DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Approaches to understand the role of *CLE* genes in xylem development using trans-activation

Patrícia Filomena Alves Caldeirinha

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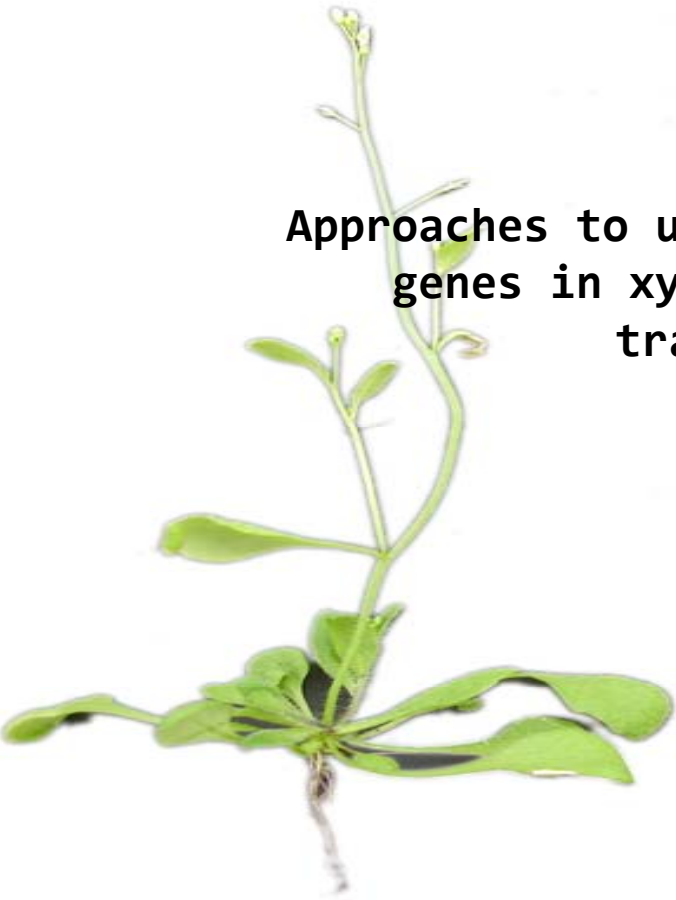# Approaches to understand the role of *CLE* genes in xylem development using trans-activation

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To my father, you'll always be my guiding line

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Abstract

Molecular and genetic studies of the vascular cambium activity and secondary growth in *Arabidopsis* are quite relevant due to the economic and ecological importance of wood.

Despite the fact that *Arabidopsis* is not an economical woody species, it has been used for molecular analysis of wood production because it is possible to stimulate secondary growth by repeatedly removing the inflorescences, hence stimulating vegetative growth. The use of molecular approaches, such as trans-activation, gives the possibility to study genes responsible for wood formation and development. In the future, this will become tools necessary to obtain plants producing more wood in a shorter period of time, therefore occupying a reduced area of land with advantages for the ecosystem and economy.

One other advantage of *Arabidopsis* is its small size, rapid generation time, and high number of seeds produced per plant. However, results of these studies can be extrapolated into woody plants given the high similarities of anatomical and genomic level regarding to wood formation in *Arabidopsis* and *Populus* or pine. These studies have significant applications in economically important tree species, and may have an impact in the economy of the rural areas namely in Portugal.

The capacity to introduce and express, or inactivate specific genes in plants by transformation methods, provides a powerful experimental tool, allowing direct testing of some hypotheses in plant physiology that have been exceedingly difficult to resolve using other biochemical approaches.

This work relies on plant transformation, *Agrobacterium*-mediated using binary plasmids containing the construction *10Op::gene of interest* into *Arabidopsis* plants, by floral dip method, where the genes of interest belong to the *CLE* and *DVL* gene families. Overall, these genes have important roles in plant development. We have selected transformants, established homozygous lines and as a future perspective, the crosses of these lines with the two activator lines *ATHB8* and *ATHB15*, specific to vascular cambium in stems and leaves, are going to be performed. F1 progeny will be screened and analysis of the action of several *CLE* genes of the *CLV3/ENDOSPERM SURROUNDING REGION* (CLE) family will be made.

Key Words: *Arabidopsis thaliana*, *CLE* gene family, trans-activation, xylem production.

Resumo

Em *Arabidopsis*, estudos genéticos e moleculares sobre o desenvolvimento do câmbio vascular e formação de madeira são muito relevantes devido à importância económica e ecológica desta espécie

Apesar de não ser uma planta lenhosa, *Arabidopsis* é utilizada em análises moleculares para a produção de lenho sendo possível estimular o crescimento secundário do caule removendo repetidamente as inflorescências, promovendo assim o crescimento secundário vegetativo. O uso destes estudos moleculares, como por exemplo a trans-activação, dá-nos a possibilidade de estudar genes envolvidos na formação de xilema secundário. Futuramente, estas serão ferramentas essenciais para a obtenção de plantas que produzem maior quantidade de madeira num menor período de tempo, ocupando uma área reduzida, oferecendo assim vantagens a nível do ecossistema bem como a nível económico.

Esta planta apresenta outras vantagens tais como o seu reduzido tamanho, curto tempo de regeneração e elevado número de sementes produzidas por planta. No entanto, os resultados destes estudos podem ser extrapolados para outras plantas lenhosas, dado a elevada similaridade a nível anatómico e genómico no que diz respeito à formação da madeira em *Arabidopsis*, no choupo (*Populus*) ou no pinheiro (*Pinus*). Estes estudos têm aplicações importantes em espécies de árvores economicamente importantes o que pode ter impacto na economia de áreas rurais, nomeadamente em Portugal.

A capacidade de introduzir e expressar, ou inactivar genes específicos em plantas através de métodos de transformação fornece-nos uma ferramenta poderosa, permitindo testar directamente hipóteses em desenvolvimento vegetal, o que tem sido difícil de resolver com outras abordagens bioquímicas.

Este trabalho baseia-se na transformação de *Arabidopsis* usando a via *Agrobacterium* contendo as construções *10Op::gene de interesse*, pelo método *floral dip*, em que os genes de interesse pertencem às famílias de genes *CLE* e *DVL*. De modo geral, estes genes apresentam funções importantes no desenvolvimento vegetal. Seleccionámos transformantes, estabilizámos linhas homozigóticas e, como perspectiva futura, pretende-se cruzar estas linhas com duas linhas activadoras *ATHB8* e *ATHB15*, que são específicas do câmbio vascular em caules e folhas. A geração F1 será rastreada e analisada a acção dos vários genes *CLE* pertencentes à família *CLV3/ENDOSPERM SURROUNDING REGION* (CLE).

Palavras-chave: *Arabidopsis thaliana*, família de genes *CLE*, produção de xilema, trans-activação.

CHAPTER I
INTRODUCTION

1.1 - *Arabidopsis thaliana*

Arabidopsis thaliana (L.) Heynh. is an annual herb belonging to the Brassicaceae family, also known as Cruciferae, mustard or cabbage family. It is native of the northern temperate regions in Europe, Asia, and northwestern Africa, and reaches maximal diversity around the Mediterranean area (Fig. 1.1). This worldwide distribution is probably due to its high capacity to colonize large geographic places that might be related to its life cycle, especially with the time for seed germination and the beginning of flowering time. *A. thaliana* occurs in open or disturbed habitats, sandy soil, river banks, roadsides, waste places, cultivated ground, slightly alkaline flats, under shrubs and open areas from the sea level up to 4250 m.

Figure 1.1 Worldwide distribution of *Arabidopsis thaliana*.
(Adapted from http://en.wikipedia.org/wiki/File:Arabidopsis_thaliana_distribution.png)

The plant can reach 15 to 20 cm when mature. The basal leaves are green to purplish, shortly petiolate and rosulate (Fig. 1.2, b) and covered with trichomes (Fig. 1.2, c) forming a characteristic rosette where an erect floral stem emerge (Fig. 1.2, a). The flowers are usually 3 mm, white, and arranged in a corymb (Fig. 1.2, d). The fruits are siliques, linear, the valves have a distinct midvein and each one may contain 20 to 30 seeds (Fig. 1.2, e). Seeds are ellipsoid, plump and light to reddish brown (Fig. 1.2, e). Flowering and fruiting occurs from January to June (or it can go until October). (Al-Shehbaz, and O'Kane, 2002)
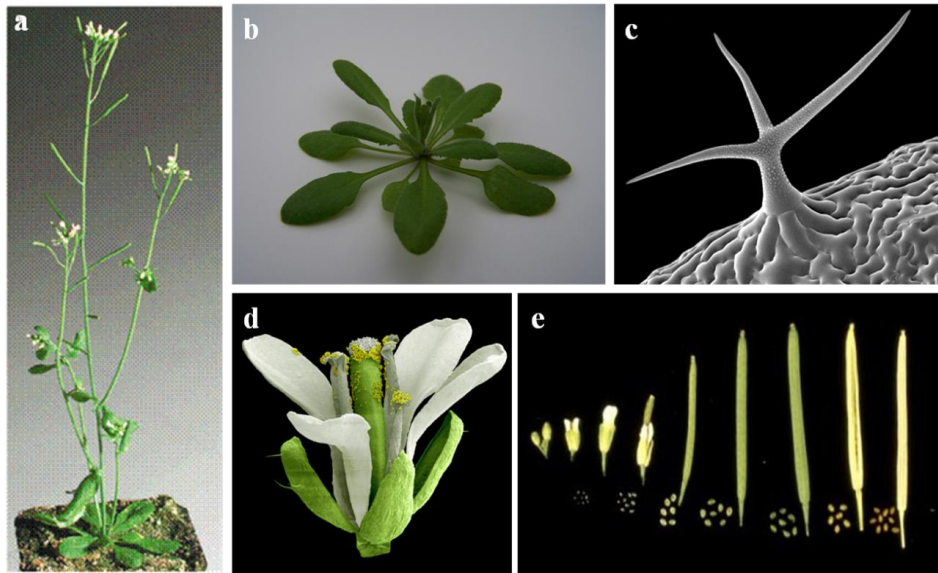
Figure 1.2 Several aspects of *Arabidopsis thaliana*:

a) almost mature plant showing the rosette leaves and the floral stems (Adapted from Page,

D. and Grossniklaus, U., 2002);

b) rosette (Adapted from http://wistep.wisc.edu/researchstudents.html);

c) trichomes (Adapted from http://www.planttrichome.org/trichomedb/species.jsp);

d) flower (Adapted from http://ec.europa.eu/research/quality-of-life/arabidopsis.html);

e) siliques and seeds (Adapted from http://www.seedgenes.org/Tutorial.html).

A. thaliana has a well known life cycle that can be divided in 5 phases: seed germination; vegetative growth (rosette stage); stem growth (inflorescence stage); flowering and seed formation (Fig. 1.3 a-e). This cycle (from germination till seed maturation) takes about 6 weeks under controlled conditions. However, in nature the cycle is annual and only one generation occurs *per* year
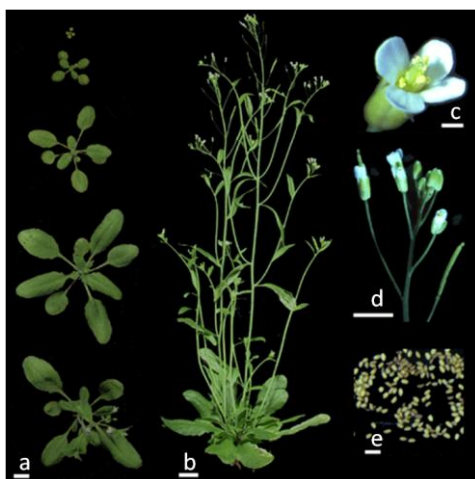
Figure 1.3 Complete life cycle of *Arabidopsis*:
a) Vegetative stage, before flowering and growth of the floral stalk (bottom left); **b)** Adult plant at full flowering/seed set; **c)** Flower; **d)** Floral stem; **e)** Mature seeds.
(Adapted from
http://www-ijpb.versailles.inra.fr/en/arabido/arabido.htm)

1.1.1- *Arabidopsis thaliana* Ecotypes.

In laboratory studies, the most commonly used ecotypes of *Arabidopsis* are the Landsberg *erecta* (*Ler*), and Columbia (Col). Other background lines less-often cited in the scientific literature are Wassilewskija (Ws), C24, Cape Verde Islands (Cvi), Nossen, among others. (Delatorre and Silva, 2008; Meinke et al., 1998; NASC, The European Arabidopsis Stock Centre).

Landsberg ecotype (Ler), named by Will Feenstra, is commonly used as wild type because it is shortest and it has a more erect stem, which makes it more manageable in the laboratory. Originally, it was George Redei that received a stock of seed from Laibach with the name Landsberg from which he performed some X-ray mutagenesis experiments. It was from this irradiated population that Landsberg *erecta* was achieved. Columbia ecotype was also selected by Rédei within the same original population as Ler, but from the seeds that weren't irradiated. Columbia is the ecotype used for sequenced in the Arabidopsis Genome Initiative (NASC, The European Arabidopsis Stock Centre).

1.1.2- *Arabidopsis thaliana* as a Model Plant

A. thaliana shows several characteristics that lead the scientific community to consider it as a model system for research in plant biology:
Due to its small size, it is possible to growth a large amount of plants in a reduced space. Thus, one single plant can be grown to maturity in 1 cm^2 in a growth chamber (Valvekens *et al.*, 1988).
Under controlled conditions of light, temperature, humidity, soil and nutrients, the plant shows a considerable fast growth - six weeks to complete its entire life cycle. The plant reproduces by self-fertilization but cross fertilization can be easily accomplished. Each plant can produce a large number of seeds, about 6000 *per* plant, in only one generation. Moreover, Arabidopsis was the first plant to have its genome entirely sequenced in 2000 by the "Arabidopsis Genome Initiative" (AGI), just after the complete genome sequence of *Caenorhabditis elegans* (1998) and *Drosophila melanogaster* (2000) (Meinke et al., 1998).

The nuclear genome of this plant is small comprising 114.5 Mb/125 Mb total and possessing around 25000 genes distributed by 5 chromosomes (n=5) (Meinke et al., 1998) (Fig. 1.4). Methods for Arabidopsis genetic transformation have been developed and the plant can be transformed efficiently using *Agrobacterium tumefaciens* making it a suitable organism for transformation and therefore, for developmental studies (Clough and Bent, 1998).

Due to its amiability for transformation, a large collection of mutant lines generated by x-ray irradiation, chemical mutagenesis, and insertional mutagenesis with T-DNA and transposons that can be use for developmental studies is available. In order to identify functions of genes involved in the development of the plant, researchers use either forward and/or reverse genetics approaches for gene function studies.
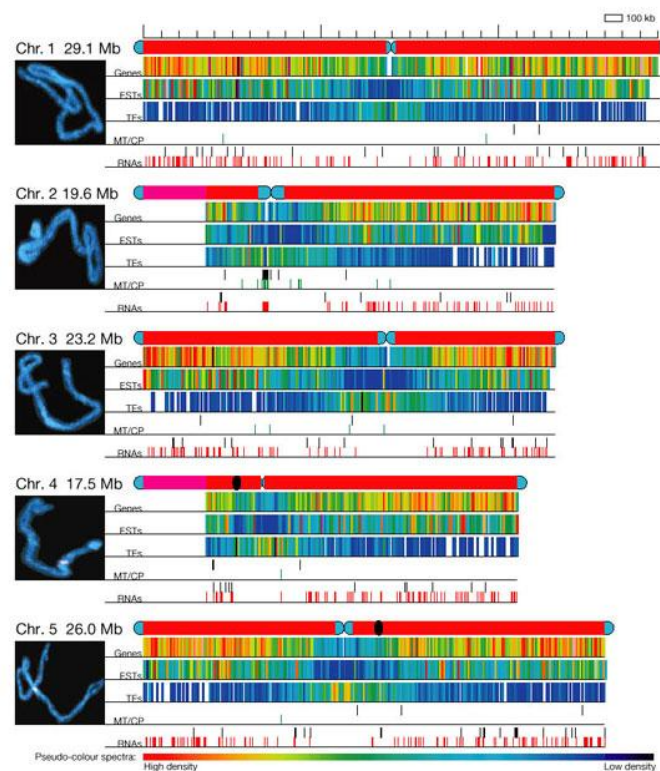
Figure 1.4 Representation of the five *Arabidopsis* chromosomes. (Adapted from 'The Arabidopsis Genome Initiative', 2000, Nature, 408).

Why we study genes involved in the vascular cambium in *Arabidopsis* and not in a tree that produces more wood and especially has more economical importance?

Several investigators proposed the genus *Populus*, in particular the species *P. trichocarpa* as a model to understand tree development. *Populus* has a larger genome,

485 million base pairs in 19 chromosomes; was the first tree to be completely sequenced in 2006 by the Joint Genome Institute, USA; has genuine commercial value as a tree for timber, plywood, pulp and paper, however, reaches reproductive maturity at 4-6 years; it's dioecious, so the selfing and back-cross manipulations are impossible; long time-to-flower is a limitation, among others (Baucher et al., 2007; Nieminem et al., 2004; Risopatron *et al.*, 2010; Taylor, 2002). The results from comparative research about the conservation of genes involved in secondary xylem development between woody and herbaceous seed plant revealed that genetics mechanisms behind processes of xylem development may potentially be highly conserved between them (Nieminem et al., 2004; Risopatron *et al.*, 2010). So, performing this type of studies in *Arabidopsis* seems logical and perfectly supported by many papers and investigators (Chaffey *et al.*, 2002; Delatorre and Silva, 2008; Meinke *et al.*, 1998; Nieminem *et al.*, 2004; Taylor, 2002).

Although *Arabidopsis* is not a plant with an agronomic or economical importance, it offers us advantages for basic research in genetics and molecular biology, especially in comparison to trees. As trees are adapted to grow for a long period of time, the study of important features characteristic of tree, such as wood development, is almost impossible. In this regard, *Arabidopsis* presents a huge advantage because it is possible to induce secondary growth (Chaffey *et al.*, 2002; Meinke *et al.*, 1998; Nieminem *et al.*, 2004; Risopatron *et al.*, 2010; Taylor, 2002) and follow the mutants during a very short period of time (several weeks). There are studies showing that *Arabidopsis* possess many xylem-forming genes that are also present in wood-forming tissues of pine (Taylor, 2002). Recent studies also show that gene expression in the cambial zone of *Arabidopsis* and *Populus* reveal more genes involved in secondary growth.

1.2 - Meristems

Almost all of the tissues in a mature plant are derived from specialized groups of cells that never stop to grow, called meristems and that are present at the tips of roots and shoots (Fig. 1.5) (Barton and Poething, 1993). There are four types of meristems: **apical**, in the apex of stem and root; **axilary**, in the leaf axils, forming the axilary buds;

lateral or **secondary**, in the adult regions of the root and stem (vascular cambium and phellogen) and the **intercalary** meristems, between the adult tissues, in the internodes region. Here we will focus on a short introduction to shoot apical meristem (SAM) and on the secondary meristem.
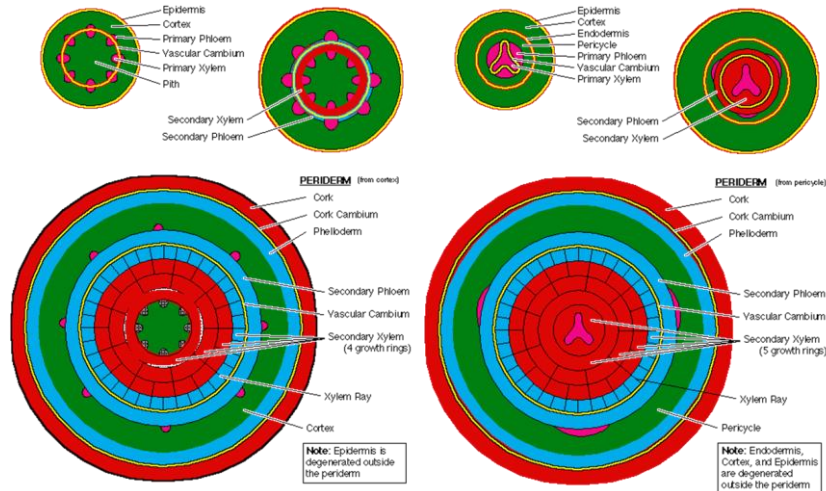
Figure 1.5 Cross section of a woody dicotyledonous secondary plant body. Stem anatomy on the left and root anatomy on the right.

(Adapted from http://plantphys.info/plant_biology/secondary.shtml)

The shoot apical meristem (SAM) is responsible for producing the above ground portions of the plant; stems, leaves and flowers (Fig. 1.6, a, b). In angiosperm, the SAM is organized into two regions - the tunica (L1 and L2) and the corpus (L3). Cell divisions in the tunica are exclusively anticlinal, allowing the maintenance of the layer arrangement in the tunica. On the other hand, the cells in the corpus may divide anticlinally, periclinally or obliquely resulting in a jumbled arrangement of cells (Fig. 1.6, c) (Barton and Poething, 1993; Fletcher, 2002; Leyser and Furner, 1992).
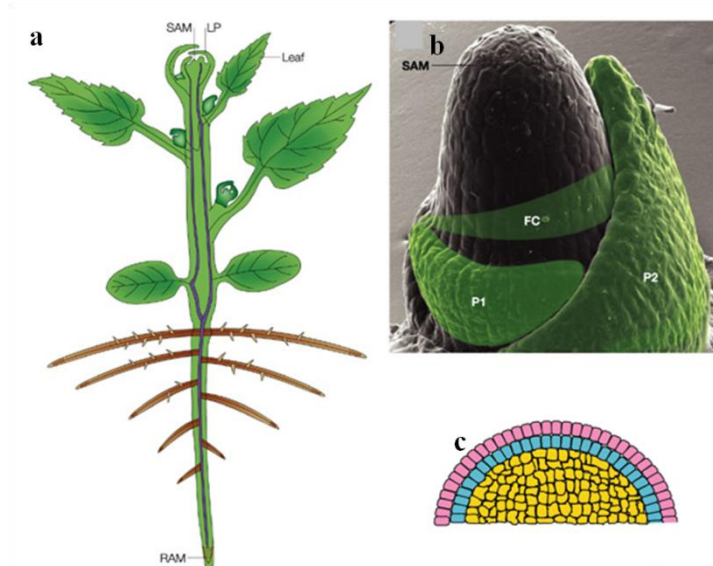
Figure 1.6 a) diagram of a plant showing the shoot apical region where the SAM is located at the growing tip above-ground and the root apical region, containing the root apical meristem (RAM) at the growing tip below-ground. LP indicates a leaf primordium; **b)** scanning electron image of a young maize apex showing the SAM with the two youngest leaf primordial (P1 and P2, in green); FC indicates the founder-cell population that will give rise to the next leaf primordium, also in green. (Adapted from Tsiantis and Hay, 2003); c) tunica corpus model for SAM structure: L1 in pink; L2 in light blue and L3 in orange. (Adapted from Tax and Durbak 2006)

Zonation patterns are uniform among angiosperm but apparently only when the meristem is active and are based on cell division: the central zone that includes cells from L1, L2 and L3 (Fig. 1.7); the peripheral zone, arising from cells of the central zone and extends around the meristem in a doughnut or inner-tube shape (Fig. 1.7) has for its main function the formation of lateral organs (mainly leaf primordia) and the rib zone arranged in longitudinal files and contributing to tissues in the central portion of the stem (Fig. 1.7) also derived from the central zone.(Fletcher, 2002; Medford, 1992).
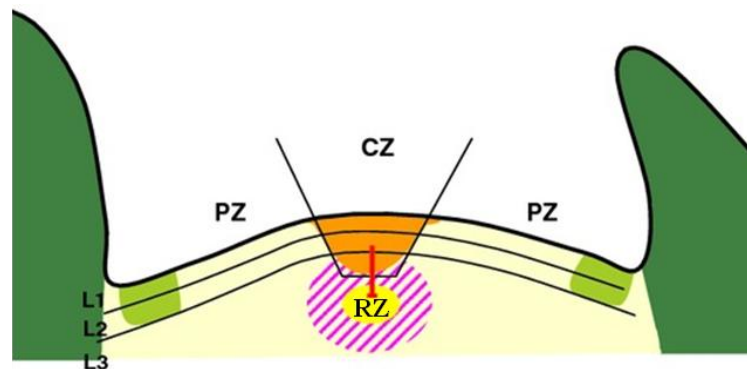
Figure 1.7 Schematic representation of the shoot apical meristem. CZ indicates the central zone that harbors the stem cells; PZ indicates the peripheral zone where organ primordia are initiated and RZ the rib zone that acts like the organizing center of the SAM. (Adapted from Wang and Fiers, 2010)

The number of cells in SAM is maintained by a balance between cell division and cell differentiation promoted by genes that are expressed in the apical meristem during plant development. In *Arabidopsis* the balance is controlled by a feedback regulation loop where CLV complex (*CLV1*, *CLV2* and *CLV3*) is required to regulate the size of the stem cell population in the SAM by controlling the rate of cell division

by repressing *WUS* expression, a homeobox transcription factor expressed in the L3 of the central zone in the SAM and promotes stem cell identity (Fig. 1.8) (Fiers *et al.*, 2005; Fiers *et al.*, 2007; Fletcher, 2002; Risopatron *et al.*, 2010; Wang and Fiers, 2010).

CLV1 and CLV2 form a heterodimer that acts as the receptor for CLV3 ligand. Mutations on *CLV* genes lead to ectopic accumulation of stem cells in the shoot and floral meristem resulting in an enlarged vegetative and inflorescence meristem (Diévart *et al.*, 2003; Fiers *et al.*, 2007; Fletcher, 2002; Ni and Clark, 2006).

CLAVATA1 (*CVL1*) encodes a membrane-bound leucine-rich repeat receptor-like kinase (LRR-RLK) which has an essential role in plant development expressed in L3 layer of the SAM (Fig. 1.8), just beneath the *CLV3* expression domain (Fiers, 2007; Fiers *et al.*, 2005; Fiers *et al.*, 2007; Fletcher, 2002; Jun *et al.*, 2008; Kinoshita *et al.*, 2007; Ni and Clark, 2006; Sharma *et al.*, 2003).

CLAVATA2 (*CLV2*) encodes a leucine-rich repeat receptor-like protein (LRR-RLP) similar to CLV1, but lacks the kinase domain which expression is also detected in shoots and flowers. It has been suggested that CLV2 stabilizes CLV1 (Fiers *et al.*, 2005, 2007; Fletcher, 2002; Jun *et al.*, 2008; Kinoshita *et al.*, 2007; Ni and Clark, 2006).

CLAVATA3 (*CLV3*) belongs to the plant-specific *CLE* (*CLV3/ESR*) gene family playing an important key role in communication stem cell fate information during *Arabidopsis* development. *CLV3* encodes a putative extracellular protein mainly expressed in L1 and L2 layers the SAM (Fig. 1.8). CLV3 peptides are secreted in the stem cells and then activate the CLV1 receptor kinase complex, which suppresses the expression of WUSCHELL (*WUS)* though a signal cascade. Loss-of-function mutations in *CLV3* cause excess stem cell accumulation in shoot apical and floral meristem (Fiers *et al.*, 2005, 2007; Fletcher, 2002; Hobe *et al.*, 2003; Jun *et al.*, 2008; Kinoshita *et al.*, 2007; Ni and Clark, 2006; Sharma *et al.*, 2003; Strabala *et al.*, 2006).
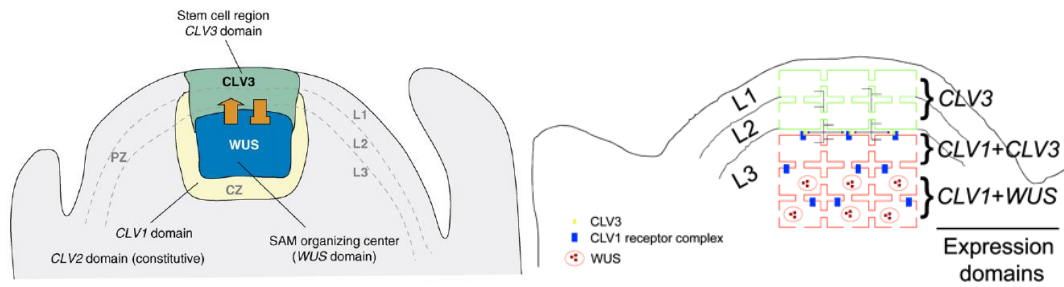
Figure 1.8 Expression domains of *WUS*, *CLV1*, *CLV2* and *CLV3*, SAM-related genes. (Adapted from Fiers *et al.*, 2007 and Rojo *et al*. 2002)

The secondary growth is promoted by the active division occurring in the vascular cambium resulting in the production of the vascular cells; the xylem on the inside of the meristem and the phloem on the outside. Although cell division activity in the cambium is most pronounced in perennial tree species, it may also occur in herbaceous plants such as *Arabidopsis* when stimulated (Caña-Delgado *et al.*, 2010). Under appropriate conditions, *Arabidopsis* undergoes secondary growth in the hypocotyl with the development of both a vascular and a cork cambium.

During the formation of fibers and vessel elements the secondary xylem of *Arabidopsis* closely resembles the anatomy of the wood of an angiosperm tree, and can be used to address basic questions about wood formation (Fig. 1.9) (Chaffey *et al.*, 2002).
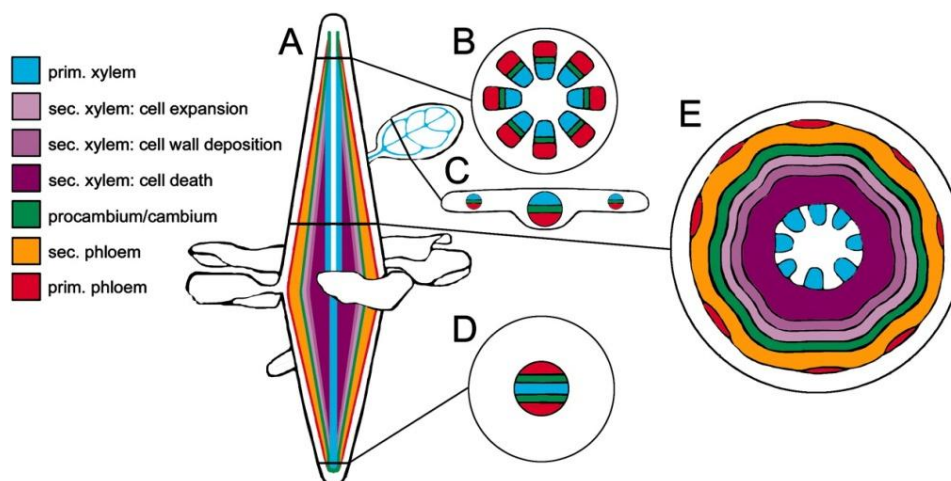
Figure 1.9 Organization of the primary and secondary vascular tissues in *Arabidopsis*. A) longitudinal view; B) cross section of the shoot apex; C) cross section of the leaf; D) cross section of the root tip; E) organization of the vascular tissues in the inflorescence stem during the secondary phase of vascular development. (Adapted from Nieminem *et al.*, 2004)

1.3- Gene Family Involved in Vascular Cambium

1.3.1- The *CLV3/ESR* Gene Family

Genes that regulate the SAM have also been found to be expressed in the vascular cambium.

There are several gene families that have been shown to be involved in the proliferation and identity of vascular tissues and vascular bundle patterning. Recently several putative peptide ligands have been identified in plants that are involved in plant-pathogen interaction, cell division, anther-stigma interaction and stem cell maintenance (Fiers, 2007; Jun *et al.*, 2008; Sharma *et al.*, 2003; Wang and Fiers, 2010). In this work we will give a special attention *CLV3* related genes.

CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR)-related (CLE) constitute one of the larger peptide ligand family of signaling polypeptides named after the first two founder genes *CLV3* and *Endosperm Surrounding Region* (*ESR*) that have at least 45 members in *Arabidopsis*. *ESR* genes were first identified in maize as being expressed in the endosperm regions surrounding the embryo. The *CLE* genes share two characteristics with CLV3 and the ESR proteins; they all encode a small protein (<10kD) with a putative secretion signal at their N-termini and contain a conserved 14-AA motif (CLE-motif) at or near their C-termini. (Fiers, 2007; Fletcher, 2002; Jun *et al.*, 2008; Strabala *et al.*, 2006). In *Arabidopsis*, these proteins are probably exported through the secretory pathway to the plasma membrane or extracellular space and acting as ligands for RLKs. The *CLE* genes are overall transcribed during the life of the plant, many widely expressed, but others are specific to some tissues (Sharma *et al.*, 2003).

CLE proteins share common characteristics such as length, charge and hydrophilicity, but diverge at their amino acid sequence level that could result of either the conserved domain being associated with diverse unrelated protein domains and/or extensive sequence diversification occurring within the CLE family (Fig. 1.10) (Cock and McCormick, 2001; Jun *et al.*, 2008; Ni and Clark, 2006).

There are studies showing controversial results on *CLE* genes overexpression. For instance, Strabala (2008) showed that overexpression of *CLE42* produced no

change in root growth but according to Kinoshita *et al.* (2007) *CLE42* leads to root growth stimulation. This opposite results can be explained by differences in experimental approaches but the main conclusion is that CLE peptides appear to play opposite roles in plant growth and development (Kinoshita *et al.*, 2007; Strabala, 2008).
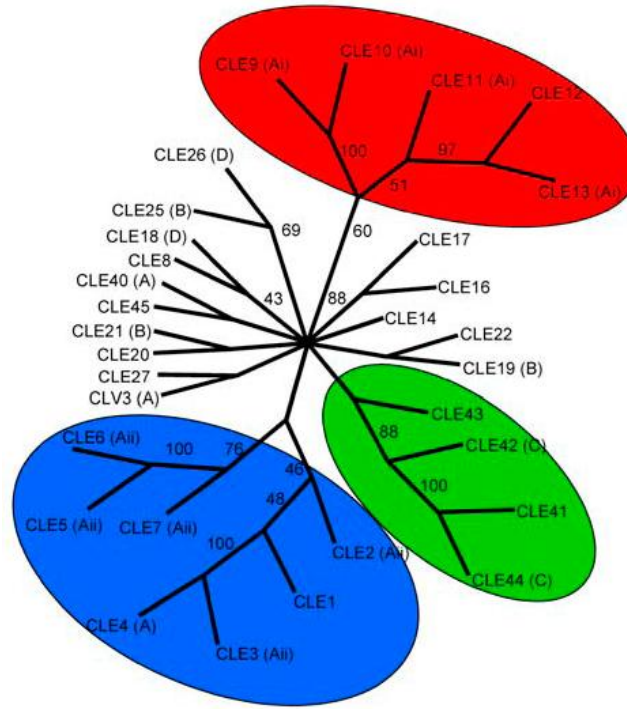
Figure 1.10 Phylogenetic tree showing the analysis of the CLE family predicted protein sequences. In red are the overexpressed genes that show *wus*-like phenotypes; in green, the shrub-like phenotypes; in blue are the dwarf phenotypes and the other *CLE* genes were grouped in long-root phenotype. (Adapted from Strabala et al., 2006)

In the present work, we have focused on five *CLE* genes: *CLE21*, *CLE27*, *CLE41*, *CLE42* and *CLE 43*. Several studies are published about these genes describing expression patterns, in terms of tissues distribution, stages of development and their roles in plant development (Cock and McCormick, 2001; Fukuda *et al.*, 2007; Kinoshita *et al.*, 2007; Sharma *et al.*, 2003; Strabala, 2008).

CLE21 transcripts are present in leaves, stem shoot apices, flowers and siliques at high levels, but in roots the level transcripts were low (Fig. 1.11) (Sharma *et al.*, 2003). Overexpression of *CLE21* showed root stunting, dark green/ purplish leaves suggesting anthocyanin overproduction, miniature rosettes and inflorescence and

developmental timing delays which may suggest possible roles in the regulation of plant stature and stress responses (Jun *et al.*, 2008; Strabala, 2008: Strabala *et al.*, 2006).

CLE27 transcripts increase during xylogenesis and are highly expressed in shoot apices, but in mature roots, leaves, flowers and siliques, the transcripts are very low (Fig. 1.11) (Sharma *et al.*, 2003). Kinoshita *et al.* (2007) observed that treatments with CLE27 peptide produced vegetative-like SAMs indicating that this peptide has mild SAM-repressing effects or function as inhibitor of flowering induction (Kinoshita *et al.*, 2007).

Previous works showed that *CLE41* is highly expressed in roots, on inflorescence stems, seedlings, developing siliques and less expressed in rosette leaves (Strabala *et al.*, 2006) (Fig. 1.11). *CLE42* expression pattern shows higher rates in seedlings and at shoot apex inflorescence meristem, but lower then *CLE41*, and reduced expression levels on other tissues tested (Fig. 1.11) (Strabala *et al.*, 2006).

CLE41 and *CLE42* are homologous probably having a specific role in suppressing xylem differentiation, by promoting maintenance and/or proliferation of procambial cells, in other words, they can repress differentiation of procambial cells into tracheary elements. Given these indicators, these cells must possess a receptor for CLE41 and CLE42 peptides. (Fukuda *et al.*, 2007; Jun *et al.*, 2008; Strabala, 2008; Strabala *et al.*, 2006). *CLE42* overexpression also exhibited development timing delays, rosettes that lack apical dominance with small and rounded leaves, however, floral development and fertility were unaffected. These results suggest that *CLE42* may have redundant roles in regulating apical dominance and organ size (Jun, 2008; Strabala *et al.*, 2006).

The study of Strabala *et al.* (2006) also shows that the expression of *CLE43* gene was very similar to *CLE42*, with higher transcript rates in seedlings shoot apex vegetative meristem and young leaves, but low expression levels in inflorescence stems, developing siliques, rosette leaves and roots (Fig. 1.11) (Strabala *et al.*, 2006).
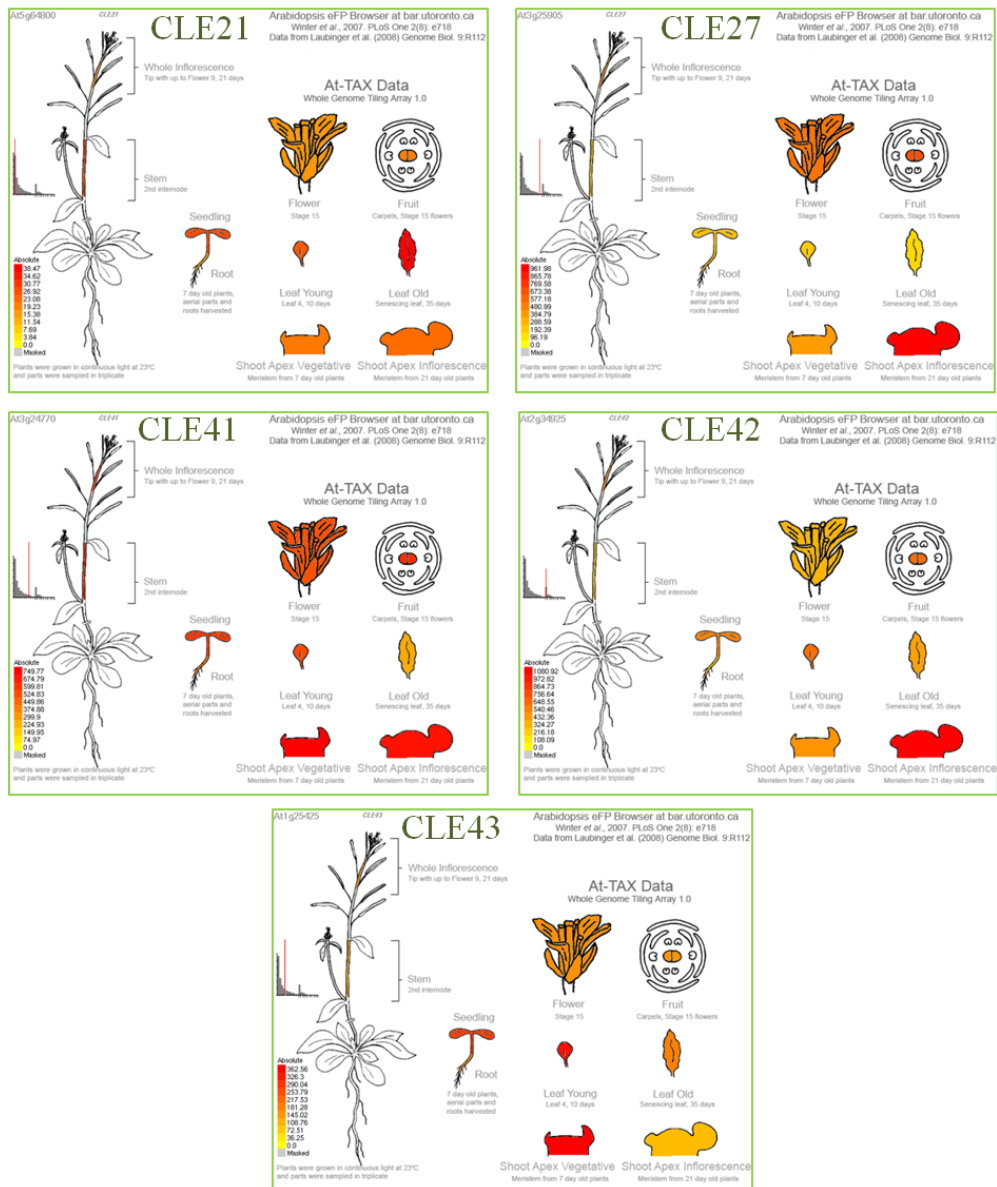
Figure 1.11 Expression patterns of *CLE21*, *27*, *41*, *42* and *43* in *Arabidopsis*. Red color shows higher rates of gene expression, while orange or yellow shows less expression on the same organ. *CLE 21* shows higher expression levels in 7-days-old plants in aerial and root parts, also in senescing leaf with 35 days but less in young leaves with 10 days; *CLE 27* has highest expression level in the shoot apex inflorescence meristem form 21 days old plants; *CLE 41* shows higher expression levels in the meristem of the shoot apex vegetative (7-days-old), also in seedlings and in young leaves with 10-days-old plants; *CLE 42* has higher gene expression in the meristem of the soot apex inflorescence from 21-days-old plants; *CLE43* shows highest levels of gene expression in the shoot apex vegetative meristem from 7-days-old plants, at seedlings and young leaves with 10-days-old. (Adapted from http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi)

1.3.2- The *DVL* Gene Family

DEVIL (DVL) is an angiosperm-specific gene family of small polypeptides encoding approximately 50 amino acids suggesting it may be involved in growth and development processes specific to angiosperm plants. With 21 members in *Arabidopsis* genome they share sequence homology in their C-terminal half. There are few studies about the expression pattern of these genes. Walker (2011) and after RT-PCR in 20 of 21 *DVL* genes, conclude that probably the *DVL* genes shows overlapping functions. When overexpressed, *DVL* genes affect the development of multiple plant organs while the gain-of-function of DVL alleles resulted in all lateral organs affected, being shorter than those of wild-type plants (Bowman, 2006; Walker, 2011; Wen *et al.*, 2004). Here we will focus in only too genes of this family: *DVL4* and *DVL16*.

DVL16 (*ROT4*) is a member of a novel gene family, ROTUNDIFOLIA FOUR LIKE/DEVIL (RTFL/DVL) family homologs (Horiguchi *et al.*, 2005; Tsukaya, 2005). Overexpression experiments suggest specific reduction in cell number along the longitudinal axis leading to a decrease in cell numbers in the leaf-length direction. However, no visible phenotype has been shown for loss-of-function of an *RTFL* member, which might be explained by the high redundancy of the *RTFL* gene family (Horiguchi *et al.*, 2005; Ikeuchi *et al.*, 2011; Tsukaya, 2006; Walker, 2011). The figure 1.12 shows that this gene has higher expression pattern in the vegetative shoot apex with 7-days-old and in the inflorescence with 21-days-old plants.

Wen *et al.* (2004) found that **DVL4** (*DEVIL 4*), also called *RTFL17* (*ROTUNDIFOLIA LIKE 17*), is highly expressed in leaves and in senescing leaf of 35-days-old plants but with a reduced expression level in roots, stems, flowers, seedlings and young leaves with 10 days old (Fig. 1.12).
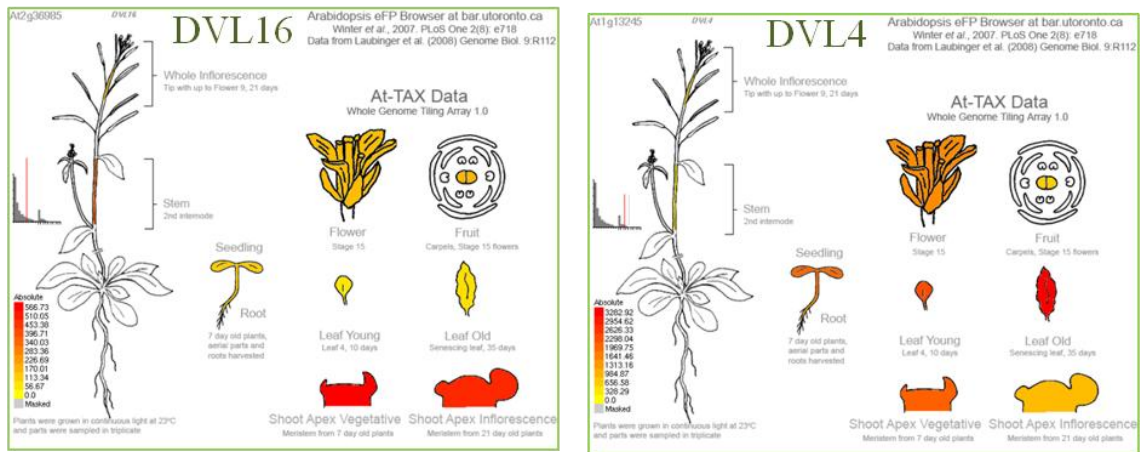
Figure 1.12 Expression patterns of *DVL4* and *DVL16* genes in *Arabidopsis*. Red color shows higher rates of gene expression, while orange or yellow shows less expression on the same organ. ***DVL4*** presents higher expression levels in old leaves (35-days-old plants) and less in seedlings and young leaves (10-days-old plants) and shoot apex vegetative meristem; ***DVL16*** has higher expression levels in the meristem of the shoot apex vegetative with 7-days-old and inflorescence, at 21-days-old plants. (Adapted from http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi)

1.3.3- The *HD-ZIP III* Gene Family

One important gene family associated with developmental processes such as embryo patterning, meristem initiation, meristem regulation, organ polarity and vascular development is the homeodomain-leucine zipper class, HD-ZIP III. Members of this family are putative transcription factors required for the establishment of the apical meristem and pattern formation in lateral organs and vascular tissues (Hawker and Bowman, 2004). In *Arabidopsis*, this family encompasses five members: 1) *PHABULOSA/ATHB14* (*PHB*); 2) *PHAVOLUTA/ATHB9* (*PHV*); 3) *REVOLUTA/ INTERFASCICULAR FIBERLESS 1* (*REV/IFL*); 4) *ATHB-8*, a class III homeodomain transcription factor which is expressed in collateral vascular patterning in stems and leaves and 5) *CORONA/ATHB15* (*CNA*) that has an expression pattern similar to the *ATHB8* in young leaves and in roots (Fig. 1.13) (Baucher *et al.*, 2007; Delatorre and Silva, 2008; Hawker and Bowman, 2004; Risopatron *et al.*, 2010). The *AtHB8 and 15* genes were used in this work, with the aim of studding the *CLE* genes in the vascular cambium using the trans-activation process (explained further ahead).

Figure 1.13 Phylogram illustrating the relationships between the *Arabidopsis HD-Zip III* gene family. (Adapted from Prigge *et al.*, 2005)

The expression pattern of the *ATHB8* gene is restricted to procambial cells of torpedo stage embryos and of developing organs. The *ATHB15* gene, as said before, has an expression pattern identical to *ATHB8* (Fig. 1.14) (Baucher *et al.*, 2007). Mutations within the *ATHB15* (*CNA)* gene led to dramatic defects in meristem size, organogenesis, stem cell maintenance, and in the expression of *WUS* and *CLV3*. Green *et al.* (2005) proposed that *CNA* acts in parallel with the CLV pathway to promote organ formation. Overexpression of *ATHB8* resulted in increased production of xylem, but results from *ATHB15* overexpression indicates that this gene acts as negative regulator in the production of xylem. These results are indicative that both have antagonistic roles during xylem development supporting Prigge *et al.* (2005) results (Baucher,*et al.*, 2007; Risopatron *et al.*, 2010). The *ATHB15* gene shows distinct functions of *REV* and *ATHB8* (Prigge *et al.*, 2005).
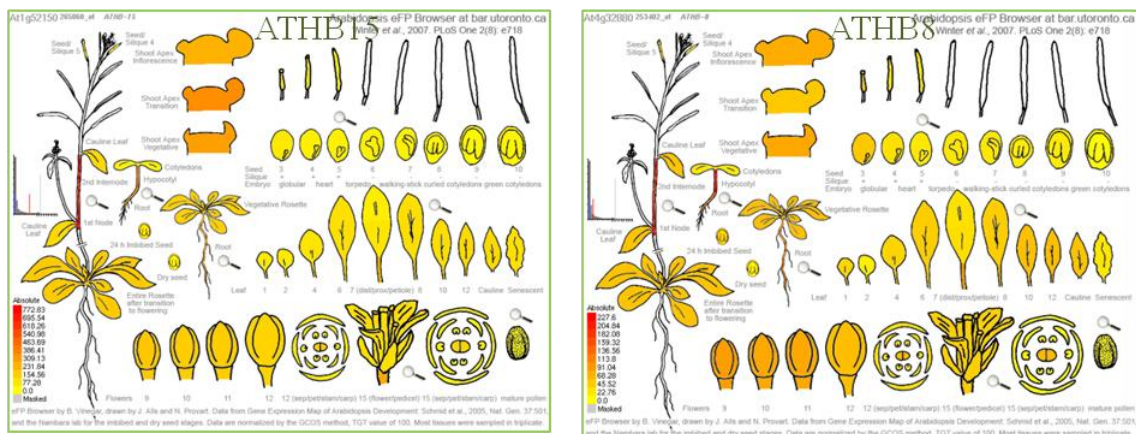
Figure 1.14 Expression patterns of *ATHB8* and *ATHB15* genes in *Arabidopsis*. Red color shows higher rates of gene expression, while orange or yellow shows less expression on the same organ. *ATHB8* shows higher levels of gene expression in the hypocotyl and in the second internode of the stem; *ATHB15* has similar gene expression in the second internode of the stem but reduced expression levels in the hypocotyl then *ATHB8*. (Adapted from http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi)

1.4 – Trans-activation

Trans-activation is a process that consists of inserting a trans-activator gene into a particular place of the genome. This gene will express a transcription factor that binds to a specific promoter. When this binding occurs, drives the transcription of the gene that is associated to the specific promoter. Multiple genes can be activated by one trans-activator gene, inducing or repressing the genes in parallel as long as they have a specific promoter region attached. A reported gene can also be attached, for example, *GUS* gene, in tandem with the promoter and our gene of interest allowing the expression pattern of the gene of interest to be seen when the trans-activation sequence is being expressed which is useful to allow a researcher to determine the locations and developmental stages of transcription factor expression in the plant (Laplaze *et al.*, 2005; Moore *et al.*, 1998; Samalova *et al.*, 2005).

The trans-activation provides us with several advantages such as the fact that our gene of interests can be expressed under the action of an endogenous promoter; it can be used to study the function of promoters; the expression of our gene can be specific to the cells under study and/or in specific stages of development and allows the use of reporter genes. It also has potential uses in gain-of-function, where the trans-activation can induce the expression of a gene or genes that wouldn't normally be expressed in a particular plant organ or developmental stage (ectopic expression), in studies of loss-of-function, the trans-activation can be used to suppress expression of certain genes in some plant organs/cells or developmental stages, and coordinating expression of multi-gene pathway is also very interesting as it has cumulative effect. Genes in a biosynthetic pathway may be introduced or up-regulated in a coordinate manner while product genes may be simultaneously down-regulated (Laplaze *et al.*, 2005; Moore *et al.*, 1998; Samalova *et al.*, 2005).

1.4.1 - Trans-activation Systems

Trans-activation systems operate using a transcriptional "enhancer" that can be involved in up or down-regulation of genes and consist of two cassettes; one with a transcription activator (trans-activator), and the other with a minimal promoter. Without

the proper activator, the transcription does not occur. In *Arabidopsis* trans-activation vectors are inserted into the plant via any method of transformation, for example, by *Agrobacterium*-mediated transformation.

There are several methods of trans-activation available today:

The **pOp/LhG4 trans-activation system** (Fig. 1.15) is based on a chimaeric transcription factor, the LhG4, comprising a transcription-activation domain-II from GAL4 of the yeast, *Saccharomyces cerevisiae*, fused to an *E. coli* DNA-binding mutant lac repressor that binds its operator with high-affinity. This molecule activates transcription from an upstream minimal promoter, pOp (Craft *et al.*, 2005; Moore *et al.*, 1998; Rutherford *et al.*, 2005; Samalova *et al.*, 2005). The pOp promoter is apparently silent when introduced into reporter lines that lack LhG4 but is activated in specific cells of F1 plants when reporter lines are crossed with activator lines that express LhG4 from appropriate tissue-specific promoters. Although the pOp/LhG4 system offers tissue-specific control over transgene expression through the use of tissue-specific promoters, it provides minimal temporal control (Craft *et al.*, 2005). The LhG4/pOp system allows the expression of multiple genes products and restricts transgene phenotypes into the F1 generation (Craft *et al.*, 2005; Moore *et al.*, 1998; Samalova *et al.*, 2005). It also offers spatially regulated gene expression in the whole plant growing under normal conditions. It is therefore possible to use tissue-specific promoters to restrict the activity of transgenes to certain tissues when using a specific promoter driving LhG4.

This system has been used to study various aspects of *Arabidopsis* biology including embryogenesis, cytokinin metabolism, and meristem control. It is important when a gene of interest needs to be studied in a variety of selected cell types and when the expression of the transgene compromises plant viability or fertility (Craft *et al.*, 2005).
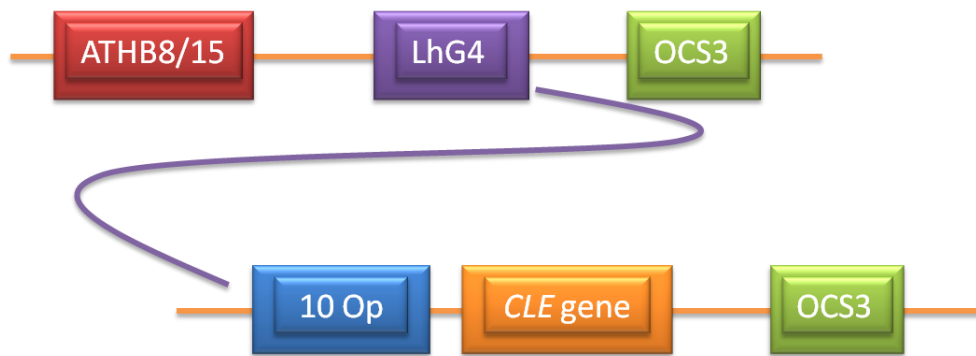
Figure 1.15 pOp/LhG4 system in *Arabidopsis thaliana* used in this work. The *ATHB8* and *ATHB15* promoter activates the transcription of the *LhG4* gene, and its products binds to the *10Op* binding site with high affinity, promoting the transcription of the *CLE* gene in the tissues where the *ATHB8/15* is specific, vascular cambium.

Besides the pOp/LhG4 system, other trans-activation systems have been used in several studies. Lössl, *et al.* (2005) studied the **ethanol-inducible trans-activation system** that regulates transcription of the *phb* operon in plastids. This system consists of a nuclear-located, ethanol-inducible *T7RNA polymerase* (T7RNAP) which is targeted to the plastids harboring the *phb* operon under the control of *T7* regulatory elements (Fig. 1.16) (Lössl *et al.*, 2005).
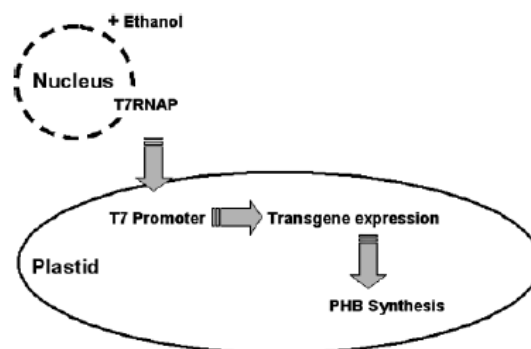
Figure 1.16 Inducible trans-activation in plastids. The T7RNAP was expressed under the control of an inducible promoter. Induction by ethanol application leads to the import of the T7RNAP into the plastids. Transcription of the plastid transgenes under control of polymerase-specific regulatory elements occurs and the PHB pathway becomes established in the chloroplasts. (Adapted from Lössl *et al.*, 2005)

GAL4/UAS system was first developed in *Drosophila* and is another example of a trans-activation gene expression. Laplaze, *et al.* (2005) used this system of two

components for molecular and cellular bases of lateral root development approaches. *GAL4* is a heterologous transcription factor from yeast that can be driven by a variety of promoters depending on the special and temporal expression pattern. GAL4 can act on the upstream activating sequence (UAS) that is cloned, along with minimal promoter, upstream of the gene of interest (Fig. 1.17). GAL4-mediated trans-activation is now widely used to elucidate the cell-specific functions of known genes, for targeted cell ablation and for conventional genetic approaches, as the basis of enhancer or suppressor screens (Laplaze *et al.*, 2005; Liang *et al.*, 2006).
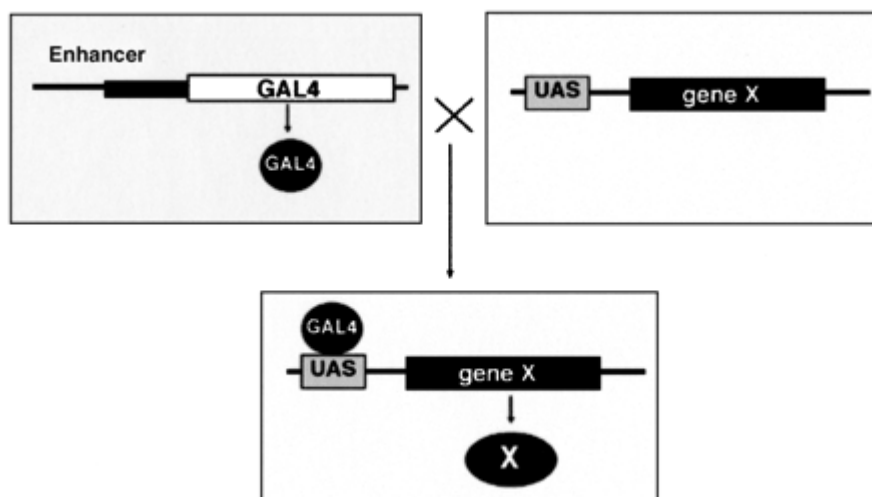
Figure 1.17 GAL4/UAS binary expression system. A plant line expressing the yeast transcription factor GAL4 in specific areas is crossed with another plant line in which the gene of interest (gene X) is under the control of a UAS element. In the progeny of this cross, GAL4 binds to the UAS sequence leading to the transcriptional activation of gene X. (Adapted from http://icb.oxfordjournals.org/content/44/4/269/F4.large.jpg)

1.5 - Steps Towards a Genetically Modified Plant

A genetically modified plant is an organism whose genetic material has been altered via non sexual through genetic engineering techniques. These techniques, known as recombinant DNA technology, use DNA from different sources, combined into one molecule to create a new set of genes that are transferred into an organism. When the

inserted DNA is originated from a different species, the organism is called transgenic. The transgenic plants allow studies of development, metabolism, biochemistry, regulation and gene expression.

There are few factors that can affect the success in plant transformation such as the integration of the DNA in the host genome, its expression, heritage and the stability of the exogenous DNA, and when explants are needed, its regeneration can be difficult, a factor that depends on the plant species.
The transformation process includes several steps as the identification of the gene of interest, the isolation, cloning and introduction of the gene into the plant, the selection of the transformed plants and the molecular analysis and the phenotype.

The cloning process uses genetic engineering techniques where the restriction enzymes or endonucleases, with their high specificity towards a base sequence, are capable of recognize and cut the DNA in a particular nucleotides sequence. Also important for this process is the DNA ligase that bonds the fragments produced by the restriction enzymes, allowing the formation of new DNA molecules (recombinant DNA). To get multiple copies of the DNA amplification needs to be performed.

The recombinant DNA must contain a promoter necessary for the correct expression of the gene in eukaryote organisms since the enzymes that work in the transcription process are able to recognize that region; a gene of interest; a transcriptional terminator important for terminate the gene transcription and a selection marker gene that will act in the selection of the transformed cells. This selective gene usually is an antibiotic or herbicide resistance gene. In order to introduce the new gene into the plant, a transportation vehicle able to carry the new DNA into the cell is needed. Such vehicles are called vectors, just like the plasmids of bacteria or viruses. The first vector used in transformation was the *Escherichia coli* plasmid. Plasmids are circular, small with only a few thousand of base pairs carrying some genes and with a single origin of replication (McBride and Summerfelt, 1990).

The cloning process involves the cut of both the DNA and plasmid with the same endonuclease. After the cutting, the DNA and the plasmid presents single-stranded or sticky ends. The DNA ligase attaches to these sticky ends forming a recombinant

plasmid that can then be inserted into the bacterial cell, usually *E. coli* by thermal shock, which integrates the plasmid and reply it with its own genome. Promoting the replication of the bacteria in an adequate medium increases the number of copies of the plasmid carrying the gene of interest (Fig. 1.18). The integration of the gene for selection is important at this stage because by adding the antibiotic or herbicide to the medium only the cells conferring resistance to the antibiotic/herbicide will be able to growth.
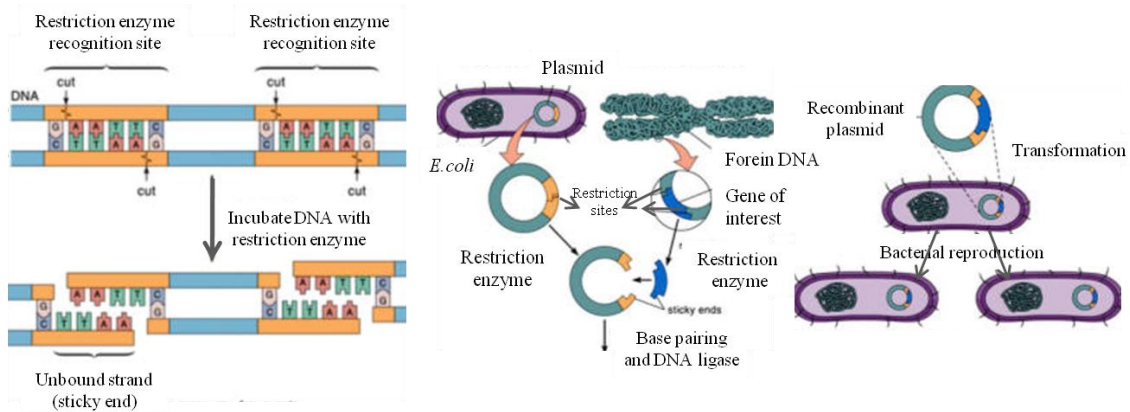
Figure. 1.18 The cloning process. These schemes show all the process described earlier, from the cut of the DNA by the restriction enzymes (left), the incorporation of the recombinant DNA into the bacteria (center) to the reproduction of the bacteria and at the same time replying the recombinant plasmid with the gene of interest (right).

(Adapted from http://academic.kellogg.edu/herbrandsonc/bio111/propagation.htm)

As mentioned before, the transformation is a process that allows the integration of foreign DNA into plant cells. Several methods were developed. These methods are based on direct and indirect processes. In the biological methods (indirect transformation), the most commonly used is the *Agrobacterium* mediated vector. The direct methods are mainly physical (particle bombardment or gene gun and electroporation) or chemical (polyethyleneglycol or $CaCl_2$) (Andrade, 2003; Birch, 1997).

The chemical methods for transformation make use of **polyethyleneglycol** (PEG) or **$CaCl_2$** to facilitate the entrance of foreign DNA through the plant cell wall, and among the physical there are the gene gun and the electroporation methods.

The **gene gun method** was developed to enable penetration of the genetic material containing a gene of interest throughout the cell wall of living cells. The steps taken are similar to the *Agrobacterium*-mediated method that includes: isolation of the gene of interest; development of the transgenic construct; incorporation into a plasmid; introduction of the transgenes into plant cells and the plant cells regeneration. Tungsten or gold particles coated with plasmid DNA are thrown through a macro-projectile, which are accelerated with air pressure and shot into plant tissue on a petri plate. The transgenes are then released from the particle surface and may be incorporated into the chromosomal DNA of the cells (Fig. 1.19) (Andrade, 2003). Selection of the transformed cells is performed with the use of selectable markers genes (Andrade, 2003).

The **electroporation method** consist of using short but high voltage electric impulses, that temporarily modifies the permeability of the plasma membrane, inducing the formation of pores and increasing the permeability of the membrane allowing the entrance of the exogenous gene (Fig. 1.19) (Andrade, 2003).

Plants transformed using chemical or physical methods generally carry copies of the entire DNA fragments. Frequently, some sections of the presented plasmid sequences are not transferred using these methods. Therefore, the boundaries of the transferred DNA will be predicted with less precision using these methods than with *Agrobacterium*-mediated transformation methods (Potrykus and Spangenberg, 1995). Despite the choice of the method, any transformation system should allow: a stable DNA integration into the host genome without structural alterations of the foreign DNA; the integration of a distinct number of copies of the exogenous DNA; heritable stability of the new phenotype over several generations.
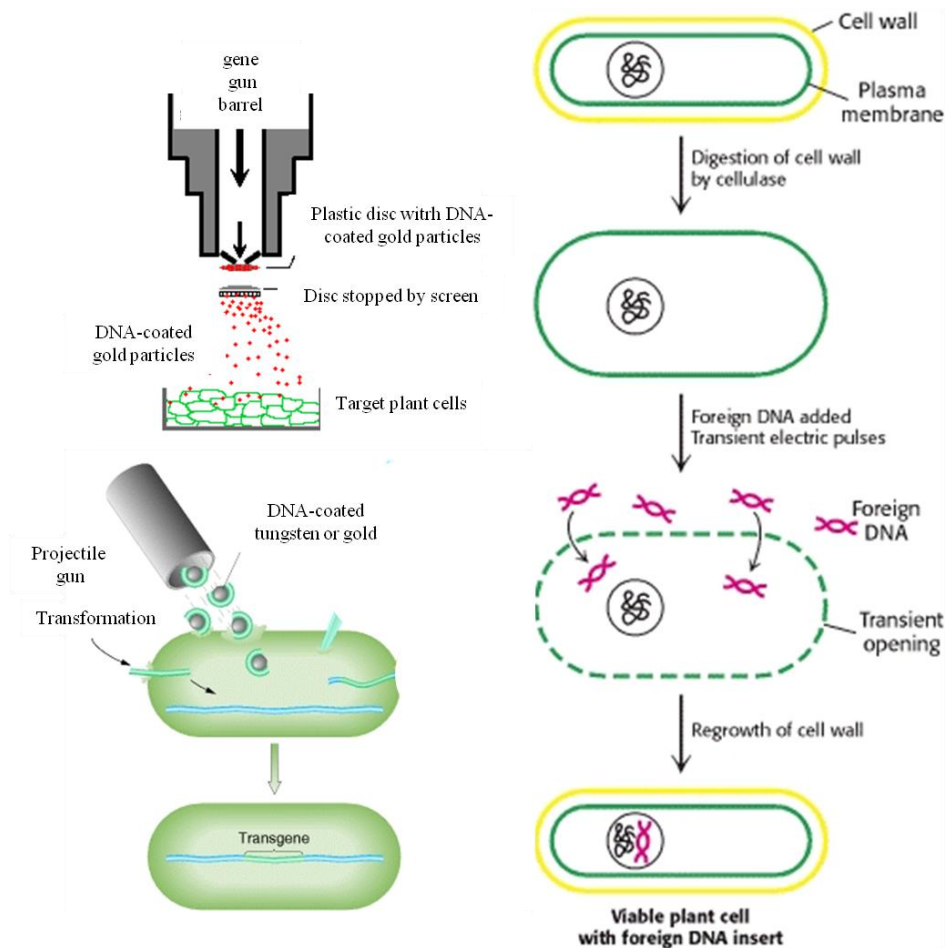
Figure 1.19 Gene gun and electroporation transformation methods. Upper left - scheme showing the gene gun method where the particles of tungsten or gold, coated with DNA are introduced into plant cells by a projectile; upper right - scheme of electroporation where the application of intense electric fields allows the plasma membranes to be transiently permeable for foreign DNA.

(Adapted from:

left figures: http://www.nepadbiosafety.net/for-regulators/resources/subjects/biotechnology (above) and http://www.ncbi.nlm.nih.gov/books/NBK21428/(below);

right figure: http://www.ncbi.nlm.nih.gov/books/NBK22390/).

The biological method more often used is the **binary vector system derived from *Agrobacterium tumefaciens***. The bacteria from these genera are phytopathogenic and have a natural ability to transfer DNA into some dicotyledonous species, inducing a disease called crown gall. The plant need to be wound so that the bacteria recognize the plant and starts the DNA transference (Fig. 1.20) (Andrade, 2003).
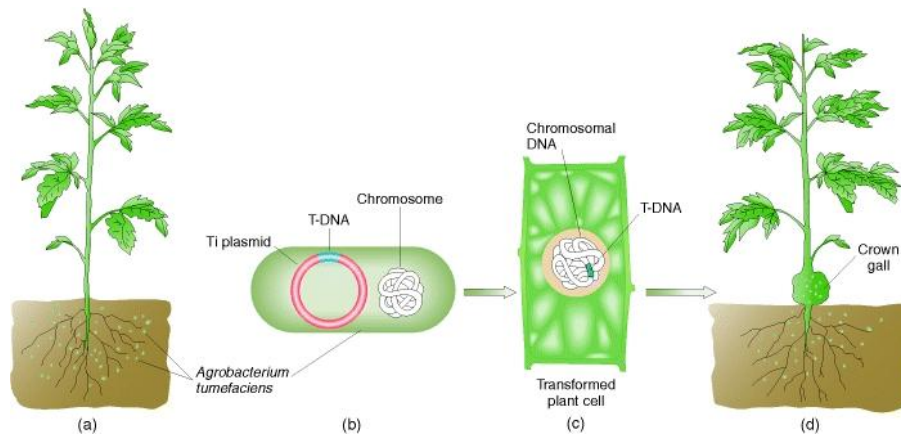
Fig. 1.20 DNA transference mediated by *Agrobacterium tumefaciens*. In the process of causing crown gall disease, the bacterium *Agrobacterium tumefaciens* transfers a part of its Ti plasmid, the T-DNA into the chromosome of the host plant. (Adapted from http://www.ncbi.nlm.nih.gov/books/NBK21428/)

The *A. tumefaciens* has a Ti-plasmid (tumor-inducing) that can be integrated into the plant genome of several species. It contains most of the genes necessary for tumor formation and development. A determinant factor for the infection to occur is the expression of the transferred DNA (T-DNA) in the plant genome in a stable way. The T-DNA is a fragment of the *Agrobacterium* DNA located outside the chromosome of the bacterium. Wounded plants exude phenolic compounds that stimulate the expression of the **virulence genes** (*vir* genes) that are also located on the Ti plasmid. The *vir* genes encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome. The T-DNA region is flanked at both ends by 25 base pairs of nucleotides (**T-DNA borders).** The left border is not essential, but the right border is indispensable for T-DNA transfer (Fig. 1.21).

The system is based on a disarmed Ti-plasmid where the genes responsible for the crown gall disease have being removed (oncogenes). The genes related to the transference (*vir* region and the DNA ends) are kept in the vector (Fig, 1.22) (Andrade, 2003; McBride and Summerfelt, 1990).
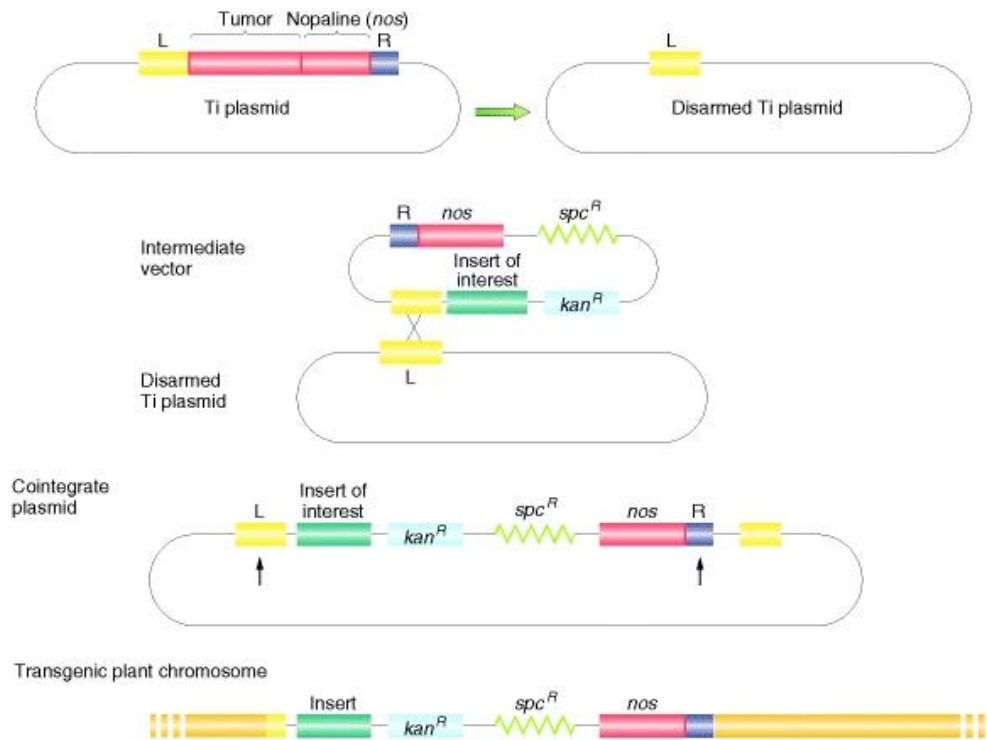
Figure 1.21 Schematic binary vector system. Co-integration structure bearing the insert of interest and a selectable plant marker (in this case, kanamycin-resistance) between the T-DNA borders. (Adapted from http://www.ncbi.nlm.nih.gov/books/NBK21428/)
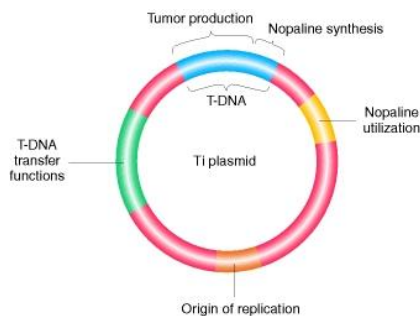
Fig. 1.22 Simplified representation of the Ti plasmid of *A. tumefaciens.* The T-DNA, when inserted into the chromosomal DNA of the host plant, directs the synthesis of nopaline that promotes uncontrolled cell division in the plant cell: tumor formation. (Adapted from http://www.ncbi.nlm.nih.gov/books/NBK21428/)

The transformation methods described earlier require preparation of plant cells or tissues, the introduction of DNA via *Agrobacterium* or particle bombardment, tissue or cell culture, the selection of transformant and regeneration of plants, and this requires time and sometimes expensive laboratory supplies (Fig. 1.23). Bechtold *et al.*, 1998 created a new method: the *Agrobacterium* vacuum infiltration. It consists in the growth of *Arabidopsis* till the flowering stage, application of *Agrobacterium* to whole plant via vacuum infiltration in a sucrose/hormone medium, replanting, seed recovery and

identification of transformed plantlets by selection media with antibiotics or herbicide (Clough and Bent, 1998; Zhang *et al.*, 2006).

Figure 1.23 Regenerating transgenic plants. The scheme shows the introduction of the plasmid into plant cell and the way to achieve transgenic plants, by regeneration of plant cells, usually applied in some *Agrobacterium*, electroporation and gene gun methods. (Adapted from http://www.ncbi.nlm.nih.gov/books/NBK21428/)

One other method for plant transformation is the **floral dip**, described by Clough and Bent, 1998 and Zhang *et al.*, 2006 where above-ground parts of the plant are submerged into an *Agrobacterium* solution for a few seconds allowing the uptake of the bacteria by the female gametes. This method requires less time and effort than the previous method. However, there are a few critical steps for a successful floral dip transformation: the dipping should occur in optimal plant stage growth; when plants contain numerous unopened floral buds (Fig. 1.24, A) and when the siliques are removed. This way, higher rates of transformed progeny are obtained. The plants should be covered with a plastic for 24 hours after the dipping in order to retain the humidity. The use of sucrose (5%) and surfactant (500µl/l of Silwet L-77) also raise the transformation rates, comparing to infiltration mediums without these components (nevertheless it is necessary to be careful with handle because the use of high levels of surfactant can cause plant tissue necrosis) (Clough and Bent, 1998; Zhang *et al.*, 2006). As described by and Zhang *et al.*, 2006, a large volume of bacteria was achieved by growing cells in liquid medium, harvested by centrifugation and resuspended in infiltration medium with sucrose and Silwet L-77 (Clough and Bent, 1998; Logemann *et al.*, 2006; Zhang *et al.*, 2006).

The *Arabidopsis* plants to be used for dipping are left to grown for 3–4 weeks until flowering stage is reached. The pots must be well watered until the first two true leaves are developed. Healthy plants are a pre-requisite for a successful transformation and the use of stronger plants promotes the production of a higher number of seeds. The dipping is achieved by inverting plants and dip the aerial parts of plants into the infiltration medium for 10 seconds with gentle agitation (Fig. 1.24, B) (Zhang *et al.*, 2006). After this step, plants should be lay down for 24 hours, covered (Fig. 1.24, C) and transported to the growth chamber until seeds are mature and ready to harvest (Fig. 1.24, D).
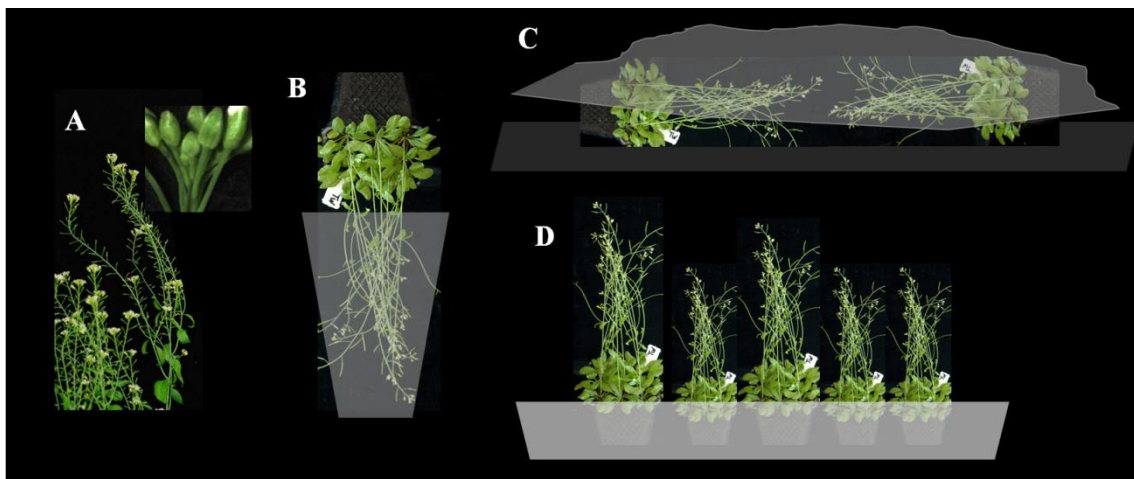
Figure 1.24 The floral dip process. **A-** flower stage of plants with unopened buttons; **B-** the plants should be inserted upside down into vessels containing infiltration medium with the bacteria suspension; **C-** after dipping, the plants should be left for 24 hours laid down and covered with plastic; **D-** the plants can now be transferred into the growth chamber.

Only a small proportion of the target cells receive the DNA and of these, only a few survives the treatment and stably integrates the **foreigner** DNA. It is therefore essential to efficiently select and regenerate the transformed cells (Birch, 1997). The presence of a selection marker in the plasmid allows identification of transformed seeds. Examples of these selection markers are the resistance to antibiotic, such as kanamycin (Kan), gentamycin (Gen), spectinomycin (Spec), carbenicilin (Carb) or to herbicide (for example, BASTA). The seed selection is a lengthy process which does not always lead to easily identifiable transformants. Selection usually takes 7 to 10 days and high seedling density and fungal contamination may result in failure to recover transformants (Birch, 1997; Harrison *et al.*, 2006).

To make sure that the seeds collected in the homozygous stage, have the construction with the gene of interest it is necessary to perform molecular, phenotypic and genetic characterization on the transformed lines. Sometimes, the target gene is silenced and transgene expression can provide ambiguous phenotypes. Phenotypic analysis is not a defining characteristic of gene transfer because many transformants have unexpressed copies of introduced sequences, which can be resulted by integration of multiple and often rearranged copies of the transferred DNA (Birch, 1997). Techniques like genes conferring resistance to a selective marker, genes conferring a phenotype allowing visual or physical screening, or polymerase chain reaction (PCR) screening to identify plants containing transferred genes can all be used to recover transformants. Unambiguous phenotypic analysis requires negative results from untransformed controls.

1.6 - Objectives

This work relies on the study of the expression of several CLE and DVL family genes; *CLE21* (At5g64800); *CLE27* (At3g25905); *CLE41* (At3g24770); *CLE42* (At2g34925); *CLE43* (At1g25425); *DVL4* (At1g13245) and *DVL16* (At2g36985) under the action of too vascular cambium genes *ATHB8* and *ATHB15* belonging to the Class III HD-ZIP family using the trans-activation process. The main goal proposed for this thesis was the study of the action of *CLE* and *DVL* genes mentioned above in cells where they are not expressed but that promote secondary growth, the procambium for xylem formation. For that, we have chosen the promoter two genes that are expressed specifically in those cells: *ATHB8* and *15* and this way, inducing the expression of the *CLE* and *DVL* genes in the same cells as *ATHB8* and *ATHB15* are expressed.

During this work, I have worked in all the process between cloning and the cross of the two plant lines. A higher number of protocols were used such as: selection of transformed *Escherichia coli* cells, preparation of competent cells of *Agrobacterium tumefaciens* and transformation, floral dipping of *Arabidopsis thaliana*, seed recovery and selection with kanamycin and the *in cross* between the activator and the reporter lines.

2.1- Starting material

The *Arabidopsis* ecotype used in this work was Landsberg *erecta ngatha* (*nga*) mutant line for the *Agrobacterium*-mediated transformation. Mutants *nga* were used because they show an incomplete fusion of the upper region of the stigma and abnormal split style facilitating to a great extend the *Agrobacterium* penetration in the unfertilized ovules. The *Arabidopsis* ecotype Colombia was grown as control in some essays, especially for seed selection with kanamycin (Kan).

The trans-activation constructions used were based on the 10Op::putative gene, being the putative genes *CLE21*, *CLE27*, *CLE41*, *CLE42*, *CLE43*, *DVL4* and *DVL16* genes.

The transformed lines *10Op*::*CLE 21* and *10Op*::*CLE 42* were already present in seeds, hence the experiments started with the seed selection in the presence of Kan until homozygous lines could be obtained.

The remaining genes of interest were already inserted in *E. coli* DH5α cells and kept in a glycerol stock.

Agrobacterium tumefaciens strain ASE was used for *Arabidopsis* transformation.

2.2 - Plant Growth

The plants were grown on a 1:1 mixture of peat and soil, autoclaved (20 min, 120ºC at 1.2 atm), cooled and then distributed into plastic cups in trays. The seeds were spread over the cups previously moistened, covered with plastic to maintain a high degree of humidity and put under 4ºC for 48 hours in the dark, for vernalization and growth synchronization. The trays were then transferred into the growth chamber (Fitoclima 10000 EHHF) at 20ºC±1 and relative humidity of 70%, under long day photoperiod of 16 h light, 8 h dark. After 1-2 weeks plastic covers were removed and the plants let to grow, watering every two days. After 6-8 weeks of sowing, seeds were collected.

2.3 - Transformation

2.3.1 - Preparation of competent cells

For genetic transformation process competent cells of *Agrobacterium* were previously prepared according to the protocol described by Weigel and Glazebrook, (2006). Briefly, ASE *Agrobacterium* strain was grown under appropriate conditions (28ºC, 48 hours under agitation) in a large volume of Luria-Bertani (LB) medium (Bertani, 1951) composed by 10g/l of Tryptone, 5g/l of Yeast extract and 10g/l NaCl, (pH=7,2) until the log phase was attained. Then, upon successive centrifugations sufficient pellet of bacteria was obtained. Then, the cells were washed with chilled water for several times and the pellet resuspended in 10% ice-cold sterile glycerol and store at -80ºC.

2.3.2 - Plasmid DNA Extraction

For plasmid DNA extraction *E.coli* cells carrying the plasmid of interest were spread on plates containing LB plus the antibiotic which, in this case was spectinomycin (Spec). The plates were kept at 37ºC for 24h. Following this period one colony was isolated with a tip and inoculated in 3ml of LB + 100 µg/ml of Spec and placed at 37ºC, for 24 hours under agitation. In a following step the High Pure Plasmid Isolation Kit (Roche) was used for plasmid DNA isolation and the DNA stored at 4ºC for a few days or at -20ºC when longer periods were required

An alternative method for DNA extraction was also performed, the Boiling Plasmid Miniprep (Holmes and Quigley, 1981). Briefly it consists of growing *E. coli* in 5ml of suspension medium and centrifuged in order to get a bacteria pellet (four centrifugations were made using the same 1,5 ml tube in order to get a higher number of cells). Cells were then resuspended in 300µl STET (8% Sucrose, 1% Triton X-100, 50mM EDTA, 50mM Tris pH 8.0) by pipeting up and down several times. 20µl of the lysozyme mixture (50mM Tris pH 8.0 with 10mg/ml lysozyme) was then added, mixed and incubated at room temperature for at least 15 seconds (can rest up to 10 minutes). The tubes were placed in boiling water for 2 minutes followed by a spin of 5 minutes at 13 000 rpm. Then, the "snot" was removed with toothpick, 325 µl of isopropanol (75%

v/v) was added, the mixture was gently inverted and centrifuged for 5 minutes. The pellet was rinsed with 70% EtOH and left to dry. Finally, the pellet was resuspended in 50 µl TE + RNase. As above mentioned, when necessary the DNA was stored at 4ºC for a few days or at -20ºC for longer periods.

2.3.3 – Polymerase Chain Reaction (PCR)

To confirm if DNA isolated from the *E. coli* stock had the right construction carrying our gene of interest a PCR reaction was carried out.

Three pairs of primers were used: 10Op forward (F) and OCS3 reverse (R); CLE F and R and *nptII* F and R. The first pair refers to the whole construction between left and right border of the plasmid. The second pair delimits the gene *CLE*, and the third pair limits the kanamycin gene, conferring resistance to the transformed plants.

The 25 ml of PCR reaction mixture was performed in a MJ Mini Personal Thermal Cycler, Bio Rad accordingly to the conditions described in Table 2.1. The reaction cycle consists of a denaturating step of 5 min. at 94ºC; 35 cycles of denaturation (94ºC, for 20 sec.), annealing (temperature depending on primers pair, for 0.40 minutes) and extension (72ºC, for 1 min.). The final extension step was carried out a 72ºC for 5 min.

Table 2.1 – Components and reagents of a polymerase chain reaction.

	Sotck	Final concentration	For each 25ul
Taq Pol Buffer	5x	1x	5 µl
dNTP's	10mM	0.4mM	1 µl
$MgCl_2$	50mM	2mM	1 µl
DNA	-	-	1 µl
Primer F	10µM	0.2µM	0.5 µl
Primer R	10µM	0.2µM	0.5 µl
Taq Polymerase	5 units	1 unit	0.2 µl

To analyze the PCR products usually it is used an electrophoresis in agarose gel. In order to prepare the gel, 1g of agarose was mixed with 100ml of 1x TBE (from the 5x TBE Stock: 0,445M Tris-base, 0,445M Boric Acid and 10mM EDTA, pH 8) solution and placed on the microwave for about 1:30 minutes till boiling point to dissolve the agarose. After cooling to 60ºC, 5mg/ml of ethidium bromide was added. The gel was poured slowly into the tank, bubbles were removed with a disposable tip and the comb was placed and checked for its correct position. The gel was left to polymerize for 0:30-1 hour. Longer periods of time gives best results due to a tighten net. It is important to use the same TBE buffer concentration that is used to prepare the gel.

To visualize the DNA, 5-10µl of each DNA sample was transferred to a microfuge tube and added 2-3µl of loading buffer. Another way to do this is to put a drop of loading buffer in a strip of parafilm, pipet 5µl of DNA, mix it with the drop and load the samples on the gel. The gel ran at 80-100V for 30-45 minutes.

Once confirmed that the construct was the correct, the assays of plant genetic transformation were initiated.

Plants for transformation should growth under mild temperature (16ºC) in order to promote a slower growth that leads to a shorter and stronger main stem facilitating the process of floral dipping. By the time plants started to produce secondary inflorescences, the plants are decapitated of the apical inflorescence to remove inflorescence dominance promoting more and stronger secondary inflorescences. With this technique we promote a higher number of flowers in the right stage susceptible for transformation (Bechtold and Pelletier, 1998).

2.3.4 – Transformation of *Agrobacterium tumefaciens* and selection

The *Agrobacterium tumefaciens* competent cells were transformed by electroporation with our plasmid of interest initially grown in *E. coli* (Weigel and Glazebrook, 2002). The transformed cells were left to grow in a 1.5 ml eppendorf with LB medium at 28ºC for 3 h with agitation. The selection was performed on plates containing LB + antibiotics for 48 h. Three antibiotics were used; spectinomycin (100µg/ml) and kanamycin (50µg/ml) for plasmid resistance, and chloranphenicol

(Chlor, 30µg/ml) for *Agrobacterium* resistance. Plates can be store at 4ºC but it is necessary to peak a colony every month to a new LB + antibiotics plate to maintain the growth. Glicerol stocks can also be made even though "fresh" *Agrobacterium* transformations should be obtained for each plant transformation.

2.3.5 - Plant transformation

One single isolated transformed *Agrobacterium* colony was chosen and grown into 20 ml of LB + antibiotics, Kan (50 µg/ml), Chlor (30 µg/ml) and Spec (100 µg/ml) at 28ºC for 24 hours, with agitation. After 24h, 20ml of bacteria mixture were transferred to 300 ml of LB + antibiotics and let to grow at 28ºC for another 24 hours, with agitation.

2.3.5.1 - Floral Dipping

When a large amount of bacteria was obtained, optical density of the mixture was measured and adjusted to be between 1.7 and 2 at 600 nm.

Meanwhile all siliques were removed of the plant to be used in the floral dipping assays. New fresh infiltration medium (IM) have to be prepared for each transformation. 500ml for each line to be transformed were used. The IM composition is shown in Table 2.2.

Table 2.2. Composition of the infiltration media.

	1L
10mM MgCl$_2$	1,3554g
5% Sucrose	50g
1x B5 Gambory Vitamin (1000xs)	2ml
0.44µM BAP	10 µl
0.03% Silwet L-77	300 µl

All the bacteria cell culture was centrifuged (15 minutes at 4.2g) until a saturated pellet was achieved, which was resuspended in the infiltration media. Plants were dipped (with the plant upside down) into that mixture for about 5 minutes. Plants were then laid down on absorbing paper, covered with plastic and left to rest for 24 hours. On the next day, plants were strengthened up and transfer into the growth chamber. After 3 to 4 weeks the seeds from these plants were collected.

2.3.5.2 - DNA extraction

Upon seed selection with kanamycin, and after at least two generations (usually called F1 and F2) is necessary to confirm whether the transformed plant contain the construct of interest by performing a PCR with the appropriate pair of primers. In this case, the *nptII* forward and reverse primer that (primer for the gene that confers resistance to kanamycin) were tested. The protocol used for the DNA extraction was the CTAB method and the constitution of the buffers can be seen in Table 2.3. About 3 inflorescences per plant were grounded with 300µl of CTAB with a pistil until a fine paste was obtained and incubated at 65ºC for 30 min. After cooling; 300µl of chloroform was added, vortex vigorously and spun for 30 sec. at maximum speed followed by the transfer of the upper aqueous phase (that contains DNA) to a clean tube. 300µl of isopropanol were added, blend softly and spun for 5 min. at maximum speed. The supernatant was then removed and the pellet washed with 500µl of EtOH 70% and spun for 1 minute at maximum speed. Finally the EtOH was removed, the pellet dried and dissolved it in 50-60µl of TE buffer.

DNA was extracted from three lines namely *10Op::CLE 21* and *10Op::CLE 42* (F2 generation), CLE 43 (F1 generation). A PCR was then carried out to confirm construct insertion. The same cycle as described before was used (see section 2.6) and the primers *nptII* (F and R) for kanamycin resistance gene 10Op (F) and CLE 21/42/43 (R) CLE 21/42/43 (F) and OCS3 (R) tested. For DNA detection an electrophoresis in agarose gel was made and the gel ran at 80-100V for 30-45 min.

Table 2.3. Constitution of buffers used in plant DNA extraction by CTAB method.

CTAB Buffer
2% CTAB
1,4M NaCl
100mM Tris-HCl
20mM EDTA
TE Buffer
10mM Tris-HCl
1mM EDTA

2.3.6 - Selection

Upon seed collection, they were dehydrated in a dry and dark environment for at least 3 weeks. After this time, the transformed seeds were selected on plates with MS medium and the corresponding antibiotics. The protocol for seed selection started with a disinfection of the seeds through a wash in a solution (1ml) of commercial bleach and EtOH (1:4) performed in 1.5 ml Eppendorf tubes for 15 minutes under agitation. In the flow chamber, too washes were performed with 1ml of EtOH 100%. After this seeds were left to dry for about 1-2 hours.

2.3.6.1 - Seed selection

The selection of the transformed seeds was carried out on plates with 50% MS medium salts (Murashige and Skoog, 1962) with pH adjusted to 5.8 accordingly to the Table 2.4. Sterilized medium was left to cool down to 60ºC, temperature that allows the addition of antibiotics without promoting its degradation. Since the plasmid confers Kan resistance to the transformed plant it was used 50µg/ml of Kan. A few seeds were spread on the plate (250/300 per plate), and placed at 4ºC for two days for vernalization before germination. After that, the plates were transferred to the growth chamber at 28±1ºC under a long day photoperiod of 16 h light/8 h dark. After 8-10 days transformed seedlings could be distinguished from the non-transformed because they showed the appearance of the small dark green cotyledons.

Table 2.4. Constitution of the modified MS medium used for seed germination.

	1L
50% Macronutrients (Stock: 50ml/l)	25ml
50% Micronutrients (Stock: 2ml/l)	1ml
50% Iron (Stock: 5ml/l)	2.5ml
50% Myo-inositol (Stock: 4ml/l)	2ml
50% Vitamins (Stock: 2ml/l)	1ml
Sucrose	10g
Phytoagar	8g

2.4 – Crosses

Crosses were hand-made by transferring pollen from the anthers of one plant (activator line) to the stigma of another plant (reporter line).

Plants of the two lines transformed with the constructions *10Op::CLE* and *ATHB8/15::LhG4* were placed to grown separately until flowering occurred. In general, in Arabidopsis, the development of the gynoecium (female part of the plant) occurs at an earlier stage than the androecium (male part) (Fig. 2.1, a). When the flower has opened, the self-pollination already occurred. To be able to make the crossing is necessary to remove the anthers (emasculation) before the process of self-pollination has occurred.

The emasculation has to be performed under a binocular lens because of the size of the *Arabidopsis* flowers. The flowers are carefully held between the fingers and with the other hand the anthers removed using tweezers (Fig. 2.1, b). After the emasculation, pollinization is carried out with pollen from the donor plant. The pollen is collected from fully mature flowers. The application of the pollen is done by removing a donor anther (from the activator line) and rubbing it on the stigma of the other plant (reporter line). The contact between the anthers and stigma is repeated until the pollen adheres to the stigma (Fig. 2.1, c). It is often necessary to transfer the pollen to a recipient with a

brush and then brushing the pollen into the stigma (Fig. 2.1, d). The inflorescences that have been polinizated must be labeled (Fig. 2.1, e), and the other inflorescences that were not in the optimal stage of pollination should be removed. After pollination, it takes about two weeks to begin embryo development and to develop the fruit, the siliqua. When the siliqua is completely dry (about 2 weeks) seeds are isolated. The seeds should be dried completely and stored in a dry place, which can take up to a few months after collecting.
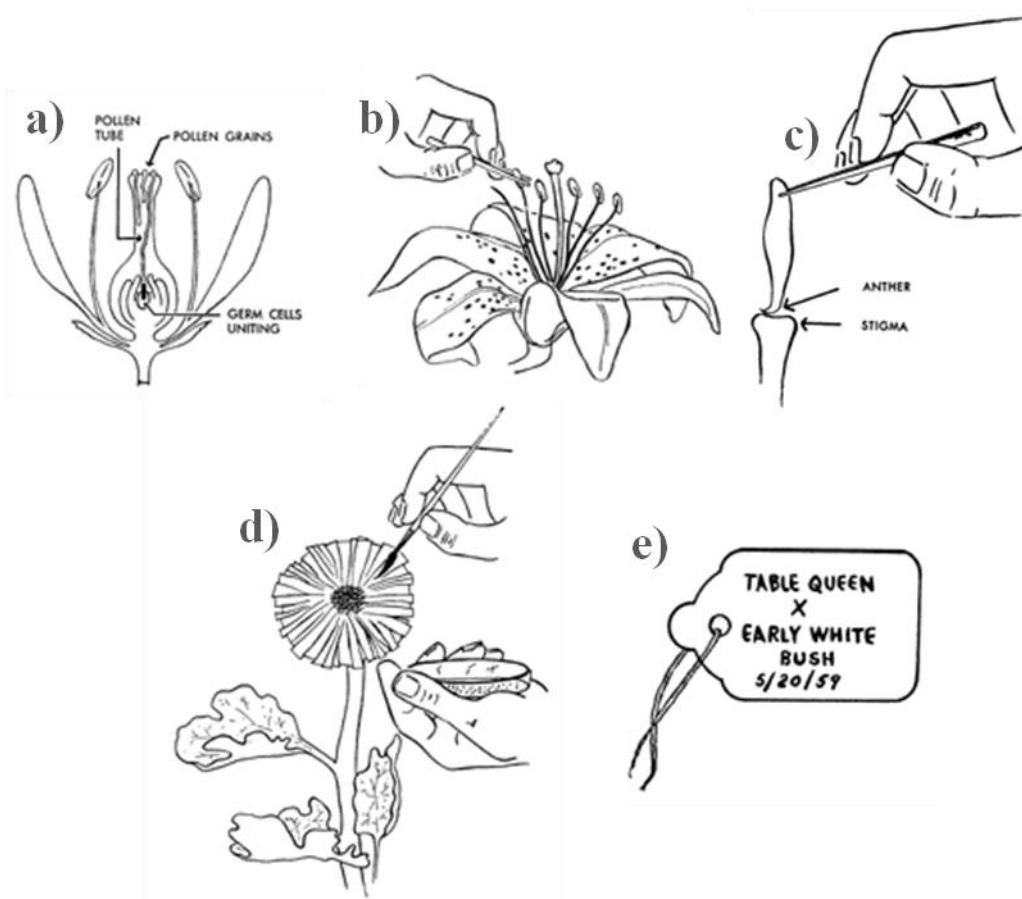
Figure 2.1 Phases of the artificial crosses performed in Arabidopsis. a) scheme of a flower showing the male (stamen) and female (pistil) reproductive organs; b) scheme of a flower emasculation, process by which all anthers or stamens are removed; c) scheme showing the cross-pollinization which consists on the removal of the stamens from the pollen parent and then, holding a stamen with tweezers, brush the anther across the stigma of the seed parent; d) cross-pollination using a brush; e) labeling of the pollinated flowers..

(Adapted from http://www.aces.uiuc.edu/vista/html_pubs/PLBREED/pl_breed.html#fund)

CHAPTER III
RESULTS

3.1- DNA Plasmid Extraction and PCR

E. coli plasmids harboring the constructs, *10Op::CLE27, 10Op::CLE41, 10Op::CLE43, 10Op::DVL4* and *10Op::DVL16* were extracted using the High Pure Plasmid Isolation Kit (Roche) and an electrophoresis was performed in order to see if the sample had enough DNA to proceed for Agro-transformation (Fig. 3.1, **A**).

Before the transformation assays, a PCR was performed using the extracted DNA to confirm whether the DNA had the construction with the gene of interest (Fig. 3.1, **B** and **C**). Verifying that the samples had enough amount of DNA, the plasmid was inserted into *Agrobacterium* by electroporation, as described in chapter 2.3.5.

The PCR performed on these lines showed that the construction *10Op::CLE43* presented one intense band around 400 base pairs (bp) for the pair of primers 10Op and CLE R (Fig. 3.1, **B**) and another one around 200 bp for the pair of primers CLE F and R (Fig. 3.1, **C**), which is consistent with the length of the gene sequence. The construction *10Op::DVL4* presented a band between the 400 and 600 bp on the agarose gel when used the pair of primers 10Op and CLE R and 200 bp for the primers CLE F and R. The line *10Op::CLE27* presented a strong band (200-400 bp) and another band of 600 bp that can be seen on the figure 3.1, **B** probably due to the hybridization of the primer 10Op to the ten Op regions present in the promoter. The bands corresponding to the construction *10Op::DVL16* were less intense and for the line *10Op::CLE41*, no band was detected for any of the two PCRs that have been performed.
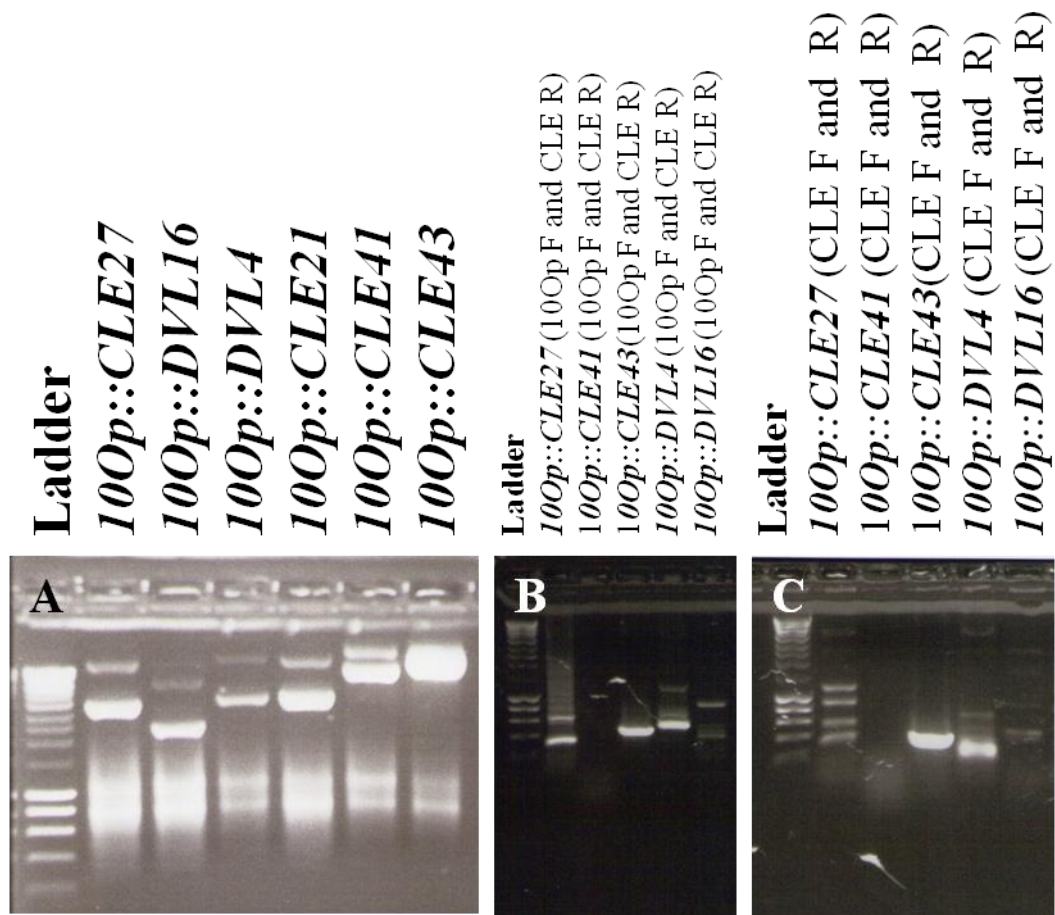
Figure 3.1 Electrophoresis gel showing: **A -** the result from the DNA plasmid extraction from *E. coli* and PCR (B and C) performed on lines *10Op::CLE27, 10Op::CLE41, 10Op::CLE43, 10Op::DVL4* and *10Op::DVL16* using the pair of primers; **B -** 10Op forward (F) and CLE reverse (R) primers and **C -** CLE F and CLE R.

The transformation with *Agrobacterium* and the selection in a selective medium (LB medium + 50μl/ml Kan + 30μl/ml Chlor + 100μl/ml Spec) resulted in only one line resistant, *10Op::CLE43*, so the transformation protocol was followed the by inserting the construction into *Agrobacterium tumefaciens*.

The *Agrobacterium* strain used for plant transformation should be checked for the presence of the Ti plasmid and the easiest way to do this is to make a miniprep and use a PCR to determine if the cells contain the construction with the gene of interest.

After trying again the DNA plasmid extraction using the kit and having no results, another miniprep protocol was performed: the Boiling Miniprep (see 2.3.2). The

boiling miniprep was then applied to the other four constructions in study; *10Op::CLE27, 10Op::CLE41, 10Op::DVL4, 10Op::DVL16.*

After DNA extraction the confirmation was done by PCR, to see if the bacteria had the constructions with the gene of interest, using two different protocols: PCR colony - cells are introduced directly into the PCR tube, and the other protocol involved a previous extraction of DNA. The results of the different PCR are shown in figure 3.2 (**B**-**E**). Only with the primers *nptII* the results from PCR were clear, showing single straight bands in the correct position (700bp). The other PCR analysis showed inconsistent results Thus in figure 3.2, **B**, *10Op::CLE27* and *10Op::DVL16* showed multiple bands when the primers CLE F+R were used. However, when the primer 10Op was used, those multiple bands resulted, as said before, from the hybridization of the primers to the several Op sequences that are in tandem.
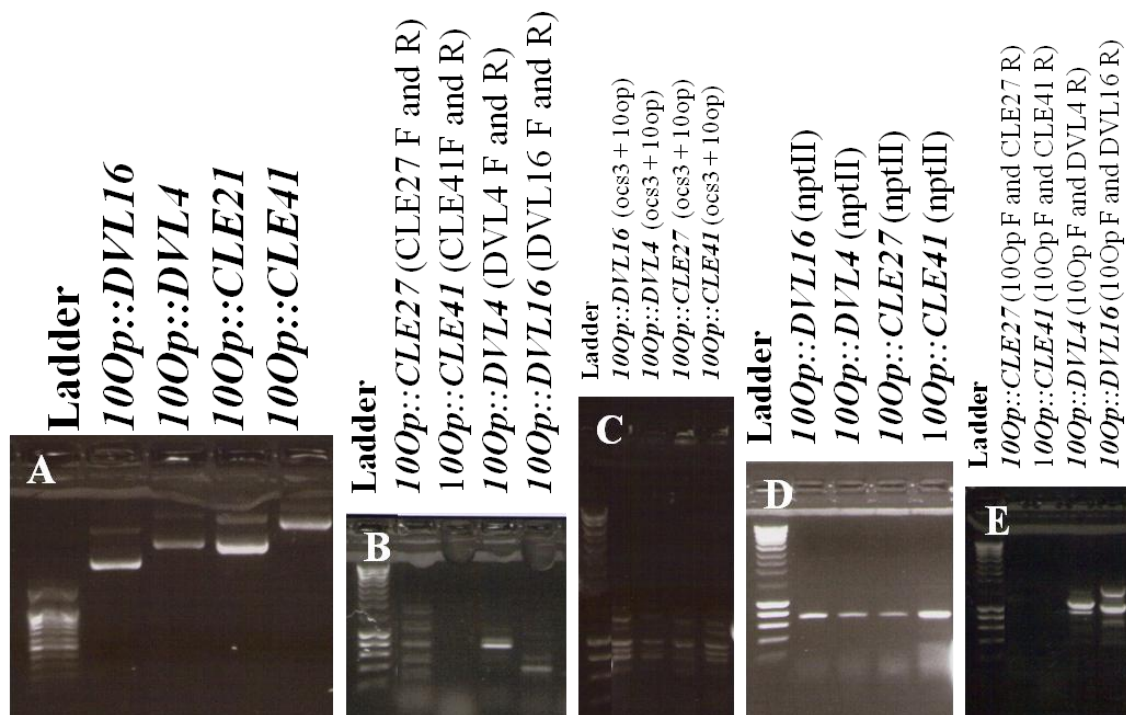
Figure 3.2 Electrophoresis gel image showing: **A -** DNA extraction from *Agrobacterium*; **B -** PCR (from DNA extracted by boiling miniprep protocol) with the primers CLE F+R and **C -** PCR colony using the primers OCS3+10Op; **D –** PCR using the primers *nptII* F+R; **E –** PCR performed to the DNA extracted using the primers 10Op+CLER, performed on *Agrobacterium* transformed with the construction *10Op::CLE27*, 10Op::CLE41, *10Op::DVL4* and *10Op::DVL16.*

3.2- Seed Selection

The *E. coli* plasmid containing the *10Op::CLE43* construction was extracted and inserted into *Agrobacterium tumefaciens*. *Arabidopsis* plants were transformed by floral dip method, each cup with four plants, let to reach maturity in the growth chamber, and then seeds were collected. Seeds of three cups were selected on Petri plates with ½ MS medium and, because plants were kanamycin resistant, this antibiotic was added (50µl/ml) to the medium. After two weeks, *Arabidopsis* plantlets were removed from the growth camber and the germinated seeds were counted. It was easy to identify the difference between the transformants that were kanamycin resistant and the seeds that were not since the non resistant ones presented a yellowish color and did not develop further than the first two leaves. In the Petri plate of Plant 1 no viable seeds were found, meaning a total absence of seed transformation (Fig. 3.3, **A**). Kanamycin resistant plants were obtained in plates 3 and 5 (Fig. 3.3, **B** and **C**, respectively)**.** The transformation rate of this line was 0,53%, as shown in Table 6 where the number of germinated seeds (in a total of 250) per plate in the transformants recovery (T) and in the first generation (F1) is shown

The average of F2 seeds *per* plate was 300 seeds and the results obtained can be seen on Table 6.
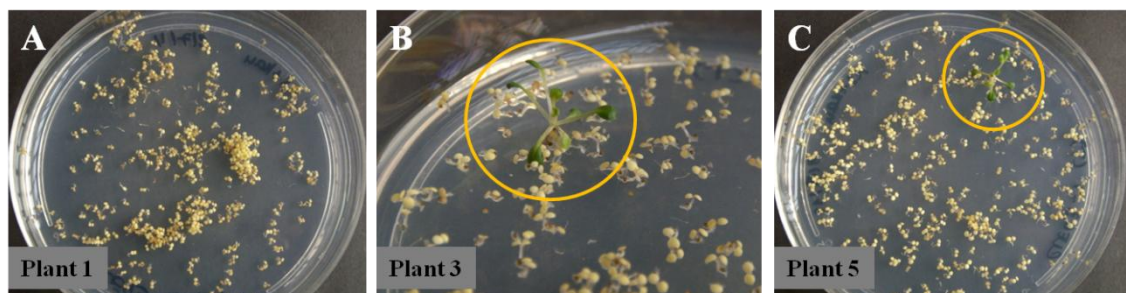
Figure 3.3 Seed selection on solid germination medium from transformed plants with the construction *10Op::CLE43*.

The two lines *10Op::CLE21* and *10Op::CLE42* were already transformed, so the transformants seeds were select in the same way as described before. The figure 3.4 shows the plantlets that were resistant to the antibiotic. In **A** no seeds germinated, but in **B** and **C**, few green plantlets can be seen meaning that they were kanamycin resistant; in

the pictures below, **D** for plant 1, **E** for plant 3 and **F** for plant 5, correspond to the *10Op::CLE42* transformants. It is possible to observe several resistant plantlets on each petri plate. The orange boxes highlight details of the transformants healthy developed. *10Op::CLE 21* showed a transformation rate of 4,93% and *10Op::CLE42* as 9,60% of transformants (Table 6).
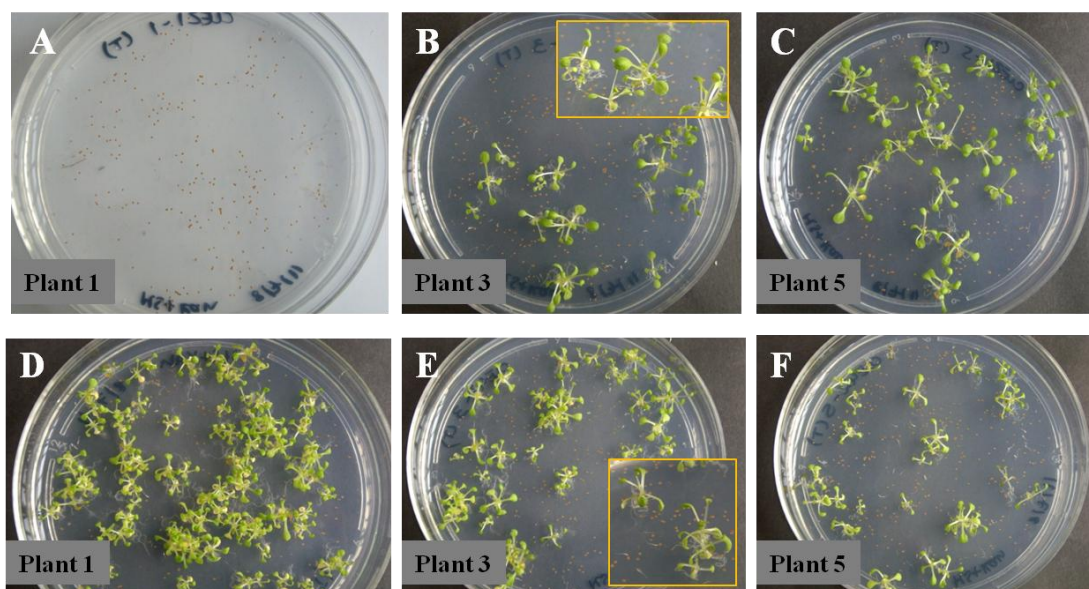
Figure 3.4 Seed selection of the transformants of *10Op::CLE21* (A, B and C) and *10Op::CLE42* (D, E and F).

Table 6. Germination rates of the three transformed lines. **T** refers to the transformants seeds, **F1** to the first generation and **F2** to the second generation.

Plants	*CLE21*			*CLE42*			*CLE43*	
	T	F1	F2	T	F1	F2	T	F1
1	0	¬	¬	0	¬	¬	0	¬
3	14	13	284	47	22	263	3	26
5	23	8	280	25	11	255	1	18
Average	12,33	10,50	282,00	24,00	16,50	259,00	1,33	22,00
% germinated seeds	4,93	4,20	94,00	9,60	6,60	86,33	0,53	8,80

Concerning the *10Op::DVL4*, transformed *Agrobacterium* could be obtained which grew well. However, during the selection phase, it was not possible to obtain any colonies, therefore plant transformation wasn't carried out with this line. The three remaining lines were used to transform the plants. After the time necessary for seeds to germinate, the result wasn't the expected since no transformants were found in all petri

plates of these lines, as can be seen in figure 3.5. These results show that the plants didn't incorporate the constructions, so they were not transformed by the *Agrobacterium* during the floral dip process
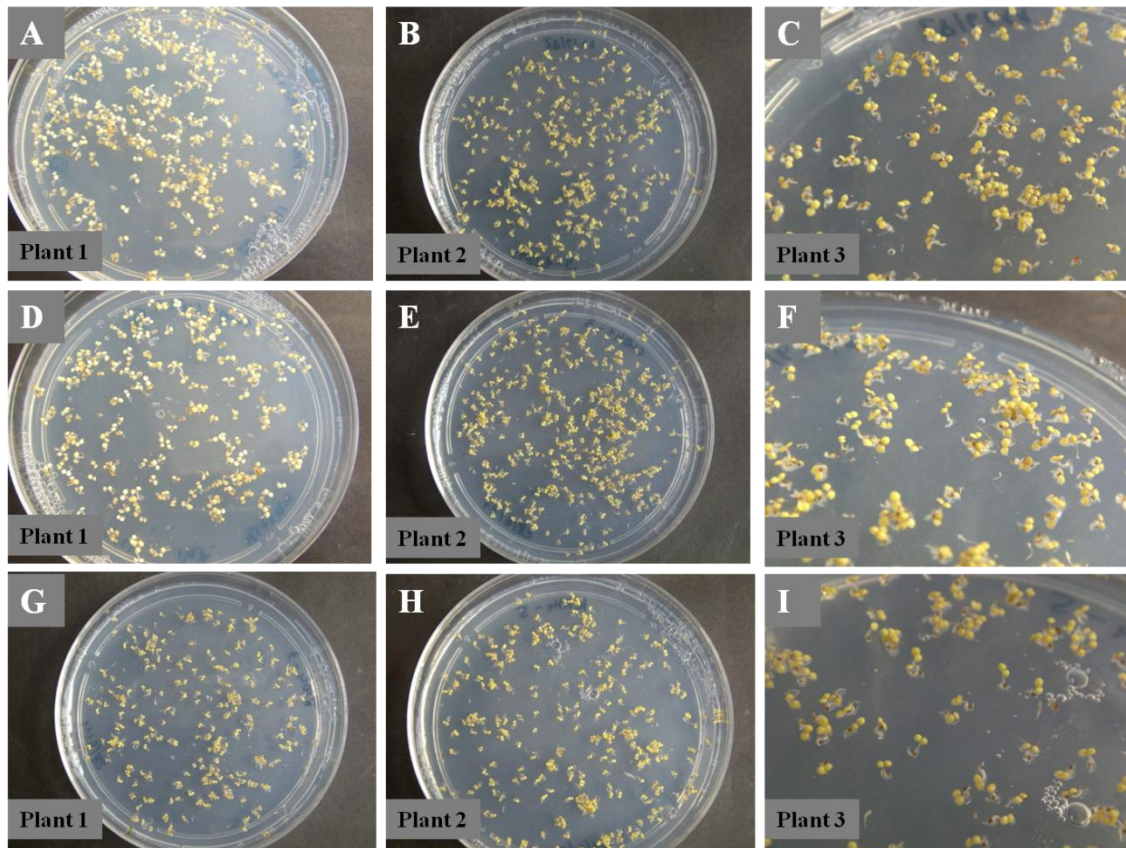
Figure 3.5 Selection of transformed seeds with the construct *10Op::CLE27* (**A, B, C**), *10Op::CLE41* (**D, E, F**) and *10Op::DVL16* (**G, H, I**). These seeds showed no signs of germination on a medium containing kanamycin. In most cases germination occurred but the developing seedlings rapidly turned yellow and died.

3.3- Homozygous Plant Production

Having already seeds with the construction inserted and willing to have the gene of interest integrated in a stable way in plants, plants were grew to maturity, seeds were harvested and selected in the petri plates with a selective medium (in this case it was used 1/2MS + 50µl/ml kanamycin), in a successive way, until 100% green plantlets could be obtained indicating that these plants that produced those seeds were already homozygous.

During the time of this work two generation of transformed *Arabidopsis* seeds were achieved carrying the construction *10Op::CLE21* and *10Op::CLE42* (F1 and F2) and one generation of the construction *10Op::CLE43* (F1). Also, seeds from F3 were collected from *10Op::CLE21* and *10Op::CLE42* and F2 seeds from *10Op::CLE43*. The figure 3.6 shows the plates with selective medium where germinated F1 seeds can be seen. The figure 3.7 shows the second generation of the *10Op::CLE21* and *10Op::CLE42* lines on the same selective medium.
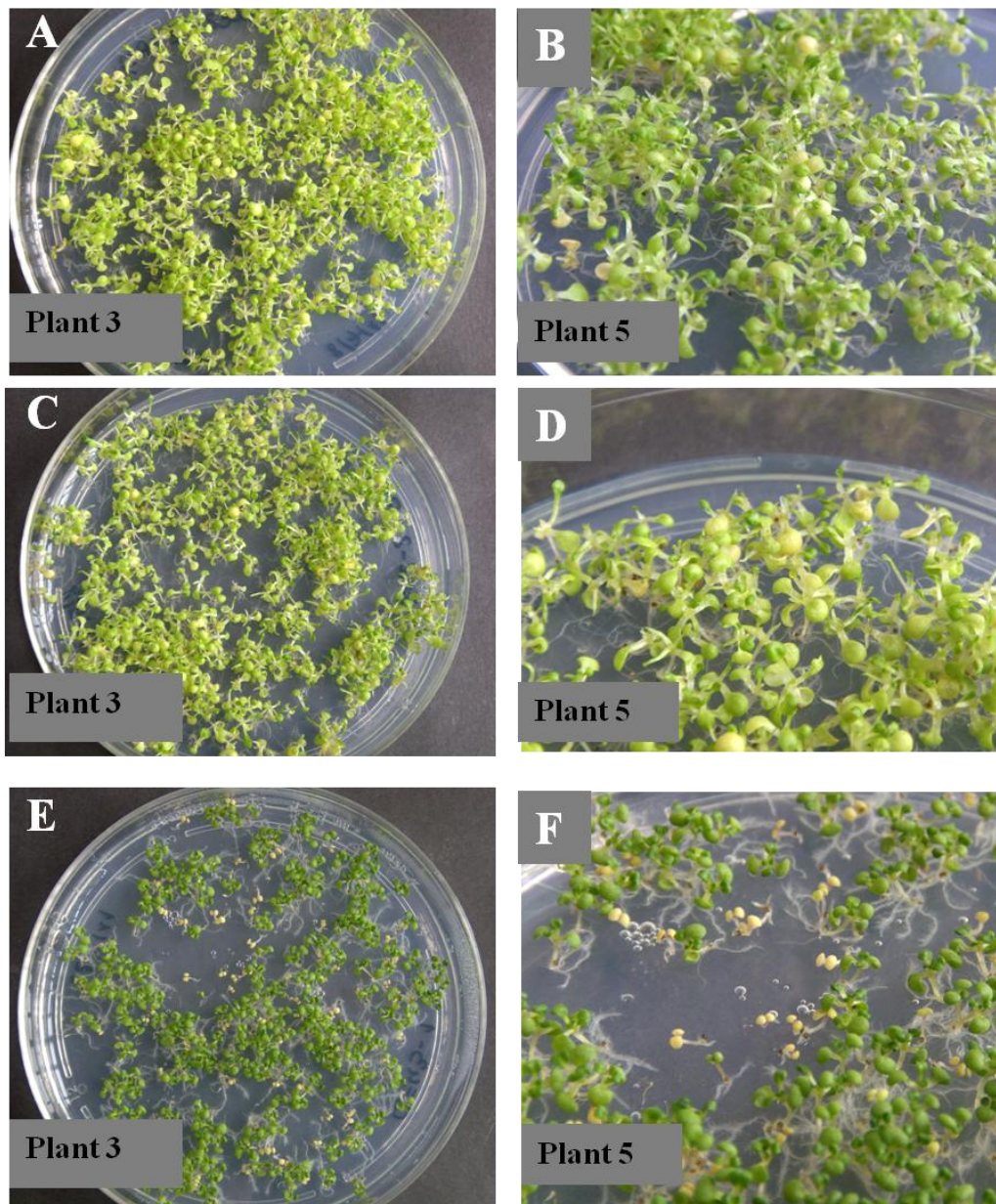
Figure 3.6 F1 generation of resistant plants obtained in petri plates with a selective medium. **A** and **B** correspond to *10Op::CLE 21* seeds; **C** and **D** to the *10Op::CLE 42* and **E** and **F** to *10Op::CLE 43*, from plants 3 and 5, that resist to seed selection.
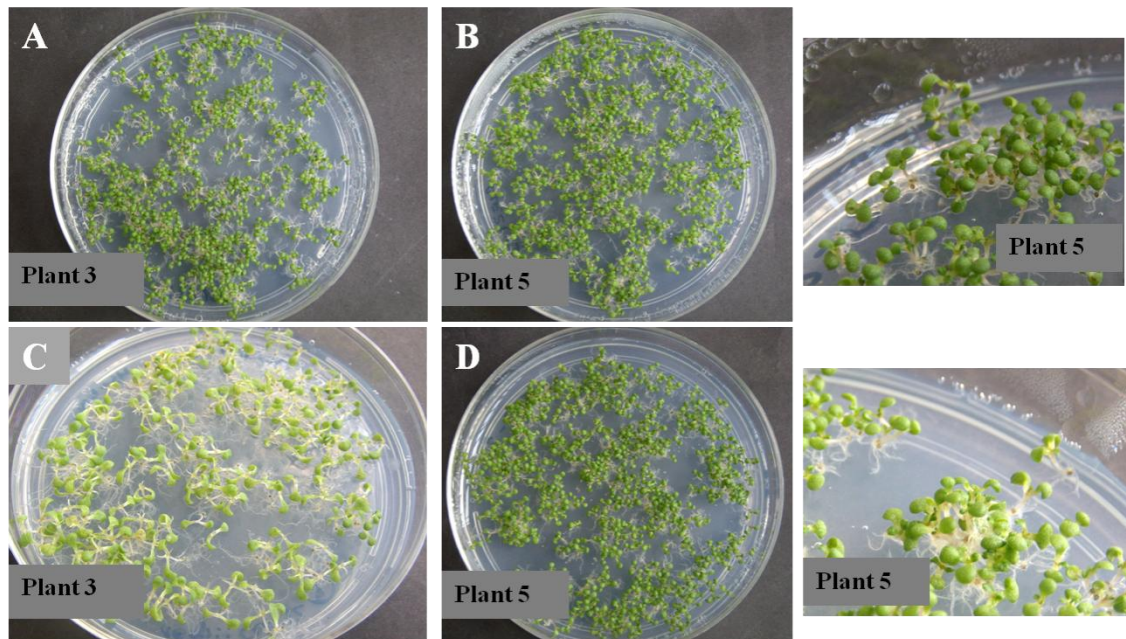
Figure 3.7 F2 generation of resistant plants. **A** and **B** correspond to *10Op::CLE 21* seeds from plants 3 and 5 (with a detail on the left); **C** and **D** correspond to the *10Op::CLE* 42 resistant seeds from plants 3 and 5, and a detail on the left of the plate corresponding to plant 5.

In all these selection experiments of homozygous production positive and negative controls were performed. For positive controls, seeds from transformed plants with the construction *6Op::GUS* from which, it was known that were resistant to kanamycin were used. These seeds were further grown on a 1/2MS medium + Kan and plants transformed with *NGATHA* gene, not resistant to kanamycin, in 1/2MS medium (without any antibiotic). For negative control, *NGATHA* seeds in 1/2MS + Kan were used. Results from these control assays can be seen in figure 3.8. As expected, in petri plates with 1/2MS + Kan, seeds from *6Op::GUS* grew (Fig. 3.8, **A**) and seeds with NGATHA germination was completely absent (Fig. 3.8, **B**). This control also indicated that the medium was the correct formulation since seeds from all the transformed plants (CLE constructions) (Fig. 3.8, **C**) and *NGATHA* seeds grew (Fig. 3.8, **D**) in 1/2MS without antibiotic.
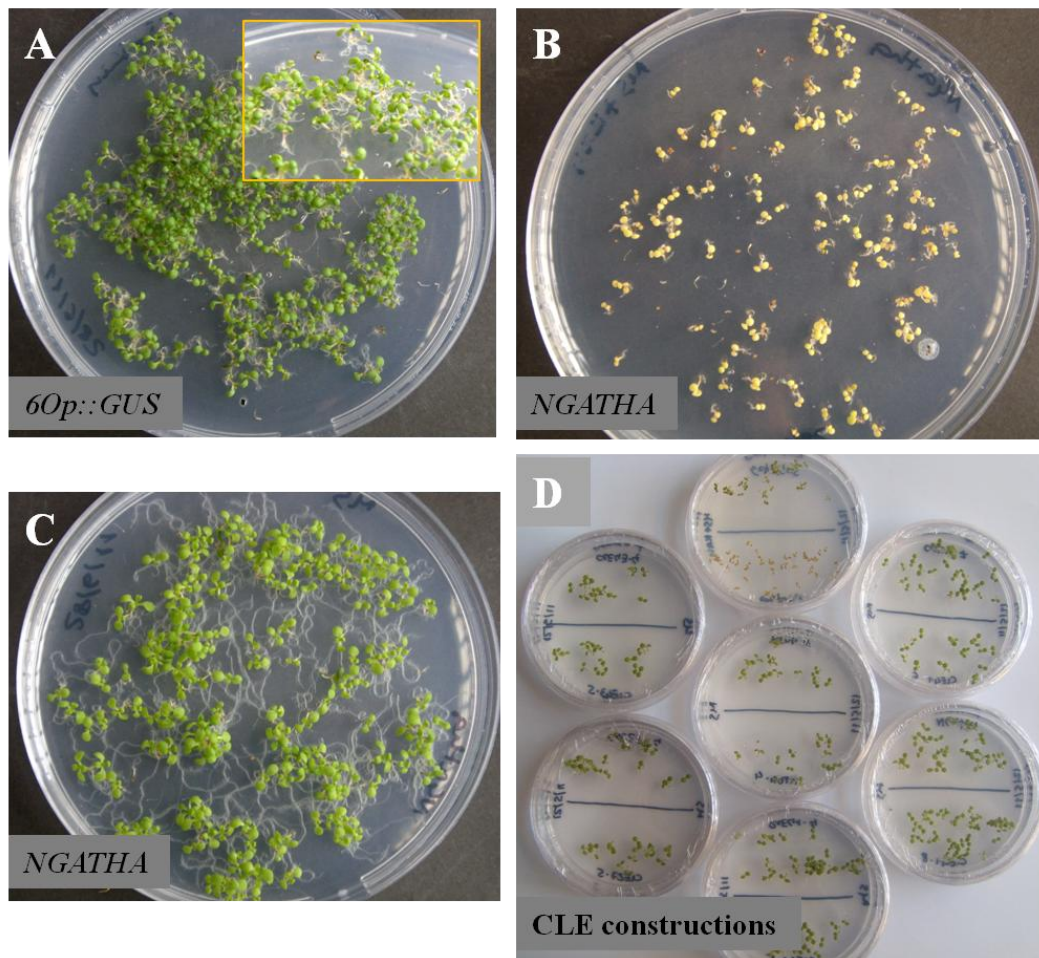
Figure 3.8 Positive: *6Op::GUS* (1/2MS+Kan), *NGATHA* (1/2MS) and *CLE* (1/2MS) and negative: *NGATHA* (1/2MS+Kan) control assays for seed selection and homozygous production.

After the plants from these lines were transformed and the seeds were selected, it is necessary to confirm whether those plants have the construction carrying the gene of interest. For that, the plants were left to grow till the inflorescence stage time at which the DNA was extract. Using the CTAB protocol as described in section 2.3.6.2, the DNA was obtained. The figure 3.6, **A** shows the result of the DNA extraction where the amount of DNA extracted can see. After that a PCR was performed in order to confirm if the DNA had the right constructions (Fig. 3.8, **B**, **C** and **D**). In **B** and **C**, the agarose gels shows that when the *nptII* primers (kanamycin resistance gene) were used on plant transformed with *10Op::CLE21*, *10Op::CLE42*, and *10Op::CLE43*, a clear band is present demonstrating therefore that the T-DNA was integrated when the infection by *Agrobacterium* occurred. When used the primers 10Op F and CLE R, two intense bands appeared corresponding to the *10Op::CLE43*, resulting from the extraction of DNA

from transformants and F1 generation plants. No visible bands were found on the agarose gel resulting from the PCR using the primer 10Op F and CLE R that can be due to changes on the hybridization of the primers to the T-DNA when it is inserted in the genome.
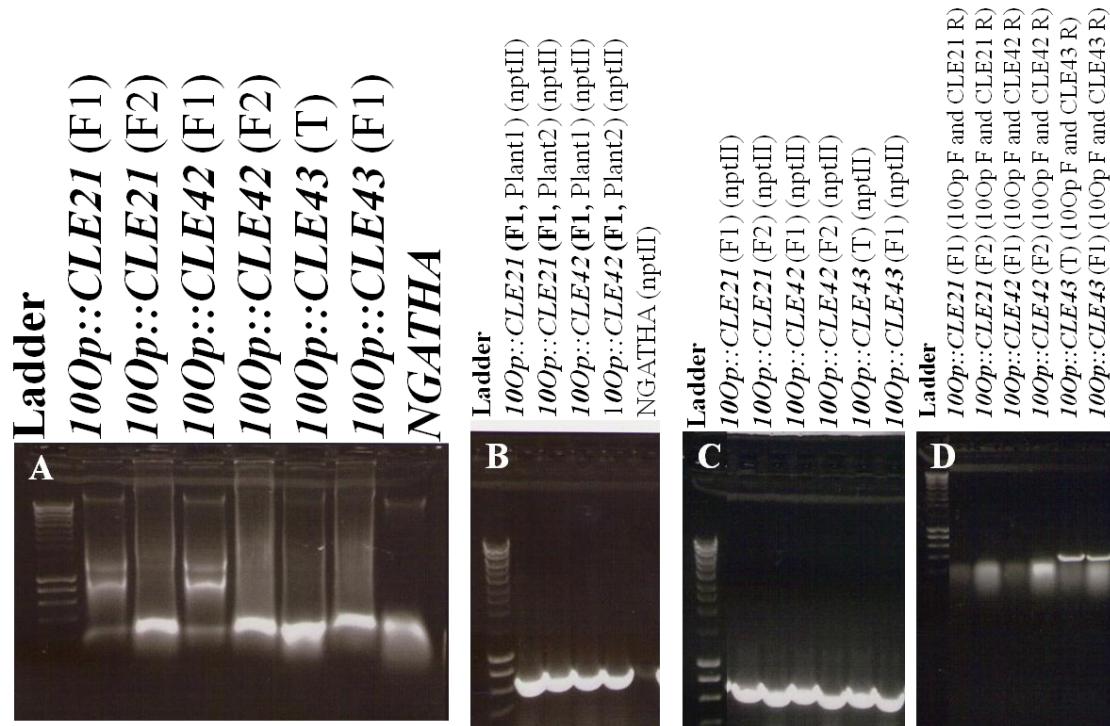
Figure 3.9 Electrophoresis gels resulting from the DNA extraction (**A**) and PCR (**B**, **C** and **D**) where we can observe that the lines in study have the constructions inserted in the genome. In **A** we can see the result from the DNA extraction and in **B** and **C** there is the PCR performed to these DNA samples using the primer *nptII* F+R, and in **D**, a PCR using the 10Op F and CLE R primers applied to the same DNA.

The crosses between those lines and the *ATHB8* and *ATHB15* transformed plants (seeds already obtained) can now be performed. For this purpose, all the plants were put to grow at the same time in order to have all the plants flowering more or less at the same time. Following the crosses seeds have to be double selected in the same selective medium with BASTA (herbicide that select the *ATHB8* and *ATHB15* seeds) and kanamycin (select the 10Op lines). These experiments have already started, however, the time for this project was limited, so no data are still available.

CHAPTER IV
DISCUSSION

In this work, three lines were transformed by floral dip method which resulted in different rates of transformation. The choice of the method equally influences the transformation rates according to some investigators. Clough and Bent (1998) found that the rate of transformants can reach 0.5 to 3% using the floral dip method (Clough and Bent, 1998) or up to 4% accordingly to Desfeux *et al.* (2000). Results with the vacuum infiltration transformation methods have reported lower rates of transformants, usually around 1% (Bent, 2000; Desfeux *et al.*, 2000).

We have achieved distinct rates of transformation of the three lines under study. The line *10Op::CLE43* showed 0,53% of transformants, while the lines *10Op::CLE21* and *10Op::CLE42*, the rates were much higher than expected showing percentages of transformation of 4,93% and 9,60%, respectively. One of the possible explanations for this difference on the transformation rates might be due to the stage of the plant development that should present unopened floral buds. In the case of the line *10Op::CLE21* it is possible that the plants used for transformation were not exactly at the best stage, a condition that often occurs. Other limitations could be related to the infiltration medium that contains high sugar concentration and/or to the inclusion of a surfactant which is also important for an efficient *Agrobacterium* infection at the time of floral dipping. Plant covering for 24h following dipping may also be a reason for the higher transformation rates obtained, as suggested previously by Clough and Bent (1998). With so many factors influencing the transformation rates, the variations observed between different laboratories or experiments are expectable.

When sowing the transformats after each generation in order to obtain homozygous plants, a F1 generation consisting of 25% kanamycin-resistant plants was expected. From these homozygous plants a F2 generation consisting of 100% of resistant plants must be obtained, however, these expected results could not be obtained with the lenes *10Op::CLE21* and *10Op::CLE42*. In the F1 generation, we observed that the two lines showed a lower percentage of germinated seeds (4,20% and 6,60% respectively). The stabilization of the transgene in the plant influences these rates as some can incorporate the T-DNA when the infection occurs but are unable to keep it in the genome. As for *10Op::CLE43* the results were as expected as the percentage of resistant seeds was 22%, quite around the 25% expected. For the F2 generation the

resistant seeds follow the expected with around 90% of resistant seeds from *10Op::CLE21* and *10Op::CLE42*.

To make sure that the transgene is stably integrated in plant we should achieve 100% of resistant seeds, likely to be achieved in the next generation (F3) and use these plants to cross them with the activator lines (*ATHB8* and *ATHB15*). We need to use homozygous plants to ensure each line transmit to the offspring one copy of the T-DNA necessary to promote the trans-activation.

During this work we came across to some setbacks. We tried several times the electroporation in the lines to be transformed with no success, despite the fact that electroporation could be used efficiently on nearly all cell and species. Miller and Nickoloff (1995) show that in transformation of *E. coli* by electroporation, 80% of the cells received the foreign DNA. The amount of DNA required is smaller. However, wrong length or ineffective intensity of the pulses may cause some pores to become larger or fail to close hence causing cell damage or rupture. Also, the material that enters or goes out of the cell during the time of permeability is relatively nonspecific. This may result in improper cell function and cell death (Weaver, 1995). Anyone of these factors could be the reason for failure on transformation. Despite these unsuccessful factors, the lines *10Op::CLE27, 10Op::CLE41 and 10Op::DVL16* could be transformed. The seeds that were not resistant in selective medium in some lines of transformants could be caused by an inappropriate stage of plants to be transformed or by other cause related to the process of *Agrobacterium* infection.

The DNA extraction of *Agrobacterium* using the High Pure Plasmid Isolation Kit (Roche) wasn't successful after several repetitions of the protocol so an artisanal way to extract DNA (boiling miniprep) showed best results. This probably happens because *Agrobacterium* cells are larger than *E.coli* cells (cells for which the Roche protocol is recommended) clotting the columns, retaining the DNA and preventing it from being eluted.

Investigations involving genetic approaches on plant development studies always carry several problems associated with them. Besides those setbacks we have mentioned earlier, we have had other constrains. This master thesis is integrated in a, larger project being impossible in the time designated for a master study to achieve

solid results on plant development. Time was consumed on protocol that didn´t worked or when plants had pass beyond their optimal stage and we had to wait for another set of plants, when we couldn't go further because a PCR result wasn't the expected, etc. However all the work we have done is very important for the further development of the project. The next experiments include the hand-cross of the activator and reporter lines in order to obtain a generation seeds where trans-activation occurs and as these plants grow which phenotype will be analyzed with the use of techniques such as histology and histochemistry. Induction of secondary growth will also be performed on plants obtained after hand-cross because according to Ito et al. (2006), a B-type *CLE* gene, *CLE41* used in this study suppress tracheary development or in other words, inhibits xylogenesis. By over-expressing the gene *CLE41* in procambium cells because by trans-activation, the promoter driving the expression of *CLE41* is the *AtHB8* and *15* (both procambium specific genes- see introduction), we expect a reduction of xylem production or even its complete inhibition. Therefore, we can expect a shrub, dwarf phenotype, similar to what was observed by Strabala et al. (2006).

Concerning the gene *CLE27*, being an A-type *CLE* gene, is main action is on root development by inhibiting its growth (Kinoshita et al., 2007). Once we will be over-expressing it on the vascular system of the stem, we do not expect phenotypes but those results need to be confirmed by *in situ* immunolocalization.

Several studies have demonstrated that these trans-activation systems are efficient in different species and with different goals:

Moore *et al.* (1998) published their work relying on the development of the pOp/LhG4 system, which was the one we used on this work. The reporter plants contain the plasmid where we have cloned the gene of interest downstream of the Op promoter that has binding sites for a transcription factor, not present in plants (pOp-GUS). In tobacco, crosses between pOp-GUS plants and pLh-G4 generate F1 seedlings resistant to hygromycin and methotrexate and testing GUS activity, enzyme activity was observed in all organs, and in fact, this system was effective in three consecutive generations of transgenic plants (Moore *et al.*, 1998).

Laplaze *et al.* (2005), in order to study mechanisms of lateral root development in *Arabidopsis*, screened F1 GAL4-GFP lines showing GUS activity in root pericycle cells in front of the xylem pole. Two lines were isolated and they turned out to be two enhancers trap lines that will be useful to study molecular and cellular mechanisms of lateral root development with the use of GAL4/UAS trans-activation system (Laplaze *et al.*, 2005).

Johnson *et al.* (2005) and Liang *et al.* (2006) also used a trans-activation system for to analyze gene expression in specific types of cells in rice and its physiological function. The system was based on two cassettes, one with the promoter::UAS::gene of interest::GUS and the other with GAL4::GFP enhancer trap lines. The results showed that GFP inflorescence levels were positively correlated with GUS staining intensity which means that the transcriptional activator GAL4 activated the gene of interest, downstream of UAS (Johnson *et al.*, 2005; Liang *et al.*, 2006).

Hawker and Bowman (2004) tested the expression pattern of Class III HD-Zip and four *KANADI* genes in a trans-activation system with a *pKAN::LhG4*, *pIII HD-Zip::LhG4* and *6Op::GUS* constructions, resulted in the expression at low levels of the four genes in the phloem of the root. This suggests that both genes families are expressed in complementary patterns in developing lateral roots playing functional roles in lateral root formation (Hawker and Bowman, 2004).

The studies mentioned above demonstrate the applicability of the trans-activation and its specificity at cellular level. It has been demonstrated that the genes of interest being analyzed were only expressed in cells where the promoters are also induced. The results of other groups give us confidence for the applicability of our system. The lines already obtained in this study will be subjected to further analysis and the studies will continue.

CHAPTER V
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