lux operon transformation of plastids in higher plants

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Memoria de la tesis doctoral presentada por Aranzazu Balfagón Martin para optar al grado de Doctora por la *Universitat Internacional de Catalunya.*

Trabajo realizado en el Departamento de Biología Molecular y Celular, bajo la dirección de los Doctores Agustí Fontarnau Riera y Alberto T. Estévez.

Proyecto subvencionado por *Fundació La Caixa, INCASOL (Generalitat de Catalunya)* y *MICINN.*

Doctoranda	**Director**	**Co-director**
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Cuando realmente quieres una cosa,

el Universo conspira para ayudarte a conseguirla.

Mi Universo, mi familia

A mis peludos

Kimet, es nuestra

Lo último que uno sabe es por dónde empezar.

B. Pascal

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No tenía miedo a las dificultades: lo que la asustaba era la obligación de tener que escoger un camino. Escoger un camino significaba abandonar otros. . . .

El Alquimista- P. Coelho

JUSTIFICATION

JUSTIFICATION

This work is part of the research in genetics of the Group of Research GENETIC ARCHITECTURES, a transversal and interdisciplinary group of the *Universitat Internacional de Catalunya*, recognized as Consolidated in Research (*ref. 2009 SGR 862*) by the *Generalitat*, the regional government of Catalonia (Spain). The global objective of the group is to investigate on new architectonic forms and materials to solve, in a sustainable and caring way, some of the most important human needs, such as light, heat and habitat, to reduce, at the same time, the human impact on natural environment. Its research covers two different areas of interest: a) the biodigital area, to generate new forms of inhabitable spaces, using a digital methodology and looking for inspiration in biological structures; b) the biotechnological area, to obtain new materials and organisms with added values in energetic or structural aspects and with interest in architecture.

The aim of the group is to present a development model based on the management and conservation of heritage, bringing scientific advances in urban planning, taking into account human welfare and pioneering in the study of digital and biotechnological issues.

In the context of the biotechnological area of the group, we present here our approach to the expression of the *lux* operon from bacterial sources in chloroplasts of higher plants, in order to make them able to emit visible light in an autonomous way, with no need of any external stimuli o supply of energy.

It is worth to note that this work was also a pioneering attempt to transfer a whole multigenic process in chloroplasts. This metabolic pathway is due to the coordinated work of five genes in a sequential manner. Achieving this objective would also amplify the possibilities of genetic engineering technologies for others metabolic pathways.

This research was supported by research competitive grants from *Fundació La Caixa*, *INCASOL* (*Generalitat de Catalunya*) and the *Ministry of Science and Innovation*-Spain (MICINN).

Ja hauràs pogut comprendre què volen dir les Ítaques
Kavafis- L.Llach

MAIN OBJECTIVES

OBJECTIVES

The **main objective** is to perform a genetic transformation of ornamental plants with an operon responsible of bioluminescence phenotype, *lux* operon, achieving a level of intensity in the emitted light that make transformed plants as useful elements in architecture and urban spaces.

- The first partial objective is to obtain organogenesis from leaves of selected ornamental plants, in which organogenesis is not an optimized process, in order to obtain good candidates for chloroplast transformation.

- The second partial objective is to obtain a suitable chloroplast transformation vector with *lux* operon as genes of interest which could express the bioluminescent phenotype in the target specie.

- The third partial objective is to introduce and express our genes of interest in the model specie *N.tabacum* and in ornamental species of interest, as a final target species.

La teoría es asesinada tarde o temprano por la experiencia

A. Einstein

GENERAL INTRODUCTION

1. General introduction

Life on earth is related to oxygenic photosynthesis in almost all higher forms of life. This process is linked to the use of light energy to synthetize the chemical metabolites NADPH and ATP and the subsequent released of oxygen and water. This process is driven by the photosystems I and II which are included in one organelle, the chloroplast, were most of the atmospheric oxygen was produced (Nelson and Ben-Shem 2005).

Plants are also in the base of the food chain and in the origin of agriculture, 11,000 years ago, and therefore linked to the creation of settled, sedentary communities. This shift into an agricultural lifestyle allows the rise of all the great civilizations of recent human history (Zohary et al., 2012) and current crops are the result of domestication in ancient times. Since domestication, farmers have been altering the genetic makeup of the crops in order to improve some features such as faster growth, sweeter fruits or pest resistance by hybridization and selection. Nowadays, the biotechnology allows modifying in plants characteristics by genetic modification.

It was in the early 1980s when the first fertile transgenic plants were created by four groups working independently at Washington University and Monsanto Company (St. Louis, Missouri), the Rijksuniversiteit (Ghent, Belgium) and the University of Wisconsin (Madison, Wisconsin). Since then, the market of GMCs and the hectares occupied by GMCs are rising exponentially. In fact, for the period between 1996 and 2011, biotech crops reached a surface of 1.25 billion hectares (James 2011). Use of plant biotechnology for the production of high-value products is now one of the goals for biotechnology, and use of plants as a molecular farming has the potential to provide a cheap and accessible source of pharmaceutical products and, nowadays, bioplastics and other biomaterials (Somleva et al., 2013).

1.1. Nuclear transformation

1.1.1. Nuclear transformation by *Agrobacterum tumefaciens*

Since Chilton et al. shown in 1977 the possibility to incorporate in the plant nuclear genome a part of a virulence plasmid carried by *Agrobacterium*

tumefaciens (Chilton et al. 1977), a lot of plant species have been transformed. This interkingdom gene transfer is done mediated by *Agrobacterium tumefaciens,* gram-negative soil bacteria (Smith and Townsend 1907) that, in virulent strains, harbors the tumor-inducing Ti plasmid.

In short, this transformation process starts with recognition and attachment to plant cell. This process is mediated by phenolic compounds, basically acetosyringone, released by the plant. Then, this plant signals are recognized by VirA, a membrane integrated protein, that mediated VirG activates the *vir* gene region. Following, this plant signals are recognized by VirA, a membrane integrated protein, which through VirG activates the *vir* gene region. Finally, an ssDNA, named T-DNA, is generated. This T-DNA, will be finally exported into the plant and finally imported to nucleus and integrated in the host nuclear genome. The genes that codify the proteins implicated in this process are codified in the Ti plasmid (Tzfira et al., 2004).

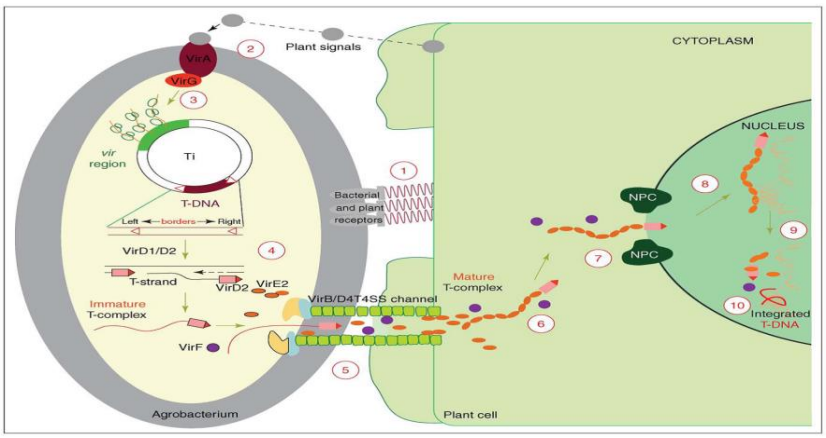


Figure 1.- *Agrobacterium*-mediated genetic transformation process. Extracted from Tzfira and Citovsky , 2006. (1) recognition and attachment to the host cells, (2) sensing of plant signals by VirA/VirG, (3) *vir* gene region (4) T-DNA generated by VirD1/D2 protein complex, (5) VirD2–DNA complex is delivered into the host-cell cytoplasm (6,7) T-complex is transported and actively imported into the host-cell nucleus , (8,9) T-DNA is recruited to the point of integration, (10) and integrated into the host genome.

The knowledge about the mechanism of T-DNA transfer led to the design of vectors for genetic transformation of plants using *Agrobacterium* species. As only the border sequences of T-DNA are required for the transfer , it allows to the development of vectors to introduce foreign genes into plants

(Garfinkel et al., 1981) by replacing genes that code for auxin synthesis of opines for the genes of interest.

1.1.2.PEG-mediated DNA uptake and electroporation

The first attempt to DNA delivery into plant protoplast was reported in 1980 by Davey and colleagues (Davey et al., 1980). Protoplasts are plant cell without their cell wall, which was removed by enzymatic degradation. This removal generates singles cells with no barrier to DNA uptake and PEG causes permeabilization of plasmatic membrane allowing the pass of macromolecules into the cell.

Another method to incorporate DNA into protoplast is the electroporation. The protoplasts are subjected to electrical pulses that again permeabilize the plasma membrane for the macromolecules.

These inexpensive methods help to overcome the handicap of host range limitations of *Agrobacterium*-mediated transformation because this could be adapted to a wide range of plant species and tissue sources. However, there are some disadvantages. On the one hand, there are not routine methods for protoplast culture for some species or there are technically difficulties to establish and maintain this cell culture and regenerate whole plant from them. On the other hand, electroporation improved the simplicity of the technique and the reproducibility of high-frequency DNA delivery but there are the same limitations and led to produce transient expression systems rather than stable transgenic lines (Bates et al., 1990).

2. Nuclear transformation vs. chloroplast transformation

Despite the boom of transgenics, there are several disadvantages in nuclear transformation. As shown in *Table 1*, the number of transgenes integrated in nuclear genome is low and the accumulation of foreign protein is often a limitation. Furthermore, the random integration process could trigger position effect, variation of expression exhibited by identical transgenes that are inserted into different regions of a genome, frequently observed in nuclear transgenic lines (Daniell et al., 2002).

Most of this drawback has been solved by chloroplast transformation, done by first time in 1990 by Svab et al. As a result of it, a new transformation target is allowed: plastids. Then, plants carrying a transgenic plastome will be termed transplastomics (Svab et al., 1990). Site-specific integration into the chloroplast genome by homologous recombination of flanking chloroplast DNA sequences present in the chloroplast vector eliminates the concerns of position effect. Also, there are solved the effect of transgene silencing. In fact, the accumulation of transcripts could arrive at a level 169-fold higher than in nuclear transgenic plants (Lee et al., 2003). This transcripts accumulation is related to the ability to accumulate large amounts of foreign protein at levels up to 46% of total leaf protein when the transgene is stably integrated (De Cosa et al. 2001). In part, this is due to the polyploidy of the cpDNA with up to 10,000 copies in each plant cell, resulting in a very high number of functional gene copies (Bendich 1987).

	Chloroplast genome	Nuclear genome
Transgene copy number	Many transgene copies (up to 10,000) per cell	Few copies of the transgene per cell
Level of gene expression	Polyploidy results in high transcripts and foreign proteins accumulation	Accumulation of foreign protein is often a limitation
Gene arrangement	Multiple transgenes can be introduced and expressed in a single transformation event	Each transgene is independently inserted and transcribed into a monocistronic mRNA
Position effect	Site-specific insertion eliminates position effects on transgene expression	Random insertions result in variable transgene levels of expression
Gene silencing	Not reported	Transcriptional and post-transcriptional gene silencing have been reported
Gene containment	Maternal gene inheritance in most crop plants results in natural gene containment	Paternal transgene inheritance results in outcrossing among crops and weeds
Folding and disulfide bond formation	Chloroplasts form disulfide bonds and correctly fold human proteins	For disulfide bond formation, proteins are targeted to the endoplasmic reticulum
Toxicity of foreign proteins	Adverse effects might be minimized by chloroplast compartmentalization	Toxic proteins accumulating within the cytosol might result in serious pleiotropic effects
Transgenic lines	Uniform gene expression	Highly variable gene expression
Homogeneity at ploidy level	Homoplasmy is mostly achieved by repetitive selection and regeneration	Homozygosity is achieved either by selfing or crossing

Table 1.- Comparison of chloroplast and nuclear genetic engineering. Adaptation from Daniell et al., 2002, showing the main important differences between the genetic transformation in chloroplast genome (left) and nuclear genome (right).

Another important advantage of plastid transformation relative to nuclear transformation with respect to concerns of outcrossing of transgenic pollen is that plastid genomes are very rarely transmitted in pollen in most angiosperm plant species, with chloroplast maternally heritage (Svab and Maliga 2007). Therefore, it may be possible to prevent the transmission of transgenes to other plants using stable chloroplast transformation instead of nuclear transformation. In short, all of these advantages of chloroplast transformation listed on *Table 1* might allow becoming a more widely practiced transformation technique.

3. Chloroplast structure and function

Plastids, the plant cellular organelles, have their own genome and transcription and translation machinery that share almost all characteristics with prokaryotic mechanisms (Kuroda and Maliga 2001). Chloroplast, the green plasts presents in leaves, are semi-autonomous organelles that possess its own DNA and machinery of transcription and translation. The most accepted theory for its origin is the endosymbiont theory first proposed by Lyyn Margulis at 1967 in which paper showed the features shared between chloroplasts and its prokaryote antecessor (Howe et al., 2003).

The chloroplasts are a 5-10 µm diameter organelles found in the cytosol of leaf cells. It presents different regions: thylakoids and stroma, all surrounded by a double membrane. Thylakoids are the intern membranous system that has a very organized network of vesicles. In their membranes are inserted the four major multisubunit protein complexes involved in photosynthesis: PSI, PSII, the cytochrome b6f complex and the ATP synthase complex (Wollman et al., 1999) formed by nearly 100 proteins (Friso et al. 2004) .This photosystems absorb photons through their core pigment molecule, primarily chlorophylls. Then, this energy pass through protein complexes of the electron transport chain to be finally will be used to generate a hydrogen proton gradient across the membrane. The ATP synthase complex, embedded in thylakoid membrane, drives the photophosphorylation of one ADP molecule. The released electrons are ultimately used to reduce NADP to NADPH, the final electron acceptor. This chemical energy is used in the dark phase to CO_2 fixation in Calvin's cycle (Berg et al., 2006).

Most of the soluble proteins presents in the chloroplast stroma are encoded in 2,100-3,600 nuclear genes (Leister 2003), synthetized in cytosol and post-translationally imported to stroma and only a small fraction are synthetized by chloroplast ribosomes (Maul et al., 2002). These ribosomes differ in size and molecular composition from their cytoplasmic counterparts and are similar but not identic in certain protein composition to prokaryotic (Manuell et al., 2007).

3.1. cpDNA

The chloroplast genome is a double stranded and circular DNA molecule with a size that varies between 120-160 kb in higher plants (Bendich 1987) that encodes about 120 genes, most of them organized in operons. cpDNA of each organelle is found in several copies attached to membranes (Kobayashi et al., 2002) and clustered in so-called plastid nucleoids (Kuroiwa and Suzuki 1981) . The number of cpDNA molecules per organelle and the number of plastids per cell is highly variable and depends on the cell type (Bendich 1987). For reference, each leaf cells in *N.tabacum* contains 100 chloroplasts, each chloroplast containing 100 cpDNA molecules. That gives 10,000 cpDNA per cell (Thomas and Rose 1983). Almost all the higher plants cpDNA share the same structure: a circular, double stranded molecule, with two fragments of single sequence, the Small Single Copy (SSC) of about 15 to 25 kb and the Large Single Copy (LSC) of about 80 to 100 kb separated by two Inverted Repeats regions (IRa and IRb) that are usually 20 to 30 kb in size (Palmer 1983). Similarly, the chloroplast nucleotide sequence is highly conserved (Douglas 1994), specially within IRs. Most of the IRs sequences are genes that encode for rRNAs and certain tRNAs. This region has the higher evolutionary conservation due the slower rate of point mutations accumulation (Perry 2002).

Higher plant plastid genomes have generally relatively high levels of adenine and thymine (high-AT) in its codons. For expression of foreign proteins, a change in GC content of native sequence for one more AT-rich resulting in approximately 1.5–2-fold gains in protein accumulation. This is generally less improvement than has been observed in *E. coli* suggesting that the plastid genome is able to express transcripts derived from diverse sources including non-AT-rich sequences (Reddy et al., 2002).

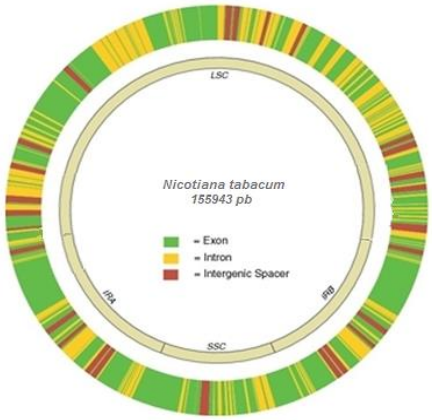


Figure 2 Structure of the chloroplast genome of *Nicotiana tabacum* showing the LSC, SSC and IR regions. The green color shows coding regions and the non-coding regions are yellow for introns and red for spacer regions, representing about 40% of the genome. Extracted from Wakasugi et al., 1998

3.2. Chloroplast gene expression

The chloroplast genome, has we talk previously, share common properties with both prokaryotic and eukaryotic systems. Like prokaryotes, they have σ^{70} type promoters, operons, Shine-Dalgarno-like sequences and 70S ribosomes. Like eukaryotes, some of their genes have introns and produce highly stable mRNAs.

3.2.1.Chloroplast gene expression regulation

For plant survival, it is crucial that the gene chloroplast expression has a proper regulation in response to environmental and development cues (Baecker et al., 2009). This regulation can be divided in two main processes: transcription, that determines which gene is used as a template for mRNA production, and post-transcriptional processes, which include physical modification of mRNA (editing, splicing and cleavage), RNA stability and translation. Gene expression levels in plastids are predominately determined by promoter and 5´-UTR (untranslated regions) elements (Gruissem and Tonkyn 1993).

3.2.2.Plastids RNA polymerases

In chloroplast there are two types of RNA polymerases: NEP (nuclear-encoded polymerases) and PEP (plastid-encoded polymerases). PEP is a

cyanobacterial-type RNA polymerase that forms a multisubunit complex which is the predominant transcriptional active species in mature chloroplasts. PEP recognize the typical prokaryotic promoters consensus sequences TTGACA (-35) and TATAAT (-10) which can be founded upstream of plastids operons (Liere et al., 2011). The holoenzyme depends on the addition of sigma factors, encoded by nuclear genes (Fujiwara et al. 2000).

POSITION DISTRIBUTION

	-35						-10					
	T	T	G	A	C	A	T	A	T	A	A	T
A	0	5	2	35	7	30	1	55	14	36	40	1
T	58	49	1	4	13	12	59	5	27	7	7	56
G	0	2	51	6	14	7	0	0	9	12	2	1
C	0	2	4	13	24	9	0	0	10	5	11	2
Chloroplast	100	84	88	60	41	52	98	92	45	60	67	93
Procaryotes	82	84	79	64	54	45	81	95	44	59	51	96

Table 2.- Distribution of Bases at each Position in Promoters of Chloroplast Genes.
Dates from alignment of 60 promoters sequences compiled given in overall a statistically similar conserved degree than procaryotic promoters. Modify from Kung and.Lin 1985

The presence of NEP was suggested by Ellis and Hartey in 1971 (37) and, in fact, plastid genes were still transcribed when PEP genes are deleted in chloroplast of *N.tabacum* but leads to off-white phenotypes suggesting a complex functional integration of PEP and NEP into the genetic system of the plant cell (Legen et al. 2002).

3.2.3.Chloroplast operon promoter

In general, in prokaryotic systems the promoters consist of two regions of conserved sequences, located about 10 and 35 bp upstream of the transcription start-point and separated by an optimal distance of 17 bp (Hawley and McClure 1983). Most of the plastid promoters, conform to its cyanobacterial origin, contain the -35 (TTGaca) and -10 (TAtaaT) consensus sequences of typical eubacterial σ^{70}-type promoters (Liere and Borner 2007). These σ^{70}-type promoters are also termed PEP promoters because they are used by PEP polymerases. Link demonstrated that the upstream regions are required for efficient in vitro transcription and subsequently the importance of the distance between them was indicated. In fact, only one base pair insertion in maize *rbcL* promoter reduced the level of transcription drastically

(Kung and Lin 1985; Link 1984). Despite this, two other types of plastid promoters were discovered.

Type-II promoters lack a consensus motif around position -35 and differ completely in sequence and organization from Type-I promoters. They are called 'non-consensus' NEP promoters (NCII) (Miyagi et al., 1998).

The third type-identified is thought to be an internal promoter of a subpopulation of chloroplast tRNA genes. Very few examples of each type of chloroplast promoter have been analyzed and very little is known about the function and regulation (Klein et al., 1994).

In order to obtain high-level protein accumulation from expression of the transgene, the first requirement is a strong promoter to ensure high levels of mRNA. For this reason, the strong σ^{70}-type plastid rRNA operon promoter (P*rrn*) is usually used to drive transgene expression. This P*rrn* promoter has binding sites for both the NEP and PEP polymerases. Another usual promoter is the *psbA* promoter (P*psbA*), whereas P*psbA* contains only a PEP transcription start site (Allison et al., 1996). Protein accumulation from the same (P*rrn*) promoter may vary as much as 10,000-fold depending on the choice of translation control signals. Protein accumulation from the transgene depends on the 5'-UTR inserted upstream of the open reading frame encoding the genes of interest. To enable translation, P*rrn* is fused with the 5' UTR of plastid or other prokaryote-type genes.

3.2.4.Regulatory sequences

Stability of the transgenic mRNA is ensured by the 5'-UTR and 3'-UTR sequences flanking the transgenes.

Shine-Dalgarno (SD) sequence is a Ribosome Binding Site, located at 5' UTR, found in prokaryotic mRNAs. It forms a base paired region with its complementary sequence at the 3' terminus of 16S rRNA prior to initiation of translation (Bonham-Smith and Bourque 1989). In *E. coli*, SD sequences remain highly conserved, having the consensus sequence AGGA, which displays perfect complementarity to the 3 'terminus of 16S rRNA. Mutations in the Shine-Dalgarno sequence can reduce translation due to a reduced mRNA-ribosome pairing efficiency. SD-like sequences are found in the leaders of many , but not all, mRNAs from chloroplasts, having the consensus

sequence GGAGG and competition assays confirmed that also interacts with the 3' terminus of chloroplast 16S rRNA. However, this 5'UTR are hypervariable in location, size, and base composition compared to those in *E. coli* (Hirose and Sugiura 2004).

The 3' regulatory region, located immediately downstream of the stop codon, encodes the mRNA 3' UTR, typically harbors a stem-loop-type RNA secondary structure that facilitate RNA maturation and prevent degradation of the RNA by ribonucleases (Stern et al., 2010). The 3' regulatory region is important for mRNA stability (Monde et al., 2000). 3' UTRs used to regulate foreign genes in plastids are usually derived from the plastid *psbA*, *rbcL*, and *rps16* genes and, nowadays, the pair of 5'-UTR and 3'-UTR most commonly used is *psbA/*T*psbA* (Kittiwongwattana et al. 2007).

3.2.5.Operon organization

Most of the genes encoded in higher plant chloroplasts are organized as operons (Barkan 1988) and, as shown in *Table 3*, genes that bear part of related functions are usually clustered in operons and transcribed as polycistronic units. It facilitates the coordinate and stoichiometric accumulation of subunits that belongs to the same metabolic pathway (Mullet 1993). Then, these primary transcripts are further modified to produce functional mRNAs. In higher plants, post-transcriptional modifications include RNA cleavage of pre-existing RNAs, RNA stabilization, intron splicing and RNA editing.

Operon	Gene products
16S-trnI-trnA-23S-4.5S-5S rRNA	rRNAs, tRNAs
rpl23-rpl2-rpslg-rpl22-rps3-rpl16 -rpl14-rps8-infA-rpl36-rpsl1-rpoA	ribosomal proteins, initiation factor 1, RNA pol subunit α
rpoB-rpo1 -rpoC2	RNA polymerase subunits
psbl-psbK-psbD-psbC-orf62- trnG	PSII subunits, orf62, tRNA
psb B-psb H-pet B-pet D	PSII and Cyt subunits
psaA-psaB-rpsl4	PSI subunits, ribosomal protein
atp I-atp H -atp F-atpA	ATP synthase

Table 3.- Gene composition of selected plastid operons. Extract from Mullet 1993 showing the eight main operons present in chloroplasts.

4. Chloroplast transformation

The plastid transformation started with the discovered of biolistic delivery by John Sanford and colleagues during the late 1980s. Nowadays, biolistic delivery is the preferred system in most laboratories for chloroplast transformation that allows transformation of leaves, cotyledons, or cultured cells in tissue culture and requires less experience than the alternative PEG treatment of protoplasts. The first target to be transformed was *Chlamydomonas reinhardtii* chloroplasts (Boynton et al. 1988) and soon was followed by *Nicotiana tabacum*, *Arabidopsis thaliana* (Sikdar et al. 1998) and more recently, several plants are attempted to transform (Sidorov et al. 1999).

The biolistic process, as shown in *Figure 3* for *N. tabacum*, starts with the biolistic delivery of plasmid DNA that contains a Gene of interest (GOI) and selectable marker and finished with obtaining homoplasmic secondary shoots.

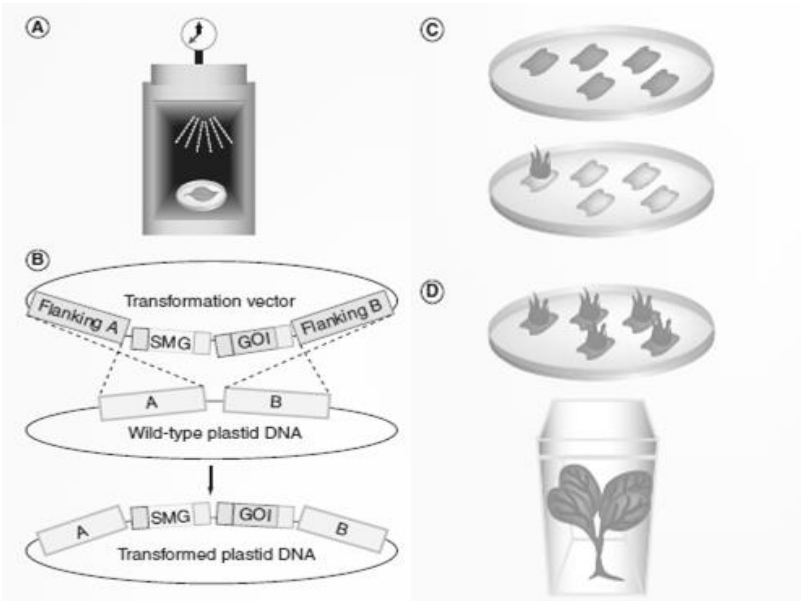


Figure 3.- Schematic representation of the plastid transformation process in higher plants. (A) Biolistic delivery of plasmid DNA containing the transgene of interest and a selectable marker gene (B) Integration of the expression cassette in cpDNA by homologous recombination (C) Regeneration of heteroplasmic primary shoots on selective medium (D) Regeneration of homoplasmic secondary shoots. Extracted from Cardi et al., 2010.

4.1. **Plastids transformation vectors**

Plastid transformation vectors are *E. coli* plasmid derivatives with cloned cpDNA sequences flanking the chloroplast expression cassette. The vector carries origin of replication (ori) sequences that allow the plasmid to replicate in bacteria and a bacterial resistance gene facilitating the transformation in bacteria and the production of large quantities of plasmid. The plastid vector propagated in *E. coli*, is then introduced into plastids. The expression cassette is integrated in the targeted region by two homologous recombination events and the *E. coli* vector part is lost done given their absence of a plastid replication origin.

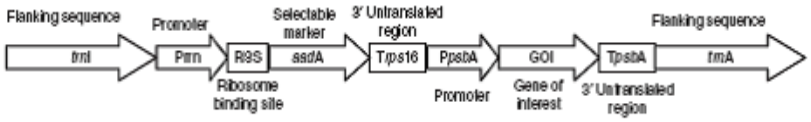


Figure 4.- Schematic representation of the chloroplast integration and expression cassette. This map of chloroplast-expression vector shows the flanking sequence, promoter, regulatory elements, selectable marker gene and the gene of interest (Verma et al., 2008).

As shown in *Figure 4*, the chloroplast expression cassette contains several critical elements: a chloroplast operon promoter, ribosome-binding sequences, a selectable marker gene (e.g., antibiotic resistance), a 3'-UTR, gene promoter, a 5'-UTR, the gene of interest and its 3'-UTR all of them between two flanking sequences (Verma et al., 2008).

4.2. Homologous recombination and flanking sequences

The homologous recombination process that occurs in chloroplast is well documented, however, little is known about the molecular mechanism involved in its recombination or their biological significance.

In bacteria, RecA is a crucial component in homologous recombination and recombination at DNA repair (Kowalcykowsky 2000). Mutation of *recA* confers a dramatic reduction not only in the efficiency of homologous recombination but also in the extent of cellular tolerance to DNA damage. Chloroplast homologues of bacterial RecA proteins have been identified in

Chlamydomonas reinhardtii (Inouye et al. 2008) and higher plants (Lin et al. 2006) and have been suggested that the major function of the highly active recombination machinery is maintenance and repair of cpDNA. In *C. reinhardtii* the repair and recombination of cpDNA was suppressed when a dominant mutant version of *E. coli* RecA was targeted to the chloroplasts (Cerutti et al. 1995). Similarly, a plant *Arabidopsis thaliana* cDNA encoding for a bacterial RecA homolog had been isolated (Cerutti et al. 1992) and was found to be targeted to chloroplasts and seems that the process by which damaged DNA is repaired in bacteria has been retained in their endosymbiotic descendent, the chloroplast (Rowan et al., 2012).

This homologous recombination is the base for integration of foreign sequences in the plastid genome. In spite of that it is possible to achieve integration without 100% sequence identity between the vector and plastid genome sequence. The recombination and hence transformation efficiency decreases when sequences are divergent. (Ruhlman et al. 2010).The chloroplast transformation vector contains flanking sequences which are used for foreign gene insertion. These flanking regions, with 1–2 kb in size, are homologous to the desired site of integration in host plastid genome and facilitate site-specific recombination and define the final integration site of the transgene.

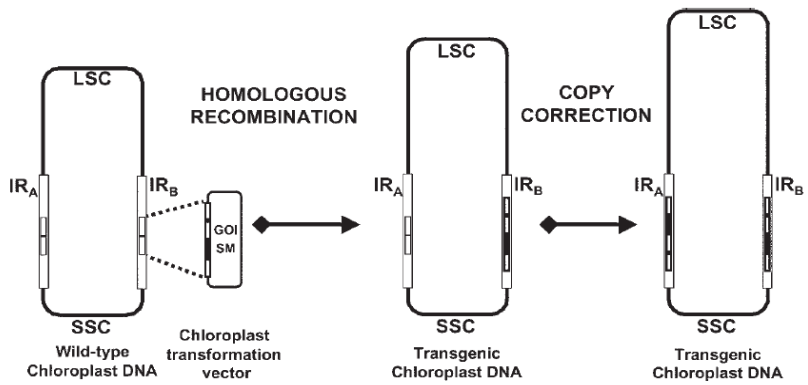


Figure 5.- Schematic representation of chloroplast homologous recombination and copy correction phenomenon. Modified from Daniell and Chase 2004.

Transgenes integration sites could be transcriptionally silent spacer regions, transcriptionally active spacer regions or read-through spacer regions. This is followed by transgene integration into the chloroplast genome via homologous recombination facilitated by a RecA-type (Cerutti et al., 1992) system between the plastid-targeting sequences of the transformation vector and the targeted region of the plastid genome. Chloroplast transformation vectors are thus designed with homologous flanking sequences on either side of the transgene cassette to facilitate double recombination. Targeting sequences have no special properties other than that they are homologous to the chosen target site and are generally about 1 kb in size. Both flanking sequences are essential for homologous recombination. Transformation is accomplished by integration of the transgene into a few genome copies, followed by 25 to 30 cell divisions under selection pressure to eliminate untransformed plastids, thereby achieving a homogeneous population of plastid genomes. If the transgene is targeted into the IR region, integration in one IR is followed by the phenomenon of copy correction that duplicates the introduced transgene into the other IR as well. Chloroplast vectors may also carry an origin of replication that facilitates replication of the plasmid inside the chloroplast, thereby increasing the template copy number for homologous recombination and consequently enhancing the probability of transgene integration. Also, *oriA* is present within the *trnI* flanking region (Kunnimalaiyaan and Nielsen, 1997; Lugo et al., 2004), and this might facilitate replication of foreign vectors within chloroplasts (Daniell et al., 1990), enhance the probability of transgene integration, and achieve homoplasmy even in the first round of selection (Guda et al., 2000).

Integration of foreign DNA in intergenic regions of the plastid genome had been accomplished at 16 sites but the most commonly used are *trnV-3'rps12*, *trnI-trnA* and *trnfM-trnG* (Maliga 2004). The *trnV-3'rps12* and *trnI-trnA* sites are located in the 25 kb inverted repeat (IR) region of cpDNA and thus a gene inserted into these sites would be rapidly copied into two copies in the IR region. The *trnfM-trnG* site is located in the large single copy region of the cpDNA, and the gene inserted between *trnfM* and *trnG* should have only one copy per cpDNA. These two tRNAs are located between the small (*rrn16*) and large (*rrn23*) rRNA subunit genes and the operon is transcribed from promoters upstream of rrn16. The polycistronic *rrn* operon mRNA is

efficiently processed, releasing the transgenic mRNA inserted between the two tRNAs.

The region most commonly used is the transcriptionally active spacer region between *trnI* and *trnA* genes. This region is located within the rRNA operon, where the *16S* rRNA promoter drives transcription of six genes and each spacer region within this operon is transcriptionally active. The *trnI* gene intron also contains a chloroplast origin of replication, which might facilitate replication of foreign vectors within chloroplasts and enhance the probability of transgene integration. This region also offers the unique advantage that transgenes lacking promoters or 5'- or 3'-UTRs can be inserted and expressed.

4.2.1.Homoplasmy and antibiotic resistance markers

Transplastomic shoots regenerated from leaves after bombardment are always chimeras than, in selective media, cells with plastids with the expression cassette integrated, containing antibiotic resistance markers, shows preferential propagation (Moll et al., 1990). The preferred method to obtain homoplastomic tobacco plants is regenerating new shoots from the transplastomic sectors, which are then rooted (Svab et al., 1990b; Svab et al., 1990a).

Done cpDNA is present in many copies, when one or few cpDNA is transformed, primary markers are used for selectively resistance. This critical process involves gradually diluting plastids carrying non-transformed copies on a selective medium and sorting out of non-transformed plastids because wild-type proplastids are antibiotic sensitive and divide more slowly (Maliga 2004).

The most common antibiotics used are spectinomycin, streptomycin and kanamycin, which inhibit protein synthesis on prokaryotic-type plastid ribosomes and inhibiting finally greening, cell division, and shoot formation in tobacco culture. Then, shoot formation is used to identify transplastomic clones on a selective medium. The *aadA* gene encoding aminoglycoside 3-adenylyltransferase that inactivates spectinomycin and streptomycin (GenBank X02340, M10241) was used as a selection marker gene. The transformation with *aadA* gene dramatically improved the recovery of

plastid transformants to a rate of, on average, about one transplastomic line in a bombarded leaf sample (Svab and Maliga 1993).

The initial chloroplast transformation event involves the change of only a single (or at most a few) out of several thousand plastid genome copies in a leaf cell. During subsequent cell and organelle divisions, the presence of high concentrations of the selecting antibiotic favors multiplication of chloroplasts containing transformed genomes, whereas chloroplasts harboring only wild-type genomes may be eliminated effectively. However, individual chloroplasts may still contain a mixed population of wild-type and transformed plastid genome molecules (intraorganellar heteroplasmy). In additional rounds of plant regeneration on selective medium, gradual sorting out of residual wild-type genomes is achieved, eventually leading to cells with a homogeneously transformed population of plastid genomes commonly referred to as ``homoplasmic'' or ``homoplastidic''. Formation of homoplastomic cells is accelerated by chloroplast to proplastid dedifferentiation, with a concomitant reduction in cpDNA number in tissue culture cells following by a rebuilding in regenerated plants. (Thomas and Rose 1983).

Así debéis hacer vosotros: mantenecos locos, pero compertaos como personas normales.
Torred el riesgo de ser diferentes, pero aprended a hacerlo sin llamar la atención.

Verónica decide morir- P. Coelho

HYPOTHESIS

HYPOTHESIS

Considering the broad benefits of chloroplasts transformation as an expression system to express foreign proteins and the ability of this system to express an entire operons, the main hypothesis of the project, in which this thesis is based, is that should be possible to express the *lux* Operon in chloroplast of higher plants.

Therefore, our experiments will be conducted to demonstrate that it is possible to achieve expression of the *lux* operon in chloroplasts of *N. tabacum* as a model specie and in ornamental plant as a target specie.

- For this reason, our first hypothesis is that is possible to obtain a good organogenesis rates in ornamental species, as will be explained in Chapter I.

- Our second hypothesis is that lux operon can be expressed in chloroplast of higher plants, which will be explained in Chapter II.

Elige un trabajo que te guste y no tendrás que trabajar ni un día de tu vida.

Confucio

EXPERIMENTAL PROCEDURES

EXPERIMENTAL PROCEDURES

1. *In vitro* plant culture and regeneration experiments

For **plant species selection**, a commercial analysis was done considering the ornamental value. This study was performed following the commercial standards and criteria of *Corma S.L.,* company leader in ornamental plant. Other considerations were: maternal inheritance of pollen and phylogenetic distance between the selected species with *N.tabacum*. All selected species received a high score considering these three inputs. Plants were divided in herbaceous and woody plants specimens.

As shown in *Table 4*, **plant culture media** were MS or MS ½ containing 3% of sucrose and 0.8% phytoagar, pH 5.8. Cultures were maintained in a growth chamber under short day conditions (8 h light (150 $\mu molm^{-2}s^{-1}$) 16 h darkness, 24±2 °C).

A

Micronutrients	µM	Macronutrients	mM
KI	5.00	NH_4NO_3	20.61
H_3BO_3	100.27	KNO_3	18.79
$MnSO_4.4H_2O$	100.00	$MgSO_4$	1.50
$ZnSO4.7H_2O$	29.91	$CaCl_2.aq$	2.99
$Na_2MoO_4.2H_2O$	1.03	KH_2PO_4	1.25
$CuSO_4.5H_2O$	0.10	Vitamins	µM
$CoCl_2.6H_2O$	0.11	Nicotinic acid	4.06
FeNaEDTA	100	Thiamine · HCl	0.30
		Pyridoxine · HCl	2.43
		Glycine	26.64
		Myo-inositol	554.94

B

Micronutrients	µM	Macronutrients	mM
KI	5.00	NH_4NO_3	10.3
H_3BO_3	100.27	KNO_3	9.6
$MnSO_4.4H_2O$	100.00	$MgSO_4$	0.75
$ZnSO4.7H_2O$	29.91	$CaCl_2.aq$	1.5
$Na_2MoO_4.2H_2O$	1.03	KH_2PO_4	0.65
$CuSO_4.5H_2O$	0.10	Vitamins	µM
$CoCl_2.6H_2O$	0.11	Nicotinic acid	4.06
FeNaEDTA	100	Thiamine · HCl	0.30
		Pyridoxine · HCl	2.43
		Glycine	26.64
		Myo-inositol	554.94

Table 4.- Plant media composition. (A) Murashige and Shoog medium (MS) (B) Half strength Murashige and Shoog medium (MS½)**.** Extracted from Murashige and Skoog 1962.

In vitro culture media were supplemented with concentrated **plant growth regulators** (PGRs) solutions as necessary. Hormone solutions were 0.22 µm filter sterilized and stored at -20ºC. GA_3 (# 77-06-5), IAA (# 87-51-4) and 2,4-D (# 94-75-7; Sigma) were prepared at a concentration of 1 $mg.ml^{-1}$ in bi-distilled water plus 0.05% EtOH96, necessary to achieve its complete

dissolution. BAP (# 1214-39-7), NAA (N 0903) were dissolved with 500 µL HCl 0.5N and TDZ (# 51707-55-2) plus 0.05% 1M NaOH. All growth regulators were from Duchefa unless otherwise indicated.

For the establishment of *in vitro* woody plant lines, five mature healthy plants from selected woody species growing in a greenhouse under controlled environment in non-sterile conditions were used as explant donors. Healthy leaves and internodal segments were excised using a sharp blade from the basal part of plants.

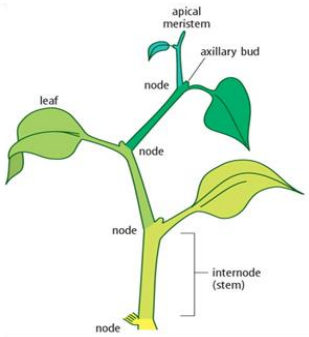


Figure 6.- Schematic representation of explant donors parts in a plant.

For **disinfection** of leaves and internodal segments in order to establish axenic cultures, four selected protocols, with increasing degree of hardness, were tested.

For Protocol 1, leaves and internodal segments were washed for 10 minutes in running water, surface-sterilized by immersing in 70% ethyl alcohol for 1 min. Then, 25% (v/v) commercial bleach sodium hypochlorite containing 0.1% (v/v) Tween 20 was added for 20 min, gently mixing by inverting and then rinsed three times in sterile distilled water. For Protocol 2, leaves and internodal segments were maintained in 25% commercial bleach sodium hypochlorite for 30 min, for Protocol 3 and 4, an additional incubation step with 0.01 % $HgCl_2$ and 0.1 % $HgCl_2$, respectively, was done for 15 min.

For **callus induction** two different media were used: MS supplemented with 2 $mg.l^{-1}$ 2,4-D (MSI) and MS supplemented with 2 $mg.l^{-1}$ 2,4-D and 1 $mg.l^{-1}$ BAP (MSII). Once callus was obtained, indirect regeneration experiments with different auxin/cytokinin ratios were arranged in a completely randomized design (CRD). Explants were kept in culture room under white fluorescent lamps (150 $\mu molm^{-2}s^{-1}$ with 16 h light/8 h dark cycle at 26±2 ºC) for two months.

For **establishment of *in vitro* plant lines from seeds**, seeds of *N.tabacum Wisconsin38*, gently given to us from IRTA collection seeds, ornamental seeds, obtained from Semillas Fitó (Barcelona, Spain), and *Codariocalyx*

motorious seeds, obtained from Pépinières Karnivores (Colmar, France), were disinfected.

Seeds disinfection was performed according three protocols with increasing degree of hardness: Soft disinfection, medium disinfection and hard disinfection. For soft disinfection, seeds were surface-sterilized by immersing in 70% ethyl alcohol for 1 min, then in 25% commercial bleach sodium hypochlorite (v/v) in water containing 0.1% Tween 20 (v/v) for 10 min, gently mixing by inverting and then rinsed three times in sterile distilled water. For Medium disinfection seeds were maintained in 25% commercial bleach sodium hypochlorite for 30 min and for hard disinfection a previous incubation step in 0.001 % $HgCl_2$ 10 min was added.

For **seeds germination**, a random experiment was done. Three replicates with 10 seeds per species were inoculated in a Petri dish in three different media. The media used were MS0 (MS without PGRs), MS with 1 $mg.l^{-1}$ GA_3 and incubation at 30ºC for 24h in water with 1 $mg.l^{-1}$ GA_3 following by MS0 medium. Petri dishes were kept in culture room under white fluorescent lamps (150 $\mu molm^{-2}s^{-1}$ with 16 h light/8 h dark cycle at 26 ºC). Seeds were kept on growth chamber for two weeks. A multivariable protocol for non-germinating seeds was also performed. Seeds placed on MS0 and MS supplemented with 1 $mg.l^{-1}$ GA_3were kept on darkness at 12, 18, 22 ºC and 26ºC for two months.

In **regeneration experiments**, leaf explants were cut into 0.5 x 0.5 cm squares, except for *D. caryophyllus* that was 0.4 x 0.4 cm, with a sterile scalpel (avoiding large leaf veins and any damaged areas). The leaf pieces are then transferred (adaxial side up) to MS medium supplemented with different concentrations of auxin and cytokinin hormones. Treatments were arranged in a completely randomized design (CRD). Explants were maintained at 24±2ºC under white fluorescent lamps (150 $\mu molsm^{-2}s^{-1}$) with a photoperiod of 16 h light/8h darkness or only darkness. Each experiment was conducted for 15 weeks. All treatments consisted of three replicates and each replicate contained 10 explants. Callus induction was analyzed with the subsequent code: - callus compact and browning, + callus no friable; ++to callus friable; +++ callus friable and pro-organogenic. Regeneration was analyzed with the subsequent code: - for no regeneration, + at least 1 shoot

in at least 1 explant; ++ at least 1 shoot in at least 6 explants; +++: at least 1 shoot in every explant. Root formation was reported also.

In vitro raised shoots were excised from leaf explant with a sharp blade and placed on a MS0 or MS ½ media for rooting under white fluorescent lamps (150 µmolsm^{-2}s^{-1}) with a photoperiod of 16 h light/8h darkness.

2. Vector construction

The **genotypes** of *Escherichia coli* strains used for cloning procedures was ***E.coli* DH5α:** F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1 . (Takara #9057) and ***E.coli XL1-Blue****:{recA1endA1gyrA96thihsdR17supE44relA1lac[F´proABlacIqZΔM15Tn10(Tetr)]}(Stratagene).*

Vibrio fischeri strains used for genomic DNA isolation were *V.fischeri* BB170, BB152, BB886 and NRRL-B-11177. These strains were gently given to us by Dr. Rodrigo Horacio González (University of Buenos Aires).

Culture media were supplemented by selective antibiotic as described by Sambrook *et al.* 1989 with concentrated solutions of the following antibiotics in order to select and maintain plasmid-containing *Escherichia coli* strains and transformant plants. Antibiotic solutions were sterilized by filtration and stored at −20 ºC. Ampicillin (Ampicillin sodium, # A0104.0025 Duchefa) and Spectinomycin (#50188.0025 Duchefa) were used with according concentrations. *V. fischeri* was cultured as described previously in seawater tryptone medium **(**Boettcher and Ruby 1990) at 26 ºC, o/n, 250 rpm.

Media	Content
Luria-Bertani medium	Bacto Tryptone 10 g·l^{-1}, Yeast Extract 5 g·l^{-1},NaCl 10 g·l^{-1}, pH 7
Luria-Bertani agar + X-Gal	LB + x-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; #R0941, Fermentas). Final concentration : 40 µg·ml^{-1}.
SWT Medium	NaCl 23 g/L Na_2HPO_4 , 15,5 g/L NaH_2PO_4 ,2g/L Nutrient Broth Oxoid #2 10 g/L pH 7.8 with NaOH 1M Difco agar (BD): 15 g.l^{-1}.

Table 5.- Culture media used in this study. Luria-Bertani (LB) and seawater tryptone medium (SWT) are shown.

The primers for **cloning procedures** were designed on the target sequence provided to analyze and by applying the first tool Blast database of the NCBI and checked with *Lasergene* program. This tool designed primers under specific conditions of temperature and dissociation length thereof and the length of amplicon. Additionally the specificity of the primer was verified with a blast on the sequence of the desired organism. DNA amplifications were carried out using Taq polymerase with the buffer supplied using standard protocols for a BioRad Thermal Cycler.

Primer name	Sequence 5'-3'
Lux CD R	TTAATAAGGATCCTTAGGCTAATTCCAATAGCTCATTCTC
LuxCD F	CGCGCGCCCGGGGAGGAATCATTAATGATTAAATGTATTCCGAT
Lux EF-F	GCCTTAATCTAGAGGAGGGAACCATGACTAATCATATTGAATATAAAAAAAATCAAA
Lux EF-R	AATTTAAAGCGGCCGCTTATATGTATGCAAAAGCATCGGC
LuxAB F	GCCTAA GGATCCTTATTAAGAAGGAATAGAGATAATGAA GTTTGG
LuxAB R	CCTCCTCTAGATTAAGGCAGATTCTTTTCGATTTTCT

Table 6.- Primers used for cloning procedures. Forward (F) and reverse (R) 5'-3' sequence of primers are shown.

Unless otherwise stated, PCR volumes were made up to 100 µl with sterile deionised water and the PCR products were stored at 4°C. All PCR were properly optimized for each gene. For transplastomic plants, Phire Plant Direct PCR Kit (# F-130, Thermo Scientific) was used for screening of recent shoots of putative transplastomic *N.tabacum* plants and PCRs were performed according manufacturer's instructions. This kit allows to amplify by PCR 0.50 mm punches from leaves and control positive primers directly into 50 µl PCR without tissue waste.

For bacterial DNA, DNeasy® Blood & Tissue Kit (#69506 QIAgen) was used with specific modifications for Gram negative bacterial sources.

Sequences to be digested were checked with the programs JustBio and Lasergene. Restriction endonuclease enzymes (FastDigest NotI #FD0593, BamHI #FD0054, XbaI # FD0684, SmaI # FD0663, EcoRI #FD0274, and HpaI # FD1034 all from Fermentas) were used with the buffers supplied and manufacturer's instructions always followed and deactivated by incubation at 75°C for 5 min. Plasmids were dephosphorylated with FastAP Thermosensitive Alkaline Phosphatase #EF0651 from Fermentas following

manufacturer's instructions to avoid self religation. Digested samples were analyzed by agarose gel electrophoresis.

DNA products from isolated plasmid or genomic DNA, cDNA, PCR and restriction enzyme digestions were visualized after electrophoresis in agarose gels. Around 5-10 µl of samples were loaded with 3 µl of 6x DNA loading dye (Fermentas) to 0.8 to 1% (w/v) agarose gels with 0.05 $\mu g.ml^{-1}$ ethidium bromide added. The resolution capacity of this method was adjusted by the concentration of agarose in the gel and it was carried out in a horizontal tank containing TAE buffer and run at 100 V for 1 h. For size estimation, 2.5 µl of an appropriated molecular weight marker (GeneRuler 1kbPlus DNA Ladder #SM1333, GeneRuler 100 Pb DNA Ladder #SM0321 and GeneRuler 1kb DNA Ladder #SM0311 from Fermentas) were loaded. Gels were imaged using an UV trans-illuminator Imager.

Fragments amplified by PCR or digested can be purified for further sub-cloning from the gel after agarose gel electrophoresis. QIAquick Gel Extraction Kit #28704 was used for purification of the fragments from the gel. In this method the agarose gel is dissolved and DNA fragments bind to the matrix of the spin column and fragments between 50bp – 10Kb can be finally eluted with low salt buffer or water.

Ligations between the insert and the vector were performed with the enzyme T4 DNA ligase (Roche Applied Science, catalog number 10481220001). Reaction conditions were established as recommended by the manufacturer following the molar ratio between insert and vector in the reaction mixture. It depends on the size of fragments to be ligated. Similar sized fragments are ligated with a recommended molar ratio insert: vector of 10:1. The ratio used for multiway ligation was 2:1. The concentration of DNA was between 10 and 20 $ng.ml^{-1}$ and the final volume must not exceed 25 ml. All ligations were incubated 16 h and a gradual temperature from 4 to 16 º C. After ligation, the enzyme was inactivated by incubation for 10 min at 65 ° C. For easy ligations, Rapid Ligation Kit #K1422 from Fermentas was used following manufacture's recommended conditions.

Transformation of *E. coli* competent cells with foreign DNA was performed according to the method described by Cohen *et al.*,1972. After

transformation, cells were grown at 37°C in LB medium supplemented by selective antibiotic as described by Sambrook *et al.* 1989. For rapid screening of putative transformed bacterial colonies, colony PCR was done by picking bacterial colonies using sterile pipete tips and resuspending the cells in 10 µl sterile deionised water. 1 µl of the supernatant was transferred to the PCR mixture and the PCR was run according to the above protocol. For transformation with *E. coli* DH5α, the α-complementation system was used to differentiate strains containing recombinant pUC19 plasmids (white) from those did not (blue) (Sambrook et al., 1989) by white/blue screening. For this purpose, 20 $mg.ml^{-1}$X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to transformation plates.

Plasmid extraction was performed using Wizard® Plus Minipreps DNA Purification System (Promega, #A7100) and eluted in 100µl nuclease-free water.

In order to check the correct vector design, a **bioluminescence assay** was performed using Synergy™ HT Multi-Mode Microplate Reader (Biotek®) and white opaque microplates. Samples were diluted independently to different final concentrations (1:1, 1:10, 1:100 v/v) with 1 $mg.ml^{-1}$ BSA. 20 µl of each dilution were pipetted into wells of a microplate in duplicates. The plate was then studied by the Synergy™ HT Multi-Detection Microplate Reader for bioluminescence detection. The plate was allowed to adapt to dark at 37ºC for 10 minutes. This dark adaptation period allows the microplate to dissipate any residual autoluminescence, resulting from energy absorption by the plate itself. For incomplete Lux Operon vectors, the bioluminescent reaction was initiated by dispensing 1 to 5 µl of n-decanal, substrate of the reaction. Instead, no substrate was required in *lux* Operon vectors. The luminescence of each well was then mixed and bioluminescence was measured. Finally, data for all measurements were then exported to Microsoft Excel for subsequent analysis. All samples were normalized in front of control samples (LB media) and in front of absorbance to normalize density.

3. Biolistic transformation of chloroplasts

In order to generate transplastomic lines, biolistic PDS-1000/He (Bio-Rad) equipment was used. Settings were: 1,100 psi rupture disks, a fixed distance of 12 cm between the bombardment capsule containing the microprojectils and transformation plates and 0,6mm gold particles. All devices were properly disinfected.

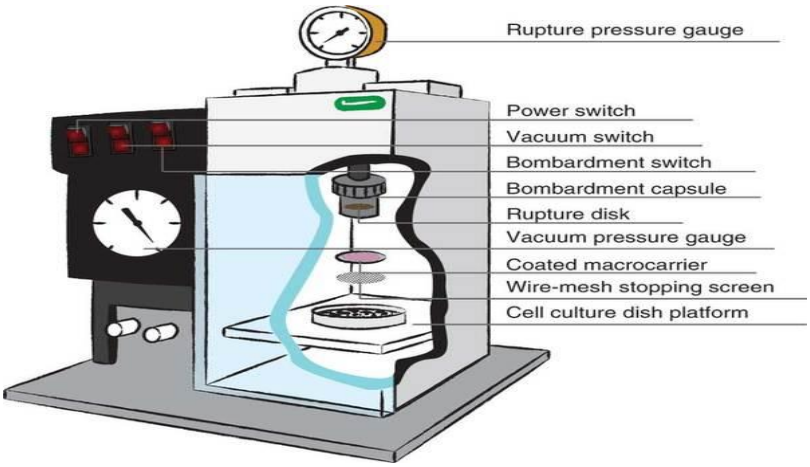


Figure 7.- PDS-1000/He Biolistic® system (Biorad). Extracted from Wu et al., 2011

For **coating gold particles with DNA,** 50 mg of gold particles were added in a siliconized 1.5-ml Eppendorf tube and washed with 1 ml molecular grade 100% ethanol following to 70% v/v ethanol. The mixture was incubated at room temperature for 15 min and mixed intermittently by gentle shaking. Finally, it was washed and resuspended in 1 ml of sterile 50% v/v glycerol and stored at -20 ºC until use. For one bombardment, corresponding to five plates, were added to a 50 µl gold particles suspension: 5 µg of DNA (1µg/µl), 50 µl of 2.5 M $CaCl_2$ and 20 µl spermidine 0.1M (Spermidine, S1369, Duchefa). Mixture was stirred for 3 minutes and then washed twice with 250 µl of 100% ethanol. Finally, it was resuspended in 50 µl 100% ethanol. DNA-coated gold particles can be stored on ice for 2–3 h and should be used as soon as possible.

In order to perform **leaf tissue bombardment** experiments, tobacco leaves were harvested at 5 to 7 leaf stage and placed on sterile Whatman 70-mm circle filter disk on RMOP medium (Svab, et al, 1975). Leaves were placed with the adaxial side facing to the filter disk. DNA delivery was performed using a standard particle bombardment method as described by Standford et *al*., 1987. After 48 hours of incubation in the dark, bombarded leaves were cut into small pieces and placed in RMOP medium with spectinomycin selection (500 $mg.l^{-1}$). Plates were cultured in a culture room for 4-6 weeks in order to wait putative transgenic green shoots appeared from bleached leaves. Once plantlets were achieved 5 to 7 leaves stage, they were propagated *in vitro* twice in RMOP to achieve homoplasmy and finally propagated using Microbox tissue vessels in order to allow increasing concentration of gas exchange from 9,87 GR / day to 81,35 GR / day (volumetric gas exchange coefficient : number of gas remplacements in the vessel per time unit). Finally, when roots were established, the roots were thoroughly washed to remove the phytoblend or agar and transferred to a greenhouse in pots containing substract #5 with automatic dispensing of fertirigation solution diluted 1:60, as shown in *Table 7,* in a transparent plastic box to maintain high relative humidity. The box was kept in a CRAG greenhouse chamber #11 (large day conditions, 25±2 ºC light, 21±2 ºC dark with ambient humidity) and was progressively opening until box was completely open. Then, plants were grown in the same chamber until seeds were produced.

A

Substract # 5	
N	250 $mg{\cdot}l^{-1}$
P_2O_5	150 $mg{\cdot}l^{-1}$
K_2O	270 $mg{\cdot}l^{-1}$
pH	5,5 – 6,0

B

Element	Concentration	
NO_3K	8.4 mM	0,85 $g{\cdot}l^{-1}$
NH_4NO_3	1,2 mM	0,096 $g{\cdot}l^{-1}$
K_2HPO_4	1,2 mM	0,209 $g{\cdot}l^{-1}$
KH_2PO_4	3,6 mM	0,489 $g{\cdot}l^{-1}$
$Ca(NO_3)_2 . 4H_2O$	2,5 mM	0,596 $g{\cdot}l^{-1}$
$MgSO_4 . 7H_2O$	0,7 mM	0,172 $g{\cdot}l^{-1}$
SO4Fe . 7H2O	0,6 mM	0,166 $g{\cdot}l^{-1}$
Kelamix Complex	-	35 $mg{\cdot}l^{-1}$
Microelements	-	0,4 $g{\cdot}l^{-1}$

Table 7.- Greenhouse media. (A) Substract composition for acclimatation pots **(**B) Fertirigation solution elements for acclimatation. Hoagland and Arnon's 1938 solution (modified by Jonson et al., 1957).

4. Molecular tools

4.1. ptDNA assays

For **genomic DNA isolation** from plants, 100mg wet weight from leaves were disrupted using N_2 liquid and ground with a mortar and pestle. Samples were processed with DNeasy® Plant Kit (#69104, QIAgen) following manufacturer's instructions and stored at -20ºC until use.

For **cpDNA isolation,** tobacco chloroplasts were isolated according to manufacturer's instructions (Sigma # CPISO-1KT). Briefly, 20 g of leaves without midribs (wet weight) of 48h light deprived plants were homogenized using a mortar and pestle in 1x CIB-BSA Buffer. The macerate was passed through a filter mesh, centrifuged and the supernatant collected and centrifuged again at 1000g for 7 min. The resulting chloroplast pellet was resuspended in 1x CIB-BSA Buffer and treated with proteinase K at 37◦ C for 30 min. DNA from isolated chloroplasts was extracted using DNeasy Blood & Tissue Kit (# 69504, QIAgen) with some modifications: chloroplasts were centrifuged for 5 min at 300xg. The pellet was resuspended in 200 µl PBS (50 mM potassium phosphate, 150 mM NaCl; pH 7.2) and 20 µl of proteinase K , 200 µl Buffer AL, 0.7% N-lauroylsarcosine and 0.5% SDS (v/v) were added and incubated at 25 ºC for 12h. Finally, DNA was precipitated and concentrate with sodium acetate 3M pH 5.2 method. Concentration and purification was checked using Gene5 program and Synergy™ HT Multi-Mode Microplate Reader (Biotek®).

Specific primers design, as shown in *Table 8*, and cloning procedures for **ptDNA amplifications** were performed with standard tools as explained before.

Primer name	Sequence 5'-3'
3P	AAAACCCGTCCTCAGTTCGGATTGC
3M	CCGCGTTGTTTCATCAAGCCTTACG
C+ F	AGTTCGAGCCTGATTATCCC
C+ R	GCATGCCGCCAGCGTTCATC

Table 8.- Primers used for ptDNA amplifications. Forward (F) and reverse (R) 5'-3' sequence of primers are shown.

4.2. SDS gel electrophoresis and Western blot

SDS-PAGE and WB were performed using standard methods. 100mg wet weight from leaves from *in vitro* plants were disrupted using liquid N_2 and ground with a mortar and pestle. Sample lysis was performed by sonication with cold lysis buffer, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40 with protease and phosphatase inhibitors and immediately used or stored at −20 ºC. Then, a Bradford assay was using and the absorbance displacement between 465-595 nm was measured using Synergy™ HT Multi-Mode Microplate Reader (Biotek®) and Bradford reagent (#500-006, BioRad). A convenient standard curve was done using increasing concentrations of bovine serum albumin (BSA).

SDS-PAGE was performed using "Mini-Protean 3 Cell" System (BioRad) and 7.5% Mini-PROTEAN® TGX™ Precast Gel (#456-1021, BioRad) and 12% Mini-PROTEAN® TGX™ Precast Gel (#456-1041, BioRad) following manufacturer's instructions. Equal amounts of protein were prepared by adding Loading Buffer (10mM Tris-HCl, 0.4% SDS (v/v), 20mM DTT, 25 % Glicerol (v/v), pH=6.8) and subsequently boiled at 95 ºC for 5 min. Samples and commercial molecular weight marker ("Precision Plus Protein Standard-Kaleidoscope"; #161-0375, BioRad) were run in Electrophoresis Buffer (25mM Tris, 0.2M Glicine, 0.2% SDS (v/v)) at constant voltage of 100-120 V. Then, resolved proteins were transferred with constant amperage of 400mA from the polyacrylamide gel to Polyvinylidene difluoride Hybond-P (PDVF) activated membranes using "Mini Trans-Blot Electrophoretic Transfer Cell" (#170-3930, BioRad) during 1 h at 4 ºC in Transfer Buffer (25 mM Tris, 0.2 M Glicine, 10% MetOH (v/v), pH=8.3). Membranes were blocked for 1 hour with constant agitation with blocking buffer (5% of skimed milk (p/v) dissolved in TBS- Tween (5mM Tris, 15mM NaCl, 10mM KCl, 0.2% Tween (v/v), pH= 7.4).
Membranes were incubated o/n at 4ºC with the correspondent primary antibody, as shown in *Table 9*.

Antibody	Origin	Dilution	Reference	Type
LuxA-HRP	Rabbit polyclonal	1:500	ab390	Ab - HRP
LuxA custom	Rabbit polyclonal	1:1000	#35434 Genemed	Ab primary
Rubisco	Rabbit polyclonal	1:2000	ab62391	Ab primary
Anti Rabbit (IgG) HPR	Goat polyclonal	1:5000	401315 Calbiochem	Ab secondary - HRP

Table 9.- Antibodies used in this study. Origin, dilution, commercial reference and type were also listed.

Primary antibodies were diluted in TBS- Tween, with 5% (v/v) of BSA and 0.01 % of sodium azide. Membranes were then washed three times for 10 minutes with TBS-Tween and were incubated with the correspondent secondary antibody conjugated to peroxidase (HPR), diluted 1:5.000 in Blocking Buffer for 1 hour at room temperature with continuous agitation. Membranes were washed three times with washing buffer. Finally membranes were revealed with ECL chemiluminiscent reactive (Millipore). For LuxA-HRP, some modifications were performed. Membranes were incubated 1h at 37ºC in darkness with continuous agitation with LuxA-HRP antibody diluted 1:500 in blocking buffer without sodium azide. Membranes were then washed three times for 10 minutes with TBS-Tween and finally were revealed with ECL chemiluminiscent reactive (Millipore).For deshibridation, membranes were incubated with Stripping Buffer (Biorad) for 45 minutes at 55ºC , washed three times with TBS-Tween and a standard immunodetection was done.

4.3. Real Time PCR

RNAs from leaves were isolated by the TRIzol® RNA isolation kit (Invitrogen Life Technologies). 100 mg of weight leaves tissue were mechanically disrupted in nitrogen liquid to a fine powder during 30" and nitrogen was allowed to evaporate. Then, the material was transferred to the Trizol extraction buffer (1ml·mg^{-1}) and the suspension was homogenized by shaking moderately for several seconds and incubated at room temperature for 2 min. 200 µL of chloroform were added to each ml of cell suspension, samples were mixed on the vortex for 30 seconds and phases were separated by centrifugation at 12.000 rpm for 15 min at 4ºC. The upper aqueous phase (500-600 µL) was transferred to a new eppendorf, and 500 µL of ice-cold-2-propanol was added to each tube, and samples were allowed to precipitate overnight at -20ºC. After that, samples were centrifuged at 14.500 rpm for 15 min at 4ºC. Supernatant was decanted and the obtained pellet was washed with 500 µL of EtOH75, mixed in a vortex for 30 seconds, and centrifuged at 14.500 rpm for 10 min at 4ºC. Supernatant was decanted again, and pellet RNA was dried at 37ºC for 15 min. Finally, RNA was resuspended in 25 µL of RNAase free water and solubilized at 55-60ºC for 15 min. RNA purity and concentration was quantified in a spectrometry micro plate reader (Biotek Synergy HT). Optimal purity values were considered

between 1,8-2,1 (Abs260/280 nm).1µg of the total obtained RNA was retrotranscribed using 5X iScript cDNA Synthesis Kit (Bio-Rad, #170-8890), following the manufacturer's instructions. The reaction protocol used was 5 min at 25ºC, 30 min at 42ºC and 5 min at 85ºC. For quantitative amplification, 250 ng of reverse-transcribed cDNA products were amplified with *SYBR® Green Kit* (Biorad) following manufacturer's instructions and specific primers shown in *Table 10*.

Primer name	Sequence
LucP F	AGGTCGCATCTCTGAGGAGT
LucP R	CAATAGCGGCAGTTCCTACA
Actin F	CCTGAGGTCCTTTTCCAACCA
Actin R	GGATTCCGGCAGCTTCCATT
18SrRNA F	GGTGGAGCGATTTGTCTGGT
18SrRNA R	CAGGCTGAGGTCTCGTTCGT

Table 10.- Primers used for Real Time amplification. Forward (F) and reverse (R) 5'-3' sequence of primers are shown.

The reaction protocol used was 5 min at 95ºC, and 35 cycles with 95ºC for 1 min, 62ºC for 3 min and 71ºC for 3 min and final melting curve with increment of 0,5ºC every 5 sec. *Actin* and *18S* genes were used as housekeeping controls. Experiments were carried out in three technical replicates for each sample. Data were analyzed by standard ΔΔCt method.

4.4. IVIS Spectrum *In vivo* bioluminescence assay

Plants in sterile boxes were properly surface disinfected and placed on Imaging chamber.

Figure 8.- IVIS Spectrum from Xenogen for *In vivo* and *in vitro* optical imaging. Image extracted from IVIS manual.

In Living Image Software, exposure time, f-stop and pixel binning can be optimized based on the expression level of the sample. Then, luminescence was selected as Imaging Mode. Exposure time was selected between 3 and 300 second. F/stop, which controls lens aperture size, adjusting the amount of light received by the CCD was selected between f/1 and f/4, being f/1 used for low light resolution luminescence and f/2 or f/4 used for brighter. Smaller F/stop means larger aperture and thereby increased sensitivity. Larger F/stop means smaller aperture and gives less sensitivity but better resolution. Binning improves the signal of noise ratio to read noise, but also reduces spatial resolution and medium (8 pixel) and large (16 pixel) were selected. As shown in *Figure 9*, IVIS Spectrum has a total of 18 emission filters with 20nm bandwidth and a total of 10 excitation filters with a 35nm bandwidth. Different combinations were selected in order to detect luminescence.

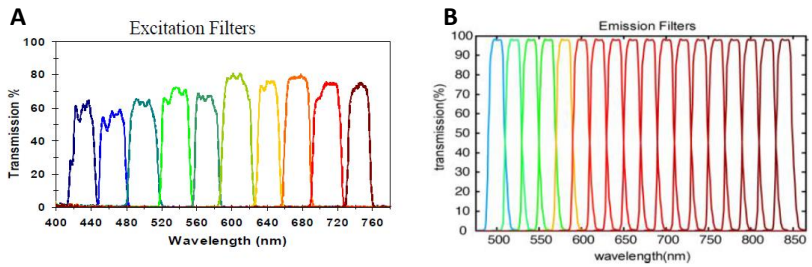


Figure 9.- Filters for IVIS Spectrum. (A)Excitation filters (B) Emission Extracted from IVIS Spectrum manual.

Chapter I: In vitro regeneration of ornamental plants

La tierra se ríe en flores.

Edward Estlin Cummings

INTRODUCTION

In vitro regeneration of ornamental plants

An important part of cells of any higher plant species possess the characteristic of totipotency: the capability of independent development up to a whole, mature new plant, if proper external conditions are provided (White 1954). This regeneration capability of a whole organism from cells of adult somatic tissue is a well-known phenomenon. Compared with animals, plants have a profound capacity to regenerate organs from their differentiated somatic tissues through the manipulation of plant hormones.

1. Micropropagation

Micropropagation is the name given to clonal propagation and it can be used for propagation of species and varieties, to maintain specific lines free from pathogens and it has applications in genetic breeding programs (Tombolato and Costa 1998).

1.1. Stages of micropropagation

There are four states for micropropagation of plants.

Stage I is characterized by the establishment of axenic cultures. It starts by the disinfection of explants followed by the initiation of shoot growth. This shoot formation could start in pre-existing meristems or in adventitious meristems depending on the type of explant. According to Mantell et al., 1994, there is a range of explants that can be as large as seedlings and organs (such as in ovule or embryo culture) or as small as isolated cells and protoplasts. The main objective of this stage is to obtain free-contamination lines with a controlled environment.

Stage II is characterized by shoot production and multiplication. Newly formed shoots could be subcultured in order to obtain multiple plantlets.

Stage III is characterized for shoot elongation and rooting and could include the hardening of plants to allow its future *ex-vitro* acclimation.

Stage IV is characterized for the final transference of plants to soil under natural conditions, process that is named acclimation. It includes the

progressive modification of leaves anatomy (stomata and epicuticular layer), the increase of photosynthesis rate and the progressive adaptation to real environmental conditions with lower humidity, higher illumination and variable temperatures (Davey and Anthony 2010), a hardening process that should allow the plant to be able to survive *ex vitro*.

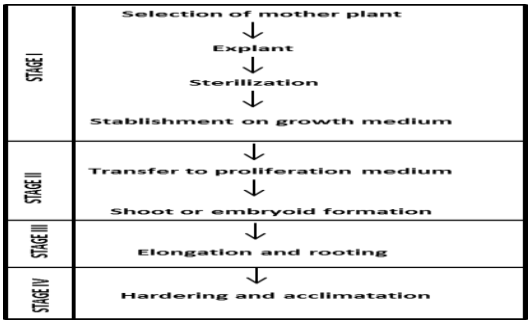


Figure 10.- Major stages of micropropagation. Modified from Chawla et al., 2004.

2. Plant morphogenesis *in vitro*

Morphogenesis is the process that results in the formation of discrete organs or whole plants from somatic cells of isolated tissues.

There are two ways to achieve plant morphogenesis *in vitro*, embryogenesis and organogenesis, and morphogenesis is the result of an organized division and differentiation of cells through patterns of expression of certain genes (Fehér et al., 2003). In short, the production of stems is independent from roots in organogenesis while in embryogenesis the embryos result from a process that originates a stem and a radicle simultaneously, like in seeds.

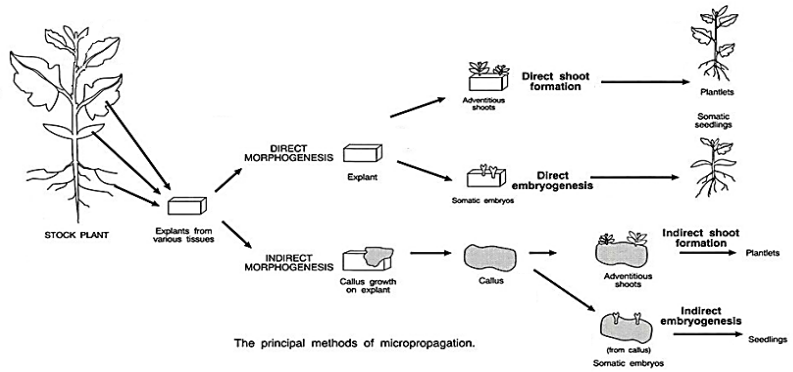


Figure 11.- Principal methods of morphogenesis showing direct and indirect morphogenesis.

2.1. Somatic embryogenesis

In somatic embryogenesis, a new plant with both root and shoot axes arises from actively dividing cells, but it does not form any direct vascular connections with the original tissue (Hicks 1980). Once initiated, the somatic embryo develops in a similar manner that a zygotic embryo does it from a seed (Meinke 1995).

Embryo formation begins with cell divisions that establish the apical-basal axis. Further divisions elaborate, on this basic plan, pro-embryo and globular stages, heart-shaped, torpedo and cotyledonary embryo stages that finally will form the cotyledons, as well as the apical meristems of root and shoot. Embryogenesis can be direct or indirect. When embryos are started from unorganized tissue, callus, it is referred as indirect embryogenesis while direct embryogenesis occurs when embryos are started directly from explant tissue, without formation of a callus intermediate. This embryogenic callus, named type I, has a number of morphologic characteristics: it is compact, highly organized, pale white to yellow in color and with slow growing rate. Frequently, this callus is surrounded by a callus type II, soft, friable and with higher proliferation rate (Vasil and Vasil 1984). It could be maintained *in vitro* for long periods (Armstrong and Green 1985).

1.2. Somatic organogenesis

In contrast to somatic embryogenesis, the organogenic pathway to obtain a whole plant, either by direct or indirect way, requires a sequential culture in different media. This necessity is due to media that promote development of shoots inhibit root formation, and vice versa.

Pioneering work has shown that a high auxin/cytokinin ratio induces root regeneration, whereas a low ratio promotes shoot induction (Skoog and Miller 1957). This indicates that auxin and cytokinin might have a cross-talk during *in vitro* organogenesis. So far, the molecular mechanism of such interaction between auxin and cytokinin in formation of meristems *in vitro* remains mostly unknown.

Buds can be formed directly from the explant (direct organogenesis) or indirectly from callus. In contrast to embryogenesis, vascular connections are

present. In indirect organogenesis, callus is produced and adventitious buds and shoots develop with vascular connection to vascular tissue. Compared to indirect type, direct organogenesis do not produce callus and adventitious organs originate in the cut surface of the explant.

3. Plant tissue culture

Tissue cultures represent the major experimental systems used for plant genetic engineering as well as micropropagation, and it has also become an important part of the commercial propagation of many plants because of its advantages as a multiplication system (Iliev et al., 2010). It involves asexual methods of propagation and it is the reference name given to all types of plant cultures like callus, cell, protoplast, meristem and embryo cultures, among others (George 1993).

3.1. Plant media

There is no single formula that can promote and/or maintain optimum growth of different tissues, cells and organs, scilicet there are no universal media for *in vitro* culture. Each different plant species and cultivars have its specific requirements with regard to different components of the medium (Saric et al., 1995) but it must contain organic substances and mineral elements.

Formulas by Murashige and Skoog, Quorin and Lepoivre, White and Gamborg have been used as starting points in several plant species and significant alterations are made in the components to meet specific cases. Sucrose or any other sugar source is the only component that is always present in the culture media and its concentration varies from 3 to 12% (Do Valle and Faria 2001).

3.1.1.Plant hormones

The importance of the levels of cytokinins and auxins in callus induction and organogenesis have been demonstrated for a large number of plant species since Skoog and Miller discovered in 1957 that both auxin and cytokinin were synergistically required to induce cell division and growth in plant tissue cultures (Skoog and Miller 1957).

The existence of antagonistic and additive interactions between these two plant hormones are a fact, but generalizations about using plant growth regulators in plant cell culture media cause some difficulties because of the great differences in culture response among species.

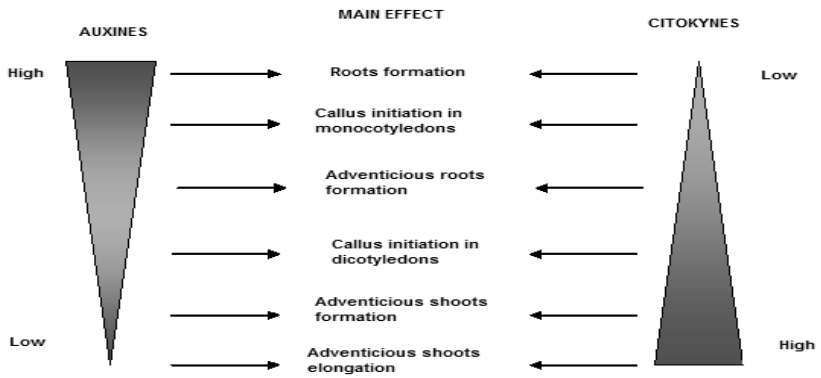


Figure 12.- Main effect of relative concentrations of auxins and cytokinins in *in vitro* culture.

Auxins positively influence cell enlargement, bud formation and root initiation. The most important naturally occurring auxin is indole-3-acetic acid (IAA), but it is unstable to both heat and light. In *in vitro* culture, synthetic auxins as 1-Naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) are commonly used. Cytokinins, a large group of structurally related purine derivatives, influence cell division and shoot formation. 6-Benzylaminopurine (BAP), Thidiazuron (TDZ) and Zeatin (Z) are commonly used alone or in combination with auxins. Gibberellins are involved in regulating cell elongation, germination, dormition and senescence. The most common gibberallin used in plant tissue culture media is Gibberellic acid (GA_3).

4. Ornamental plant tissue culture

The production of flowers and ornamental plants in Europe continues to increase the current valuation of 19.000 million euros. It represents 40% of the world's production.

In 1991, 156 different ornamental genera were propagated through tissue culture worldwide (Rout et al., 2006).

This ornamental plant sector is continuously looking for product improvement, either as new varieties or by way of added value of existing varieties, such as resistance to drought (Xianjun et al., 2011), to diseases (Azadi et al., 2011) or novel flower colors (Nishira et al., 2011).

4.1. Ornamental species used in this study

The plants used for this study are grouped by their phylogeny following the APGIII classification, as shown in *Figure 13*. This is a molecular-based system for plant taxonomy that bear in mind the interrelationships of some orders and families of core eudicots.

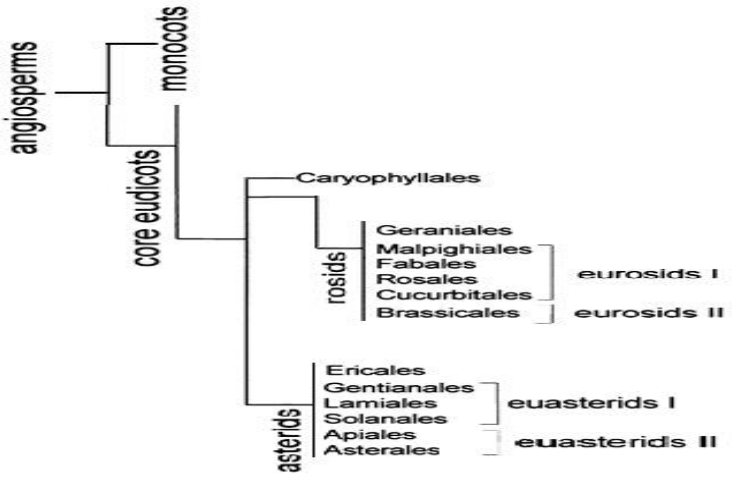


Figure 13.- APG3 classification. Modified from the Botanical Journal of the Linnean Society 2009.

Phyllostachys aurea is a Monocotyledoneae that belongs to ***Poaceae order***. There are about 88 genera and 1400 recorded species of bamboo in the world, 34 genera and 534 species of which are in China (Wu and Raven 2006).

Dianthus caryophyllus resides in the ***Caryophyllales order*** as well as *Mirabilis jalapa* and *Bougainvillea glabra* which contains 86 genera and 2,200 species

(Bittrich 1993). *D. caryophyllus* (carnation) is native mainly to Europe and Asia. Carnation is used as an economically important cut flower and a bedding plant due to perpetual flowering (Mii et al. 1990) and the presence of single and multicolor cultivars (Dole and Wilkins 1999). *M. jalapa* is a bushy perennial that belongs to *Caryophyllales* order**,** it is original from South America and is commonly used as a medicinal plant as well as an ornamental plant (Aoki, et al., 2008). *B.glabra*, a woody ornamental plant with aesthetic and economical values, is native from Latin America and commonly used for grown in gardens and boundary walls (Shah et al., 2006).

The **rosids** are members of a large clade of flowering plants, containing about 70,000 species, containing a quarter of all angiosperms (Wang et al., 2009) including the *Geraniales*, *Malpighiales Fabales, Rosales*, *Cucurbitales and Brassicales* orders*.*

Pelargonium spp., commonly known as geraniums, belong to the ***Geraniales Order*** and are among the most economically important bedding and pot plants in North America with yearly sales in excess of $100 million.

Viola spp. is an herbaceous annual that belongs to ***Malpighiales Order*** and is present throughout much of Europe and Asia (Lagerberg 1948; Mossberg et al. 1992). It is predominantly outcrossing (Lagerberg 1948; Skogsmyr and Lankinen 1999).

Fabales order, which contains about 350 plant species, is the order of *Codaryocalyx motorious* and *Celosia argentea*. *C. motorious* (syn. *Desmodium motorium* or *D. gyrans*), is a potential ornamental plant owing to its capacity of movement of its lateral leaflets under the influence of white light of various intensities. *Celosia argentea* is a tender annual that is often grown in gardens. Its leaves and flowers are edible and grown for this use in Africa and Southeast Asia (Grubben and Denton 2004).

Ficus benjamina belongs to ***Rosales*** order and is a species of flowering plant native to south and south-east Asia and Australia (Berg and Hijmann 1989). It is commonly used as ornamental plant.

Begonia, the flowering plant genus, belongs to a ***Cucurbitales order***. This genus is widely used as ornamental houseplant and it has pantropical

distribution. Plastomic phylogenies indicated that extant Begonia lineages first diversified in Africa and then in America and Asia. B*. semperflorens-cultorum* was originated from Brazil. This wax type begonia is a bedding plant and is often used to create flowerbeds and gardens.

Mathiola incana belongs to the ***Brassicales order*** and it is used as a cut flowers and a bedding plant. This plant prefers cold temperatures and it is a good competitor in the winter market of cut flowers.

Asterids, a clade according to the APG III system for the classification of flowering plants, belongs to *Ericales, Gentaniales, Lamiales, Solanales Apiales* and *Asterales* order.

Cobaea and *Cyclamen persicum* belongs to ***Ericales* order**. *C. scandens* is native to the tropical America and widely cultivated for its highly ornamental, bell-shaped flowers, which change from white to purple (Hoyos 1998), and *C. persicum,* an important flowering pot plant native from Asia Minor (Abu-Qaoud 2004). *Primula hortensis* is one of the most important commercial pot flowers, perennial and well-appreciated (Widmer 1891).

Nerium oleander belongs to ***Gentianales Order*** and is an evergreen ornamental plant valued for evergreen foliage and terminal flowers, available in different colors (Isabel Santos et al., 1994).

Lamiales order includes approximately 11,000 species divided into about 20 families. *Antirrhinum majus*, *Coleus x hybridus, Mentha viridis* and *Origanum vulgare* belongs to this order. *A. majus* is a herbaceous short-lived perennial plant characterized by a patchy distribution in southern Europe. Its geographic distribution is centered over the Pyrenees, between north-eastern Spain and south-western France (Khimoun et al., 2011). *C. hybridus (Solenostemon scutellarioides)* is a perennial plant that grows as annual plant outside tropical areas and is widely used as a houseplant or landscape for this good foliage color. *Mentha viridis* and *Oreganum vulgare* are perennial herbaceous plants well-known for its aromatic, ornamental and medicinal value.

Ipomoea purpurea, Petunia grandiflora and *Petunia hybrida* belong to **Solanales order**. *Petunia spp.* is an economically important ornamental plant species. It is greatly diversified and available in a range of colors (Knapp 2002b). Ornamental plants are produced exclusively for their esthetic values. The improvement of quality attributes such as flower color and longevity, plant shape, architecture, and creation of novel variation are important economic goals (Burchi et al., 1995). *I. purpurea* is one of the largest genus in number of species of family Convolvulaceae in number of species. It is distributed all over the world having about 500 species. Members of this are distributed in tropical, subtropical and temperate regions (Bhellum 2012).

Hedera helix belongs to ***Apiales order*** and it is an evergreen woody perennial with high ornamental value for use in walls and gardens and native for Europe (Ackerfield and Wen 2002).

Zinnia elegans belong to ***Asterales Order*** and is the most well-known of the 20 or so species in the *Zinnia* genus. The wild form is a coarse, upright, bushy annual, 80 cm high, with solitary daisy-like flowers on long stems and opposite, sand-papery, lance shaped leaves (Mahmoodzadeh et al., 2010).

RESULTS

RESULTS

1. Establishment of *in vitro* woody plant lines using leaves of greenhouse's plants as a source

First approach to obtaining of *in vitro* woody plant lines was using a greenhouse's plants as an explant source. Because of their non-sterile condition, first of all a sterilization procedure was necessary.

1.1. Disinfection of leaves and nodal explants

As shown in *Figure 14*, leaves of adult specimens of *Bougainvillea glabra, Hedera helix, Nerium oleander, Phyllostachys aurea* and *Ficus benjamina* growing in a controlled environment of a greenhouse were used to establish *in vitro* plant lines.

Figure 14.- Adult specimens in a greenhouse's used as a source of explants. Photography done with Nikon D80 camera in a greenhouse.

The disinfection procedures were numbered as explained in Experimental Procedures, following an increase of hardness. As shown in *Table 11*, for *B. glabra* leaves disinfection, disinfection procedure number 1 was shown to be enough for sterile explant culture initiation and 3 and 4 disinfection causes necrosis on the explants. For *B. glabra* nodal segments, the disinfection procedure number 2 was necessary to obtain sterile explants because disinfection procedure number 1 was not enough strong and procedures 3 and 4 tends to cause necrosis in the explant. For *F. benjamina* leaves, disinfection protocol number 1 was shown to be enough for sterile explant culture initiation and 2, 3 and 4 will produce necrosis; no shoot disinfection was obtained for *F. benjamina.* For *H. helix*, the strong procedures 3 and 4

were needed for leaves and nodal segments disinfection. For *N. oleander* leaves, procedure number 4 did not show any contamination but it necrosed some explants and contamination occurs with 1, 2 and 3. No disinfection was achieved for shoots.

	B.glabra		*H.helix*		*N.oleander*		*F. benjamina*	
	Shoot	Leaf	Shoot	Leaf	Shoot	Leaf	Shoot	Leaf
1	Cont	+	Cont.	Cont.	Cont.	Cont	Cont.	+
2	+	+	Cont.	Cont.	Cont.	Con.	Cont.	-
3	-	-	+	+	Cont.	+	-	-
4	-	-	+	+	Cont.	+/-	-	-

Table 11 .- Disinfection results for greenhouse's explants. Procedures 1, 2, 3 and 4 were shown**.** Cont. for contamination in explants, + for disinfection achieved and – for contamination achieved but necrosis associated.

1.2. Callus induction from leaves

Next step to obtain free-contamination lines was to get callus induction in the selected species. Callus induction started in MS supplemented with 2,4-D 2 $mg.l^{-1}$ for *B. glabra* and *H. helix* in three weeks of continuous culture. As shown in *Figure 15*, for *B. glabra*, good friable and pro-organogenic callus was obtained in six weeks of *in vitro* culture; root induction was achieved. Browning and compact callus was obtained in six weeks of *in vitro* culture for *H. helix* and root induction was observed. *N. oleander* and *F. benjamina* were placed in MS supplemented with 2,4-D 2 $mg.l^{-1}$ + 1$mg.l^{-1}$ BAP for callus induction and a compact callus, with non-pro-organogenic appearance was obtained for *N. oleander* but no callus induction was obtained in *F. benjamina.*

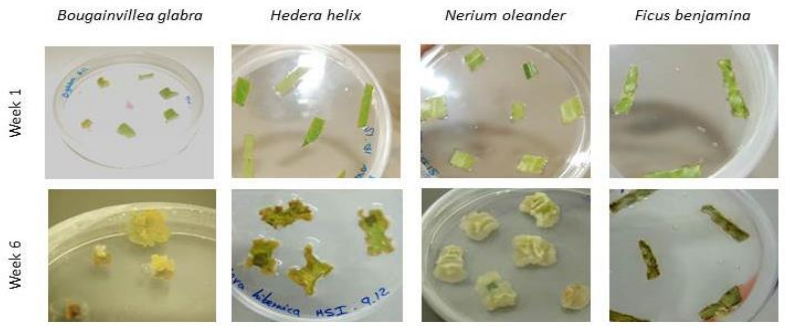


Figure 15.- Callus induction and regeneration from leaves of *B. glabra, H. helix, N. oleander* and *F. benjamina* at weeks 1 and 6. Photography done with Nikon D80

The effect of $HgCl_2$ disinfection in callus induction was tested to determine if $HgCl_2$ causes toxicity and inhibition on regeneration. As shown in Figure 16, for *N.oleander* no difference was observed. These results are comparable to *H. helix* (data not shown).

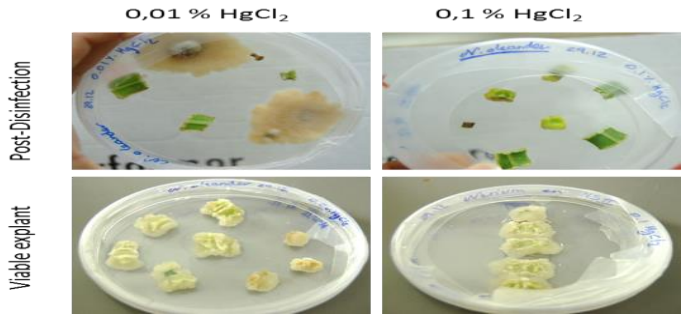


Figure 16.-Percentage of $HgCl_2$ effect on callus induction viability in *N.oleander.* Free-contamination and healthy callus was replicated in MSII in order to test $HgCl_2$ influence. Photography done with Nikon D80

1.3. Callus induction from internodal shoots

Shoots of *B. glabra* and *H.helix* in which the disinfection was achieved, were placed on MSI in order to obtain a callus induction. As shown in Figure 17, callus induction was obtained in MS supplemented with 2,4-D 2 $mg.l^{-1}$ for *B. glabra* shoots in three weeks of continuous culture and browning and non-friable callus was obtained in six weeks of *in vitro* culture. For *H. helix* shoots in MS supplemented with 2,4-D 2 $mg.l^{-1}$, few callus was obtained in three weeks of *in vitro* culture.

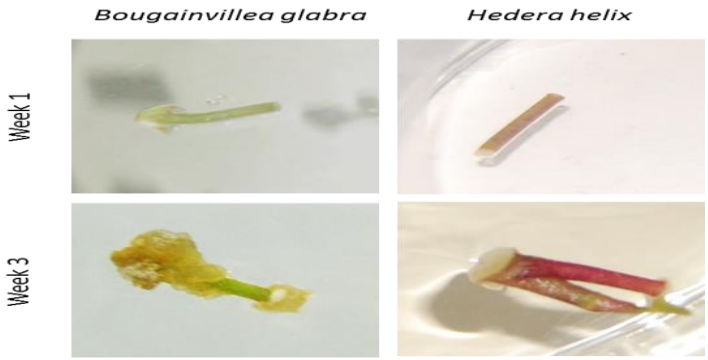


Figure 17.-Callus induction and regeneration from nodal shoots. Photography done with Nikon D80 .

1.4. Indirect regeneration from callus

Type II callus from *B. glabra* leaves and *H. helix* shoots, obtained with MS supplemented with 2,4-D 1 $mg.l^{-1}$, and *N. oleander* leaves in MS supplemented with 2,4-D 2 $mg.l^{-1}$ + 1$mg.l^{-1}$ BAP maintained *in vitro* were used to start a regeneration experiment as shown in *Table 12*. Only root formation was obtained from *B. glabra*, *H. helix* and *N.oleander* callus in all tested media.

	B. glabra	*N. oleander*	*H. helix*		*B. glabra*	*N. oleander*	*H. helix*
0.1 $mg.ml^{-1}$ AIA + 0.1 $mg.ml^{-1}$ BAP	-	-	-	0.1 $mg.ml^{-1}$ ANA + 0.1 $mg.ml^{-1}$ BAP	-	-	-
0.1 $mg.ml^{-1}$ AIA + 0.3 $mg.ml^{-1}$ BAP	-	-	-	0.1 $mg.ml^{-1}$ ANA + 0.3 $mg.ml^{-1}$ BAP	-	-	-
0.1 $mg.ml^{-1}$ AIA + 0.5 $mg.ml^{-1}$ BAP	-	-	-	0.1 $mg.ml^{-1}$ ANA + 0.5 $mg.ml^{-1}$ BAP	-	-	-
0.1 $mg.ml^{-1}$ AIA + 0.7 $mg.ml^{-1}$ BAP	-	-	-	0.1 $mg.ml^{-1}$ ANA + 0.7 $mg.ml^{-1}$ BAP	-	-	-
0.1 $mg.ml^{-1}$ AIA + 0.9 $mg.ml^{-1}$ BAP	-	-	-	0.1 $mg.ml^{-1}$ ANA + 0.9 $mg.ml^{-1}$ BAP	-	-	-
0.3 $mg.ml^{-1}$ AIA + 0.1 $mg.ml^{-1}$ BAP	-	-	-	0.3 $mg.ml^{-1}$ ANA + 0.1 $mg.ml^{-1}$ BAP	-	-	-
0.3 $mg.ml^{-1}$ AIA + 0.3 $mg.ml^{-1}$ BAP	-	-	-	0.3 $mg.ml^{-1}$ ANA + 0.3 $mg.ml^{-1}$ BAP	-	-	-
0.3 $mg.ml^{-1}$ AIA + 0.5 $mg.ml^{-1}$ BAP	-	-	-	0.3 $mg.ml^{-1}$ ANA + 0.5 $mg.ml^{-1}$ BAP	-	-	-
0.3 $mg.ml^{-1}$ AIA + 0.7 $mg.ml^{-1}$ BAP	-	-	-	0.3 $mg.ml^{-1}$ ANA + 0.7 $mg.ml^{-1}$ BAP	-	-	-
0.3 $mg.ml^{-1}$ AIA + 0.9 $mg.ml^{-1}$ BAP	-	-	-	0.3 $mg.ml^{-1}$ ANA + 0.9 $mg.ml^{-1}$ BAP	-	-	-
0.5 $mg.ml^{-1}$ AIA + 0.1 $mg.ml^{-1}$ BAP	-	-	-	0.5 $mg.ml^{-1}$ ANA + 0.1 $mg.ml^{-1}$ BAP	-	-	-
0.5 $mg.ml^{-1}$ AIA + 0.3 $mg.ml^{-1}$ BAP	-	-	-	0.5 $mg.ml^{-1}$ ANA + 0.3 $mg.ml^{-1}$ BAP	-	-	-
0.5 $mg.ml^{-1}$ AIA + 0.5 $mg.ml^{-1}$ BAP	-	-	-	0.5 $mg.ml^{-1}$ ANA + 0.5 $mg.ml^{-1}$ BAP	-	-	-
0.5 $mg.ml^{-1}$ AIA + 0.7 $mg.ml^{-1}$ BAP	-	-	-	0.5 $mg.ml^{-1}$ ANA + 0.7 $mg.ml^{-1}$ BAP	-	-	-
0.5 $mg.ml^{-1}$ AIA + 0.9 $mg.ml^{-1}$ BAP	-	-	-	0.5 $mg.ml^{-1}$ ANA + 0.9 $mg.ml^{-1}$ BAP	-	-	-
0.7 $mg.ml^{-1}$ AIA + 0.1 $mg.ml^{-1}$ BAP	-	-	-	0.7 $mg.ml^{-1}$ ANA + 0.1 $mg.ml^{-1}$ BAP	-	-	-
0.7 $mg.ml^{-1}$ AIA + 0.3 $mg.ml^{-1}$ BAP	-	-	-	0.7 $mg.ml^{-1}$ ANA + 0.3 $mg.ml^{-1}$ BAP	-	-	-
0.7 $mg.ml^{-1}$ AIA + 0.5 $mg.ml^{-1}$ BAP	-	-	-	0.7 $mg.ml^{-1}$ ANA + 0.5 $mg.ml^{-1}$ BAP	-	-	-
0.7 $mg.ml^{-1}$ AIA + 0.7 $mg.ml^{-1}$ BAP	-	-	-	0.7 $mg.ml^{-1}$ ANA + 0.7 $mg.ml^{-1}$ BAP	-	-	-
0.7 $mg.ml^{-1}$ AIA + 0.9 $mg.ml^{-1}$ BAP	-	-	-	0.7 $mg.ml^{-1}$ ANA + 0.9 $mg.ml^{-1}$ BAP	-	-	-
0.9 $mg.ml^{-1}$ AIA + 0.1 $mg.ml^{-1}$ BAP	+[1]	+[1]	+[1]	0.9 $mg.ml^{-1}$ ANA + 0.1 $mg.ml^{-1}$ BAP	+[1]	+[1]	+[1]
0.9 $mg.ml^{-1}$ AIA + 0.3 $mg.ml^{-1}$ BAP	-	-	-	0.9 $mg.ml^{-1}$ ANA + 0.3 $mg.ml^{-1}$ BAP	-	-	-
0.9 $mg.ml^{-1}$ AIA + 0.5 $mg.ml^{-1}$ BAP	-	-	-	0.9 $mg.ml^{-1}$ ANA + 0.5 $mg.ml^{-1}$ BAP	-	-	-
0.9 $mg.ml^{-1}$ AIA + 0.7 $mg.ml^{-1}$ BAP	-	-	-	0.9 $mg.ml^{-1}$ ANA + 0.7 $mg.ml^{-1}$ BAP	-	-	-
0.9 $mg.ml^{-1}$ AIA + 0.9 $mg.ml^{-1}$ BAP	-	-	-	0.9 $mg.ml^{-1}$ ANA + 0.9 $mg.ml^{-1}$ BAP	-	-	-

Table 12.- Indirect regeneration test from *B. glabra, N.oleander* and *H. helix* callus. + for aerial part organogenesis achieved, $+^{1}$ for root organogenesis achieved and – for any change observed.

2. Establishment of *In vitro* plant lines using seeds as a source

2.1. Disinfection of seeds

Three disinfection procedures were used to establish *in vitro* plant lines from seeds. Results of germination rate and contamination are shown in *Table 13*.

Species list	Soft disinfection	Medium disinfection	Hard disinfection
Anthirrinum majus	9/10	5/10	-
Begonia semperflorens	10/10	7/10	-
Cobaea scandens	8/10	5/10	-
Codariocalyx motorious	Cont	9/10	-
Coleus hybridus	Cont	5/10	-
Celosia argentera	9/10	7/10	-
Cyclamen persicum	1/10	0/10	-
Dianthus caryophyllus	10/10	6/10	-
Ipomaea purpurea	Cont	5/10	0/10
Mathiola incana	9/10	5/10	-
Mentha viridis	9/10	6/10	-
Mirabilis jalapa	Cont	5/10	-
Origanum vulgare	4/10	4/10	-
Pelargonium zonale	9/10	7/10	-
Petunia grandiflora	Cont	Cont	0/10
Petunia hybrida	Cont	Cont	0/10
Primula hortensis	Cont	Cont	0/10
Viola odorata	Cont	0/10	0/10
Viola tricolor	Cont	0/10	0/10
Zinnia elegans	9/10	5/10	-

Table 13.- Effect of disinfection on germination rate. Values are the average of three replicates with 10 seeds each. - not applicable, Cont: contamination.

Soft disinfection was shown as a good disinfection procedure for *A. majus*, *B. semperflorens, C. scandens, C. persicum, D.caryophyllus, M. incana, M. viridis, O. vulgare, P. zonale* and *Z. elegans*. Medium disinfection was shown as a good disinfection procedure for seeds of *C. hybridus, I. arborescens, C. motorious, V. odorata*, *V. tricolor* and *M. jalapa.* For seeds of*, P. grandiflora, P. hybrida* and *P. hortensis,* hard disinfection was the procedure that eliminates fungal and bacterial contamination but with hard disinfection no germination was obtained in 2 months.

2.2. Seed germination

Seed germination rate was measured as the time needed for at least one of the seeds to start germination. As shown in *Table 14*, three different medium were used for germination.

Species list	MS0	MS + 1 mg.l^{-1} GA_3	30 ºC+ MS + 1 mg.l^{-1} GA_3
Anthirrinum majus	2 weeks	2 weeks	-
Begonia semperflorens	2 weeks	2 weeks	-
Cobaea scandens	n.o.	n.o.	2 weeks
Codariocalyx motorious	n.o.	n.o.	1 week
Coleus hybridus	n.o.	n.o.	1 week
Celosia argentera	1 week	1 week	-
Cyclamen persicum	n.o.	1 week	-
Dianthus caryophyllus	1 week	1 week	-
Ipomaea purpurea	1 week	1 week	-
Mathiola incana	1 week	1 week	-
Mentha viridis	1 week	1 week	-
Mirabilis jalapa	1 week	1 week	-
Origanum vulgare	2 week	2 week	-
Pelargonium zonale	1 week	1 week	-
Petunia grandiflora	n.o.	n.o.	n.o.
Petunia hybrida	n.o.	n.o.	n.o.
Primula hortensis	n.o.	n.o.	n.o.
Viola odorata	n.o.	n.o.	n.o.
Viola tricolor	n.o.	n.o.	n.o.
Zinnia elegans	2 weeks	2 weeks	-

Table 14.- Effect of media in germination rate. - not applicable; n.o.: no germination obtained.

For *A. majus, B. semperflorens, C. persicum, D. caryophyllus, I. arborescens, M. incana, M. viridis, M. jalapa, O. vulgare* and *P. zonale*, MSO and MS +1mg.l^{-1} GA_3 didn't show any special benefit in germination rate. Seeds of *C. scandens*, *C. hybridus and C. motorious* needed previous removal of dormancy by soaking them for 24 h in water with 1 mg.l^{-1} GA_3, at 30 ºC, and germinated after one week either in MS0 or MS with 1 mg.l^{-1} GA_3. Without the soaking treatment, germination had not been successful after two months. No germination was obtained in *P. grandiflora, P. hybrida, P. hortensis, V. odorata* and *V. tricolor* in any of the selected mediums after two months. Seeds with germination rates lower than 40% were started on a multi-variable germination experiment. Three media (MSO, MS + 1 mg.ml^{-1} GA_3 and previous soaking in 1 mg.ml^{-1} GA_3) were tested with different incubation temperatures and darkness periods (12, 18, 22 ºC in darkness and

26ºC darkness; data not shown). Germination rate was not improved in any case.

2.3. Callus induction and regeneration

Once the plants have reached the stage of 5-7 leaves, a hormone battery was tested in all the species that achieved the minimum rate of germination accepted, 50%, and the rest of species were all discarded. As it is explained in Experimental Procedures, ten leaves explants per plate were placed in MS0 with different rates of auxin/cytokinin and auxin or cytokinin alone. All experiments consist of three replicates, giving a total of 30 explants per rate.

Callus induction was analyzed considering the consistency and appearance of callus formation given - for non-callus or compact and brown callus in the explant, + given to callus no friable; ++ to callus friable and +++ to callus friable and pro-organogenic with bud structures.

Shoot regeneration was analyzed considering the number of shoots in the explants, given the subsequent code: - for no regeneration, + at least 1 shoot in at least 1 explant; ++ at least 1 shoot in at least 6 out of 10 explants; +++: at least 1 shoot in every explant. Root formation was reported also with $+^{1}$ code. All experiments lasted for 15 weeks and results are detailed in *Tables 15 to 25*.

2.3.1.Callus induction and regeneration in *D. caryophyllus*

As shown in *Table 15*, good friable calli were induced from all auxin/cytokinin ratios when auxin was NAA and cytokinin was BAP, the most appropriate being the 0.8:1 ratio.

	Callus induction	Regeneration
0.2 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	+
0.4 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	+
0.6 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	++
0.8 mg l^{-1} NAA + 1 mg l^{-1} BAP	++	+++
0.5 mg l^{-1} AIA + 1 mg l^{-1} BAP	-	+
0.7 mg l^{-1} AIA + 1 mg l^{-1} BAP	-	+
1 mg l^{-1} 2,4 D	+	-

Table 15.- Effect of hormone concentration on callus production and regeneration in leaves of *D. caryophyllus*

*2.3.2.*Callus induction and regeneration in *M. jalapa, C. scandens and C. hybridus*

No callus induction and thus no regeneration were obtained for *M. jalapa, C. scandens and C. hybridus* in any of the selected medias as shown in *Table 16*. All the explants had shown no changes or even a small degree of necrosis through time.

	Callus induction	Regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.5 mg l^{-1} NAA +1 mg l^{-1} BAP	-	-
0.7 mg l^{-1} NAA +1 mg l^{-1} BAP	-	-
0.9 mg l^{-1} NAA +1 mg l^{-1} BAP	-	-
1 mg l^{-1} 2,4-D	-	-
0.5 mg l^{-1}AIA +1 mg l^{-1} BAP	-	-
0.7 mg l^{-1}AIA +1 mg l^{-1} BAP	-	-

Table 16.- Effect of hormone concentration on callus production and regeneration in leaves of *M. japala, C. scandens and C. hybridus*

2.3.3.Callus induction and regeneration in *P.zonale*

For *P. zonale*, callus induction was obtained for 1:2 auxin/cytokinin ratio but a strong necrosis was finally developed in all produced calli and, subsequently, no regeneration was obtained.

	Callus induction	Regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.2 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.4 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.5 mg l^{-1} NAA +1 mg l^{-1} BAP	++	-
0.6 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.7 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.8 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.9 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
1 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.5 mg l^{-1} AIA + 0.9 mg l^{-1} BAP	-	-
1 mg l^{-1} 2,4-D	-	-

Table 17.- Effect of hormone concentration on callus production and regeneration in leaves of *P.zonale*

2.3.4.Callus induction and regeneration in *C. motorious*

For *C. motorious*, as shown in *Table 18*, good friable calli were induced with lower auxin/cytokinin ratios, being the most appropriate 0.1:1 and 0.3:1 but callus induction was observed in all selected media. For regeneration of aerial parts, the most appropriate ratio was 0.1:1, which shown the higher regeneration rate. For higher auxin/cytokinin ratio, the regeneration product were predominately roots.

	Callus induction	Regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	++	++
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	++	+
0.5 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.7 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.9 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
1 mg l^{-1} 2,4-D	+	-
0.4 mg l^{-1}ANA +0.8 mg l^{-1} BAP	+	+[1]
0.3 mg l^{-1}ANA +0.9 mg l^{-1} BAP	+	+[1]
0.5 mg l^{-1}AIA +1 mg l^{-1} BAP	+	+[1]
0.7 mg l^{-1}AIA +0.8 mg l^{-1} BAP	+	+[1]
2 mg l^{-1} BAP	+	-

Table 18.- Effect of hormone concentration on callus production and regeneration in leaves of *C. motorious.*

2.3.5.Callus induction and regeneration in *C. argentera*

As shown in *Table 19*, good callus induction were obtained with increasing ratios of auxin/cytokinin, peaking with 0.9:1 but there was no regeneration for any of the selected medias, neither aerial nor roots.

	Callus induction	Regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.5 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.7 mg l^{-1} NAA +1 mg l^{-1} BAP	++	-
0.9 mg l^{-1} NAA +1 mg l^{-1} BAP	+++	-
1 mg l^{-1} 2,4-D	+	-
0.5 mg l^{-1}AIA +1 mg l^{-1} BAP	+	-
0.7 mg l^{-1}AIA +1 mg l^{-1} BAP	+	-

Table 19.- Effect of hormone concentration on callus production and regeneration in leaves of *C. argentera*

2.3.6.Callus induction and regeneration in *B.semperflorens*

As shown in *Table 20*, for *B.semperflorens*, callus were induced in every media used, being the auxin/cytokinin ratios 1:2 and 1:3 in darkness the most appropriate to obtain good friable calli. Aerial part regeneration was shown only in darkness and best media was the auxin/cytokinin ratio 1:3. Ratio 1:2 had shown strong root regeneration predominance. Explants placed on light conditions shown a strong necrosis, that increase in parallel with the increase of hormone concentration.

	Callus induction	Shoot regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.2 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.4 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.5 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.6 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.7 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.8 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.9 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
1 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.4 mg l^{-1} NAA + 0.8 mg l^{-1} BAP	++	$+^{1}$
0.3 mg l^{-1} NAA + 0.9 mg l^{-1} BAP	+++	++
2 mg l^{-1} BAP	++	+
0.7 mg l^{-1} NAA + 1.5 mg l^{-1} BAP+0.1 mg l^{-1} TDZ	+	+
1 mg l^{-1} 2,4 D	+	-

Table 20.- Effect of hormone concentration on callus production and regeneration in leaves of *B. semperflorens.*

2.3.7.Callus induction and regeneration in *M. incana*

For *M. incana*, as shown in *Table 21*, callus were induced from auxin/cytokinin rate 0.4:1, being the most appropriate to obtain a good friable callus 1:2 rate. The aerial part regeneration was shown in the same media that resulted in good callus induction from ratio 0.4:1, being the most appropriate 1:2.

	Callus induction	Regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.2 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.4 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	+
0.5 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	+
0.6 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	+
0.7 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	+
0.8 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	+
0.9 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	+
1 mg l^{-1} NAA + 1 mg l^{-1} BAP	++	++
0.4 mg l^{-1} NAA + 0.8 mg l^{-1} BAP	+++	+++
0.3 mg l^{-1} NAA + 0.9 mg l^{-1} BAP	++	++
0.5 mg l^{-1} AIA + 1 mg l^{-1} BAP	+	$+^{1}$
0.7 mg l^{-1} AIA + 1 mg l^{-1} BAP	-	-
2 mg l^{-1} BAP	-	-
0.7 mg l^{-1} NAA + 1.5 mg l^{-1} BAP+0.1 mg l^{-1} TDZ	-	-
1 mg l^{-1} 2,4 D	+	-

Table 21.- Effect of hormone concentration on callus production and regeneration in leaves of *M. incana*.

2.3.8.Callus induction and regeneration in *A. majus*

Type II calli induction were obtained with increasing rates of auxin/cytokinin, peaking at 0.9:1, but no regeneration was obtained in any of the selected media as shown in *Table 22*. Same results were obtained with light deprivation explants and neither necrosis nor browning of calli was observed.

	Callus induction	Regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.5 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.7 mg l^{-1} NAA + 1 mg l^{-1} BAP	++	-
0.9 mg l^{-1} NAA + 1 mg l^{-1} BAP	+++	-
1 mg l^{-1} 2,4-D	+	-
0.5 mg l^{-1} AIA + 1 mg l^{-1} BAP	+	-
0.7 mg l^{-1} AIA + 1 mg l^{-1} BAP	+	-

Table 22.- Effect of hormone concentration on callus production and regeneration in leaves of *A. majus*

2.3.9.Callus induction and regeneration in *M. viridis*

As shown in *Table 23*, *M. viridis*, good friable calli were induced from ratio 0.6:1, being the most appropriate 1:1. Explants with low concentration of NAA shown a strong necrosis which is not avoided with light deprivation (data not shown). For the aerial part regeneration the most appropriate ratio was not correlated, being the 1:2 ratio the average value that shown higher regeneration rate.

	Callus induction	Regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.2 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	-	-
0.4 mg l^{-1} NAA +1 mg l^{-1} BAP	-	-
0.5 mg l^{-1} NAA +1 mg l^{-1} BAP	-	-
0.6 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.7 mg l^{-1} NAA +1 mg l^{-1} BAP	+	+
0.8 mg l^{-1} NAA +1 mg l^{-1} BAP	++	+
0.9 mg l^{-1} NAA +1 mg l^{-1} BAP	+++	+
1 mg l^{-1} NAA + 1 mg l^{-1} BAP	+++	+
0.3 mg l^{-1} NAA + 0.9 mg l^{-1} BAP	+	-
0.1 mg l^{-1} NAA + 0.25 mg l^{-1} BAP	-	-
0.4 mg l^{-1} NAA + 0.8 mg l^{-1} BAP	++	++
0.4 mg l^{-1} NAA + 0.8 mg l^{-1} BAP	++	-
1 mg l^{-1} 2,4-D	-	-
2 mg l^{-1} BAP	-	-
0.1 mg l^{-1}NAA +0.1 mg l^{-1} TDZ+ 1.5 mg l^{-1} BAP	-	-
0.4 mg l^{-1}TDZ +25% CW	++	-
0.5 mg l^{-1}AIA +1 mg l^{-1} BAP	-	-

Table 23.- Effect of hormone concentration on callus production and regeneration in leaves of *M. viridis.* Dark squares represent light deprivation.

2.3.10. Callus induction and regeneration in *I. purpurea*

As shown in *Table 24*, callus was induced with all auxin/cytokinin ratios, except for auxin alone. The most appropriate to obtain a good friable callus was the higher ratio for both NAA and AIA. Instead, no aerial part

	Callus induction	Regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	$+^{1}$
0.5 mg l^{-1} NAA +1 mg l^{-1} BAP	+	$+^{1}$
0.7 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.9 mg l^{-1} NAA +1 mg l^{-1} BAP	++	-
1 mg l^{-1} 2,4-D	-	-
0.4 mg l^{-1}AIA +1 mg l^{-1} BAP	+	-
0.6 mg l^{-1}AIA +1 mg l^{-1} BAP	++	-
0.8 mg l^{-1}AIA +1 mg l^{-1} BAP	++	-

Table 24.- Effect of hormone concentration on callus production and regeneration in leaves of *I. purpurea.*

regeneration was shown in any of the tested media.

2.3.11. Callus induction and regeneration in *Z.elegans*

As shown in *Table 25*, callus induction was obtained with increasing rates of auxin/cytokinin, peaking at 0.9:1. No aerial parts regeneration was obtained in any of the selected medium, obtaining instead root formation in 0.7 or 0.9:1 ratios.

	Callus induction	Regeneration
0.1 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.3 mg l^{-1} NAA + 1 mg l^{-1} BAP	+	-
0.5 mg l^{-1} NAA +1 mg l^{-1} BAP	+	-
0.7 mg l^{-1} NAA +1 mg l^{-1} BAP	+	+[1]
0.9 mg l^{-1} NAA +1 mg l^{-1} BAP	++	+[1]
1 mg l^{-1} 2,4-D	+	-
0.5 mg l^{-1}AIA +1 mg l^{-1} BAP	+	-
0.7 mg l^{-1}AIA +1 mg l^{-1} BAP	++	-

Table25.- Effect of hormone concentration on callus production and regeneration in leaves of *Z. elegans*.

Figure 18.- Ornamental plants regeneration. Upper row: Calli induction from leaves explants. Lower row: Regeneration induction. Photographs taken with Nikon SMZ745T and NIS element capture program. Objective magnifications used are indicated at each photograph.

1.3. Elongation and rooting

For all the regenerated species, the newly formed shoots were successfully transferred to a MS0 medium for elongation and rooting.

Half strength MS medium (MS1/2) did not show any specific benefit in any of the species tested, and *C. motorious* plants had shown worse appearance of leaves and chlorosis.

DISCUSION

DISCUSSION

To settle a breeding program for future biotechnological approaches like plant transformation, the establishment of an *in vitro* micropropagation technology is required as the first step.

One of the most important condition and usually a bottleneck for plant transformation is to possess an efficient and easy procedure for regeneration. In plants, the capacity to obtain somatic organogenesis is a paramount important tool for *in vitro* studies of plant species, and a *sine qua non* requirement for successive steps in genetic transformation.

For this reason, a 19 ornamental non-woody species and four woody species were pre-selected by their commercial value and taking also into consideration other aspects like chloroplast pollen heritability and the ability to grow under Mediterranean environmental conditions.

A first approach was directed to test a protocol to use as source of material leaves and nodal explants directly from greenhouse's plants, in order to avoid the germination and shoot elongation time.

Disinfection of leaves was achieved in 4/5 species tested but endogenous fungal contamination didn't allow axenic culture of *P. aurea* in any of the disinfection procedures tested. *P.aurea* showed high sensitivity to standard disinfection treatments. The high bacterial and fungal contamination observed, indicated the need for disinfection strategies alternatives, combining chemical biocides, such as PPM, with diluted commercial bleach and / or the addition of ASA in the culture medium. For *B. glabra*, soft disinfection was enough to establish an axenic culture and for *H.helix* and N.*oleander* the standard method of ethanol/NaClO was insufficient for the *in vitro* establishment of these species. An additional washing step with 0.001 %$HgCl_2$ was needed in order to obtain a sterile explant due a strong bacterial contamination shown in soft and medium disinfection protocols. For *F. benjamina,* soft disinfection seems to be an aggressive procedure and explants undergo necrosis with time. NaOCl was effective but this treatment cause loss of explants due to browning and by leaching of chlorophyll and it prevents the success of subsequent steps.

For nodal tips, stronger protocols were needed and our experiments revealed that $HgCl_2$ was more potent for effective disinfection of nodal explants.

Callus induction was obtained from *B. glabra, H. helix* and *N.oleander*. Good callus induction was obtained in *N. oleander* with 2,4-D and BAP supplemented medium (Santos et al., 1994) but no direct regeneration was obtained. It could be, in concordance with Santos et al results, because only callus from young leaves is embryogenically competent. Callus from *B. glabra* and *H. helix* was successfully induced in MS supplemented with 2,4-D. No previous work, as far as we know, has been done to induce callus from leaves in this species. Calli induced from nodal shoots were only obtained in *B. glabra* and *H. helix.* However, the callus appearance was not friable and developed browning and necrosis for *B. glabra*. That contradicts Shah et al. for *B. glabra*, because although a good callus was formed, browning prevented subsequent organogenesis (Shah et al., 2006). For *H. helix*, little calli were obtained, in concordance with Banks et al. (1979), when show the low organogenic potencial of callus of that species ((Banks et al., 1979)).

Regeneration experiments done with friable callus from *B. glabra* and *N. oleander* leaves and *H. helix* shoots did not show any aerial part regeneration in any of our experimental conditions tested. It could be due to the low organogenic capacity from leaves of mature plants. (Souzal et al., 2006) but an extensive hormonal test was done in order to test it. Finally, taking into consideration this important handicap, our efforts were directed to obtain good generation rates in newly germinated plants, to avoid the low regeneration capability in older plants.

For this reason, the second approach was directed to obtain an *in vitro* sterile line starting from seeds. The procedure however, that eliminates any source of external contamination, could affect the percentage of germination, phenomenon that is seems to be proportionate to the hardness of disinfection procedure and treatment times (Dempsey and Walker 1978).

However, the effects of sodium hypochlorite on the germination of seed from different species are conflicting. Thus, sodium hypochlorite has been reported to promote (Macit 1981), to inhibit (McCollumand Linn 1955) and to reduce the rate of germination (Cantllffe and Watkins 1983).

On one hand, it is proposed that a suitable concentration of sodium hypochlorite treatment mimics the effect of acid scarification and as a result seeds will be more porous to gas exchange and GA_3(,) penetration and increasing sensitivity to light treatment and protect to the mortality of the seedlings. However, prolonged sodium hypochlorite treatment resulted in either poor germination or even seed disintegration (Hsreo 1980).

Duration of treatment with the disinfectant is a very critical step and it is important to strike a balance between the mortality due to the excessive disinfectant treatment and contamination due to incomplete disinfections.

For this reason, two disinfection procedures with raising treatment times and a step with sodium hypochlorite are tested and one additional using $HgCl_2$. A positive treatment was the weaker procedure that allows no contamination with at least 50% of germination rate in the corresponding media. This experiment allowed to elucidate the direct effect of the disinfection protocol on germination capability on our species tested. Soft disinfection was the common procedure used in the bibliography. The surface disinfection with ethanol reduce surface contamination and a second step of 10 minutes with 25% commercial bleach sodium hypochlorite (v/v) as a disinfectant and Tween20 as a wetting agent added reduce surface tension allow better surface contact and the elimination of fungal spores or bacterial contamination. This protocol was selected for *A. majus, B. semperflorens, C. scandens, C. argentera, C. persycum, D. caryophyllus, M. incana, M. viridis, O. vulgare* and *P. zonale* and is in concordance with the related bibliography, when times and concentration of NaClO differs from differents authors but are in the same range (Espino et al., 2004).This disinfection seems strong enough to eliminate any kind of contamination and doesn't inhibit the germination ability of seeds. This could be related to the frailness of the seeds, which are not able to resist a hard protocol.

Mercuric chloride has shown as a very effective sterilizing agent. The chlorine gas released from $HgCl_2$ could penetrate and destroy the microorganisms present in most tissues of the explant but this product is also toxic to explant tissues. Therefore concentration of the sterilizing agent and duration of the treatment should be optimum to minimize tissue mortality of explants due to over sterilization (Young 1919). Seeds germination are influenced not only

by disinfection procedures but also for the hormone (media) composition of media and temperature (Finkelstein 2004).

For this reason, seeds were placed on different conditions in order to obtain a successful germination rate that we establish as 90 out of 100. Germination was considered not to have occurred if seedlings were contaminated or nonviable.

Seeds dormancy, a temporary block of a viable seed to complete germination under physical favorable conditions (Baskin and Baskin 2004) can be broken by the combination of NaOCl, GA_3, and light, indicating a high degree of variability in germination responses to various sets of conditions (Bewley and Black 1982). Dormancy in some species required a cold period of incubation in order to promote germination and its period is often overcome by gibberellins (particularly GA_3, GA_4 and GA_7) (Kermode 2005) changing hormone biosynthesis and degradation toward a low ABA/GA ratio as ABA controls embryo dormancy and GA embryo germination. For this reason, MS was supplemented with GA_3 in order to elucidate if it was necessary to overcome dormancy. In the case of germination ratios below 40% the additional procedures of cold period of incubation tested didn't show any specific benefit.

Due to the great need of leaf explants for future regeneration experiments, as had been established previously, the minimum rate of regeneration to be accepted in subsequent protocols was 50% and all the species that had ratios of germination below were discarded. Although *Petunia sp*. was a strong candidate for genetic transformation for its commercial value, good regeneration and germination rates and previous transformation work done by other groups which ensures its functionality (Abu –Qaoud et al., 2010), no germination was obtained under our experimental conditions. The cause could be the strong protocol used to eliminate contamination which contains $HgCl_2$ that, as explained in a previous section, could be toxic for the embryos. For this reason, *O. vulgare*, although seems a good candidate (Kumari and Saradhi 1992; Arafeh et al., 2006) in our experimental conditions, with 40% germination rate, was discarded for future regeneration experiments. Similar handicap occur in *C. persycum*. Although regeneration procedures were described previously (Abu-Qaoud 2004), the lower germination rate

obtained (10%) made us discard this species for further regeneration experiments.

P. hortensis, V. odorata and *V. tricolor* are discarded for this lack of germination although good procedures for regeneration have been reported previously (Naeem et al., 2013).

Tissue explants in presence of a particular concentration of auxin, proliferate and produce an undifferentiated mass of cells, a callus. However, further growth of the callus depends upon the availability of cytokinin, because the callus by itself cannot synthesize cytokinins. Callus cells can be further induced to develop into shoots, roots or both by providing auxins and cytokinins in a defined ratio. As shown in figure 12, at high ratio of auxin to cytokinin callus produces only shoots, at lower ratio the callus induces only roots, but at an intermediate ratio both shoots and roots develop.

Regeneration was, in all tested species, the next step after callus formation and the relation between both was almost quantitatively: a good regeneration rate was the following up of a good callus formation, except for *M. incana* using AIA instead of NAA that acceptable regeneration could be obtained directly from the explants without a previous callus formation.

In *D. caryophyllus*, best results were obtained in media containing 1 mg L^{-1} NAA + 1 mg L^{-1} BAP, shown in *Table 15*, avoiding hyperhydricity. Casas et al., 2010 show that adventicious shoot formation is induced with BAP 1mg-l^{-1} + NAA 0.2mg-l^{-1} in leaves that remain attached to the axillary bud and is in that basal region were new meristems are formed. Pareek et al. 2003 obtain somatic embryogenesis and embryo germination without an intervening callus phase from leaves, but several steps are needed with the subsequent time (needed) and costs associated. (Pareek et al., 2004). In *D. chinesis*, Kantia et al .2002, shows that high concentrations of BAP and NAA (1:3 or 1:6 ratio) produce good regeneration in leaf explants. We obtain a good regeneration rate when auxin and cytokinin acts synergistically at 1:1 ratio (Kantia and Kothari 2002). Hyperhydricity, or vitrification, is one induced physiological disorder that consists of thick and glassy appearance in *in vitro* plants. This is one of the main problems for carnation in *in vitro* culture (Kharrazi et al., 2011).

Cytokinins have been shown to induce vitrification in a concentration dependent manner (Leshem 1988). Our results shown that 1:1 ratio auxin/cytokinin avoiding hyperhydricity and its effects are increasing with less quantity of auxin. To avoid vitrification, lower concentrations of BAP, in concordance to Kharrazi et al. 2011, are suggested to be more suitable to obtain normal plantlets with a minimum vitrification rate.

As shown in *Table 16*, neither callus induction nor regeneration was obtained from *C. hybridus, C. scandens* and *M. jalapa*. For *C. hybridus*, no previous bibliography exists (was obtained), but for nearly species reported, two steps were needed for regeneration. For *C. forskonhlii*, (Reddy et al., 2001) optimal callus was produced from mature leaves with BAP 0.5mg.l^{-1} and for shoots regeneration with MS medium supplemented with BAP 1 mg.l^{-1}+ NAA 0.1 mg.l^{-1}. This callus mediated organogenesis needed two steps, one for callus induction and another for shoot regeneration from callus. And for *C. blumei*, callus induction was obtained from mature leaves in MS supplemented with BAP 2 mg·l^{-1} and NAA 1 mg·l^{-1} and shoot tips were produced from previous callus with BAP 4 mg·l^{-1} and NAA 0.5 mg·l^{-1} and rooted with MS supplemented with IBA 2 mg·l^{-1} (Jing et al., 2008). For *C. scandens*, to our knowledge no previous work has been done and the nearest specie described (founded) was *P. paniculata*, with which it shares the same family (Polemoniaceae). For *P. paniculata*, shoot regeneration was induced from leaf explants with MS supplemented with BAP 1,5 mg.L^{-1} + AIA 0,5 mg.L^{-1} (Jain et al., 2002). For *C. scandens*, in our experimental conditions, neither callus induction nor regeneration was obtained. For *M. jalapa*, Zaccai et al., 2007 reported consistent shoot regeneration from nodal segments in MS plus BAP 2 mg l^{-1}, Z 2 mg l^{-1} and AIA 1 mg l^{-1} (Zaccaia et al., 2007 ; Xu et al.,2005) show that regeneration was achieved, in all the explant type tested, only from cotyledons with MS plus IAA 1mg.L^{-1} and TDZ 1mg.L^{-1} with 1 week in darkness and subsequently placed on MS + TDZ 2 mg.L^{-1} under day/night conditions.

It is well known, from studies of regeneration of other species, the importance of explant source (Hemphill et al., 1998). One possible reason for the failure to obtain callus induction and thus adventicious shoot regeneration is the poor intrinsic ability from leaves to regenerate. Anyway, a large hormone battery test could be necessary to confirm this hypothesis.

For *P. zonale*, our experimental results show a good callus induction for 0.5:1 auxin/cytokinin ratio but no regeneration was obtained with NAA and BAP growth regulators. Previous work with *P. capitatum* show that two steps are required to obtain shoot organogenesis from mature leaf tissues. The protocol involved pre-culture of leaf sections in MS medium supplemented with TDZ 2.2 $mg.l^{-1}$ + BAP 1 $mg.l^{-1}$ +1 NAA $mg.l^{-1}$ and then subcultured without TDZ (Muhammad et al., 2012). TDZ was related as an important plant growth regulator for induction of somatic embryogenesis in a wide range of species including *Pelargonium* (Murthy et al., 1998) ; Murthy et al., 1996; Visser et al., 1992) and previous work demonstrated that TDZ may possess an auxin-like property or may modify the biosynthesis or metabolism of endogenous auxins.

In some cultivars, shoot organogenesis has been improved by a reduction of the mineral concentration of MS medium (Hildebrandt and Harney, 1988) and by optimized plant growth regulator concentrations (Desilets et al., 1993).

The choice of explant has also been shown to significantly affect regeneration efficiency having the best regeneration capability seedlings, shoots and protoplast-derived callus (Dunbar and Stephens 1991); Qureshi and Saxena 1992) .

In *C. motorious*, good callus induction and regeneration were obtained in MS supplemented with low auxin/cytokinin ratio (NAA 0.1 $mg.L^{-1}$ + BAP 1 $mg.L^{-1}$). Previous work showed somatic embryogenesis from cotyledon segments with IAA 0.5 $mg\text{-}l^{-1}$ + BAP 1 $mg\text{-}l^{-1}$ (Chitra Devi and Narmathabai 2011) or two steps regeneration from seedlings using MS supplemented with NAA 0.1 $mg\text{-}l^{-1}$ + BAP 2 $mg\text{-}l^{-1}$ for callus induction and NAA 0.05 $mg\text{-}l^{-1}$ + BAP 2 $mg\text{-}l^{-1}$ for shoot regeneration (Mao et al., 2010) These results are in concordance with another work with *D. affine* and *D. uncinatum* from leaves (Rey and Mroginski 1977).

In *B. semperflorens*, the best bud differentiation and shoot regeneration medium was MS + BAP 0.9 mg l^{-1} +NAA 0.3 mg l^{-1} + sucrose 30 g l^{-1} and we found that light was a strong inhibitor of regeneration and induced necrosis at the explants. Our results are in concordance to Mendi et al. 2009 (Mendi

et al., 2009) for *B. elatior* were the best morphogenetic response was obtained when the auxin/cytokinin ratio was 1:2. In fact, in our experimental conditions, the 1:3 auxin/cytokinin ratio has shown to be the most effective proportion to obtain regeneration, followed by 1:2 ratio auxin/cytokinin. As it is well known, NAA is a strong auxin and the increase of its concentration minimizes the regeneration effect or even inhibits plant regeneration. In contrast, Espino et al., 2004, found for *B. semperflorens*, that best ratio was obtained for 1:1 auxin/cytokinin ratio (Espino et al., 2004). But in all cases, the best morphogenetic response was obtained when the citokynin was BAP.

Also, our results showed that light induced necrosis of explants and regeneration was negatively affected. It could be done the effect of Plant Growth Regulators (PGRs). As Heide showed in 1968, in *Begonia* light intensity is able to influence the capacity to form adventitious buds and that could be the reason to our increase of necrosis and inhibition of regeneration of explants in high light conditions.

The browning of explants due to the oxidation of phenolic compounds could be related to the enzymatic activity of explant, like polyphenoloxidase and peroxidase (Pizzocaro et al. 1993; Down et al. 1995; Whitaker et al. 1995) when the contents of cytoplasm and vacuoles are mixed due to the damage of tissue. Oxidized compounds affect negatively the *in vitro* cultures by lethal darkening of explants (Laukkanen et al. 1999). To avoid the browning of explants, some solutions were founded: activated charcoal that seems to adsorb PGRs, frequent subculturing, the addition to the medium of some antioxidants such as citric acid and ascorbic acid, PVP (polyvinyl pyrolidone) and sodium chloride can also reduce phenolic oxidation and contribute to regeneration of explants (Pizzocaro et al. 1993). But, in concordance to our results and previous results of Bouman and Klerk in 2001 for *B. hiemalis*, dark incubation could be an inexpensive method that avoids the necessity to use additives.

For *M. incana*, as shown on *Table 21*, the results of our investigations show good rates of regeneration from young leaves in MS plus 0.4 mg L^{-1} NAA + 0.8 mg L^{-1} BAP. These results differ with Gautman et al., 1983 (Gautamet al., 1983) that show organogenesis from cotyledonary explants of *M. incana* in MS plus 1 $mg.L^{-1}$ of BAP alone. Regeneration was observed also for Hesar et al., 2011 and Kaviani et al., 2011, with shoot tips with 0.5 to 2 $mg.l^{-1}$ of

Kinetin alone. We reproduced similar results with leaves from young *in vitro* plants. This allowed us to avoid a continuous germination of seeds, saving time and reducing costs.

For *A. majus*, previous work showed callus induction from internode, seedling shoot tips or hypocotyl and regeneration only from hypocotyl in MS supplemented with NAA 0.25 $mg.l^{-1}$+ 10% CW (Atkinson et al., 1989) . We consider important to mention that, according to George, 1993 (George 1993) that some authors rutinary used coconut milk to improve regeneration (Maddock et al., 1983; Mathias and Simpson, 1986; Nasib et al., 2008), a product that contains many nitrogenous and carbon sources, inorganic compounds, organic acids, vitamins and growth regulators such as cytokinin and auxin. Their studies had shown regeneration from hairy roots with ½ MS without growth regulators (Cui et al., 2001). In our experimental conditions, good callus induction was obtained with high ratios of auxin/cytokinin, being the best response with 1:1 auxin/cytokinin ratio but no regeneration was obtained in any case. It could be due to the poor organogenic response of leaves compared with good organogenic response in hypocotyl and seedling shoot tips.

For *M. viridis*, previous work reported direct shoot regeneration for leaves explants cultured on MS supplemented with BAP 3 $mg.l^{-1}$ + 2, 4-D 1 $mg\text{-}l^{-1}$ and root induction with MS supplemented with IBA 1.5 $mg.l^{-1}$ (Senthil, and Kamaraj 2012). Our work show good callus induction with high ratios of auxin/cytokinin from 0.7:1 NAA/BAP on and the best callus induction followed by good regeneration was obtained for the 1:2 ratio with a 8-16 h photoperiod. This was correlated with previous auxin/cytokinin ratios 1:3 but with callus phase intervening in our experimental conditions and effective rooting was done without hormones. These results were successful only in leaves explants from recently *in vitro* germinated plants and no regeneration was obtained from micropropagated plants. This may be due to the age of the explant. It is well known, for a wide range of species, that age of the explants strongly influences organogenesis, demonstrating a strong organogenic response in young leaves than in older ones (Souzal et al., 2006).

For *I.purpurea*, our results show good callus induction with high auxin/cytokinin ratios but no regeneration was obtained in our experimental conditions. The nearest specie reported in literature is *I. batatas* when two-step procedure was needed for petiole organogenesis regeneration using MS supplemented with 4-FA and Z (Lou et al., 2006).

In *Z. elegans*, previous work has shown callus induction and regeneration from shoots using MS supplemented with TDZ 0.02mg.l^{-1} (Mahmoodzadeh et al., 2010) but, to our knowledge, no previous work with zinnia leaves have been reported. Our experimental results show good callus induction with high auxin/cytokinin ratios but only root induction was obtained in these media. Probably, a change in growth regulators is needed to obtain aerial regeneration from callus. According to Mat Taba 2012 for *C. cristata*, (Taha and Wafa 2012), our results show good callus induction for *C. argentera* with high auxin/cytokinin ratio but no regeneration was obtained. It could be due to the poor organogenic response from leaves since good regeneration rates were obtained from shoot tips.

For the initiation of organogenic callus in dicot plants a combination of high concentration of auxins with low concentration of cytokinins was widely used (Caboni et al. 2000; Haliloglu et al. 2006) and sometimes cytokinins alone (Yam et al. 1990). We found that the synergic effect of both, with high concentration of cytokinins with low concentrations of auxins promote not only the organogenic callus but also regeneration itself.

The *in vitro* protocol for regeneration reported in this study could be used for genetic transformation methods.

Shoot elongation requires a combination of cell division and enlargement of the cells laid down by the shoot apical meristem (SAM), located at the apex of the stem. This shoot elongation depends on endogenous hormones but can be stimulated by addition to media of low concentrations of BAP and NAA. In our experimental conditions, shoot elongation was successful and external PGRs were not added.
Root elongation is the result of the enlargement of new cells being formed by cell divisions in apical meristems (Torrey 1956) and is a vital process to obtain plants that could be successfully established in the soil.

For this purpose, shoots were treated with MS0 media and half strength MS in order to obtain a successful rooting. No difference was observed between both treatments and the root rate was effective. For this reason, although some authors report the necessity to add to the media some PGRs as the auxin IBA to influence root proliferation, our experimental results showed that there is no need for that in our experimental conditions (Awamy et al., 2002).

CONCLUSIONS

CONCLUSIONS

We report an efficient, inexpensive and easy method to obtain good rates of organogenesis from mature leaves, *in vitro* germinated, of four plants with ornamental value: *Begonia semperflorens cv. hybrida, Codariocalyx motorious, Dianthus caryophyllus and Matthiola incana* in only one step.

This method reduces costs and working hands-on timing avoiding the necessity of transferred to another supplemented media and allows us to have a good protocol for our future biolistic experiments for chloroplast transformation.

Chapter II: Plastidial transformation with *lux* operon

Sólo una cosa vuelve un sueño imposible: el miedo a fracasar

INTRODUCTION

Plastidial transformation with *lux* operon

1. Bioluminescence

The first reference about phosphorescence in the sea was described as early as year 500 BC by Aristotle (384-322 BC), who talked about 180 marine species that emitted "cold light". The term bioluminescence was first referred by E. Wiedemann in 1888. In nature, there are a range of bioluminescent organisms: some species of bacteria, fungi, dinoflagellates and insects, amongst others (Harveyi 1957).

Bioluminescence is the emission of visible light from living organisms through enzymatic catalysis. There are strong differences among the reactions in light-emitting systems as well as in the enzymes (luciferases) or substrates (luciferins). The main characteristic shared is the needed of oxygen for the bioluminescent reaction (Meighen 1991).

1.1. Bioluminescence in bacteria

Three bacterial genera possess almost all luminous bacteria: *Photobacterium*, *Vibrio* and *Xenorhabdus* (Campbell 1989).

1.1.1.Vibrio fischeri

Vibrio fischeri is a Gram negative bacteria bioluminescent that lives both as a symbiont of *Euprymna scolopes*, one marine squid, and as a free-living organism (Ruby and McFall-Ngai 1992). *V. fischeri* colonizes the developing light organ of the squid and is responsible for the animal's ability to become bioluminescent (Wei and Young 1989).

1.1.2.Photorhabdus luminescens

P.luminescens, previously called *Xenorhabdus luminescens*, is a terrestrial motile gram-negative bacteria belonging to the family *Enterobacteriaceae* that establish a mutualistic association with nematodes in the soil environment (Forst and Nealson 1996).

One difference between both bacteria is the optimal temperature of luciferases, being 30ºC for *V. fischeri* and 37°C for *P. luminescens* and the presence of *LuxG* in *V. fischeri*, as shown in *Figure 19* (see further in the text the function of *LuxG*). Another difference is the decay rate of light intensity.*:* luciferase from *P. luminescens* has slow decay rates and luciferase of *V. fisheri* has rapid decay rates. This slow decay can be converted in fast decay with the aminoacid substitution E175G (Glu^{175}→Gly) (Hosseinkhani et al., 2005).

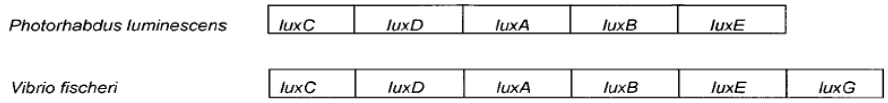


Figure 19.- Comparison between bioluminescence genes from *V. fischeri* and *P. luminescens*. Adapted from Forst and Nealson 1996.

2. *lux o*peron

In bacteria, the bioluminescent reaction involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long-chain fatty aldehyde according to the following reaction, with the emission of blue-green light at λ_{max} = 490-505 nm (Lin et al., 1993):

$$FMNH_2 + O_2 + R\text{-}CHO \rightarrow FMN + H_2O + R\text{-}COOH + h\nu\ (490\ nm)$$

The genes encoding this metabolic pathway are contained in the *LuxCDABE* operon. Three of these genes, *LuxC, LuxD,* and *LuxE*, encode for proteins involved in the synthesis of the aldehyde, while *LuxA* and *LuxB*, respectively, encode the α and β subunits of luciferase, the enzyme that drives the reaction (Meighen 1988). The bacterial luciferase is a flavin monooxigenase, an heterodimeric enzyme of 77KDa composed by a α- and β-subunits with molecular masses of 40 and 37 kDa, respectively. These subunits appears to have arisen by gene duplication and they share 32% sequence identity being α subunit 31 amino acid residues longer and having the enzymatic properties (Baldwin and Ziegler 1992).

lux C, *luxD* and *luxE* genes encode for enzymes with reductase, transferase, and synthetase activity, respectively and act together as a multienzyme fatty acid reductase complex of 500 KDa. These polypeptides have molecular masses of 54 kDa (reductase), 42 kDa (synthetase), and 33 kDa (transferase) and form a complex consisting of a central tetramer of reductase subunits, each interacting with a synthetase subunit that in turn bind weakly to transferase subunit, giving a functional quaternary structure of 12 polypeptides (Boylan et al., 1989).

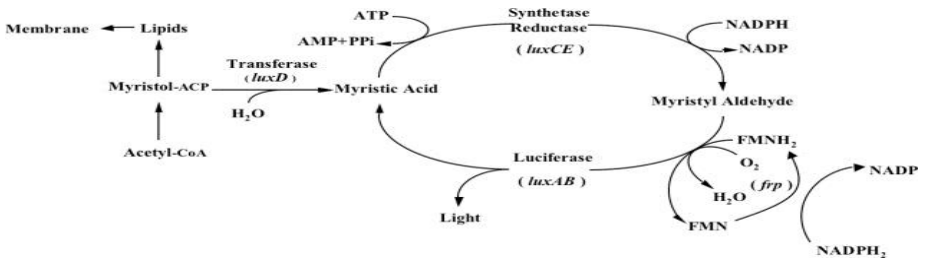


Figure 20.- Bioluminescent reaction catalyzed by the bacterial luciferase operon. Extracted form Close et al., 2009.

As shown in *Figure 20*, the transferase subunit catalyzes the transfer of activated fatty acyl groups to water with the enzyme, being acylated during the course of the reaction. Synthetase activates the fatty acid, resulting in the formation of a fatty acyl-AMP intermediate that is tightly bound to the enzyme. Then, the reductase enzyme is responsible for the NADPH-dependent reduction. This long chain fatty aldehyde in the presence of $FMNH_2$ and O_2 is used by the luciferase to give light (Rodriguez and Meighen 1985). The preferred fatty acid used as a substrat are acyl derivatives with chain length of fourteen carbons, named tetradecanal, for which transferase has a high specificity *in vivo*. However, differences in aldehyde specificity do exist among different bacterial luciferases (Ulitzur and Hastings 1978).The $FMNH_2$ could be provided NAD(P)H and FMN. It is catalyzed by flavin reductase by the following enzymatic reaction.

$$NAD(P)H + H^{+} + FMN \rightarrow NAD(P)^{+} + FMNH_2$$

This reduced FMN ($FMNH_2$) is a key component of the bioluminescent reaction and it is oxidized by the oxygen in a non enzymatic reaction to give hydrogen peroxide and FMN (Inouye 1994). In bioluminescent bacteria, this free $FMNH_2$ is joined to luciferase.The deduced amino acid sequences of *luxG*

are similar to the flavin reductase, Fre, found *in E. coli* (Ingelman et al., 1999). Therefore, it has been postulated that the *luxG* gene product is a flavin reductase that provides the FMNH substrate for the luciferase reaction (Nijvipakul et al., 2008).

RESULTS

1. pLD*LuxCDABE* transformation in *N.tabacum*

1.1. *pLDluxCDABE* vector design

First step in chloroplast transformation is the design of an efficient chloroplast transformation vector. Taking advantage of the existing pLD backbone vector gently given to us (Dr. Daniell, University of Central Florida) a pLD*luxCDABE* was designed and constructed.

The first approach was used a pXen13 vector (pSK*luxCDABE*, 8802bp; Xenogen) in order to isolate *luxCDABE* operon. This vector, as shown in *Figure 21*, contains 5 of the genes responsible for bioluminescence in *Photorhabdus luminescens*.

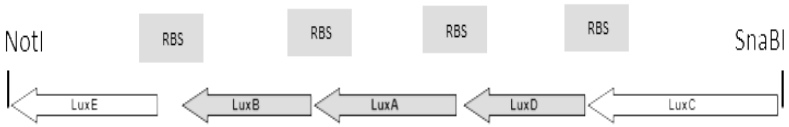


Figure 21.- *luxCDABE* map showing restriction sites to isolate *luxCDABE* operon and RBS. Linear map using SeqBuilder (Lasergene) and BitGene

First, pXen13 was analyzed using bioinformatics tools (Lasergene program; SeqBuilder and MegAlign) in order to detect **suitable restrictions sites**, gene and RBS sequences previous to each gene in order to optimize transcription in chloroplast.

Lux operon was analyzed with the free software BitGene (www.bitgene.com) in order to check the **AT/CG content**, given results as GC content of 37.34%.

BitGene software was used also to analyze **target site accessibility** to predict the gene expression level by evaluating the interaction between ribosomes and RBS. The score will quantify the accessibility of local mRNA fragments the higher the score the target site will be easier to be accessed. This score was high for all the genes analyzed.

Once checked the suitability of pXen13, it was digested using SnaBI and NotI and the 5.000 pb fragment corresponding to *luxCDABE* operon was extracted from gel as shown in *Figure 22*.

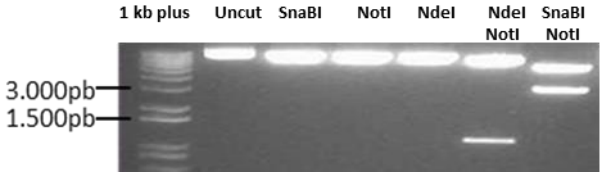


Figure 22.- pXen13 restriction map. Plasmid size: 8.801 pb. Pattern and sizes checked with lasergene program. *NdeI-NotI : 7673 + 1135 pb; SnaBI +NotI : 5830 + 2936 pb*

pLD backbone was digested with SnaBI and NotI, dephosphorylated and ligated with *lux* operon fragment to generate pLD*luxCDABE* and finally transformed in *E.coli* XL-10.

As evidenced by *Figure 23*, *E. coli* colonies of transformation were checked by colony-PCR with DV146 and DV258 and the result of PCR was run in 1% gel in order to confirm the 715 fragment size expected. In order to check the correct pattern of the vector, colonies were re-confirmed by enzyme restriction map, as shown in *Figure 24*.

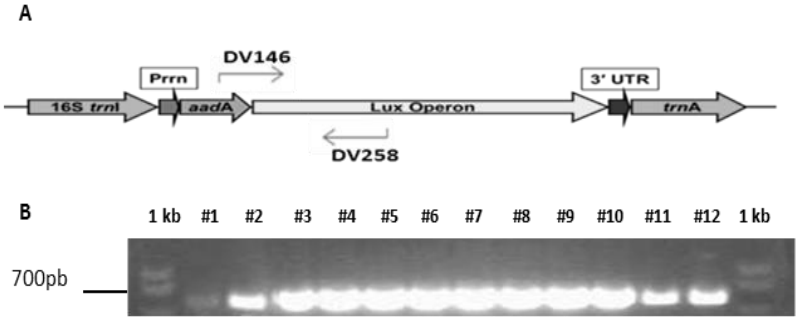


Figure23.-. Colony PCR of transformants A Colonies were PCR amplified with DV146 and DV258 primers (primers sequences specified in Experimental procedures) **B** 12 putative transformants analyzed by colony PCR were run in a 1% agarose gel to confirm the 715 fragment presence.

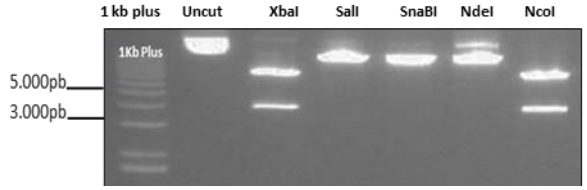


Figure 24.- Restriction map of pLD*luxCDABE*. Plasmid size: 11.788 pb. Pattern and sizes checked with lasergene program. *NcoI* : 4145 + 7573 pb.

The pLD*luxCDABE* vector was checked for correct expression of the operon by bioluminescence assay and the bioluminescence was also visible by naked eye. As noted in *Figure 25*, the Relative Units of Luminescence (RLU) were measured in a liquid culture, referred to $OD_{0,8}$ and the autofluorescence from media was subtracted.

A

	RLU ($OD_{0,8}$)
pXen13	785.225
pLDLux	1.360.160

B

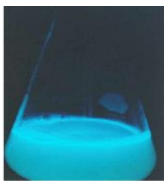


Figure 25.- Bioluminescent assay. (A) Comparative bioluminescent assay using Synergy™ HT Multi-Detection Microplate Reader (B) Photograph made with a common Nikon D80 camera in a dark room without special filters.

1.2. Tranformation by biolistic bombardment in *N.tabacum*

pLD*luxCDABE* was extracted from *E.coli-pLDluxCDABE,* precipitated to increase its concentration and cleaned by a Qlaquick PCR purification kit to eliminate any contamination that could interfere with a chloroplast transformation. As shown in *Figure 26*, gold particles were coated in gold particles as described in Experimental procedures and in order to check the correct coating of the particles a sample was loaded into a gel.

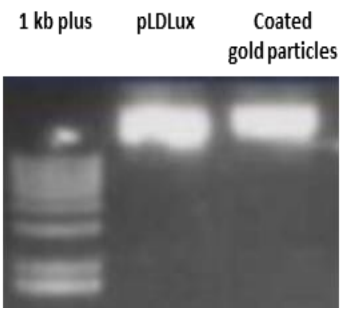


Figure 26.- Gold particle coated. Gold particle coating with pLD*luxCDAB*E were checked by DNA electrophoresis.

A bombardment experiment was carried out with *N.tabacum* WT leaves of an *in vitro* plant of 5-7 leaves stage and pLD*luxCDABE* vector as explained in Experimental Procedures. As depicted in *Figure 27*, two selected putative transplastomic shoots were emerged in three weeks of culture in RMOP selective media. Multiples shoots were emerged from positive control and explants in negative control were died as expected.

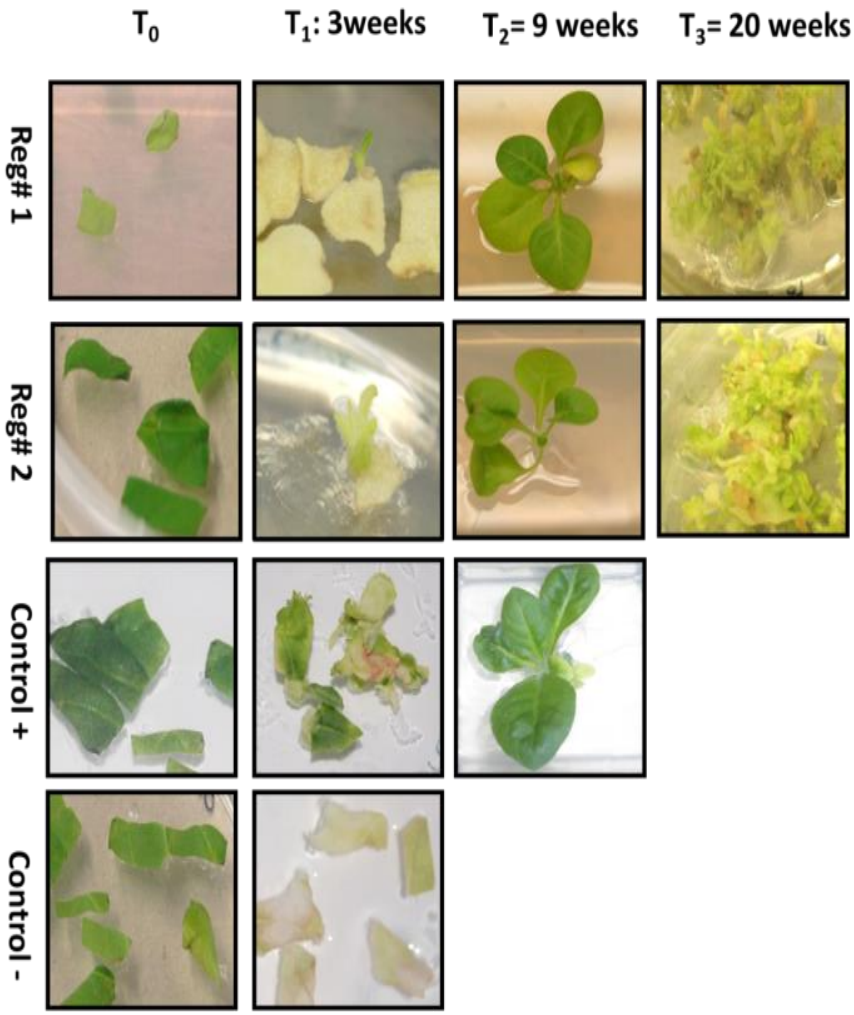


Figure 27.- Bombardment and regeneration in *N.tabacum* with pLD*luxCDABE*. The experiment consists of 5 bombarded plates with one positive and one negative control. Photographs were taken with a binocular microscope at different stages of regeneration until 20 weeks from bombardment.

Emerged shoots were excised from surrounded died explant and placed on MS selective media in order to allow growing. Once achieved the 5 to 7 leaves stage, at week 9, explants from leaves were placed in selective RMOP to achieve regeneration that, as shown in *Figure 27*, was profuse in both putative transplastomic *N.tabacum* plants at week 20.

1.3. Transgene integration check

Once plants achieved 9 weeks stage, the putative transplastomic shoots were screened by PCR for transgene integration in order to confirm a correct integration in cpDNA and distinguish from spontaneous mutation to spectinomycin resistance. As shown below, transgene integration was confirmed by PCR with 3P and 3M primers.

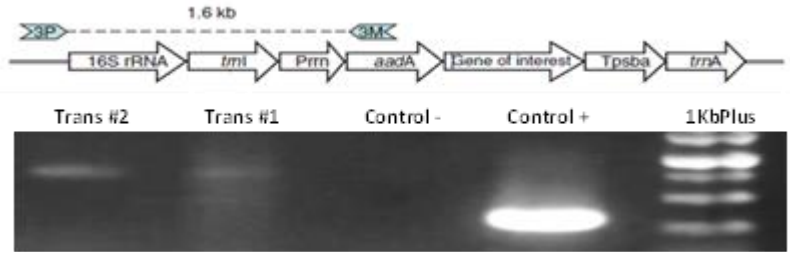


Figure 28.- Transgene integration check. Trangene integration analyzed by PCR with 3P and 3M primers. Both transplastomic plants, WT as negative control and positive control were analyzed.

1.4. RNA expression

Primers for RT-PCR were designed against a 149 pb fragment in *luxA* region. RNA expression was detected by Real-Time PCR with SYBR® Green dye. No relative RNA expression was detected when values were analyzed by ΔΔCt method as described in Experimental Procedures but when the product of Real Time was run in a 1% agarose gel a specific amplification was shown for both transplastomic *N.tabacum* samples and inespecific amplification was shown for *N.tabacum*-WT, the negative control as shown in *Figure 29 A*.

In order to see if this amplification was consistent with monocistronic RNA expression, a PCR from cDNA was done and as shown in *Figure 29B*, actin, *luxA* and *addA-luxA* levels were corroborated for both transplastomic *N.tabacum* and no amplification was shown for either *LuxA* or *addA-LuxA* in *N.tabacum-WT* (data not shown).

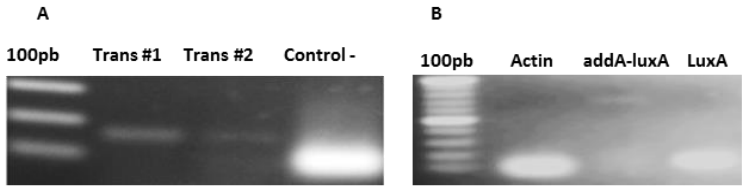


Figure 29.- RNA expression. A PCR amplification from cDNA with primers against *luxA* in *N.tabacum* transplastomic #1, #2 and WT as a negative control. **B** PCR amplification from cDNA against *luxA, Actin* and *addA-luxA* in *N.tabacum* transplastomic #2

1.5. Protein expression assay

To elucidate if there is an expression of luciferase in transplastomic plants, we used the antibody against LuxA region as described in Experimental Procedures on wild type and transplastomic plants protein extracts. As shown in *Figure 30* , luciferase was detected on transplastomic leaves and no expression was detected on wild type leaves extract.

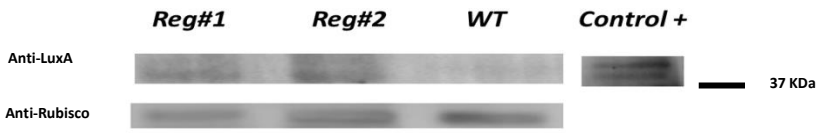


Figure 30.- Western Blot of leaves extracts probed either with ant-LuxA and anti-Rubisco antibodies. 30 µ of protein were loaded in each lane except for positive control (1 µg of pure luciferase; Sigma-Aldrich, #L8507).

These results suggested a weak luciferase expresion in transplastomic plants.

1.6. IVIS bioluminescence assay

In order to check the bioluminescent expression of transplastomic *N.nicotiana* an IVIS bioluminescence assay was performed. Both transplastomic *N.tabacum* plants and *N.tabacum*-WT as a control were analyzed in *IVIS-Spectrum* (Molecular Imaging Platform at CIBBIM-Nanomedicine- Vall d'Hebron Institut de Recerca) under the direction of Dr. Fernández Amurgo.

As shown below, at time 0, photons emission was registered with excitation filter block. In order to check if its photons are provided by chlorophyll, two measurements were done: one with filter (excitation filter 675 /emission filter 720) and another with excitation filter block and emission filter at 500 at darkness for 30 minutes in order to eliminate residual chlorophyll emission of photons. No differences were obtained between transplastomic and WT *N. tabacum* and no detectable bioluminescence was obtained.

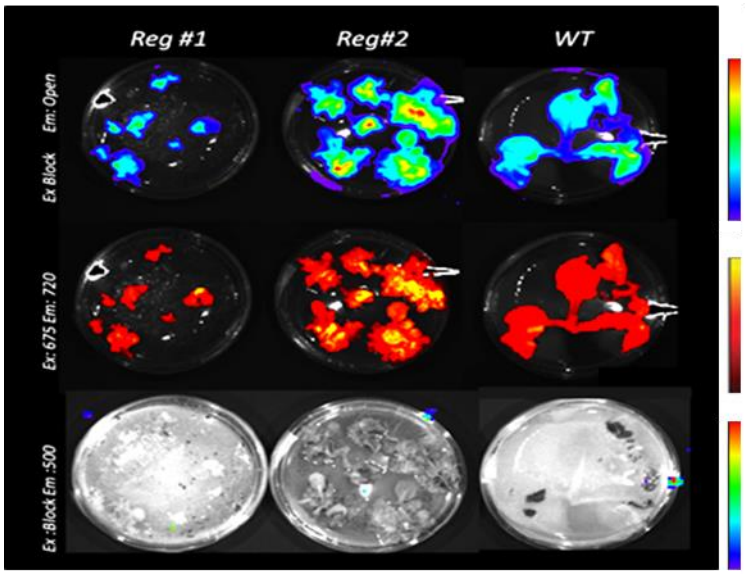


Figure 31.- IVIS Bioluminescence Assay. Bioluminescent was provided as a RLU, established in each sample the maximum and minimum relative and relativized it in a color range.

1.7. Rooting, acclimation and seed production

N.tabacum plantlets were rooted in MS0 and pre-acclimated in selective MS0 in succesive *in vitro* boxes with filters with increasing Gas Exchange Rate to try to achive a better greenhouse's acclimation rate. *N.tabacum*-WT and transplastomic *N.tabacum*-pLD*luxCDABE* were succesfully transplanted to white filter (9,87 GE/day), red filter (15,58 GE/day) and green filter (81,35 GE / day). All transplastomic plants were successfully pre-acclimated and two specimens of each transplastomic plant were successfully transferred to a pot containing autoclaved soil in a greenhouse. The seed pods were matured and collected for further growing of transplastomic plants. Seeds were successfully germinated *in vitro* in selective MS.

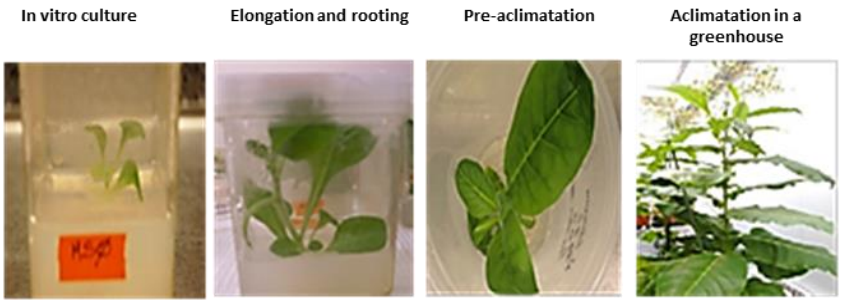


Figure 32.- Steps of *N. tabacum* transformation showing *in vitro* culture, elongation and rooting, pre-acclimation and acclimation in a greenhouse until seeds were produced.

Done the low efficiency of *pLDluxCDABE* in *N.tabacum*, the second approach was designed a pLD*luxCDABEG* vector. This vector, as shown in *Figure 33*, differs from pLD*luxCDABE* in two main characteristics: pLD*luxCDABEG* contains an extra gene, LuxG, which provides for a flavin reductase enzyme and the source of whole *Lux* operon which in this case comes from *Vibrio fischeri*.

2. Design a pLD*luxCDABEG* transformation vector

The selected design was performed using bioinformatics tools (Lasergene program; SeqBuilder and MegAlign). Due to the lack of **suitable restrictions sites** and RBS upstream to each gene, a pair of primers with synthetic compatible restriction sites at each end and RBS upstream that didn't had were designed to clone it from genomic DNA.

Figure 33.- pLD*luxCDABEG* expression cassette showing the synthetic restriction sites and RBS upstream every pair of genes.

V. fischeri cultures were grown and genomic *V. fischeri* DNA was extracted as previously described in Experimental Procedures. The Lasergene program was used in order to detect enzymes that not cut *luxAB* fragment but that had restriction sites in the rest of genomic DNA to facilitate its further cloning. Genomic DNA was digested with HpaI and was check in a 1% agarose gel as shown in Figure 34.

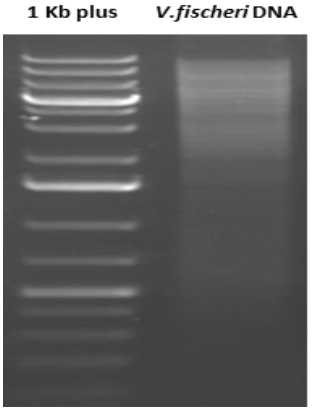


Figure 34.- Genomic DNA digested with HpaI run in a 1% agarose gel to check integrity.

Primers designed as previously explained were used to clone the *LuxAB* fragment and digested with BamHI and XbaI, the new restriction sites formed. Simultaneously, pBSKS was digested with the same enzymes, dephosphorylated to avoid religation and both insert and vector were quantified with ImageJ, extracted from gel and transform in *E.coli* DH5α.

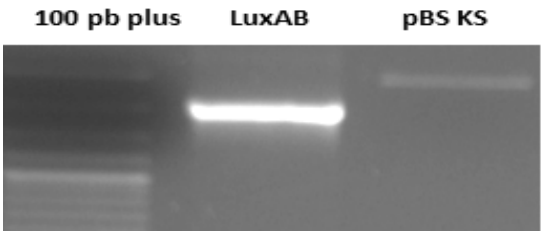
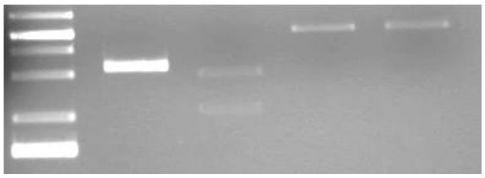


Figure 35.- *luxAB* and pBSKS digested with BamHI and XbaI.

The result of transformation was analyzed using the blue/white screening by adding X-gal to LB-Ampicillin plates. White colonies were selected and colony-PCR was performed.
For positive colonies, two additional tests were performed: a restriction map and a bioluminescent assay in order to detect correct expression of luciferase. As noted in *Figure 36*, a correct pattern was obtained in a restriction map and a consistent bioluminescence was obtained in bioluminescent assay when the substrate, n-decanal, was added. As may be seen, bioluminescence was substrate-dependent.

A



B

	$RLU_{OD_{0,8}}$
E.coli-pBSLuxAB + 1 µL decanal	2128
E.coli-pBSLuxAB + 5 µL decanal	5029

Figure36.- pBS*luxAB* transformation testing. A Restriction map with BamHI and XbaI **B** Bioluminescent assay.

Once pBS*luxAB* was obtained, a cloning procedure to obtain *luxCD* and *luxEG* was performed. In this case, genomic DNA from *V. fischeri*, analyzed as explain before by Lasergene program, was digested with EcoRI. *luxCD* and *luxEG* were cloned, as shown in *Figure 37,* with specific primers designed explained previously. pBS*luxAB* was digested with SmaI-BamHI, *luxCD* was digested with SmaI-BamHI and *luxEG* was digested with XbaI-NotI. Simultaneously, pLD*luxCDABE* was digested with NotI-SmaI and pLD vector was isolated from gel.

A multiway ligation was performed with *luxAB*, *luxCD*, *luxEG* fragments and pLD vector backbone. Possible clones were selected for its bioluminescence at naked eye and a restriction map was done for clones 1, #4, #6 and #10. Clone #10, which showed a good pattern as noted in *Figure 20* was checked again for SmaI-NotI in order to confirm it.

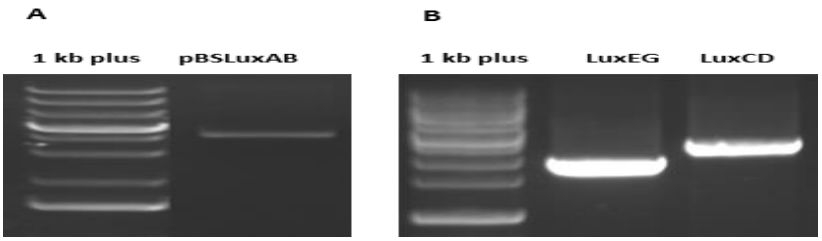


Figure 37.- PCR results. A pBSLuxAB digested with BamHI- SmaI**. B** *luxEG* and *luxCD* fragments **.** Expected size: *luxCD* 2400 and *luxEG* 1800: 12.150 pb

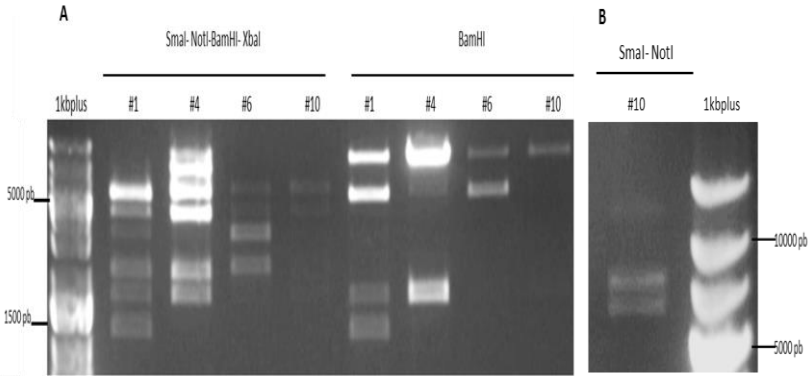


Figure 38.- Restriction map for four clones of transformation. A Clones #1, #4, #6 and #10 digested with SmaI-NotI-BamHI-XbaI and BamHI. **B** Clone #10 digested with SmaI-NotI.

In order to quantify its bioluminescence, a bioluminescent assay was performed as explain in Experimental Procedures. As shown in *Table 26*, clone #10 had the highest bioluminescence in terms of Relatives Units of Luminescence (RLU). In each sample, autoluminescence from media was subtracted and the results are the media of three replicates.

	$RLU_{OD_{0,8}}$
Tranformant #1	620
Tranformant #4	6456
Tranformant #6	1198
Tranformant #10	8306
E.coli- pLDLux	1360
E.coli- pXen13	785

Table 26.- Bioluminescent assay.

3. Biolistic transformation of *LuxCDABE* in ornamental plants

The final aim was the biolistic transformation with *lux* Operon of ornamental plants. Once effective regeneration protocols from leaves were established in *B. semperflorens, M. incana, C. motorious* and *D. caryophyllus,* an exhaustive analysis of biolistic bombardment possibilities was performed.

3.1. Flanking regions analysis

In order to analyze if pLD could be a suitable vector, a nucleotide-nucleotide BLAST (Basic Local Alignment Search Tool) analysis between *trnI* and *trnA* fragment present in pLD backbone and *trnI* and *trnA* chloroplast genes was done. Given that the cpDNA was only sequenced for few species, two representative species were selected for the following orders.

Specie	Homology trnI	Homology trnA	Family		
Silene latifolia	0,97	0,96	CARYOPHYLLALES		
Celosia cristata	0,97	0,96	CARYOPHYLLALES		
Pelargonium hortorum	0,97	0,96	GERANIALES		ROSIDS
Francoa sonchifolia	0,99	0,97	GERANIALES		
Populus alba	0,98	0,97	MALPIGHIALES	Eurosids I	
Salix nigra	0,97	0,96	MALPIGHIALES		
Lotus japonicus	0,92	0,93	FABALES		
Pisum sativum	0,89	0,93	FABALES		
Fragaria vesca	0,99	0,97	ROSALES		
Rosa hybrid	0,99	0,97	ROSALES		
Cucumis melo	0,99	0,97	CUCURBITALES	Eurosids II	
Cucurbita pepo	0,99	0,96	CUCURBITALES		
Brassica napus	0,96	0,96	BRASSICALES		
Arabidopsis thaliana	0,94	0,94	BRASSICALES		
Franklinia alatamaha	0,99	0,99	ERICALES		ASTERIDS
Gordonia lasianthus	0,99	0,99	ERICALES		
Asclepias albicans	0,98	0,98	GENTIANALES	Euasterids I	
Nerium oleander	0,99	0,99	GENTIANALES		
Antirrhinum majus	0,99	0,99	LAMIALES		
Mentha x piperita	0,99	0,99	LAMIALES		
Ipomoea purpurea	0,99	0,97	SOLANALES		
Solanum lycopersicum	0,99	0,98	SOLANALES		
Anthriscus cerefolium	1	0,98	APIALES	Euasterids II	
Daucus carota	1	0,98	APIALES		
Artemisia frigida	0,99	0,99	ASTERALES		
Chrysanthemum indicum	0,98	0,99	ASTERALES		

Table 27.- Homology between pLD *trnI* and *trnA* (Flanking Regions) and *trnI* and *trnA* from two representative species of each Order.

The homology found between *trnI/trnA* regions was higher than 94% for *Caryophillales*, *Malpighiales* and *Brassicales* Orders. This homology is lower for *Fabales* Order.

3.2. Biolistic bombardment

A biolistic experiments with *pLDluxCDABE* was performed for *B. semperflorens, M. incana* and *D. caryophyllus*. *C. motorious* was discarded done its lower *trnI-trnA* homology.

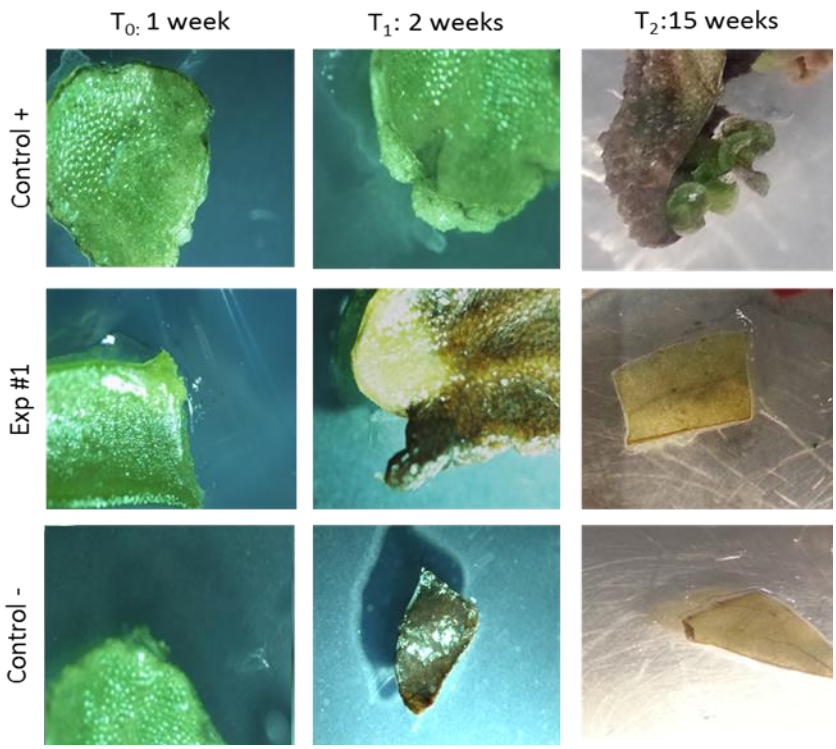


Figure 39.- Biolistic experiment in B. semperflorens The experiment consists of 5 bombarded plates with one positive and one negative control. Photographs were taken with a binocular microscope at different stages of regeneration until 15 weeks from bombardment.

As shown in *Figure 20* for *B. semperflorens*, any transplastomic plant was obtained for 25 bombardment plates.

For *M. incana* and *D. caryophyllus,* similar results were obtained for 20 and 15 bombardment plates respectively.

DISCUSION

DISCUSION

As described previously, first need in chloroplast transformation is to obtain a good organogenesis protocol. Once obtained that, next step is the obtainment of a suitable chloroplast transformation vector.

Through our ultimate goal is the *lux* operon expression in ornamental species, first approach is the lux operon expression in a plant model: *N. tabacum.* For this reason, pLD*luxCDABE* was designed. In order to obtain a suitable expression cassette, *pXen13* was analyzed to know if it could be used to isolate *lux* operon. Due to mutations in RBS can reduce the efficiency of translation in chloroplast (Hirose and Sigura 2004), the expression cassette was analyzed in order to check that comprises at least one RBS upstream of each of the polynucleotide sequences of *luxA*, *luxB*, *luxC*, *luxD* and *luxE* genes. Once the presence of a RBS valid for expression in the chloroplast was tested, the AT/CG content was analyzed. The GC content overall falling well below 50 % for all plastid genes, being the CG content in *Vicia faba*, for example , about 37.3% which is slightly but significantly different from that of nuclear DNA (39.4%) and it was maintained in all the analyzed species until date (Smith 1977). We also considered codon usage as a potential limitation to optimal translation efficiency in plastid transformants (Shimada and Sugiura, 1991). But it is important to mention that transcripts derived from genes of diverse sources were stable in plastids, including bacterial genes with relatively high levels of adenine and thymine (Reddy et al. 2002). This suggests the compatibility of the plastid's machinery with mRNAs from different sources and it could avoid the necessity to construct synthetic genes for plastid expression.

If we talk about the structure of the expression cassette, is important to note that *pLDluxCDABE* was designed without immediate promoter before. It was done because multiple attempts to introduce a promoter in pLD*luxCDABE* vector were unsuccessful transformed in *E.coli*, It could be done due to high energy load this cassette of expression that could be was inconsistent with cell survival. In fact, quantum yield of bacterial luciferase is 0.1, equivalent with 60 ATP per photon (Koncz et al., 1990) and excessive overexpression of this system could be unfeasible. Then, the *lux* operon and *addA* gene were

under the control of the same promoter. This gene cluster was under the control of *Prrn* promoter that normally drives the transcription of the plastid ribosomal RNA (*rrn*) operon (Vera and Sugiura, 1995). This strong, σ^{70}-type rRNA operon promoter has a GTGGGA sequence located at −35 box that works as an essential sequence required for promoter activity and its activity is not strong light regulated. Other possible promoters, as the tobacco native psbA 5'UTR that could facilitate high levels of foreign protein accumulation was discarded because is well known that translation of *psbA* is highly regulated by light. In order to ensure mRNA stability, the presence of a 3'UTR regulatory region is a key factor. For this reason, we use one of the most commonly used, the derived from the plastid *psbA* genes (Maliga 2003).

Once a proper vector was designed, created and checked as shown in *Figures 21* to *23*, a first analysis was performed. A bioluminescent assay in *E.coli* was performed and emission of visible light was detected both by the naked eye as by luminescent assay. Done that correct expression in *E.coli* should attested the future plastid's ability to properly interpret the prokaryotic expression signals, a bombardment experiment was performed.

Positive regenerated plants were screened for transgene integration in order to discard spontaneous mutation. This is done because although the plastid marker genes are designed for expression in plastids, spontaneous mutation to spectinomycin resistance is very common in all species tested so far, including tobacco. This could be avoided if kanamycin resistance was used because no spontaneous mutation for has been found in tobacco (Fromm et al., 1987). These positive transplastomic plants were analyzed by Real-Time PCR in order to check the levels of RNA expression but the ΔΔCt didn't give significative results. These is done because, as shown if *Figure 29-A*, there are some unspecific amplification in *N.tabacum* Wild Type. The specific amplification was shown for *N.tabacum-pLDluxCDABE* transplastomic plants. Then, the results of Real-Time PCR are no valid for SyBR-green primers and TaqMan Real-Time PCR will be necessary to be performed, in order to avoid the detection of inespecificities.
Transcription of operons should result in a polycistronic RNA encoding all the gene products: addA, LuxA, LuxB, LuxC, LuxD and LuxE. In order to shown if a polycistronic RNA was result, a retrotranscripction of RNA and posterior PCR

against *aadA-luxA* fragment was performed as shown in *Figure 29-B.* These results suggest a weak but present expression of one polycistronic RNA.

There are several studies that did not correlate transcript abundance with translation efficiency (Dhingra et al., 2004). This lack of correlation between the increased of transcript levels and translation efficiency seems to suggests than protein stability is more important that transcript abundance (Kuroda and Maliga, 2001). For this reason, a LuxA protein analysis was performed to detect if these polycistronic RNA is properly translated. As shown in *Figure 30*, there is LuxA protein expression in both *N.tabacum-pLDluxCDABE* transplastomic plants. This LuxA expression is not detected in *N.tabacum* Wild Type as expected. Also, high overexpression of proteins may cause protective inclusion bodies formation and thus insoluble aggregates of misfolded and nonfunctional proteins (Fernandez-San Millan et al., 2000 ; Lafolla et al., 2008) and for our purpose this phenomenon need to be avoided.

Once the integration, RNA presence and LuxA protein presence was confirmed, an IVIS bioluminescent assay was performed. Once of the main problems to which we have confronted was chlorophyll emission. As it well-known, in plants, light energy is absorbed by chlorophyll, carotenoids and other pigment molecules present in the thylakoid membranes (Falkowski & Raven, 2007). The absorption wavelength was between 480 and 650 nm and the emission wavelength was between 650 to 800 nm. For this reason a multivariable experiment with IVIS Spectrum was performed, with different combination of excitation and emission filters as shown in *Figure 31*. Since bacterial luciferase reaction has a peak wavelength of about 490 nm and chlorophyll needs previous light excitation, image was acquiring with a dark incubation of 30 minutes and first emission filter selected (500nm). This image suggest no difference between *Wild-Type* and transplastomic plants which brings us to discard the bioluminescence emission, at least at levels above the sensitivity of IVIS.

Several reasons could produce this lack of bioluminescence. Once of them could be the weak expression levels of the enzymes involved in luciferase metabolic pathway in chloroplast but if levels are detectable by western blot this hypothesis is probably incorrect. Other possibility was the incorrect folded of luciferase or the pH 8 of stroma influence in protein stability.

But, a work published in 2010 by Krichevsky et al., demonstrating the *lux* operon expression in plants, and thus the correct folded of enzymes involved in the reaction. These results tend us to discard this hypothesis and propose that the absence of bioluminescence is due to the lack of *luxG* gene in our system. Moreover, the pH of the *E. coli* cytosol is thought to be around 7.5 (Rey et al., 2005). The oxygen availability could be other impediment. But O_2 is produced in photosynthesis by thylakoids in photosynthetic electron transport chain (PETC) in which process to water molecules are oxidized two in O_2. This oxygen is released from thylakoids to citosol thorough the stroma. This *lux* pathway reaction consumes reducing power ($FMNH_2$) and oxygen but it should be capable to use it before released, as previously reported (Krichevsky et al., 2010).

As explained before, luxG is a flavin reductase involved in the turn-over of FMN that supplies reduced flavin mononucleotide ($FMNH_2$) for bacterial luminescence. Levels of $FMNH_2$ are abundant in cytoplasm of prokaryotic *E. coli* and in mitochondria of eukaryotic cells. In fact, a homologue of *LuxG*, *Fre* was observed with *E. coli* (Fieschi et al., 1995). This could be the reason for our previous observations of a strong bioluminescence in *E. coli*. In chloroplast, although FMN hydrolases were found, at least in our knowledge there are no significant levels of $FMNH_2$. These supposed endogenous low levels could be not sufficient for in situ detection of luciferase activity. In fact, the availability of $FMNH_2$ was identified as the limiting bioluminescence substrate in the mammalian cell transformed with *luxCDABEG* and $FMNH_2$ supplementation led to a 151-fold increase in bioluminescence (Close et al., 2010). Thus, several attempts need to be performed to confirm this hypothesis but several experimental problems were difficult it. A chemically reduced FMN should not be added to culture media to provide it exogenously *in vivo* because chloroplast membranes are impermeable to FMN (Allen, J. 1978). Then, *in vitro* experiments to add $FMNH_2$ should involve the isolation and subsequent lysis of chloroplast to allow the contact between reduced $FMNH_2$ and the rest of enzymes of the metabolic pathway.

Other factor that might be involved could be the temperature. If is certain that *Photorhabdus luminescens* is thermostable at mammalian cells optimal temperature (Westerlund-Karlsson et al., 2002) this is not the normal temperature range for plant cells . In contrast, a robust expression of

luxCDABE cloned from *P. luminescens* was shown for a broad range of temperature from 10 to 40 °C (Song et al., 2009). This suggests that the temperature should not be a problem.
These results made us consider the design of a new chloroplast transformation vector that includes *luxG*. The selected source was *V. fischeri* to avoid possible problems with luciferase optimal stability temperature due to luciferase from *V. fischeri* has an optimal temperature of 26ºC (Scheerer et al., 2006).

Acclimation of *N.tabacum* plants was optimized with the use of *in vitro* micropropagation boxes that had filters that allow the gas exchange. This successive increase of GER (Gas Exchange Rate), allow the reduction of ambiental humity avoiding the contamination of plantlets. The impaired function of stomata of *in vitro* leaves is proportional to water stress and the high evotranspiration that might cause desiccation under *ex vitro* conditions is diminished with this strategy (Donelly and Tisdall 1993). This pre-acclimation of plantlets grown in GER boxes in relatively air-exchange cultivation vessels perform a first hardening process that allows acclimatization with higher success rate. In fact, the four specimens acclimated survived and successfully produce vigorous plants that flowered and produced seeds. This seeds were successfully germinated *in vitro* in selective MS, which demonstrates the correct presence of the expression cassette which confers resistance to spectinomycin.

Done its ease in genetic manipulations, *N. tabacum* has been widely exploited for plastid transformation. Also, the great productivity of *N. tabacum*, being one plant capable to generate a million of seeds per year (Arlen et al., 2007) make it widely used as a model specie. Other characteristic, shared by almost all the angiosperms id the paternal inheritance as a dominant mode (Birky, 1995). This characteristic is opposed in gymnosperms, which inheritance of their chloroplast genome is primarily from the paternal parent (Stine et al., 1989). Both angiosperms and gymnosperms seem to have primarily uniparental inheritance (Clément and Pacini, 2001).Moreover, recent studies have reported that escape of transgenes in tobacco is 0.0087% to 0.00024% (Svab and Maliga, 2007), making this an ideal system for use of chloroplasts as target to genetic manipulations. If we talk about the use of *N.tabacum* as a bioreactor, it has

been estimated to be more 50 times less expensive than *E. coli* fermentation systems (Kusnadi et al., 1997). Other advantatge is that the use of *N.tabacum* eliminates possible problems with genetic contamination of food chain because it is a non-feed crop. However, there are some disadvantages. *N.tabacum* is not used as an ornamental plant and, for our purpose, the characteristics of this specie are not optimum. Also, the presence of alkaloids could be a problem for safe use in landscaping (Arlen et al., 2007).

For this reason, several ornamental plants were analyzed previously and, once obtained a good protocol for organogenesis from leaf explant, the possibility to transform it with pLD*luxCDABE* was analyzed. For this reason, done that the cpDNA of our selected species is not sequenced yet, a BLAST analysis against the most proper phylogenetically specie sequenced was performed. This was done because several authors reported that the lack of homology between the flanking regions of plastid vector and plastid genome greatly affect transformation efficiency (Sidorov et al., 1999; Ruf et al., 2001). Then, although the hypothesis of a generic vector containing the *trn*A -*trn*I genes from the IR region of the *N. tabacum* cpDNA for whatever plastid transformation specie was proposed several years ago (Daniell et al., 1998), lower efficiency was observed with 98% of homology in *Petunia sp*. when *N. tabacum* vector was used to transform it (DeGray et al., 2001).

To our knowledge, any attempt to transform *B. semperflorens, D. caryophyllus* and *M. incana* by biolistic bombardment was done until date. The bombardment of *B. semperflorens, D. caryophyllus* and *M. incana* did not give any transplastomic line. Even though our analysis shown a high degree of homology between these intergenic spacer regions of the selected species, previous reports with *Solanaceae* members should be taken into account. For example, only one *Solanum tuberosum* chloroplast transplastomic line was obtained per 35 bombarded plates when compared to about 15 tobacco chloroplast transgenic lines often generated from one bombarded plate (Fernandez-San Millan et al., 2003). This low efficient for non-species specific chloroplast vectors should be avoided with the sequencing of *trnI-trnA* region and designing a specific vector. Other possibility could be increasing the number of bombardments experiments to improve the possibility of obtain a transplastomic line to overcome this limitation.

CONCLUSIONS

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The conclusions of this chapter, which aim was the obtainment of a higher plant species with a bioluminescent phenotype, are:

- The expression of pLDLuxCDABE in chloroplast of *N.tabacum* is feasible and was achieved.
- The bioluminescent phenotype is not achieved in transplastomic *N.tabacum*-pLDLuxCDABE.
- A new chloroplast transformation vector, named pLDLuxCDABEG, was designed and could overcome above limitation.

El objeto de toda discusión no debe ser el triunfo, sino el progreso.

Joseph Joubert

GENERAL DISCUSSION

GENERAL DISCUSSION

Our results, considering them as a whole, lead us to think about the need to set up an expression cassette that provides all components, $FMNH_2$ included, to ensure the correct expression of the bacterial metabolic pathway of bioluminescence. This has already been achieved and it should now be introduced into the ornamental plants chloroplasts.

Although our good organogenesis rates in *B. semperflorens, C. motorious, D. caryophyllus* and *M. incana*, an improvement of rates and the increase of target species would be an interesting method to increase the possibilities of success in biolistic bombardment. Also, as it has been explained, the design of species-specific vectors with the appropriate flanking regions should be optimum. Another improvement could be the design of a light-regulable expression vector in order to avoid the higher metabolic cost for plants. For this reason, a *cis*-regulatory nuclear element of 12 bp (GGATTTTACAGT) capable of conferring dark induction, as well as light down-regulation was obtained from Dr. Inaba (Inaba et al., 2000). It could use the nuclear transcription of luciferase subunits in nucleus. These subunits should need, then, chloroplast transit peptides to ensure the availability in chloroplast (Shashi, B. et al., 2006).

Other option could be the expression of *lux* operon in mitochondria. At present, mitochondria transformation is still unavailable in higher plants, but some efforts have been successfully done in the yeast *Saccharomyces cerevisiae* and the green alga *Chlamydomonas reinhardtii* with foreign genes using a biolistic delivery system (Fox et al., 1988, Johnston et al., 1988 and Boynton and Gillham, 1996). The mitochondrial genome is mutated in order to generate respiratory-deficient strain lacking part of mitochondrial DNA region. The DNA delivered into mitochondria is subsequently incorporated into mitochondrial DNA (mtDNA) by the highly active homologous recombination machinery and reconstruction of missing genes is achieved. Selection of mitochondrial transformants in these species is accomplished by their ability to grow in media that require active mitochondrial function. This technique has not been routinely performed to date and should need a large

study in order to start this approach. In this case, however, FMN should not be a limiting factor.

On the other hand, bioluminescence provided by *lux operon*, although visible to the naked eye, has a low intensity. One possible solution has been proposed to increase it: the use of bioluminescent genes from *Photinus pyralis*, the firefly. The intensity provided by its eukaryotic-luciferase is much higher than the bacterial one. Comparisons of kinetic parameters and quantum yields of luciferases also favors the firefly luciferase, which, in contrast to the 60 ATP for one photon emitted for bacterial luciferase, it needs only one ATP (Wood, 1995). But this system has one important disadvantage to our purposes. The metabolic pathway in which firefly luciferase works is, until date, not entirely known. This is done because the whole way to produce the subtract, Luciferin, is still unknown. Then, although the gene *Luciferin-Regenerating Enzyme* has been cloned and could be introduced to regenerate D-luciferin from oxyluciferin (Gomi & Kajiyama, 2001; Day, J. and Bailey, M. 2003), luciferin is taken up inefficiently by living cells and to enhance this uptake cells should be treated by different methods that reduce their viability as DMSO, low pH values or high concentrations of luciferin (Koncz, C, 1990).

Considering these results altogether, we could say that there is still a long way to explore to achieve bioluminescent transformed plants emitting a light intense enough to make them interesting for architectonical purposes.

And now, the end is here,
and so I face the final curtain.
my friend, I´ll say it clear,
I´ll state my case, of which I´m certain.
I´ve lived a life that´s full,
I travelled each and every highway.
and more, much more than this,
I did it my way.

F. Sinatra

MAIN CONCLUSIONS

MAIN CONCLUSIONS

- We report an efficient protocol for organogenesis from mature leaves, in only one step (one culture medium), for four plants with ornamental value: *Begonia semperflorens cv. hybrida, Codariocalyx motorious, Dianthus caryophyllus and Matthiola incana*.

- It is possible to express the *luxCDABE* operon in chloroplast of *N.tabacum* but the bioluminescent phenotype should be further studied to increase the intensity of the emitted light.

Rodéate de sabios y algo en ti se quedará

Mägo de Oz en la canción "La Danza del Fuego"

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Y al contrario y viceversa, y en la buena y en la adversa...

Serrat y Sabina

SUMMARY IN SPANISH

I. JUSTIFICACIÓN

Nuestro proyecto se enmarca tratando de abarcar una de las necesidades humanas, la necesidad de continuar con la calidad de vida que proporciona el urbanismo y la vida en grandes ciudades, el hecho de cubrir necesidades tales como luz, calor y habitabilidad, pero tratando de llegar a un modelo eco-sostenible. Dentro de este planteamiento el Grupo de Investigación Consolidado Arquitecturas Genéticas trata de unir los conocimientos transversales de arquitectura y el diseño de nuevas estrategias biotecnológicas.

Por este motivo, dado el amplio consumo en iluminación presente en las ciudades y la necesidad de alternativas sostenibles, se plantea el uso de la bioluminiscencia natural presente en la naturaleza en un sistema eucariota, los vegetales superiores.

II. HIPÓTESIS Y OBJETIVOS

El objetivo principal que aborda el presente trabajo es la obtención de plantas ornamentales que expresen los genes de bioluminiscencia de manera visible y eficaz para su uso en la arquitectura. Este objetivo se desglosa en los tres objetivos siguientes:

- El ***primer objetivo*** es la obtención de tasas óptimas de organogénesis desde explantes foliares de plantas ornamentales.
- El ***segundo objetivo*** es la obtención de vectores de transformación cloroplásticos diseñados para introducir el operón bacteriano *lux* en el especie modelo *N.tabacum*.
- El ***tercer objetivo*** es introducir genes de interés en especies ornamentales de interés.

Estos objetivos se abordaran en el capítulo I, primer objetivo, y en el capítulo II, segundo y tercer objetivo.

III. INTRODUCCIÓN GENERAL

La vida en la tierra ha estado ligada desde siempre a las plantas. De ellas se extrae no sólo el oxígeno necesario para la vida sino que también son la fuente de sustento para las poblaciones humanas. De hecho, la agricultura fue lo que determinó el establecimiento de poblaciones asentadas, dejando atrás los tiempos nómadas. El hombre, desde aquellos inicios agricultores, ha modificado las propiedades de sus cultivos para lograr mejoras en sabor,

productividad y resistencia a plagas, entre otros. Aquellas modificaciones, que se iniciaron mediante el cruce y selección de especies con mejores características, se continúan realizando hoy en día, si bien la biotecnología ha abierto puertas a la manipulación genética. De hecho, desde 1996, las hectáreas ocupadas por cultivos genéticamente modificados (GMCs) ha aumentado de manera exponencial y se prevé que este crecimiento continúe (James 2011) e incluso aumente.

Que el ser humano requiere de alimentación es un hecho, pero las plantas actualmente no sólo están sufriendo ingeniería genética para su consumo humano, sino que se está viendo su potencial como biofactorías para proveer de productos farmacéuticos a gran escala y bajo coste, nuevos bioplásticos y otros biomateriales (Somleva et al., 2013).

La transformación de vegetales superiores se inició gracias a que Chilton et al., demostraron, en 1977, la posibilidad de incorporar en el genoma nuclear un fragmento del plásmido de virulencia de la bacteria *Agrobacterium tumefaciens*. Esta bacteria, causante de la enfermedad *Agalla de la corona* en plantas, posee el plásmido Ti es capaz de incorporarse al DNA de la planta huésped gracias a unas secuencias bordes que recombinan y permiten insertar un fragmento. Esta capacidad llevó al desarrollo de vectores con capacidad de insertar genes foráneos (Garfinkel et al., 1981) al reemplazar aquellos genes que codifican para la síntesis de auxinas y opinas.

En 1980, gracias a Davey et al., se ampliaron los métodos para incorporar DNA foráneo en el genoma nuclear de la planta mediante el uso de polietilenglicol (PEG) en protoplastos de células vegetales. Estos métodos son capaces de superar una de las limitaciones de la transformación mediante *A. tumefaciens*, la limitación de posibles especies a transformar.

Si bien la limitación de especie ha sido solventada, existen otros problemas que son inherentes a la transformación nuclear. Estas desventajas son el bajo porcentaje de expresión de proteínas foráneas insertadas en el núcleo, los efectos de posición debido a la integración al azar en el genoma nuclear (Daniell et al., 2002), el silenciamiento y el escape de transgenes.

Gracias a Svab et al., 1990, aparece una nueva técnica que solventa los problemas debidos a la transformación nuclear: la transformación cloroplástica. En este tipo de transformación las plantas con su DNA cloroplástico, a partir de ahora transplastómicas (Svab et al., 1990), integran el DNA foráneo en sus cloroplastos mediante recombinación sitio-específico, lo que evita los efectos de posición. Además la acumulación de transcritos

en vegetales transplastómicos es capaz de llegar a 169 veces el acúmulo en vegetales transgénicos (Lee et al., 2003) y la proteína foránea puede llegar a representar el 46% del total proteico del ejemplar (De Cosa et al., 2001). Esto es debido a la gran poliploidía del DNA cloroplástico (cpDNA) descrita por Bendich en 1987. Además, se puede evitar el silenciamiento de los genes insertados, la expresión génica es uniforme (Daniell et al., 2002) y se puede minimizar el posible escape de transgenes dada la no presencia de cloroplastos en el polen en la mayoría de Angiospermas (Svab y Maliga 2007).

Los cloroplastos poseen su propia maquinaria de transcripción y replicación y comparten con los procariotas la mayor parte de sus características (Kuroda y Maliga 2001). Los cloroplastos son orgánulos de 5-10 µm de diámetro que presentan diferentes regiones diferenciadas: los tilacoides, el sistema membranoso interno, y el estroma, siendo todo rodeado por una doble membrana. Es en las membranas de estos tilacoides donde se encuentra las proteínas que forman el complejo fotosintético (Wollman et al., 1999).

El cpDNA es un genoma de doble cadena, circular cuyas medidas varían entre 120-160kb (Bendich 1987) y posee unos 120 genes, la mayoría en forma de operones, que codifican para una pequeña parte de las proteínas del cloroplasto. La mayor parte de las proteínas del estroma están codificadas en genes nucleares (Leister 2003) y serán posteriormente importadas. Cada orgánulo, si tomamos como referencia una hoja de *N.tabacum*, posee aproximadamente 100 cloroplastos y 10 copias del cpDNA, dando un total de 1.000 cpDNA por célula (Thomas y Rose 1983). La estructura del cpDNA consta de dos fragmentos de secuencia simple: uno de 15 a 25 kb (SSC) y otro de 80 a 100 kb (LSC) separados ambos por dos regiones iguales e inversas (Ira e IRb). La secuencia dentro del cpDNA es altamente conservada, especialmente entre las IRa e IRb (Douglas 1994), donde se encuentran los genes que codifican para rRNAs y tRNAs. Las características de la expresión génica en cloroplastos, como ya se ha comentado previamente, se asemeja a la de procariotas. Como ellos, posee promotores del tipo σ^{70}, secuencias Shine-Dalgarno (SD) y operones. Sin embrago, también posee ciertas características eucarióticas, ya que algunos genes poseen intrones y son capaces de producir mRNAs muy estables.

La transcripción de los genes en plastidios está regulada por la región promotora situada en 5'. De los tres tipos de promotores existentes (Miyagi et al., 1998; Klein et al., 1994), nos centraremos en los promotores del tipo σ^{70}, promotores fuertes que comparten las secuencias consenso situadas en -35 y -10 con los promotores procarióticos (Liere and Borner 2007). Estos promotores son también llamados promotores PEP dado que son reconocidos por la polimerasas codificadas en plastidios (PEP), necesitando este holoenzima de factores sigma codificados en el genoma nuclear (Fujiwara et al., 2000).

Otro factor importante para la expresión del mRNA son las regiones SD, situadas en 5' y la región 3'. La secuencia SD es un lugar de unión del ribosoma (RBS) que se haya en los mRNAs procarióticos y se complementa con el 16S RNA previamente al inicio de la traducción (Bonham-Smith y Bourque 1989). Estas secuencias también se hayan en los extremos 5' de los genes de cloroplastos, si bien estos 5'UTR son más variables en cuanto a localización, tamaño y composición (Hirose and Sigiura 2004). La región 3'UTR da lugar a una estructura secundaria que forma un bucle que favorece la estabilidad, previniendo el ataque por ribonucleasas (Stern et al., 2010).

Los plámidos usados para la transformación cloroplástica poseen una estructura básica: un promotor, un RBS, un gen marcador y un 3'UTR seguido de otro promotor, RBS, Gen de interés (GOI) y 3'UTR (Verma et al., 2008), todo ello flanqueado por dos regiones homólogas al genoma del cpDNA. Esta homología permite el fenómeno de recombinación homóloga innato en el cloroplasto, donde se han encontrado homólogos de la proteína *RecA* (Lin et al., 2006). Se cree que la función de esta maquinaria de recombinación es la de mantener y reparar el cpDNA. Esta recombinación disminuye de manera paralela a la disminución de la homología entre las regiones flanqueantes y el cpDNA (Ruhlman et al., 2010). Otro mecanismo que se da en cloroplastos es la corrección de copia, donde una vez insertado un fragmento en una de las regiones IR, este fragmento se copia y queda insertado en ambas (Daniell and Chase 2004). Para mejorar la posibilidad de recombinación, los plásmidos usados para la transformación cloroplástica poseen un origen de replicación que permite la replicación del plásmido en el estroma del cloroplasto (Daniell et al., 1990). Actualmente, 16 lugares diferentes han sido usados como lugares de integración. De éstos, los más comúnmente utilizados son trnV-3'rps12, trnI-trnA y trnfM-TrnG (Maliga 2004).

En la transformación por biolística, una vez bombardeada la hoja y dispuesta en medio específico para lograr la organogénesis suplementado con el antibiótico de resistencia se obtienen brotes, que son quimeras (Moll et al., 1990). Éstos poseen una, o unas pocas, copias de su cpDNA transformadas y es mediante sucesivas tandas en medio de selección que se obtiene el enriquecimiento en cpDNA transformados, hasta lograr la homoplasmia, es decir la totalidad de las copias de cpDNA transformadas (Maliga 2004).

IV. CAPÍTULO I: Regeneración de especies ornamentales

A. Introducción

Las células de un organismo multicelular presentan un fenómeno llamado totipotencia, que expresa la posibilidad de que cada célula sea capaz de un desarrollo independiente si se proporcionan las condiciones externas adecuadas (White 1954). Esta capacidad de regeneración de un organismo completo a partir del tejido somático adulto es un fenómeno bien conocido que en plantas puede ser logrado a través de la manipulación de hormonas vegetales.

La micropropagación es el nombre dado a la propagación clonal y puede ser utilizado para la propagación de especies y variedades, para el mantenimiento de una línea libre de patógenos y para posteriores aplicaciones en programas de mejora genética (Tombolato y Costa, 1998).
Esta micropropagación puede dividirse en cuatro etapas secuenciales: la etapa I se caracteriza por el establecimiento de cultivos axénicos a partir de diferentes explantes (Mantell et al.1994). El principal objetivo de esta fase es la obtención de líneas libres de contaminación, con un ambiente controlado.
La etapa II se caracteriza por la producción y multiplicación de brotes, siendo la etapa III la caracterizada por la elongación de los brotes y el enraizamiento. La IV y última etapa se caracteriza por la transferencia definitiva de las plantas al suelo en condiciones naturales, que se denomina proceso de aclimatación, donde las plantas deben sufrir un endurecimiento que incluye la modificación progresiva de la anatomía de sus hojas, el aumento de la tasa fotosintética y la adaptación progresiva a las condiciones ambientales reales (Davey y Anthony 2010).

La morfogénesis es un proceso por el cual da como resultado la formación de órganos discretos o plantas enteras a partir de células somáticas de tejidos aislados y es el resultado de una división organizada y de cambios en la expresión de ciertos genes (Fehér et al, 2003). Existen dos maneras de obtener morfogénesis in vitro: la embriogénesis y la organogénesis.

En la embriogénesis somática, no se observan conexiones vasculares directas con el tejido original (Hicks 1980) y el embrión somático desarrolla de una manera similar a un embrión cigótico de una semilla (Meinke 1995). Esta embriogénesis puede ser directa o indirecta. Cuando los embriones se inician a partir de tejido desorganizado, o callo, el nombre se le conoce como embriogénesis indirecta mientras embriogénesis directa se produce cuando los embriones se inician directamente del explante. El callo embriogénico, o callo tipo I, es compacto, muy organizado, blanco pálido y con una tasa de crecimiento lento. Con frecuencia, este callo está rodeado de callo de tipo II, suave y con una mayor tasa de proliferación (Vasil y Vasil, 1984).
En contraste con la embriogénesis somática, la vía organogénica, ya sea por vía directa o indirecta, requiere medios de cultivo secuenciales. Skoog y Miller, en 1957, demostraron que una alta relación auxina/citoquinina inducía el desarrollo de raíces, si bien una baja relación promovía la inducción de brotes.
Los cultivos de tejidos representan los principales sistemas experimentales utilizados para la ingeniería genética de las plantas, así como la micropropagación se ha convertido en una parte importante de la propagación comercial de muchas plantas, debido a sus ventajas como sistema de multiplicación (Iliev et al., 2010).
No existen medios universales para el cultivo in vitro ya que cada especie posee sus requisitos específicos con respecto a los diferentes componentes del medio (Saric et al., 1995), si bien existen fórmulas que se han utilizado como puntos de partida, como las propuestas por Murashige y Skoog.

Como ya se ha comentado, la organogénesis depende de la concentración relativa de auxinas y citoquininas. Las auxinas influyen positivamente en la formación de yemas y el inicio de la raíz, siendo sus mayores representantes el ácido indol-3-acético (IAA), el ácido 1-naftalenacético (ANA) y EL ácido 2,4-diclorofenoxiacético (2,4-D). Las citoquininas influyen en la división celular y

la formación de brotes. Las citoquininas más comunmente utilizadas son la 6-Bencilaminopurina (BAP), el tidiazurón (TDZ) y la zeatina (Z).

B. Resultados y discusión

Para obtener una planta transplastómica es necesario pasar por un proceso de organogénesis, proceso mediante el cual se obtiene la regeneración de parte aérea y raíces de manera secuencial a partir de un explante. Este fenómeno tiene sus orígenes en los trabajos de Skoog y Miller en 1957, donde observaron que una ratio auxina/citoquinina baja promueve la inducción de regeneración de parte aérea mientras que una ratio alta promueve el desarrollo de raíces.
Si bien se ve claro que existe una interrelación entre las concentraciones de auxinas y citoquininas durante la organogénesis, cada especie posee sus propios requerimientos específicos en cuanto a la sinergia de éstas hormonas y sus concentraciones. Es por este motivo, y dado que actualmente el mercado ornamental es un mercado en alza que busca la incorporación de nuevas variedades o características (Nishira et al., 2011; Azadi et al., 2011), que, tras realizar un análisis de mercado con ayuda de una compañía líder en el sector, Corma S.L., se planteó lograr la organogénesis de 25 especies, seleccionadas por su potencial ornamental, la presencia de herencia cloroplástica materna y una distancia filogenética a *N.tabacum* , la especie modelo, no demasiado alta. Para ello , las plantas utilizadas para este estudio se agrupan por su filogenia siguiendo la clasificación APGIII.

El estudio se inició tratando de obtener organogénesis desde explanto foliar o intermodal de ejemplares adulos crecidos en invernadero de *Bougainvillea glabra, Hedera helix, Nerium oleander, Phyllostachys aurea* y *Ficus benjamina.* Para tal fin se desinfectaron los explantes con cuatro protocolos con orden creciente de dureza y se dispusieron en medio inductor de callo, excepto para *P. aurea*, donde ninguno de los protocolos de infección permitió la desinfección efectiva. Tal contaminación fúngica endógena no permitió cultivo axénico de *P. aurea* en cualquiera de los procedimientos de desinfección ensayados y mostró una alta sensibilidad a los tratamientos habituales de desinfección. Se debería plantear el uso de estrategias de

desinfección alternativas, tales como PPM (Plant Preservative Mixture™) que no repercutan en la viabilidad del explante.

Tampoco se logró la desinfección efectiva de los segmentos internodales de *Nerium oleander, Phyllostachys aurea* o *Ficus benjamina*. La inducción de callo fue efectiva para los explantos foliares de *B. glabra, H. helix* y *N. oleander* y para los segmentos internodales de *B. glabra* y *H. helix.* Estos segmentos internodales requirieron desinfección con $HgCl_2$ protocolos más fuertes y nuestros experimentos revelaron que fue más potente para la desinfección eficaz de explantes nodales. El cloruro de mercurio se ha demostrado como un agente de esterilización muy eficaz ya que el gas liberado podría penetrar y destruir los microorganismos presentes en la mayoría sin embargo, también se ha reportado toxicidad para los tejidos explante. Por lo tanto la concentración del agente esterilizante y la duración del tratamiento debe ser óptima para reducir al mínimo la mortalidad de los explantes de tejido debido a un exceso de esterilización (Young 1919).

La inducción de callo se obtuvo de *B. glabra, H. helix* y *N.oleander* y éstos se sometieron a un experimento de regeneración indirecta.
Para *N. oleander*, la inducción de callo se obtuvo en medio suplementado con 2,4-D y BAP (Santos et al., 1994) pero no se obtuvo la regeneración directa. Esto podría ser debido, en concordancia con dicho autor, ya que solo el callo formado a partir de hojas jóvenes es embriogénicamente competente.

Para hojas de *B. glabra* y tallos de *H. hélix*, el callo se indujo con éxito en MS suplementado con 2,4-D. Con este callo pro-organogénico en apariencia se llevó a cabo un experimento de regeneración indirecta a partir de callo con una batería de concentraciones de auxina y citoquininas

Pese a que el único, bajo nuestro conocimiento, reporte sobre el tema gracias a Shah y su equipo muestra buena formación de callo para *B. glabra* a partir de tallo, en nuestro caso éste fue escaso y su ennegrecimiento pudo impedir la posterior organogénesis (Shah et al., 2006). Similar fue lo que ocurrió con el callo formado a partir de explanto foliar. Para *H. helix* se obtuvieron poco callo, en concordancia con Banks et al. (1979), donde mostraron el bajo potencial organogénico de callo de esa especie.

Pese a la extensa batería hormonal realizada, no se logró la regeneración de parte aérea, si bien la inducción de raíces se logró con altas ratios auxina/citoquinina. Podría ser debido a la baja capacidad de organogénica de las hojas de las plantas maduras (Souzal et al., 2006) y la dureza de los protocolos de desinfección necesarios para obtener explantos estériles. Teniendo en cuenta esta importante desventaja, nuestros esfuerzos se dirigieron a obtener buenas tasas de generación en las plantas recién germinadas, para evitar la baja capacidad de regeneración de las plantas más maduras.

Para ello, 20 especies fueron seleccionadas según los criterios anteriores. La desinfección de semillas se llevó a cabo mediante tres procedimientos, llamados suave, medio y duro, con órdenes crecientes de dureza. La desinfección suave fue efectiva para las siguientes especies: *A. majus, B. semperflorens, C. scandens, C. persicum, D.caryophyllus, M. incana, M. viridis, O. vulgare, P. zonale* y *Z. elegans*. La desinfección media resultó efectiva para C*. hybridus, I. arborescens, C. motorious, V. odorata, V. tricolor* y *M. jalapa*. Sin embargo, *P. grandiflora, P. hybrida* y *P. hortensis* necesitaron la desinfección más agresiva.

En cuanto a la tasa de germinación, para *A. majus, B. semperflorens, C. persicum, D. caryophyllus, I. arborescens, M. incana, M. viridis, M. jalapa, O. vulgare* y *P. zonale* , ambos medios MSO y MS +1mg l^{-1} GA_3 mostraron tasas iguales de germinación, situándose entre 1 y dos semanas según la especie.

La germinación en *C. scandens*, *C.hybridus* y *C. motorious* necesitó de una incubación previa en 1 mg l^{-1} GA_3, at 30 ºC durante 24 horas para después germinar, tanto en MS0 o MS suplementado con 1 mg l^{-1} GA_3.

No se obtuvo germinación en *P. grandiflora, P. hybrida, P. hortensis, V. odorata* y *V. tricolor* en ninguno de los medios estudiados. Por ello se inició un experimento multivariable en los tres medios estudiados con diferentes temperaturas y condiciones de luz (12, 18, 22 y 26ºC en oscuridad) sin obtener germinación en ningún caso. Esto se planteó ya que la dormición en algunas especies requiere un período de incubación en frío con el fin de

promover la germinación.- Además el uso de GA_3 ayuda a superar la dormición (Kermode 2005).
El procedimiento para eliminar cualquier fuente de contaminación externa, podría afectar el porcentaje de germinación, fenómeno que se parece ser proporcional a la dureza del procedimiento de desinfección y tiempos de tratamiento (Dempsey y Walker 1978).Sin embargo, los efectos de hipoclorito de sodio sobre la germinación de las semillas de diferentes especies son contradictorios y se ha informado tanto de la capacidad para promover (Macit 1981), para inhibir (McCollumand Linn 1955) y para reducir la tasa de germinación (Cantllffe y Watkins 1983).Esta capacidad de promover sería el resultado de el mimetismo con el efecto de la escarificación con ácido que daría como resultado semillas más porosas para intercambio de gases y GA_3. Debe tenerse en cuenta la duración del tratamiento con el desinfectante encontrando un equilibrio entre la mortalidad por el tratamiento y la contaminación debido a la desinfección incompleta.

Aquellas especies cuya tasa de germinación se situó por encima del 50%, una vez alcanzado el estadío de 5-7 hojas, se sometieron a un experimento de regeneración. Este consistió en una bacteria hormonal con diferentes ratios de auxinas/citoquininas. Cada experimento consitió en diez explantes por placa, con un total de tres replicados, dando un cómputo de 30 explantes por especie y ratio.

La inducción de callo a partir de explanto foliar se analizó bajo el siguiente código: - para ausencia de inducción de callo, + para obtención de callo no friable, ++ para callo friable y +++ para callo de apariencia friable que acaba mostrando estructuras pro-organogénicas.
La regeneración de parte aérea se analizó considerando el número de tallos, bajo el siguiente código: - para ausencia de regeneración, + para un mínimo de 1 tallo en al menos un explante, ++ para al menos un tallo en un mínimo de 6/10 explantes y +++ para un mínimo de un tallo en cada explante. Todo el experimento se llevó a cabo durante 15 semanas.

En *D. caryophyllus*, se obtuvieron mejores resultados en medios que contenían 1 mg L-1 de NAA + 1 mg L-1 de BAP evitando la vitrificación. Ésta es un desorden fisiológico inducido que consiste en apariencia gruesa y

cristalina de las hojas. Este es uno de los principales problemas en el género *Dianthus* en el cultivo in vitro (Kharrazi et al., 2011). Se ha visto que las citoquininas inducen vitrificación de una manera dependiente de la concentración (Leshem 1988). Nuestros resultados muestran que una proporción de 1:1 auxina / citoquinina es capaz de evitarla.
Casas et al., 2010 muestra la formación de brotes adventicios con BAP 1 mg.l^{-1} + ANA 0,2 mg.l^{-1} y Pareek et al. 2003 obtiene embriogénesis somática y germinación de embriones sin una fase de callo previa, pero son necesarios varios pasos. En *D. chinesis*, Kantia et al .2002, mostraron que se obtiene una buena tasa de regeneración, cuando auxina y citoquinina actúan sinérgicamente con una relación de 1:1.

En *C. motorious*, se obtuvo buena inducción de callo y regeneración posterior en MS suplementado con baja relación auxina / citoquinina (NAA 0,1 mg.L^{-1} + BAP 1 mg.L^{-1}). Trabajos anteriores mostraron embriogénesis somática a partir de segmentos de cotiledones con IAA 0,5 mg.l^{-1} + BAP 1 mg.l^{-1} (Chitra Devi y Narmathabai 2011) o tras dos etapas de regeneración utilizando MS suplementado con NAA 0,1 mg.l^{-1} + BAP 2 mg.l^{-1} para la inducción del callo y NAA 0.05 mg.l^{-1} + BAP 2 mg.l^{-1} para la regeneración de brotes (Mao et al., 2010). Estos resultados están en concordancia con otro trabajo con *D. afinne* y *D. uncinatum* (Rey y Mroginski 1977).

En *B. semperflorens*, el medio más favorable para la regeneración de brotes fue MS suplementado con BAP 0,9 mg.l^{-1} y ANA 0,3 mg.l^{-1}. Nuestros resultados están en concordancia con Mendi et al. 2009 (Mendi et al., 2009) para *B. elatior* eran se obtuvo la mejor respuesta morfogenética cuando la relación auxina / citoquinina fue de 1:2. De hecho, en nuestras condiciones experimentales, la relación auxina / citoquinina 1:3 ha demostrado ser la proporción más eficaz para obtener la regeneración, seguido de la ratio 1:2 auxina / citoquinina. Como es bien sabido, NAA es una auxina fuerte y el aumento de su concentración reduce al mínimo el efecto de regeneración o incluso inhibe la regeneración de la planta. En contraste, Espino et al., 2004, encontrado para *B. semperflorens*, la mejor relación se obtuvo para la ratio auxina / citoquinina 1:1 (Espino et al., 2004). Pero en todos los casos, se obtuvo la mejor respuesta morfogenética cuando el citoquinina era BAP.

La luz pareció actuar como fuerte inhibidor de la regeneración e inducir necrosis en los explantes. Heide mostró en 1968, que la luz era capaz de influir en la capacidad para formar yemas adventicias. El oscurecimiento de los explantes debido a la oxidación de compuestos fenólicos podría estar relacionado con la actividad enzimática de explante, como polifenoloxidasa y peroxidasa (Pizzocaro et al 1993;. Abajo et al 1995;.. Whitaker et al 1995) cuando el contenido del citoplasma y vacuolas se mezclan debido al daño de tejido. Estos compuestos oxidados afectan negativamente a los *cultivos in vitro* (Laukkanen et al. 1999) y existen alternativas para evitarlo, tales como carbón activado, subcultivo frecuente o la adición al medio de algunos antioxidantes (Pizzocaro et al. 1993). Pero, en concordancia con nuestros resultados y los resultados anteriores de Bouman y Klerk en 2001 para *B. hiemalis*, la deprivación de luz podría ser un método de bajo costo que evita la necesidad de usar aditivos.

Para *M. incana*, los resultados de nuestras investigaciones muestran buenas tasas de regeneración a partir de hojas jóvenes en MS suplementado con 0,4 mg L^{-1} NAA + 0,8 mg L^{-1} de BAP. Estos resultados difieren con Gautman et al., 1983 que muestra organogénesis de cotiledones en MS con 1 mg.L^{-1} de BAP. Hesar et al., 2011 y Kaviani et al., 2011, mostraron resultados similares en ápices foliares en MS con 0,5 a 2 mg.l^{-1} de kinetina. Nuestros resultados fueron concordantes con hojas de jóvenes plantas *in vitro*. Esto nos permitió evitar una germinación continua de semillas, ahorrando tiempo y reduciendo costos.

Estas especies fueron correctamente elongadas y enraizadas tanto en MS0 como en MS½. Este último fue desfavorable para *C. motorious*. La elongación de los brotes requiere una combinación de la división celular y la ampliación de las células establecidas por el meristemo apical del brote (SAM), situado en el ápice del tallo. Esta elongación depende de las hormonas endógenas, pero puede ser estimulada por la adición a los medios de comunicación de bajas concentraciones de BAP y NAA. En nuestras condiciones experimentales, elongación de los brotes fue un éxito y no se añadieron PGRs externos. Por otra parte, la elongación de las raíces es el resultado de la ampliación de las células nuevas que se están formando por divisiones celulares en meristemos apicales (Torrey 1956) y es un proceso vital para obtener plantas que podrían establecerse con éxito en el suelo. Aunque

algunos autores informan de la necesidad de añadir algunos PGRs como la auxina IBA para influir en la proliferación de la raíz, nuestros resultados experimentales demostraron que no hay necesidad de que en nuestras condiciones experimentales (Awamy et al., 2002).

Para el resto de especies, es bien conocida la importancia de la fuente de explante (Hemphill et al., 1998). Una posible razón para la no obtención de la inducción de callos y/o regeneración de brotes es la escasa capacidad intrínseca de las hojas de regenerarse. De todos modos, una ampliación de la batería hormona grande podría ser necesaria para confirmar esta hipótesis en las especies restantes ya que, aunque para la iniciación de callo en plantas dicotiledóneas una combinación de alta ratio de auxinas/citoquininas se (Caboni et al 2000;.. Haliloglu et al 2006) o citoquininas en solitario (Yam et al 1990) es ampliamente utilizada, se han encontrado grandes variaciones en cuanto a la concentración, relación y tipo de PGRs a utilizar.

C. Conclusiones

El protocolo *in vitro* para la regeneración eficiente en las siguientes especies: *Begonia semperflorens cv. hybrida, Codariocalyx motorious, Dianthus caryophyllus* y *Matthiola incana* planteado en este estudio podría ser utilizado para los métodos de transformación genética.

V. CAPÍTULO II: Expresión del operón *lux* en cloroplastos de vegetales superiores

A. Introducción

La bioluminiscencia es la emisión de luz visible a partir de organismos vivos a través de la catálisis enzimática. Aunque existen grandes diferencias entre las reacciones en los diferentes organismos capaces de producirla, todas las enzimas reciben el nombre de luciferasas y los sustratos de luciferinas. La principal característica común es la necesidad de oxígeno para la reacción bioluminiscente (Meighen 1991).

Tres géneros bacterianos poseen casi todas las bacterias luminosas: *Photobacterium, Vibrio* y *Xenorhabdus* (Campbell 1989). *Vibrio fischeri* es una bacteria Gram negativa bioluminiscente que vive tanto como un simbionte de Euprymna scolopes, un calamar marino, y como organismo de vida libre (Ruby y McFall-Ngai 1992).

Photorhabdus luminescens, sin embargo, es una bacteria terrestre móvil, gram negativas, perteneciente a la familia (Forst y Nealson 1996). Las principales diferencias entre ambas bacterias son la temperatura óptima de las luciferasas, siendo 30 º C para *V. fischeri* y 37 ° C para *P. luminescens* y la presencia de *luxG* en *V.fischeri*.

En las bacterias, la reacción bioluminiscente implica la reducción de fosfato de riboflavina ($FMNH_2$) y la oxidación de un aldehído graso de cadena larga de acuerdo con la siguiente reacción, con la emisión de luz verde-azulada en λmax = 490-505 nm (Lin et al., 1993).

Los genes que codifican esta vía metabólica se encuentran en el operón *luxCDABE*. Tres de estos genes, *luxC, luxD, y luxE*, codifican para proteínas implicadas en la síntesis del aldehído, mientras que *luxA* y *luxB*, respectivamente, codifican las subunidades α y β de la luciferasa (Meighen 1988). La luciferasa bacteriana es una flavina monooxigenasa, una enzima heterodimérica de 77KDa compuestas por una α-y β-subunidades con masas moleculares de 40 y 37 kDa, respectivamente. Estas subunidades parece haber surgido por duplicación de genes y comparten 32% de identidad de secuencia siendo la subunidad α 31 residuos más larga y poseyendo las propiedades enzimáticas (Baldwin y Ziegler 1992). El substrato de la luciferasa, preferentemente un ácido graso de cadena larga, en presencia de $FMNH_2$ y O_2 se utiliza por la luciferasa para dar luz (Rodríguez y Meighen 1985).

Los genes *luxC, luxD* y *luxE* codifican para enzimas reductasa, transferasa, y sintetasa, respectivamente, y, actúan juntos como un complejo multienzimático ácido graso reductasa de 500 KDa. Estos polipéptidos forman un complejo que consta de un total de 12 polipéptidos (Boylan et al., 1989).

El FMN reducido ($FMNH_2$) es un componente clave de la reacción bioluminiscente y se oxida por el oxígeno molecular en una reacción no enzimática para dar peróxido de hidrógeno y FMN (Inouye 1994). Las secuencias de aminoácidos deducidas de *luxG* son similares a la flavina reductasa, *fre*, encontrada en *E. coli* (Ingelman et al., 1999). Por lo tanto, se ha postulado que el producto del gen *luxG* es una flavin reductasa que proporciona $FMNH_2$ para la reacción de luciferasa (Nijvipakul et al., 2008).

B. Resultados y discusión

El primer objetivo planteado en este capítulo fue expresión en cloroplasto del operón *lux* en la especie modelo *N. tabacum*. Dado que el primer paso para la transformación cloroplástica es la obtención de un vector que contenga el cassette de expresión deseado, el vector pLD*luxCDABE* fue diseñado. El esqueleto, pLD, que contiene los elementos imprescindibles para la transformación en *E.coli* y posterior integración en el cloroplasto fue gentilmente proporcionado por el Dr. Daniell de la *University of Central Florida*.

Para el diseño del cassette de expresión, se usó el vector pXen13 (pSK *luxCDABE*, 8802bp; Xenogen) para aislar el operón *luxCDABE*. Este vector contiene 5 genes responsables de la bioluminiscencia *Photorhabdus luminescens.* Para ello, primeramente se analizó mediante programas bioinformáticos (programa Lasergene; SeqBuilder y MegAlign) para detector lugares de restricción y la presencia de RBS. Debido a mutaciones en RBS pueden reducir la eficiencia de la traducción en el cloroplasto (Hirose y Sigura 2004), el casete de expresión se analizó con el fin de comprobar que comprende al menos un RBS "aguas arriba" de cada uno de los genes. Una vez fue comprobada la presencia de RBS, se analizó el contenido en AT/CG mediante el software BitGene (www.bitgene.com) siendo éste de 37.34%.
El contenido en CG en los cloroplastos suele situarse por debajo del 50%, siendo en *Vicia faba* de 37,3%, ligeramente pero significativamente menor al del ADN nuclear (39,4%). Ésta relación se mantiene en las especies analizadas hasta la fecha (Smith 1977). Sin embargo, es importante mencionar que los transcritos derivados de los genes de diversas fuentes se mantuvieron estables en cloroplasto, incluyendo genes bacterianos con

niveles relativamente altos de adenina y la timina (Reddy et al. 2002). Esto sugiere la compatibilidad de la maquinaria del cloroplasto con los ARNm de diversas fuentes y podría evitar la necesidad de construir genes sintéticos para la expresión en este orgánulo.

Una vez comprobada la utilidad de pXen13, se digirió con SnaBI y NotI y se aisló el fragmento correspondiente al operón *LUXCDABE*, DE 5.000pb. Estos enzimas fueron también utilizados para digerir el esqueleto pLD, que fue defosforilado previamente. Se realizó una ligación y posteriormente una transformación en *E. coli* XL-10. Las colonias resultantes fueron analizadas por una PCR de colonias con los *primers* DV146 and DV258 y la presencia del fragment de 715 pb fue confirmada. El patrón del vector fue también confirmado mediante un mapa de restricción y se comprobó la emisión de bioluminiscencia, es decir de la vía metabólica completa, mediante un ensayo de bioluminiscencia. Se detectó emisión de luz visible tanto a ojo desnudo como mediante el ensayo luminiscente. Dado que la expresión correcta en *E. coli* debería ir en paralelo con la capacidad de los cloroplastos para expresar transcribir y traducir correctamente el operón, se realizó un experimento de bombardeo.

Respecto a la estructura del vector *pLDluxCDABE* es importante destacar que fue diseñado sin promotor inmediatamente "aguas arriba" del operón bacteriano. Esto se hizo debido a que los múltiples intentos de introducir un promotor no tuvieron éxito y no se obtenían *E. coli* en la transformación. Esto podría ser debido a la alta carga de energía este cassette de expresión que podría ser incompatible con la supervivencia celular. De hecho, el rendimiento cuántico de la luciferasa bacteriana es de 60 ATPs por fotón (Koncz et al., 1990) y la sobreexpresión excesiva de este sistema podría ser inviable. Finalmente, se optó por situar al operón *lux* inmediatamente después del gen *addA* bajo el control del promotor *Prrn* (Vera y Sugiura, 1995). Este es un promotor fuerte del tipo σ^{70} cuya actividad no se encuentra elevadamente influenciada por la luz. De cara a asegurar la estabilidad del mRNA, se utilizó uno de los 3'-UTR más utilizados, el 3'-UTR del gen *psbA* (Maliga 2003).

Una vez comprobado, se procedió a realizar un bombardeo con partículas de oro con el vector resultante, pLD*luxCDABE*. El experimento se llevó a cabo con hojas de *N.tabacum* WT en estadio de 5-7 hojas. Éstas se incubaron en medio RMOP, usando espectinomicina como antibiótico de selección. Se incluyó también un control positivo, correspondiente a una hoja no bombardeada en medio RMOP y un negativo correspondiente a una hoja no bombardeada en RMOP-Espectinomicina. Tras tres semanas de cultivo en RMOP-Espectinomicina, dos putativos brotes transplastómicos emergieron. En el control positivo, emergieron múltiples brotes y el control negativo presentó enblanquecimiento, tal y como se esperaba. Éstos brotes transplastómicos fueron escindidos del explante y se pusieron en MS-espectinomicina para permitir su elongación. Una vez alcanzaron el estadio de 5-7 hojas, se realizó otra regeneración desde explante foliar en RMOP-Espectinomicina. A la semana 20 se constató una profusa regeneración.

Debido a que se han reportado casos de mutación espontánea que confieren resistencia a la especitinomicina, es necesario comprobar la correcta integración en el cpDNA del cassette de expresión. Esto podría evitarse si se utiliza la resistencia a kanamicina, para la que ninguna mutación espontánea para se ha encontrado en tabaco (Fromm et al., 1987). La integración fue comprobada mediante los *primers* 3P and 3M.

Una vez comprobada la integración en el cpDNA, se analizó la expresión mediante *Real-Time PCR* de los niveles de expresión del mRNA. Se diseñaron primers contra un fragmento en la región del gen *luxA*. Como *housekeeping* se utilizaron *primers* contra la actina y el 16S. La expresión de mRNA mediante SYBR® Green dye no mostró niveles de expresión significativos respecto al control, *N. tabacum* WT, al analizarlo mediante el método de ΔΔCt. Sin embargo, cuando el producto de la Real-Time PCR se corrió en un gel se pudo ver amplificación específica a la altura esperada para los ejemplares transplastómicos y una amplificación inespecífica a una altura menor para el control negativo. Esto pone de manifiesto la necesidad de optimizar tal estudio, siendo los *primers TaqMan*® una alternativa posible ya que evitan la detección de inespecificidades.

Hay varios estudios que no se correlacionan con la abundancia de transcripción eficiencia de traducción (Dhingra et al., 2004). Esta falta de

correlación entre el aumento de los niveles de transcripción y la eficiencia de traducción parece sugiere que la estabilidad de la proteína es más importante que la abundancia de transcripción (Kuroda y Maliga, 2001).

Para detectar si el mRNA es traducido correctamente, se realizó un western blot utilizando un anticuerpo contra la proteína codificada en el gen *luxA* con los extractos proteicos de *N. tabacum WT* y ambas transplastómicas. Se observó una expresión débil de ésta proteína en ambas *N. tabacum* transplastómicas y ausencia de expresión en *N. tabacum WT*. Esta expresión es baja, si bien la sobreexpresión de proteínas foráneas puede causar la formación de cuerpos de inclusión y agregados insolubles de proteínas mal plegadas y no funcionales (Fernández-San Millan et al, 200; Lafolla et al, 2008) y para nuestro propósito este fenómeno que debe evitarse.

Una vez que se confirmó la integración, la presencia de mRNA y la presencia de proteína *luxA*, se realizó un ensayo IVIS de bioluminiscencia. Una de los principales problemas fue la emisión de clorofila. Como se conoce bien, en las plantas, la energía luz es absorbida por la clorofila, carotenoides y otras moléculas de pigmentos presentes en las membranas de los tilacoides (Falkowski y Raven, 2007). Por esta razón se llevó a cabo un experimento multivariable con IVIS Spectrum, con diferente combinación de filtros de excitación y emisión. Desde reacción de la luciferasa bacteriana tiene una longitud de onda máxima de alrededor de 490 nm y la clorofila necesita, la imagen se adquirió con una incubación de 30 minutos de oscuridad y un filtro de emisión (500 nm). El experimento sugirió la falta de diferencia entre las plantas de tipo salvaje y transplastómica que nos lleva a descartar la emisión de bioluminiscencia, al menos en los niveles sobre la sensibilidad de IVIS. Hay varias razones que pueden producir esta falta de bioluminiscencia. Una de ellas podría ser los niveles de expresión de las enzimas implicadas en la ruta metabólica, pero si los niveles de luciferasa son detectables por western blot esta hipótesis es probablemente incorrecta.

Otra posibilidad podría ser el incorrecto plegamiento de la luciferasa o la influencia del pH del estroma en la estabilidad de las proteínas. Sin embargo, un trabajo publicado en 2010 por Krichevski et al., demostraron la expresión del operón *lux* en las plantas, y por lo tanto el correcto plegado de las enzimas que participan en la reacción tienden a descartar esta hipótesis y

nos lleva a creer que la diferencia es la falta del gen *luxG* en nuestro sistema. Por otra parte, se cree que el pH del citosol de *E. coli* se encuentra en valores alrededor de 7,5 (Rey et al., 2005).

La disponibilidad de oxígeno podría ser otro impedimento. Pero el O_2 es producido en la fotosíntesis en cadena de transporte de electrones fotosintética (PETC) en Tilacoides, en cuyo proceso las moléculas de agua se oxidan en O_2, y éste es liberado.

EL producto del gen luxG es una flavin reductasa implicada en el *turn-over* de FMN a $FMNH_2$. Los niveles de $FMNH_2$ son abundantes en el citoplasma de *E. coli* y en las mitocondrias de las células eucariotas. De hecho, un homólogo de *luxG*, *fre* se observó con *E. coli* (Fieschi et al., 1995). Esto podría ser la razón de nuestras observaciones anteriores de una fuerte bioluminiscencia en *E. coli*. En cloroplasto, bajo nuestro conocimiento, no existen niveles significativos de $FMNH_2$. Estos supuestos bajos niveles endógenos podrían no ser suficientes para la detección *in situ* de la actividad luciferasa.
De hecho, la disponibilidad de $FMNH_2$ fue identificado como el sustrato limitante para la bioluminiscencia en la célula de mamífero transformada con *luxCDABEG* ya que la suplementación con $FMNH_2$ dió lugar a un aumento de 151 veces en la bioluminiscencia (Close et al., 2010).

Por lo tanto, para confirmar esta hipótesis deberían plantearse una serie de experimentos. El $FMNH_2$, reducido químicamente, no podría ser añadido a medios de cultivo para ser proporcionado exógenamente debido a que las membranas de los cloroplastos son impermeables al FMN (Allen, J. 1978). Entonces, los experimentos *in vitro* para añadir $FMNH_2$ deben implicar el aislamiento y la posterior lisis de cloroplasto para permitir el contacto entre el $FMNH_2$ y el resto de las enzimas de la ruta metabólica.

Otro factor que podría estar implicado podría ser la temperatura. Si es cierto que *Photorhabdus luminescens* es termoestable en células de mamífero (Westerlund-Karlsson et al., 2002), este no es el intervalo de temperatura normal para las células vegetales. En contraste, una expresión robusta de *luxCDABE* clonado a partir de *P. luminescens* se observa para un amplio rango de temperaturas de 10 a 40 ° C (Song et al., 2009). Esto sugiere que la temperatura no debería ser un problema.

Estos resultados nos hicieron considerar el diseño de un nuevo vector de transformación de cloroplastos que incluye *luxG*. La fuente seleccionada fue *V. fischeri* para evitar posibles problemas de estabilidad por temperatura ya que la luciferasa de *V. fischeri* tiene una temperatura óptima de 26 º C (Scheerer et al., 2006).

La aclimatación de *N.tabacum* se optimizó con el uso de magentas que poseían filtros que permiten el intercambio de gases. Este incremento sucesivo de GER (Gas Exchange Rate), permite la reducción de la humedad ambiental evitando la contaminación de las plántulas. Con esta estrategia se logra disminuir la evotranspiración, ya que la disfunción de los estomas es proporcional a ésta y podría ocasionar la desecación en condiciones *ex vitro* (Donelly y Tisdall 1993). Esta pre-aclimatación de las plántulas permite realizar un primer proceso de endurecimiento que permite la aclimatación con una mayor tasa de éxito. De hecho, los cuatro especímenes aclimatadas sobrevivieron y produjeron con éxito plantas vigorosas que produjeron semillas. Estas semillas germinaron con éxito en MS-Espectinomicina, lo que demuestra la presencia correcta del casete de expresión que confiere resistencia a la espectinomicina.

Por los motivos mencionados anteriormente, se planteó un segundo enfoque, el diseño del vector pLD*luxCDABEG*. La diferencia respecto al anterior reside en la fuente del operón bacteriano, *Vibrio fischeri*, y la presencia del gen *luxG*. Del mismo modo que anteriormente se analizó la secuencia mediante programas bioinformáticos. Dado la falta de lugares de restricción apropiados y de RBS de uso preferencial en cloroplastos, se clonaron los genes con primers que contenían la secuencia de restricción y RBSs a partir del genoma de *V. fischeri* para construir el vector pLD*luxCDABEG*. Este vector fue comprobado mediante ensayos de bioluminiscencia y mapas de restricción para garantizar el correcto patrón. Estevector está actualmente disponible para su uso.

Dada su facilidad de manipulación genética y la productividad en la producción de semillas (Arlen et al., 2007), la especie *N. tabacum* ha sido ampliamente utilizada para la manipulación genética. Otra característica común a casi todas las angiospermas es de la herencia paterna del cpDNA

como modo dominante (Birky, 1995). Esta característica se opone en gimnospermas, donde la herencia es principalmente de paterna (Stine et al., 1989). Ambos angiospermas y gimnospermas parecen tener herencia uniparental principalmente (Clément y Pacini, 2001). Por otra parte, estudios recientes han informado de que el escape de transgenes en el tabaco es 0,0087% a 0,00024% (Svab y Maliga, 2007), haciendo de este un sistema ideal para los el uso de los cloroplastos como objetivo a las manipulaciones genéticas. Si hablamos de la utilización de N.tabacum como un biorreactor, se ha estimado como 50 veces menos costoso que los sistemas de fermentación de *E. coli* (Kusnadi et al., 1997). Sin embargo, existen algunas desventajas. *N.tabacum* no se utiliza como una planta ornamental y, para nuestro propósito, las características de esta especie no son óptimas. Además, la presencia de alcaloides podría ser un problema para un uso seguro en la jardinería (Arlen et al., 2007).

Por ello, una vez establecidos protocolos efectivos de regeneración en *B. semperflorens, M. incana, C. motorious* y *D. caryophyllus*, se procedió a analizar la factibilidad de ser transformada con el vector *pLDluxCDABE*. Para ello, se realizó un BLAST (Basic Local Alignment Search Tool) entre ellas regions flanqueantes del cassette de expression presente en el esqueleto pLD y los genes *trnI* y *trnA* del cpDNA. Dado que el cpDNA solo se encuentra secuenciado en algunas especies, se seleccionaron dos representantes de cada orden. La homología presente entre las regions *trnI*/*trnA* resutó mayor del 94% para los órdenes *Caryophillales*, *Malpighiales* y *Brassicales*. Esta homología resultó menor en el orden *Fabales*. Esto se hizo debido a que varios autores informaron que la falta de homología entre las regiones flanqueantes del vector y el cpDNA afecta en gran medida a la eficiencia de transformación (Sidorov et al, 1999;.. Ruf et al, 2001). Entonces, aunque la hipótesis de un vector genérico que contenga los genes *trnI/trnA* de la región IR del cpDNA de *N. tabacum* para cualquier especie se propuso hace varios años (Daniell et al., 1998), se observó una menor eficiencia con 98 % de homología en *Petunia sp*. cuando se utilizó el vector de *N. tabacum* para transformarla (DeGray et al., 2001).

Hasta donde sabemos, ningún intento de transformar *B. semperflorens, D. caryophyllus* o *M. incana* mediante Biolística se ha realizado hasta la fecha. El bombardeo *de B. semperflorens, D. caryophyllus* y *M. incana* no resultó en

ninguna línea transplastómica. A pesar de que nuestro análisis muestra un alto grado de homología entre esta región espaciadora intergénica, se deben tener en cuenta las revisiones anteriores con los miembros de *Solanáceas*. Por ejemplo, sólo se obtuvo una línea transplastómica para *Solanum tuberosum* por 35 placas bombardeadas en comparación con las 15 líneas transgénicas de tabaco que suelen generarse en por placa bombardeada (Fernández-San Millán et al., 2003). Esta baja eficiencia usando vectores no especie-específicos podría ser evitada mediante el diseño de un vector específico o el aumento del número de bombardeos para aumentar la posibilidad de obtener una línea transplastómica.

C. Conclusiones

Las conclusiones de este capítulo, cuyo objetivo era la obtención de de plantas con un fenotipo bioluminiscente, son: la expresión de pLD*luxCDABE* en el cloroplasto de *N.tabacum* es factible y fue conseguido y comprobado, sin embargo el fenotipo bioluminiscente conseguido en *E. coli* transformada con pLD*luxCDABE* no se logra en *N.tabacum*-pLD*luxCDABE*. El vector pLD*luxCDABEG* está listo para introducido en *N.tabacum* y podría superar la limitación anterior. Se necesitarán varios intentos de introducir pLD*luxCDABE* o pLD*luxCDABEG* en cloroplastos de plantas ornamentales por la subóptima homología entre cpDNA de *M. incana*, *D. caryophyllus* y *B. semperflorens* y las regiones flanqueantes del vector pLD.

VI. Discusión

Los resultados del presente trabajo nos llevan a pensar en la necesidad de crear un cassette de expresión que proporcione todos los componentes, $FMNH_2$ incluido, para garantizar la correcta expresión de la vía metabólica bacteriana causante de la bioluminiscencia en cloroplastos. Esto ya se ha logrado y deberá introducirse en los cloroplastos de las plantas ornamentales.

A pesar de que se han obtenido buenas tasas de organogénesis en *B. semperflorens, C. motorious, D. caryophyllus* y *M. incana*, una mejora de las tasas y el aumento de las especies sería un interesante método para incrementar las posibilidades de éxito. También el diseño de vectores especie-específicos con las regiones flanqueantes apropiadas debe ser óptimo. Otra mejora podría ser el diseño de un vector de expresión de luz

regulable con el fin de evitar el alto costo metabólico más alta que deben soportar las plantas. Por esta razón, se plantea el uso del elemento regulador nuclear de 12 pb (GGATTTTACAGT) capaz de conferir la inducción por oscuridad, obtenido por cortesía del Dr. Inaba (Inaba et al., 2000). Este podría ser usado para regular la transcripción de las subunidades de luciferasa en el núcleo, implicando una doble transformación, cloroplástica y nuclear. Estas subunidades requerirían de péptidos de tránsito para garantizar la disponibilidad en el cloroplasto (Shashi, B. et al., 2006).

Otra opción podría ser la expresión del operón *lux* en las mitocondrias. En la actualidad, la transformación de las mitocondrias aún no está disponible en plantas superiores, pero algunos esfuerzos se han hecho con éxito en la levadura *Saccharomyces cerevisiae* y la alga verde *Chlamydomonas reinhardtii* utilizando un sistema de biolística (Fox et al., 1988, Johnston et al., 1988 y Boynton y Gillham, 1996). El genoma mitocondrial se encuentra con el fin de generar la cepa deficiente en respiración carece de parte de la región del ADN mitocondrial (mtDNA). Esta deficiencia se corregiría mediante la recombinación con un vector que contendría el gen defectuoso, además del GOI, permitiendo la selección de transformantes. De todos modos, esta técnica no es rutinaria hasta la fecha y se precisaría de un gran estudio previo con el fin de iniciar este enfoque. En este caso, sin embargo, la presencia de $FMNH_2$ no debería ser un factor limitante.
Por otro lado, la bioluminiscencia proporcionada por operón *lux*, aunque visible para el ojo humano, tiene una intensidad baja. Se han propuesto dos soluciones posibles para aumentarla. Uno de ellos es el uso de genes bioluminiscentes de la luciérnaga *Photinus pyralis* ya que la intensidad proporcionada por la luciferasa eucariota es mucho más alta que la bacteriana. Las comparaciones de los parámetros cinéticos y los rendimientos cuánticos favorece a la luciferasa de luciérnaga, que, en contraste con los 60 ATP requeridos para un fotón emitido por la luciferasa bacteriana, necesita sólo uno (Wood, 1995). Sin embargo, este sistema tiene una desventaja importante para nuestros propósitos. La vía metabólica de *P. pyralis* es, hasta la fecha, desconocida parcialmente ya que no se conoce el mecanismo productor del substrato, la luciferina. Entonces, aunque el gen *LRE* ha sido clonado y podría ser introducido para regenerar la D-luciferina a partir de oxiluciferina (Gomi y Kajiyama, 2001; Día, J. y Bailey, M. 2003), se sabe de la incorporación extracelular deficiente para la luciferina y para

mejorar tal absorción las células deben ser tratadas v por métodos que reducen su viabilidad, tales como DMSO, valores bajos de pH o altas concentraciones de luciferina (Koncz, C, 1990).
Teniendo en cuenta estos resultados en conjunto, podríamos decir que existe un largo camino por explorar para lograr plantas con fenotipo bioluminiscentes que emitan una luz suficiente para que sean interesantes para propósitos arquitectónicos.

VII. Conclusiones

Las conclusiones finales del presente estudio pueden resumirse en las siguientes:

- Se presenta un protocolo eficiente para la organogénesis de las hojas maduras, en un solo paso, de cuatro plantas con valor ornamental: *Begonia semperflorens cv. hybrida, Codariocalyx motorious, Dianthus caryophyllus* y *Matthiola incana.*

- Es posible expresar el operón *luxCDABE* en cloroplastos de N.tabacum.

- El fenotipo bioluminiscente para *N.tabacum* debe estudiarse más a fondo para aumentar la intensidad de la luz emitida.

Nos sobran los motivos...

J. Sabina

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Así que, a ti que lo lees, GRACIAS.

Quan surts per fer el viatge cap a Ítaca,

has de pregar que el camí sigui llarg,

ple d'aventures, ple de coneixences.

Has de pregar que el camí sigui llarg,

que siguin moltes les matinades

que entraràs en un port que els teus ulls ignoraven,

i vagis a ciutats per aprendre dels que saben.

Tingues sempre al cor la idea d'Ítaca.

Has d'arribar-hi, és el teu destí,

però no forcis gens la travessia.

És preferible que duri molts anys,

que siguis vell quan fondegis l'illa,

ric de tot el que hauràs guanyat fent el camí,

sense esperar que et doni més riqueses.

Ítaca t'ha donat el bell viatge,

sense ella no hauries sortit.

I si la trobes pobra, no és que Ítaca

t'hagi enganyat. Savi, com bé t'has fet,

sabràs el que volen dir les Ítaques.

Ha sido un gran camino… GRACIAS