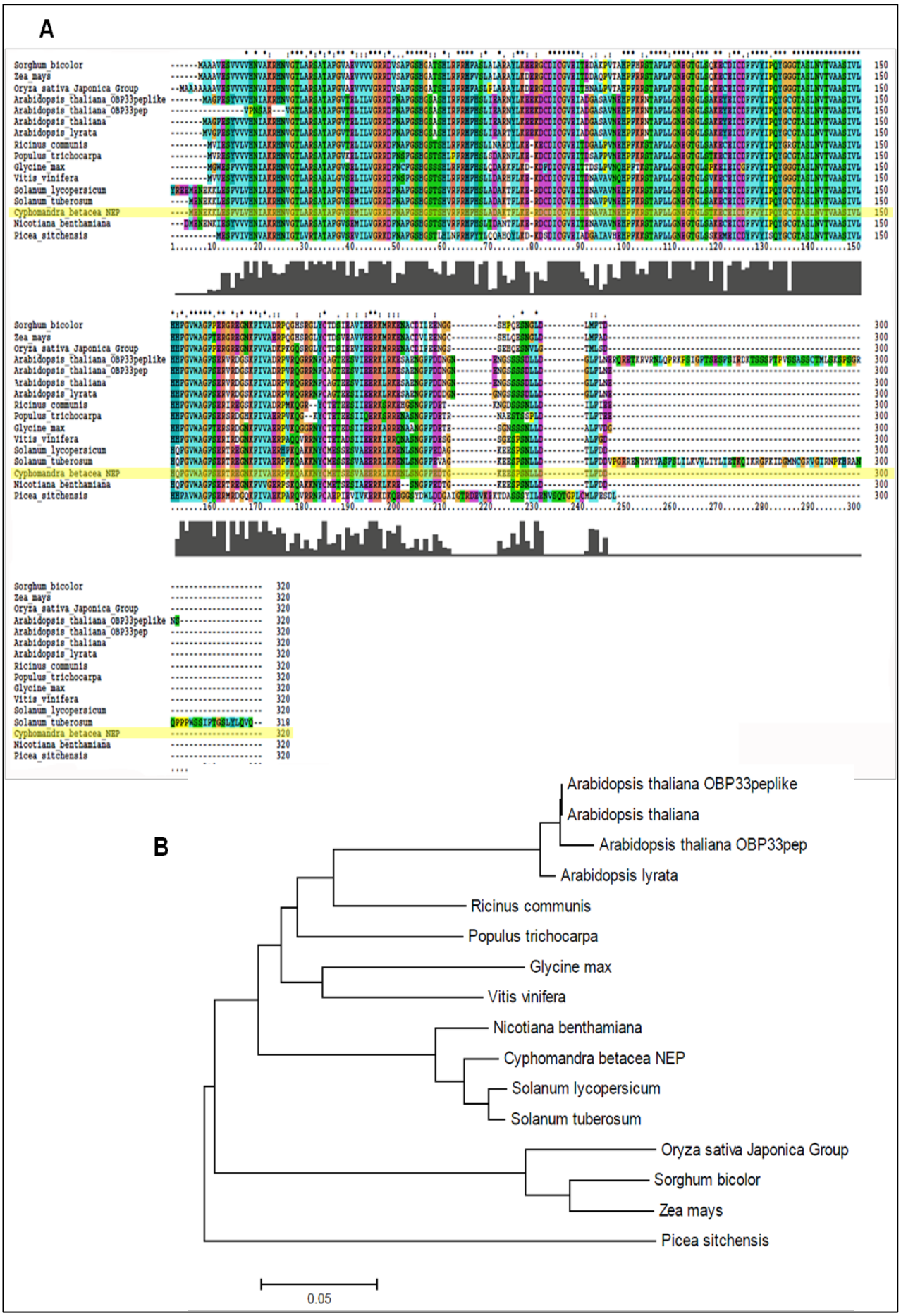
A
Sorghum_bicolor
Zea_mays
Oryza_sativa_Japonica_Group
Arabidopsis_thaliana_OBP33peplike
Arabidopsis_thaliana_OBP33pep
Arabidopsis_thaliana
Arabidopsis_lyrata
Ricinus_communis
Populus_trichocarpa
Glycine_max
Vitis_vinifera
Solanum_lycopersicum
Solanum_tuberosum
Cyphomandra_betacea_NEP
Nicotiana_benthamiana
Picea_sitchensis
B
Arabidopsis thaliana OBP33peplike
Arabidopsis thaliana
Arabidopsis thaliana OBP33pep
Arabidopsis lyrata
Ricinus communis
Populus trichocarpa
Glycine max
Vitis vinifera
Nicotiana benthamiana
Cyphomandra betacea NEP
Solanum lycopersicum
Solanum tuberosum
Oryza sativa Japonica Group
Sorghum bicolor
Zea mays
Picea sitchensis
0.05

Figure 3 - Tamarillo NEP25 sequence alignment and similarity relationships of the most closely related proteins (obtained from Blast results). **(A)** ClustalX alignment of NEP25 deduced amino acid sequence with identical proteins (>75% identity) of other species, showing the degree of similarity. Gaps (-) were introduced to maximize alignment. Identical (*), conserved (:) and semi-conserved (.) residues in all aligned sequences are indicated. Sequences from other species were extracted from GenBank - *Arabidopsis lyrata* (XP_002870233.1), *Arabidopsis thaliana* OBP33pep (AAA79704.1), *Arabidopsis thaliana* (NP_193287.1), *Arabidopsis thaliana* OBP33 pep like protein (CAB45991.1), *Glycine max* (ACU13639.1), *Oryza sativa* Japonica group (NP_001054604.1), *Picea sitchensis* (ABK25846.1), *Populus trichocarpa* (XP_002312722.1), *Ricinus communis* (XP_002516828.1), *Sorghum bicolor* (XP_002440562.1), *Vitis vinifera* (XP_002278797.1) and *Zea mays* (NP_001151868.1), and from SGN (SOL Genomics Network) Unigene sequences – *Nicotiana benthamiana* (SGN-P570505), *Solanum lycopersicum* (SGN-P704302), *Solanum tuberosum* (SGN-P73740). **(B)** Neighbour-joining tree of NEP25 and similar proteins corresponding to the sequences represented in the alignment.

The phylogenetic analyses of NEP25 and the selected sequences (Fig. 3B) showed an expected division into three major evolutionary lineages, with the major clade containing the eudicot proteins. The most distant sequence, an unknown *Picea sitchensis* protein, corresponds to the only conifer sequence identified in BLAST search. Also monocot species formed a subcluster distinct from those of eudicot species. *Cyphomandra betacea* NEP25 sequence was clustered with the other solanaceous species, being more related to the other Solanaceae species (potato and tomato).

NEP25 predicted amino acid sequence was analyzed with several online available bioinformatics tools that contributed to a better perspective of this putative protein's characteristics, modifications and location. ProtParam tool allowed confirming that NEP25 encodes 221 amino acids, with a theoretical mass of 24.742 kDa and a theoretical isoelectric point of 6.09. It also classified the protein as unstable, with an instability index (II) of 41.05 and an estimated half-life period of 30 hours. With WoLF PSort software we could predict the NEP25 cellular location as most likely to be cytoplasmatic (0.450 certainty), although a not clear identification of a SKL motif (signal for peroxisomal protein) was also taken into account, due to a 0.397 certainty. Trying to identify potential sites of post-translational modifications in NEP25, NetPhos 2.0 and NetNGlyc 1.0 software were used and the analysis predicted 7 possible phosphorilation sites (6 serines and 1 threonine) and 2 potencial motifs for N-glycosylation, respectively.

A

```
           1       10        20        30        40        50        60        70        80        90       100       110       120       130
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AT4G15520.1 QERY---LESFVLVHNIAKRHNVGTLARSATAFGVSEMILVGRRDFNAFGSHGSTSHVRFRHFHSLAESVVHNIAKRHNVGTLARSATAFGVEILVGRRDFNAFGSHGSSHRFRHFHSLSBCTFESY---
AT5G15390.1       QERYVLVHNIAKRHNVGTLARSATAFGVSEMILVGRRDFNAFGSHGSTSHVRFRHFHSLV-------------------NRSAAGVR-----------------------NSGSBCT-------
AT2G19870.1 QERYEKKLESFVLVHNIAKRHNVGTLARSATAFGVSEMILVGRRDFNAFGSHGSTSHVRFRHEKVN-------------------GRSAFGR-----------------------SSSBCTEEKYSLW
AT4G17610.1
AT4G38020.1
  Consensus .......e..vlvhniakrhnvgtlarsatafgvsemilvgrrdfnafgshgstshvrfrh.....................a.g.....................s.sbct.......

           131   140       150       160       170       180       190       200       210       220       230       240       250       260
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AT4G15520.1 VYVHNIAKRHNVGTLARSATAFGVTELILVGRRDF-----NAFGSHGSASHIRFRHFHSLIQERYDAKTFLKERDCDICGVEITENAVAINEHPFKRSTAFLLGNEGTGLSTKECEICDFFVYIALKEDC
AT5G15390.1 LVVEGLSDFGNISAAFRSADALGIQSVHVVSCDSSKRYNGNRHVSMGAEKWLDIEFWDTPQERYADAKTFLKERDCDICGVEITENAVAINEHPFKRSTAFLLGNEGTGLSTKECEICDFFVYILKRI--
AT2G19870.1 VALDEVTDPQNLGAIIRSAYFFGATGVYVCAKNSAPL---SAVVSKASAGSLEVMEQERYFHSLADAKTFLKERDCDICGVEITENAVAINEHPFKRSTAFLLGNEGTGLSTKECEI--FLEGAVANE--
AT4G17610.1                                                                                                  QERYKTFLKERDCDICGVEITENAVAINEHPFKRSTAFLLGNEGTGLSTKECEICDFFVYI--K---
AT4G38020.1
  Consensus .........n.....rsa...g.....................s.....................daktflkerdcdicgveitenavainehpfkrstafllgnegtglstkeceicdffvyi......

           261   270       280       290       300       310       320       330       340       350       360       370       380       390
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AT4G15520.1 DICGVEIAANEHPFKRT--AFLLGNEGGLSKEEICDFFVYISBCTEARNYLKEEKDCDICGVEIADGASAVNEHPFKRNTAFLLGNEGSGLSAKEYEICDFFVYIQERYPQYGCGTASLNYTVAASIVLH
AT5G15390.1 -------------------VITAGNEGG--SEDISBCTKECFKVLKS-------RGYRIATTHLGMDTVSIYDMDWSCPTAIVYGNEGRGISDEALELSDLRCSIQERYPQYGCGTASLNYTVAASIVLH
AT2G19870.1 ---------------------TLGNEGTGLSBCTLRYCKNMMQFLEA----SAENGWRVVGGSVSPKAVALNEVLPGSPTILVLGNEGTGLRPLVERSQERYCDFFVYIPQYGCGTASLNYTVAASIVLH
AT4G17610.1 -------------------FLIGETNVFTLGEGIDISBCTKLFLEK----KKREGFSILGLEQTANSVSLDKYQFPKKTVLVLGREKEGIPVDIIHILDACIEIQERYPQYGCGTASLNYTVAASIVLH
AT4G38020.1                                                                                  QERYENAVAINEHPFKRSTAFLLGNEGTGLSTKECEICDFFVYI----PQYGCGTASLNYTVAASIVLE
  Consensus ..................  ....gneg.................le.........g..i.g.e....ava.ne..f...Ta.vlGnEg.Gls....ei.*....iqeryPQYGCGTASLNYTVAASIVLh

           391   400       410       420       430       440       450       460       470       480       490       500       510       520
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AT4G15520.1 QFGVWAGFSERTREGNKFIVAERPFKQAKKNYCMETSESPQYGC-GTASLNVTVAASIVLHFGVWAGFSERRGKFIVARPQNCTESSBCTPQYGCGTASLNVTVAASIVLHHFGVWAGFSERVRDGSKFI
AT5G15390.1 QFGVWAGFSERTREGNKFIVAERPFKQAKKNPGSNVVARI-HGS-EERESBCTPMNGMVDSFNVSVAAGILMHHAVSDRTTRLGSHGDLSE------AEKEILMAEFSLRHSRSSQERYYCMCSBCTICI
AT2G19870.1 QFCV---ISLNVVAALHSBCTCTDLVRISGNMPNEV--AV-TES-DDAEGEGFRSFLAVESLNVSVAAGLFLHHL
AT4G17610.1 QFPQ---GSLNVVILSBCTPQLGVVRSLNVHVSGAIALWEY
AT4G38020.1 AEPLGEGGLSCVIPGSLNVYAILSBCTEFAQSLAEKPLCLILGSEGNGLSEQARKVCVLVSIPMAGDFESLNVSVAGGIFLQERYHSBCTY
  Consensus qf.v..g.s..v..g....va.........n...e.......gs...a..........v.s..v.....................................................................

           521   530       540       550       560       570       580       590       600       610       620       630       639
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AT4G15520.1 VADRPVRQGRRNFCAGTEESQERYVAEERRLKKENLSNGFFEDTGKEESPSNLLDTLFVEERLKENGFFDGESSLLDLFSBCTVIEERKLRKESAENGFFDDNGNENGSSSSDLLDGLF
AT5G15390.1
AT2G19870.1
AT4G17610.1
AT4G38020.1
  Consensus .....................................................................................................
```

B

AT Gene	GenBank	Aminoacids	MW (kDa)	p.I.	Tissue specific expression (eFP Browser data)
AT4G17610	NM_117869	1850	207.926	6.6	Shoot apex (inflorescence and transition levels), heart embryo (cotyledons)
AT2G19870	NM_179661	589	65.559	8.6	No data
AT5G15390	NM_121543	350	38.853	7.1	Heart and torpedo embryos (cotyledons), SAM (rib meristem level)
AT4G38020	NM_119963	352	38.460	8.3	Torpedo embryo (cotyledons), SAM (central zone)
AT4G15520	NM_117642	222	24.327	6,2	Shoot apex (inflorescence and transition levels)

Figure 4 - *Arabidopsis thaliana* members of the SpoU rRNA Methylase family. **(A)** Amino acid sequence alignment (MutiAlin tool) of five RNA methyltransferases of *Arabidopsis* (obtained from TAIR). Blue and red sequences represents conserved domains. Boxed sequences indicate the presence of a conserved peptide IPQYGCTASLN, also present in tamarillo NEP25. **(B)** Chart summarizing the data available for the members of the *Arabidopsis thaliana* RNA Methyltransferase family. Tissue specific expression data indicated in the chart match to the tissues highlighted in eFP Browser showing higher expression levels for each of the considered proteins.

3D modulation of NEP25 conformation (Fig. 5) was a useful tool to better visualize the exposed regions of the protein and to select the most appropriate sequences for polyclonal antibody production against the protein.

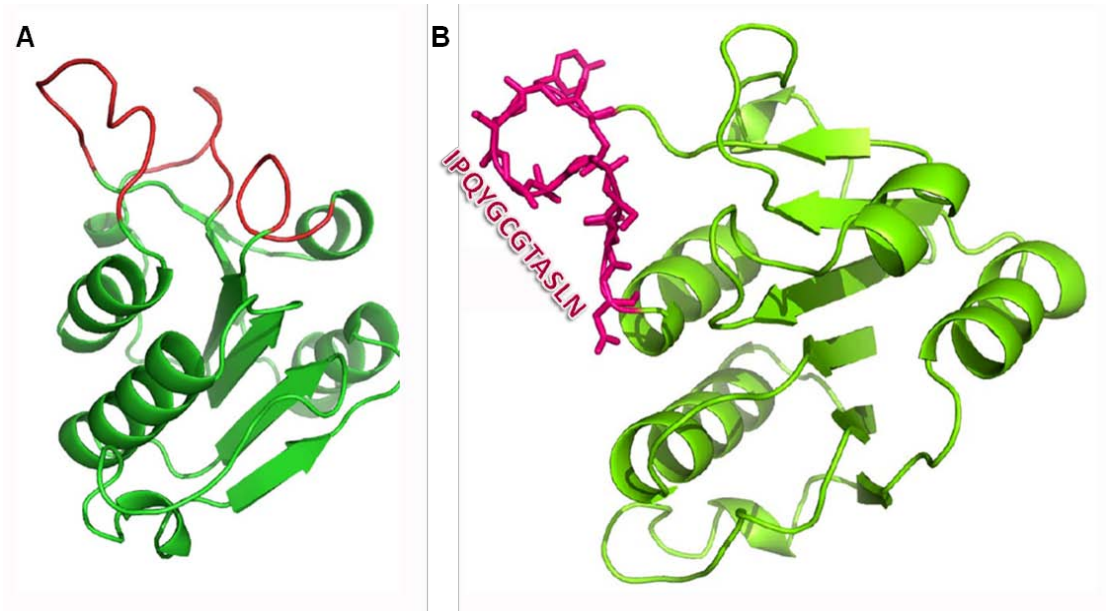

Figure 5 - Three-dimensional modulation of NEP25 conformation using the software Swiss-Model. **(A)** 3D predicted structure with exposed domains (red loops). **(B)** 3D structure showing the peptide sequence against which a polyclonal antibody was made.

4.4.3. Tamarillo somatic embryogenesis induction protein profiles

The determination of the protein levels present in the different samples was crucial for further analyses. The applied extraction protocol utilizes TCA/acetone, and is one of the most widely used because protein degradation and interaction with non-protein compounds are minimized. Through this method, high yields of protein per g of fresh mass weight could be obtained (Table 1) from the samples tested (Figs. 6A and 7A). Non cultured zygotic embryos and induced zygotic embryos showed the highest amount of total protein extracted, with values of 16.7±2.9 and 27.4±7.1 mg/g FW, respectively, which means they released five and seven times more less-soluble proteins, when compared to leaf explants. Also, for EC and NEC, samples obtained from zygotic embryos had higher protein levels than the ones obtained from leaves. Despite the type of explants used for SE induction, EC samples always yielded higher protein levels than NEC samples. EC from zygotic embryos explants had the highest levels of total protein, 7.1±0.4 mg/g FW.

Table 1 – Yields of protein extracted in the different explants and types of *calli* used in the experiments. Values for protein are given in mg per g of fresh weight (FW) ± standard error (SE).

	Fresh mass weight (mg ± SE)		Total protein concentration (µg/µl ± SE)		Protein quantification (mg/g FW ± SE)	
	Leaves	Zygotic embryos	Leaves	Zygotic embryos	Leaves	Zygotic embryos
Initial explants	326.3±50.4	101.7±8.3	1.2±0.1	1.7±0.3	3.6±0.3	16.7±2.9
Induced explants	430.5±34.5	112.3±19.5	1.7±0.2	3.1±0.8	3.9±0.5	27.4±7.1
Embryogenic callus	511.7±1.5	508.7±3.4	2.7±0.3	3.6±0.2	5.2±0.6	7.1±0.4
Non-embryogenic callus	1066.0±35.2	1008.0±31.3	2.9±0.4	3.4±0.1	2.7±0.4	3.4±0.1

Our first approach in order to investigate NEP25 expression was to analyze 10% SDS-PAGEs of the protein profiles from non-induced and induced leaves (Fig. 6B) and zygotic embryos (Fig. 7B), as well as from the correspondent ECs and NECs, obtained after the induction period. With this preliminary assay we intended to study in detail the expression patterns put in evidence in the profiles and also to verify the presence of the band of 26.5kDa, previously described by Faro *et al.* (2003) as NEP25.

The protein profiles of ECs and NECs, obtained either from leaves of from zygotic embryos, showed similar patterns of protein expression, with a high number of protein bands, most of them faint and with mobilities between 10 and 150 kDa. This means that the expression patterns during SE induction and callus formation were not affected either by the auxin (2,4 or Picloram) or by the type of explant (leaves or zygotic embryos). Nevertheless, some qualitative differences between the two types of *calli* were detected, namely in terms of the intensities of some bands and in the restricted presence of others in a particular type of callus. One of those differences was the presence of a protein band around 25 kDa in NEC but not in EC samples. The exception was the ZEEC sample which showed a faint band with an identical molecular weight.

Interestingly, in our assay, this band does not have an apparent molecular weight of 26.5 kDa, but it is slightly below 25 kDa, which conforms with the molecular weight of the predicted amino acid sequence reported by Faro *et al.* (2003). NEC protein profiles are also characterized by the high expression of a ~20 kDa protein band which showed to be much less intense in EC samples. Differences between ZEEC and ZENEC profiles where mostly related to a 35 kDa protein. Differences in the same protein could only be hardly detected between LEC and LNEC samples. As for EC samples, proteins exclusively expressed in ZEEC were not found, but two bands around 30 kDa were sharply evidenced in EC than in NEC.

Time-course protein patterns analysis revealed that the initial explants have very peculiar profiles, easily distinguishable from the induction or from the callus stages (Figs. 6B and 7B). Leaf profiles were characterized by the presence of two intense bands, ~26 and ~52 kDa, and four less intense bands with mobilities between those limits. Zygotic embryos also displayed a distinct profile, with three very pronounced bands below 50 kDa. This analysis also revealed that the ~25 kDa protein band is not exclusive of EC samples, as it appears through all the induction stages, including the initial stage. This is true for both types of explants, but more evident in those derived from leaves. A more detailed scrutiny of the protein profiles indicated the presence of bands that specifically appeared in the later stages of induction (T6 – T12), and that were not detected in the initial explant neither on the callus stages. These bands were a ~40 kDa in leaves and a ~30-32 kDa in zygotic embryos .

4.4.4. NEP25 expression during somatic embryogenesis induction in tamarillo

To unequivocally determine NEP25 expression during the SE induction process, a polyclonal antibody against it was produced. The selected peptide is a predicted exposed region of the protein including a set of amino acid residues, with high antigenicity, that enhance antibody-antigen reaction. LNEC samples were used to verify serum specificity by comparison with pre-immune serum reaction, and antibody titre (data not shown).

Immunodetection of NEP25 confirms previous observations made in SDS-PAGE profiles, namely the non exclusive presence of NEP25 in NEC samples. Immunoblots from leaf-derived (Fig. 6C) and from zygotic embryo-derived (Fig. 7C) explants, indicated that the expression of a protein of ~25 kDa is only absent on EC.

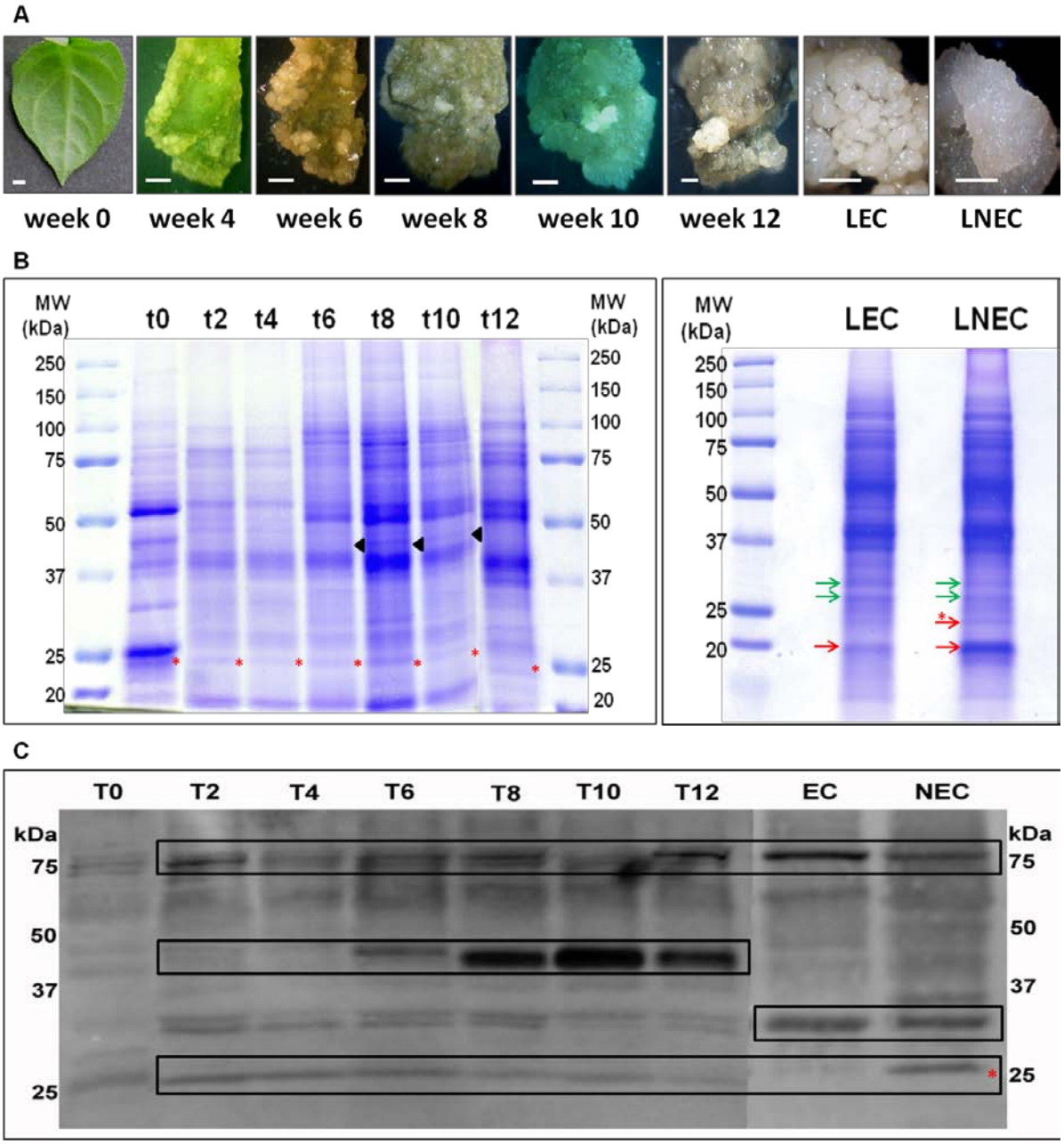

Figure 6 - SDS-PAGE protein profiles and immunoblot detection of tamarillo NEP25 on leaf-derived SE induced explants. **(A)** Leaves (week 0) and induced leaves (week 4 to 12) were removed from the induction medium at 2-week intervals for proteomic analysis. LEC and LNEC were obtained after the 12 week induction period, and subcultured separately for 1 month before analysis. Bars 1mm. **(B)** Protein SDS-PAGE profiles of the time-course and of LEC and LNEC samples. Comassie blue was used as stain. Red arrows (→) represent protein bands exclusively or more intensively expressed on LNEC, and green arrows (→) represent the protein bands more expressed in LEC than in LNEC. (*) Indicates the presence of the ~25kDa protein band, and (◄) points to a protein band that only appears in the later stages of the induction period. **(C)** Western blotting analysis of proteins extracted from the various samples. Twenty micrograms of extracted protein were loaded into each well for SDS-PAGE, blotted, and reacted with the antibody raised against NEP. Boxes put into evidence proteins (~25, ~32, ~40 and ~75kDa) differently expressed throughout the induction period.

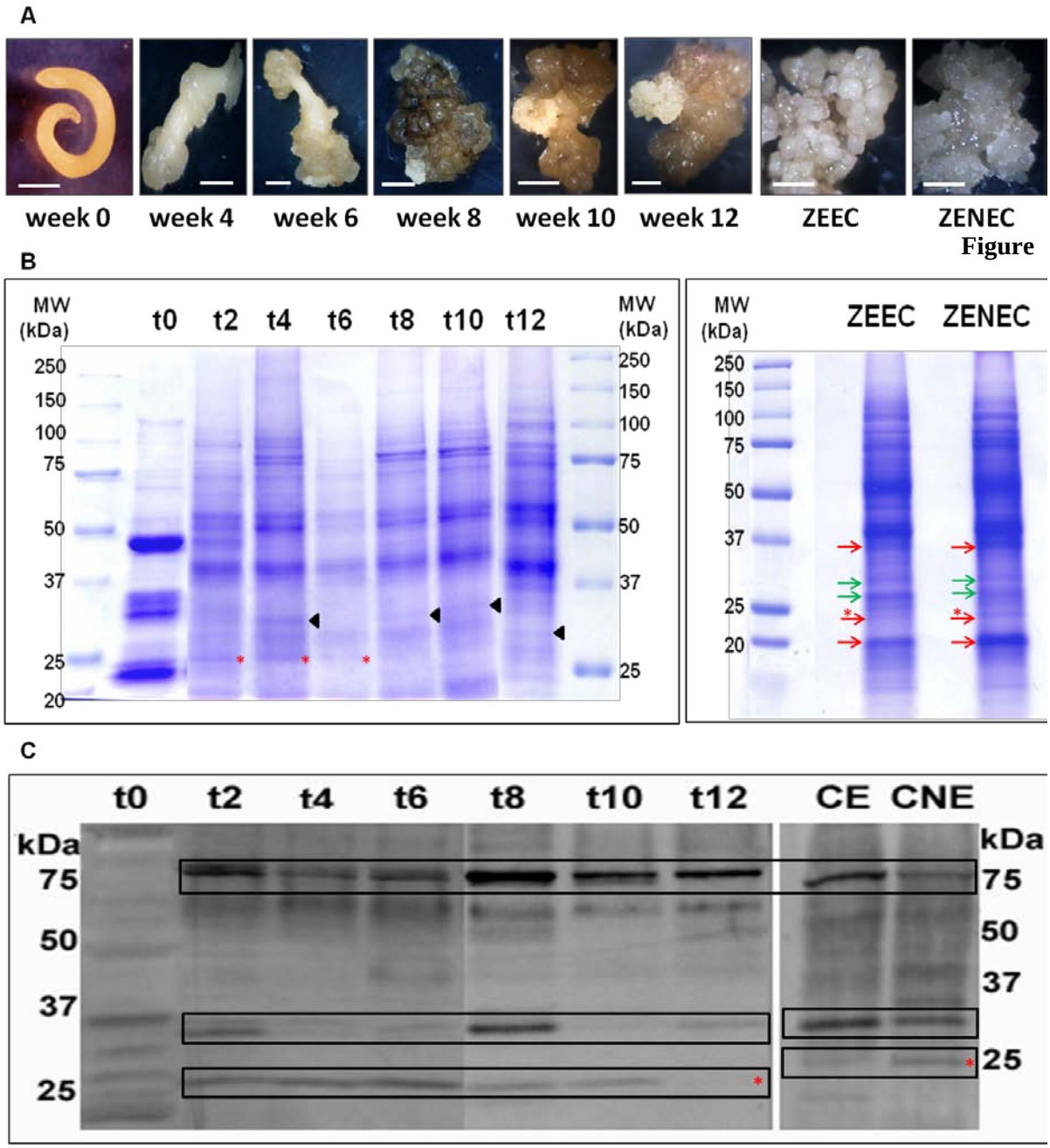

Figure 7 - SDS-PAGE protein profiles and immunoblotting ofNEP25 in zygotic embryo (ZE)-derived SE induced explants. **(A)** ZE (week 0) and induced ZE (week 4 to 12) were removed from the induction medium at 2-week intervals for proteomic analysis. ZEEC and ZENEC were obtained after the 12 week induction period, and subcultured separately before analysis. Bars 1mm. **(B)** Protein SDS-PAGE profiles of the time-course and of ZEEC and ZENEC samples stained with Comassie blue. Red arrows (→) represents protein bands exclusively or more expressed on ZENEC, and green arrows (→) represents the protein bands more expressed in ZEEC than in ZENEC. (*) points to the presence of the ~25kDa protein band, and (◄) indicates a protein band appearing after week 4 of the induction period. (C) Western blotting analysis of proteins extracted from the various samples. Twenty micrograms of extracted protein were loaded into each well for SDS-PAGE, blotted, and reacted with the antibody raised against NEP. Boxes highlight the most pronounced protein differences (~25, ~32 and ~75kDa) detected during the induction period.

In fact, besides NEC samples, the 25 kDa protein is more expressed in the initial explants and earlier induction stages and seems to decrease when embryogenic areas start to appear in induced explants. The data also showed that the antibody recognized proteins with other molecular weights, in particular a ~32-35 kDa protein, present both in EC and NEC, but more evidenced in ECs, where the ~25 kDa band was absent. Moreover, a ~75 kDa protein is expressed through all the stages of the process, varying its expression according to the variation of other detected forms.

An enhanced immunodetection of a ~40 kDa protein in the late stages of SE induction from leaves, as well as of a ~32 kDa protein in the late stages of SE induction from zygotic embryos, were in accordance to what previously found by SDS-PAGE analysis, although high levels of expression occurred.

The qRT-PCR analysis (Fig. 8) confirmed that *NEP25* transcripts are abundant in stage T0 decreasing hereupon. Although high transcript values were expectable in LNEC, the equivalent level detected on LEC was not in conformity to the immunoblot expression analysis.

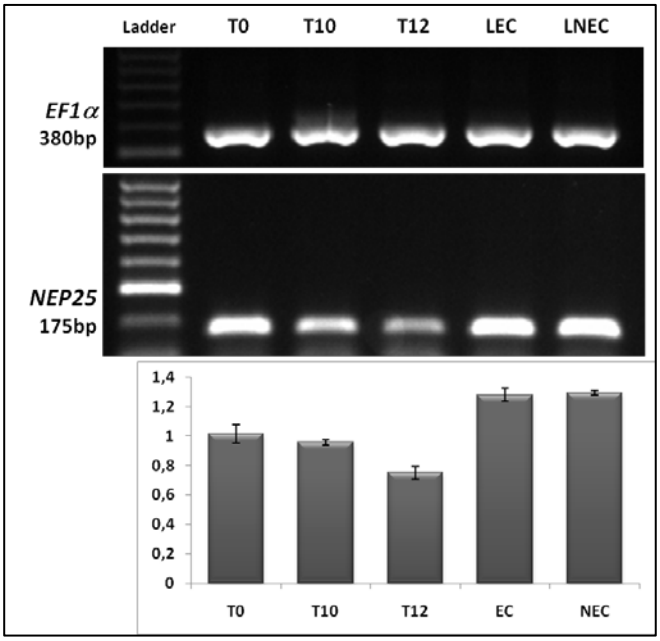

Figure 8 - Expression of NEP25 in the late stages of somatic embryogenesis induction, and callus stages, measured using qRT-PCR. Expression was estimated using ImageJ software and normalization was made to EF1α expression. Bars represent SE (n =3).

4.4.5. Enhanced levels of somatic embryogenesis induction from *Arabidopsis* NEP knock-out lines

To study the functional significance of NEP25 in the somatic embryogenesis process, we have selected an *Arabidopsis* T-DNA insertion for *AT4G15520* from the Salk Institute Genomic Analysis Laboratory Collection (Alonso *et al.*, 2003). The

SALK_027418 line, described as a homozygous insertion line, with a T-DNA inserted into an intron of the *AT4G15520* gene (Fig. 9A), was used for these studies. The progeny of self-fertilized kanamycin-resistant plants grown under non-selective conditions was analyzed by PCR in order to select true homozygous plants. We confirmed that the T-DNA insertion is located in the third intron, downstream of the start codon (Fig. 9A). One band was observed in the homozygous lines, and its size is consistent with the insertion of a single T-DNA (Fig. 9B). The absence of *AT4G15520* mRNA from the mutant line was also verified by RT-PCR (Fig.10B).

The comparison between wild-type plants and the knock-out mutants (KO) did not revealed any particular phenotype under regular growth conditions (Fig. 9C). Morphogenetic processes, such as rosette and inflorescence development, as well as rooting (Fig. 9D) were carefully analyzed, and no differences could be found.

Somatic embryogenesis was induced in immature zygotic embryos of 8-week-old plants. After one month on the induction medium explants were observed paying specific attention to those aspects described in literature (Pillon *et al.*, 1996; Gaj *et al.*, 2001; Ikeda-Iwai *et al.*, 2002) during somatic embryogenesis induction in *Arabidopsis*, namely: callus formation, primary somatic embryo development, germinated zygotic embryos and non-responsive explants (Fig. 10A). A high percentage of explants (about 60%) showed callus formation without further differentiation of somatic embryos. No significant differences were found between the wild type and knocked-out lines of *Arabidopsis* in terms of callus formation. In the same way, some explants were non-responsive and a few precociously germinated instead of forming callus or differentiating into somatic embryos (Fig. 10A). Primary somatic embryos were produced directly from immature zygotic embryos and started to appear after about 20 days of culture, developing in green primary embryos possessing two or fused cotyledon-like structures (Fig. 10A). Most of the somatic embryos formed, both in wild type and KO lines showed abnormalities similar to those observed by other authors in this species. The cotyledon-like structures did not possess trichomes thus confirming it's embryogenic rather than organogenic origin. The frequency of primary somatic embryo formation was usually below 25% and it was interesting to verify that the knock-out line for the *AT4G15520* gene showed higher rates of primary somatic embryo formation (23%) than the wild type line (14%).

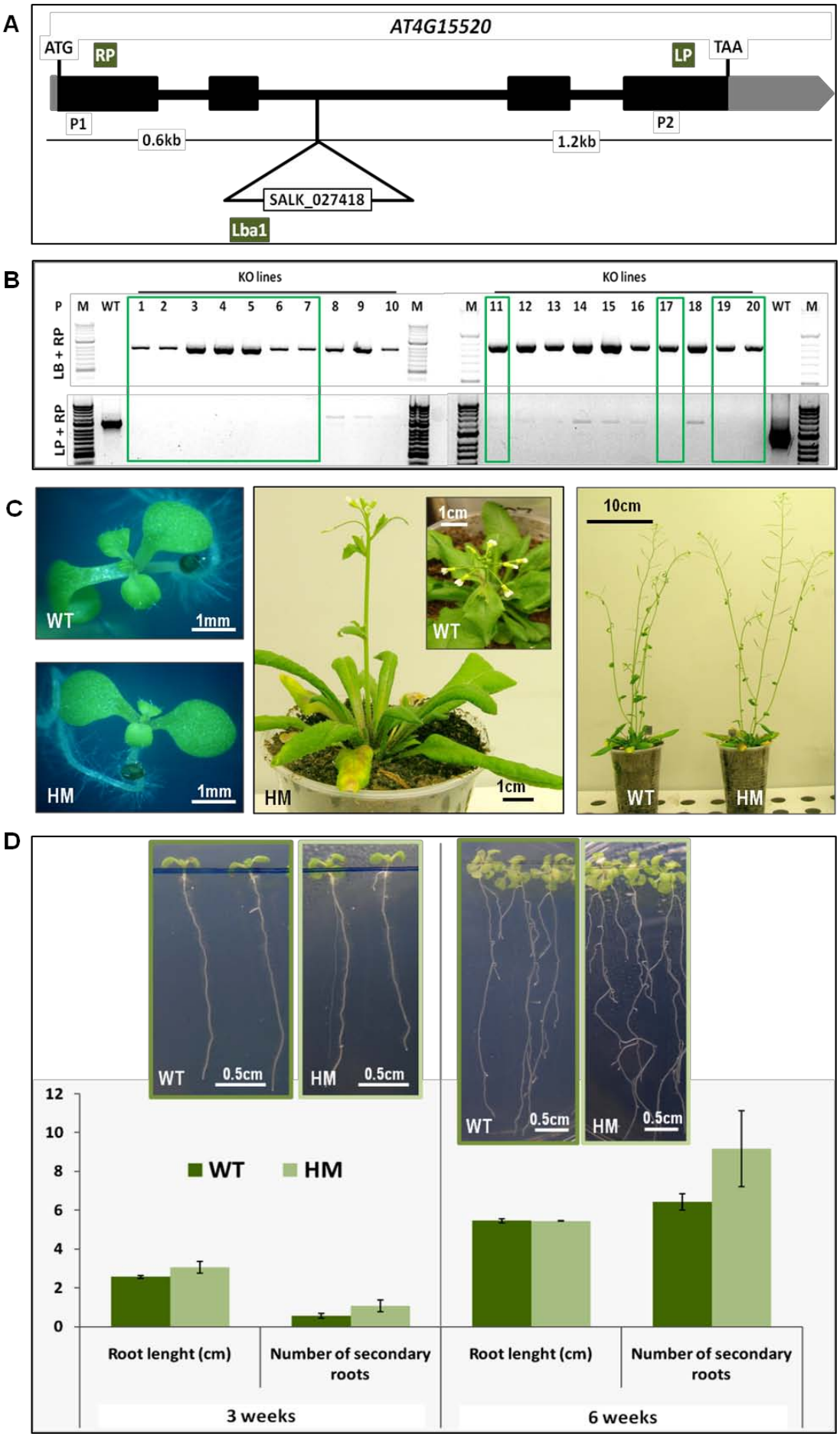
A
AT4G15520
ATG
RP
LP
TAA
P1
P2
0.6kb
1.2kb
SALK_027418
Lba1
B
KO lines
KO lines
P
M
WT
1 2 3 4 5 6 7 8 9 10 M
M 11 12 13 14 15 16 17 18 19 20 WT M
LB + RP
LP + RP
C
WT
1mm
HM
1mm
1cm
WT
HM
1cm
10cm
WT
HM
D
WT
0.5cm
HM
0.5cm
WT
0.5cm
HM
0.5cm
12
10
8
6
4
2
0
WT
HM
Root lenght (cm)
Number of secondary roots
Root lenght (cm)
Number of secondary roots
3 weeks
6 weeks

Figure 9 - Phenotypic and molecular analyses of *Arabidopsis* wild-type (Col-0) and *AT4G15520* mutant plants. **(A)** Schematic representation of the gene structure of *AT4G15520*, and position of the T-DNA insertion. Black boxes and thick lines indicate the positions of exons and introns, respectively. The triangle represents the T-DNA (not drawn to scale) inserted in position +600 of the *AT4G15520* genomic sequence. The positions of the primers used for RT-PCR (P1 and P2) and for PCR screening of homozygous plants (RP, LP and Lba1) are indicated. **(B)** PCR screening of homozygous (HM) plants (green boxes). **(C)** Comparative analysis of morphogenetic aspects of WT and HM plants development, showing no noticeable differences. **(D)** Rooting development in half-strenght MS medium revealed no differences between root length or number of secondary roots in WT and HM plants.

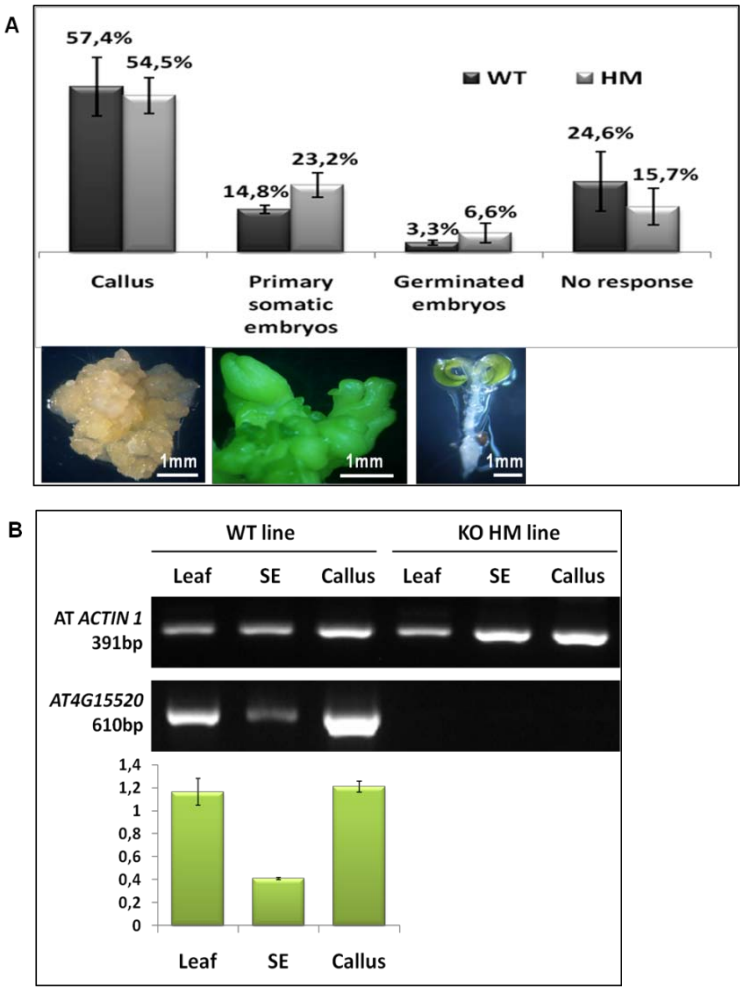

Figure 10 - Somatic embryogenesis induction in knock-out plants of *Arabidopsis thaliana* for *NEP25* orthologous. **(A)** Percentage of callus formation, primary somatic embryo formation, germination and non responsive explants in cultured immature zygotic embryos of *Arabidopsis thaliana* wild-type (WT) and knocked-out homozygous line (HM), after 1 month of culture in B5 induction medium, supplemented with 4.5 µM of 2,4-D. Pictures show the main morphological aspects observed. **(B)** RT-PCR analysis of *AT4G15520* transcripts in leaves, embryogenic explants and callus explants. *Arabidopsis ACTIN 1* expression was used as a control to assess the quality of the mRNA and the efficiency of the RT-PCR. Expression values are the result of 3 replicates ±SE, analyzed by Imaje J software.

RT-PCR demonstrated that *AT4G15520* mRNA was absent from the homozygous insertion line (Fig.10B) but was clearly detectable in the wild-type Columbia ecotype (Col-0). It is worth mentioning that, in embryogenic explants, the expression levels were 3-fold lower than in leaves or callus explants, hence indicating a likely inhibitory role of this protein in somatic embryogenesis induction.

4.4.6. NEP25 down-regulation in tamarillo transgenic plants

To verify the results obtained with the *Arabidopsis* insertion mutant using an independent experimental approach, we examined whether identical changes could be observed in tamarillo plants in which the expression of *NEP25* had been deliberately down-regulated using a post-transcriptional gene-silencing strategy. For this purpose, a 264 bp PCR fragment of NEP25 cDNA sequence (Fig. 11B), was inserted via recombination into pK7GWIWG2(I), a vector that generate hpRNA constructs (Karimi *et al.*, 2002). PCR and restriction analysis with HindIII (Figs. 11C and D) confirmed the construction (Fig. 11A) before incorporation into *Agrobacterium tumefaciens* LBA4404 strain.

Agrobacterium-mediated transformation using one-month induced leaves as explants was found to be suitable to produce large numbers of independently transformed transgenic lines in tamarillo. After cocultivation with *Agrobacterium* harboring the pNEP25 construct (Fig. 11A), the explants were grown in induction selection medium to induce somatic embryogenesis. Simultaneous application of a selection pressure (presence of a set of 3 antibiotics) combined with embryogenic induction conditions were found to be suitable for the production of kanamycin-resistant clumps of embryogenic cells, within 10-12 weeks (Fig. 11A). Nevertheless, induction rates were very low, only 2 from a total of 106 explants (an average of 21 explants in 5 replicate treatments), cocultivated with *Agrobacterium*, developed embryogenic tissues. These were carefully isolated and placed in fresh induction selective medium, where they proliferated, hence increasing their mass (Fig.11E). These kanamycin-resistant embryogenic masses developed into somatic embryos after 2-3 weeks in development selective medium. Also, the putative transgenic embryos were grown to plantlets (Fig. 11E), using a protocol similar to that followed for regeneration of normal plantlets but containing kanamycin (100 µg/ml) in the medium.

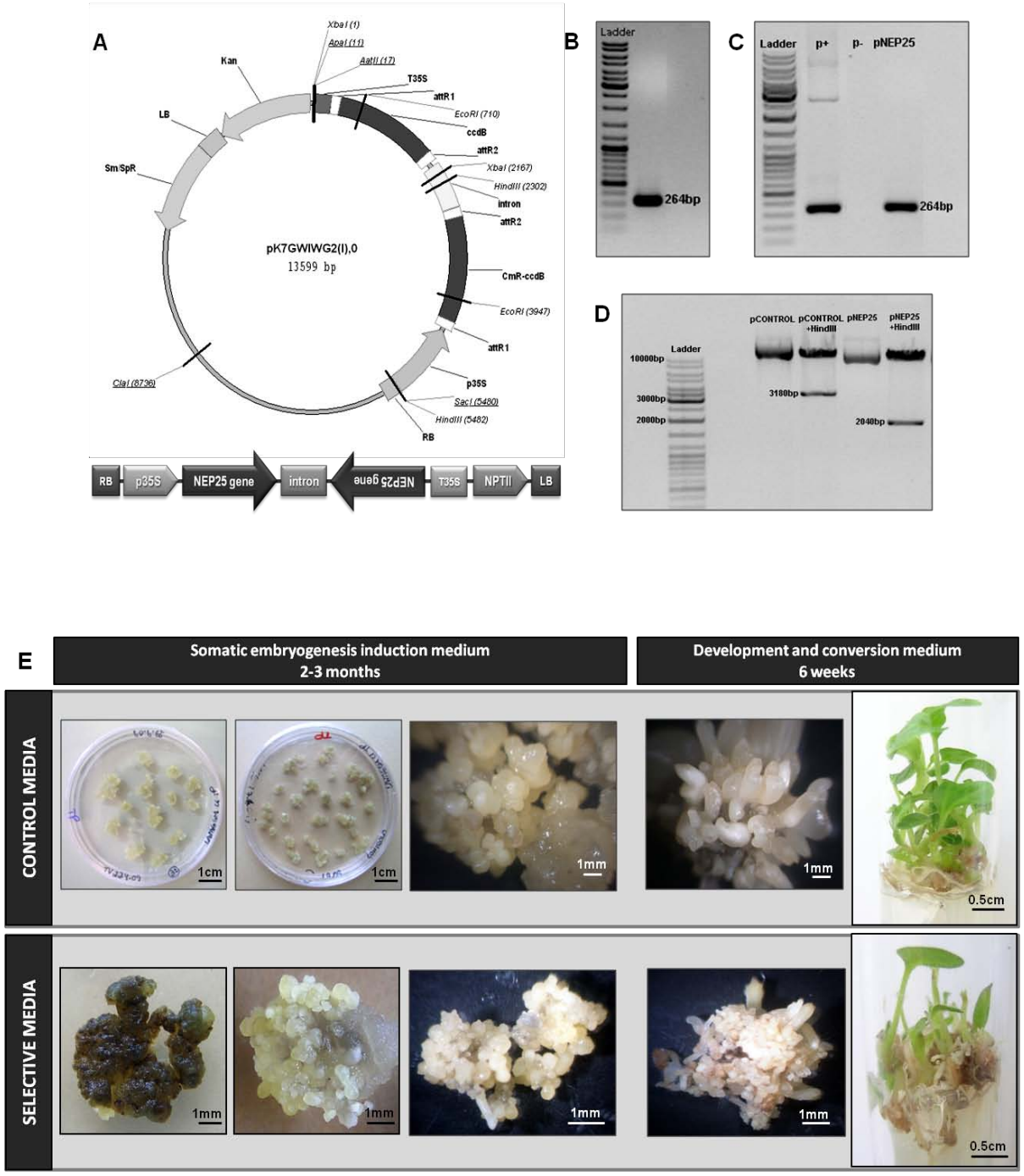

Figure 11 - Post-transcriptional gene silencing of *NEP25* gene in tamarillo. **(A)** Construction of a GATEWAY™ vector (Invitrogen). The final product of recombination contains one spliceable intron and *nptII* as plant selectable marker gene, and produces double-stranded RNA - hairpin RNA - from the *NEP25* gene sequence, triggering post-transcriptional gene silencing. **(B)** NEP25 PCR products were generated with attB1 and attB2 recombinant sites, producing a 264bp fragment. **(C) (D)** The construct sequence was verified by PCR and restriction analysis, before being incorporated into the *Agrobacterium* strain. **(E)** Regeneration of kanamycin-resistant plants through somatic embryogenesis. All the regeneration process of transformed explants was followed by a control in which no antibiotics were added to the culture medium, to insure the embryogenic ability of the explants used.

The young plantlets were placed on MS basal medium, with kanamycin (50µg/ml) selective pressure, where they grew very quickly with well-developed roots. A total of 80 kanamycin-resistant somatic embryo- derived plantlets developed on selection medium, in test tubes, from an initial 20 mg mass of resistant embryogenic callus. Half of the individual antibiotic resistant plants were selected randomly for preliminary PCR screening of stable transformation. The genomic DNA isolated from those kanamycin-resistant plants was positive for *nptII* gene in 75% of them (Fig. 12A), confirming their genetic modification. There was no PCR product observed when non-transformed control plants genomic DNA was used for amplification.

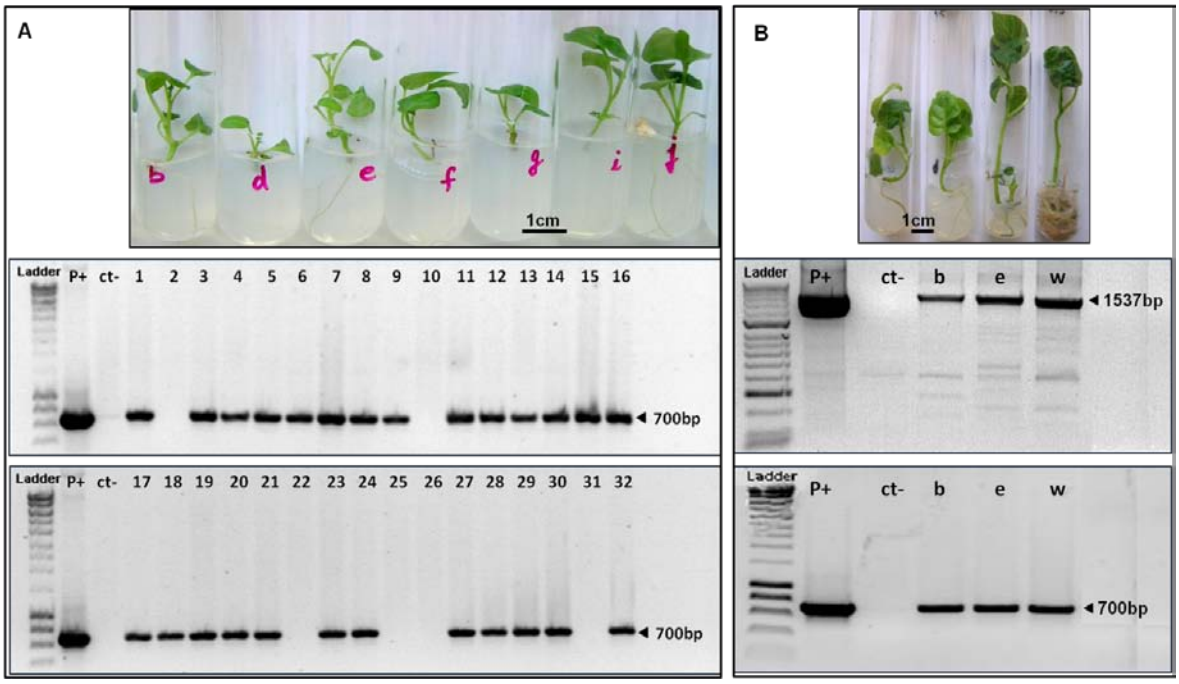

Figure 12 - Electrophoretic analysis of the PCR products of 36 putative transgenic tamarillo plants. **(A)** PCR-screening of almost half of the 80 self-rooted plants obtained, through the amplification of a 700bp fragment with primers for *npt*II. Some of the plants inside the test tubes are showed. **(B)** Analyses of 3 selected putative transgenic plants (lines b, e and w). The upper gel shows the amplification of a 1537bp fragment, with primers for the construct intron and P35S. In the lower gel, a 700bp fragment from *npt*II is amplified. The positive control was pNEP25 plasmid (p+) and non-transformed plants were used as negative control (ct-).

RT-PCR was used to compare NEP25 expression levels in *A. tumefaciens* transformed plants. Three plants transformed with pNEP25 vector (plants b, e and w) were randomly selected from the *nptII* positive plants together with a control plant, for RT-PCR analysis. Quantitative RT-PCR demonstrated that the abundance of transcripts encoding NEP25 was substantially (reaching 50%) reduced in at least one of the down-regulated lines (line e, Fig. 13A). Nevertheless, no clear phenotypical differences were

observed between the down-regulated and the control plants (Figs. 12B and 13B). Even in terms of somatic embryogenesis induction rates, no significant differences were observed between the percentage of ECs formed in the control line and the percentage formed by the silenced lines, namely for line e (Fig. 13C). Line b was the one with higher induction levels, even though it wasn't the one displaying the highest gene down-regulation levels.

RT-PCR and immunoblotting of the induction process in line e also confirmed gene down-regulation, with a significantly reduced expression of NEP25 transcripts throughout the process. Interestingly the immunoblot results revealed the most striking difference in the expression of the ~40 kDa protein, and not of the ~25 kDa protein by the end (12 weeks) of the induction period.

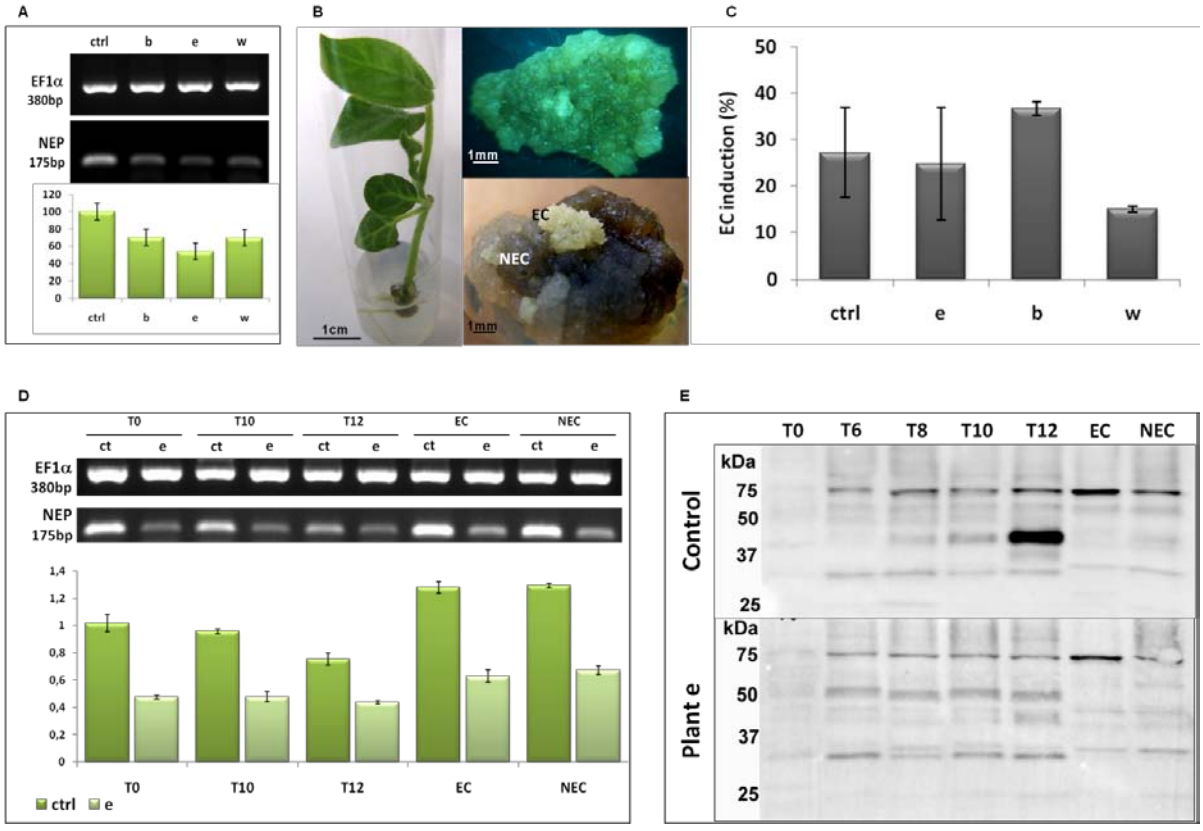

Figure 13 - Evaluationof NEP25 gene silencing. **(A)** RT-PCR analysis of the transcript level of NEP25 in 3 transformed lines and in a control plant. Primers annealing outside of the NEP25 construct were used. The expression of EF1α was tested as a control. Non-embryogenic callus was used to determine NEP25 mRNA levels in the analyzed plants. PCR was performed for 35 cycles for both genes. **(B)** Somatic embryogenesis was induced in leaf explants of the lines tested. The explants reacted in similar ways for control and silenced plants, and after 12 weeks in culture, embryogenic (EC) and non-embryogenic (NEC) areas were evident. **(C)** Percentage of EC formed per total of explants induced. **(D)** For line e (highest levels of gene silencing) a comparative time-course analysis of NEP25 expression was performed, by RT-PCR (same conditions as previously described) and **(E)** immunoblot analysis, with the antibody produced against NEP25 exposed peptide.

4.5. DISCUSSION

The present work was carried out to evaluate the putative role, if any, of a previously identified protein found in non-embryogenic *calli* of tamarillo that has been named NEP 25. The full length of *NEP25* cDNA was previously isolated and sequenced from a non-embryogenic callus library (Faro *et al.*, 2003) as a result of a comparative analysis of SDS-PAGE profiles of ECs and NECs of tamarillo (Ferreira *et al.*, 1998).

The predicted 221 amino acid protein encoded by *NEP25* contains a significant sequence similarity to proteins of the SpoU Methylase family, which is the second largest group of RNA methyltransferases (MTases). All of these enzymes are dimmers, with the catalytic site formed at the interface of two monomers, which modify RNA by a methyltransfer reaction in which S-adenosyl-methonine (SAM) is the donor of the methyl group. Although methylation of nucleic acids by SAM occurs spontaneously, MTases enhance the reaction rates by promoting favorable orientation and spatial proximity between RNA and the methyl group donor (Motorin and Helm, 2011). RNA methylation is important for biophysical stabilization of the RNA structure (Lapeyre *et al.*, 2005) and previous reports suggest that rRNA and tRNA anticodon loop methylations might have a role in the fidelity of mRNA decoding by the ribosome (Decatur *et al.*, 2002). More recent reports referred that hypomodified tRNA species may result from the cellular response to oxidative stress conditions, as was recently described for tRNA methylation in *Saccharomyces cerevisiae* (Chan *et al.*, 2010). These observations reinforce the perspective that the dynamic nature of RNA post-transcriptional modifications may be crucial for the control of gene expression. Our results associate (for the first time) a putative member of the SpoU Methylase family protein with the somatic embryogenesis induction process, which is a morphogenic process strongly associated with stress conditions (Zavattieri *et al.*, 2010; Takáč *et al.*, 2011), a situation that could be also observed in tamarillo, by the identification of several stress-related proteins (Chapter 5).

Analyzing NEP25 expression in a more detailed way through the induction process in leaves or in zygotic embryos, it was found that, opposite to what was previously observed, NEP25 is not exclusively expressed in NECs. Immunoblot analysis shows that the protein is expressed in the early stages of SE induction (until T8) starting to diminish afterwards, and is completely absent in ECs. Despite these observations, and although *NEP25* transcript analysis was in accordance to protein data,

in what concerned NEP25 expression in non-induced explants and in NECs, it surprisingly revealed an equivalent expression level in ECs and NECs.

To evaluate the role of this putative RNA methyltransferase in tamarillo somatic embryogenesis, a functional genomics approach was adopted, trying to access gene function through its silencing. Also, the high similarity (76% identity) between NEP25 amino acid sequence and a tRNA/rRNA methyltransferase in *Arabidopsis thaliana* led us to consider the analysis of knock-out mutants for *AT4G15520*, the orthologous gene of *NEP25*.

Reverse genetics has been playing a very important role in functional genomics. In recent years, complete genome studies and gene sequencing from a wide variety of plant species together with an array of bioinformatic tools have produced a massive amount of information about DNA and gene sequences. A major issue for plant biologists is how to convert this huge information into an understandable picture about the role of genes in controlling physiological and developmental processes. Methods for post-transcriptional gene silencing (PTGS), through double-strand RNA-mediated suppression have been developed (Waterhouse *et al.*, 1998; Helliwell and Waterhouse, 2003; Small, 2007). In particular, hairpin RNA (hpRNA) molecules that initiate the synthesis of small interfering RNA, are very potent PTGS inducers in plant cells (Smith *et al.*, 2000). This PTGS transgenic loss-of-function is usually achieved via targeting specific RNAs for degradation (2006; Karami *et al.*, 2007). Studies in plants and other eukaryotic organisms have shown that inverted-repeat transgenes (especially if they are separated by an intron) provide a reliable and highly efficient method for suppression of gene expression (Chuang and Meyerowitz, 2000; Smith *et al.*, 2000). Other approaches for studying the functions of genes in plants include loss-of-function mutations that result from T-DNA or transposon insertions, or from chemical or radiation-induced DNA variations (Wang and Waterhouse, 2001).

In our work, we have developed a reproducible and highly efficient regeneration system for transformed tamarillo plants, based on somatic embryogenesis in tamarillo. Our transformation procedures used *Agrobacterium tumefaciens* (LBA4404 strain) and followed a classical protocol that involved (1) cocultivation of pre-induced tissues (1-month induced leaves) with *Agrobacterium* carrying the T-DNA of interest; (2) embryogenic callus induction in the presence of a selective agent (kanamycin); (3) embryo development, also in the presence of a selective agent; and (4) rooting in the presence of the same selective agent. A period of preculture of explants before

cocultivation activates cell division, thus increasing cell competence for T-DNA integration (Busov *et al.*, 2010). Although regeneration of transformed plants have already been reported for tamarillo (Atkinson and Gardner, 1993; Cohen *et al.*, 2000), those works were based on organogenesis rather than somatic embryogenesis. Somatic embryogenesis based transformation offers several advantages over other transformation methods, because of the single-cell origin of the somatic embryo (Merkle *et al.* 1995), thus reducing the development of chimeric plants, which is often the main disadvantage when using the shoot tip method of transformation (Wilkins *et al.*, 2004). Although detailed histological studies in tamarillo have not yet been performed, preliminary data (not published) indicate that each proembryogenic mass of tamarillo embryogenic tissue arises from the division of a single cell. Although the percentage of somatic embryogenesis induction following tissue transformation was very low (2%), the potential to easily multiply the transformed callus lines, and the high rates of somatic embryo/plantlets development from one single embryogenic mass (3000 positive transformed plants per g of callus) considerably improves the efficiency of the process.

In what concerns our target gene silencing, a RT-PCR analysis of the randomly selected transformed plants, revealed a maximum of 50% for *NEP25* down-regulation obtained in line e. This quantitative analysis was possible due to the normalization of the obtained values against the expression levels of a possible reference gene of tamarillo. That gene was *ELONGATION FACTOR 1* (*EF1*), described as a stable reference gene in *Solanum tuberosum* (Nicot *et al.*, 2005) during biotic and abiotic stresses, of which sequence is highly conserved also in *Solanum lycopersicum* and *Arabidopsis thaliana*. The conserved sequences in *S. tuberosum EF1* cDNA were used to design a pair of primers that amplified a 380 bp product in tamarillo, which has the same length that the one for *S. tuberosum,* confirming that this gene could also be conserved in tamarillo.

Despite the obtained down-regulation levels, no evident phenotypical differences could be observed, neither in terms of normal morphogenesis processes or in somatic embryogenesis, when compared to the control plants. This observation was also true for *A. thaliana* homozygous knock-out plants *vs.* wild-type plants although a slightly higher somatic embryogenesis induction rate was registered for *A. thaliana* knock-out lines. In fact, while in *A. thaliana* knock-out lines gene silencing is total, in tamarillo *NEP25* gene expression is down-regulated, which means that the expressed protein, even at

lower levels, could ensure its function. On the other hand, this absence of specific phenotypes related to NEP25 down-regulation called our attention to other proteins of this family. Immunoblot analysis strongly suggests a temporal involvement of different members of a putative SpoU MTases family. *Arabidopsis* databases search for members of this family revealed several described tRNA/rRNA methyltransferases with molecular weights that are in accordance to the bands present in tamarillo immunoblots. It is worth noting that those proteins are differentially expressed in several *A. thaliana* tissues (data from eFP Browser), and that most of them are highly expressed in developing embryos and SAM, which associates these proteins with important morphogenic plant developmental processes.

In fact, the difficulty in relating individual genes to phenotypes (Wang and Waterhouse, 2001), and the substantial gene redundancy that many plant gene families present (Busov *et al.*, 2010), have already been referred as relevant limitations for the dissection of individual gene function(s) through these functional genomics approaches. Genetic redundancy is a major problem in all plant species. Plant genomes possess a very large number of repeated, functional genes (Busov *et al.*, 2010). For example, it was found that the genome of *Arabidopsis* is duplicated (Vision *et al.*, 2000), and *Populus* has around 30% duplicated genes (Busov *et al.*, 2010). Therefore single loss-of-function mutations often do not have obvious phenotypic effects in most experimental environments, which could explain our results in tamarillo.

Also, the fact that the SpoU MTase family includes several proteins with a strongly conserved domain, as observed in *A. thaliana*, could also be true in tamarillo. From the analysis of *A. thaliana* MTases amino acid sequences, the presence of the peptide, against which NEP25 antibody was produced, is evident. Also, judging by the correlation between molecular masses weight of these proteins and the bands detected in our immunoblot analysis, it is very likely that tamarillo also expresses proteins of this family during the somatic embryogenesis induction process. Nevertheless, the immunoblot detection of a ~75 kDa band, which does not correlate with the *A. thaliana* MTases molecular weights, could possibly be related to the presence of the NEP25 protein in a multimeric form, given the lack of a total denaturation. This is supported by the literature, since SpoU RNA MTases are reported to be functional as dimmers (Motorin and Helm, 2011), eg. in a multimeric form. Yet, this is an assumption that requires experimental support.

The results obtained show that NEP25 could, not only be a relevant key regulator for somatic embryogenesis in tamarillo, regardless of the explant origin or the hormone used during the process, but also a member of an important regulatory protein family of RNA MTases. Recently, more attention is being devoted to modifications in new RNA species, and some authors sustain that they could establish the links to various cellular mechanisms including gene silencing, transcriptional modulation of gene expression, stress response and, possibly, development and epigenetics (Motorin and Helm, 2011).

Several genes have been reported as being involved in the process of somatic embryogenesis induction, like *LEC* (*LEAFY COTYLEDON*) (Gaj *et al.*, 2005) or *SERK* (*SOMATIC EMBRYOGENESIS RECEPTOR- LIKE KINASE*) (Aker *et al.*, 2006), both being considered essential for the acquisition of embryogenic competence, thus enhancing the embryogenic competence of the explant. As far as we know, no inhibitory genes related to the induction of somatic embryogenesis have been reported before. The molecular mechanisms through which the expression of the NEP25 gene can negatively interfere with somatic embryogenesis are not known. A decreased methylation activity would make tRNA and rRNA less stable, thus compromising the efficiency of the translational process. It is known that protein synthesis is critical for somatic embryogenesis induction (Takáč *et al.*, 2011), a situation that could also be observed in tamarillo (Chapter 5 of this Thesis). In this context, the hypothesis that NEP25 could be a non-functional RNA MTase associated with non-embryogenic stages of the somatic embryogenesis process seems reasonable. Other MTases (~40kDa) may also have important roles when embryogenic cells differentiate, namely when protein synthesis activity is higher and needs efficient RNA stabilization.

Further assays with other *Arabidopsis* mutant lines, complimentary immunodetection tests in *Arabidopsis* and additional information about *NEP25* expression in other stages of tamarillo SE could bring more information about NEP25 role and other putative RNA MTases during somatic embryogenesis in tamarillo.

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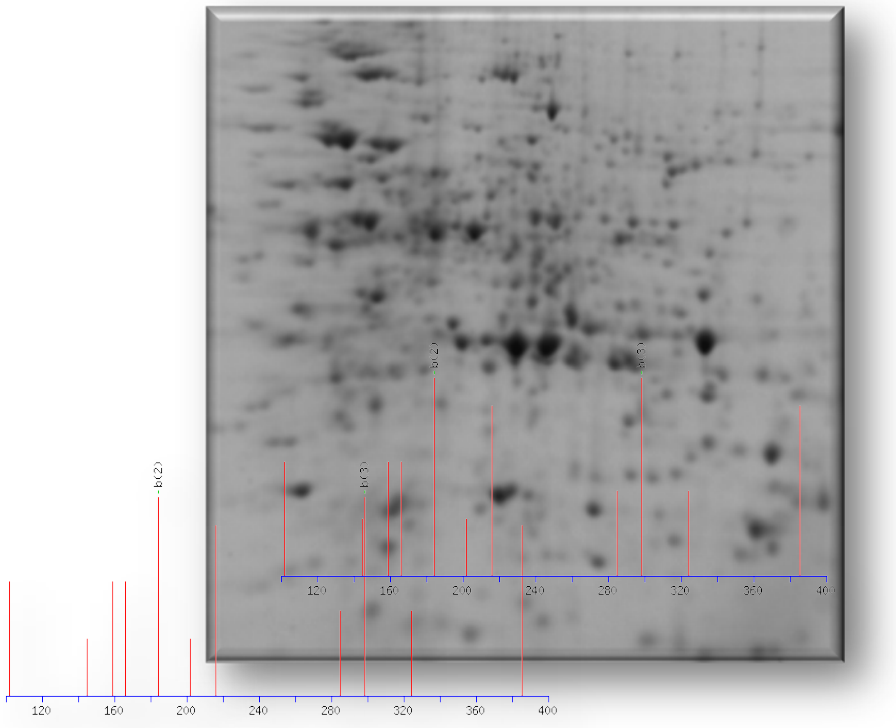

CHAPTER 5

SOMATIC EMBRYOGENESIS INDUCTION IN THE SOLANACEOUS TREE *Cyphomandra betacea* – A PROTEOMIC ANALYSIS

5. SOMATIC EMBRYOGENESIS INDUCTION IN THE SOLANACEOUS TREE *Cyphomandra betacea* – A PROTEOMIC ANALYSIS

5.1. ABSTRACT

Cyphomandra betacea (tamarillo) is a small tree which produces edible high nutritional fruits. In tamarillo, somatic embryogenesis is achieved through a two-step process in which embryogenic tissues are first produced (induction phase) on an auxin-rich medium and further on developed into embryos following transfer to an auxin-free medium (development phase). During the induction stage both embryogenic (EC) and non-embryogenic *calli* (NEC) arise from the same explant (young leaves or zygotic embryos) in the presence of either picloram or 2,4-dichlorophenoxyacetic acid. Trying to find embryogenic-specific proteins, we performed a comparative analysis of the proteome of tamarillo's EC and NEC. Analysis of 2-dimensional electrophoresis (2-DE) gels revealed 422 spots, from which 154 were only found in EC. Until now 22 of these proteins have been identified by LC-MS/MS. Proteins exclusively or predominantly expressed in the embryogenic tissue include enolases, adenylate kinases, fumarate hydratases, treonine synthases whereas pathogenesis-related proteins such as fructokinases occur mainly in NEC. A number of additional differentially expressed proteins involved in various functional categories were also identified, like heat-shock and ribosomal proteins. Cytoplasmic isozymes like fructose-bisphosphate aldolases were expressed in both EC and NEC samples. The data so far obtained indicate that for the same type of *calli* (embryogenic tissue or non-embryogenic callus) a strong resemblance occurs between proteins in spite of the auxin (2,4 or picloram) or explant (young leaves or zygotic embryos) tested.

Keywords: 2-D electrophoresis; LC-MS/MS; embryogenic callus; non-embryogenic callus; stress-related proteins; metabolism; plant proteomics.

5.2. INTRODUCTION

In recent years proteomics studies have emerged as leading tools for the study of many different aspects of plant physiology and development, making important contributions to better understand molecular processes so distinct such as meristem organization, polar auxin transport, flowering, seed maturation and fruit growth, just to name a few (Takáč *et al.*, 2011).

The role of proteomic characterization, particularly two dimensional gel electrophoresis (2-DE)-based protocols, in large-scale research of complex biological systems cannot be undervalued. While genomic/transcriptomic analysis indicate the potential for accumulation of specific enzymatic and structural proteins, these data are insufficient for defining the nature and dynamics of proteome profiles. In addition, as the characterization of cellular chemistry enters the field of large-scale experimentation (e.g., metabolomics), proteomics will play a central role in establishing the essential links between genes and metabolism (Hurkman and Tanaka, 2007). 2-DE, in combination with mass spectrometry (MS), is one of the most powerful techniques for plant proteomic analysis. Global protein identification together with analysis of the intrinsic patterns and relationships of protein populations has provided new insights into the interrelated biochemical processes of specific proteomes (Lippert *et al.*, 2005; Winkelmann *et al.*, 2006).

Proteomics has been successfully applied to the systematic analysis of protein expression during somatic embryo formation and development in various plant species (Takáč *et al.*, 2011). Following the pioneering studies in carrot (Reinert, 1958; Steward, 1958), somatic embryogenesis (SE) has been considered not only as an efficient system for *in vitro* clone propagation, but also an outstanding model system quite appropriate to better understand totipotency in higher plants as well as embryo development, considering the difficulties that have been encountered to analyse the early stages of zygotic embryos during their development inside the ovule tissues (Komamine *et al.*, 2005). Following embryogenic induction, modifications in the patterns of gene expression must occur to redirect cells to a new developmental process, resulting in the synthesis of new mRNAs and proteins specifics of the different stages through which embryo development proceeds (Chugh and Khurana, 2002). The SE pathway of cell differentiation has been investigated in some species, such as carrot (*Daucus carota*) and alfalfa (*Medicago sativa*), with description of morphological events and underlying

mechanisms of gene expression (reviewed by Chugh and Khurana 2002; Yang and Zhang, 2010). More recently, improvements in high resolution 2-DE and mass spectrometry (Hurkman and Tanaka, 2007) have contributed to the large-scale profiling and identification of proteins involved in plant development, particularly in somatic embryogenesis (Hochholdinger *et al.*, 2006).

A clue that seems to come up from recent studies concerning proteomic studies during SE is that stress-related signaling appears to play an important role for somatic embryo formation. Studies carried out in rice showed that 10 defense-associated proteins were observed to be expressed specifically during the differentiation of seed-derived morphogenic callus (Yin *et al.*, 2008). Also in the seedless cultivar Thompson of *Vitis vinifera*, a large number of the proteins specifically expressed in embryogenic callus are stress-related (Marsoni *et al.*, 2008; Zhang *et al.*, 2009). Altogether these data seem to support the viewpoint that adaptation to tissue culture-induced stress is a key factor in the reprogramming of the proteome and in further commitment of cultured cells to an embryogenic pathway (Feher *et al.*, 2003).

Although SE may occurs throughout a direct pathway, the most common induction method by indirect embryogenesis cannot be dissociated from initial cell division, a crucial step for growth and development of plants, regulated via hormonal (mainly auxins and cytokinins) signaling (Takáč *et al.*, 2011). For somatic embryogenesis induction, the levels of plant growth regulators at which cells are submitted are artificially modified with the purpose of redirect cell fate. The use of proteomics in SE systems could help to find proteins which are regulated by hormone action. For instance, changes in the relative amounts of cytokinin and auxin used to promote differentiation in rice callus led to marked modifications in their protein profile, namely in proteins related with carbohydrate and energy metabolism as well as stress/defense (Yin *et al.*, 2008). Curiously, these functional groups of proteins were also detected in *Vanilla planifolia calli* during shoot organogenesis (Palama *et al.*, 2010), which seems to indicate that distinct morphogenic processes might require similar groups of proteins to progress. Also, Chitteti *et al.* (2008) reported protein synthesis, changes in the chromatin structure, cytoskeleton reorganization, and prevalent down-regulation of chloroplast proteins on dedifferentiating *Arabidopsis* cotyledon cells treated with kinetin and 2,4-dichlorophenoxyacetic acid (2,4-D).

Despite all the accumulated data on protein expression changes during SE induction, the precise mechanism underlying this process has not yet been explained.

In an effort to better characterize the SE induction in tamarillo (*Cyphomandra betacea*) and as an attempt to identify somatic embryogenesis related proteins we have conducted a comparative proteomic analysis of leaf and zygotic embryo-derived embryogenic callus (EC) and non-embryogenic callus (NEC) cultures.

Cyphomandra betacea is a small solanaceous tree which the main economic interest lies on its edible fruits (Meadows, 2002). Protocols for the *in vitro* regeneration of different cultivars of tamarillo have been established at our lab, including axillary shoot proliferation, organogenesis induction (Guimarães *et al.*, 1996) and somatic embryogenesis (Guimarães *et al.*, 1988; Lopes *et al.*, 2000; Canhoto *et al.*, 2005). In tamarillo, somatic embryogenesis is achieved through a two-step process in which embryogenic tissues are first produced (induction phase) on an auxin-rich medium and further developed into embryos following transfer to an auxin-free medium (development phase). During the induction stage both EC and NEC arise from the same explant (young leaves or zygotic embryos) in the presence of either picloram or 2,4-D and high sucrose levels. These two types of tissues are easily distinguishable and can be isolated and grown separately in the same culture medium (Correia *et al.*, 2011).

In order to understand the biochemical and molecular events underlying EC differentiation, a 2-D gel electrophoresis associated with Liquid Chromatography together with Tandem Mass Spectrometry (LC-MS/MS) approach was used to quantify and identify proteins that were differentially expressed during tamarillo somatic embryogenesis. Samples of ECs and NECs obtained from different explants and in the presence of two auxins (2,4-D and Picloram) were used. Special attention was given to proteins that were simultaneously expressed in both types of ECs or NECs tested and differentially expressed between the two groups, since their expression was not a consequence of the induction conditions or the type of explant tested. As far as we know this is the first proteomic analysis of embryogenic and non embryogenic tissues originated from the same explant a system that can give important insights about the proteins and the cellular events involved on the first phases of somatic embryogenesis induction.

5.3. MATERIAL AND METHODS

5.3.1. Plant material

Embryogenic (EC) and non-embryogenic callus (NEC) cultures were established following the methodology described by Lopes *et al.* (2000), using as initial explants leaves excised from *in vitro*-cloned shoots of tamarillo (red cultivar) and mature zygotic embryos. The most apical expanding leaves were collected from 6 weeks developed shoots, randomly punctured and placed (abaxial side down) in test tubes containing induction medium. Zygotic embryos were isolated from seeds of an adult red tamarillo tree previously disinfected in a 7% (w/v) calcium hypochlorite solution, with a few drops of Tween 20 for 20 minutes, rinsed in sterilized water and placed in test tubes containing induction medium. For both types of explants, MS basal medium (Murashige and Skoog, 1962) plus 0.25 M sucrose, 0.25% (w/v) gelrite and pH 5.7 was used. In the case of leaves 20 µM Picloram, were used to induce embryogenic tissue formation whereas 9 µM 2,4-D, was used with zygotic embryos. Cultures were incubated at 24±1ºC in the dark for 12 weeks. After this incubation period, embryogenic and non-embryogenic areas were isolated and subcultured at 4 weeks intervals on the same medium. Samples for proteomic analysis were collected from the central part of the *calli* during active growth, which means 15 days after the last subculture (Fig.1A-D). EC samples were identified as LEC (Leaf Embryogenic Callus) and ZEEC (Zygotic Embryo Embryogenic Callus), and NEC samples as LNEC (Leaf Non-Embryogenic Callus) and ZENEC (Zygotic Embryo Non-Embryogenic Callus). At least three replicates of 0.5 g of ECs and of 1 g of NECs were collected and fast-frozen in liquid nitrogen before being kept at -80ºC for further proteomic analysis. To confirm the differences in embryogenic potential of the different *calli*, pieces of them were transferred to a development medium – consisting of the MS basal medium (Murashige and Skoog, 1962) plus 0.07 M sucrose, 0.6% (w/v) agar and pH 5.7.

5.3.2. Protein extraction

For total protein extraction a modified procedure based on the work described by Zhang *et al.* (2009) was applied. This protein extraction protocol, which utilizes

TCA/acetone, is used to minimize protein degradation and to avoid the interference of and non-protein components that may affect protein separation during electrophoresis.

The EC and NEC samples were ground to a fine powder in liquid nitrogen. While still frozen, the powder was suspended in 4 ml of cold acetone containing 0.2% (w/v) DTT and 10% (w/v) TCA. The suspension was then transferred to cleaned centrifuge tubes, incubated overnight at -20ºC and centrifuged for 30 min at 20,000 ×g (4ºC). The supernatant was carefully decanted, and the remaining protein pellet was washed twice in cold acetone (containing 0.2% DTT), incubated for 30 min at -20ºC, centrifuged 30 min at 20,000 ×g (4ºC) and vacuum-dried. The resulting pellet was re-suspended in 500 µl per tube of an isoelectric focusing IEF solubilization buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% (w/v) DTT, and 1% (v/v) IPG buffer]. The suspension was sonicated and incubated at room temperature for 2 h in a rotary shaker, and then centrifuged at 20,000 ×g for 1 h to remove the insoluble material. The total protein concentration was assessed using the 2-D Quant Kit (GE Healthcare; Amersham Biosciences) according to the manufacturer's guidelines, and using BSA as standard. Samples were aliquoted and stored at -20ºC until further processing.

5.3.3. 2-D Electrophoresis and image analysis

Before sample loading, 13 cm-long IPG strips (pH 3–10 NL;GE Healthcare; Amersham Biosciences) were hydrated overnight on IEF solubilization buffer. The extracted proteins (250 µg) of EC and NEC samples were individually loaded onto the strips with a cup-loading system (Bio-Rad). Isoelectric focusing was performed using the Protean IEF cell (Bio-Rad) programmed as follows: 500 V for 1 h, 8,000 V for 2 h (applying an increasing gradient) and 8000 V for 4h. The strips were then equilibrated twice successively in an equilibration solution (50 mM Tris–HCl, 6 M urea, 10% (v/v) glycerol, 2% (w/v) SDS, and trace amounts of bromophenol blue), in which the first equilibration contained 2% (w/v) DTT and the second 2.5% (w/v) iodoacetamide. The second-dimension SDS-PAGE was performed in 10% acrylamide gels employing the BioRad System. Proteins were stained using colloidal Coomassie Blue G-250 dye (Candiano *et al.*, 2004), and the stained gel images were acquired with a Spot Cutter (Bio-Rad) in the white-trans mode. Gel images were imported into PDQuest^{TM} 8.0 and the spots were detected and matched through the entire matchset. After matching, gel

images were normalized using the "Local Regression Model" algorithm, available in PDQuest^{TM} 8.0. In order to find significant differences between the groups of samples under study, protein spots intensities were subjected to One-Way ANOVA Bonferroni adjustment ($p < 0.05$). Spots of varied intensities in EC and NEC gels were excised manually. When a spot was present only in EC or NEC samples, identification was carried out with the specific sample; otherwise, parallel samples from EC and NEC were subjected to identification separately.

5.3.4. Protein Identification by Liquid Chromatography combined with Tandem Mass Spectrometry (LC-MS/MS)

Gel spots were destained with 50 mM ammonium bicarbonate and 30% acetonitrile. In-gel digestion was performed overnight at room temperature with 30µL of trypsin (10 ng/µL) in 10 mM ammonium bicarbonate. Peptides were extracted with 30%, 50%, and 98% acetonitrile in 1% formic acid, pooled, dried by rotary evaporation under vacuum, and resuspended in 2% acetonitrile and 0.1% formic acid. Protein identification was carried out on a hybrid quadrupole/linear ion-trap mass spectrometer (4000 QTrap; Applied Biosystems/MDS Sciex) using an nanoelectrospray source and a dual gradient pump (Ultimate 3000; Dionex). The mass spectrometer was programmed for information dependent acquisition scanning full spectra, followed by an enhanced resolution scan to determine the ion charge states, and set the appropriate collision energy for fragmentation. The IDA cycle was programmed to perform 6 MS/MS on multiple charged ions (+1 to +4) and performed two repeats before adding ions to the exclusion list for 60s (mass spectrometer operated by Analyst 1.4.1). Peptides were eluted into the mass spectrometer (Ultimate 3000, Dionex) with a binary gradient (250 nL/min 2% acetonitrile, 0.1% formic acid to 98% acetonitrile, 0.1% formic acid in a multiple step gradient for 50 min), using a nanoelectrospray source. Peptide identification was performed with MASCOT and Protein Pilot software (v2.0.1, Applied Biosystems/ MDS Sciex) (Le Blanc *et al.*, 2003 and Shilov *et al.*, 2007) against the Swiss-Prot or the NCBI non-redundant (nr) protein databases. Positive identifications were considered when Mascot ID protein score was above 32 and/or Protein Pilot ID was above 1.3, for confidence >95% (Song *et al.*, 2008). Protein identification based on single peptide hit had a minimum individual score of 95% and a minimum sequence tag of 3 amino acids (4 consecutive peaks in the MS/MS spectrum).

5.4. RESULTS

5.4.1. Somatic embryogenesis induction

Both explants used (leaves and zygotic embryos) yielded two types of tissues on the callus induction medium. Callus from zygotic embryos (Figs. 1A and 1B) or from wounded leaves (Figs. 1D and 1E) started to appear by the 6th week of culture and 2-3 weeks later, areas of whitish, compact embryogenic cell masses could be distinguished among the more friable and fast growing non-embryogenic callus. These small embryogenic areas continued to grow and could be easily separated from the surrounding non-embryogenic callus. EC separated from the NEC and subcultured on the same medium were slow growing, and formed compact whitish globular masses of cells of about 50-200 µm in size (Figs. 1A and 1D) whereas NEC, when subcultured onto the same medium were soft, fast growing and translucent (Figs. 1D and 1E). Both tissue types unvaryingly maintained their characteristics in successive subcultures. A more detailed analysis of these results is described in chapter 2.

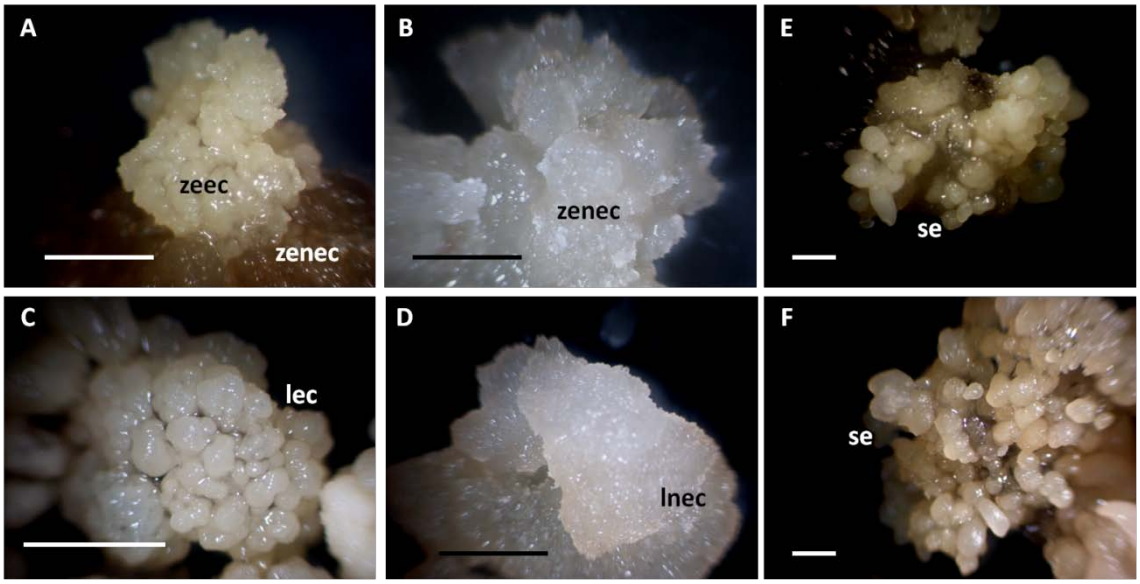

Figure 1 - Macroscopic aspects of embryogenic (EC) and non-embryogenic (NEC) *calli* of tamarillo obtained from zygotic embryos (**A** and **B**, respectively) and leaf explants (**C** and **D** respectively) following a 12 weeks culture period in the presence of an auxin. **E**, **F** – Somatic embryo development from zygotic embryo-derived EC (c) and from leaf-derived EC (f), after 3 weeks on auxin-free medium. lec – leaf embryogenic callus; lnec – leaf non-embryogenic callus; se – somatic embryo; zeec – zygotic embryo embryogenic callus; znec – zygotic embryo non-embryogenic callus. Bars: 1mm.

The inoculation of ECs and NECs on embryo development and germination medium (MS medium, 0.07 M sucrose, 0.6% (w/v) agar, pH 5.7) confirmed the identity of the two different samples. ECs developed into somatic embryos (Figs. 1E and 1F) and NECs remained undifferentiated becoming completely necrotic after 3-4 weeks (data not shown).

5.4.2. Comparison of 2D gels and protein identification

The protein extraction protocol used yielded an average of 3.4 mg of protein per 0.5 g of fresh EC, and about 2.3 mg of protein from 1 g of fresh NEC. It was also seen that the proteins in the IEF on the immobilized linear pH gradient gel strip were distributed in the range between pH 3.0 and pH 10.0. The 2-DE images of ECs and NECs, obtained either from leaves or from zygotic embryos, and using 10% SDS-PAGE in the second dimension of the electrophoresis, showed that the same type of *calli* displayed a similar spot distribution (Fig. 2). The number of spots that could be distinguished among the *calli* was variable with the highest number (420) in ZEEC, followed by LEC (420) and then LNEC (322) and ZEEC (313) (Fig. 2). The statistical analysis showed that 155 of the spots displayed significantly different ($p < 0.05$) expression levels when the two types of *calli* were compared (embryogenic vs non-embryogenic). From these differentially expressed proteins 32 were further selected (Fig. 3) based on the resemblance between them in LEC and ZEEC or in LNEC and ZENEC, which means that their expression was tissue specific and not affected by the auxin (2,4 or picloram) or by the type of explant (young leaves or zygotic embryos) tested. The numbers of those 32 spots can be cross-referenced with the corresponding protein expression data (Fig.4) and annotations plus associated information, as listed in Table 1.

The selected 32 spots were picked from the gels and submitted to LC-MS/MS analysis. Following LC-MS/MS analysis and database search, 14 differentially expressed protein spots were precisely identified. Table 1 provides the putative names of proteins, the plant organism from which the protein has been identified, the Swiss-Prot and NCBInr databases accession numbers, the values for theoretical molecular mass and pI, the protein score (unused score) and the number of peptide matches (≥95%), corresponding to protein spots shown in Fig. 3. Eight protein spots (3005, 4008, 9301, 4001, 8507, 8109, 8801 and 1101) were identified with only 1 peptide

sequence matched. No homology to any known peptide sequence in available protein databases was perceived for 10 protein spots. The identified proteins were classified into 7 functional groups according to the annotation in the MIPS Functional Catalogue Database (http://mips.helmholtz-muenchen.de/proj/funcatDB, Table 1; Fig. 5). Most of the identified proteins can be included in functional groups involved in the following cellular processes: 1) metabolism (35%), 2) cell rescue, defense and virulence (31%), and 3) protein synthesis (13%) or fate (folding, modification, destination) (4%). The remaining identified spots were proteins involved in transcription (4%), cell cycle and DNA processing (4%), and proteins with binding function or cofactor requirement (9%,).

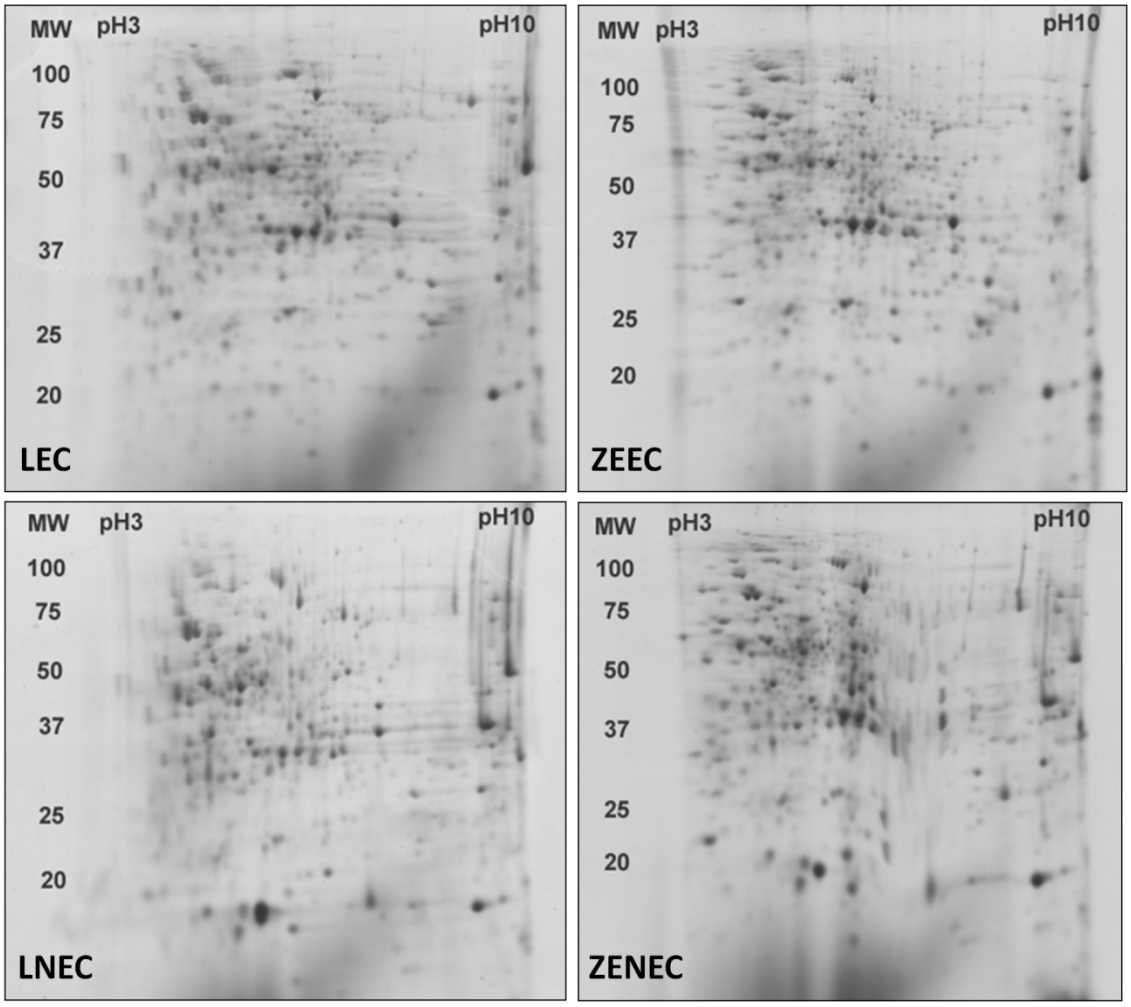

Figure 2 - Two-dimensional gels of ECs (LEC and ZEEC) and NECs (LNEC and ZENEC) proteins extracted using the TCA/acetone protocol; 250 µg of proteins are separated in the first dimension on an immobilized non-linear 3-10 pH gradient and in the second dimension on a 10% acrylamide-SDS gel. The gels shown were stained with colloidal Coomassie brilliant blue. The spots were analysed by PDQuest^{TM} 8.0 and a master gel was obtained. The molecular weight of protein standards is indicated on the left of each figure.

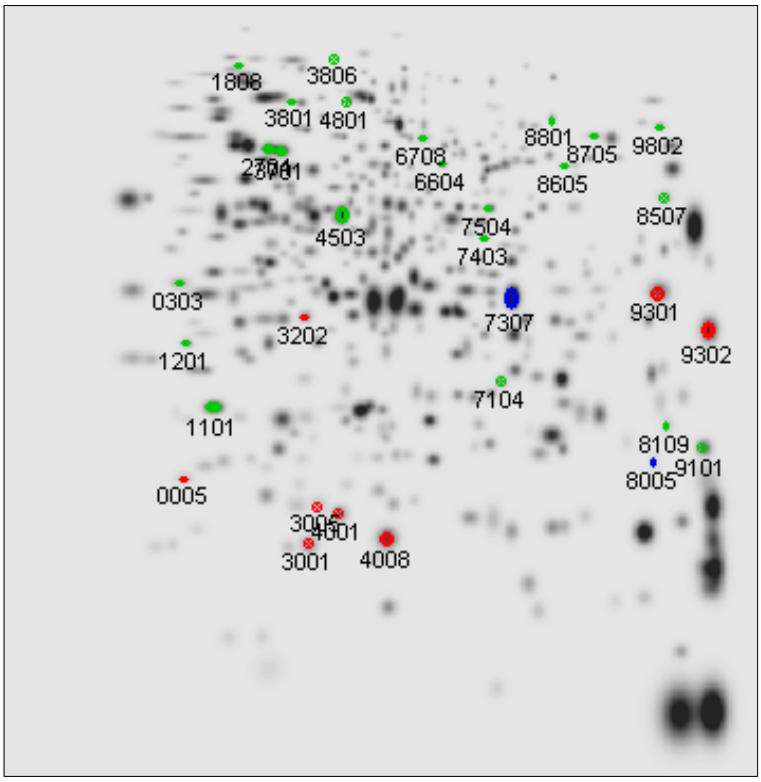

Figure 3 – Master 2-DE proteome map from the comparative analysis of LEC, LNEC, ZEEC and ZENEC gels. Proteins represented with colors and spot number were up-regulated in NECs (red marked spots) or ECs (green marked spots), or their presence was constant in both ECs and NECs (blue marked spots). These proteins were selected for further processing by LC-MS/MS.

Although the *Cyphomandra betacea* genome has not been sequenced, more than 70% of the proteins were identified using sequence data from various other Solanaceous, such as tomato and potato (64%), or *Capsicum* species (9%). Most of the other identifications were based on the sequences from the known genomes for *Arabidopsis thaliana* and *Oryza sativa*.

Figure 4 – Expression levels of the proteins selected from ECs and NECs gels. The relative ratio of proteins shown was determined with PDQuest™ 8.0 software. The amount of a protein spot was expressed as the volume of that spot, defined as the sum of the intensities of all pixels constituting the spot. From the 32 proteins selected, 3 were equally present in ECs and NECs (blue box), and 29 were differentially expressed in ECs and NECs, from which 8 spots were up-regulated in LNEC and ZENEC (red box) and 21 spots were up-regulated in LEC and ZEEC (green box). Data are shown as the average of three experiments ± SD. ANOVA was used to test the significance of the difference of protein volume between samples. Different letters indicate significant difference. *Bonferroni test $p<0.05$; **Bonferroni test $p<0.01$.

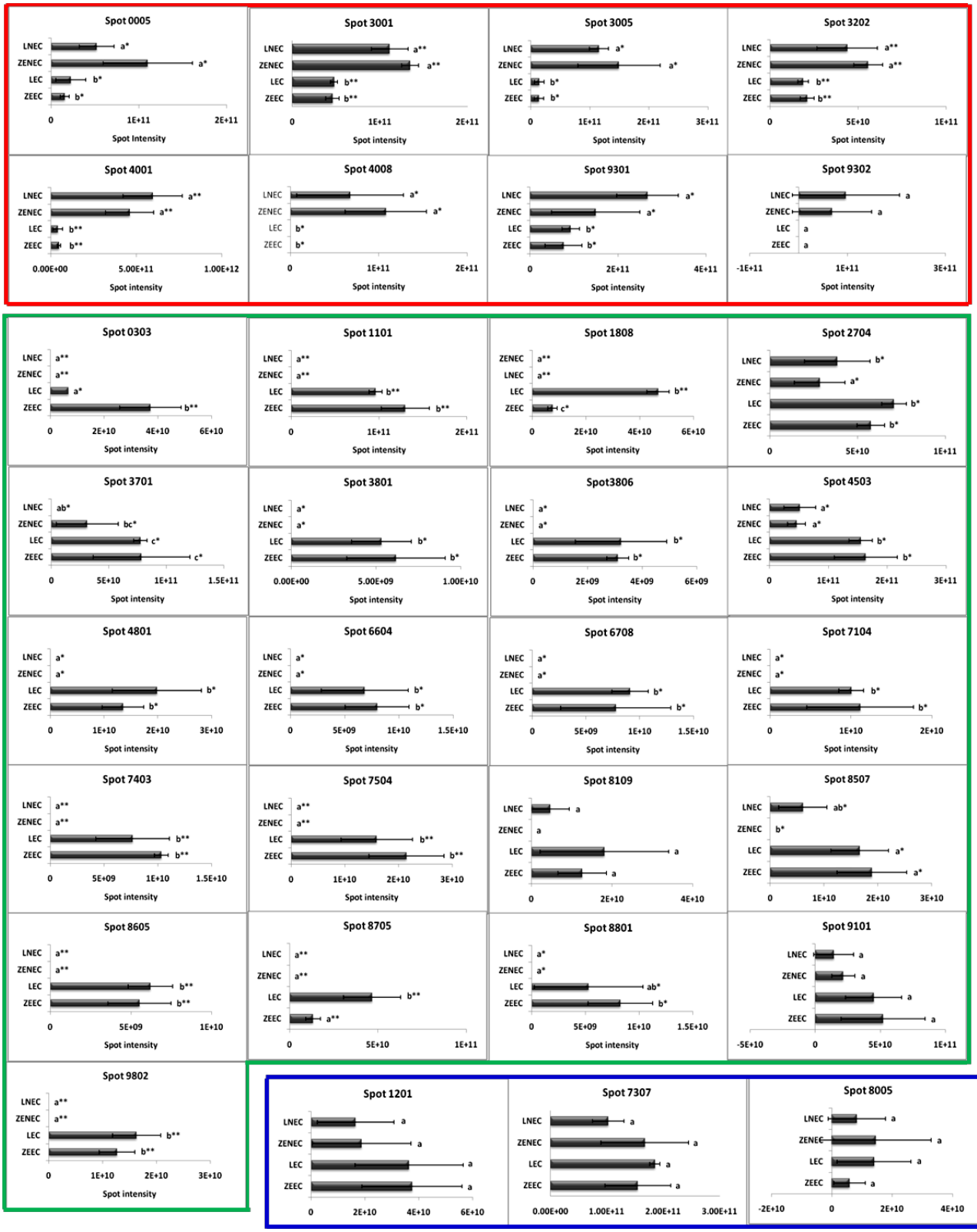
Spot 0005
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 1E+11 2E+11
Spot Intensity
Spot 3001
LNEC a**
ZENEC a**
LEC b**
ZEEC b**
0 1E+11 2E+11
Spot intensity
Spot 3005
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 1E+11 2E+11 3E+11
Spot intensity
Spot 3202
LNEC a**
ZENEC a**
LEC b**
ZEEC b**
0 5E+10 1E+11
Spot intensity
Spot 4001
LNEC a**
ZENEC a**
LEC b**
ZEEC b**
0.00E+00 5.00E+11 1.00E+12
Spot intensity
Spot 4008
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 1E+11 2E+11
Spot intensity
Spot 9301
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 2E+11 4E+11
Spot intensity
Spot 9302
LNEC a
ZENEC a
LEC a
ZEEC a
-1E+11 1E+11 3E+11
Spot intensity
Spot 0303
LNEC a**
ZENEC a**
LEC a*
ZEEC b**
0 2E+10 4E+10 6E+10
Spot intensity
Spot 1101
LNEC a**
ZENEC a**
LEC b**
ZEEC b**
0 1E+11 2E+11
Spot intensity
Spot 1808
ZENEC a**
LNEC a**
LEC b**
ZEEC c*
0 2E+10 4E+10 6E+10
Spot intensity
Spot 2704
LNEC b*
ZENEC a*
LEC b*
ZEEC b*
0 5E+10 1E+11
Spot intensity
Spot 3701
LNEC ab*
ZENEC bc*
LEC c*
ZEEC c*
0 5E+10 1E+11 1.5E+11
Spot intensity
Spot 3801
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0.00E+00 5.00E+09 1.00E+10
Spot intensity
Spot3806
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 2E+09 4E+09 6E+09
Spot intensity
Spot 4503
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 1E+11 2E+11 3E+11
Spot intensity
Spot 4801
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 1E+10 2E+10 3E+10
Spot intensity
Spot 6604
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 5E+09 1E+10 1.5E+10
Spot intensity
Spot 6708
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 5E+09 1E+10 1.5E+10
Spot intensity
Spot 7104
LNEC a*
ZENEC a*
LEC b*
ZEEC b*
0 1E+10 2E+10
Spot intensity
Spot 7403
LNEC a**
ZENEC a**
LEC b**
ZEEC b**
0 5E+09 1E+10 1.5E+10
Spot intensity
Spot 7504
LNEC a**
ZENEC a**
LEC b**
ZEEC b**
0 1E+10 2E+10 3E+10
Spot intensity
Spot 8109
LNEC a
ZENEC a
LEC a
ZEEC a
0 2E+10 4E+10
Spot intensity
Spot 8507
LNEC ab*
ZENEC b*
LEC a*
ZEEC a*
0 1E+10 2E+10 3E+10
Spot intensity
Spot 8605
LNEC a**
ZENEC a**
LEC b**
ZEEC b**
0 5E+09 1E+10
Spot intensity
Spot 8705
LNEC a**
ZENEC a**
LEC b**
ZEEC a**
0 5E+10 1E+11
Spot intensity
Spot 8801
LNEC a*
ZENEC a*
LEC ab*
ZEEC b*
0 5E+09 1E+10 1.5E+10
Spot intensity
Spot 9101
LNEC a
ZENEC a
LEC a
ZEEC a
-5E+10 0 5E+10 1E+11
Spot intensity
Spot 9802
LNEC a**
ZENEC a**
LEC b**
ZEEC b**
0 1E+10 2E+10 3E+10
Spot intensity
Spot 1201
LNEC a
ZENEC a
LEC a
ZEEC a
0 2E+10 4E+10 6E+10
Spot 7307
LNEC a
ZENEC a
LEC a
ZEEC a
0.00E+00 1.00E+11 2.00E+11 3.00E+11
Spot 8005
LNEC a
ZENEC a
LEC a
ZEEC a
-2E+10 0 2E+10 4E+10

Table 1 – Differentially expressed proteins in ECs and NECs of *Cyphomandra betacea* identified by LC-MS/MS. Each entry contains a spot number which corresponds to the protein spots marked in Figs. 3 and 4.

Spot ID	Protein ID	Species	Assession number	Database	Hyp mass (kDa) / pI	Protein score (M / P)	Peptide matches (≥95%) (M / P)	Functional Group
S3001	Pathogenesis-related protein STH-2	*Solanum tuberosum*	PRS2_SOLTU	SwissProt	17398 / 5.66	235 / 5.34	5 / 3	
S3005	Putative pathogenesis-related protein	*Capsicum chinense*	gi|58531054	NCBInr	17302 / 5.22	58 / --	1 / --	**CELL RESCUE, DEFENSE and VIRULENCE**
S4008	Pathogenesis-related protein STH-21	*Solanum tuberosum*	PRS1_SOLTU	SwissProt	17199 / 5.73	-- / 2.31	-- / 1	
S3202	Fructokinase	*Solanum tuberosum*	SCRK_SOLTU	SwissProt	33972 / 5.47	299 / 5.21	9 / 2	
	Probable fructokinase-4	*Arabidopsis thaliana*	SCRK4_ARATH	SwissProt	35250 / 5.21	292 / --	9 /--	**METABOLISM**
S9301	AdoMet-dependent rRNA methyltransferase SPB1	*Magnaporthe grisea*	SPB1_MAGGR	SwissProt	99800 / 8.74	46 / --	1 / --	
S0005	Copper chaperone	*Arabidopsis thaliana*	gi|15228869	NCBInr	13105 / 4.91	62 / 2.18	2 / 1	**PROTEIN WITH BINDING FUNCTION or COFACTOR REQUIREMENT**
S4001	Eukaryotic translation initiation factor 5A-1/2	*Solanum tuberosum*	IF5A1_SOLTU	SwissProt	17649 / 5.66	44 / --	1 / --	**PROTEIN SYNTHESIS**
S4503	Enolase	*Solanum lycopersicum*	ENO_SOLLC	SwissProt	48054 / 5.68	143 / --	4 / --	
S7104	Adenylate kinase 1	*Arabidopsis thaliana*	KAD1_ARATH	SwissProt	27143 / 6.91	141 / --	3 / --	
S7403	Fumarate hydratase 1, mitochondrial	*Arabidopsis thaliana*	FUM1_ARATH	SwissProt	53479 / 8.01	67 / --	3 / --	**METABOLISM**
S7504	Threonine synthase, chloroplastic	*Solanum tuberosum*	THRC_SOLTU	SwissProt	58003 / 6.54	91 / --	3 / --	
S3701	Heat shock 70 kDa protein, mitochondrial	*Solanum tuberosum*	HSP7M_SOLTU	SwissProt	73317 / 6.37	330 / 9.30	14 / 5	**CELL RESCUE, DEFENSE and VIRULENCE**
	Heat shock 70 kDa protein, mitochondrial	*Phaseolus vulgaris*	HSP7M_PHAVU	SwissProt	72721 / 5.95	267 / --	11 / --	

S8507	Heat shock 70 kDa protein, mitochondrial	*Solanum tuberosum*	HSP7M_SOLTU	SwissProt	73317 / 6.37	46 / 2.00	1 / 1	
S2704	Heat shock 70 kDa protein, mitochondrial	*Solanum tuberosum*	HSP7M_SOLTU	SwissProt	73317 / 6.37	320 / 16.16	17 / 7	**CELL RESCUE, DEFENSE and VIRULENCE**
S8109	Glucan endo-1,3-beta-glucosidase, basic isoform 2	*Solanum tuberosum*	E132_SOLTU	SwissProt	39848 / 6.32	77 / 2.01	1 / 1	
S6604	Poly(A)-binding protein	*Daucus carota*	gi|13560783	NCBInr	72114 / 6.94	110 / --	2 / --	**PROTEIN WITH BINDING FUNCTION or COFACTOR REQUIREMENT**
	Probable inactive purple acid phosphatase 1	*Arabidopsis thaliana*	PPA1_ARATH	SwissProt	65696 / 6.17	-- / 2.08	-- / 1	
S9101	60S ribosomal protein L12	*Capsicum annuum*	gi|40287508	NCBInr	17818 / 8.81	286 / --	3 / --	
	60S ribosomal protein L12	*Prunus armeniaca*	RL12_PRUAR	SwissProt	17986 / 9.02	73 / 13.03	3 / 7	**PROTEIN SYNTHESIS**
	60S ribosomal protein L12-1	*Arabidopsis thaliana*	RL123_ARATH	SwissProt	17946 / 9.02	63 / --	2 / --	
S8801	Ribonucleoside-diphosphate reductase large subunit	*Arabidopsis thaliana*	RIR1_ARATH	SwissProt	92669 / 6.24	51 / --	1 / --	**CELL CYCLE and DNA PROCESSING**
S1101	Pentatricopeptide repeat-containing protein At2g03880, mitochondrial	*Arabidopsis thaliana*	PP147_ARATH	SwissProt	72850 / 8.24	36 / --	1 / --	**TRANSCRIPTION**
S1201	40S ribosomal protein	*Glycine max*	RSSA_SOYBN	SwissProt	24406 / 8.49	159 / 11.10	6 / 7	
	40S ribosomal protein	*Arabidopsis thaliana*	RSSA2_ARATH	SwissProt	30654 / 5.04	141 / 1.68	4 / 4	**PROTEIN SYNTHESIS**
	Spermidine synthase	*Solanum lycopersicum*	SPDE_SOLLC	SwissProt	38111 / 4.81	187 / --	8 / --	
	Spermidine synthase	*Nicotiana sylvestris*	SPDE_NICSY	SwissProt	34889 / 5.21	152 / --	6 / --	
S7307	Fructose-1,6-bisphosphate aldolase	*Solanum lycopersicum*	gi|14484932	NCBInr	22651 / 7.82	185 / --	6 / --	**METABOLISM**
	Fructose-bisphosphate aldolase, cytoplasmic isozyme 2	*Pisum sativum*	ALF2_PEA	SwissProt	38638 / 6.77	81 / --	3 / --	
	Fructose-bisphosphate aldolase, cytoplasmic isozyme	*Zea mays*	ALF_MAIZE	SwissProt	39036 / 7.52	74 / --	3 / --	
S8005	Peptidyl-prolyl cis-trans isomerase	*Solanum lycopersicum*	CYPH_SOLLC	SwissProt	18184 / 8.83	72 / 6.77	1 / 4	**PROTEIN FATE (folding, modification, destination)**

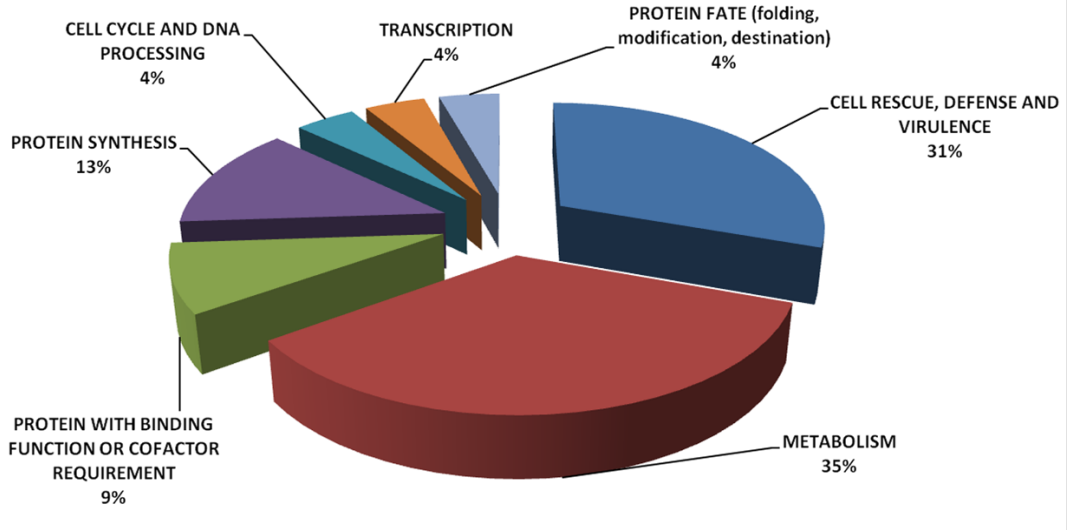

Figure 5 – Distribution by functional group (MIPS Functional Catalogue Database) of the proteins identified by LC-MS/MS analysis and database search.

5.5. DISCUSSION

Cyphomandra betacea is a solanaceous tree of which there isn't much information about its close relatives in the available genomic/proteomic databases, where most of the current data are from model plants such as *Arabidopsis thaliana* or *Populus trichocarpa* or from economically important species such as rice, coffee and eucalypts. Even so, it was possible to identify 22 of the 32 analyzed spots, representing a percentage of about 70%. Identification of tamarillo proteins that could be unique of this species or specifically modified may have failed due to the lack of analogs in the databases In this way, *de novo* interpretation of mass spectrometry data independently of database information could be helpful in future projects, as sustained by Hoehenwarter *et al.* (2008).

The results with tamarillo show that the proteins produced by the *in vitro* cultured explants fell mostly in three distinct groups, namely 1) metabolism-related proteins, 2) cell rescue, defense and virulence, and 3) protein synthesis and fate. In the following sections particular aspects of these different types of proteins are discussed emphasizing the perspective of somatic embryogenesis or *in vitro* morphogenesis

5.5.1. Metabolism-related proteins

Embryogenic competence must be accompanied by active metabolic changes (Fehér *et al.*, 2003). About 35% of the identified proteins were associated with metabolism (see Fig. 5), in particular with carbon and phosphate metabolism. Some of them were proteins not differentially expressed in ECs and NECs, like fructose-1,6-biphosphate aldolase (S7307) or spermidine synthase (S1201). This seems to point to housekeeping functions rather than to particular pathways directly related with somatic embryogenesis. Fructose-1,6-biphosphate aldolase is a glycolitic enzyme, belonging to the class I fructose-bisphosphate aldolase family, involved in the reversible reaction that converts triosephosphate into fructose-1,6-bisphosphate. In the mesophyll cells, this enzyme is located in the chloroplast (starch synthesis) and in the cytosol (sucrose biosynthesis, Páez-Valencia *et al.*, 2008). In the case of tamarillo only cytosolic enzymes could be identified.

Spermidine is a polyamine (triamine) that has been already identified with a positive effect on the embryogenic ability of some species, such as *Citrus sinensis* where spermidine synthase expression seems to precede the formation of globular embryos (Pan *et al.*, 2009). Other studies have also demonstrated a correlation between increase in polyamine levels and cell division, as well as their involvement in interactions of cell wall components that enhance cell wall rigidity and cell-to-cell adhesion (Bais and Ravishankar, 2002). In our assays, the highly variable values of expression obtained for spermidine synthase showed no significantly differences between ECs and NECs samples, although their levels were consistently higher in ECs, according to what has been suggested in the literature.

Regarding the proteins involved in energy mobilization, besides fructose-1,6-biphosphate aldolase, our results showed other proteins that were differentially expressed in ECs and NECs. Enolase (S4503), a glycolitic enzyme, and mitochondrial fumarate hydratase 1 (S7403), involved in the tricarboxylic acid cycle, were found to be significantly enhanced in ECs. Enzymes related to the glycolysis and tricarboxylic acid cycle have been correlated with the cellular utilization of ATP in higher plants (Wang *et al.*, 2009). Nevertheless, glycolysis represents the most prominent physiological process in both tissues. Since embryogenesis is a developmental process connected with biosynthesis of many compounds, cell division and cell elongation, glycolysis is

important for energy supply and formation of several metabolic intermediaries (Rode *et al.*, 2011). An increase in proteins involved in carbohydrate metabolism during SE was previously reported (Winkelmann *et al.*, 2006; Lyngved *et al.*, 2008).

Proteins involved in phosphate metabolism are proteins exhibiting phosphotransferase activity. In our experiments those are fructokinase (S3202), up-regulated in NECs, and adenylate kinase 1 (S7104) up-regulated in ECs. Fructokinase plays an important role in maintaining the flux of carbon towards starch formation since it specifically catalyzes the transfer of a phosphate group from ATP to fructose, as the initial step of starch synthesis. The data showed that fructokinase, was significantly higher expressed in NECs. And we hypothesize that this may be related to the high amounts of starch grains often found in cytological analyses of the non-embryogenic callus of tamarillo (data not shown). Adenylate kinase is a small ubiquitous enzyme essential for maintenance and cell growth. It is a phosphotransferase enzyme that catalyzes the interconversion of adenine nucleotides (ATP, ADP and AMP). This protein was exclusively expressed in ECs presumably indicating the high energy state of these cells and their intense metabolic activity.

5.5.2. Cell rescue, defense and virulence proteins

Stress response proteins were identified as one of the largest differentially expressed protein group in this study (31%). Among these, pathogenesis-related (PR) proteins and heat-shock proteins (HSPs) were found to be present both in NECs and ECs. These types of proteins have been frequently reported in dividing cells and tissues (Takáč *et al.*, 2011).

One of the identified PR proteins was a glucan endo-1,3-β-glucosidase, basic isoform 2 (S8109), that was up-regulated in EC, and was not expressed in ZENEC. Plant β-1,3-glucanases are classified as members of the PR-2 family and catalyze the hydrolysis of β-1,3-glucan (Simmons, 1994). They play a direct role in fungal defense by hydrolyzing fungal cell walls and an indirect role by generating oligosaccharide elicitors (Klarzynski *et al.*, 2000; van Hengel *et al.*, 2001). In tree species glucanases are reported in *Castanea*, *Fagus*, *Jatropha*, *Picea* and *Populus*, with enhanced expression during fungal elicitation, wounding, drying, and flooding stresses (Veluthakkal *et al.*, 2010). Nevertheless, the up-regulation of glucanases has also been observed in the

absence of pathogen attack or stress in various plant tissues due to the enzyme's ability to cleave 1,3;1,6-β-D-glucans present in the cell walls (Akiyama *et al.*, 2004). A previous work concerning SE in *Picea glauca*, showed that the transcript of a β-1,3-glucanase gene was abundant expressed in embryogenic tissues gradually decreasing during somatic embryo formation with the lowest levels occurring by the globular stage of embryo development (Dong and Dunstan, 1997). Previous studies had already reported the relevance of chitinase-modified AGPs (arabinogalactan proteins) of the extracellular matrix in the control or maintainance of plant cell fate, namely, an embryogenic cell fate (van Hengel *et al.*, 2001). Also Ko *et al.* (2003) described β-1,3-glucanase as being involved in spatial and temporal cell wall loosening, hence facilitating cell elongation during fruit development. All these data could reflect what is occurring, in terms of cell proliferation and differentiation, when this enzyme is expressed during EC formation in tamarillo. In our study, β-1,3-glucanase expression was highly variable, and it was also expressed in LNEC, even though it was more abundant in LEC and ZEEC.

Another group of stress-related proteins includes heat-shock proteins (HSPs), which were significantly up-regulated in NECs. The eventual involvement of these proteins during tamarillo somatic embryogenesis is discussed below.

The prevalence of biotic and abiotic stress responsive proteins in *C. betacea* may be related to the stress conditions at which the cells are submitted when cultured *in vitro*. Moreover, callus induction in tamarillo involves wounding of explants (in particular of leaves,) and inoculation on induction media. The induction media contain the synthetic auxins 2,4-D or Picloram both also known by their herbicide effects that cause considerable stress in plant cells (Kitamiya *et al.*, 2000).

5.5.3. Proteins involved in protein synthesis and fate

Physiological and metabolic changes during cell reprogramming require the synthesis, assembly and stabilization of newly synthesized proteins, as well as the modification and/or removal of peptides (Bian *et al.*, 2010). Interestingly, one of the largest functional groups found in ECs was that of proteins involved in protein biosynthesis, what might suggest that protein metabolism is a key factor for somatic embryogenesis induction. In line with this, pentatricopeptide repeat-containing proteins

(S1101), known by their role in protein–protein interactions related to a large variety of functions (chaperoning, transcription, etc.), were exclusively found in ECs, an aspect that deserves further attention.

Another protein related with protein synthesis was identified in spot 9101, a 60S ribosomal protein L12, which is required for the assembly and/or stability of the 40S ribosomal subunit. This protein is necessary for the processing of the 20S rRNA-precursor to mature into 18S rRNA during a late step of 40S ribosomal subunits maturation.

5.5.4. Up-regulated proteins in NEC

Pathogenesis-related (PR) proteins (S3001, S3005 and S4008) were the most abundant proteins detected in NEC gels, and significantly more expressed in those *calli* than in ECs. One of these PRs (STH-21, S4008) was found to be exclusively present in NECs. Recent results reported by Marsoni *et al.* (2008) also showed that two PR proteins (BetVI family) were the only up-regulated stress response proteins in non-embryogenic *calli* of *Vitis vinifera* (Marsoni *et al.*, 2008). PR protein synthesis has been analysed in many crop species, including rice, wheat, barley, corn, tomato, potato, beans, pea, and strawberry where they have also been extensively reviewed (Datta and Muthukrishnan, 1999; Broekaert *et al.*, 2000; van Loon *et al.* 2006). Their accumulation is usually triggered by pathogen attack, abiotic stress, hypersensitive response (HR), and systemic acquired resistance (SAR) and they play a major role in natural defense against pests and pathogens (Veluthakkal *et al.*, 2010). Their synthesis during *in vitro* cultures could be related with the adaptation of the plants cells to new environmental conditions which, in a first phase, may be perceived by the cells as unfavorable, hence resulting in the activation of defense mechanisms probably not directly related with a specific morphogenic pathaway such as somatic embryogenesis. As an alternative it can be hypothesized that the stress response of plant cells may activate signaling pathways triggering cellular events leading to the formation of embryonary structures.

A copper chaperone (S0005) was also up-regulated in NECs. Chaperone proteins are overexpressed under several types of stress, including maintenance of cells during early culture or during initiation of *in vitro* plant development (Imin *et al.*, 2005; Marsoni *et al.*, 2008). Chaperone proteins are also known for their roles in the

maturation of protein complexes and in facilitating the folding process of newly synthesized proteins. As previously stated cell reprogramming and differentiation require the synthesis, assembling and stabilization of proteins (Bian *et al.*, 2002; Marsoni *et al.*, 2008). Therefore, it is not surprising that a translation initiation factor 5A-1/2 (S4001) was also expressed in NECs. The precise function of eIF-5A in protein biosynthesis is not known but its role seems to be related with the formation of the first peptide bond. Besides, this protein seems to be the only eukaryotic protein to have an hypusine residue which is a post-translational modification of a lysine by the addition of a butylamino group (from spermidine).

An interesting differentially expressed protein was identified in spot 9301, an AdoMet-dependent rRNA methyltransferase SPB1 from *Magnophorthe grisea* (strain of blast fungus) a fungi that causes diseases in rice. This protein is required for proper assembly of pre-ribosomal particles during the biogenesis of the 60S ribosomal subunit. This result deserves further investigation since it correlates with our work (chapter 4).

5.5.5. Up-regulated proteins in ECs

Proteins involved in sugar, glucoside, polyol and carboxylate catabolism were highly expressed in ECs, like the mitochondrial fumarate hydratase 1 (S7403), which is involved in the tricarboxylic acid cycle and whose expression was only detected in EC.

Enolases (S4503) are enzymes of the glycolysis pathway, which catalyze the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP). PEP is not only important as a substrate for a further glycolytic reaction but also represents a precursor for the biosynthesis of fatty acids (Andriotis *et al.* 2010). Our results showed a significant increase in enolase expression in ECs, when compared to NECs. The high amounts of sucrose mobilization and utilization presumably necessary for EC growth are probably responsible for the activity of this enzyme found in the embryogenic tissue. The involvement of these enzymes during *in vitro* embryogenic and organogenic processes has been reported (Winkelmann *et al.*, 2006). It must be stressed out that cells of the tamarillo EC *calli* are much less vacuolated and possess a much higher number of organels than their non-embryogenic counterparts what could explain the higher expression of this kind of proteins.(Winkelmann *et al.*, 2006).

Heat shock proteins (HSP) are a class of functionally related proteins whose expression increases when cells are exposed to high temperatures or other stresses. Most of HSPs are known as molecular chaperones whose biological role is to maintain and shield the unfolded state of newly synthesized proteins thus preventing them from misfolding or aggregating (Vierling, 1991; Wang *et al.*, 2004). Based on molecular weight, these chaperones can be categorized into large HSPs and small HSPs (from 12 to 43 kDa). Various members of the HSP70 molecular chaperones have also been reported to be involved in protein import and translocation into chloroplasts and mitochondria (Wang *et al.*, 2004). Several HSP 70 proteins (S2704, S3701, S8507) were identified in this study and all of them were expressed at higher levels in ECs than in NECs which is in accordance with a report concerning somatic embryogenesis induction in grapevine (Marsoni *et al.*, 2008; Zhang *et al.*, 2009). Also in carrot, the expression of HSPs during somatic embryogenesis, in response to hormones, such as 2,4-D, had already been referred (Kitamiya *et al.*, 2000). The functions of HSP70 in preventing aggregation and in assisting refolding of non-native proteins, as well as their involvement in protein import and translocation processes, and in facilitating the proteolytic degradation of unstable proteins by targeting the proteins to lysosomes or proteasomes (Frydman, 2001) seems consistent with their up-regulation in ECs.

This study is a first approach to clarify the particularities of EC and NEC in tamarillo through proteomic analysis. The results point towards an increasing metabolism during somatic embryogenesis as well to the prevalence of stress-related proteins. The examination of differentially expressed proteins between ECs and NECs suggests that the embryogenic status of EC cells could be related to a better ability to regulate the effects of stress conditions, namely through the action of heat-shock proteins action.

A hypothesis is that the expression of totipotency in cultured somatic cells is part of a general stress adaptation process. An oxidative burst, probably triggered by the auxin present in the induction medium, may be the key event resulting in the acquisition of embryogenic competence by some callus cells and leading to the programmed death of others. This implies a fine regulation of auxin and stress signaling resulting in the restart of cell division and embryogenic competence acquisition. Our results indicate that this can occurs in spite of the auxin (2,4-D or Picloram) or explant (leaves, zygotic embryos) used. The observation that embryogenic tissues of different origin and

obtained with different auxins display similar protein profiles points to a general behaviour of cellular metabolism that can gave important insights about the mechanisms triggering and controlling somatic embryo formation.

The future progress in this field of knowledge requires new and/or complementary approaches including cell fractionation, specific chemical treatments, molecular cloning and subcellular localization of proteins combined with more sensitive methods for protein detection and identification. The use of laser-capture microdissection tools (Casson *et al.*, 2008) is one of those technical improvements, that could overcome the limitations in tissue accessibility allowing more accurate molecular profiles of isolated embryogenic tissues.

5.6. REFERENCES

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CHAPTER 6

CONCLUDING REMARKS

The optimization of somatic embryogenesis protocols for an efficient use in woody species is of utmost importance for the developing of new cultivars and faster propagation of elite trees. Rejuvenation, in particular in woody trees, is another aspect in which somatic embryogenesis can have a positive role. Understanding the molecular regulation of somatic embryogenesis induction may help to develop the necessary tools to optimize these protocols and also to improve regeneration/transformation systems in woody species. It could also bring important knowledge about the process underlying the regulation of the zygotic embryogenesis. In this way, the search for molecular markers for the critical stages of somatic embryogenesis is a promising field of research that has been gaining relevance due to the new tools that molecular biology offers.

However, several limitations restrain a wider application of SE, namely the recalcitrance of adult material to SE induction, the asynchronous embryo development, the continuous *calli* proliferation and the presence of anomalous/poorly developed somatic embryos. Our work was conducted in order to uncover those limiting steps of SE and to try to identify markers that could be related to this process, which would allow achieving more efficient results, not only with tamarillo, but also with other woody species. To achieve our goals, three strategies were followed, namely 1) the improvement of induction and somatic embryo development protocols, 2) the investigation of a putative SE inhibitory protein and 3) a comparison between proteomic profiles of embryogenic and non-embryogenic *calli*.

We were able to establish an effective system for regeneration of tamarillo selected adult plants through somatic embryogenesis from *in vitro* rejuvenated explants (Chapter 2). Although juvenile explants, such as those obtained from seedlings or young plants are good explants for somatic embryogenesis induction, their genotypes are unknown thus impairing the effectiveness of the micropropagation process. So, it is extremely important the development of these systems that allow to clone selected elite-plants. Nevertheless, it cannot be underestimated the relevance of different genotypes to undergo SE. Thus it is imperative that several genotypes are evaluated and efforts are being carried out to induce SE in other cultivars beyond the red one.

In what concerns somatic embryo development we found that, in spite of the high number of embryos that can be formed, most of them display some kind of morphological abnormality that, however, do not impair the effectiveness of somatic

embryo conversion (Chapter 3). We have also shown that the manipulation of the sucrose concentration in the development medium and dark conditions before germination increase the number of normal somatic embryos (Chapter 3). Besides, the observation of an inefficient accumulation of storage compounds in tamarillo somatic embryos could explain the low rates of normal somatic embryos obtained. Further analyses should highlight the effect of different maturation treatments tested in relation to the storage compounds accumulated in the embryo cells, since the improvement of maturation conditions could be very important to the yield of the somatic embryogenesis process in this species.

Understanding the role of specific conditions or compounds in promoting somatic embryogenesis and improving somatic embryo development is crucial for the establishment of effective regeneration protocols. Tamarillo has several features that can make it an interesting species to characterize somatic embryogenesis in woody angiosperms, since 1) several organs can undergo somatic embryogenesis, such as zygotic embryos, hypocotyls and leaves, 2) several auxins, such as NAA, 2,4-D, picloram or dicamba, can promote somatic embryo formation, 3) long-term embryogenic *calli* can be maintained in culture without loss of embryogenic potential, 4) somatic embryo conversion into plantlets is easy and several plants can be obtained from a single callus, 5) somatic embryogenesis can be induced in explants derived from adult trees, and 6) embryogenic and non-embryogenic cell lines can be obtained from the same explant. With the knowledge of specific markers it is possible to identify the most appropriate conditions, and transfer that knowledge from model species to related ones. Based on previous molecular assays, in which a protein (NEP25), associated with non-embryogenic callus of tamarillo, was identified and sequenced, we have followed two different approaches aimed at finding its role on somatic embryogenesis induction in tamarillo (Chapter 4).

Trying to elucidate the role of NEP25, which shows a sequence orthologous to a tRNA/rRNA methyltransferase of *Arabidopsis thaliana*, we have first checked changes occurring during somatic embryogenesis induction in knock-out lines of *A. thaliana* and also in down-regulated tamarillo transformed plants. The process followed to obtain tamarillo transformed plants was a new *Agrobacterium*-mediated transformation protocol using embryogenic plant material, hence distinguishing it from previous

protocols in which organogenesis was used for regeneration. Our results pointed, for the first time, at the involvement of a protein family of RNA MTases in the SE induction process. NEP25 could be a member of this protein family in tamarillo, acting as a SE inhibitor. Nevertheless, the involvement of other members of this protein family in the process is also strongly supported by our immunoblot data and bioinformatics analysis.

The few data available about tRNA/rRNA methylation in eukaryotes emphasizes the role of this mechanism in RNA stabilization, allowing an efficient protein synthesis. It is known that protein synthesis is critical for somatic embryogenesis induction, and active protein synthesis could also be observed in tamarillo (Chapter 5). Thus RNA MTases may help RNA stabilization during formation of embryogenic cells when protein synthesis activity occurs at higher rates. Further assays should be conducted to verify this hyphothesis, namely enzyme activity analysis that could confirm NEP25 function in RNA methylation. Also complimentary immunodetection tests in *Arabidopsis* and additional information about *NEP25* expression in other stages of tamarillo SE could bring more data about NEP25 role and other putative RNA MTases during somatic embryogenesis in tamarillo.

The proteomic profiles analysis provided new insights into the somatic embryogenesis induction process in tamarillo. The results pointed out towards an increasing metabolism during somatic embryogenesis as well as to the prevalence of stress-related proteins in spite of the auxin (2,4-D or picloram) or explant (leaves, zygotic embryos) used. The observation that embryogenic tissues of different origin and obtained with different auxins display similar protein profiles points to a general behavior of cellular metabolism that can gave important insights about the mechanisms triggering and controlling somatic embryo formation.

Some of the identified differentially expressed proteins deserves further analysis because they could have the potential to be useful markers for the embryogenic ability of the explants tested. A possible good candidate for that evaluation is the group of heat-shock proteins detected, that could explain the differences in stress responses between embryogenic and non-embryogenic cells. Also, the future progress in this field of knowledge requires new and/or complementary approaches including cell fractionation, specific chemical treatments, molecular cloning and subcellular localization of proteins combined with more sensitive methods for protein detection and identification. The application of laser-capture microdissection techniques, associated

with the transcriptome analysis of mRNA profiles in cells at different stages, would be an interesting future approach to the study of of the embryogenesis induction process in tamarillo. This approach could permit the identification of very specific and localized changes in the abundance of major classes of transcripts expressed in regulatory pathways, which would allow the construction of models to describe the changes associated with cell differentiation in somatic embryogenesis.