DOCTORADO EN CIENCIAS Y BIOTECNOLOGIA DE PLANTAS

Estudios moleculares durante la embriogénesis somática en *Coffea arabica*

Tesis que para obtener el grado de Doctor en Ciencias presenta:

Rafael Antonio Rojas Herrera

Centro de Investigación Científica de Yucatán A. C

Mérida, Yucatán, México

2002

A MAMA, que desde el cielo me bendice y porque esta Tesis sería todo su orgullo.

A MAMI y a PAPI, que me dieron la vida y me guiaron para que fuera lo que hoy soy.

A SILVIA, por supuesto, que me dio su vida y su amor.

- Pero yo no quiero andar entre locos -observó Alicia.

- ¡Ah!, no podrás evitarlo -dijo el gato-: aquí estamos todos locos. Yo estoy loco. Tú estás loca.

-¿Cómo sabes que estoy loca? -dijo Alicia.

- Tienes que estarlo -dijo el gato- o no habrías acudido aquí.

* * *

- Podría contarles mis aventuras... a partir de esta mañana -dijo Alicia con cierta timidez-; sería inútil referirme a las de ayer, porque yo era entonces una persona distinta.

Lewis Carroll. Alicia en el país de las maravillas.

It would be sad if all our biological knowledge is based in man, Arabidopsis and yeast.

Appel, Bellstedt & Gresshoff. J. Plant Physiol. Vol. 154. 1999.

Reconocimientos

Este trabajo se realizó en la Unidad de Bioquímica y Biología Molecular de Plantas del Centro de Investigación Científica de Yucatán A. C., bajo la dirección del Dr. Víctor Manuel Loyola Vargas y fue financiado por el Consejo Nacional de Ciencia y Tecnología (4123P-N y 31816-N) y una beca de Doctorado (117155) para Rafael Rojas Herrera.

La foto de la portada de esta tesis fue gentilmente donada por Marcela Méndez Zeel.

Agradecimientos

La consecución de este doctorado ha sido quizá la meta más difícil que me he trazado, hasta ahora, en la vida y por supuesto que no ha sido fácil y no lo hubiera logrado de no haber contado con el apoyo de muchas personas que de una u otro manera me tendieron la mano cuando lo necesité; así que ante todo quiero decirles que esta es la tesis de todos nosotros.

El Dr. Víctor M. Loyola Vargas fue el asesor de este trabajo, gracias a sus consejos y experiencia en la investigación científica recibí menos tropezones de los que merecía; gracias por todo Víctor.

Hay personas a las que uno nunca les estará suficientemente agradecido porque su trabajo no lo hacen por obligación sino por amor y cuando te ayudan lo hacen con el corazón: Miriam Monforte González, más que echarme la mano y permitirme aprender un poco de su enorme experiencia en el laboratorio me regaló lo más preciado que da un ser humano; su amistad. Gracias Monfi. Marcela Méndez Zeel con sus manos de oro para el cultivo de tejidos, su paciencia y sonrisa eterna para todo, contribuyó a que yo no tuviera que estar siglos sentado en las campanas y que mis experimentos de CTV fueran de verdad exitosos. Gracias Marce. Rosa Ma. Galaz Ávalos y Luis Carlos Gutiérrez Pacheco siempre me apoyaron con sus consejos y experiencia técnica cada vez que los necesité. Gracias Rosy y Luigi. Francisco Quiroz Figueroa y Felipe Sánchez Téyer más que amigos y compañeros de laboratorio fueron parte fundamental de esta tesis, sus ideas y las buenas discusiones que nos aventábamos a cada rato, quedaron plasmadas aquí; la amistad no se las puedo agradecer chavos, esa se las debo de por vida.

Mis agradecimientos especiales a la Dra. Teresa Hernández Sotomayor y a todo su grupo por el apoyo que me brindaron y por todos los reventones que nos aventamos, a Ileana y Manuel por toda su amistad y por haberme concedido el honor de ser el padrino de Luis Manuel, a los miembros de mi comité tutoral a lo largo de todo este tiempo (Dras. Lourdes Miranda Ham, Nancy Santana Buzzy y Magdalena Segura Nieto) que con sus buenos consejos contribuyeron a que esta tesis viera la luz.

Especialmente le estoy muy agradecido a la Dra. Nancy Santana (mi querida Ñuñú) por todos sus consejos y por sacarme de mis peores depresiones. A todos mis compañeros del postgrado y a los integrantes del Loyola´s Team (actuales y pasados), a los profesores que generosamente me brindaron un poco de su experiencia, a todos les estaré eternamente agradecido por su apoyo. A María Solís y Silvia Pasos por echarme la mano cada vez que lo necesité van mis muy especiales agradecimientos.

A esta gran nación que me recibió como un hijo, donde encontré una nueva y preciosa familia (los Solís Vargas y todas sus ramas) y porque nunca me hicieron sentir extranjero: GRACIAS MÉXICO.

Index

Resumen

La embriogénesis somática (ES) fue descubierta en zanahoria (*Daucus carota*), en la década de los 50s y desde entonces, éste ha sido el modelo más empleado para estudiar dicho fenómeno. Hasta la fecha se han clonado varios genes cuya expresión varía a lo largo de la inducción de la ES y el desarrollo de los embriones. Debido al amplio espectro de los productos codificados por estos genes, se hace difícil un análisis que integre los resultados publicados, así como la propuesta de un modelo que explique la reprogramación de las células somáticas y su canalización hacia la formación de un embrión somático. Básicamente, los genes que participan en este fenómeno pueden actuar en el inicio de esta reprogramación, en el tránsito de las estructuras embrionarias a través de diferentes estadios de desarrollo donde se establecen los patrones de órganos y tejidos de la futura planta, y en la maduración de los embriones, estadio del que se han clonado varios genes LEA y/o regulados por ABA, así como aquellos que pueden no tener un papel directo, pero que pueden ayudar en la formación de los embriones.

Debido a su importancia económica y dado que la ES se obtuvo en este cultivo desde 1970, el café (*Coffea spp.*) puede ser un buen modelo para estos estudios en plantas tropicales perennes. En el presente trabajo, el objetivo fundamental fue estudiar la expresión diferencial de genes durante la inducción de la ES en *Coffea arabica*. Con este fin se utilizó el despliegue diferencial de mensajeros, clonándose aquellos fragmentos de genes que mostraban expresión diferencial en los estadios tempranos de la inducción del proceso, en explantes foliares y suspensiones celulares. Un total de 23 fragmentos fueron clonados, entre los cuales se encontraron incrementos y disminuciones de los niveles de expresión. Para eliminar los falsos positivos obtenidos en los despliegues diferenciales, se desarrolló una metodología de "dot blot" inverso que permitió realizar estos análisis rápida y eficientemente. A partir de los fragmentos de genes clonados, se pudo constatar la complejidad de la ES y la diversidad de genes que participan en este proceso.

Entre los fragmentos de genes clonados se encontró uno (AR-52), del que se pudo traducir conceptualmente un polipéptido altamente similar a quitinasas ácidas clase III. En otros modelos se ha observado que estas enzimas pueden tener un papel muy importante en el establecimiento de la ES. En café, los resultados obtenidos sugieren la participación de estas enzimas en los estadios tempranos de la inducción de la ES así como una regulación diferencial por herida. Los datos zimográficos mostraron la existencia de tres isoenzimas con regulación diferencial en condiciones de embriogénesis y no embriogénesis.

Abstract

Somatic embryogenesis (SE) was discovered in carrot (*Daucus carota*) in the 50´s and it has been the most used model for studying this phenomenon in plants. To date, several genes, whose expression varies through SE and the development of embryos, have been cloned. Due to the high diversity of products encoded by those genes, it is very difficult to make an integrating analysis of the published results and then, to propose a plausible model to explain the reprogramming of somatic cell and its rerouting toward the formation of a somatic embryo. Genes participating in SE can act at the onset of this reprogramming, in the passing of embryos throughout different developmental stages where organs and tissues are established and during maturation. Other cloned genes seem to have not a direct role in SE but can serve as helpers in assisting the cellular process during embryo formation.

In coffee (*Coffea* spp.), SE was established in 1970. It has proven to be a good model to conduct molecular and biochemical studies of morphogenesis in perennial and tropical plants. In the present work, the main purpose was to study the differential gene expression during the induction of SE in *Coffea arabica*. A differential display analysis approach was used and those genes that were differentially expressed during the early stages after induction in leaf explants and suspension cultures were cloned. A total of 23 gene fragments that showed variations in their expression were re-amplified and cloned. To eliminate false positives obtained in this approach, a reverse dot blot assay was developed allowing fast and reliable results. Cloned fragment allowed us to confirm the complexity of SE and the diversity of genes participating in this process.

In other model systems, the participation of chitinases in the establishment of SE had been described. A partial cDNA encoding a putative chitinase was cloned in the present study. Our results suggest the participation of these enzymes in the early stages of SE induction in coffee, as well as the existence of a differential regulation by wounding. Zymography of chitinases suggest that there

are three isoenzymes which are differentially regulated in embryogenic and nonembryogenic conditions.

Introduction

Somatic embryogenesis (SE) is the process by which a somatic cell can be converted into a bipolar structure quite similar to an embryo. This was discovered in the 1950s by several groups (Krikorian and Simola, 1999), and due to similarities to zygotic embryogenesis, it gave an excellent opportunity to study embryogenesis in higher plants. In general, embryogenesis can be divided into two fundamental stages: the "morphogenetic" stage, where the perception of an "embryogenic signal" by a somatic cell initiates a genetic program that, in turn, activates the cellular division to form a globular embryo. Thereafter, the embryo passes through different stages when organ patterns are specified. At this point embryogenesis "in *stricto sensu*" is completed and somatic embryos undergo some preparation for germination that corresponds to the "metabolic" stage.

A developing embryo consists of a growing population of cells, whose fate in forming a functional organism depends on their position inside the embryo (Laux and Jürgens, 1997), which implies a coordinated action of several genes and/or their products to determine the function of particular cells. The isolation and characterization of the genes and proteins involved in determining such biochemical and molecular events may help to shed light on the mechanisms that give rise to a somatic embryo. It is not known yet which molecular and/or biochemical events are necessary for a cell to develop its embryogenic potential (De Jong et al., 1992) and the ability to form a bipolar structure, such as a somatic embryo.

Carrot (*Daucus carota*) has been the most studied model for molecular and biochemical studies on SE, whereas in zygotic embryogenesis *Arabidopsis thaliana* has recently been widely employed based on the facility to obtain embryo-defective mutants (Dodeman et al., 1997). Only a few of the cloned genes during SE are related to the earliest stages of embryo development. Many of them are expressed late during embryogenesis or other stages of plant development. A large number of such genes are believed to be related to the maturation process,

rather than to the first developmental stages. On the other hand, mutants can be valuable tools in the study of embryo development; nevertheless, mutations that affect embryogenesis can arrest embryo morphogenesis. Many of the corresponding proteins are involved in basic cellular functions and probably do not have direct regulatory roles. In some cases, mutated genes may be involved in embryogenic pattern formation, and sometimes even not to function specifically during embryogenesis. Mutations can also occur in the final stages of embryogenesis, such as those related to the maturation and desiccation of embryos or affecting postembryonic events. Moreover several of those genes have pleiotropic effects and a double mutant cannot be obtained because of the epistatic nature of the mutation (Lotan et al., 1998). It has been determined, based on statistical extrapolations that around 40 genes appear to control the formation of embryo axis pattern elements in *A. thaliana* (Dodeman et al., 1997).

The diversity of the nature, habitats and environmental conditions of plants does not always allow for extrapolation from published data. Several findings in *D. carota* are not applicable to other systems, especially among tropical plants. Thus, it was decided to use coffee as a model.

Objectives, justification and guidelines of the project

Coffee is mainly produced from two species of the *Coffea* genus: *Coffea arabica* and *Coffea canephora*. They are grown in nearly 50 countries around the world being Brazil, Colombia, Indonesia, Viet nam and México the major producers.

Somatic embryogenesis in coffee was first reported in *C. canephora* by Staritsky (1970) and in *C. arabica* by Söndahl and Sharp (1977). Yasuda et al., (1985) reported the induction of SE in leaf explants using benzyl aminopurine (BA) as sole growth regulator. Embryogenesis in Coffea may require elaborate protocols. They can involve only one step (Yasuda et al., 1985) or more (Van Boxtel and Berthouly, 1996; Sreenath et al., 1995; Neuenschwander and Baumann, 1992; de Garcia and Menéndez, 1987). Among the employed explant

sources are young leaves (Van Boxtel and Berthouly, 1996; Bieysse et al., 1993; Neuenschwander and Baumann, 1992; Hatanaka et al., 1991; de García and Menéndez, 1987; Yasuda et al., 1985), anthers (Ascanio and Arcia, 1994), immature embryos (Sreenath et al., 1996), ovules (Sreenath et al., 1995; Lanaud, 1981), internodes (Staritsky, 1970) and protoplasts from embryogenic calli (Tahara et al., 1994; Schöpke et al., 1987).

Generally, SE in coffee has been carried out from embryogenic cultures and proceeds in three steps, depending on culture conditions. Söndhal and Sharp (1977) established the production of embryos at low frequency in a first phase (LFSE) and at a high frequency in a second phase (HFSE), whereas Neuenschwander and Baumann (1992) demonstrated a third phase of embryogenesis in coffee cultures known as self controllable SE (SCSE). SE in coffee has also been directly induced from leaf explants (Dublin, 1981).

Among the factors that have not been studied and affect SE in coffee are ethylene levels (Hatanaka et al., 1995), auxin concentrations in tissues used as explant source, the supply of carbon and nitrogen, and the level of calcium. Molecular and biochemical aspects of SE in coffee are particularly underexplored. However, Menéndez et al., (1994) reported that embryogenic and non-embryogenic calli differ in some polypeptides.

In the present thesis, the main objective was to contribute to the understanding of molecular and biochemical events that take place during the onset of SE in coffee, through the evaluation of the gene expression patterns during the earliest stages of SE using differential display analysis, as well as the cloning of cDNAs that were differentially expressed and when possible, to identify them by sequence homology. For this, a system in which somatic embryos are obtained from leaf explants placed on benzyladenine-containing medium was employed. Samples were taken since day 0 up to the observation of the embryonic structures (preglobular embryos), accomplished within the first four weeks of culture. After differential displays, all bands consistently showing differential

expression in at least two experiments were cloned and sequenced and some of them further studied.

In chapter I, an examination of the published results coping with molecular studies during somatic embryogenesis is presented, paying special attention to the onset, stage transition and maturation.

As a consequence of the analysis of the gene expression patterns during SE, a total of 23 gene fragments were cloned. A modified dot blot assay was developed to assess their levels of expression and rule out false positives. Sequence analyses showed that some of them have homologies to genes/proteins reported in databases, whereas others have not or were false positives. Six cloned cDNAs consistently showed to be differentially regulated during the development of SE in coffee. These data are presented and discussed in Chapter II.

In Chapter III a more detailed study of one of the cloned cDNA is presented. Among the cloned fragments there was a class III chitinase-homologue, which is up-regulated during somatic embryogenesis in coffee. Due to the existence of several reports dealing with a possible role for chitinase during somatic embryogenesis in other species, it was interesting to us to determine an eventual correlation between the expression pattern of this cDNA and the chitinolytic activity during SE in coffee. Northern blot analysis and zymographic patterns showed differential regulation of this putative chitinase by wounding and somatic embryogenesis.

Finally, in Chapter IV, a general discussion of the results obtained in the present thesis is presented. Taking together our results and those published in the literature, a theoretical model is proposed seeking to explain the main events taking place during SE in coffee explants.

REFERENCES

Ascanio, E.C.E. and Arcia, M.M.A. (1994). Efecto del estado de desarrollo de las anteras y de un shock térmico sobre la androgénesis en *Coffea arabica* L. var. Garnica. Café Cacao Thé *38*, 75-80.

Bieysse, D., Gofflot, A., and Michaux-Ferrière, N. (1993). Effect of experimental conditions and genotypic variability on somatic embryogenesis in *Coffea arabica*. Can. J. Bot. *71*, 1496-1502.

de García, E. and Menéndez, A. (1987). Embriogénesis somática a partir de explantes foliares del cafeto 'Catimor'. Café Cacao Thé *XXXI*, 15-22.

De Jong, A.J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., Van Kammen, A., and De Vries, S.C. (1992). A carrot somatic embryo mutant is rescued by chitinase. Plant Cell *4*, 425-433.

Dodeman, V.L., Ducreux, G., and Kreis, M. (1997). Zygotic embryogenesis *versus* somatic embryogenesis. J. Exp. Bot. *48*, 1493-1509.

Dublin, P. (1981). Embryogenèse somatique directe sur fragments de feuilles de caféier Arabusta. Café Cacao Thé *25*, 237-242.

Hatanaka, T., Arakawa, O., Yasuda, T., Uchida, N., and Yamaguchi, T. (1991). Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. Plant Cell Rep. *10*, 179-182.

Hatanaka, T., Sawabe, E., Azuma, T., Uchida, N., and Yasuda, T. (1995). The role of ethylene in somatic embryogenesis from leaf disks of *Coffea canephora*. Plant Sci. *107*, 199-204.

Krikorian, A.D. and Simola, L.K. (1999). Totipotency, somatic embryogenesis, and Harry Waris (1893-1973). Physiol. Plantarum *105*, 348-355.

Lanaud, C. (1981). Production of *Coffea canephora* plantlets by somatic embryogenesis obtained by *in vitro* culture of ovules. Café Cacao Thé *XXV*, 231-236.

Laux, T. and Jürgens, G. (1997). Embryogenesis: A new start in life. Plant Cell *9*, 989-1000.

Lotan, T., Ohto, M., Matsudaira, Y.K., West, M.A.L., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (1998). Arabidopsis *leafy cotyledon1* is sufficient to induce embryo development in vegetative cells. Cell *93*, 1195-1205.

Menéndez, A., de Garcia, E.G., and Nieto, M.S. (1994). Comparative study of protein electrophoretic patterns during embryogenesis in Coffea arabica cv Catimor. Plant Cell Rep. *13*, 197-202.

Neuenschwander, B. and Baumann, T.W. (1992). A novel type of somatic embryogenesis in *Coffea arabica*. Plant Cell Rep. *10*, 608-612.

Schöpke, C., Mueller, L.E., and Kohlenbach, H.W. (1987). Somatic embryogenesis and regeneration of plantlets in protoplast cultures from somatic embryos of coffee (*Coffea canephora* P. ex Fr.). Plant Cell Tiss. Org. Cult. *8*, 243-248.

Söndahl, M.R. and Sharp, W.R. (1977). High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z. Pflanzenphysiol. *81*, 395-408.

Sreenath, H.L., Muniswamy, B., Naidu, M.N., Dharmaraj, P.S., and Ramaiah, P.K. (1996). Embryo culture of three interspecific crosses in coffee. J. Plant. Crops. *20*, 243-247.

Sreenath, H.L., Shanta, H.M., Babu, K.H., and Naidu, M.M. (1995). Somatic embryogenesis from integument (perisperm) cultures of coffee. Plant Cell Rep. *14*, 670-673.

Staritsky, G. (1970). Embryoid formation in callus tissues of coffee. Acta Bot. Need. *19*, 509-514.

Tahara, M., Yasuda, T., Uchida, N., and Yamaguchi, T. (1994). Formation of somatic embryos from protoplasts of *Coffea arabica* L. HortScience. *29*, 172-174.

Van Boxtel, J. and Berthouly, M. (1996). High frequency somatic embryogenesis from coffee leaves - Factors influencing embryogenesis, and subsequent proliferation and regeneration in liquid medium. Plant Cell Tiss. Org. Cult. *44*, 7-17.

Yasuda, T., Fujii, Y., and Yamaguchi, T. (1985). Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. Plant Cell Physiol. *26*, 595-597.

Chapter I

MOLECULAR ANALYSIS OF SOMATIC EMBRYOGENESIS: AN OVERVIEW[^]

R. Rojas-Herrera[1, 2], F. Quiroz-Figueroa[1], L. Sánchez-Teyer[1] and V. M. Loyola-Vargas*

[1]Unidad de Bioquímica y Biología Molecular de Plantas. Centro de Investigación Científica de Yucatán. Calle 43 No. 130, Col. Chuburná de Hidalgo, Mérida, Yucatán, México.

[2]Departamento de Genética y Mejoramiento. Instituto Nacional de Ciencias Agrícolas, Gaveta Postal 1, San José de las Lajas, La Habana, Cuba.

*Corresponding author email: vmloyola@cicy.mx

ABSTRACT

Somatic embryogenesis (SE) was discovered in the 50´s and it has been regarded as a promissory tool for massive propagation of commercial crops and a potential model to study cellular differentiation in plants. The first evidence of differential gene expression during SE arose in the 80´s and up to date, a large number of genes whose expression varies during the induction and development of somatic embryos have been cloned and studied.

The objective of the present review is to make an analysis of the available molecular evidence of somatic embryogenesis, paying special attention to three fundamental stages: i) the onset; where reported results suggest the existence of a signalization/reception process leading the somatic cell to a reprogramation leading to a new embryogenetic course, ii) the transition of embryos through the characteristic stages where organs are formed and the body plan is established, and iii) maturation where a large number of LEA and ABA-regulated genes have been cloned and studied. Finally, an analysis of other cloned genes, such as those coding for heat shock proteins that can assist in the formation of a somatic embryo, is presented and some basic unexplored aspects of somatic embryogenesis are discussed.

[^] Accepted for publication in Physiology and Molecular Biology of Plants.

INTRODUCTION

Embryogenesis in gametophytes of higher plants corresponds to the beginning of a new sporophytic generation. Seven cells constitute the female gametophyte: the haploid egg cell, two synergids, the diploid central cell and three antipodal cells. Dual fertilization of the central cell and the egg cell lead to the formation of the triploid endosperm and the diploid zygote, respectively. Almost immediately after, the first cellular division occurs in the zygote, resulting in an upper rounded cell of dense cytoplasm and a basal, enlarged and vacuolated cell. Subsequently, the embryo proper will form from the upper cell, and the suspensor will differentiate from the lower.

Nevertheless, embryogenesis is not exclusive of gametes. In 1958, Steward and coworkers reported that if explants from carrot roots were cultured in a liquid medium, isolated cells that were able to grow and multiply appeared in the culture medium after some time and, under certain conditions, these developed into embryo-like structures (Steward et al., 1958a; Steward et al., 1958b). This phenomenon was later known as somatic embryogenesis (SE). It is now recognized that SE was co-discovered in the 1950´s by several groups (Krikorian and Simola, 1999).

To date, SE has been described in numerous angiosperm and gymnosperm species. Its success depends on several factors, such as the genotype of the species, the source of the explant, nutrients and growth regulators present in the culture medium, subculture status, etc (Carman, 1990). It has been recognized as an important pathway for the regeneration and propagation of plants of commercial interest and a potential model to study regulatory and morphogenetic events that have an effect during embryogenesis in higher plants (Zimmerman, 1993).

For research purposes, SE represents a model system in which the lack of material and accessibility that are limiting factors in its zygotic counterpart has been overcome. Nevertheless, similarities between zygotic and somatic embryos demonstrate that the genetic program is present in the somatic cell and can

proceed independently of the presence of gene products from mother cells (Goldberg et al., 1989; Zimmerman, 1993) or fertilization signals. Moreover, the development of a complete embryo in the absence of the seed suggests that a signal from the non-embryogenic tissue of the seed is not needed to complete this process, or that it can be replaced by some component(s) of the culture medium (Goldberg et al., 1989).

The embryogenic process can be divided into two main and fundamental phases: a "morphogenetic" stage where the proper embryo is formed and the organs and tissues of the future plant are specified, and a "metabolic" stage, characterized by the storage of reserves (carbohydrates, proteins, lipids) in preparation for germination. As many excellent reviews have focused on zygotic and somatic embryogenesis and comparisons between them (Goldberg et al., 1989; Dudits et al., 1991; Sterk and De Vries, 1992; Thomas, 1993b; De Jong et al., 1993a; Zimmerman, 1993; Goldberg et al., 1994; Wilde et al., 1995; Laux and Jürgens, 1997; Mordhorst et al., 1997; Dodeman et al., 1997), our analysis will be centered on gene expression through the course of SE, paying particular attention to the three stages mentioned above. We also discuss the role of heat shock genes which are not embryo-specific, but are differentially expressed during SE and may help to form the somatic embryo.

THE ONSET OF SE: THE VERY BEGINNING

While a zygote is destined to be transformed into an embryo and therefore, can be defined as an embryogenic cell, it is not very clear which changes a somatic cell must undergo to develop its embryogenic potential and to be able to generate a whole embryo (Mordhorst et al., 1997). According to De Jong et al., (1993a) the term embryogenic must be applied to those cells that have completed the transition from its somatic status to a new condition where no further external stimuli are required (e. g. the application of exogenous growth regulators) to generate an embryo. The transition from a proliferative condition of callus cells to a new status, where embryos can develop, may require the turn off of the expression of some genes in callus cells (Lin et al., 1996). Moreover, molecular and

biochemical changes may take place to shelter the change that is taking place to ensure the success of the newly initiated program. The instauration of a new genetic program is then assumed, that not only specifies an embryogenetic pathway, but also inhibits any other process that will not be required.

In carrot and other species, several genes with enhanced expression during the early stages of SE have been cloned (Table I) (Lin et al., 1996; Dong and Dunstan, 1996a; Dong and Dunstan, 1999). However, many of such studies in carrot are biased towards the analysis of genes expressed after the acquisition of the embryogenic competence. This can be explained based on the way in which SE is induced in this model system, e. g., exposure of the suspension cultures to high concentrations of 2,4-D which produce clusters of small isodiametric cells, known as proembryogenic masses (PEMs) (Halperin, 1966). The subsequent elimination of auxins from the culture medium allows the completion of embryogenesis (De Vries et al., 1988; Komamine, 1992). It is now accepted that, in the continuous presence of auxins, proembryogenic masses synthesize the gene products required to complete the globular stage of embryogenesis, as well as the mRNAs and proteins that stop the somatic program. The depletion of auxins will result in the inactivation of a certain number of genes, allowing the embryogenetic program to proceed. The transition to the later developmental stages may require new gene products (Zimmerman, 1993). It has been observed that the exposure of carrot cell cultures to high concentrations of auxins can induce DNA hypermethylation (Lo Schiavo et al., 1989) which may, in turn, inhibit the transcription of methylated genes (Carman, 1990).

The presence of specific proteins in somatic embryos of carrot was first observed by Sung and Okimoto (1981a), who compared the electrophoretic patterns of total protein from somatic embryos and from calli. They observed that both, the calli and the embryos synthesize almost the same polypeptides except for a few qualitative and quantitative differences and that 2,4-D may modulate these differences (Borkird et al., 1986). Likewise, Wilde et al., (1988) noticed that the mRNA population of PEMs present less differences to that of torpedo embryos

compared to other mature tissues. These results could be taken as a demonstration that there is differential gene expression throughout SE.

Molecular and biochemical mechanisms underlying the transition from a somatic to an embryogenic state have not been established. It is presumed that a signaling/reception process may lead the somatic cell to a new genetic program, which specifies the embryogenic pathway.

The *Arabidopsis* gene LEAFY COTYLEDON1 (LEC1) specifies the identity of cotyledons of zygotic embryos and acts during embryo maturation. The LEC1$^-$ mutant produces embryos in which cotyledons possess trichomes and numerous plastids (regularly found in leaves), but no protein and lipid bodies (typical of cotyledons). Moreover, mutant seeds are viviparous and intolerant to desiccation (Meinke, 1992). Their germination is inhibited by ABA showing its sensitivity towards this growth regulator (Meinke et al., 1994). It has also been shown that LEC1 embryos express genes characteristic of embryogenesis along with some characteristic of germination (West et al., 1994).

LEC1 is expressed in preglobular and heart-shaped zygotic embryos, as well as in the endosperm, but not in maturing embryos. It is homologous to CCAAT box-binding factors, and induces the spontaneous formation of somatic embryos in leaves of transgenic plants when it is ectopically expressed, which leads to the suggestion that "LEC1 is a major embryonic regulator that mediates the switch between embryo and vegetative development" (Lotan et al., 1998). A second LEC gene (LEC2), encoding a B3 domain transcription factor, has been cloned very recently. It is expressed during seed development and, like its counterpart LEC1, also provokes somatic embryo formation after ectopic expression (Stone et al., 2001). Hence, LEC1 and LEC2 may have a role in the acquisition of embryogenic competence and may also activate genes that function in the initiation of somatic embryogenesis.

Table I. Cloned genes with enhanced expression during early stages of SE.

Gene	Homology/ Feature	Expression	References
DC5	Unknown	Detectable in hypocotyls and leaves and increased in PEMs, globular and torpedo SE.	(Wilde et al., 1988)
DC13	Unknown	Expressed in hypocotyls and leaves and slightly in PEMs and globular SE. More noticeable in torpedo SE.	
CEM-6	Gly-rich protein	In PEMs one day after removing 2,4-D to globular stage and lesser in heart and torpedo stages in cotyledon and plantlets hypocotyls.	(Sato et al., 1995)
ASET1[1]	Highly hydrophobic	Expressed in PEMs of embryogenic genotypes, but not in nonembryogenic ones or mature SE.	(Giroux and Pauls, 1997)
ASET2[1]	Putative membranal protein	Highly expressed in PEMs from embryogenic cultures.	
Met1	DNA methyl transferase	Accumulated in suspension-cultured cells, PEMs and to a lesser extent in developing SE. Highly expressed in apices and much less in other mature tissues.	(Bernacchia et al., 1998)
Met2	DNA methyl transferase		
PcGER1[2]	Germin-like	Highly expressed in embryogenic lines and quiescent ZE, but absent in non-embryogenic calli, or during germination.	(Neutelings et al., 1998)
Dcarg-1	Auxin-regulated genes	Expressed in hypocotyls after auxin treatment, but not during SE development.	(Kitamiya et al., 2000)
SERK	Receptor-like kinase	Expressed in hypocotyls in the presence of 2,4-D and up to the 100-celled globular embryo. In plants it is expressed in flowers 3 and 20 days after pollination.	(Schmid et al., 1997)

Cloned and studied in: [1]*Medicago sativa* and [2]*Pinus caribaea*. SE: somatic embryos; ZE: zygotic embryos.

Conversely, de Vries (1998) argued that due to the observation of enhanced somatic embryogenesis in other *Arabidopsis* mutants, many different genes may be involved in the acquisition of embryonic capacity by somatic cells and proposed that the effect of the ectopic expression of LEC1 may be due to a prolonged embryonic state and a delay in activating post embryonic programs. This last suggestion contradicts the observation of concomitant expression of genes characteristic of embryogenesis and germination mentioned above.

Homeosis in plants often acts in a coordinated fashion through functional interactions between homeotic genes to specify organ identity. In *Arabidopsis*, for example, the developmental program that follows shoot meristem formation, to produce either flowers or vegetative shoots, is controlled by a delicate equilibrium amongst several genes (Weigel and Meyerowitz, 1994; Pidkowich et al., 1999). It is probable that the ectopic expression of LEC genes provokes an imbalance among the products of those genes responsible for the acquisition of embryogenic competence by somatic cells and those that specify the somatic program in such cells.

Future studies on the interrelationship between LEC genes and other embryonic genes will clarify this point. It would be very helpful to identify the target genes for LEC proteins and how these genes are activated.

A gene encoding a protein homologous to a leucine-rich repeat containing a receptor-like kinase was cloned from embryogenic carrot cultures. The deduced protein possessed characteristics of a protein kinase and when it was expressed in bacteria, it was able to autophosphorylate. The gene (named SERK, for Somatic Embryogenesis Receptor Kinase) was expressed 7 days after culture of the hypocotyls in the presence of 2,4-D and up to the 100-celled globular embryos, whereas in plant organs it is only expressed in flowers between 3 and 20 days following pollination (Schmid et al., 1997). In *Dactylis glomerata*, SERK is continuously expressed in the shoot apical meristem, transiently expressed in the protoderm, coleoptile and coleorhiza; whereas in embryogenic cultures, it was not expressed beyond the globular stage nor in nonembryogenic cultures (Somleva et al., 2000). As stated by Schmid et al. (1997), SERK may belong to a mechanism involved in the embryogenic competence acquisition by somatic cells. Therefore, it is not incongruous, to think that a transcription factor is needed to allow the expression of the genes responsible to shelter the change from a somatic to a zygotic program. This transcription factor may be activated after perceiving a signal by a protein with similar characteristics to SERK. Unfortunately, it is still unknown which proteins can be phosphorylated by SERK and what signal (ligand) provokes their action.

STAGE TRANSITION: PATTERNING AND ORGAN FORMATION

Once the acquisition of embryogenic competence is completed, the embryogenic cells begin to divide and organize to establish the embryo pattern. While cell division is well defined in zygotic embryogenesis (Mordhorst et al., 1997), it appears that cells can either divide, symmetrically or asymmetrically, during somatic embryogenesis (De Jong et al., 1993a; Quiroz-Figueroa et al., 2002).

A developing embryo consists of a growing population of cells whose ability to form a functional organism depends on their relative position inside the embryo (Laux and Jürgens, 1997). It requires the coordinated action of several genes and/or their products to establish the particular cell function/differentiation program. Early embryogenic cells appear not to be strongly determined and they are vulnerable to physiological or biochemical perturbations (Carman, 1990). Therefore, the completion of successful embryogenesis and seed maturation depends on the expression and modulation of a large number of genes. In *Arabidopsis thaliana*, for example, more than 40 genes appear to control the formation of the zygotic embryo axis (Dodeman et al., 1997). The transition from a developmental stage towards others with a higher degree of differentiation (from globular to heart-shaped or from heart-shaped to torpedo, for example), requires the activation of a set of genes that guarantee the specificity and the implementation of such processes.

The so-called "master regulatory genes" are considered as the initiators of an activating cascade, resulting in biochemical and molecular changes occurring during embryogenesis (Wurtele et al., 1993). Homeobox-containing genes belong to such a class of genes that start the body plan and control the development of many eukaryotic organisms. The homeobox encodes a polypeptide of about 60 amino acids with a specific DNA binding function (Gehring, 1987). Kawahara et al. (1995) isolated six homeobox-containing genes from carrot: one of them (CHB2) accumulated specifically at the heart-shaped and early torpedo stages during SE, and in cotyledons of zygotic embryos. Thus, CHB2 expression was arrested by the

addition of 2,4-D, a potent inhibitor of embryogenesis in carrot. Three other genes, CHB3, CHB4 and CHB5, were located in the innermost cell layer of the torpedo embryos; whereas CHB6 was detected in immature vascular cells from heart-shaped embryos to postembryonic stages. It is probable that these homeobox-containing genes are involved in specifying the identity of vascular tissue (Hiwatashi and Fukuda, 2000). Similarly, in soybean, a homeobox-containing gene was cloned. It was present during proliferation of somatic embryos and early development, particularly during embryo elongation and cotyledon initiation, implying a role as a transcription factor which controls the formation of vascular tissues (Ma et al., 1994). Other genes with putative regulatory functions during stage transition include CUS1 (a MADS-box gene) (Filipecki et al., 1997) and DcDB1 (a chromo box gene) (Kiyosue et al., 1998).

EP2 encode a 12.5 kDa protein, similar to several lipid transfer proteins (LTP). It is expressed in embryogenic cultures of carrot, as well as in proliferating somatic embryos, independent of cellular density and the presence on 2,4-D. However, no expression of EP2 was observed in non-embryogenic cultures. In plants, EP2 was only detected in the shoot apex, inflorescences and in maturing seeds. In developing zygotic embryos, EP2 is expressed in the suspensor and protodermal cells of embryos. EP2 expression occurs very early in zygotic embryos; in later stages, high levels of expression are observed when protoderm had became epidermis. This expression pattern is restricted to the epidermis of developed cotyledons and it is completely absent in mature seeds. EP2 seems to be limited to the epidermis during the early development of particular organs and tissues, and to the periphery of globular and heart-shaped somatic embryos, specifically in protodermal cells (Sterk et al., 1991). The correlation that exists between EP2 expression and the embryogenic potential of cellular cultures of carrot is very high and has lead to the suggestion that the expression of EP2 marks the acquisition of, and is a measure for, the embryogenic competence in suspension cultures of carrot.

The gene AtLTP1 (*A. thaliana* lipid transfer protein 1), homologous to EP2, has been studied in *Arabidopsis*. Expression of the construct AtLTP1:luciferase in

transgenic carrot totally and faithfully reflects the endogenous expression of the EP2 carrot gene, under tissue culture conditions. A clear correlation between the expression of the construct and the development of SE was observed (Toonen et al., 1997), suggesting a universal mode of action for this lipid transfer protein. Additionally, in maize, the expression of a LTP mRNA has also been observed in early zygotic embryos, in the endosperm and during early germination, where it can function in the synthesis of storage lipids and membrane biogenesis of the newly-formed tissues (Sossountzov et al., 1991).

It has been proposed that LTP is involved in protoderm formation (Sterk et al., 1991; Thoma et al., 1994). Consequently the expression of this protein is expected to appear wherever the protoderm is formed, independent of the occurrence of embryogenesis.

Another cloned gene, EP3, has a putative function in the stage transition of developing somatic embryos. It encodes a 32 kDa extracellular endochitinase isolated from embryogenic suspension cultures of carrot by its ability to rescue the temperature-sensitive mutant ts11, in which embryos are arrested at the globular stage (De Jong et al., 1992). Ts11 was first described as defective on the glycosylation of extracellular proteins (Lo Schiavo et al., 1990). The main morphological effect of EP3 in ts11 embryos appeared to be in the restoration of protoderm which was altered when embryogenesis was induced at the non-permissive temperature of 32°C (Baldan et al., 1997). The defect appeared to be the result of an inappropriate quantity of EP3 secreted by the mutant line, rather than to differences in the physico-chemical properties of the endochitinase (De Jong et al., 1995). It has been suggested that in the mutant ts11, the regulation of the gene coding for the chitinase is altered. Cytohystological analysis showed alterations in the cell division planes and in the organization in the protoderm. Alterations were also observed in the endomembranal system and in the protein secretory pathways (Baldan et al., 1997).

It has been proposed that a basic endochitinase cloned from *Picea glauca* showing a transient expression along the course of the embryogenic process, could be involved in SE in this species (Dong and Dunstan, 1997a).

Ts11 can also be rescued by a Nod factor (De Jong et al., 1993b), whereas in *P. abies,* Nod factors stimulate somatic embryogenesis, mimicking the effect of growth regulators in the promotion of cell division of protoplasts (Dyachok et al., 2000). On the other hand, chitinases can cleave Nod factors in a very specific manner (Schultze et al., 1998), but to date, no active Nod factors or N-acetyl glucosamine oligomers that may provide a substrate for chitinases have been reported in plants (Van Hengel et al., 2001).

The presence of glucosamine in arabinogalactoproteins (AGPs) was first reported by van Holst et al. (1981b). These AGPs can reinitiate embryogenic cell formation in carrot (Kreuger and Van Holst, 1993) and reestablish somatic embryo formation in non-embryogenic cell (McCabe et al., 1997). Conversely, Domon et al. (2000), have reported that a 48 kDa basic chitinase-like protein can hydrolyze AGPs from embryogenic tissues or seeds. It is attractive to postulate that AGPs can be the substrates for chitinases and then liberate lipochitooligosaccharide-like molecules, which can lead to the transition from globular to heart-shaped somatic embryos. However, as reported by van Hengel et al. (2001), chitinase probably activates AGPs after cleavage, which suggests that the whole molecule may be the signal rather than a resulting chitinaseous molecule.

The data analyzed here draws attention to an alternative role for chitinases in plants, in addition to its earlier attributed function in defense. Biochemical implications of chitinase participation during a morphogenetic process are obvious, and most components of a signal transduction mechanism have been established. For example, chitinase genes can be induced by N-acetyl chitooligosaccharides, depending on the size and structure of the elicitor molecule (Nishizawa et al., 1999) and a putative receptor for that elicitor molecule has been identified in the plasma membrane of rice cells (Ito et al., 1997). It is still unknown if there is a direct relationship between this receptor and the activation of chitinase genes by

chitooligosaccharides. Additional work is needed to shed light on the function of chitinases during somatic embryogenesis and to determine its position on a signal transduction cascade required for the transition of embryos from globular to heart-shaped stages through the correct positioning of cellular division planes.

MATURATION

Several genes preferentially expressed during SE code for proteins, which are characteristic of the late stages of zygotic embryos and seed development (Table II). The most studied proteins of this class belong to the LEA family.

LEA (Late Embryogenesis Abundant) proteins are very hydrophilic and abundantly expressed in the course of late stages of zygotic and somatic embryogenesis of numerous species. An important characteristic of LEA genes is the presence of ABA Responsive Elements (ABRE) which suggests a role in the protection of cellular structures during the embryo desiccation (Dure et al., 1989). ABA can exquisitely modulate the promoter activity of some genes (Vivekananda et al., 1992), while other LEA-like genes show stereospecific preference for natural ABA enantiomers (Dong and Dunstan, 1997b).

Thomas (1993b) proposed the existence of a bipartite promoter which allows a hierarchical regulation of ABREs located in its proximal and distal regions. In wheat, ABRE possesses a CACGTGGC sequence that is bound by a leucine zipper present in the transcription factor EMBP-1 (Guiltinan et al., 1989). Other classes of ABRE found in LEA genes are fairly similar to that in wheat with a consensus nucleus ACGT, frequently associated to the leucine zipper motifs of plant transcription factors (Schindler et al., 1992).

The DC8 gene (Choi et al., 1987) encodes a 60.2 kDa protein with an estimated isoelectric point of 6.47. In its sequence, there is a potential glycosylation site (Asn-X-Thr/Ser). Hydropathic analyses have shown that DC8 codes for a hydrophobic protein (Franz et al., 1989). DC8 protein was localized in the cytoplasm, protein bodies and cell walls of developing zygotic embryos and in the endosperm of carrot seeds. In developing young tissues, it was predominantly

located in vacuoles, vesicles and components of the endomembranal system, while in mature tissues it was present in protein bodies and cell walls. No DC8 was found in the nucleus, lipid bodies, plastids or mitochondria (Franz et al., 1989), in mature leaves (Hatzopoulos et al., 1990; Cheng et al., 1996), or in the root cortex, apical meristems or petioles, even after the addition of ABA. Only tissues with embryogenic competence or embryo-derived tissues (such as cotyledons) accumulated DC8 protein. In such tissues, ABA could increase the expression levels of DC8 up to 100 fold in 24 hours (50% of this increment was accomplished within 15 minutes) (Hatzopoulos et al., 1990).

The effect of ABA in DC8 mRNA accumulation suggests the existence of transcriptional regulation (Hatzopoulos et al., 1990). The DC8 promoter is ABA-inducible in young plants, but not in older plants or leaves, probably due to the loss of a seed specific factor required for ABA responsiveness. Activation of the DC8 promoter has been observed in tobacco and *Arabidopsis* seeds, but not in ABA-treated leaves (Cheng et al., 1996).

Regardless of the estimated DC8 promoter length (2,600 bp), a 305 bp fragment located upstream of the transcription start, is enough to promote its expression and the correct spatial and temporal regulation during development (Cheng et al., 1996). Methylation of the DC8 promoter diminishes the expression of the gene; whereas treatments with 5-Aza C (a methylation inhibitor) increase its expression levels (Zhou et al., 1996).

Induction of DC8 expression by ABA can be regulated by two elements located in the promoter which are required for reaching a high expression level and an increment in stimulation by ABA. One of those elements is located at -170 and -51 and contains three copies of the sequence ACGT, associated to leucine zipper binding elements in transcription factors (see above). The other one is located further upstream (-505 and -301) and its function can be substituted by sequences upstream of base -505 (Goupil et al., 1992).

Table II. Some cloned genes with enhanced expression during later stages of SE.

Gene	Homology/Feature	Expression	References
DC8	LEA	Expressed in ZE and SE, young and mature tissues and poorly in calli. ABA inducible in SE and young plants.	(Choi et al., 1987; Franz et al., 1989; Hatzopoulos et al., 1990; Cheng et al., 1996)
DC3	LEA	Poorly expressed in calli and highly in PEMs and SE. Inducible by ABA in SE and vegetative tissues.	(Wilde et al., 1988; Vivekananda et al., 1992)
EMB-1	LEA	Detectable in calli and accumulated in SE and seeds.	(Wurtele et al., 1993)
ECP31 ECP40	LEA	Both abundantly expressed in PEMs and detectable in SE. Highly expressed in ZE at heart stage. ABA-inducible in SE.	(Kiyosue et al., 1992; 1993)
PgEMB12[1] PgEMB14 PgEMB15	LEA	Accumulated at high levels in cotyledons of SE and differentially regulated by ABA enantiomers.	(Dong and Dunstan, 1996a; 1997b)
Mat1[2]	Lipoxygenase	Expressed in desiccated SE and ZE. Decreased after rehydration.	(Liu et al., 1994)
PM 2.1[3]	Metallothionein	Expressed in SE at the cotyledonary stage. In ZE it is preferentially expressed in mid-embryogenesis and in somatic tissues of seedlings. ABA, osmoticum and metal ions modulate its expression in SE.	(Chatthai et al., 1997)
PM2S1, 2, 3 and 4[3]	Seed storage protein	Expressed during the development of ZE. Highly expressed in seeds. In SE maximal expression is at the cotyledonary stage. ABA and osmoticum modulate its expression in SE.	(Chatthai and Misra, 1998)
C-ABI3	Homologous to ABI from *Arabidopsis* and VP1 of maize	Expressed in developing SE and ZE as well as in seeds.	(Shiota et al., 1998)
Gea8	Globulin-like	Strongly induced at globular and heart stages of SE. Detectable in embryogenic calli. In ZE, the pattern is similar to SE, but detectable in all stages of ZE development. It is also expressed in the endosperm.	(Lin et al., 1996; Lin and Zimmerman, 1999)

Cloned and studied in: [1]*Picea glauca*, [2]*Glycine max*, [3]*Pseudotsuga menziesii*; SE: somatic embryos; ZE: zygotic embryos.

The expression of DC8 in young plants has led to the suggestion that it is more closely related to the growth rate than to the developmental stages of embryos (Cheng et al., 1996). In general, LEA genes of carrot isolated to date, are highly homologous and their proteins are structurally similar (Dure et al., 1989), suggesting a conserved function for these proteins.

Many other LEA genes are highly expressed in embryogenic cells during the induction of somatic embryogenesis, which may be due to the high levels of endogenous ABA detected in the embryogenic cell clusters (Kiyosue et al., 1992).

HELPING TO FORM A SOMATIC EMBRYO

Some genes, coding for low molecular weight heat shock proteins (LMWHSP), are expressed during the induction of somatic embryogenesis. Early embryogenesis overtakes a developmental period, in which the regulation of heat shock proteins (HSP) changes from a transcriptional to a translational level (Apuya and Zimmerman, 1992) and thereafter, during advanced stages of development, it returns to the transcriptional level. Superimposed on this change in the regulation level, there is a dramatic and precise arrest of embryo development in response to heat shock (Zimmerman et al., 1989).

Exposure of carrot globular embryos to a heat shock of 37°C for two to three hours can permanently block their development. Apart from an increase in the size of embryos, they were unable to progress beyond the heart-shaped stage. A negligible accumulation of the heat shock protein (HSP) HS 17.5 mRNA was observed in globular embryos, less than in calli or other embryo stages. However, despite of this, translation seems to be much more efficient in globular embryos allowing them to synthesize the same amount of HSP 17.5 as calli do (Zimmerman et al., 1989). A heat shock-independent expression of a gene coding for a LMWHSP was observed during the development of somatic embryos in alfalfa. This gene showed a transient expression and was no longer detectable in torpedo embryos; whereas in non-induced suspension cultures, it was only expressed as a response to a heat shock (Györgyey et al., 1991).

In *Picea glauca*, two genes homologous to LMWHSP were cloned (PgEMB 27, coding for hsp 17.0 and PgEMB 29, coding for hsp 17.1). Both belong to class II or cytoplasmic HSP. A third gene, PgEMB 22 (coding for hsp 23.5) is a putative mitochondrial protein encoded by a nuclear gene. After exposure for 2 h at 37°C, HSP 17.1 mRNA increased 365 fold and HSP 23.5 mRNA increased between 5 and 6 fold at temperatures between 37 and 43°C compared to levels at 22°C. Expression of both genes was also increased when embryos were treated with ABA or PEG (Dong and Dunstan, 1996b).

The HSP are evolutionarily conserved, suggesting that they may be involved in fundamental cellular processes and in protecting the cell from stress damages. While HSP70 is predominant in most eukaryotes, the most abundant HSPs in plants are proteins of low molecular weight (10 to 30 kDa) (Dong and Dunstan, 1996b). The LMWHSP of plants have been implicated in the acquisition of thermotolerance (reviewed by Yeh et al., 1994) and in the protection of mitochondria from oxidative injury (Banzet et al., 1998). An attractive role for LMWHSP in assisting protein trafficking between cytoplasm and membranes of organelles has been hypothesized by Coca et al., (1994).

Based on the function as chaperones, that has been assigned to HSPs (see Waters et al., 1996), they could be acting during the synthesis of macromolecules needed during embryogenesis. The existence of factors mimicking those elements needed for the activation of HS genes could be an optional explanation on why HS gene expression at some stages during a morphogenetic process is altered.

On the basis of sequence divergences of heat shock transcription factors, the regulation of HS genes by signals different from heat stress has been proposed by Waters et al. (1996). A protecting role of zygotic embryo structures during desiccation has been attributed to HSP given the coordinated accumulation of LEA and LMWHSP transcripts and its induction by ABA and osmotic agents (Almoguera and Jordano, 1992; Coca et al., 1996). In tobacco, a gene coding for a LMWHSP was induced in pollen by starvation, pollen development and embryogenesis. In pollen-derived embryos, expression was only observed during early development,

that is, in globular and heart-shaped stages, but not in early cotyledonary embryos (Zarsky et al., 1995). Finally, a gene was cloned from carrot that encodes an LMWHSP, whose expression is modulated by 2, 4-D (Kitamiya et al., 2000).

More research is needed to shed light on the role of these genes during embryogenesis, in order to answer questions like: What is the true function of HSP during embryogenesis? Why HSPs are sometimes expressed in the absence of heat shock? What is the nature of the factors, if any, mimicking HS elements? How are HS genes activated during embryogenesis?

FUTURE PERSPECTIVES

Although SE was first described more than 40 years ago, it is still a poorly understood process where empiric manipulations have played a crucial role (Santana, 1993). Somatic embryos do differ from zygotic embryos, often lacking a suspensor and being raised from morphologically dissimilar cellular clusters (Toonen et al., 1994), which demonstrates that the development of somatic embryos implies more variables than that of zygotic embryos (Mordhorst et al., 1997).

Emerging data on the expression of genes needed during embryogenesis, and on their regulation will be very helpful to the understanding of this morphogenetic process. As discussed in the present review, many genes with embryo-enhanced expression have been cloned and the products encoded by those genes are quite variable. This makes it difficult to integrate the results obtained into a reliable model to explain the acquisition of embryogenic competence and embryo development. Several questions dealing with somatic embryogenesis remain to be determined and more molecular and biochemical research is needed to understand the basic aspect of morphogenesis.

Finally, the understanding of the somatic embryogenesis process will impulse the development of new biotechnological approaches, such as the massive propagation of F1 hybrids and more efficient methods for the regeneration of transgenic plants.

ACKNOWLEDGEMENTS

Authors are indebted to Consejo Nacional de Ciencia y Tecnología, México by grants 4123P-N and 31816-N and post-graduated scholarships to RRH (117155), FQF (116916) and LST (118132).

REFERENCES

Almoguera, C. and Jordano, J. (1992). Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNA. Plant Mol. Biol. *19*, 781-792.

Apuya, N.R. and Zimmerman, J.L. (1992). Heat shock gene expression is controlled primarily at the translational level in carrot cells and somatic embryos. Plant Cell *4*, 657-665.

Baldan, B., Guzzo, F., Filippini, F., Gasparian, M., LoSchiavo, F., Vitale, A., De Vries, S.C., Mariani,P., and Terzi,M. (1997). The secretory nature of the lesion of carrot cell variant ts11, rescuable by endochitinase. Planta *203*, 381-389.

Banzet, N., Richaud, C., Deveaux, Y., Kazmaier, M., Gagnon, J., and Triantaphylidès,C. (1998). Accumulation of small heat shock proteins, including mitochondrial HSP22, induced by oxidative stress and adaptive response in tomato cells. Plant J. *13*, 519-527.

Bernacchia, G., Primo, A., Giorgetti, L., Pitto, L., and Cella, R. (1998). Carrot DNA-methyltransferase is encoded by two classes of genes with differing patterns of expression. Plant J. *13*, 317-329.

Borkird, C., Choi, J.H., and Sung, Z.R. (1986). Effect of 2,4-dichlorophenoxyacetic acid on the expression of embryogenic program in carrot. Plant Physiol. *81*, 1143-1146.

Carman, J.G. (1990). Embryogenic cells in plant tissue cultures: Occurrence and behavior. In Vitro Cell. Dev. Biol. *26*, 746-753.

Chatthai, M., Kaukinen, K.H., Tranbarger, T.J., Gupta, P.K., and Misra, S. (1997). The isolation of a novel metallothionein-related cDNA expressed in somatic and zygotic embryos of Douglas-fir: regulation by ABA, osmoticum and metal ions. Plant Mol. Biol. 34: 243-254.

Chatthai, M. and Misra, S. (1998). Sequence and expression of embryogenesis-specific cDNAs encoding 2S seed storage proteins in *Pseudotsuga menziesii* [Mirb.] Franco. Planta *206*, 138-145.

Cheng, J.C., Seeley, K.A., Goupil, P., and Sung, Z.R. (1996). Expression of DC8 is associated with, but not dependent on embryogenesis. Plant Mol. Biol. *31*, 127-141.

Choi, J.H., Liu, L.S., Borkird, C., and Sung, Z.R. (1987). Cloning of genes developmentally regulated during plant embryogenesis. Proc. Natl. Acad. Sci. (USA) *84*, 1906-1910.

Coca, M.A.,Almoguera, C and Jordano, J (1994). Expression of sunflower low-molecular-weight heat-shock proteins during embryogenesis and persistence after germination: localization and possible functional implications. Plant Mol. Biol. *25*, 479-492.

Coca, M.A., Almoguera, C., Thomas, T.L., and Jordano, J. (1996). Differential regulation of small heat-shock genes in plants: Analysis of a water-stress-inducible and developmentally activated sunflower promoter. Plant Mol. Biol. *31*, 863-876.

De Jong, A.J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., Van Kammen, A., and De Vries, S.C. (1992). A carrot somatic embryo mutant is rescued by chitinase. Plant Cell *4*, 425-433.

De Jong, A.J., Heidstra, R., Spaink, H.P., Hartog, M.V., Meijer, E.A., Hendriks, T., Lo Schiavo, F., Terzi, M., Bisseling, T., Van Kammen, A., and De Vries, S.C. (1993b). *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. Plant Cell *5*, 615-620.

De Jong, A.J., Hendriks, T., Meijer, E.A., Penning, M., Lo Schiavo, F., Terzi, M., Van Kammen, A., and De Vries, S.C. (1995). Transient reduction in secreted 32 kD chitinase prevents somatic embryogenesis in the carrot (*Daucus carota* L.) variant ts11. Devel. Genet. *16*, 332-343.

De Jong, A.J., Schmidt, E.D.L., and De Vries, S.C. (1993a). Early events in higher-plant embryogenesis. Plant Mol. Biol. *22*, 367-377.

De Vries, S.C. (1998). Making embryos in plants. Trends Plant Sci. *3*, 451-452.

De Vries, S.C., Booij, H., Meyerink, P., Huisman, G., Wilde, H.D., Thomas, T.L., and Van Kammen, A. (1988). Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta *176*, 196-204.

Dodeman, V.L., Ducreux, G., and Kreis, M. (1997). Zygotic embryogenesis *versus* somatic embryogenesis. J. Exp. Bot. *48*, 1493-1509.

Domon, J.M., Neutelings, G., Roger, D., David, A., and David, H. (2000). A basic chitinase-like protein secreted by embryogenic tissues of *Pinus caribaea* acts on arabinogalactan proteins extracted from the same cell lines. J. Plant Physiol. *156*, 33-39.

Dong, J.Z. and Dunstan, D.I. (1996a). Expression of abundant mRNAs during somatic embryogenesis of white spruce [*Picea glauca* (Moench) Voss]. Planta *199*, 459-466.

Dong, J.Z. and Dunstan, D.I. (1996b). Characterization of three heat-shock-protein genes and their developmental regulation during somatic embryogenesis in white spruce [*Picea glauca* (Moench) Voss]. Planta *200*, 85-91.

Dong, J.Z. and Dunstan D.I. (1997a). Endochitinase and β-1,3-glucanase genes are developmentally regulated during somatic embryogenesis in *Picea glauca*. Planta *201*, 189-194.

Dong, J.Z. and Dunstan, D.I. (1997b). Characterization of cDNAs representing five abscisic acid- responsive genes associated with somatic embryogenesis in *Picea glauca*, and their responses to abscisic acid stereostructure. Planta *203*, 448-453.

Dong, J.Z. and Dunstan, D.I. (1999). Cloning and characterization of six embryogenesis-associated cDNAs from somatic embryos of *Picea glauca* and their comparative expression during zygotic embryogenesis. Plant Mol. Biol. *39*, 859-864.

Dudits, D., Bögre, L., and Györgyey, J. (1991). Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. J. Cell Sci. *99*, 473-482.

Dure, L.I., Crouch, M., Harada, J., David-Ho, T., Mundy, J., Quatrano, R., Thomas, T., and Sung, Z.R. (1989). Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol. Biol. *12*, 475-486.

Dyachok, J.V., Tobin, A.E., Price, N.P.J., and Von Arnold, S. (2000). Rhizobial Nod factors stimulate somatic embryo development in *Picea abies*. Plant Cell Rep. *19*, 290-297.

Filipecki, M.K., Sommer, H., and Malepszy, S. (1997). The MADS-box gene *CUS1* is expressed during cucumber somatic embryogenesis. Plant Sci. *125*, 63-74.

Franz, G., Hatzopoulos, P., Jones, T.J., Kraus, M., and Sung, Z.R. (1989). Molecular and genetic analysis of an embryonic gene, DC 8, from *Daucus carota* L. Mol. Gen. Genet. *218*, 143-151.

Gehring, W.J. (1987). Homeo boxes in the study of development. Science *236*, 1245-1252.

Giroux, R.W. and Pauls, K.P. (1997). Characterization of somatic embryogenesis-related cDNAs from alfalfa (*Medicago sativa* L). Plant Mol. Biol. *33*, 393-404.

Goldberg, R.B., Barker, S.J., and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. Cell *56*, 149-160.

Goldberg, R.B., De Paiva, G., and Yadegari, R. (1994). Plant embryogenesis: Zygote to seed. Science *266*, 605-614.

Goupil, P., Hatzopoulos, P., Franz, G., Hempel, F.D., You, R., and Sung, Z.R. (1992). Transcriptional regulation of a seed-specific carrot gene, DC8. Plant Mol. Biol. *18*, 1049-1063.

Guiltinan, M.J., Thomas, J.C., Nessler, C.L., and Thomas, T.L. (1989). Expression of DNA binding proteins in carrot somatic embryos that specifically interact with a *cis* regulatory element of the french bean phaseolin gene. Plant Mol. Biol. *13*, 605-610.

Györgyey, J., Gartner, A., Németh, K., Magyar, Z., Hirt, H., Herbele-Bors, E., and Dudits,D. (1991). Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol. Biol. *16* , 999-1007.

Halperin, W. (1966). Alternative morphogenetic events in cell suspensions. Am. J. Bot. *53*, 443-453.

Hatzopoulos, P., Fong, F., and Sung, Z.R. (1990). Abscisic acid regulation of DC8, a carrot embryonic gene. Plant Physiol. *94*, 690-695.

Hiwatashi, Y. and Fukuda, H. (2000). Tissue-specific localization of mRNA for carrot homeobox genes, *CHBs*, in carrot somatic embryos. Plant Cell Physiol. *41*, 639-643.

Ito, Y., Kaku, H., and Shibuya, N. (1997). Identification of a high-affinity binding protein for N-acetylchitooligosaccharide elicitor in the plasma membrane of suspension-cultured rice cells by affinity labeling. Plant J. *12*, 347-356.

Kawahara, R., Komamine, A., and Fukuda, H. (1995). Isolation and characterization of homeobox-containing genes of carrot. Plant Mol. Biol. *27*, 155-164.

Kitamiya, E., Suzuki, S., Sano, T., and Nagata, T. (2000). Isolation of two genes that were induced upon the initiation of somatic embryogenesis on carrot hypocotyls by high concentrations of 2,4-D. Plant Cell Rep. *19*, 551-557.

Kiyosue, T., Shiota, H., Higashi, K., Kamada, H., and Shinozaki, K. (1998). A chromo box gene from carrot (*Daucus carota* L.): its cDNA structure and expression during somatic and zygotic embryogenesis. Biochim. Biophys. Acta *1398*, 42-46.

Kiyosue, T., Yamaguchi-Shinozaki, K., Shinozaki, K., Higashi, K., Satoh, S., Kamada, H., and Harada, H. (1992). Isolation and characterization of a

cDNA that encodes ECP31, an embryogenic-cell protein from carrot. Plant Mol. Biol. *19*, 239-249.

Kiyosue, T., Yamaguchi-Shinozaki, K., Shinozaki, K., Kamada, H., and Harada, H. (1993). cDNA cloning of ECP40, an embryogenic-cell protein in carrot, and its expression during somatic and zygotic embryogenesis. Plant Mol. Biol. *21*, 1053-1068.

Komamine, A. (1992). Mechanism of SE in cell cultures: physiology, biochemistry and molecular biology. In Vitro Cell. Dev. Biol. *28*, 11-14.

Kreuger, M. and Van Holst, G.J. (1993). Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta *189*, 243-248.

Krikorian, A.D. and Simola, L.K. (1999). Totipotency, somatic embryogenesis, and Harry Waris (1893-1973). Physiol. Plantarum *105*, 348-355.

Laux, T. and Jürgens, G. (1997). Embryogenesis: A new start in life. Plant Cell *9*, 989-1000.

Lin, X.Y., Hwang, G.J.H., and Zimmerman, J.L. (1996). Isolation and characterization of a diverse set of genes from carrot somatic embryos. Plant Physiol. *112*, 1365-1374.

Lin, X.Y. and Zimmerman, J.L. (1999). Expression of a globulin-like protein gene, *Gea8*, in somatic and zygotic embryos. J. Exp. Bot. *50*, 1139-1147.

Liu, W., Hildebrand, D.F., Moore, P.J., and Collins, G.B. (1994). Expression of desiccation-induced and lipoxygenase genes during the transition from maturation to the germination phase in soybean somatic embryos. Planta *194*, 69-76.

LoSchiavo, F., Giuliano, G., De Vries, S.C., Genga, A., Bollini, R., Pitto, L., Cozzani, F., Nuti-Ronchi, V., and Terzi, M. (1990). A carrot cell variant temperature sensitive for somatic embryogenesis reveals a defect in the glycosylation of extracellular proteins. Mol. Gen. Genet. *223*, 385-393.

LoSchiavo, F., Pitto, L., Giuliano, G., Torti, G., Nuti-Ronchi, V., Marazziti, D., Vergara, R., Orselli, S., and Terzi, M. (1989). DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor. Appl. Genet. *77*, 325-331.

Lotan, T., Ohto, M., Matsudaira, Y.K., West, M.A.L., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (1998). *Arabidopsis leafy cotyledon1* is sufficient to induce embryo development in vegetative cells. Cell *93*, 1195-1205.

Ma, H., McMullen, M.D., and Finer, J.J. (1994). Identification of a homeobox-containing gene with enhanced expression during soybean (*Glycine max* L.) somatic embryo development. Plant Mol. Biol. *24*, 465-473.

McCabe, P.F., Valentine, T.A., Scott, F.L., and Pennell, R.I. (1997). Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot. The Plant Cell *9*, 2225-2241.

Meinke, D.W. (1992). A homeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. Science *258*, 1647-1649.

Meinke, D.W., Franzmann, L.H., Nickle, T.C., and Yeung, E.C. (1994). Leafy cotyledons mutants of Arabidopsis. Plant Cell *6*, 1049-1064.

Mordhorst, A.P., Toonen, M.A.J., and De Vries, S.C. (1997). Plant embryogenesis. Crit. Rev. Plant Sci. *16*, 535-576.

Neutelings, G., Domon, J.M., Membré, N., Bernier, F., Meyer, Y., David, A., and David,H. (1998). Characterization of a germin-like protein gene expressed in somatic and zygotic embryos of pine (*Pinus caribaea* Morelet). Plant Mol. Biol. *38*, 1179-1190.

Nishizawa, Y., Kawakami, A., Hibi, T., He, D.Y., Shibuya, N., and Minami, E. (1999). Regulation of the chitinase gene expression in suspension-cultured rice cells by *N*-acetylchitooligosaccharides: differences in the signal transduction pathways leading to the activation of elicitor-responsive genes. Plant Mol. Biol. *39*, 907-914.

Pidkowich, M.S., Klenz, J.E., and Haughn, G.W. (1999). The making of a flower: control of floral meristem identity in *Arabidopsis*. Trends Plant Sci. *4*, 64-70.

Quiroz-Figueroa, F. R., Fuentes-Cerda, C. F. J., Rojas-Herrera, R., and Loyola-Vargas, V. M. Histological studies on ontogenesis, development stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. Plant Cell Rep. 2002. In Press

Santana, N. (1993). Embriogénesis somática en el cultivo del cafeto (*Coffea* sp.). (La Habana: INCA), pp. 1-154.

Sato, S., Toya, T., Kawahara, R., Whittier, R.F., Fukuda, H., and Komamine, A. (1995). Isolation of a carrot gene expressed specifically during early-stage somatic embryogenesis. Plant Mol. Biol. *28*, 39-46.

Schindler, U., Beckmann, H., and Cashmore, A.R. (1992). TGA1 and G-box binding factors: two distinct classes of *Arabidopsis* leucine zipper proteins compete for the G-box-like element TGACGTGG. Plant Cell *4*, 1309-1319.

Schmid, E.D.L., Guzzo, F., Toonen, M.A.J., and De Vries, S.C. (1997). A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development *124*, 2049-2062.

Schultze, M., Staehelin, C., Brunner, F., Genetet, I., Legrand, M., Fritig, B., Kondorosi, E., and Kondorosi, A. (1998). Plant chitinase/lysozyme isoforms show distinct substrate specificity and cleavage site preference towards lipochitooligosaccharide Nod signals. Plant J. *16*, 571-580.

Shiota, H., Satoh, R., Watabe, K., Harada, H., and Kamada, H. (1998). *C-AB*13, the carrot homologue of the *Arabidopsis AB*13, is expressed during both zygotic and somatic embryogenesis and functions in the regulation of embryo-specific ABA-inducible genes. Plant Cell Physiol. *39*, 1184-1193.

Somleva, M.N., Schmidt, E.D.L., and De Vries, S.C. (2000). Embryogenic cells in *Dactylis glomerata* L. (Poaceae) explants identified by cell tracking and by SERK expression. Plant Cell Rep. *19*, 718-726.

Sossountzov, L., Ruiz-Avila, L., Vignols, F., Jolliot, A., Arondel, V., Tchang, F., Grosbois, M., Guerbette, F., Miginiac, E., Delseny, M., Puigdomenech, P., and Kader, J.-C. (1991). Spatial and temporal expression of a maize lipid transfer protein gene. Plant Cell *3*, 923-933.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. The Plant Cell *3*, 907-921.

Sterk, P. and De Vries, S.C. (1992). Molecular markers for plant embryos. In Synthetic Seeds. Applications of Synthetic Seeds to Crop Improvement, K.Redenbaugh, ed. (Boca Ratón: CRC Press), pp. 115-132.

Steward, F.C., Mapes, M.O., and Mears, K. (1958b). Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Am. J. Bot. *45*, 705-708.

Steward, F.C., Mapes, M.O., and Smith, J. (1958a). Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. Am. J. Bot. *45*, 693-703.

Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (2001). Leafy cotyledon encodes a B3 domain transcription factor that induces embryo development. Proc. Natl. Acad. Sci. (USA) *98*, 11806-11811.

Sung, Z.R. and Okimoto, R. (1981). Embryonic proteins in somatic embryos of carrot. Proc. Natl. Acad. Sci. (USA) *78*, 3683-3687.

Thoma, S., Hecht, U., Kippers, A., Botella, J., De Vries, S., and Somerville, C. (1994). Tissue-specific expression of a gene encoding a cell wall-localized lipid transfer protein from *Arabidopsis*. Plant Physiol. *105*, 35-45.

Thomas, T.L. (1993). Gene expression during plant embryogenesis and germination: An overview. Plant Cell *5*, 1401-1410.

Toonen, M.A.J., Hendriks, T., Schmidt, E.D.L., Verhoeven, H.A., Van Kammen, A., and De Vries, S.C. (1994). Description of somatic-embryo-forming single cells in carrot suspension cultures employing video cell tracking. Planta *194*, 565-572.

Toonen, M.A.J., Verhees, J.A., Schmidt, E.D.L., van Kammen, A., and De Vries, S.C. (1997). AtLTP1 luciferase expression during carrot somatic embryogenesis. Plant J. *12*, 1213-1221.

van Hengel, A.J., Tadesse, Z., Immerzeel, P., Schols, H., Van Kammen, A., and De Vries, S.C. (2001). N-acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis. Plant Physiol. *125*, 1880-1890.

van Holst, G.J., Klis, F.M., De Wildt, P., Hazenberg, C.A.M., Buijs, J., and Stegwee, D. (1981b). Arabinogalactan protein from a crude cell organelle fraction of *Phaseolus vulgaris* L. Plant Physiol. *68*, 910-913.

Vivekananda, J., Drew, M.C., and Thomas, T.L. (1992). Hormonal and environmental regulation of the carrot LEA-class gene Dc3. Plant Physiol. *100*, 576-581.

Waters, E.R., Lee, G.J., and Vierling, E. (1996). Evolution, structure and function of the small heat shock proteins in plants. J. Exp. Bot. *47*, 325-338.

Weigel, D. and Meyerowitz, E.M. (1994). The ABC of floral homeotic genes. Cell *78*, 203-209.

West, M.A.L., Yee, K.M., Danao, J., Zimmerman, J.L., Fischer, R.L., and Goldberg, R.B. (1994). Leafy cotyledon is an essential regulator of late embryogenesis and cotyledon identity in *Arabidopsis*. Plant Cell *6*, 1731-1745.

Wilde, H.D., Nelson, W.S., Booij, H., De Vries, S.C., and Thomas, T.L. (1988). Gene-expression programs in embryogenic and non-embryogenic carrot cultures. Planta *176*, 205-211.

Wilde, H.D., Seffens, W.S., and Thomas, T.L. (1995). Gene expression in somatic embryos. In Biotechnology in Agriculture and Forestry. Vol. 30. Somatic Embryogenesis and Synthetic Seed I, Y.P.S.Bajaj, ed. (Berlin: Springer-Verlag), pp. 41-52.

Wurtele, E.S., Wang, H., Durgerian, S., Nikolau, B.J., and Ulrich, T.H. (1993). Characterization of gene that is expressed early in somatic embryogenesis of *Daucus carota* . Plant Physiol. *102*, 303-312.

Yeh, K.-W., Jinn, T.-L., Yeh, C.-H., Chen, Y.-M., and Lin, C.-Y. (1994). Plant low-molecular-mass heat-shock proteins: their relationship to the acquisition of thermotolerance in plants. Biotech. Appl. Biochem. *19*, 41-49.

Zarsky, V., Garrido, D., Eller, N., Tupy, J., Vicente, O., Schöffl, F., and Heberle-Bors, E. (1995). The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Environ. *18*, 139-147.

Zhou, Y.X., Magill, J.M., Magill, C.W., and Newton R.J. (1996). DNA methylation and Dc8-GUS transgene expression in carrot (*Daucus carota* L). Plant Cell Rep. *15*, 815-818.

Zimmerman, J.L. (1993). Somatic embryogenesis: a model for early development in higher plants. The Plant Cell *5*, 1411-1423.

Zimmerman, J.L., Apuya, N., Darwish, K., and O´Carroll, C. (1989). Novel regulation of heat shock genes during carrot somatic embryo development. Plant Cell *1*, 1137-1146.

Chapter II

DIFFERENTIAL GENE EXPRESSION DURING SOMATIC EMBRYOGENESIS IN *Coffea arabica* L., REVEALED BY RT-PCR DIFFERENTIAL DISPLAY[0]

R. Rojas-Herrera[1, 2], F. Quiroz-Figueroa[1], M. Monforte-González[1] L. Sánchez-Teyer[1] and V. M. Loyola-Vargas[1] *.

[1]Unidad de Bioquímica y Biología Molecular de Plantas. Centro de Investigación Científica de Yucatán, Apartado Postal 87, Cordemex, Mérida, Yucatán. México.

[2]Departamento de Genética y Mejoramiento. Instituto Nacional de Ciencias Agrícolas. Gaveta Postal 1, San José de las Lajas, La Habana, Cuba.

*Corresponding author. email: vmloyola@cicy.mx

ABSTRACT

Molecular and biochemical studies of somatic embryogenesis may help to shed light on the mechanisms governing this phenomenon. In the present paper, a differential display analysis approach was employed to investigate the changes taking place during the induction of somatic embryogenesis in leaf explants and suspension cultures of coffee. Cloned fragments showed homologies to several proteins reported in databases, but only one of then has been previously described as regulated during somatic embryogenesis. By a modified reverse dot blot assay the expression pattern of such fragments was evaluated.

Key words: *Coffea arabica*, somatic embryogenesis, gene expression, differential display.

Abbreviations: SE: somatic embryogenesis, DD: differential display, RT-p DB: reverse transcription probed dot blot, MS: Murashige and Skoog basal medium, lmwHSP: low molecular weight heat shock proteins.

[0] Published in Molecular Biotechnology 20 (1): 43-50. 2002.

INTRODUCTION

Somatic embryogenesis (SE) has been recognized as an important model for studying the morphogenetic and regulatory events that take place during embryogenesis in higher plants (Zimmerman, 1993), as well as for regenerating and propagating plants of commercial significance.

While a zygote is intended to develop into an embryo and hence, it can be defined as an embryogenic cell, what is less understood are the changes a cell must accomplish to become embryogenic and thus, express the ability to form a complete embryo (Mordhorst et al., 1997). Since the first report of differential gene expression during carrot SE (Sung and Okimoto, 1981), several genes, expressed in developing embryos coding for diverse products, have been isolated (Dudits et al., 1995; Choi et al., 1987; Wilde et al., 1988; Kawahara and Komamine, 1995; Aleith and Richter, 1990). Most of them are preferentially expressed in the late stages of embryogenesis (see Thomas, 1993 and Wilde et al., 1995).

Based on statistical extrapolation, it has been calculated that in *A. thaliana*, more than 40 genes could be involved in the control of the formation of embryo axis (Dodeman et al., 1997). This idea is supported in the cloning of a large number of different genes with embryo-enhanced expression in *Daucus carota* (Lin et al., 1996), *Picea glauca* (Dong and Dunstan, 1996a) and *Medicago sativa* (Giroux and Pauls, 1997; Fowler et al., 1998).

Although coffee represents a highly valuable commercial crop and SE has been established since 1970 (Staritsky, 1970), there are no molecular or biochemical studies aimed at understanding and controlling SE in this species. In coffee, two embryogenetic methodologies have been described: the first one consists of several steps, which use different culture media and thus, embryos are produced from callus tissue after several months (Söndahl and Sharp, 1977). In the second one, a single culture medium supplemented with 5 µM benzyladenine is used (Yasuda et al., 1985), a minimum callus growth is observed and embryos are produced directly from the edge cells of explants (Quiroz-Figueroa et al., 2002).

In this paper, we present a survey by DD analysis, and the cloning of several gene fragments showing differential expression during the induction of both embryogenetic methodologies in *C. arabica*.

MATERIALS AND METHODS

Plant material. Embryos from seeds of *C. arabica* var. "Caturra Rojo" were germinated in MS medium (Murashige and Skoog, 1962), supplemented with thiamine (29.6 μM), myo-inositol (0.56 μM), biotin (0.41 μM), L-cysteine (0.15 μM), glucose (166.48 mM), NAA (0.53 μM), Kin (2.32 μM) and Gelrite® (0.25% w/v). Plantlets were grown in the same medium mentioned above, but glucose was substituted for sucrose (87.64 mM) and cultured under 16 h photoperiod at 25 ± 2°C.

Direct induction of SE: Leaves of plantlets were cut into fragments of approximately 0.25 cm^2 and inoculated in a medium containing 5 μM benzyladenine as sole growth regulator (Yasuda et al., 1985). Development of the embryos was scored under a stereoscope.

Indirect embryogenesis induction. Calli were obtained from plantlets as previously described by Söndahl and Sharp (1977). Suspension cultures were obtained by inoculating one or two g of calli in 50 ml of the same medium. The resulting suspensions consisted of clusters of cells (>60 μm to <5000 μm). These suspensions were maintained on a gyratory shaker at 100 rpm and 25 ± 2°C in the dark. To induce SE, the suspension cultures were transferred to the induction medium (Söndahl and Sharp, 1977) at a density of 10^{-3} g/ml and maintained on a gyratory shaker at 100 rpm and 25 ± 2°C in the dark.

Total RNA extraction. RNA was extracted from 100 mg of tissue using Tripure reagent (Boehringer). The protocol followed was that recommended by the supplier. RNA integrity was checked by electrophoresis in agarose gels and stained with ethidium bromide.

RNA DD. From 2 μg of total RNA extracted at 0 and 16 d, the first strand of

cDNA was synthesized using 2.5 µM of oligo$(dT)_{13}$VN as the anchor primer. The reaction mix contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 20 µM each dNTP and 200 U of M-MLV RT (Life Technologies, Maryland, MD, USA) and was incubated for 1 h at 37°C. PCR was performed using 2.5 µl of the first strand reaction in a mix, containing 1 µM of the same anchor primer used to synthesize the first strand of cDNA and 1 µM of a 10 mer arbitrary primer of (Operon Technology), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 µg/ml gelatin, 1.5 mM $MgCl_2$, 20 µM each dNTP and 1 µCi [α ^{32}P]dCTP per sample and 2.5 U Taq Polymerase (Life Technologies, Maryland, MD, USA). PCR was performed in a GENEAMP 9600 thermocycler (Perkin-Elmer). The program was 94°C, 30 s (one cycle), followed by 94°C, 30 s; 42°C, 2 min; 72°C, 30 s (40 cycles) and a final extension of 5 min at 72°C. The amplification products (2 µl) were loaded onto a 5% polyacrylamide sequencing gel. After drying, the gels were exposed to Hyperfilms (Kodak) in cassettes with intensifier screen at -80°C. After 24 to 48 h, the films were developed and re-exposed with a radioactive ink spot in the corner to orientate the gel and the bands of interest were cut off. Cut bands were eluted as describe by Chen et al. (1997), and reamplified as mentioned above, but doubling the concentration of dNTPs.

Cloning, sequencing and analysis of cloned fragments. Reamplified bands were cloned in pGEM-T-Easy Vector System II (Promega, Madison, Wi, USA) and introduced into *E. coli* (strain DH5α). Cloning and transformation was as recommended by the supplier. The purification of plasmid DNA was done as described elsewhere (Sambrook et al., 1980). Sequencing of clones was performed in an automated sequencer ABI PRISM 310. Sequence comparison was done using BLASTP (Altschul et al., 1990). Multiple sequence alignment was made using ClustalW (Jeanmougin et al., 1998) 1.7 and BOX-SHADE.

RT-p DB. Five µg of each clone-containing plasmid were dotted, fixed on positively charged nylon membranes and probed with amplified cDNA from both embryogenic systems. The first strands of selected day of culture were synthesized using a mix of those anchor primers (0.8 µM each) used in DD (see Table II). The

reaction settings were as mentioned above. Five µl of the first strand were amplified under the same conditions mentioned above, but using a mix of primers (0.5 µM of each anchor and 0.75 µM of each decamer used in DDs; see Table II). The reactions were labeled with 25 µCi [α^{32}P]dCTP. Hybridization was performed at 42°C in the presence of 30% formamide. The final washes were 30 min at 65°C in SSC 0.1X and SDS 0.1%. Autoradiographies were made in cassettes with an intensifier screen using Hyperfilm (Kodak), at -80°C.

RESULTS AND DISCUSSION

DD analysis, developed by Liang and Pardee (1992), has become a feasible way to obtain probes for molecular studies (Appel et al., 1999). It allows the analysis of gene expression in several samples run in parallel. In this work, we used eight combinations of primers among which three gave differences in displayed bands (Table I). There were up-regulated and down-regulated genes and others were expressed transiently during the development of the embryogenetic process. It was remarkable that using the same combination of primers, a larger number of fragments with differential expression was observed when embryogenesis was induced from suspensions cultures. It presumably reflected a major representation of mRNA in the suspension system, which might be attributable to a larger number of cells entering into the embryogenetic process.

Differentially expressed bands were excised from gels, re-amplified and cloned. Among them, 9 cDNAs (42% of those cloned) showed to be false positives after dot blotting (Table I), so they were not further studied. Analyses of the deduced amino acid sequences of some cloned bands showed homologies to diverse proteins reported in the databases (Table II). A smaller group of bands (5) did not show homologies to any reported gene/protein sequence. They were short sequences (less that 100 bp) and contained several stop codons suggesting that they are 3'UTR (data not shown). None of the clones were full length and most of them represented the 3 terminus of their corresponding mRNA. Clones were named as shown in Table II.

Table I. Data from differential display gels. Since PCR were repeated at least 3 times, cloned bands were those observed at least in two different gels

Primer combination	Total bands displayed	Up-regulated bands	Down-regulated bands	Cloned and sequenced bands	False positives*
$(dT)_{13}$GC/GAAACGGGTG	44	4 (9%)	3 (6.8%)	1	0
$(dT)_{13}$AA/CAGCACCCAC	74	7 (9.4%)	4 (5.4%)	5	3
$(dT)_{13}$CG/GAAACGGGTG	97	12 (12.4%)	7 (7.2%)	15	6

*Determined by reverse dot blot.

DDB-1 was observed as an up-regulated band during the induction of SE in leaf explants (Fig.1, upper panel). Its length was 292 nucleotides and contained a polyA tail of 12 residues. A polypeptide of 52 amino acids could be conceptually translated from it (Table II). Sequence comparison showed a 82% identity to an oxygenase from *Nicotiana tabacum* (AJ007630). An identical fragment was cloned when embryogenesis was induced from suspension cultures (Fig. 1, lower panel), which might imply a major role of this gene during embryogenesis in coffee. RT-p DT showed that DDB-1 was up-regulated during the induction of SE in coffee (Fig. 2).

Clones ERKI3 and ERKI8, which deduced amino acid sequences exhibited homology to phosphoglycerate kinase, were nearly identical within their coding region (Fig. 3 A), but shared a low homology on their 3´ UTR (Fig. 3 B). Although both clones seem to code for the same protein and were up-regulated during SE in coffee, ERKI3 was a less abundant transcript than ERKI8 (Fig. 2), which may be attributed to either different factors regulating the expression of each gene or to differential locations inside the cell. This evidence suggest that ERKI3 and ERKI8 represent two isogenes coding for phosphoglycerate kinase. Their participation in SE or in processes associated with cellular division/differentiation remains to be elucidated.

Table II. Primer used for DD analysis and some characteristics of the obtained clones.

Clone name	Primer combination	Length (nt)	Deduced polypeptide length (aa)	Homology	Accession number
DDB-1	$(dT)_{13}$GC/GAAACGGGTG	292	52	Oxygenase	AF343970
AR- 60	$(dT)_{13}$AA/CAGCACCCAC	405	135	ADP-ribosylation factor	AF343969
ERKI3	$(dT)_{13}$CG/GAAACGGGTG	341	28	Phosphoglycerate kinase	AF343967
ERKI8	$(dT)_{13}$CG/GAAACGGGTG	356	25	Phosphoglycerate kinase	AF343968
UBI 9	$(dT)_{13}$CG/GAAACGGGTG	314	51	Ubiquitin	AF297089
ERSH15	$(dT)_{13}$CG/GAAACGGGTG	362	55	Imw HSP	AF343966

Involvement of phosphoglycerate kinase, particularly in embryogenesis, is unlikely to be due to the participation of this enzyme in a central metabolic pathway (glycolisis). It probably reflected an enhanced metabolic activity of cells entering the embryogenetic process. In carrot, genes involved in important metabolic pathways were also regulated during SE (Lin et al., 1996).

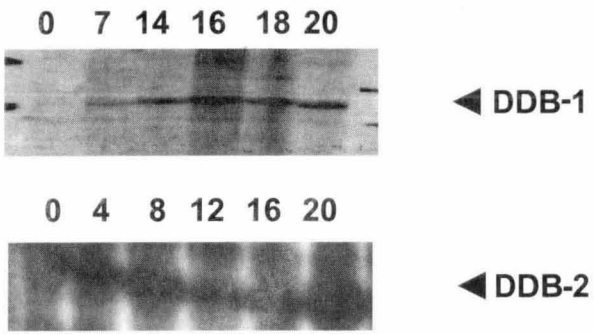

Figure 1. DD pattern of clone DDB1 during the induction of direct SE (upper) and indirect SE (lower). Two µg of amplification products were loaded on polyacrylamide sequencing gels. Numbers on top represent days after induction of SE.

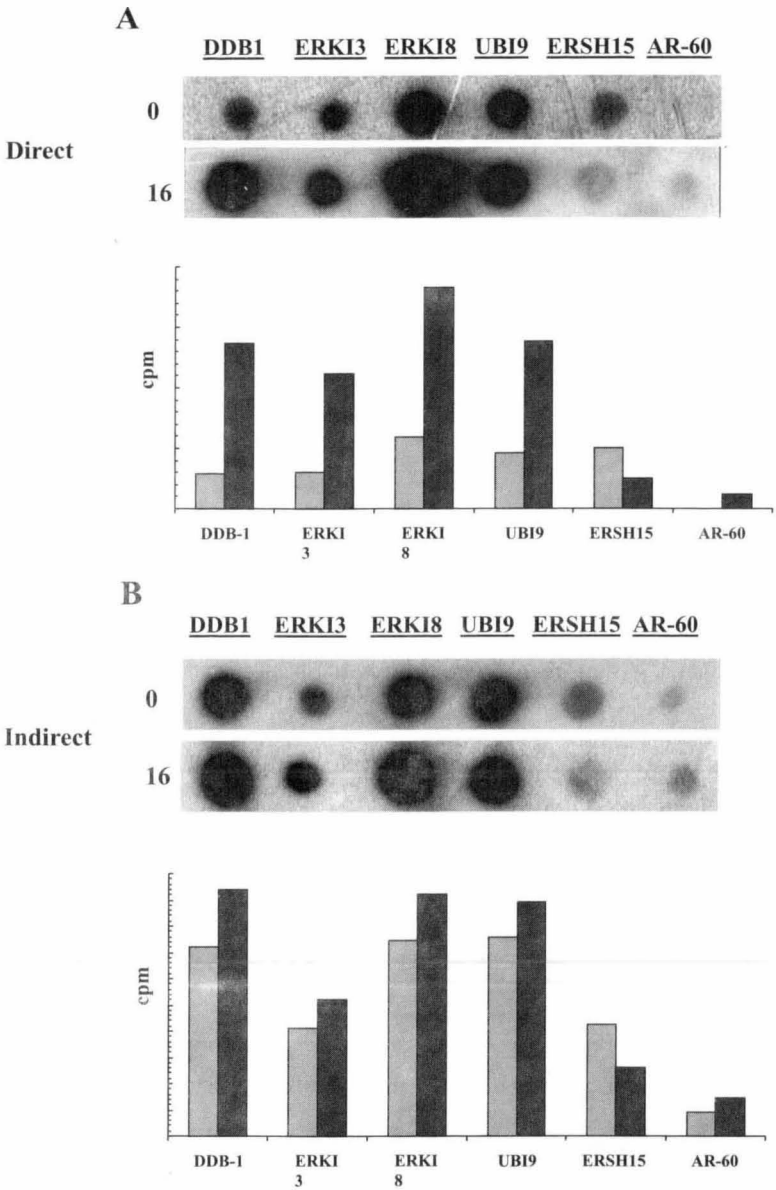

Figure 2. RT-p DB analysis of the expression of cloned fragments during the induction of direct SE (A) and indirect SE (B). Clones were dotted directly on nylon membranes and probed with amplified cDNA from days 0 (▒) and 16 (■) after induction of embryogenesis. Quantification was performed by densitometric analysis of films using a phosphorimager.

The clone UBI9 showed a high similarity (over 90% identical amino acids) to ubiquitin and contains the major part of the reading frame of the ubiquitins (not shown). It was up-regulated during direct SE and to a lesser extent during indirect embryogenesis (Fig. 2). Ubiquitin is involved in the regulation of the cell cycle promoting proteosome-dependent degradation of cyclines, and thus preventing further divisions (Stals et al., 2000). An active cellular division that was strongly reduced after embryos reached the heart-shaped stage characterized the onset of embryogenesis. In both, the direct and the indirect systems, most globular embryos were formed by day 16 (Quiroz-Figueroa et al., 2002) and consequently, cellular division could be reduced.

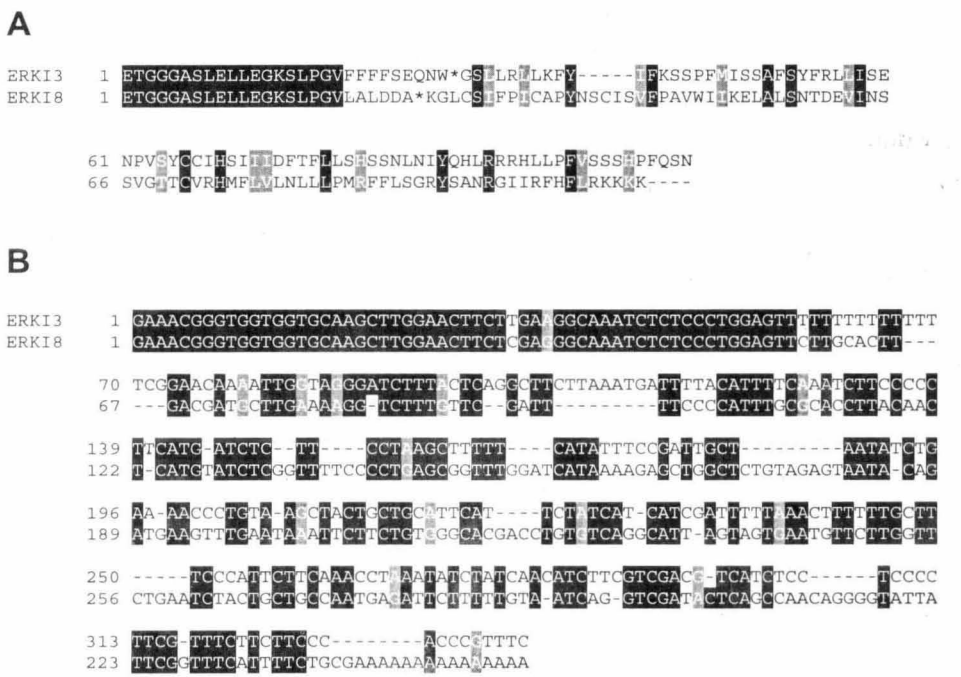

Figure 3. Alignment of clone ERKI 3 and ERKI 8. Regardless of the high identity observed in the coding region between the clones, their 3´UTR showed a low homology in the deduced amino acids (A) or nucleotides (B) sequences. The first stop codon in amino acid sequences (A) is represented by an asterisk.

The clone ERHS15 was 362 nucleotides long and a polypeptide of 55 amino acids upstream of the first stop codon was conceptually translated. It showed a high homology to several lmwHSP from different plants, and included the consensus region I in its deduced amino acid sequence (P-X_{14}-G-V-L), typical of these proteins (Waters et al., 1996) (Fig. 4). ERSH15 was down-regulated at day 16 after induction of SE in coffee (Fig. 2). Heat shock protein coding genes have been previously reported to be up regulated during SE in *Daucus carota* (Zimmerman et al., 1989), *Medicago sativa* (Györgyey et al., 1991) and *Picea glauca* (Dong and Dunstan, 1996b). Regulation of HS genes by signals other than heat stress has been proposed by Waters et al., (1996). A concomitant accumulation of LEA and lmwHSP transcripts and their induction by ABA and osmotic agents, had led to propose a role of HSP in protecting cellular structures of zygotic embryos from damage during desiccation (Almoguera and Jordano, 1992; Coca et al., 1996). Nevertheless, as ERSH15 generally seemed to be expressed early during SE in coffee, an alternative role should be attributed to this protein. In tobacco, the expression of a gene coding for a lmwHSP was observed only during the early development of pollen-derived embryos (Zarsky et al., 1995). An interesting role for these proteins in the assistance of protein trafficking between cytoplasm and membranes of organelles has been hypothesized by Coca et al., (1994).

The insert we had named AR-60 was observed as an up-regulated band in DD analysis during the induction of direct embryogenesis in leaf explants. It was 405 nucleotides long and a polypeptide of 135 amino acids could be translated from it. No stop codons were observed along the sequence, suggesting that it corresponds to a region of the gene faraway from the 3´UTR. Sequence comparison showed 44% identical amino acids (69% similarity) to an ADP-ribosylation factor from *Arabidopsis thaliana* (AC025290). AR-60 was not detected in leaf explants at day 0 but only after 16 days of culture, a slight expression was observed (Fig. 2 A). In suspension cultures, AR-60 was readily expressed; nevertheless after SE induction, its expression is increased (Fig. 2 B).

The RT-p DB technique proved to be suitable for assessing the expression pattern obtained by DD. It allowed the probing of several fragments at the same time using a minimal amount of total RNA and circumventing purification of the polyA RNA, which made it a very attractive technique for surveying cloned fragments by DD and to rule out false positives. Moreover, while northern blot allows the analysis of one fragment on each membrane at a time, using RT-p DB several fragments could be probed at the same time.

Figure 4. Deduced amino acid sequence from clone ERSH 15 (A) and sequence alignment with several ImwHSP from plants (B). The consensus sequence P-X_{14}-G-V-L, typical of plants ImwHSP, is underlined in the deduced amino acid sequence (A) and mark (▽) in the sequence alignment (B). Accession number of ImwHSP sequences used for multiple sequence alignment are: *C. arabica* (AF343966), *L. peruvianum* Hsp 20.1 (CAA12387), *L. esculentum* HSP 17.6 kD (AAD30454), *G. max* HSP 18.5 (P05478), *M. sativa* HSP 18.2 (P27880), *H. annus* HSP 18.6 (AAB63310) and *N. tabacum* Nthsp 18p (CAA50022).

The change in the expression pattern between days 0 and 16 is comparatively more dramatic when SE is induced from leaf explants, than the one

observed when SE is induced in suspension cultures. Similar results have been reported for carrot (Wilde et al., 1988; Wilde et al., 1995) and probably could reflect the triggering of a genetic program in proembryogenic masses, which may be already present in non-induced suspension cultures: minor changes would then allow the completion of embryogenesis, whereas in leaf explants, the embryogenic program initiated from the onset.

Embryogenesis is the result of active cellular division, from the beginning of the embryo arrangement until the end of the globular stage, followed by a differentiation process that culminates in the formation of a complete structure, possessing apex and root meristems, vascular tissue, epidermis and cotyledons. Dissection of the processes taking place specifically during embryogenesis can be a cumbersome task due to biochemical and molecular events that are concomitantly occurring during embryogenesis. In tissue or cell suspension cultures, cellular division normally take place in many cells that will never differentiate into embryos, but control of the division must be undertaken by the same mechanism that regulates the division in those giving rise to an embryo. Results shown in the present paper strengthened the evidence of the molecular and biochemical complexity of embryogenesis.

Unraveling the mechanisms governing this process will lead to the advancement in the comprehension of SE and set the basis for future uses in commercial propagation, genetic breeding and genetic manipulation. SE can be an effective way for the regeneration of genetically modified cells as well as a valuable tool for multiplication of elite F_1 progenies, resulting from breeding programs.

ACKNOWLEDGEMENTS

Authors are indebted to Q.F.B. Marcela Méndez-Zeel for technical help and the Consejo Nacional de Ciencia y Tecnología, México [grants 4123P-N and 31816-N and post-graduated scholarships to RRH (117155), FQF (116916) and LST (118132)].

REFERENCES

Aleith, F. and Richter, G. (1990). Gene expression during induction of somatic embryogenesis in carrot cell suspensions. Planta *183*, 17-24.

Almoguera, C. and Jordano, J. (1992). Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNA. Plant Mol. Biol. *19*, 781-792.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403-410.

Appel, M., Bellstedt, D.U., and Gresshoff P.M. (1999). Differential display of eukaryotic mRNA: Meeting the demands of the new millennium? J. Plant Physiol. *154*, 561-570.

Chen, W., Hardy, P., and Wilce, P.A. (1997). Differential expression of mitochondrial NADH dehydrogenase in ethanol-treated rat brain: Revealed by differential display. Alcohol. Clin. Exp. Res. *21*, 1053-1056.

Choi, J.H., Liu, L.-S., Borkird, C., and Sung, Z.R. (1987). Cloning of genes developmentally regulated during plant embryogenesis. Proc. Natl. Acad. Sci. (USA) *84*, 1906-1910.

Coca, M.A., Almoguera, C. and Jordaño J. (1994). Expression of sunflower low-molecular-weight heat-shock proteins during embryogenesis and persistence after germination: localization and possible functional implications. Plant Mol. Biol. *25*, 479-492.

Coca, M.A., Almoguera, C., Thomas, T.L., and Jordano, J. (1996). Differential regulation of small heat-shock genes in plants: Analysis of a water-stress-inducible and developmentally activated sunflower promoter. Plant Mol. Biol. *31*, 863-876.

Dodeman, V.L., Ducreux, G., and Kreis, M. (1997). Zygotic embryogenesis *versus* somatic embryogenesis. J. Exp. Bot. *48*, 1493-1509.

Dong, J.Z. and Dunstan, D.I. (1996a). Expression of abundant mRNAs during somatic embryogenesis of white spruce [*Picea glauca* (Moench) Voss]. Planta *199*, 459-466.

Dong, J.Z. and Dunstan, D.I. (1996b). Characterization of three heat-shock-protein genes and their developmental regulation during somatic embryogenesis in white spruce [*Picea glauca* (Moench) Voss]. Planta *200*, 85-91.

Dudits, D., Györgyey, J., Bögre, L., and Bakó, L. (1995). Molecular biology of somatic embryogenesis. In *In vitro* Embryogenesis in Plants, T.A. Thorpe, ed. (Dordrecht: Kluwer Academic Publishers), pp. 267-308.

Fowler, M.R., Ong, L.M., Russinova, E., Atanassov, A.I., Scott, N.W., Slater, A., and Elliott, M.C. (1998). Early changes in gene expression during direct somatic embryogenesis in alfalfa revealed by RAP-PCR. J. Exp. Bot. *49*, 249-253.

Giroux, R.W. and Pauls, K.P. (1997). Characterization of somatic embryogenesis-related cDNAs from alfalfa (*Medicago sativa* L). Plant Mol. Biol. *33*, 393-404.

Györgyey, J., Gartner, A., Németh, K., Magyar, Z., Hirt, H., Herbele-Bors, E., and Dudits, D. (1991). Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol. Biol. *16* , 999-1007.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G., and Gibson, T.J. (1998). Multiple sequence alignment with Clustal X. Trends Biochem. Sci. *23*, 403-405.

Kawahara, R. and Komamine, A. (1995). Molecular basis of somatic embryogenesis. In Biotechnology in Agriculture and Forestry. Vol. 30. Somatic Embryogenesis and Synthetic Seed I, Y.P.S. Bajaj, ed. (Berlin: Springer-Verlag), pp. 30-40.

Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*, 967-971.

Lin, X.Y., Hwang, G.J.H., and Zimmerman, J.L. (1996). Isolation and characterization of a diverse set of genes from carrot somatic embryos. Plant Physiol. *112*, 1365-1374.

Mordhorst, A.P., Toonen, M.A.J., and De Vries, S.C. (1997). Plant embryogenesis. Crit. Rev. Plant Sci. *16*, 535-576.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum *15*, 473-497.

Quiroz-Figueroa, F. R., Fuentes-Cerda, C. F. J., Rojas-Herrera, R., and Loyola-Vargas, V. M. (2002). Histological studies on ontogenesis, development stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. Plant Cell Reports . In press.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1980). Molecular Cloning. A Laboratory Manual., C.Nolan, M.Ferguson, and N.Ford, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), p. I-I.47.

Söndahl, M.R. and Sharp, W.R. (1977). High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z. Pflanzenphysiol. *81*, 395-408.

Stals, H., Casteels, M.P., van Montagu, M., and Inzé, D. (2000). Regulation of cyclin-dependent kinases in *Arabidopsis thaliana*. Plant Mol. Biol. *43*, 583-593.

Staritsky, G. (1970). Embryoid formation in callus tissues of coffee. Acta Bot. Need. *19*, 509-514.

Sung, Z.R. and Okimoto, R. (1981). Embryonic proteins in somatic embryos of carrot. Proc. Natl. Acad. Sci. (USA) *78*, 3683-3687.

Thomas, T.L. (1993). Gene expression during plant embryogenesis and germination: An overview. Plant Cell *5*, 1401-1410.

Waters, E.R., Lee, G.J., and Vierling, E. (1996). Evolution, structure and function of the small heat shock proteins in plants. J. Exp. Bot. *47*, 325-338.

Wilde, H.D., Nelson, W.S., Booij, H., De Vries, S.C., and Thomas, T.L. (1988). Gene-expression programs in embryogenic and non-embryogenic carrot cultures. Planta *176*, 205-211.

Wilde, H.D., Seffens, W.S., and Thomas, T.L. (1995). Gene expression in somatic embryos. In Biotechnology in Agriculture and Forestry. Vol. 30. Somatic Embryogenesis and Synthetic Seed I, Y.P.S.Bajaj, ed. (Berlin: Springer-Verlag), pp. 41-52.

Yasuda, T., Fujii, Y., and Yamaguchi, T. (1985). Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. Plant Cell Physiol. *26*, 595-597.

Zarsky, V., Garrido, D., Eller, N., Tupy, J., Vicente, O., Schöffl, F., and Heberle-Bors, E. (1995). The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Environ. *18*, 139-147.

Zimmerman, J.L. (1993). Somatic embryogenesis: a model for early development in higher plants. Plant Cell *5*, 1411-1423.

Zimmerman, J.L., Apuya, N., Darwish, K., and O'Carroll, C. (1989). Novel regulation of heat shock genes during carrot somatic embryo development. Plant Cell *1*, 1137-1146.

Chapter III

INDUCTION OF A CLASS III ACIDIC CHITINASE IN FOLIAR EXPLANTS OF *Coffea arabica* L. DURING SOMATIC EMBRYOGENESIS AND WOUNDING[!]

R. Rojas-Herrera[1] and V. M. Loyola-Vargas *

Unidad de Bioquímica y Biología Molecular de Plantas. Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, CP 97200, Mérida, Yucatán, México.

[1]Departamento de Genética y Mejoramiento, Instituto Nacional de Ciencias Agrícolas. Gaveta Postal 1, San José de las Lajas, La Habana, Cuba.

*Corresponding author email vmloyola@cicy.mx

ABSTRACT

A gene fragment, denominated AR-52, was cloned by differential display analysis during the induction of somatic embryogenesis in foliar explants of *Coffea arabica*. It is homologous to several class III chitinases. Northern blots show that it is up regulated during somatic embryogenesis and scarcely expressed in embryos at any developmental stage. AR-52 is also induced by wounding leaves of *in vitro* cultured plantlets, but not in a non-embryogenic suspension culture. Zymography revealed the existence of at least three-chitinolytic bands, also differentially regulated during somatic embryogenesis and wounding.

Key words: *Coffea arabica*, coffee, chitinases, gene expression, somatic embryogenesis.

Abbreviations: AGPs = arabinogalactan proteins; SE = somatic embryogenesis; SSC = sodium chloride and sodium citrate buffer.

[!] The AR-52 sequence has been deposited in the Gene Bank under the accession number AF125189. This chapter was accepted for publication in Plant Science.

INTRODUCTION

The formation of a somatic embryo is not the result of a fertilization process, which demonstrates that the factors determining embryogenesis are totally contained within the cell and that they can function even in the absence of gene products from the maternal environment (Zimmerman, 1993; Goldberg et al., 1994) or a fertilization signal. This information along with the knowledge that either, a symmetric or an asymmetric cell division can occur at the beginning of somatic embryogenesis (De Jong et al., 1993a; Mordhorst et al., 1997; Quiroz-Figueroa et al., 2002), makes this process a very attractive model for studying factors involved in division planes organization, polarity establishment, and cellular fate. Molecular and biochemical studies have revealed that a differential expression of genes and proteins during embryo development takes place. Genes encoding a broad diversity of proteins have been cloned, among these are the chitinases (Dong and Dunstan, 1996; Lin et al., 1996; Wilde et al., 1995; Jain et al., 1995).

Chitinases are proteins mainly related to the response of plants to fungal pathogen attacks (Graham and Sticklen, 1994). The rescue of the carrot temperature-sensitive (ts11) mutant (Lo Schiavo et al., 1990) by an acidic endochitinase, named EP3, has been described (De Jong et al., 1992). A possible role in the restoration of the protodermis and endomembranal system has been attributed to that protein (Baldan et al., 1997). EP3 is an extracellular protein secreted by both the wild type and the mutant line; nevertheless when the latter is cultivated at the non-permissive temperature (32°C), there is not enough secreted protein during the phenocritical period (De Jong et al., 1995). No structural differences between those chitinases produced by the wild type line or by the mutant line have been found regarding their physical or chemical properties, suggesting that in the mutant ts11, the regulation rather than the structure of the EP3 gene is altered (Baldan et al., 1997).

Chitinases have also been shown to be expressed during embryogenesis in other species such as *Hordeum vulgare* (Swegle et al., 1992), *Picea glauca* (Dong

and Dunstan, 1997) and *Pinus caribaea* (Domon et al., 2000), which may indicate an important role for these proteins along this process.

In the present paper, we describe the cloning and expression analysis partial cDNA encoding a polypeptide which is highly homologous to class III acidic chitinases. The cloned fragment hybridizes with an mRNA that is regulated during SE and wounding in foliar explants of *Coffea arabica* L. Zymography revealed that at least three particular isozymes are also differentially expressed along SE and wounding.

MATERIALS AND METHODS

Plant material and SE induction: *In vitro* coffee plantlets were obtained by germination of isolated zygotic embryos in MS medium (Murashige and Skoog, 1962) without growth regulators. Leaves of plantlets were cut into pieces of 0.25 cm^2 and incubated in a medium containing 5μM benzyladenine as the sole growth regulator (Yasuda et al., 1985). Samples of the explants were taken at 0, 4, 8, 12 and 16 days of incubation and stored at -80°C for further analysis. Embryos at different developmental stages were manually collected. Non-embryogenic suspension cultures were induced from calli obtained from cotyledonary leaves from the *in vitro* plantlets mentioned above, following the methodology described by Söndahl and Sharp (1977) and maintained as previously described (Quiroz-Figueroa et al., 2002).

Total RNA extraction: For total RNA extraction, 100 mg of tissue were frozen in liquid nitrogen, ground with mortar and pestle, and then extracted using Tripure reagent (Boehringer). The protocol followed was as recommended by the supplier. RNA integrity was checked by electrophoresis in agarose gels and ethidium bromide staining.

RNA differential display: The first cDNA strand was synthesized from 2 μg of total RNA using 2.5 μM of oligo $(dT)_{13}$ AA as anchor primer. The reaction mix contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 20 μM each dNTP, and 200 U M-MLV RT (Life Technologies, Maryland, MD, USA) and was incubated for 1 hour at 37°C. PCR was performed using 2.5 μl of the first

strand in a reaction mix containing 1 µM oligo$(dT)_{13}$AA and 1 µM OPA13 (CAGCACCCAC) as primers, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 µg/ml gelatin, 1.5 mM $MgCl_2$, 20 µM each dNTP, 1 µCi [α ^{32}P]dCTP per sample and 2.5 u Taq Polymerase (Life Technologies, Maryland, USA). PCR was performed in a GENEAMP 9600 thermocycler (Perkin-Elmer) using the following program: 94°C/5 minutes (one cycle), and 40 cycles of 94°C/30 seconds, 42°C/2 minutes, and 72°C/30 seconds, and a final extension of 72°C/5 minutes. The amplification products were loaded onto a 5%T polyacrylamide-sequencing gel and run for 3 hours at 52°C then attached to filter paper and dried at 80°C for 2 hours. Autoradiography was performed at -80°C in cassettes with intensifier screens using Hyperfilms (Kodak).

After 24 to 48 hours, these films were developed and re-exposed, with radioactive ink spots in the corners to orientate the gel, and the bands of interest were cut. Cut bands were eluted as describe by Chen et al., (1997). The bands were reamplified under the same conditions as PCR, but doubling the concentration of dNTPs.

Cloning, sequencing and analysis of cloned fragments: Re-amplified bands were cloned in pGEM-T-Easy Vector System II (Promega, Madison, WI, USA) and introduced into *E. coli* (strain DH5α). The protocol followed for cloning and transformation was as recommended by the supplier. Purification of plasmid DNA was as described in Sambrook et al., (1980). Clone sequencing was performed in an ABI PRISM 310 automated sequencer. Sequence comparison was made using BLASTP (Altschul et al., 1990). Multiple sequence alignment used ClustalW 1.7 (Jeanmougin et al., 1998) and BOX-SHADE.

Northern and Southern blot: Total RNA (10 µg) was fractionated in formaldehyde-agarose gels and transferred to nylon membranes. The probe (AR-52) was labeled with 50 µCi [α ^{32}P]dCTP) by random primer. Hybridization was performed at 42°C in SSC buffer containing 30% formamide. Final washes were made for 30 minutes (twice) at 65°C in 0.1x SSC and 0.1% SDS. For Southern blot, DNA was extracted according to Dellaporta et al. (1983) and 10 µg were

digested with EcoRI, HindIII, SacI and SmaI. The digestion products were separated in 0.8% agarose gels and transferred to nylon membranes. AR-52 was labeled with 50 µCi [α ^{32}P] dCTP by random priming. Hybridization was carried out in a standard hybridization buffer containing 30% formamide at 42°C for 24 hours. Final washes were made at 65°C for 30 minutes with SSC 0.1x and SDS 0.1%. After washing, membranes from Northern and Southern blots were exposed to autoradiographic films (Kodak X-Omat) for 48 h at –80°C.

Protein electrofocusing and chitinase zymography: Protein extracts were prepared from 100 mg of tissue with 0.2 ml of extraction buffer (40 mM Tris-HCl, 8 mM EDTA, 2 mM $CaCl_2$ and 0.1 mM β-mercaptoethanol) and 30% poly vinyl poly pyrrolidone (PVPP). The samples were centrifuged at 20,000x g for 20 minutes at 4°C. The protein concentration was determined by the bicinchoninic acid method (Pierce, Illinois, USA). Polyacrylamide gels were loaded with 50 µg of protein per lane and electrofocused, essentially as described by Robertson et al., (1987). Chitinase activity was detected using an overlay gel containing 0.01% of glycolchitin (Trudel and Asselin, 1989). Overlay gels were photographed under UV illumination.

RESULTS AND DISCUSSION

By using differential display analysis (Liang and Pardee, 1992), we have identified a gene fragment, named AR-52, observed as an up-regulated band during SE induction in leaf explants of coffee (not shown). After cloning and sequencing, it was found that AR-52 is a 506 bp long fragment and contains a HindIII recognition site from nt 164 to 169. From frame +2, a polypeptide of 123 amino acids, 54.5% identical and 66.6% homologous to several acidic class III chitinases from different species, was conceptually translated (Fig. 1). The polypeptide contains a putative glycosylation site (Asn-Leu-Ser) that is not conserved among the other proteins used for comparison (Fig. 1).

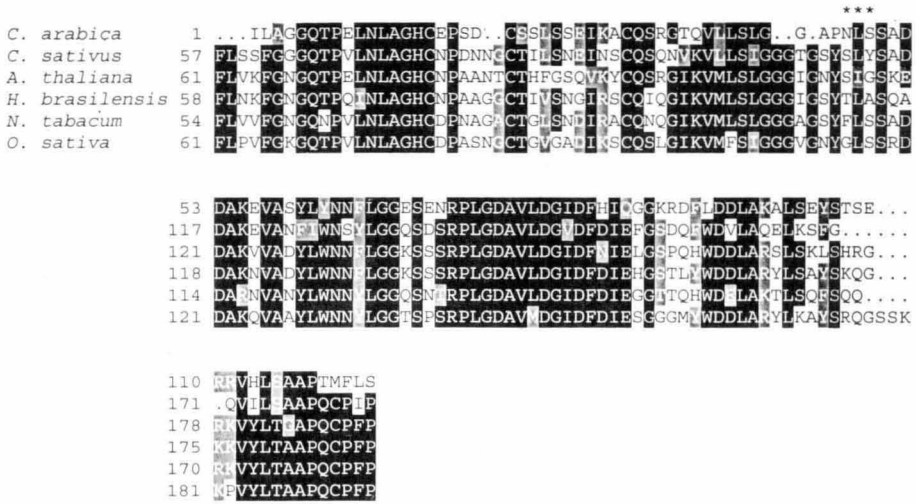

Figure 1. Alignment of AR-52 with several sequences of chitinases from different species. Identical amino acids are boxed in black and homologs are boxed in gray. A triple asterisk on top of the sequence indicates a putative glycosylation site. Dots were introduced for optimal alignment.

To investigate the genomic structure of AR-52, it was hybridized with blots that contained genomic DNA from *in vitro* cultured coffee plantlets, previously digested with four different restriction enzymes, including HindIII which have a restriction site inside the probe sequence. Results obtained showed that only one hybridization signal was observed in the SmaI digested DNA, whereas two bands were obtained for EcoRI and SacI, and four major bands for HindIII digested DNA suggesting that AR-52 is coded by two genes (Fig. 2). Minor bands might represent other chitinase genes with a lower homology to AR-52.

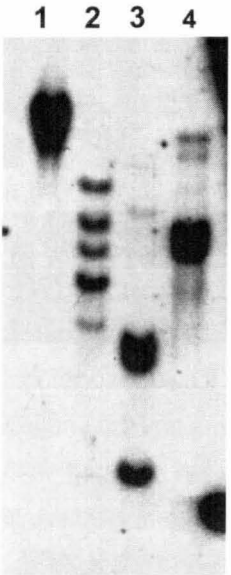

Figure 2. Southern blot analysis of AR-52. In each lane 10 µg of genomic DNA from *C. arabica* plantlets were loaded after overnight digestion with SmaI (lane 1), HindIII (lane 2), SacI (lane 3) and EcoRI (lane 4).

To reduce the possibility of a false positive signal during differential display, the expression pattern was assessed by Northern blot. The probe recognized a single mRNA of approximately 1.35 kb. As can be seen in figure 3, AR-52 was indeed up-regulated during the induction of SE in coffee leaves. It was undetectable at day 0 and scarcely expressed 4 days after induction, but from day 8 to 16, a 4-fold increase was observed. In this model system, embryos were evident after three weeks and histological studies have demonstrated that the onset of embryogenesis begins during the second week (Quiroz-Figueroa et al., 2002). To detect whether the expression of AR-52 was restricted only to the beginning of embryogenesis, embryos at different developmental stages were collected and total RNA was extracted. Northern blots demonstrated that AR-52 expression was low in embryos at any developmental stage (Fig. 3 lanes G, T and P) and was not detected in a non-embryogenic suspension culture (data not shown). Recently Passarinho et al. (2001a) have found that the AtEP3/AtchitIV

endochitinase gene is expressed during SE in the "nursing" cells surrounding the somatic embryo but not in the embryo itself.

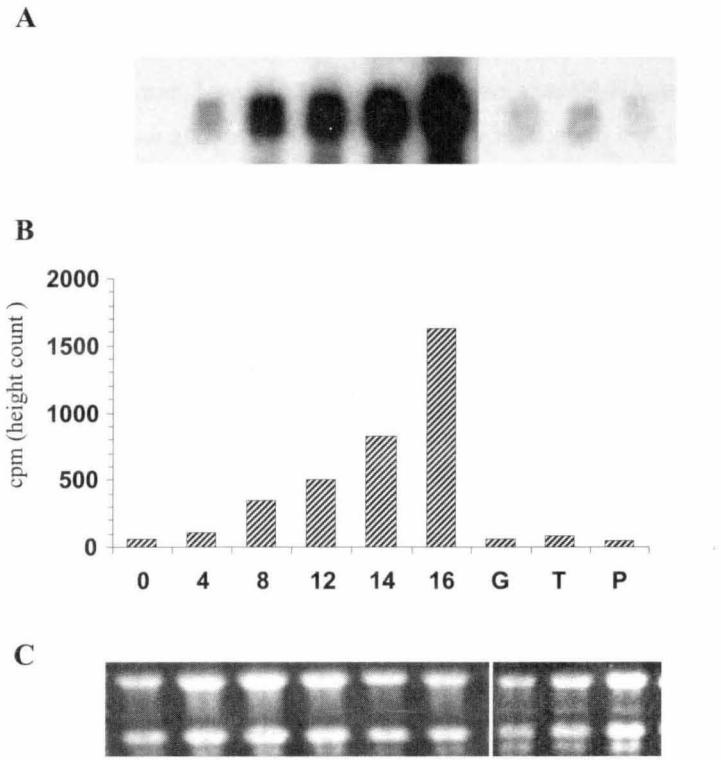

Figure 3. Northern blot analysis of AR-52 expression during the induction of direct SE in leaf explants of *C. arabica*. 10 µg of total RNA per lane were loaded. A: Film after 48 h of exposure. B: Hybridized membrane was exposed in the Imager (Screen G25) for 15 h. Lanes 0-16, days of induction in Yasuda's medium. G: globular embryos, T: torpedo-shaped embryos and P: plantlets. C: Ethidium bromide staining of membranes used for Northern blot, showing ribosomal RNA as loading control.

During the early hours after explant wounding (6, 12 and 24 h) there was no expression of AR-52 (data not shown). Nevertheless, to evaluate if wounding could induce the expression of AR-52 later, leaves of *in vitro* cultured plantlets were scissors-wounded and samples were taken at 0, 1, 2, 12 and 16 days. Northern

blots showed that expression was detectable at day 2 and reached the highest level on day 16 (Fig. 4A). It was observed at day 16, by exposure of hybridized membranes to the Phosphorimager, that the expression level due to embryogenesis induction was over 50% higher compared to that due to wounding (Fig. 4B). It is important to draw attention to the fact that through the induction of embryogenesis, AR-52 was weakly expressed at day 4, but the expression was 5 fold higher just two days after wounding. As these results were consistent in three independent experiments, it is doubtful that this disparity in expression could be attributed to differences in the amounts of RNA loaded in the gels (Figs. 3 and 4).

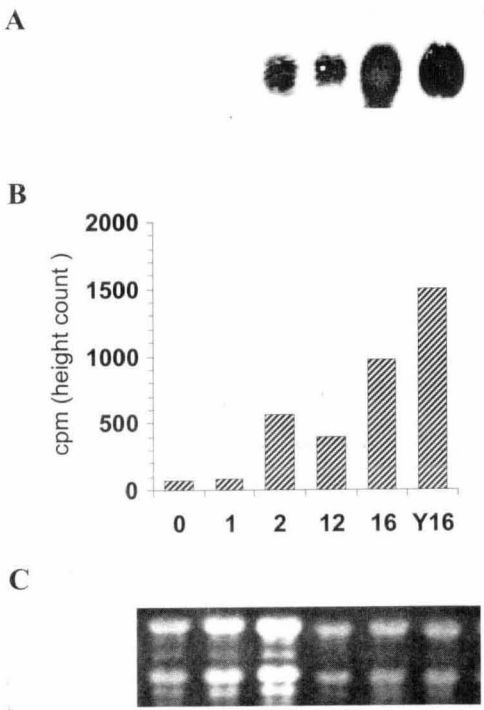

Figure 4. Northern blot analysis of AR-52 expression after wounding. 10 µg of total RNA per lane were loaded. A: Film after 48 h of exposure. B: Hybridized membrane was exposed in the Imager (Screen G25) for 15 h. Lanes 0-16, days after wounding. Y16, day 16 after SE induction in Yasuda´s medium. C: Ethidium bromide staining of membranes used for Northern blot, showing ribosomal RNA as loading control.

Since chitinases can be induced by biotic stresses like fungal, bacterial and viral infections (Graham and Sticklen, 1994), leaves infected with *Cercospora cafeicola* and *Mycena citricolor*, two pathogenic fungi, were collected from trees field and tested for the presence of chitinases. Northern hybridization of RNA samples showed no expression of AR-52 in response to infection (not shown).

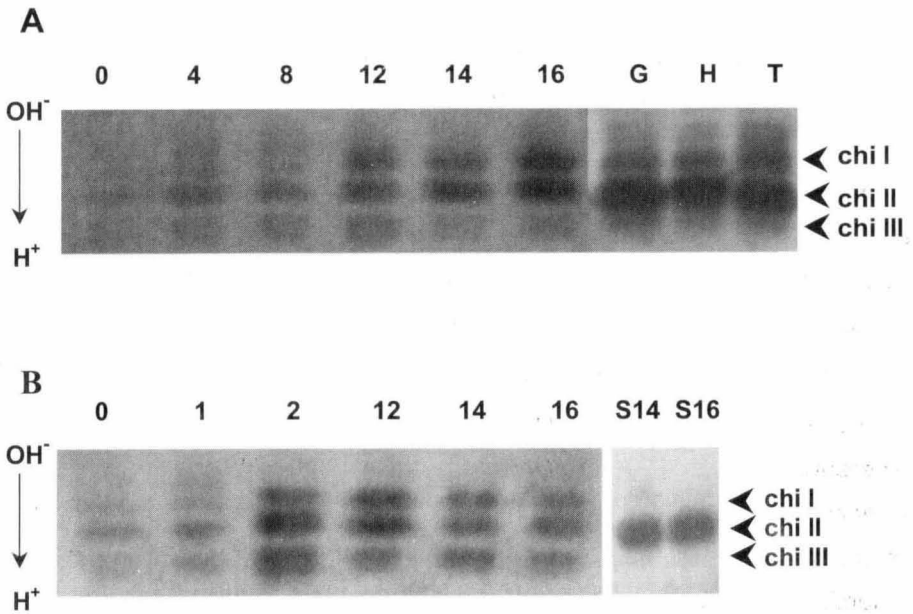

Figure 5. Chitinolytic activities in overlay gels after isoelectric focusing of 50 µg of total protein from A: induced explants after 0, 4, 8, 12, 14, 16 days and embryos at different developmental stages (G: globular, H: heart-shaped, T: torpedo) and B: wounded plants after 0, 1, 2, 12, 14 and 16 days and non-embryogenic line suspension cultures after 14 (S14) and 16 (S16) day of culture.

To detect any possible correlation between the chitinolytic activity and the expression pattern of AR-52, chitinase activity was evaluated in glycolchitin-containing gels overlayed on the gels where samples were previously electrofocused. The best separation of the different isoforms of chitinase was

achieved using a pH gradient between 3 and 5. Eleven bands of chitinolytic activity were detected in coffee leaf explants, most of them with an acid isoelectric point (not shown).

Only three bands (chi I, chi II and chi III) of chitinolytic activity varied during SE induction and wounding (Fig 5). Chi II accounted for the major chitinolytic activity in embryos at different developmental stages (Fig. 5A). During SE induction, chi I and II seemed to have similar patterns after day 12; although from day 0 to 8, chi II was more intense. This could also be observed for wounded explants (Fig. 5B), but a higher contribution of chi III was observed, particularly at day 2, whilst at day 16 chi I decreased significantly, contrasting to day 16 after SE induction (Fig. 5A). Although chi I, II and III were induced during SE and wounding, it was also very noticeable that there was a differential regulation of their activities as a consequence of the treatments. Moreover, in the non-embryogenic suspension cultures, where no expression of AR-52 was observed, chi II accounted for almost the total chitinolytic activity.

Despite that an unambiguous relationship between the expression pattern of AR-52 and the chitinolytic activity was not observed, there are an obvious differential gene expression and a differential regulation of at least three acidic chitinases after induction of SE and wounding. It is thus tempting to postulate the existence of a hierarchic regulation of AR-52, either by wounding or by SE induction. This may be supported by a higher expression of AR-52 at day 16 after SE induction, and also observed at the level of enzymatic activity reflected in a higher intensity of chi I band at day 16 after SE induction compared with that of day 16 after wounding (Fig. 5). The expression of AR-52 is restricted to those stages where active cellular division occurs, being less important after embryos have already formed. Chi II appears to be more involved with stress associated to tissue culture environment, as demonstrated by its higher activity in non-embryogenic suspension cultures and embryos. Environmental stresses such as flooding, drying and wounding (also provoked by tissue culture) caused increasing expression of a transcript encoding a basic chitinase, which is also up-regulated during SE in *Picea glauca* (Dong and Dunstan, 1997).

Regarding the role of chitinases during embryogenesis, it is not very clear whether they act by releasing or activating a signal molecule. The temperature-sensitive mutant of carrot ts11 could be rescued by adding a Nod factor (lipochitooligosaccharide), having a similar effect to that of EP3 (endochitinase). However, another Nod factor, differing only in a double bond in the fatty acid chain, showed less activity, which might suggest a central role for the fatty acid moiety (De Jong et al., 1993b) and a high specificity for the signal molecule. In *Picea abies* it has been reported that Nod factors stimulate embryo formation at low inoculum densities and can mimic the effect of growth regulators in promoting cell division of protoplasts (Dyachok et al., 2000). Conversely, chitinases can cleave Nod factors in a very specific manner (Schultze et al., 1998; Goormachtig et al., 1998). Nevertheless, the presence of active Nod factors has not been established in plants, but it has been reported that AGPs contain glucosamine (Van Holst et al., 1981), which can provide a substrate for chitinases and, in turn, liberate Nod factor-like molecules.

In carrot, AGPs can re-initiate embryogenic cell formation (Kreuger and Van Holst, 1993) and restore the formation of somatic embryos in non-embryogenic cells (McCabe et al., 1997), while Domon et al. (2000) have reported that a basic chitinase-like protein of 48 kDa can act on arabinogalatoproteins extracted from embryogenic tissues or seeds, but not on AGPs extracted from non-embryogenic tissues. One could then expect chitinases to act on AGPs, cleaving them at specific sites and to liberate a molecule, which would be structurally similar to a lipochitooligosaccharide. This molecule could be subsequently recognized by a receptor, acting as a signal and thus provoking a series of events, ending with the formation of a somatic embryo or the induction of cell division. But, as reported by van Hegel et al. (2001), the effect of chitinase seems to be on the activation of AGPs, suggesting that the entire molecule is the signal instead of a chitinaceous molecule derived from them. This was demonstrated by re-isolating the chitinase-treated AGPs by precipitation with Yariv's reagent. This fact does not provide a plausible explanation for the rescue effect of Nod factors on embryogenesis of the mutant line ts11 of carrot (De Jong et al., 1993b) and the stimulating effect on

somatic embryo development in *P. glauca* (Dyachok et al., 2000). An alternative explanation for this controversy in the role of chitinases might be that the levels of the released active molecules after chitinase action were too low to be detected by the analytical methods employed or the concomitant precipitation of lipochitooligosaccharide-like molecules with the re-isolated AGPs.

Finally, chitinases have been detected in immature carrot seeds (Van Hengel et al., 1998) and barley (Swegle et al., 1992), in the stylar transmitting tissue of tomato (Timmers et al., 1989), in the pistils, stamens and lodicules of rice (Takakura et al., 2000) and during flower formation in thin layer cell explants of tobacco (Neale et al., 1990), where roles in defense and morphogenesis have been attributed. Chitinase genes can be induced by N-acetyl chitooligosaccharides and the response depends on the size and the structures of these molecules (Nishizawa et al., 1999). A putative receptor for these elicitor molecules has been identified in the plasma membrane of rice cells (Ito et al., 1997). Nevertheless, it is not known whether lipochitoologosaccharides or chitinaceous molecules arising from the action of chitinases on AGPs, may act in the same way; if this is the case, chitinases are on top of a transduction cascade starting with the liberation of a signal molecule and ending with the formation of an embryo.

ACKNOWLEDGEMENTS

The authors are indebted to M. Sc. Miriam Monforte-González and Q.F.B. Marcela Méndez-Zeel for technical help and Francisco Quiroz-Figueroa for critical reading of the manuscript. Supported by Consejo Nacional de Ciencia y Tecnología, México (grants 4123P-N and 31816-N) and a post-graduate scholarship to RRH (117155).

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403-410.

Baldan, B., Guzzo, F., Filippini, F., Gasparian, M., LoSchiavo, F., Vitale, A., De Vries, S.C., Mariani, P., and Terzi, M. (1997). The secretory nature of the lesion of carrot cell variant ts11, rescuable by endochitinase. Planta *203*, 381-389.

Chen, W., Hardy, P., and Wilce, P.A. (1997). Differential expression of mitochondrial NADH dehydrogenase in ethanol-treated rat brain: Revealed by differential display. Alcohol. Clin. Exp. Res. *21*, 1053-1056.

De Jong, A.J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., van Kammen, A., and De Vries, S.C. (1992). A carrot somatic embryo mutant is rescued by chitinase. Plant Cell *4*, 425-433.

De Jong, A.J., Heidstra, R., Spaink, H.P., Hartog, M.V., Meijer, E.A., Hendriks, T., Lo Schiavo, F., Terzi, M., Bisseling, T., Van Kammen, A., and De Vries, S.C. (1993b). *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. Plant Cell *5*, 615-620.

De Jong, A.J., Hendriks, T., Meijer, E.A., Penning, M., Lo Schiavo, F., Terzi, M., van Kammen, A., and De Vries, S.C. (1995). Transient reduction in secreted 32 kD chitinase prevents somatic embryogenesis in the carrot (*Daucus carota* L.) variant ts11. Devel. Genet. *16*, 332-343.

De Jong, A.J., Schmidt, E.D.L., and De Vries, S.C. (1993a). Early events in higher-plant embryogenesis. Plant Mol. Biol. *22*, 367-377.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1, 19-21.

Domon, J.M., Neutelings, G., Roger, D., David, A., and David, H. (2000). A basic chitinase-like protein secreted by embryogenic tissues of *Pinus caribaea* acts on arabinogalactan proteins extracted from the same cell lines. J. Plant Physiol. *156*, 33-39.

Dong, J.Z. and Dunstan, D.I. (1996). Expression of abundant mRNAs during somatic embryogenesis of white spruce [*Picea glauca* (Moench) Voss]. Planta *199*, 459-466.

Dong, J.Z. and Dunstan, D.I. (1997). Endochitinase and β-1,3-glucanase genes are developmentally regulated during somatic embryogenesis in *Picea glauca*. Planta *201*, 189-194.

Dyachok, J.V., Tobin, A.E., Price, N.P.J., and Von Arnold,S. (2000). Rhizobial Nod factors stimulate somatic embryo development in *Picea abies*. Plant Cell Rep. *19*, 290-297.

Goldberg, R.B., De Paiva, G., and Yadegari, R. (1994). Plant embryogenesis: Zygote to seed. Science *266*, 605-614.

Goormachtig, S., Lievens, S., van der Velde, W., van Montagu, M., and Holsters, M. (1998). Srchi13, a novel early nodulin from *Sesbania rostrata,* is related to acidic class III chitinases. Plant Cell *10*, 905-915.

Graham, L.S. and Sticklen, M.B. (1994). Plant chitinases. Can. J. Bot. *72*, 1057-1083.

Ito, Y., Kaku, H., and Shibuya, N. (1997). Identification of a high-affinity binding protein for N-acetylchitooligosaccharide elicitor in the plasma membrane of suspension-cultured rice cells by affinity labeling. Plant J. *12*, 347-356.

Jain, S.M., Gupta, P.K., and Newton, R.J. (1995). Somatic Embryogenesis in Woody Plants. Vol 1 History, Molecular and Biochemical Aspects and Applications. (Dordrecht: Kluwer Academic Publishers), pp. 1-460.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G., and Gibson, T.J. (1998). Multiple sequence alignment with Clustal X. Trends Biochem. Sci. *23*, 403-405.

Kreuger, M. and Van Holst, G.J. (1993). Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta *189*, 243-248.

Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*, 967-971.

Lin, X.Y., Hwang, G.J.H., and Zimmerman, J.L. (1996). Isolation and characterization of a diverse set of genes from carrot somatic embryos. Plant Physiol. *112*, 1365-1374.

Lo Schiavo, F., Giuliano, G., De Vries, S.C., Genga, A., Bollini, R., Pitto, L., Cozzani, F., Nuti-Ronchi, V., and Terzi, M. (1990). A carrot cell variant temperature sensitive for somatic embryogenesis reveals a defect in the glycosylation of extracellular proteins. Mol. Gen. Genet. *223*, 385-393.

McCabe, P.F., Valentine, T.A., Scott, F.L., and Pennell, R.I. (1997). Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot. Plant Cell *9*, 2225-2241.

Mordhorst, A.P., Toonen, M.A.J., and De Vries, S.C. (1997). Plant embryogenesis. Crit. Rev. Plant Sci. *16*, 535-576.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum *15*, 473-497.

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner,D.R., and Peacock,W.J. (1990). Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell *2*, 673-684.

Nishizawa, Y., Kawakami, A., Hibi, T., He, D.Y., Shibuya, N., and Minami, E. (1999). Regulation of the chitinase gene expression in suspension-cultured rice cells by *N*-acetylchitooligosaccharides: differences in the signal transduction pathways leading to the activation of elicitor-responsive genes. Plant Mol. Biol. *39*, 907-914.

Passarinho, P.A., van Hengel, A.J., Fransz, P.F., and De Vries, S.C. (2001a). Expression pattern of the *Arabidopsis thaliana* AtEP3/AtchitIV endochitinase gene. Planta *212*, 556-567.

Quiroz-Figueroa, F. R., Fuentes-Cerda, C. F. J., Rojas-Herrera, R., and Loyola-Vargas, V. M. (2002). Histological studies on ontogenesis, development stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. Plant Cell Rep. In press.

Robertson, E.F., Dannelly, H.K., Malloy, P.J., and Reeves, H.C. (1987). Rapid isoelectric focusing in a vertical polyacrilamide minigel system. Anal. Biochem. *167*, 290-294.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1980). Molecular Cloning. A Laboratory Manual., C.Nolan, M.Ferguson, and N.Ford, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), p. I-I.47.

Schultze, M., Staehelin, C., Brunner, F., Genetet, I., Legrand, M., Fritig, B., Kondorosi,E., and Kondorosi,A. (1998). Plant chitinase/lysozyme isoforms show distinct substrate specificity and cleavage site preference towards lipochitooligosaccharide Nod signals. Plant J. *16*, 571-580.

Söndahl, M.R. and Sharp, W.R. (1977). High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z. Pflanzenphysiol. *81*, 395-408.

Swegle, M., Kramer, K.J., and Muthukrishnan, S. (1992). Properties of barley seed chitinases and release of embryo-associated isoforms during early stages of imibibition. Plant Physiol. *99*, 1009-1014.

Takakura, Y., Ito, T., Saito, H., Inoue, T., Komari, T., and Kuwata, S. (2000). Flower-predominant expression of a gene encoding a novel class I chitinase in rice (*Oryza sativa* L.). Plant Mol. Biol. *42*, 883-897.

Timmers, A.C.J., De Vries, S.C., and Schel, J.H.N. (1989). Distribution of membrane-bound calcium and activated calmodulin during somatic embryogenesis of carrot (*Daucus carota* L.). Protoplasma *153*, 24-31.

Trudel, J. and Asselin, A. (1989). Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal. Biochem. *178*, 362-366.

van Hengel, A.J., Guzzo, F., Van Kammen, A., and De Vries, S. (1998). Expression pattern of the carrot EP3 endochitinase genes in suspension cultures and in developing seeds. Plant Physiol. *17*, 43-53.

van Hengel, A.J., Tadesse, Z., Immerzeel, P., Schols, H., van Kammen, A., and De Vries, S.C. (2001). N-acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis. Plant Physiol. *125*, 1880-1890.

van Holst, G.J., Klis, F.M., De Wildt, P., Hazenberg, C.A.M., Buijs, J., and Stegwee, D. (1981). Arabinogalactan protein from a crude cell organelle fraction of *Phaseolus vulgaris* L. Plant Physiol. *68*, 910-913.

Wilde, H.D., Seffens, W.S., and Thomas, T.L. (1995). Gene expression in somatic embryos. In Biotechnology in Agriculture and Forestry. Vol. 30. Somatic Embryogenesis and Synthetic Seed I, Y.P.S.Bajaj, ed. (Berlin: Springer-Verlag), pp. 41-52.

Yasuda, T., Fujii, Y., and Yamaguchi, T. (1985). Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. Plant Cell Physiol. *26*, 595-597.

Zimmerman, J.L. (1993). Somatic embryogenesis: a model for early development in higher plants. Plant Cell *5*, 1411-1423.

Chapter IV

GENERAL DISCUSSION AND PERSPECTIVES

To date, a large number of genes, whose expression fluctuates during embryogenesis, either somatic or zygotic, have been identified. Gene products and their attributed roles are very diverse, making difficult the analysis of published results and to propose a plausible model to explain the mechanism by which somatic embryogenesis (SE) occurs.

Three phases can be recognized during embryogenesis (Fig. 1): the onset (phase I), where an active cell division occurs after the perception of a determined signal which in turn leads to the instauration of an embryogenic program; stage transition (phase II), which includes the formation of the globular embryo and the passing through different developmental stages; it is mainly characterized by cell differentiation and the formation of tissues and organs such as, epidermis, vascular bundles, cotyledons, etc, and a final maturation phase.

In coffee, SE can be induced directly from foliar explants (Dublin, 1981), using 5 µM 6-benzyladenine as the sole growth regulator (Yasuda et al., 1985), leaf-derived calli (Söndahl and Sharp, 1977) or suspension cultures obtained from secondary embryogenic calli (Ducos et al., 1993; Zamarripa et al., 1991; Zamarripa, 1993).

As discussed in Chapter II, the change in the expression pattern is more dramatic when SE is induced directly from leaf explants, whereas the shift in expression is more subtle in the case of suspension cultures (Chapter II fig. 2). A similar result was reported for *Daucus carota* (Wilde et al., 1988; Wilde et al., 1995), whereas Sung and Okimoto (1981) observed the presence of pro-embryos in carrot suspension cultures in the presence of 2,4-D, prior to the induction of somatic embryogenesis. These results are probably the consequence of early events in the onset of the genetic program in embryonic cells already present in the non-induced suspension cultures: after suitable culture conditions for the

expression of embryogenesis, minor changes will then allow for the completion of the morphogenetic process. In the case of direct embryogenesis, cells need to be reprogrammed towards a new "embryogenic program" and therefore, the regulation of a major number of genes is observed.

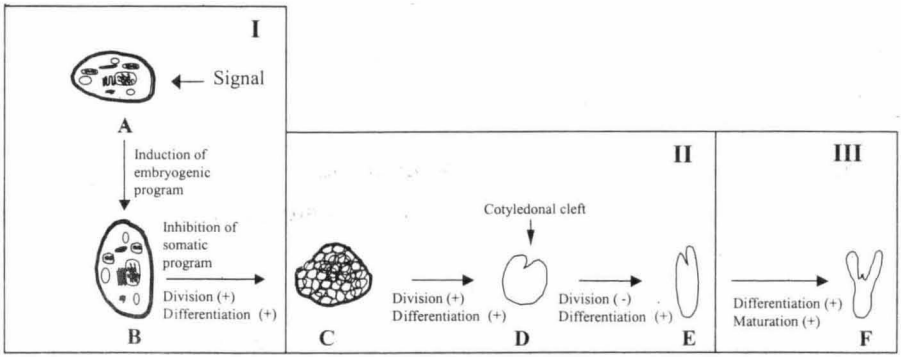

Figure 1. Diagrammatic representation of the main events occurring during SE. After receiving a signal, a somatic cell (A) can be converted into an embryogenic cell (B) through the activation of an embryogenic program. If the embryogenic program successes, embryos arise through the characteristic stages: globular (C), heart-shaped (D), torpedo (E) and cotyledonal (F). Passing throughout B to G requires activation (+) and deactivation (-) of cell division and differentiation.

Genes can be classified according to their temporal function

According to their temporal expression, genes can be expressed throughout the whole process (constitutive), at the beginning of embryogenesis (early), at the end of embryogenesis (late) and at specific stages (transients) (Goldberg et al., 1989). In addition, genes can be up-regulated (if their expression is increased) or down-regulated (if their expression decreases). Depending on their temporal functions, which is the putative function of the gene product and the moment or phenologic stage of its effect, genes expressed during the life cycle of a plant can

be involved in **universal processes (housekeeping)**, **defined processes**, **particular processes** and there are those coding for **regulatory elements**.

Genes for universal processes (housekeeping). The majority of the expressed genes during the life cycle of a plant are those required for its normal function; they encode proteins that participate in metabolism and can be regulated, depending on the metabolic status of the cell, during the cell division or by environmental factors that can affect the homeostasis of the cell. Isogenes ERKI3 and ERKI8 belong to this class. As discussed in chapter II, both genes code for putative phosphoglycerate kinase and are up-regulated during somatic embryogenesis in coffee. This may probably reflect a major demand for energy or metabolic precursors to carry out the active cell division which occurs during the induction of the morphogenetic process. In carrot, for example, housekeeping genes, whose expressions vary during embryogenesis, have been studied (Lin et al., 1996; Higashi et al., 1998; Higashi et al., 1996). This can be explained by an enhanced metabolic activity of cells committing to an embryogenic program.

Genes for defined processes. These genes are restricted to temporally defined physiological processes, for example, flowering, sporogenesis, seed maturation, etc. Organ and tissue formation are fairly general processes, not limited to embryogenesis, and the genes involved in these are not necessarily specific to embryo formation. On the contrary, storage accumulation, preparation for desiccation and germination are much more embryo-specific. As can be seen in table II (Chapter I), many of the cloned genes to date belong to this class, such as LEA, other ABA-regulated genes and those coding for seed storage protein. In this work, no such genes were cloned, mainly due to our sampling scheme, which focused mainly to the earliest stages after induction.

Genes for particular processes. The function of these genes is limited to certain "special" moments of the plant life cycle, and their eventual expression is due to the influence of external environmental factors. Some genes related to the stress response in plants are located in this group.

As a result of the expression patterns analysis during the induction of SE in coffee leaves a chitinase-homologue gene fragment was cloned (Chapter III). The mRNA corresponding to the cloned fragment, and at least three chitinase isoforms, are up-regulated by both SE and wounding, though there might be a hierarchical regulation. The involvement of oligosaccharide-generating enzymes in morphogenesis is a fascinating, and barely explored field for future research. For example, the establishment of polarity in somatic cells seems to be determined by the distribution pattern of arabinogalactan proteins in the cell wall (McCabe et al., 1997) and these molecules can be cleaved by chitinases (Domon et al., 2000). Oligosaccharides can act as signals in the cell wall (Fry et al., 1993) and regulate morphogenesis in plant cells (Spaink, 1996). A receptor for these molecules has been identified (Hahn, 1995; Hervé et al., 1996; Ito et al., 1997). All these facts lead us to consider whether chitinases are involved from the beginning in polarity determination during the onset of somatic embryogenesis.

Recently, Dyachok et al., (2002) found lipophilic chitin oligosaccharides in *Picea abies* which were capable of promoting somatic embryogenesis. Of particular interest is the fact that some chitinases can produce those signal molecules whereas others can inactivate them and thus suggesting that "different chitinases regulate embryogenesis in different ways". In Chapter III, Fig. 5, different chitinase isoforms were differentially regulated during the induction of somatic embryogenesis in coffee. Chi II seemed to be related to non-embryogenic conditions (developing embryos and non-embryogenic suspension cultures), while Chi I was highly expressed from day 12 when somatic embryos begin to form. It will be very interesting to test whether these chitin isoforms have an antagonistic action in promoting or inhibiting somatic embryogenesis in coffee.

A gene fragment (ERSH15) that encodes a low molecular weight heat shock protein (LMWHSP) was cloned in this study (Chapter II, Fig. 4). Its mRNA is down regulated during the induction of SE in coffee (Chapter II, Fig. 2). Although several LMWHSPs have been reported to be regulated during SE in *D. carota* (Zimmerman et al., 1989), *Medicago sativa* (Györgyey et al., 1991) and *P. glauca* (Dong and

Dunstan, 1996), there is not enough evidence to assign a direct role to these proteins. Most probably they may act as helpers in protein folding and trafficking between organelles (see Chapter I).

Genes coding for regulatory elements. UBI9 and AR-60 encode ubiquitin and an ADP-ribosylation factor, respectively (Chapter II). Both mRNA´s are up regulated during the induction of SE in coffee leaf explants and suspension cultures.

UBI9 is expressed in explants at day 0, but after SE induction a 2-fold increment was observed at day 16 from induction, whereas in suspension cultures this increment was only 0.2-fold (Chapter II Fig. 2). Ubiquitin promotes proteosome-dependent degradation of cyclins during the cell cycle (Stals et al., 2000). This can explain why it is activated when SE is induced in leaf explants where an active cell division is occurring headed for the cicatrisation of wounded explants and the formation of somatic embryos. In contrast, active cell division takes place in suspension cultures up to the stationary phase and a large quantity of meristematic cells is formed. Higher expression of proteins that controls the cell cycle can then be expected. As SE induction is carried out from suspension cultures under rapid growth/division conditions, the activity of cell cycle-controlling genes must be high. More detailed work to unravel the role of ubiquitin in somatic embryogenesis is presently underway in our laboratory.

Studying differential gene expression by differential display and reverse transcription probed dot blot

Differential display, first described by Liang and Pardee (1992), is a straightforward technique that allows the analysis of gene expression of samples run in parallel. This technique is very sensitive since it uses the polymerase chain reaction (PCR). Nevertheless, a certain number of problems associated with differential display have been described (see Appel et al., 1999 for an overview). Among them the high percentage of false positives obtained and the cumbersome verification of putative positives. Several alternatives have been proposed to avoid

false positives (Verca et al., 1998; Graf et al., 1997; Sung and Denman, 1997) but, due to the use of northern blot, probing for putative positives is still a time consuming and laborious step, that requires large quantities of RNA. To overcome these difficulties, we designed a reverse dot blot technique where probes are synthesized (and labeled) from 2 µg of total RNA (first strand of cDNA) and cloned fragments from differential displays are immobilized on nylon membranes. In this way, an unlimited number of clones can be probed under various experimental conditions. To achieve that, most of the first strand of the gene of interest must be hybridized, and an excess of the plasmid containing the cloned fragment has to be blotted onto the membrane. To date, we have confirmed by northern blot the expression pattern of ERKI3 and ERKI8 previously obtained by reverse transcription probed dot blot (data not show).

Final considerations: A theoretical model

SE is a very puzzling process. Dissection of an embryo-specific pathway is a cumbersome and laborious task due to the existence of several metabolic and regulatory events, occurring concomitantly to other events taking place in somatic cells that are never reprogrammed toward an embryogenic fate.

In figure 2 a theoretical model to explain SE is presented. Probably, a somatic cell acquires its embryogenic competence after perceiving a chitolipooligosaccharide-like signal result from the action of a chitinase on an arabinogalactan protein. This signal is recognized by a receptor, such as SERK, which, in turn, triggers transcription factors, e. g. LEC genes (see Chapter I) that lead to the activation of the embryogenic program. On the other hand, chitinases seem to participate in the correct positioning of the cell division planes during the epidermis formation and hence, control the passing of globular embryo to further developmental stages. Once the embryogenic competence is acquired, a series of molecular and biochemical events occur straightening the formation of the globular embryo and subsequent stages of embryo development. Active cell division at the onset of embryogenesis may involve the expression of genes, such as UBI 9, AR-60 and DDB-1, whose products can play a role in controlling the cell cycle.

Housekeeping genes should remain active during the entire process, but an increased expression may be observed during active cell division, merely reflecting an enhanced metabolic activity (see Chapter II). Other classes of genes are expressed at defined developmental stages, like homeobox-containing genes, MADS-box genes, chromo box genes, EP2, etc. (see Chapter I), whereas LEA and other ABA-regulated genes, which can be expressed early in embryogenesis, are expected to act at the late stages.

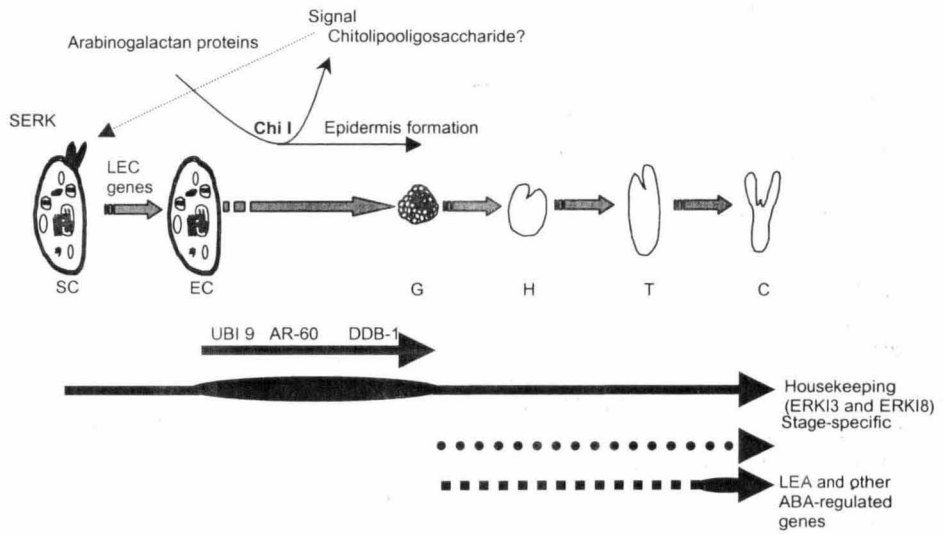

Fig. 2. Theoretical model of SE. Main molecular and biochemical events includes signal generation and perception by a somatic cell (SC) which is then changed into an embryogenic cell (EC). G, globular; H, heart-shaped; T, torpedo-shaped; C, cotyledonal.

Finally, the cloning of genes differentially regulated during SE may help to establish the basis for further biochemical studies to understand why a somatic cell may be rerouted to the formation of a somatic embryo. Results presented in the present thesis are a valuable contribution to our understanding of molecular and biochemical events taking place during SE. To our knowledge, this is the first

systematic evaluation of gene expression patterns during the earliest developmental stages in *Coffea* SE.

REFERENCES.

Appel, M., Bellstedt, D.U., and Gresshoff, P.M. (1999). Differential display of eukaryotic mRNA: Meeting the demands of the new millennium? J. Plant Physiol. *154*, 561-570.

Domon, J.M., Neutelings, G., Roger, D., David, A., and David, H. (2000). A basic chitinase-like protein secreted by embryogenic tissues of *Pinus caribaea* acts on arabinogalactan proteins extracted from the same cell lines. J. Plant Physiol. *156*, 33-39.

Dong, J.Z. and Dunstan, D.I. (1996). Characterization of three heat-shock-protein genes and their developmental regulation during somatic embryogenesis in white spruce [*Picea glauca* (Moench) Voss]. Planta *200*, 85-91.

Dublin, P. (1981). Embryogenèse somatique directe sur fragments de feuilles de caféier Arabusta. Café Cacao Thé *25*, 237-242.

Ducos, J. P., Zamarripa, C. A., Eskes, A. B., and Pétiard, V. (1993). Production of somatic embryos of coffee in a bioreactor... Montpellier, ASIC. 15^{e} Colloque de la Association Scientifique Internationale du café, pp 89-96.

Dyachok, J.V., Wiweger, M., Kenne, L. and Von Arnold, S. (2002). Endogenous Nod-factor-like signal molecules promote early somatic embryo development in Norway spruce. Plant Physiol. 128, 523-533.

Fry, S.C., Aldington, S., Hetherington, P.R., and Aitken, J. (1993). Oligosaccharides as signals and substrates in the plant cell wall. Plant Physiol. *103*, 1-5.

Goldberg, R.B., Barker, S.J., and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. Cell *56*, 149-160.

Graf, D., Fisher, A.G., and Merkenschlager, M. (1997). Rational primer design greatly improves differential display PCR (DD-PCR). Nucleic Acids Res. *25*, 2239-2240.

Györgyey, J., Gartner, A., Németh, K., Magyar, Z., Hirt, H., Herbele-Bors, E., and Dudits, D. (1991). Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol. Biol. *16*, 999-1007.

Hahn, M.G. (1995). Oligosaccharide elicitors and elicitor receptors. In Proceedings of the VIIth International Congress on Plant Tissue and Cell Culture,

M.Terzi, R. Cella, and A. Falavigna, eds. (Dordrecht, The Netherlands: Kluver Academic Publishers), pp. 37-58.

Hervé, C., Dabos, P., Galaud, J.P., Rougé, P., and Lescure, B. (1996). Characterization of an *Arabidopsis thaliana* gene that defines a new class of putative plant receptor kinases with an extracellular lectin-like domain. J. Mol. Biol. *258*, 778-788.

Higashi, K., Kamada, H., and Harada, H. (1996). The effects of reduced nitrogenous compounds suggests that glutamine synthetase activity is involved in the development of somatic embryos in carrot. Plant Cell Tiss. Org. Cult. *45*, 109-114.

Higashi, K., Shiota, H., and Kamada, H. (1998). Patterns of expression of the genes for glutamine synthetase isoforms during somatic and zygotic embryogenesis in carrot. Plant Cell Physiol. *39*, 418-424.

Ito, Y., Kaku, H., and Shibuya, N. (1997). Identification of a high-affinity binding protein for N-acetylchitooligosaccharide elicitor in the plasma membrane of suspension-cultured rice cells by affinity labeling. Plant J. *12*, 347-356.

Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*, 967-971.

Lin, X.Y., Hezari, M., Koepp, A.E., Floss, H.G., and Croteau, R. (1996). Mechanism of taxadiene synthase, a diterpene cyclase that catalyzes the first step of taxol biosynthesis in Pacific yew. Biochemistry *35*, 2968-2977.

McCabe, P.F., Valentine, T.A., Scott, F.L., and Pennell, R.I. (1997). Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot. Plant Cell *9*, 2225-2241.

Söndahl, M.R. and Sharp, W.R. (1977). High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z. Pflanzenphysiol. *81*, 395-408.

Spaink, H.P. (1996). Regulation of plant morphogenesis by lipo-chitin oligosaccharides. Crit. Rev. Plant Sci. *15*, 559-582.

Stals, H., Casteels, M.P., van Montagu, M., and Inzé, D. (2000). Regulation of cyclin-dependent kinases in *Arabidopsis thaliana*. Plant Mol. Biol. *43*, 583-593.

Sung, Y.J. and Denman, R.B. (1997). Use of two reverse transcriptases eliminates false-positive results in differential display. BioTechniques *23*, 462-468.

Sung, Z.R. and Okimoto, R. (1981). Embryonic proteins in somatic embryos of carrot. Proc. Natl. Acad. Sci. (USA) *78*, 3683-3687.

Verca, M.S.B., Verca, S.B., Rusconi, S., and Dreyer, J.L. (1998). Modification of primer design facilitates the use of differential display. BioTechniques *24*, 374-376.

Wilde, H.D., Nelson, W.S., Booij, H., De Vries, S.C., and Thomas, T.L. (1988). Gene-expression programs in embryogenic and non-embryogenic carrot cultures. Planta *176*, 205-211.

Wilde, H.D., Seffens, W.S., and Thomas, T.L. (1995). Gene expression in somatic embryos. In Biotechnology in Agriculture and Forestry. Vol. 30. Somatic Embryogenesis and Synthetic Seed I, Y.P.S.Bajaj, ed. (Berlin: Springer-Verlag), pp. 41-52.

Yasuda, T., Fujii, Y., and Yamaguchi, T. (1985). Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. Plant Cell Physiol. *26*, 595-597.

Zamarripa, C.A. (1993). Etude et dèveloppement de l'embryogenèse somatique en milieu liquide du caféier (*Coffea canephora* P., *Coffea arabica* L. et l'hybride *arabusta*. (France: Centre de Biotechnologie Végétale Francereco), pp. 1-191.

Zamarripa, C. A., Ducos, J. P., Tessereau, H., Bollon, H., Eskes, A. B., and Pétiard, V. (1991). Devéloppement d'un procédé de multiplication en masse du caféier par embryogenèse somatique en milieu liquide. .. San Francisco, ASIC. 14^{e} Colloque de la Association Scientifique Internationale du café. pp 392-402

Zimmerman, J.L., Apuya, N., Darwish, K., and O´Carroll, C. (1989). Novel regulation of heat shock genes during carrot somatic embryo development. Plant Cell *1*, 1137-1146.