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Studies on the control of cell size homeostasis, programmed cell death and gene expression in plant meristems

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ABSTRACT

Plant development relies on the control of proliferation and differentiation of meristematic stem cells. The E2F transcription factors are key components of the RB/E2F pathway, which regulates the G1/S transition of the cell cycle and coordinates stem cell maintenance and differentiation. The E2Fs act as activator or repressors of E2F-dependent genes and their interplay controls cell cycle progression as well as endoreduplication and DNA repair.

This thesis investigated in *A. thaliana* the role of activating E2Fs in two processes that are important for plant development: the emergence of floral buds at the inflorescence meristem flanks and the programmed cell death (PCD) that occurs in root meristems upon DNA damage. Overexpression of AtE2Fa in floral meristems did not alter the coordination between cell growth and DNA synthesis, and the constitutive overexpression of AtE2Fa or AtE2Fb did not change the PCD pattern induced by DNA damage in root meristems. However, co-expression with the dimerizing partner AtDPa might be necessary to promote the cell cycle progression, and possibly affect stem cell homeostasis and/or PCD in plant meristems.

Additional work for this thesis concerned also the study of regulatory elements promoting the meristematic expression of the *AtDRTS2* gene, previously shown to be negatively regulated by E2F factors, that results to be upregulated by a HEX site in the promoter and by the intron in the 5' untranslated region.

CHAPTER 1. Role of the AtE2Fa transcription factor in cell size homeostasis during floral bud formation

1. INTRODUCTION

1.1. The cell cycle and its regulators

The cell cycle is a fine event that allows cells to divide in two daughter cells. The cell division is controlled by different proteins that, through their interaction and activity, transmit endogenous and external signals to the final division.

Although animals and plants share most of the molecular complexes involved in cell cycle regulation, in plants they play an important role in modulating postembryonic development. During the entire life of the plants, new organs are formed from specialized zones known as meristems: the shoot apical meristem (SAM) and the root apical meristem (RAM). In these regions, a pool of undifferentiated and pluripotent cells provide a cell progeny that can undergo different fates. Under developmental and environmental signals, they can keep proliferating or begin a differentiation program, allowing the emergencing of new organs. Therefore, the investigation of the molecular basis of cell cycle progression is necessary to understand how plants develop and grow.

The cell cycle is usually divided in 4 phases: G1, S, G2 and M. In S phase cells duplicate their DNA, while in M phase chromosome segregation and cell division take place. G1 occurs between the S phase and the previous mitosis, whereas G2 separates the S phase and the next mitosis. In some cases, cells can also exit the cell cycle and enter a quiescent state, known as G0, or undergo endoreduplication in which DNA replication is not followed by cell division, resulting in an increase in ploidy.

The mechanism of cell cycle regulation is common in eukaryotes and involves the activity of cyclin-dependent kinases (CDKs). The CDKs belong to a class of serine-threonine protein kinases that associated to regulatory proteins, known as cyclins, phosphorylate specific substrates allowing the G1/S and G2/M transitions. In yeast, a single CDK, showing the PSTAIRE sequence in the cyclin binding domain, controls

cell cycle progression, whereas in higher eukaryotes more CDKs control the cell cycle checkpoints. Regarding plants, the sequencing of the *Arabidopsis thaliana* genome allowed the discovery of different types of CDKs. Among these, CDKA possesses the PSTAIRE domain and its mRNA and protein levels are constant throughout the cell cycle, suggesting a role in both G1 to S and G2 to M transitions (Inzè and De Veylder 2006). In mammalian cells, the G1/S transition is triggered by CDK4 and CDK6 but orthologs of these kinases have not been identified in plants. This suggests that CDKA is the only CDK active and necessary for plant G1/S transition. CDKB-type kinases have been described only in plants, in both monocotyledonous and dicotyledonous, (Hirayama et al., 1991; Joubès et al., 2000; Boudolf et al., 2001) and are divided in two subgroups, CDKB1 and CDKB2, with two member each (CDKB1;1, CDKB1;2 and CDKB2;1, CDKB2;2). In place of the PSTAIRE sequence, found only in CDKA, they possess PPTALRE and PPTTLRE sequences respectively. It has been documented that CDKB1 expression is high in S, G2 and M phases, whereas CDKB2 trascripts accumulate in G2 and M phases (Fobert et al., 1996; Magyar et al., 1997Breyne et al., 2002; Corellou et al., 2005). It has been demonstrated that CDKB1 activity is important to progress through mitosis because its down regulation, using a dominant negative mutant, blocks G2\M transition and increases the 4C content in tobacco cells (Porceddu et al., 2001).

The cyclin-dependent kinases of type C possess a PITAIRE or SPTAIRE motif and interact with CYCT. The CDKCs have been proposed to be homologous to the animal CDK9 that is not involved in cell cycle progression, but plays a role in the control of transcription (Simone et al., 2002). In plants, CDKC transcripts have been detected in epidermal and flower tissues. Moreover, it has been demonstrated that this kinase is able to phosphorylate the CTD of RNA polymerase II, confirming a role in transcription control (Barrôco et al., 2003). On the other hand, CDKE has been proposed to be homologous to animal CDK8 and its role in cell cycle progression is still unclear but has been demonstrated to be important for carpel and stamen identities in floral organs (Wang and Chen, 2004).

The cyclin-dependent kinases activity is controlled by different cell cycle regulators such as cyclins and CDK-activating kinases (CKAs), or inhibitor proteins known as

Kip-related proteins (KRP). In Arabidopsis, 32 cyclins with putative roles in cell cycle progression have been identified. Based on amino acid sequence and functional analysis, they have been organised in 4 different groups: 10 A-type, 11 B-type, 10 D-type and 1 H-type. 17 additional cyclins, classified in C, P, L, T type, are also present but their role in cell cycle progression is still unclear. The presence of so many cyclins might be linked to the extensive duplication of the Arabidopsis genome or to the plasticity of plants as sessile organisms (Simillion et al., 2002; Inzè and De Veylder 2006). Cyclins are controlled by transcriptional regulation and protein destruction mechanisms and their fluctuation mirrors the cell cycle progression. Cyclin A-type and B-type possess a destruction box that is identified as an anaphase-promoting complex target (APC), allowing APC-mediated protein degradation, whereas D-type cyclins are degraded by the ubiquitin proteasome system through interaction with the SCF complex. As A-type cyclins play a role in S phase progression, they appear at the beginning of S phase and are destroyed in G2/M, whereas B-type cyclins, controlling G2/M transition and mitosis, appear during G2 and are destroyed during anaphase. D-type cyclins control the transition from G1 to S phase and their expression has been shown to be controlled by hormones and plant growth factors (Inzè and De Veylder 2006). This is the general scenario concerning the roles attributed to cyclins in cell cycle regulation. However, in specific contexts, different functions have been reported throughout the cell cycle. For example, in *Medicago sativa* it has been reported that CYCA is involved in G2/M transition (Roudier et al., 2000), and an additional function in G2/M transition has been proposed for D-type cyclins (Kono et al., 2003). Experiments on BY-2 cell suspension have demonstrated that the tobacco CYCD3;3 and *Anthirrhinum majus* CYCD1;1 were able to stimulate both S phase and mitotic entry (Koroleva et al., 2004 Nakagami et al., 2002). In addition, a transcriptional peak for D-type cyclins in G2/M transition has been reported (Meszearos et al., 2000; Nakagami et al., 2002; Menges et al., 2005).

As mentioned above, the activity of CDKs is also regulated by a group of cyclin-dependent kinases known as CDK-activiting kinases (CAKs). Plants possess CDKD and CDKF that regulate the activity of CDKs by phosphorylation of Thr160, inducing

a conformational change allowing the association to the downstream substrates (Inzè and De Veylder 2006).

CDKs activity is also controlled by inhibitory phosphorylation, as reported for the G2/M transition in animal cells during which CDKs are negatively regulated by WEE1-like kinases and activated by CDC25 phosphatase. Plants possess a WEE1 kinase that phosphorylates CDKs at threonine-14 and tyrosine-15. A CDC25 counterpart, lacking the regulatory domain found in other CDC25 proteins, has been identified in Arabidopsis (Francis, 2011). Since Arabidopsis CDC25 is unable to complement a temperature-sensitive *cdc25*-mutant of fission yeast and a T-DNA insertional line for CDC25 develops normally (Dissmeyer et al., 2009; Spadafora et al., 2011), it is believed that the plant CDC25 does not have any role in the regulation of G2/M transition.

Moreover, plants have cyclin-dependent kinases inhibitors known as CKI or Kip related proteins (KRPs). Arabidopsis possesses 7 KRPs that are able to interact with D-type cyclins. KRP1 and KRP2 overexpression induces a decrease in petal cell number (Zhou et al., 2002) and a reduction of cell number in leaves **(**Donnelly et al., 1999), demonstrating a clear effect on cell cycle progression.

1.2. E2F/RB pathway

Many genes have a cell cycle-regulated expression profile, suggesting that the activity of specific transcription factors and precise mechanisms of transcriptional regulation are important for the control of cell cycle progression.

The E2F/Retinoblastoma pathway is crucial to trigger the transition from G1 to S phase and, although absent in fungi, is well conserved among animals and plants. The typical E2F transcription factors bind the DNA at the canonical sequence TTTSSCGS (S is either C or G) in a complex with their dimerization partners DP. In the general model proposed, the Retinoblastoma protein (pRB) is associated with E2F, repressing E2F-regulated gene transcription in G1 cells. Upon mitogenic stimuli, the cyclin-CDK complexes phoshorylate pRB, triggering the release of E2F that is now able to act as a transactivator, allowing the expression of genes involved in DNA replication and cell cycle progression (Fig. 1).

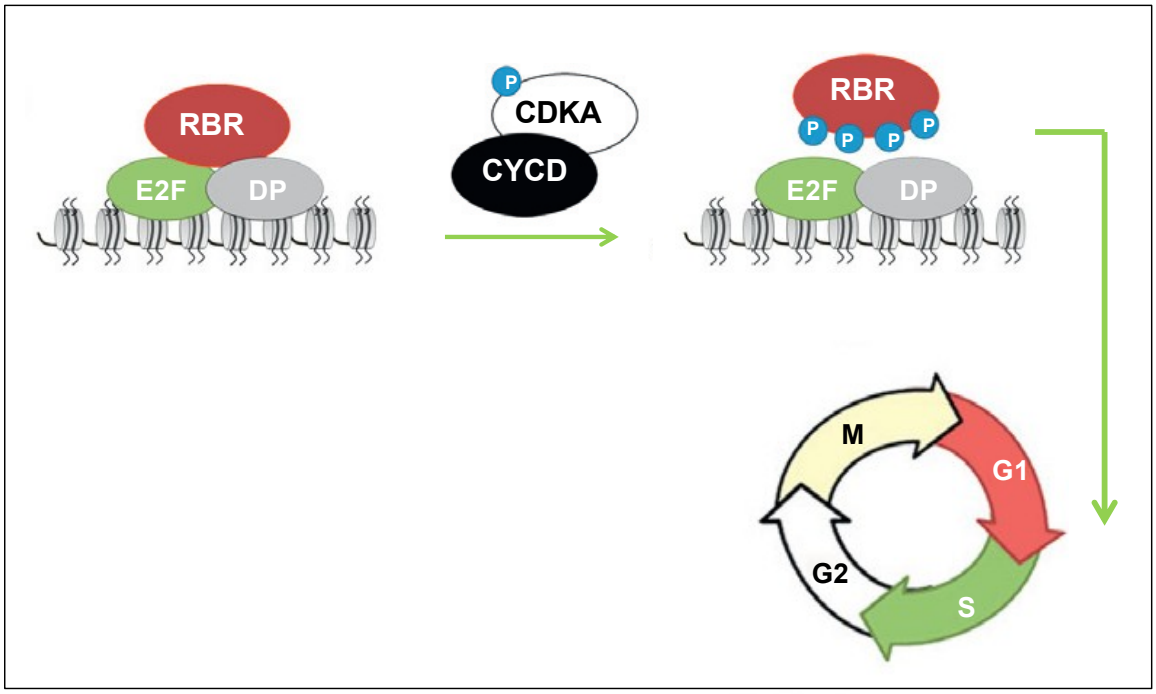


Figure 1: General mechanism of E2F/RB pathway (modified from Gutzat et al., 2011)

1.2.1. E2F transcription factors

E2Fs are an important class of transcription factors characterized by a highly conserved DNA domain. They have been discovered for the first time as an E2 promoter-binding factor, able to activate the E2 gene in adenovirus. The binding sequence is TTTCGCGC and it has been found later in several promoters of genes whose expression is necessary to entry in S phase (Inzè and De Veylder 2006).

The E2F transcription factors family is known in higher eukaryotes and found in both animals and plants. Regarding plants, they have been described in different species: wheat, tobacco, carrot, Arabidopsis and rice (Ramírez-Parra et al., 1999; Sekine et al., 1999; Albani et al., 2000; Magyar et al., 2000; Ramirez-Parra et al., 2000; Kosugi and Ohashi, 2002).

In Arabidopsis six E2F genes have been identified and divided in two groups. AtE2Fa-c, known also as typical E2Fs, belong to the first group and form heterodimers with DP proteins in order to bind the DNA. They possess a highly conserved N-terminal DNA-binding domain, followed by a DP heterodimerization domain, by a marked box and by C-terminal transactivation domain, containing the retinoblastoma protein (RB) binding region. This organization is also found in mammalian E2F1-E2F5 proteins (Shen, 2002).

The second group of E2F transcription factors (E2Fd/DEL2, E2FEe/DEL1, E2Ff/DEL3), known as atypical, has been identified first in plants and later in mammals, and differ structurally from the one described above. They possess a duplicated DNA binding domain (DBD) that allows the binding to the consensus sequence in a DP independent manner. Studies in plants and animals have shown that mutation of both DBD abolish their capacity to recognise and bind the DNA, suggesting that in atypical E2Fs the duplicated domain acts as the interface of E2F-DP heterodimers (Lammens et al., 2009). The atypical E2Fs lack the transactivaction domain and are believed to act as transcriptional repressors. This has been confirmed by different studies on animal and plants in which the knockout of atypical E2Fs caused upregulation of E2F-regulated genes (Ramirez-Parra et al., 2004; Vlieghe et al., 2005; Li et al., 2008; Zalmas et al., 2008). Moreover these E2F transcription factors lack the marked box domain that mediates the interaction between typical

E2Fs and other proteins that can affect the DNA binding specificity (Black et al., 2005).

Although the atypical E2Fs can act as inhibitor of gene transcription they are characterized by the absence of the BR binding domain, suggesting that they can act as repressors in a Retinoblastoma independent manner (Lammens et al., 2009).

So far, many studies have been designed to understand the role that E2F trascription factors might play in cell proliferation and developmental programs. Among typical E2Fs, it is well documented in Arabidopsis that AtE2Fa and AtE2Fb function in stimulating cell division, whereas AtE2Fc is involved in cell division delay and S-phase gene repression. As demonstrated by del Pozo et al. (2002), *AtE2Fc* is expressed in dividing cells as well as in differentiated cells like trichomes. Its overexpression caused a decrease in the expression of the cell cycle gene *CDC6* and affected cell division and size (del Pozo et al., 2002). On the other hand, plants with reduced AtE2Fc levels had organs with more but smaller cells and showed an increased in E2F target gene expression in developing tissues. Moreover they were characterized to possess more lateral roots, suggesting that AtE2Fc can be involved in the control of lateral root initiation (del Pozo et al., 2006).

In 2002, Rossignol et al. have investigated the role of AtE2Fa and AtDPa in stimulating cell proliferation in protoplasts from mature leaf tissues. Measuring the BrdU incorporation and checking H4 transcript levels, they demonstrated that AtE2Fa and AtDPa were able to induce cells to re-enter S phase (Rossignol et al., 2002).

The function of AtE2Fa in stimulating cell proliferation has also been investigated analysing plants overexpressing this transcription factor. These plants showed enlarged cotyledons, resulting from an increase in the number of cells, and microscopic analyses of cotyledon tissues revealed that cell differentiation was clearly delayed. Moreover, the phenotype observed in AtE2Fa overexpressing plants was enhanced by the co-expression of AtDPa. This aspect has been confirmed by the increased expression of S phase genes in AtE2Fa-AtDPa plants (De Veylder et al., 2002).

Regarding the AtE2Fb transcription factor, a study on tobacco Bright Yellow-2 (BY-2) cells has demonstrated that the overexpression of AtE2Fb is able to up-regulate both S-phase and M-phase genes, causing shortened cell cycle duration, increased proliferation and a decrease in cell size (Magyar et al., 2005).

AtE2Fb overexpression has been shown to promote cell proliferation also in transgenic Arabidopsis plants, revealing an interplay between AtE2Fa and AtE2Fb in controlling cell cycle (Sozzani et al., 2006). Plants overexpressing AtE2Fb showed upregulation of *AtE2Fa* and *RNR* expression but downregulation of *AtE2Fe* whereas plants overexpressing AtE2Fa showed increased expression of *AtE2Fb*. As *AtE2Fa* lacks an E2F cis element in its promoter, the increase of *AtE2Fa* transcript level in AtE2Fb overexpressing plants has been explained as a consequence of an overall stimulation of cell cycle (Sozzani, 2006). On the contrary, *AtE2Fb* promoter possesses a functional E2F cis element which explains the up-regulation of *AtE2Fb* and its accumulation in plant overexpressing AtE2Fa (Sozzani et al., 2006).

The role of the E2F transcription factors is not restricted to cell proliferation but includes also the control of endoreduplication, which occurs during cell differentiation and is considered a modified cell cycle in which DNA duplication is not followed by cell division. The cell decision to undergo endoreduplication or mitosis takes place at G2/M transition and depends on the presence of cellular factors known as Mitosis-Inducing Factors (MIF). AtE2Fa-AtDPa overexpressing plants displayed ectopic cell division in some tissues but endoreduplication in other tissues, indicating that AtE2Fa might be involved in endocycle regulation (Inzè and De Veylder, 2006). In fact, it has been reported that two week-old AtE2Fa-AtDPa overexpressing plants are characterized by increased ploidy levels. Moreover larger and/or more nuclei have been found in cotyledons palisade cells of AtE2Fa-AtDPa transgenic line (De Veylder et al., 2002). All together, these data suggest that AtE2Fa-AtDPa overexpression triggers S phase progression that can be followed by cell division or endoreduplication depending on the MIF presence: cells that express this factor undergo mitosis, while cells lacking MIF are stimulated to re-enter S phase that leads to increase in DNA content (DeVeylder et al., 2002).

Up to recently, it was believed that in AtE2Fa-AtDPa overexpressing plant, the elevated AtE2Fa levels escaped from RETINOBLASTOMA-RELETED protein (RBR1) repression and promoted both cell proliferation and endocycle, depending on the tissue specificity. However, recently it has been demonstrated that these plants are characterized by a strong increase of RBR1 protein levels, as well as by elevated amounts of RBR1-E2Fa complexes in proliferating tissues, suggesting that the formation of this complex is required to stimulate cell proliferation (Magyar et al., 2012). Moreover, the creation of a truncated form of AtE2Fa, in which the C-terminal amino acids necessary for both RBR binding and transactivation were removed, confirmed that AtE2Fa stimulates proliferation and endocycle in a RBR-dependent way and through a RBR-free complex respectively. An analysis of the gene targets has revealed that in proliferating cells the E2Fa-RBR1 complex is able to repress genes such as *CCS52A1* and *CCS52A2* that are necessary for endocycle onset (Magyar et al., 2012).

1.2.2. Retinoblastoma protein

The Retinoblastoma protein (pRB) has been found in higher eukaryotes but no homologues have been identified in fungi (Durfee et al., 2000). In mammals, it is well studied because of its implication in cancer development. In fact the *pRB* gene is identified as a tumor suppressor and appears to be inactivacted in more than 70% of human tumors (Gutzat et al., 2012).

In higher plants, the counterpart of mammalian pRB is known as Retinoblastoma related protein (RBR). As pRB has a role in regulating the G1/S transition, many plant studies have been focused on RBR implication in development processes. Arabidopsis possesses a single gene coding for a Retinoblastoma related protein, whereas in maize two genes (*RBR1* and *RBR2*) have been found (de Jager and Murray, 1999). Like in animals, the proteins possess two conserved domains, known as A/B pocket domains, that represent a docking site of the E2F transcription factors. Moreover, like the animal counterparts, plant D-type cyclins possess a conserved N-terminal LXCXE motif that allows the interaction of RBR with CDK-cycD complexes, resulting in RBR phosphorylation (Sabelli and Larkins, 2009).

Recently, many studies have shown the involvement of plant RBR in cell proliferation, differentiation and in the maintenance of stem cell pool. Since it is known that the loss of RBR function is gametophytic lethal (Ebel et al., 2004; Johnston et al., 2008; Johnston and Gruissem, 2009), RBR functional studies have been performed using conditional mutants obtained through virus-induced gene silencing (Park et al., 2005, Jordan et al., 2007) or inducibile RNA interference (RNAi) (Wildwater et al., 2005; Borghi et al., 2010). In *Nicotiana benthaniana*, several E2F-regulated S phase genes, like ribonucleotide reductase (*RNR*), proliferating cell nuclear antigen (*PCNA*), mini chromosome maintenance (*MDM*), histone *H1* and replication origin activation protein (*CDC6*), were upregulated upon *RBR* gene silencing, suggesting a direct involvement of RBR in the control of cell division.

It has also been demonstrated that the local reduction of *RBR* expression in Arabidopsis root led to an increase of stem cells number and prevented their differentiation in columella cells and in lateral root cap tissues (Wildwater et al., 2005), whereas a *RBR* overexpression in tobacco determined a consumption of the stem cell pool in shoot apical meristems Wyrzykowska et al., (2006). Moreover, loss of *RBR* function in the SAM caused an increase in the number of proliferating cells and influenced negatively the formation of lateral organs (Borghi et al., 2010). All together, these results suggest that RBR is important to control the "stemness" in meristems, regulating the balance between proliferation and differentiation.

1.3. Plant development

The coordination between cell proliferation, expansion and differentiation is the key event that controls organism development. Whereas in animals organogenesis occurs during embryogenesis, in plants it is a post embryonic process that happens over the entire life of the organism. Stem cell niches in the meristems are the main players of this process, as they provide a pool of undifferentiated and pluripotent cells that can turn to a differentiated status, allowing the emergence of new organs. The shoot apical meristem (SAM) and root apical meristem (RAM) are localized at the end of the main embryonic body axis, where the stem cell maintenance and the differentiation processes are coordinated by genetic activities under the control of growth hormones.

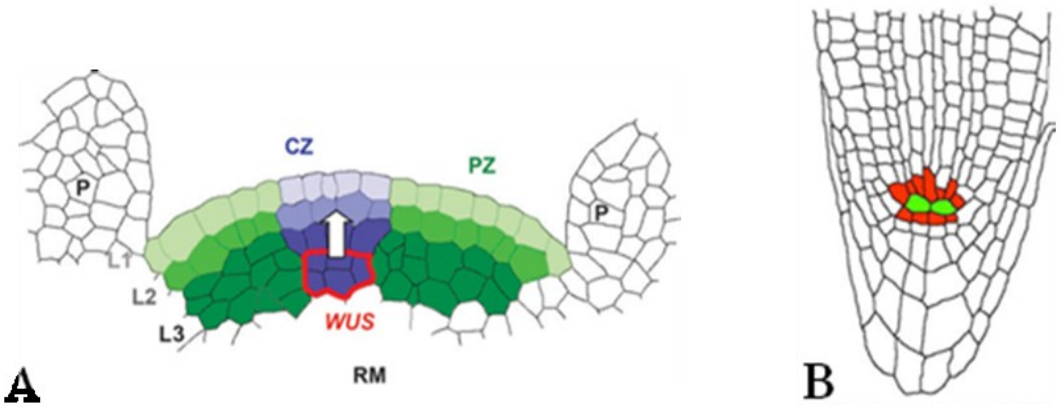


Figure 2: Structure of plant meristems.
In A shoot apical meristem organization: L1, L2, L3 represent the cell layers; the central zone (CZ) is highlight in blue and the peripheral zone (PZ) in green; in red the region where WUS is expressed; rib meristem (RM); primordial (P). In B root apical meristem organization: root inizials are marked in red; the quiescent centre (QC) is in green. (from Sablowski 2007 and Fulcher and Sablowski 2009 respectively)

1.3.1. Cell proliferation, growth and differentiation at the shoot apical meristem

During plant aerial development three different meristems are identified: the vegetative, the inflorescence and the flower meristems. Both vegetative and inflorescence meristems are indeterminate meristems that maintain a population of pluripotent stem cells and produce parts of the plant, whose size and shape depend on the local environment. On the contrary, floral meristem is determinate, as it stops producing new cells and forms floral organs (Sablowski, 2007).

The vegetative and inflorescence meristems sustain the indeterminate growth of plants through the activity of the stem cells located in the central zone (CZ). Surrounding the CZ, there is the peripheral zone in which stem cells descendents are recruited to form new organs, whereas the rib meristem (RM) is located below the central zone (Fig. 2A).

The shoot apical meristem is also organized in three layers of cells distributed parallel to the surface. L1 and L2 are the external layers in which most cell divisions are oriented tangentially to the meristem surface, whereas cells in the L3 layer divide in any direction. (Sablowski, 2007).

Upon germination, the shoot apical meristem is active as a vegetative meristem, in which the stem cells move from the central zone to switch on the differentiation program, forming leaves in the peripheral zone and stem tissues in the rib zone. Later, in response to environmental signals, the SAM switches from a vegetative meristem to an inflorescence meristem that begins to produce floral meristems on its flanks (Barton, 2010). Plant development requires the orchestration of these events that are based on the maintenance of SAM homeostasis.

The SAM homeostasis is controlled by the activity of *WUSCHEL* (*WUS*) and *SHOOT MERISTEMLESS* (*STM*) genes. They act synergistically and are required either for meristem development or for the vegetative, inflorescence and floral meristem maintenance (Sablowski, 2007).

The *WUSCHEL* gene encodes a homeodomain transcription factor that is expressed in few L3 cells in the centre of the meristem (Mayer et al., 1998) and is required to maintain the stem cell potential in all layers of the CZ (Wolters and Jurgens, 2009; Sablowski, 2007). The role of *WUS* in stem cell maintenance is performed by the

CLAVATA signalling pathway. Upon *WUS* expression, stem cells secrete the peptide CLAVATA 3 (CLV3) that binds a CLAVATA 1 receptor kinase (CLV1) and activates a feedback loop that represses *WUS*, in order to stabilize the size of the stem cell population during development (Wolters and Jurgens, 2009). In other word, WUS and CLV3 play an opposite role in shoot meristem maintenaice: WUS promotes stem cell activity while CLV3 suppress stem cell activity (Barton, 2010). It has been observed that *wus* mutants displayed a defective CZ that was not able to provide stem cells to the PZ for organ formation (Mayer et al., 1998). Moreover, ectopic expression studies have confirmed that WUS was sufficient to convert cells of organ primordia and root meristem into cells with characteristics of the shoot meristem CZ (Schoof et al., 2000; Gallois et al., 2004).

Along with these genes, *STM* (a KNOX-related gene) regulates cell number in the meristem by controlling cell differentiation and proliferation. Ectopic expression of *STM* in leaves caused the formation of outgrowths and induced the expression of the *CYCB1;1* gene in these tissues (Clark et al., 1996; Lenhard et al., 2002), whereas in strong *stm* mutants the meristem was absent at the end of embryogenesis (Barton and Poethig, 1993). Furthermore, it has been proposed that STM can delay differentiation in daughter stem cells, allowing their proliferation before being incorporated into organs (Wolters and Jurgens, 2009).

SAM organization and homeostasis demonstrate that plant development relies on the coordination between cell proliferation at the shoot apical meristem and differentiation events at its flanks. Therefore, a regulatory pathway might involve the cell cycle machinery. Concerning this, it has been observed that cells at the central zone differed from the peripheral one in terms of proliferation and growth rates. In Arabidopsis, it has been reported a cell cycle length of about 18-36 h and 36-72 h at the meristem periphery and the summit respectively (Reddy et al., 2004). Although cells didn't display any increase in division during primordium initiation, it was demostrated that cell proliferation rates increased up to three-folds after organ specification (Traas and Bohn-Courseau, 2005). To understand if organogenesis might be driven by spatial control of cell proliferation rates through the involvement

of cell cycle regulators, experiments have been set up to interfere directly with the cell cycle machinery.

In *Nicotiana tabacum*, the induction of CYCA and CDC25 at the shoot apical meristem increased the cell division frequency and locally disrupted the normal cell division pattern. Overall, the effects of the micro-inductions were limited in time and the meristem was relatively insensitive, although it has also been found that the local induction of CYCA modified the expression pattern of *NTH15*, a homeobox transcription factor of the KNOX family (Wyrzykowska et al., 2002). Moreover, the overexpression of a dominant negative CDKA caused a severe reduction in cell numbers in the shoot apical meristem but didn`t affect the size of the adult organs (Hemerly et al., 1995).

On the contrary, the overexpression of other cell cycle regulators causes effects in plant development and in some cases also in SAM organization. For example, in Arabidopsis it has been observed that the overexpression of CYCD3 caused the formation of curled leaves, a disorganised SAM and increased leaf number (Riou et al., 1999), while the overexpression of *Arabidopisis* CYCD2 in tobacco determined a significant increase in growth rate without any obvious effect on cell and meristem size (Cockcroft et al., 2000). Moreover, De Veylder et al., observed that plants overexpressing E2Fa-DPa displayed small size and an early arrest in development (De Veylder et al., 2002).

Although it is obvious that cell division is essential for plant development and to generate new organs and tissue, the results described above underline that the role of the cell cycle machinery in plant growth regulation is not completely understood and is still under debate.

Another aspect under investigation is the role of cell growth in morphogenesis. Regarding this point, it is well known that the cell wall extensibility is controlled by a family of extracellular proteins called expansins that are thought to be involved in organogenesis. Supporting this hypothesis, Pien et al. demonstrated that the local ectopic induction of expansin expression in the meristem induced morphogenesis, leading to the formation of leaves that were phenotypically similar to the one generated normally (Pien et al., 2001). Therefore, organogenesis is a complex process

that involves the coupling between cell division and cell growth, although it's not completely understood which process can trigger the other one. The molecular basis seems to involve transcriptional regulators belonging to the E2F transcription factors because trascriptome analyses have shown that the E2Fa-DPa complex can target both cells cycle genes and cell-wall-biosynthesis genes (Breyne, 2002; Vandepoele et al., 2005).

2. AIM OF THE PROJECT

The inflorescence meristem is necessary to assure growth and organogenesis during the generative phase of the plant life, since it provides cells that are recruited for bud formation. The emergence of flower primordia on its flanks implies changes in cell parameters as growth, division rates and division pattern. As E2F transcription factors are key regulators of cell cycle progression that are involved in the control of cell proliferation, differentiation and growth, in this thesis I focused on the role of AtE2Fa during bud development. Although the effects of AtE2Fa overexpression on cell proliferation have been already described, the effects of its overexpression on floral organ formation and on cell parameters have not been investigated yet. Here, to understand the relevance of AtE2Fa in these processes, transgenic lines have been created using a transactivation system that allowed the overexpression of AtE2Fa in floral meristems. Analyses of these lines using a recently developed method that combines 3D analysis of cell geometry and patterns of DNA synthesis allowed an evaluation of the role played by AtE2Fa in coupling cell proliferation and cell growth.

3. MATERIAL AND METHODS

3.1. Plant Material

Arabidopsis thaliana ecotype Landsberg was used throughout this research.

3.1.1 Seed sterilization

To sterilize 200 µl of seeds, they were put in labelled 2 ml Eppendorf tubes, placed under a tissue culture hood and 1 ml of sterilization solution (2.5 ml Dichloroisocyanuric Acid Sodium Salt 5%, 10 ml ethanol 100%, 7.5 ml dH_2O) was added. Tubes were shaken for 13 mins and, after removing the solution, the seeds were washed three times in 100% ethanol. The open tubes were left under the fume hood until seeds dried.

Approximately 20-30 µl of seeds were put in labelled 1.5 ml Eppendorf tubes and placed in a desiccator jar, under a chemical flow hood. In the jar, 3 ml hydrochloric acid ~36% was added in a becker containing 100 ml sodium hypochlorite ~10% and left for 3 hours with the open tubes to allow seeds to sterilize in chlorine gas.

3.1.2. Growth conditions

Seeds were sown directly onto germination media (GM): 4.4 g/l Murashige and Skoog salts, 1% glucose, 0.5 g/mL 4-morpholineethanesulfonic acid, 0.8% agar, pH 5.7. Media was autoclaved and left to cool before pouring into 90 mm petri dishes. For selection transgenic lines, 100 µg/ml Gentamycin (Sigma-Aldrich) was added to media after cooling to around 50°C. Plates were sealed with surgical tape 3M and placed for 48 hrs at 4°C in the dark. After stratification, plates were transferred to long day growth chambers at 21°C (16 hrs light 8 hrs dark). For plants grown to maturity, seedlings were transferred to soil after formation of the first two leaves. Plants were grown in JIC Arabidopsis soil mix consisting of Levingtons F2 compost (with Intercept) and grit at a ratio of 6:1. Seedlings were then placed into CER 51-10 under long day growth conditions (16 hrs light, day 18°C and night 20°C, humidity 80

3.1.3. Plant crosses

Crosses were carried out on flowers from primary or secondary inflorescences, using fine-pointed forceps and a binocular headband magnifier. To isolate female pollen acceptors, mature flowers and the shoot apex were removed from inflorescence meristems. The unopened floral buds were then emasculated leaving the immature carpel; this was performed on approximately 5 to 10 buds. Emasculated plants were then labelled and left for 2 days to allow carpel to mature. The carpel was then pollinated from mature flowers by dabbing anthers onto the stigma and left to mature into siliques; just before desiccation, siliques were bagged into 88mm x 120mm cellophane bags to collect seeds.

3.2. Cloning

All the enzymes and buffers used for cloning were supplied by Fermentas; Taq and dNTPs were supplied by GeneSpin. To purify the DNA the QIAquick PCR Purification Kit (Qiagen) and the QIAquick gel extraction kit (Qiagen) were used. Minipreps were performed with QIAprep Spin Miniprep kit (Qiagen).

3.2.1. *pOp::AtE2Fa* and *pOp::AtE2Fb* constructs

To clone the *AtE2Fa* cDNA into pPzp222-op-Nos, *pSTREP-AtE2Fa*, which contains the *AtE2Fa* cDNA cloned in bluescript KS-, and the pPzp222-op-Nos vector were first digested with *Xba*I and *BamH*I respectively. Both reactions were prepared as following: 10 µl DNA, 1.5 µl enzyme, 5 µl buffer 10X, 33.5 µl dH_2O and incubated at 37°C for 90 mins, followed by 65°C for 20 mins and DNA purification. To create blunt ends in both digested vectors, 40 µl of purified DNA was used in a Klenow reaction: 5 µl buffer 10X, 0.5 µl dNTPs (10 mM), 0.25 µl Klenow Fragment (10 u/µl), 4.25 µl dH_2O; incubated at 37°C for 10 mins and then at 75°C for 10 mins. The blunt-ended samples were purified and a second digestion with *Kpn*I was performed as following: 30 µl of DNA, 1.5 µl *Kpn*I, 5 µl buffer 10X, 13.5 µl dH_2O; incubated at 37°C for 90 mins and then at 65°C for 20 mins. After purification, 10 µl of both reactions were run in 0.8 % agarose gel to quantify the amount of DNA for the ligation reaction. The ligation was prepared as following: 6 µl of vector, 8 µl of insert

2 µl of T4 buffer, 1 µl of T4 ligase and incubated at 16 O/N. Ligation was used to transform DH5α cells: colonies were picked up and minipreps were produced. Plasmids were then digested to check for the insert and the right orientation.
The strategy described above was used also to clone the *AtE2Fb* cDNA (contained in *pSTREP-AtE2Fb*) in pPzp222-op-Nos.

3.2.2. *pOp::AtDPa* and *pOp::AtDPb* constructs

To clone *AtDPb* cDNA in pPzp222-op-Nos, *pSTREP-AtDPb*, that contains the *AtDPb* cDNA, and pPzp222-op-Nos were first digested with *Kpn*I as following: 10 µl DNA, 1.5 µl enzyme, 5 µl buffer 10X, 33.5 µl dH_2O and samples incubated at 37°C for 90 mins, followed by 65°C for 20 mins. DNA was purified and the second digestion with *BamH*I prepared: 40 µl of DNA, 1.5 µl enzyme, 5 µl buffer 10X, 3.5 µl dH_2O and samples incubated at 37°C for 90 mins, followed by 65°C for 20 mins. After purification, 10 µl of both reactions were run in 0.8 % agarose gel to quantify the amount of DNA for the next ligation reaction. The ligation was prepared as following: 5 µl of vector, 6 µl of insert, 2 µl of T4 buffer, 1 µl of T4 ligase and incubated at 16 O/N. Ligation was used to transform DH5α cells: colonies were picked up and minipreps were produced. Plasmids were then digested to check for the insert.
To clone the *AtDPa* cDNA in pPzp222-op-Nos a different strategy was used. *pOp::AtDPb* and *pSTREP-AtDPa* (containing the *AtDpa* cDNA) were first digested with *Sma*I as following: 10 µl of DNA, 1.5 µl enzyme, 5 µl buffer 10X, 33.5 µl dH_2O. Samples were incubated at 30°C for 90 mins. Then 1.5 µl of *Kpn*I was added and reaction temperature increased at 37°C for 90 mins, followed by 65°C for 20 mins. After purification, ligation was prepared as followed: 3 µl of *pOp::AtDPb* (KpnI/SmaI), 5 µl *pSTREP-AtDPa* (KpnI/SmaI), 2 µl of T4 buffer, 1 µl of T4 ligase and incubated at 16 O/N. Ligation was used to transform DH5α: colonies were picked up and minipreps were produced. Plasmids were then digested to check for the insert.

3.2.3. Electroporation of *E.coli* and *Agrobacterium tumefaciens*

To transform plasmid DNA into *Escherichia coli* DH5α and *Agrobacterium tumefaciens* ASE (resistant to Kanamycin 50 μg/ml and Chloramphenical 30 μg/ml), the electroporation method was used. For each transformation, 50 μl of electrocompetent cells were thawed on ice. 0.2 μl of plasmid prep was added to the cuvettes, placed on ice, and cells were then pipetted. The cuvettes were placed into a Biorad Genepulser and the following settings applied: Capacitance extender 250 μFD, Pulse controller 200 Ω, Capacitance 25 μFD, Voltage 2.5 KV and Resistence 200 OHMS for *E.coli*, 400 OHMS for *Agrobacterium.* After electroporation, 1 ml of cold LB was added to the cuvettes and mixed. The mixture was then transferred to an Eppendorf tube and incubated for 2 hrs at 37°C for *E.coli* or 28°C for *Agrobacterium.* To pellet cells tubes were spun; cells were resuspended in LB and spread in plates supplied with appropriate antibiotics. Plates were finally incubated O/N at 37°C for *E.coli* or 2 days at 28°C for *Agrobacterium.*

3.2.4. Plant transformation

The floral dipping method was used to transform Arabidopsis (Clough and Bent, 1998). For each transformation, a colony of *Agrobacterium* from transformed plate was picked up to inoculate 10 ml of LB containing Kanamycin 50 μg/ml and Chloramphenical 30 μg/ml, plus Spectinomycin 100 μg/ml The culture was incubated O/N at 28°C with shaking and then used to inoculate 500 ml LB. After incubation at 28°C O/N, the culture was ready to transform plants. Each culture was transferred into large centrifuge bottles, centrifuged at 7268 x g for 15 mins, at 20°C and the liquid poured away leaving a pellet. Infiltration media was prepared as following 4.3 g/l MS salts plus vitamins, 50 g/l sucrose, 300 μl/l silwet L-77, adjusted pH to 5.8 with KOH and kept cold. A small amount of infiltration media was first added to the bottles to resuspend cells and then the remaining was added up to 500 ml. Plants were dipped into infiltration media for 30 secs, placed on their side in a plastic bag for 24 hrs and left in the lab. The day after, they were moved to a containment glasshouse.

3.3. Genotyping

3.3.1. DNA extraction

To extract DNA from transgenic lines and amplify small DNA fragments (~ 200 bp) the follow method was used. Small leaf pieces were collected from each plant and placed in wells of 96-well plate. 50 µl NaOH 0.25 N were added and the plate was incubated at 96°C for 10 mins. After centrifugation at full speed for 5 mins, 50 µl of buffer 0.5 M Tris-HCl pH 8, 0.25% IGEPAL® CA-630 (Sigma-Aldrich) were added, followed by 50 µl HCl 0.25 N. Samples were incubated at 96°C for 10 mins, centrifuged at full speed for 5 mins and 1 µl used for PCR reaction.

3.3.2. PCR genotyping

After completing DNA extractions, PCR reactions were performed in order to confirm plants harbouring the transgene of interest. PCR reaction was prepared as following: 1 µl Genomic DNA, 1 µl Forward oligo 10 µM, 1 µl Reverse oligo 10 µM, 0.5 µl dNTPs 10 mM (Roche), 2,5 µl 10x Taq Buffer (Roche), 0.1 µl homemade Taq and 18.9 µl dH_2O. The following PCR programme was used for genotyping: 94°C for 5 mins then 30 cycles of 94°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs, followed by final 10 mins at 72°C. On completion of PCR, samples were run on 2 % agarose gel.

The primers (Sigma-Aldrich) below were used to look for Gentamycin marker:

GENT 1 FW	caagcgcgatgaatgtctta
GENT 1 REV	ggctcaagtatgggcatcat

3.4. Gene expression analysis

3.4.1 RNA extraction and reverse transcription RT-PCR

For Quantitative RT-PCR, RNA extraction was performed from 6 inflorescence apices for each line in three biological replicates. The materials were collected, placed in 1.5 ml Eppendorf tubes and frozen in liquid nitrogen. RNA was extracted using the RNEasy plant mini kit (QIAGEN) and treated with Ambion® DNA-*free* (Invitrogen), according to the manufacturer's instructions. The RNA concentration was measured using Thermo Scientific NanoDrop 1000 Spectrophotometer. 1 µg of DNAse treated RNA was reverse transcribed using 1.5 µl oligo dT (12-18) 50 µM (Invitrogen), 2 µl dNTPs 10 mM (Roche) and dH_2O to 30 µl final volume. Reaction was denatured at 65°C for 5 mins and placed back onto ice. Then the following was added: 6 µl 5X First Strand Buffer (Invitrogen), 1.5 µl DTT 0.1 M (Invitrogen), 1.5 µl RNasin RNase inhibitor (Promega), 1.5 µl Superscript III Reverse Transcriptase (Invitrogen). Samples were incubated at 50°C for 50 mins, then at 70°C for 15 mins.
To check for genomic contamination a PCR reaction was performed using the primers (Sigma-Aldrich) below:

ACT 2 FW	gcaccctgttcttctta
ACT 2 REV	aaccctcgtagattggcaca

PCR reaction was prepared as following: 1 µl cDNA, 1 µl Forward oligo 10 µM, 1 µl Reverse oligo 10 µM, 0.5 µl dNTPs 10 mM (Roche), 2,5 µl 10x Taq Buffer (Roche), 0.1 µl homemade Taq and 18.9 µl dH_2O; 1 µl of genomic DNA was amplified as control. The following PCR programme was used: 94°C for 5 mins then 35 cycles of 94°C for 30 secs, 60°C for 30 secs, 72°C for 30 secs, followed by final 10 mins at 72°C. On completion of PCR, samples were run on 2 % agarose gel.

3.4.2. Real Time PCR

Real time PCR was performed with the LightCycler 480 System and SYBR Green I (Roche) and data analysed according to Livak et al., 2001.

Below are the primers sequences (Sigma-Aldrich) for *AtE2Fa*, *AtDPa* genes; *ACTIN* and *TUBULIN* were used to normalize the data.

AtE2Fa-FW	tgatagccgtcaaagctcct
AtE2Fa-REV	tcgatgtcatggtgtcctgt
AtDpa-FW	gggcttcgtcaattcagtgt
AtDpa-REV	acacattgagcgcatcgtag
ACT 2 FW	gcaccctgttcttctta
ACT 2 REV	aaccctcgtagattggcaca
TUB 4 FW	ctgtttccgtaccctcaagc
TUB 4 REV	agggaaacgaagacagcaag

For each pair of primers an oligo mix was prepared (10 µl Forward oligo 100 µM, 10 µl Reverse oligo 100 µM, 380 µl dH_2O) and the primers efficiency tested. Real time PCR reaction was performed in three technical triplicates and prepared as following: 5 µl LightCycler SYBR Green I Master (Roche), 2 µl oligo mix, 2 µl dH_2O, 1 µl cDNA template. According to manufacturer's instructions, PCR parameters were programmed as shown in the following table:

Preincubation	1 cycle	95°C	5 mins
Amplification	45 cycles	95°C	10 secs
		Primer dependent	15 secs
		72°C	15 secs
Melting curve	1 cycle	95°C	5 secs
		65°C	1 min
		98°C	-
Cooling	1 cycle	40°C	10 secs

3.5. Shoot apex meristem imaging

3.5.1. Plant growth

Seed were sowed in soil (JIC Arabidopsis soil mix consisting of Levingtons F2 compost, with Intercept, and grit at a ratio of 6:1) with the help of a toothpick in 9x9 pots. Trays were placed in short day growth chamber (22°C, 9 hrs light, 15 hrs dark) and after about a week growth, extra seedlings were removed, leaving the healthiest one (4-5 for pot). After 4 weeks, the trays were transferred in a Sanyo growth cabinet at 16°C, 24 hrs lights level 4.

3.5.2. Apex dissection

Inflorescence apices were dissected with the help of binocular headband magnifier and using fine-pointed forceps. The dissected shoot apices, with about 5mm long pedicle, were transferred in steril boxes (5x7.5x3.5 cm) with 50 ml of growth media GM (Murashige and Skoog salts (Sigma), 1% glucose, 0.5 g/mL 4-morpholineethanesulfonic acid (Sigma), 0.8% agar, pH 5.7) and then placed in the Sanyo growth cabinet (16°C, 24 hrs lights level 4) for 45 hours to recover after cut stress.

3.5.3. EdU/Pseudo-Shiff PI protocol

After recovering for 45 hours in Sanyo growth cabinet (16°C, 24 hrs lights level 4), the apices were transferred in new boxes with GM and 10 μM EdU (5-ethynyl-2'-deoxyuridine) (Invitrogen) and left for 3h. Then the apices were dissected again to eliminate the pedicle and buds grown during recovery and processed individually in 96-wells plate with flat button (Falcon), with gentle shaking and protected by light for critical steps with fluorescent compounds. The apices were then de-hydrated in ethanol 15%, 30%, 50%, 70%, 85%, 95% for 15 mins each and then incubated in ethanol 100% overnight at room temperature. The samples were re-hydrated in the same ethanol series as before, left in water for 10 mins and then incubated for 1 day at 37°C in alpha-amylase (Sigma) 0.3mg/ml in phosphate buffer 20 mM pH 7.0, 2 mM NaCl, 0.25 mM $CaCl_2$. After rising in water, they were incubated in a solution containing 10 μM Alexa 488-azide (Invitrogen) and 100 mM Tris pH 8.5 for one hour, followed by 30 mins in 100 mM Tris; 1mM CuSO4; 10 μM Alexa 488-azide; 100 mM ascorbic acid, pH 8.5. The apices were washed in water for tree times, left 30 mins in 1% periodic acid solution and rinsed in water again twice. Freshly made Schiff reagent/PI (49 ml water; 625 μl HCl 12 N; 0.95 g sodium bisulfite; 20 μg/ml propidium iodide (PI) (Sigma)) was added and samples incubated for 2 hours. After washing in water, the apices were moved carefully on concave microscope slides (ACADEMY) and a drop of chloral hydrate solution (8 g chloral hydrate, 1 g glycerol, 2 ml water) was added until they became clear and transparent. The chloral hydrate was blotted out and replaced with a drop of Hoyer's medium (40g chloral hydrate, 100 ml water, 4 ml glycerol, 6g gum arabic) and then the coverslip (22x22 mm) was laid down carefully, avoiding bubbles. The samples were imaged the day after.

3.5.4. Microscopy

Shoot meristem imaging was performed using a Zeiss 510 Meta confocal microscope with excitation at 488 nm and emission filters set to 572-625 nm for propidium iodide and 505-600 nm for EdU. A 40X oil objective was used and Z stacks were obtained by imaging 0.5 μm sections which were averaged three times.

3.5.5. Image processing and analyses

Images were firstly imported into ImageJ64 (http://rsbweb.nih.gov/ij/download.html) and processed. The software mars-alt version 1 (http://openalea.gforge.inria.fr/doc/vplants/vtissue/doc/_build/html/user/mars_alt_v1/index.html) was used for segmentation analyses and the calculation of cell volumes. For statistical analysis the Wilcoxon`s test was used.

4. RESULTS

4.1. Overexpression of AtE2F/AtDP transcription factors in floral meristem using the pOp/LhG4 system.

To understand how E2Fs overexpression can affect cell volume and cell cycle progression through S phase during bud emergence, a tissue-specific transactivation system was used (Moore et al., 2006). This system is based on the production of effectors lines in which the gene of interest is placed under the control of a modified minimal plant promoter. The promoter possesses the elements for binding of the basal transcription apparatus and lacks binding sites for endogenous plant transcription factors, but contains a binding site for a heterologous transcription factor that can activate the promoter to high levels of transcription. When the construct is introduced in plants, the gene is transcriptionally inactive. Subsequently, the crossing of the effector lines with activactor lines expressing the heterologous transcription factor under a tissue specific promoter allows high tissue-specific expression of the gene (Fig. 3).

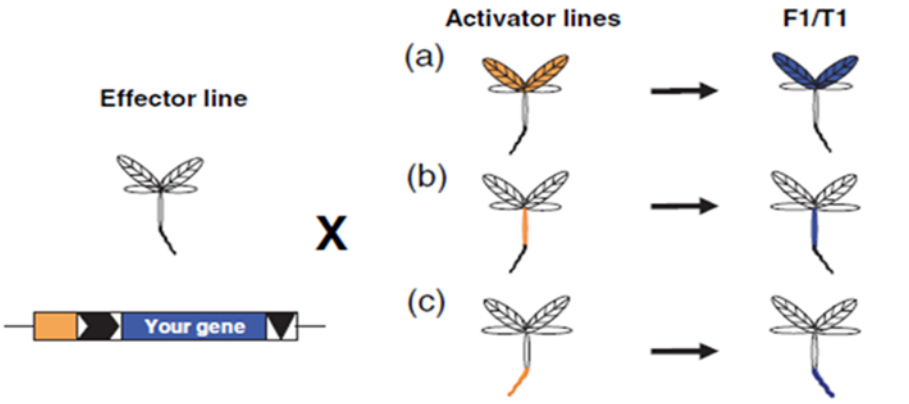


Figure 3: The principle of transactivaction system (from Moore et al., 2006)

The approach chosen made use of the pOp/LhG4 system, that comprises an artificial promoter, called pOp, possessing two ideal *lac* operators positioned upstream of a minimal CaMV 35S promoter. The pOp promoter is efficiently activated by the artificial transcription factor LhG4 that binds to its *lac* operators (Moore et al., 2006).
To achieve high expression of the E2F and DP effector lines in the floral meristems, the activactor line AP1::LhG4 was used, in which the expression of the LhG4 transcription factor is controlled by the *APETALA1* (*AP1*) promoter. This promoter allows expression of the LhG4 transcription factor in early buds, since *AP1* encodes a MADS-domain related transcription factor that is necessary and sufficient for the transition from inflorescence to floral meristem (Sablowski, 2007).
In the course of this thesis, 4 effector lines were created: pOp::AtE2Fa, pOp::AtE2Fb, pOp::AtDPa, pOp::AtDPb, and each of them was crossed with the homozygous activator line AP1::LhG4 (Emery et al., 2003). The following F1 lines were obtained: AP1-AtE2Fa, AP1-AtE2Fb, AP1-AtDPa, AP1-AtDPb.

4.2. Phenotypic analyses.

Phenotypic analyses were carried on the four F1 lines, AP1-AtE2Fa, AP1-AtE2Fb, AP1-AtDPa, AP1-AtDPb, and on the corresponding pOp lines as controls. These lines were selected on gentamicin media and after 2 weeks the seedlings were moved in soil. As expected, phenotypic analysis of each overexpressing line at the vegetative stage did not reveal any particular change compared to the controls. Later, after floral transition, the AP1-AtE2Fa and AP1-AtE2Fb plants showed longer main floral stems compared to the pOp control, while the AP1-AtDPa and AP1-AtDPb plants did not display this phenotype. Although the AtE2Fa and AtE2Fb plants developed normal flowers, the length of the floral stems suggested that their growth rate might be affected. To investigate this aspect we focused on four independent lines of AP1-AtE2Fa and the siliques of the main floral stem were counted every three day. The corresponding pOp::AtE2Fa lines were chosen as control.
Comparisons of the AP1-AtE2Fa lines with the pOp::AtE2Fa controls revealed that the growth rate increased in AtE2Fa overexpressing plants as shown in the Figure 4.

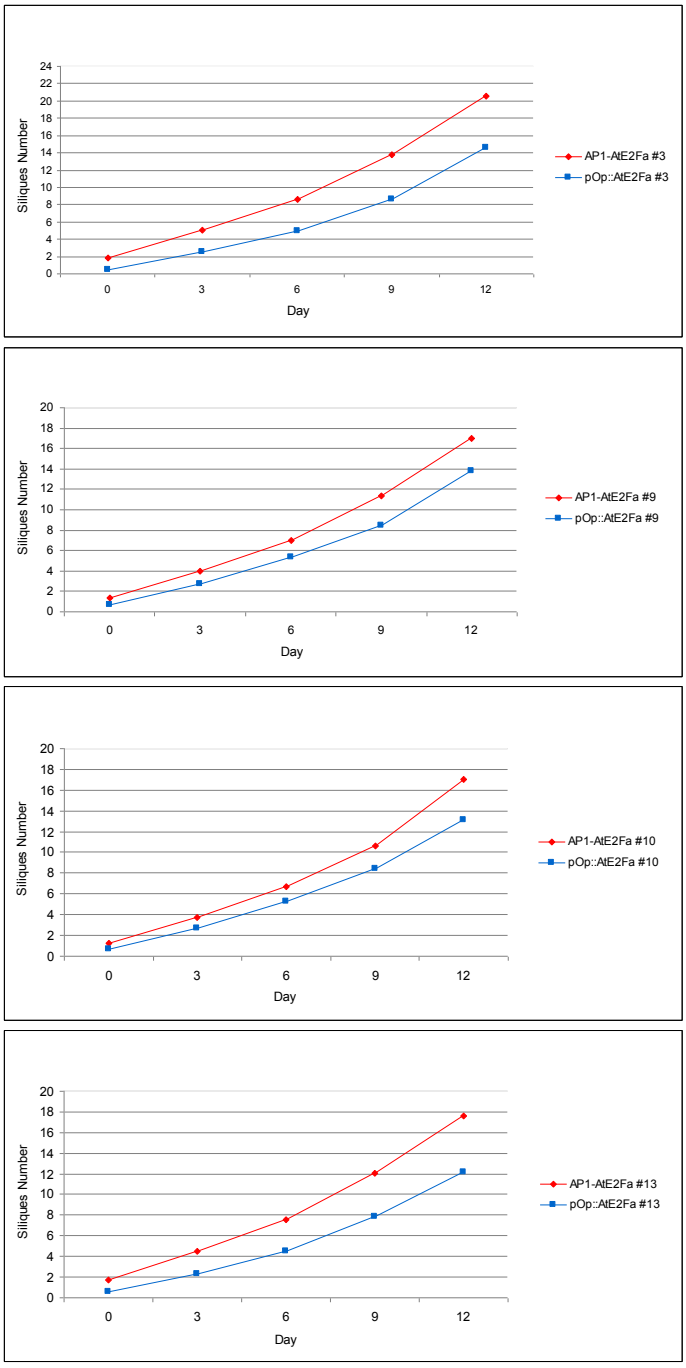


Figure 4: Graphs of silique setting of AP1-AtE2Fa independent lines germinated with antibiotic

The lines #3 and #13 appeared to be the more affected in terms of growth rate and also as final number of siliques produced. This result suggests that the overexpression of AtE2Fa in the inflorescence meristems can cause an acceleration in flower formation, displayed by the higher number of siliques.Based on this evidence, the line AP1-AtE2Fa #3 was chosen to investigate at the cellular level whether changes of cell parameters during floral bud formation are induced by this transcription factor. To proceed with microscopic analyses, it was necessary to sow the seeds directly on soil, without the selecting agent. The segregation for the gentamicin resistence gene was checked by genotyping and the positive seedlings were chosen for the microscopic analyses. Under this growth condition, we noticed that the seedlings did not display the long stem phenotype observed before, when the seeds were germinated on selection plates.

To confirm this evidence, the siliques were counted for AP1-AtE2Fa #3 and pOp::AtE2Fa #3 and the seedlings negative to gentamicin resistence gene were chosen as negative control. The siliques number was also checked in the lines #9 and #13. The results are shown in Figure 5-6 and reveal that the growth rate is not affected when the plant were sown directly in soil, suggesting that the phenotype observed earlier might be caused by the fact that the germination media was supplied with gentamicin. Despite the lack of a clear phenotype, the line AP1-AtE2Fa #3, that showed the longest stems when germinated under antibiotic selection, was chosen to perform microscopic analyses.

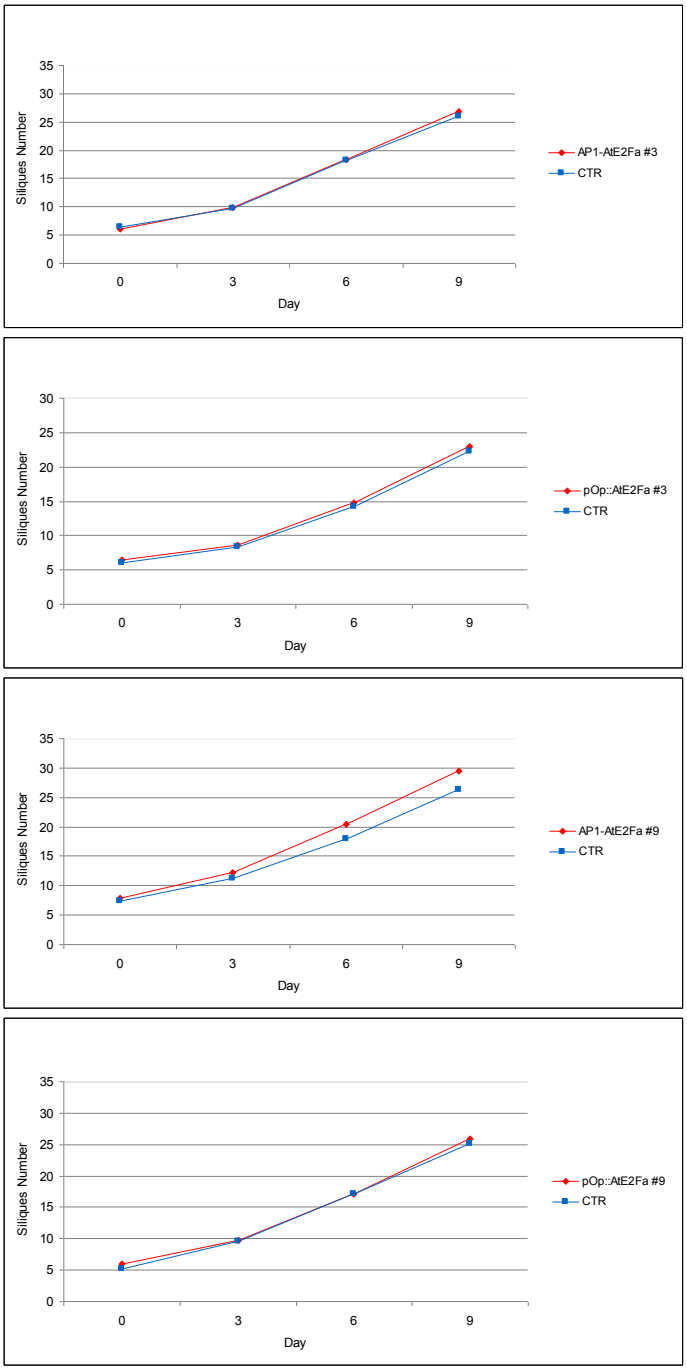


Figure 5: Graphs of silique setting of AP1-AtE2Fa and pOp::AtE2Fa indipendent lines (# 3 and #9) germinated without antibiotic

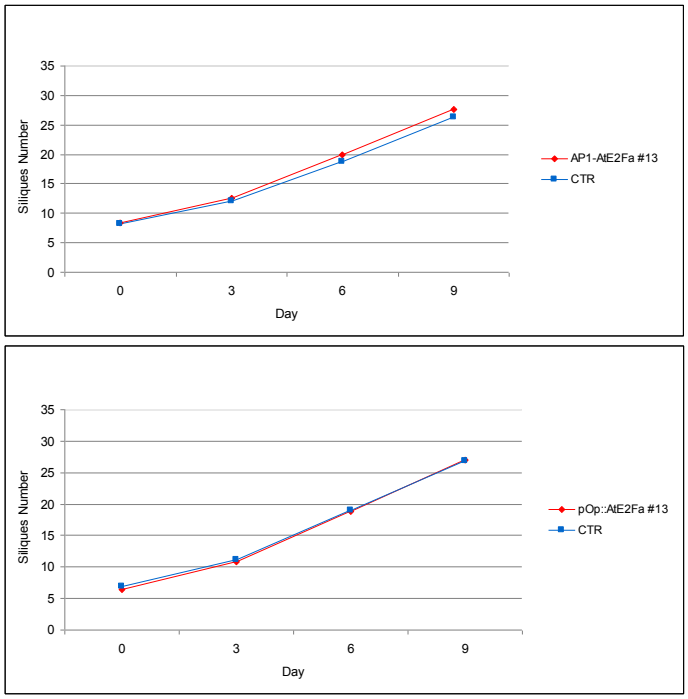


Figure 6: Graphs of silique setting of AP1-AtE2Fa # 13 and pOp::AtE2Fa # 13 germinated without antibiotic

4.3. Analyses of cell growth and DNA synthesis pattern in AP1-AtE2Fa plants by imaging acquisition of inflorescence meristems.

The S phase has been considered the growth-limiting step of the cell cycle, because inhibition of DNA synthesis stopped cell growth, affecting the patterning of the meristem (Grandjean et al., 2004). Since the E2Fa transcription factor is a key regulator in G1/S transition, and has been shown to trigger cell proliferation, we investigated the cell geometry and the DNA synthesis patterns in plants of the AP1-AtE2Fa line #3. A recent 3D approach was used to monitor the coordination between cell growth and DNA synthesis, during bud emergence.

This investigation required specific plants growth condition combined with the preparation of the inflorescence apices for confocal imaging. The plants were grown at 22°C for 4 week and then moved to a 16°C growth cabinet to develop a thick inflorescence and floral stem that allowed inflorescence dissection. The developed flowers and the old buds were removed and the dissected inflorescence apices were incubated with EdU (5-ethynyl-2'-deoxyuridine) and with PI (propidium iodide). PI allows fluorescent marking of cell walls whereas EdU is a thymidine analog that is incorporated into DNA during new DNA synthesis and can be detected by a click reaction, in which the EdU forms a stable covalent bond with an azide containing a fluorochrome. This method allowed us to differentiate cells in S phase from cells that were not synthesizing DNA. This procedure was carried out for the AP1-AtE2Fa line #3 and for pOp::AtE2Fa as a control.

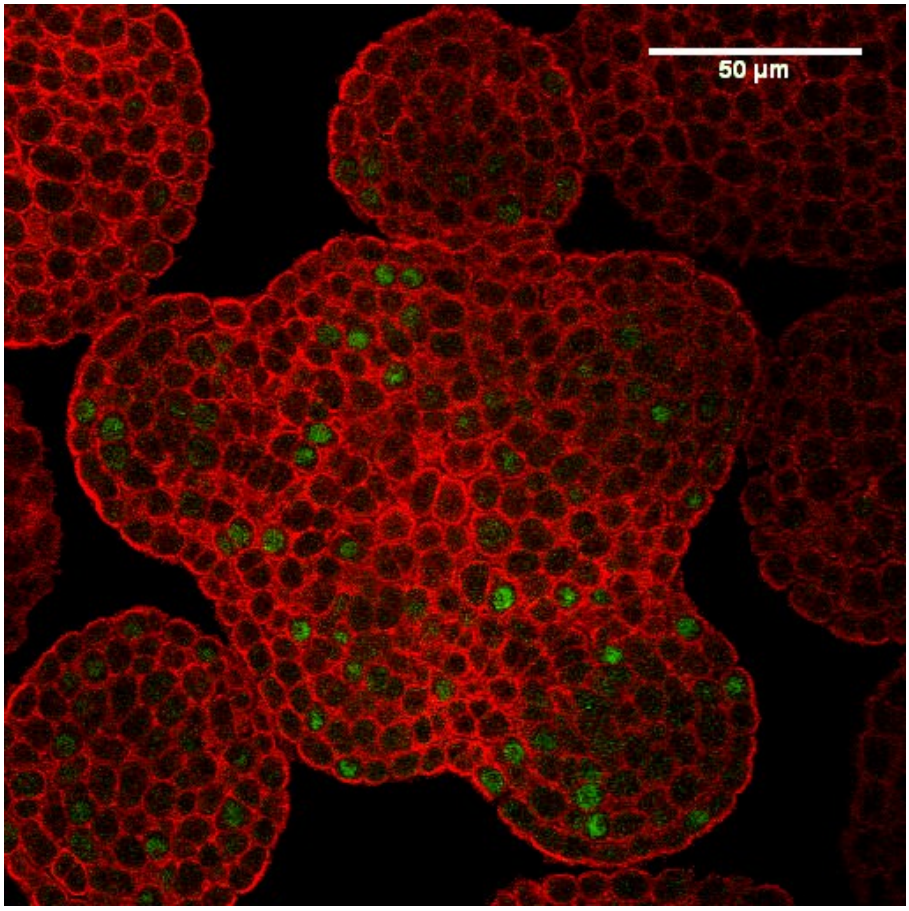


Figure 7: Single optical section through AP1-AtE2Fa line #3 inflorescence meristem

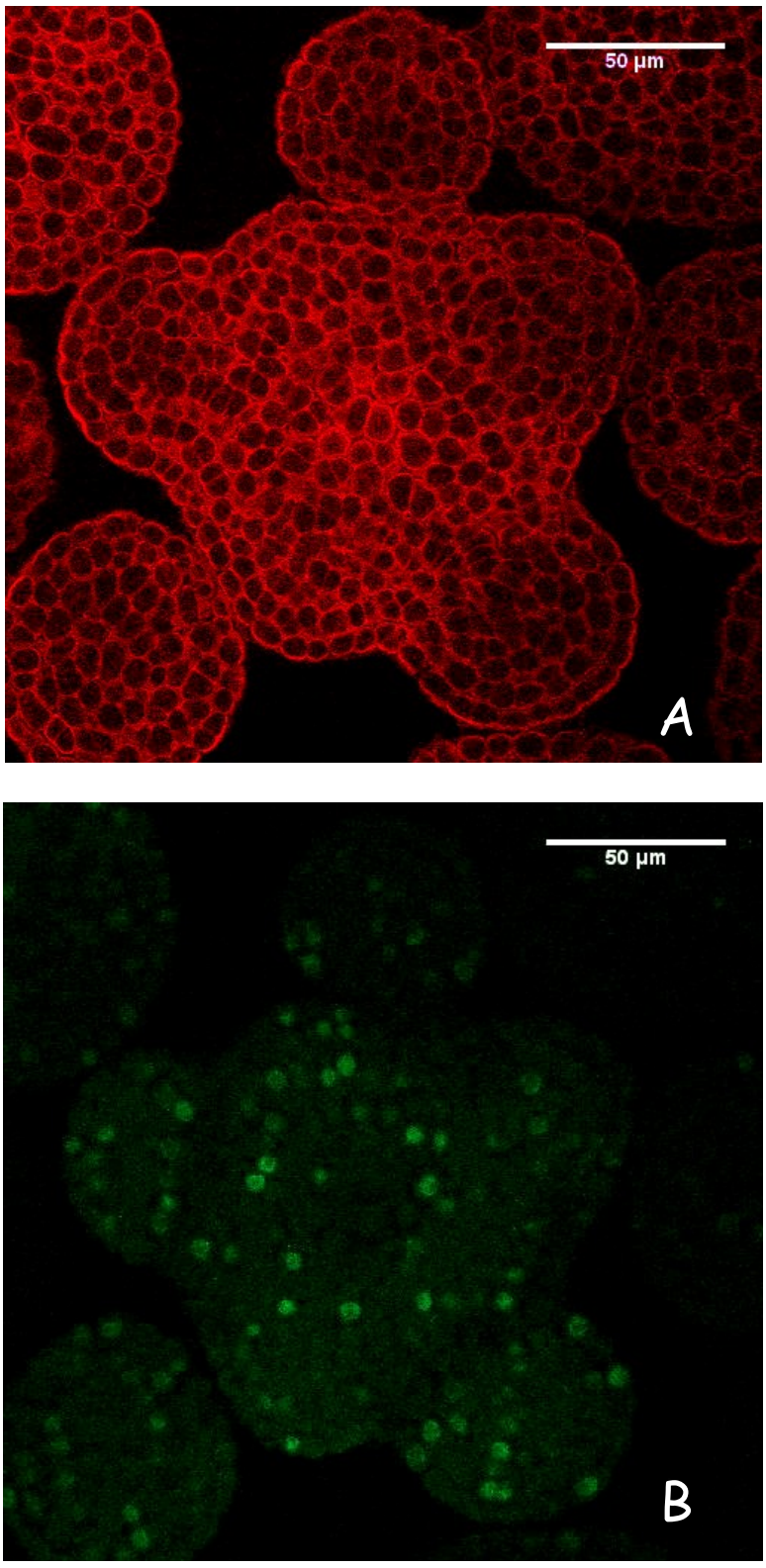


Figure 8: Single optical section through AP1-AtE2Fa inflorescence meristem: (A) red channel, PI; (B) green channel, EdU

4.4. Cell segmentation of floral bud primordia

To proceed with the 3D analysis we relied on the phyllotactic position of the buds around the inflorescence meristem. Although the *AP1* promoter is already active in earlier buds (P1, P2, P3) the border between these buds and the inflorescence meristem is not well defined. Therefore, because of the difficulty to discern the cells of the bud primordia from the inflorescence meristem ones, we decided to focus on bud P4 and P5 (Fig. 9) . The buds in the same position were chosen in pOp::AtE2Fa as control.

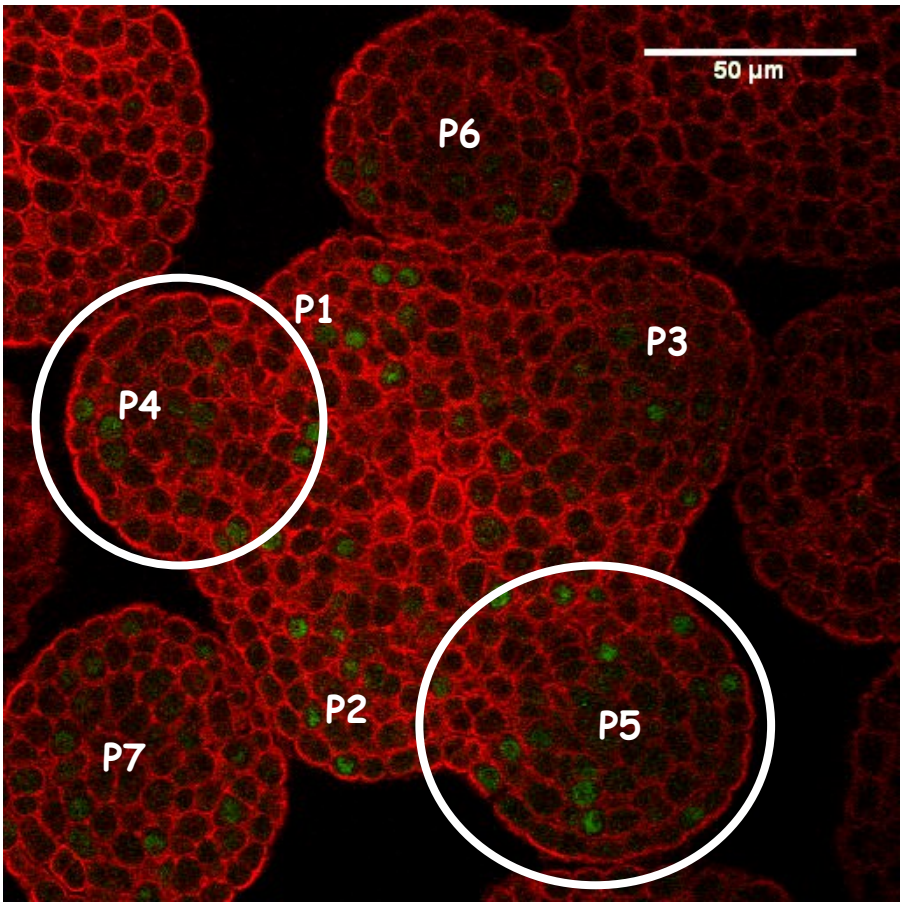


Figure 9: Single optical section through AP1-AtE2Fa line #3 inflorescence meristem showing the buds chosen for segmentation analyses.

The 3D analysis was performed with mars-alt version 1 (Fernandez et al., 2010) that provides a multiangle image acquisition, three-dimensional reconstruction and cell segmentation-automated lineage tracking (MARS-ALT). It allows accurate cell identification in three dimension and automatically tracked cell lineages through multiple rounds of cell division (Fernandez et al., 2010).

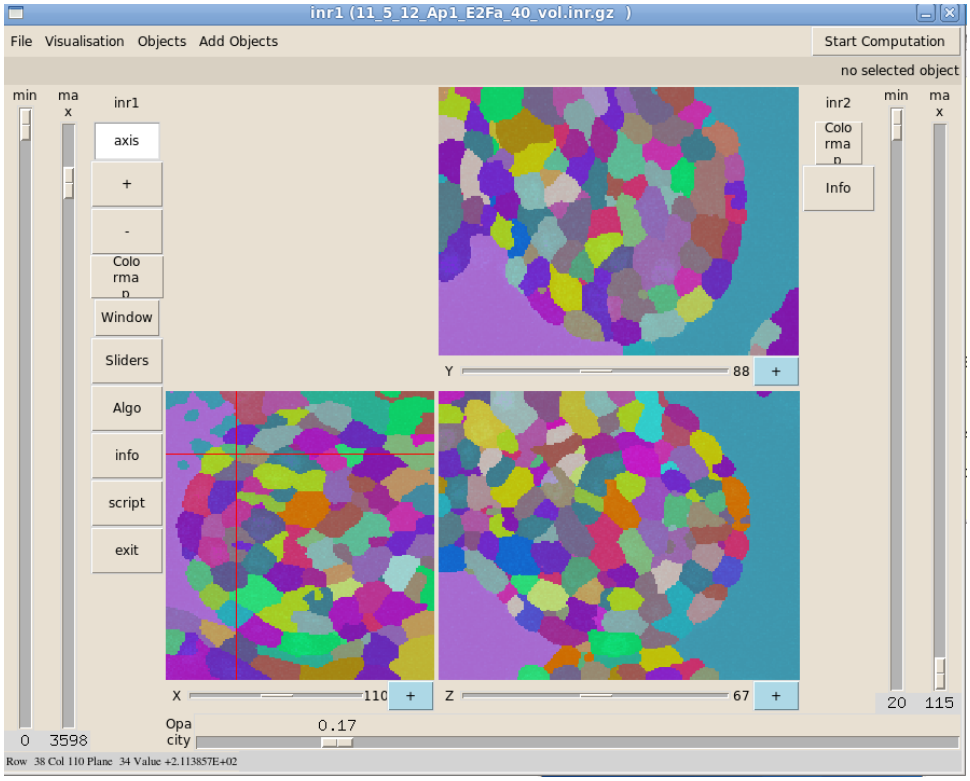


Figure 10: Virtual view of a floral bud elaborated by mars-alt version 1.

With our lines, the 3D analysis allowed us to mark cell in S phase and the neighboring control cells with no EdU signal; consequently the volume values were collected for both cell types.

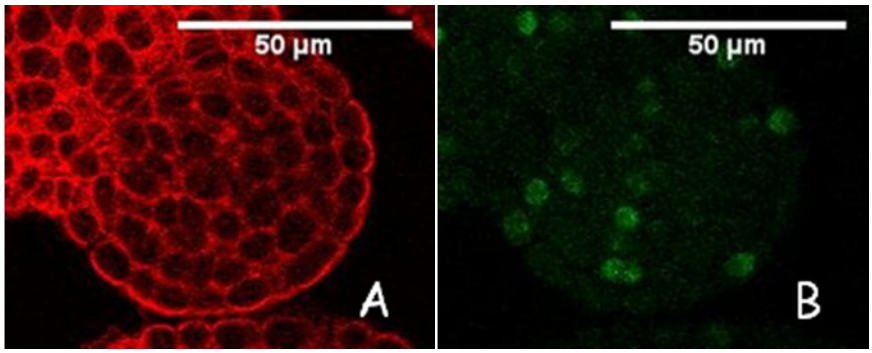


Figure 11: Single optical section through AP1-AtE2Fa line #3 P5 bud: (A) red channel, PI; (B) green channel, EdU.

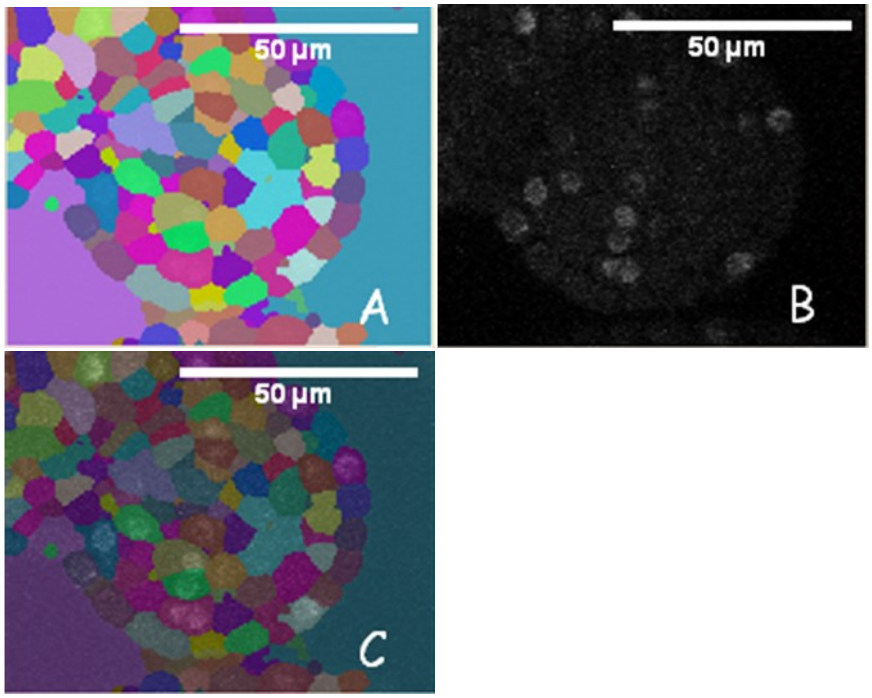
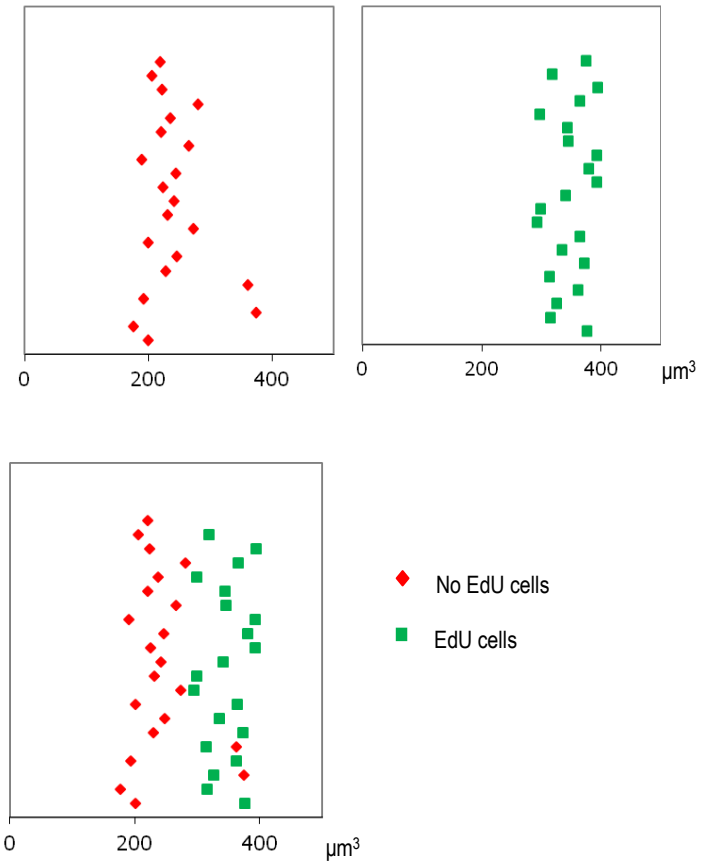


Figure 12: Virtual section corresponding to figure 11: (A) 3D segmentation, (B) EdU detection, (C) simultaneous 3D segmentation and EdU detection.

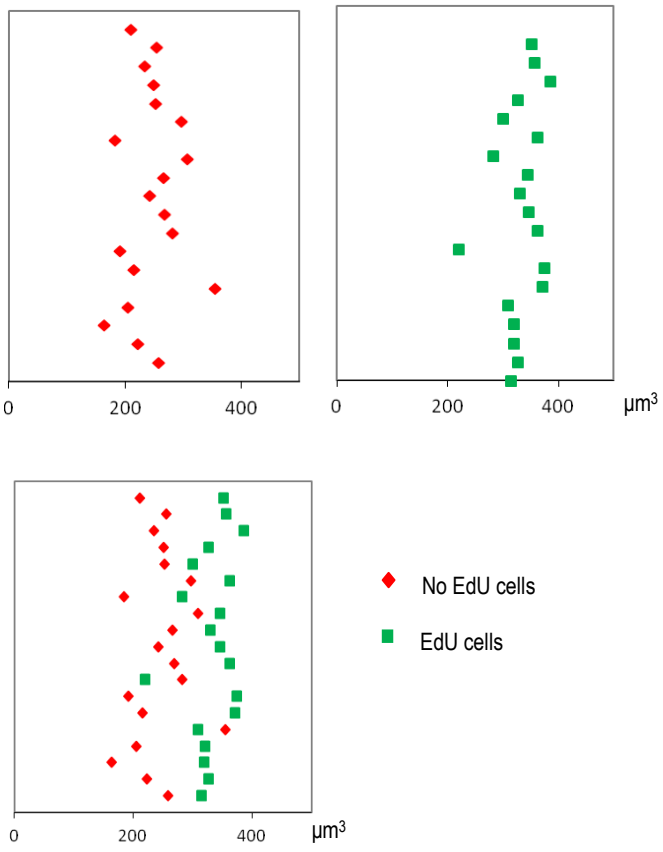
4.5. The coordination between cell growth and DNA synthesis is maintained in AtE2Fa overexpressing floral bud primordia.

To understand how cell division and cell growth are coordinated in AtE2Fa overexpressing floral bud primordia, the volumes of EdU-labeled cells (green squares) and control unlabeled neighboring cells (red diamonds) are displayed in scatterplots for the AP1-AtE2Fa line #3 P4 and P5 buds (Fig. 13 and Fig. 14) and for the same buds of pOp::AtE2Fa as a control (Fig. 15 and Fig. 16). In each scatterplot, the x axis shows cell volumes in μm^3 while the y axis does not rapresent any measurement and was used to spread out data points.



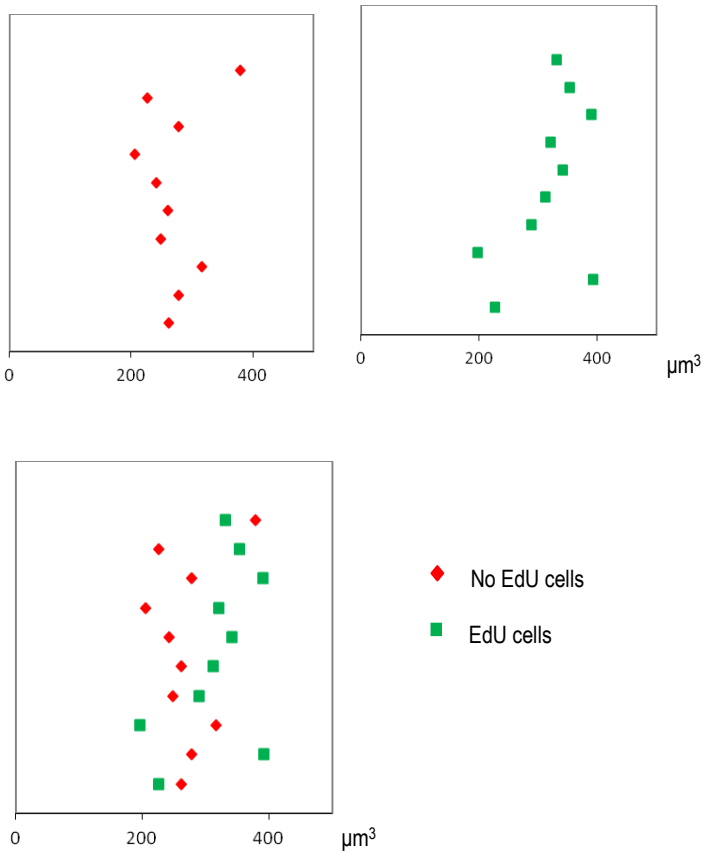
	AVERAGE	STDEV
No EdU	239.6	50.5
EdU	347.4	32.9

Figure 13: Scatterplots of the cell volumes for floral bud P4 of AP1-AtE2Fa line #3



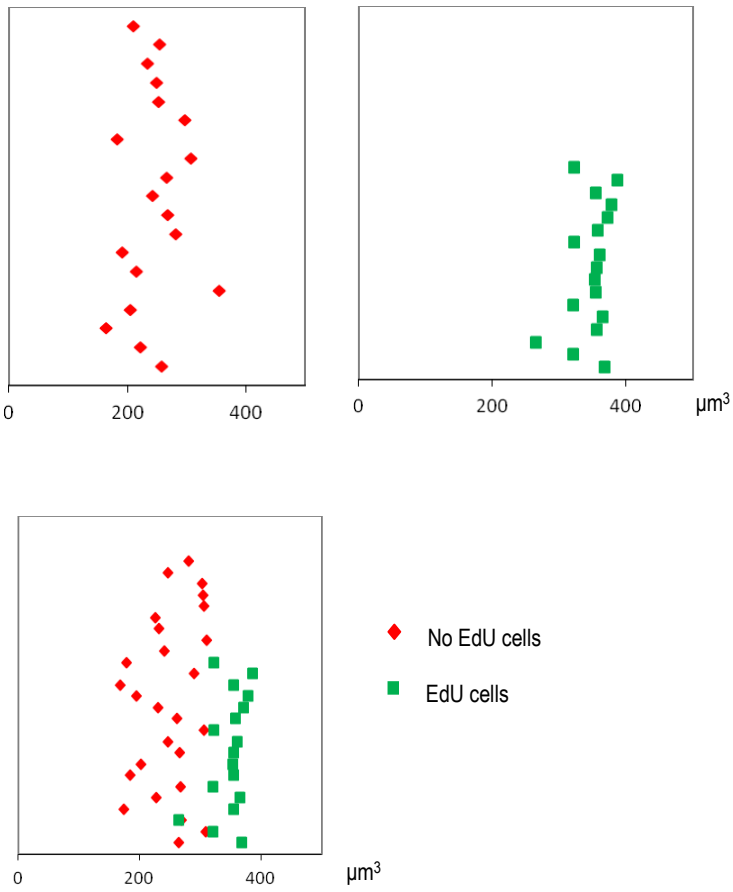
	AVERAGE	STDEV
No EdU	245.4	46.6
EdU	331.7	38.5

Figure 14: Scatterplots of the cell volumes for floral bud P5 of AP1-AtE2Fa line #3



	AVERAGE	STDEV
No EdU	269.5	49.1
EdU	315.3	63.8

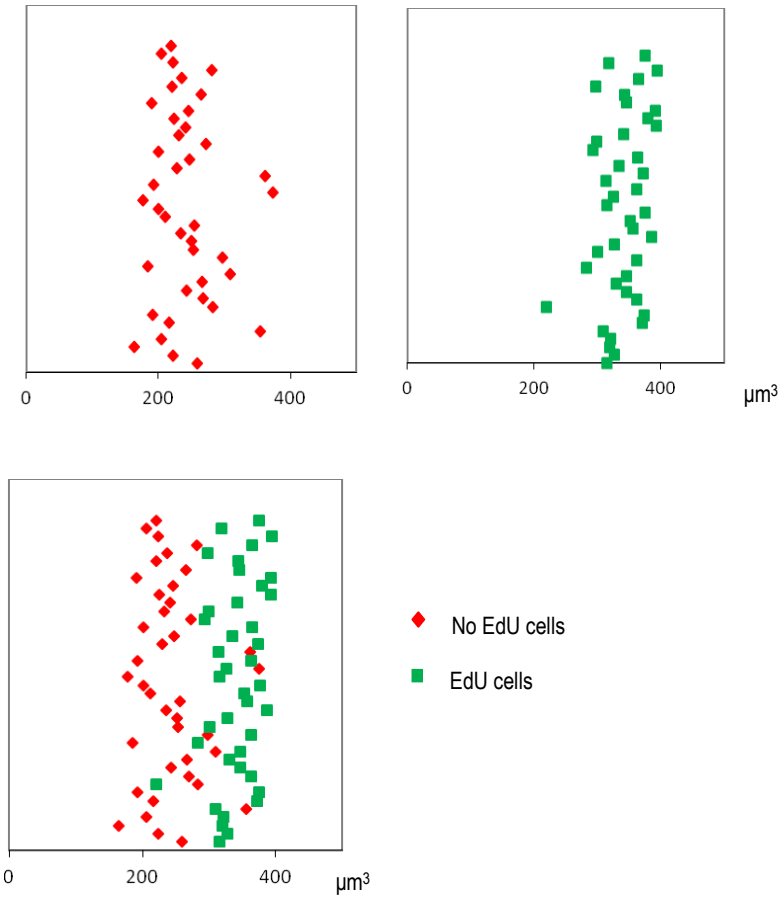
Figure 15: Scatterplots of the cell volumes for floral bud P4 of pOp::AtE2Fa



	AVERAGE	STDEV
No EdU	250	44.9
EdU	347.9	29.4

Figure 16: Scatterplots of the cell volumes for floral bud P5 of pOp::AtE2Fa

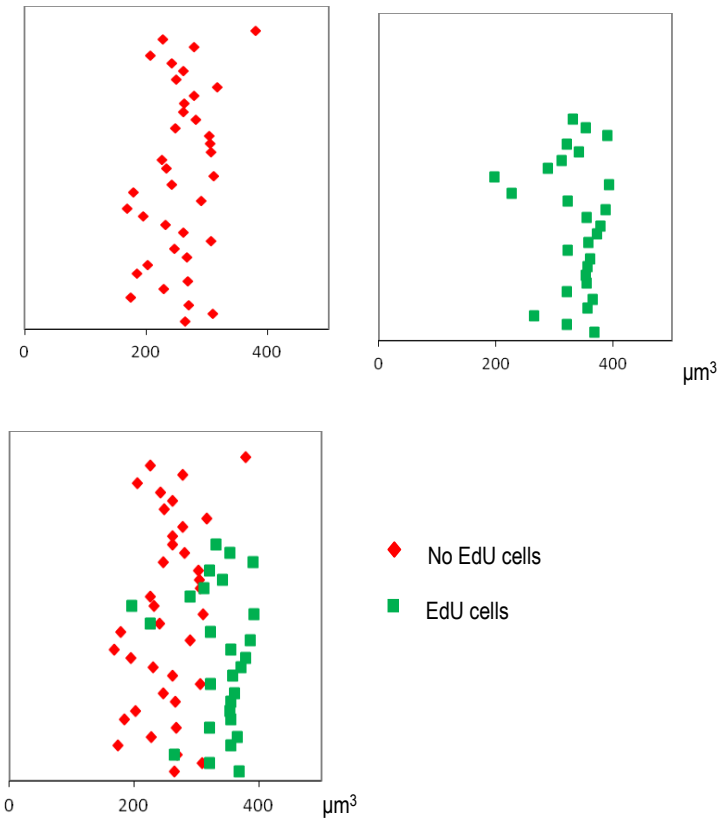
The cell volumes of P4 and P5 buds of AP1-AtE2Fa and of pOp::AtE2Fa control line are collected in a single scatterblots (Fig. 17 and Fig. 18) and the Wilcoxon's test was used for the statistical analyses. The P values equal or higher than 0.05 correspond to the null hypothesis that median cell volumes were the same for EdU-positive and negative cells. In both cases the P values was lower than 0.05 and the alternative hypothesis was accepted.



	AVERAGE	STDEV
No EdU	242.3	48.1
EdU	339.9	36.08

p = 0.002375

Figure 17: Scatterplots of the cell volumes for both P4 and P5 floral buds of AP1-AtE2Fa line #3



	AVERAGE	STDEV
No EdU	255.3	46.2
EdU	335.8	46.9

$p < 0.0001$

Figure 18: Scatterplots of the cell volumes for both P4 and P5 floral buds of pOp::AtE2Fa

In both AP1-AtE2Fa and pOp::AtE2Fa lines, the cell volumes ranged from approximately 300 to 380 μm^3 for EdU-labeled cells and from approximately 180 to 280 μm^3 for unlabeled cells. This suggest that in both AtE2Fa overexpressing and control lines, DNA synthesis occurs when cells reach a threshold volume. In other words, cell growth occurs before S phase and the AtE2Fa overexpression does not affect the coordination between cell growth and DNA synthesis.

Previous studies have demostrated that the overexpression of the dimerization partner AtDPa enhanced strongly the phenotype of AtE2Fa overexpressing plants (De Veylder et al., 2002; Inzè and De Veylder, 2006). Thus, although AP1-AtE2Fa plants did not show any clear cellular phenotype, the co-expression of the dimerization partner AtDPa might be necessary to cause macroscopic alterations and/or to promote the progression through S phase during bud emergence, and possibly affect the coordination between cell growth and DNA synthesis. To address this question, transgenic lines AP1-AtE2Fa/AtDPa, overexpressing both AtE2Fa and AtDPa in floral bud primordia, are currently being obtained by crossing the single effector lines. For this purpose, the expression level of four indipendent AP1-AtE2Fa and AP1-AtDPa lines have been checked by qPCR and the lines showing high level of expression have been chosen to make the cross (Fig. 19 and Fig. 20).

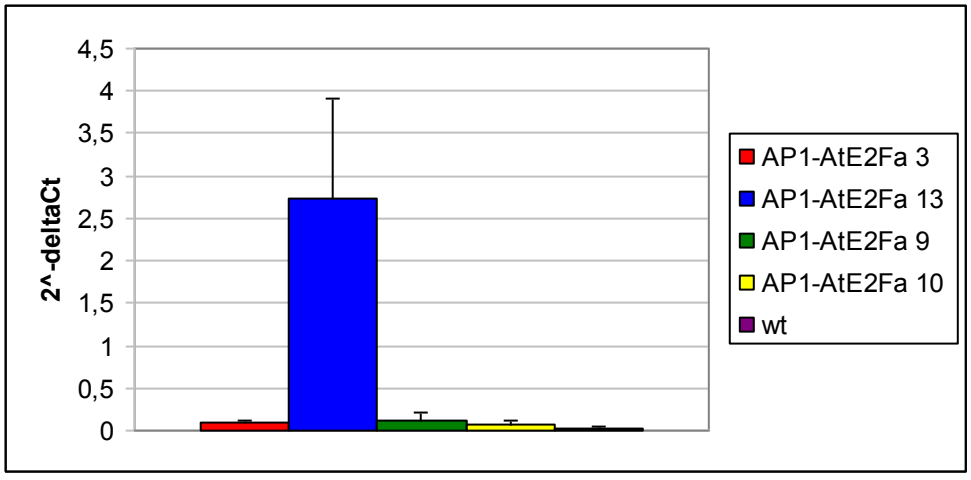


Figure 19: Expression levels of AP1-AtE2Fa relative to the *TUB* costitutive control.

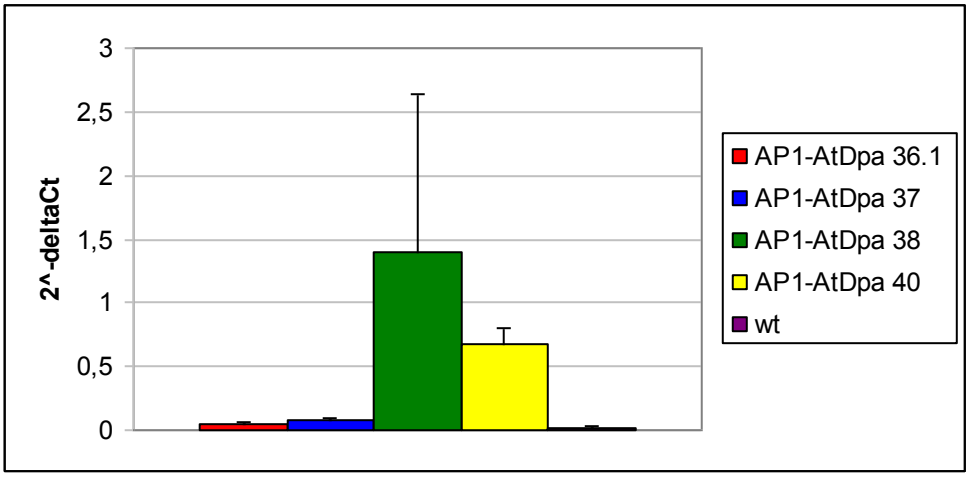


Figure 20: Expression levels of AP1-AtDPa relative to the *ACT2* costitutive control.

Among the AP1-AtE2Fa lines, the AP1-AtE2Fa line #13 has been chosen and crossed with the AP1-AtDPa line #38.

Once the F seeds will be obtained, it will possible to proceed with the phenotypic characterization by inflorescence apices dissection to investigate cell geometry and DNA synthesis in AP1-AtE2Fa/AtDPa lines.

5. DISCUSSION

The most important biological process linked to development in multicellular organisms is the control of cell growth and cell division. In order to be able to divide, cells must first duplicate their DNA and cytoplasm, and thus must activate mechanisms that assure the coordination of these events. In complex multicellular organisms, the cells are also involved in the event of organogenesis that implies an additional genetic regulation necessary to drive cells through the differentiation processes. In plants, organogenesis is a postembryonic event that occurs throughout the entire life of the organism and is assured by the activity of the apical meristems. Within the shoot and root meristems, the stem cell niches provide the precursors of differentiated cells. The study of meristem cell behaviour and the knowledge of the regulatory networks controlling cell proliferation and differentiation are the keys to understand how plants grow and develop.

In plants, cell division and differentiation cause the development of leaves and flowers on the flanks of the vegetative and inflorescence meristems respectively. Since the emergence of new organs is coupled to modification of cell parameters including growth, division rates and division pattern, the regulation of cell cycle in this context is an important issue. Although the activity of some cell cycle regulators, namely CDKA, CYCA, CDC25 in *Nicotiana tabacum* (Hemerly et al., 1995; Wyrzykowska et al., 2002) and CYCD3 in Arabidopsis (Riou et al., 2000), has been demonstrated to affect SAM organization and homeostasis, the role played by cell cycle regulation during organogenesis is still unclear. Moreover, it has been difficult to associate experimentally the activity of cell cycle genes to the changes in cell parameters linked to cell proliferation. Recently, a new approach known as MARS-ALT (multiangle image acquisition, three-dimensional reconstruction and cell segmentation-automated lineage tracking) has been developed to obtain quantitative, dynamic and three-dimensional (3D) information of cell and organ growth. This method allows the imaging of the whole organ from multiple angles, and the segmentation of this image in order to provide accurate cell identification in three dimensions (Fernandez et al., 2010).

Part of the work in this thesis focused on the role of the AtE2Fa transcription factor in controlling the cell cycle during floral bud formation. This transcription factor is a positive regulator of cell proliferation and it is known to trigger the transition from the G1 to the S phase of the cell cycle. The spatial distribution pattern of *AtE2Fa*, analysed by mRNA in situ hybridisation, confirmed that this gene is strongly expressed in actively dividing tissues, although its transcripts have been detected also in endoreduplicating tissues, suggesting an involvement in the control of the endocycle in differentiated tissues (De Veylder et al., 2002). So far, investigations of the E2F transcription factors expression in the inflorescence meristem and their contribution during floral organogenesis have not been reported yet. In addition, the functional characterization of the members of the E2Fs family in plants has relied on the study of the effect of their constitutive overexpression (De Veylder et al., 2002; Rossignol et al., 2002; Magyar et al., 2005; Sozzani et al., 2006). The work of this thesis addressed specifically the effects of AtE2Fa overexpression on the first step of *Arabidopisis* flowers development, when the early floral buds are emergencing at the inflorescence meristem flanks. The use of the pOp/LhG4 tissue-specific transactivation system (Moore et al., 2006), associated with the MARS-ALT 3D approach, allowed the analysis of cell dynamics occurring during floral bud emergence in floral meristems overexpressing AtE2Fa. Because previous studies demonstrated that the phenotype observed in plants constitutively overexpressing AtE2Fa was enhanced by the co-expression of the AtDPa dimerizing partner (De Veylder et al., 2002), we produced also plants overexpressing AtDPa that were crossed with those overexpressing AtE2Fa to generate plants overexpressing the AtE2Fa/AtDPa complex in floral meristems. These plants have not been characterised yet but will be soon subjected to further analyses by the MARS-ALT 3D approach.

Moreover, to have an overall view of the effects of the activating E2Fs on flower organogenesis, plants overexpressing AtE2Fb or AtDPb in floral meristems were also obtained. In fact, it has been demonstrated that the AtE2Fb transcription factor is also involved in the triggering of G1/S transition and an interplay of AtE2Fb with AtE2Fa has been proposed to control the balance of cell division, endoreduplication and differentiation (Sozzani et al., 2006).

The activating E2Fs are involved in the regulation of genes that are transcribed during cell proliferation and the process of organogenesis is a dynamic event that implies changes in cell division rate and patterns. Based on this consideration, we addressed whether the overexpression of the activating E2Fs could modify cell geometry and division during flower formation, and interfere with flower development. The phenotypic observation was carried on the Arabidopsis plants transformed using the pOp/LhG4 system and overexpressing independently AtE2Fa or AtE2Fb transcription factors (AP1-AtE2Fa; AP1-AtE2Fb), or the AtDPa, AtDPb dimerization partners (AP1-AtDPa; AP1-AtDPb). As expected, the AP1-AtDPa and AP1-AtDPb plants did not reveal any phenotype because the dimerization partners are not believed to be able to affect gene transcription by themselves. On the contrary, the AP1-AtE2Fa and AP1-AtE2Fb plants showed longer main floral stems compared to the control plants, suggesting that the overexpression of these transcription factors might affect the plant growth rate. Moreover, an increase in the number of siliques of the AP1-AtE2Fa plants confirmed an acceleration in flower formation. Based on these phenotypic observation, the most affected AP1-AtE2Fa line was selected to perform the MARS-ALT microscopic analysis. The procedure required the sowing of the seeds directly on soil and under this condition the line chosen for the microscopic analysis did not show anymore the growth phenotype displayed before, indicating that the longer floral stem could be a consequence of the germination in selection medium supplied with gentamicin. The reason for this discrepancy is not clear. However, the phenotype observed with the antibiotic could be linked to an effect of gentamicin selection over the activity of the overexpressed E2Fs rather than a consequence of the antibiotic itself on floral development because AP1-AtDPa and AP1-AtDPb plants germinated under gentamicin selection did not show a longer floral stem.

In AP1-AtE2Fa plants, the investigation at cellular level was necessary to understand how cell dynamics such as proliferation and growth were coordinated during flower development. It has been previously shown that constitutive overexpression of either AtE2Fa or AtE2Fb induces ectopic cell division as the size of cotyledons and the number of cells in cotyledon epidermal layers increased, whereas the cell size decreased, in seedlings overexpressing the activating E2Fs (DeVeylder et al., 2002;

Perrotta, 2011). However, it has not been investigated whether cells induced to proliferate can lower their cell volume threshold preserving the coordination between cell growth and DNA synthesis. Moreover, the S phase of the cell cycle appears to be a growth-limiting step, as it has been demonstrated that an inhibition of DNA synthesis blocks cell growth and affects SAM pattering. On the contrary, the inhibition of M phase entry does not prevent meristem and primordium growth (Grandjean et al., 2004).

Our results, using a meristem specific transactivation system, show that the cell volume threshold and the coordination between cell volume and DNA synthesis were preserved in AP1-AtE2Fa floral meristems. Moreover, an increase in cell proliferation was not induced by E2Fa overexpression in floral meristems. This result contrasts with previous data obtained in our laboratory that revealed that the constitutive overexpression of AtE2Fa or AtE2Fb affects embryo development, as shown by the formation of tricotyledonous seedlings and larger cotyledons with more cells (Perrotta, 2011). However, the constitutive espression of activating E2Fs did not appear to affect organ formation in mature plants. Therefore, an increase of AtE2Fa or AtE2Fb expression appears to affect cell proliferation only in embryonic tissues but not in the meristem established after seed germination and during plant development, which are probably subjected to a stricter hormonal control. However, both in our laboratory and by others it has been shown that the constitutive co-expression of activating E2Fs with a DP dimerization partner can affect dramatically plant development (De Veylder et al., 2002; Perrotta, 2011). In fact, plants overexpressing the complex AtE2Fa/AtDPa could not be obtained in our lab, possibly due to a severe effect on embryo development, whereas plants overexpressing constitutively an AtE2Fa/AtDPb complex in addition to an increase in tricotyledony revealed the capacity to produce multiple SAMs (Perrotta, 2011). This suggests that the endogenous level of the DP partners is clearly limiting AtE2Fa activity and only by coexpressing a DP together with AtE2Fa in floral meristem a change in cell proliferation, possibly altering the coordination between cell geometry and DNA synthesis, might be obtained. Using the MARS-ALT 3D approach, a future investigation of the floral meristems of transgenic plants co-expressing the complex

AtE2Fa-AtDPa will clarify the possible involvement of E2F transcription factors in the control of flower organ development.

CHAPTER 2. DNA damage response in root stem cells: the role of AtE2F transcription factors in programmed cell death

1. INTRODUCTION

1.1. DNA damage and repair mechanisms

All organisms have to preserve their genome from exogenous and endogenous factors that can damage the DNA and compromise the genetic transmission to the next generation. Unlike animals, plants are sessile organisms constantly exposed to environmental factors that can affect DNA integrity and potentially can have lethal consequence for the organism. These exogenous sources of DNA damage are UV radiation, ozone, desiccation and rehydration, air and soil pollutants (Waterworth et al., 2001), that display their effect as DNA double-strand breaks (DSBs), single strand DNA breaks (SSBs) or by the formation of covalent bound between adjacent pyrimidines, known as pyrimidines cyclobutane pyrimidine dimers (CPDs).

In plants, the meristems form a pool of undifferentiated cells that provide cell precursors necessary for tissues and organs formation. As these cells constantly divide, any DNA defects, in form of mutation, delection or insertion, result in clonal populations of mutant cells that compromise plant growth and viability (Waterworth et al., 2001). Upon DNA damage, cells can trigger different response mechanisms: the activation of the DNA-repair machinery, the delay or the arrest of cell cycle to activate repair mechanism before proceeding into mitosis, or the activation of programmed cell death (PCD). All of these mechanisms share two related kinases that activate the signal cascade following DNA damage and are known as the ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) proteins. Both proteins belong to the phosphatidylinositol 3-kinase family (PIKKs) and are activated in response to different types of damage. The former is involved in double-strand breaks (DSBs) response, while the latter is mostly involved in the response to single-strand breaks (SSBs) or stalled replication forks (Cools and De Veylder, 2011). In fact, it has been observed in *Arabidopsis thaliana* that *atm* mutants are highly sensitive to X-rays that induce DBSs, while *atr* mutants are sensitive to the

replication inhibitor hydroxyurea (Garcia et al., 2003; Culligan et al., 2004). Although ATM and ATR are activated by different type of damage, they display some overlaps in function that result in an integrated DNA damage response, including induction or repression of specific transcripts, histone phosphorylation and programmed cell death (Waterworth et al., 2001).

Upon DNA damage, the first step in the repair mechanism is the detection of the site that has been damaged. Regarding DSBs, two repair pathways have been identified: the non homologous end-joining pathway (NHEJ) and the homologous recombination pathway (HR). The NHEJ pathway occurs in somatic cells and involves the joining of non homologous DNA. It has been observed that this mechanism can be often imprecise, because the modification of DNA ends before joining can determine deletions or insertions at the break site (Moynahan, 2010). On the contrary, the HR pathway is responsible for repair of meiotic DSBs and acts producing a 3` single-stranded end that needs a homologous template to start repairing (Moynahan, 2010).

A complex of three proteins (MRE11, RAD50 and NBS1), known as MRN complex, is conserved in plants and is active in both pathways mentioned above (Amiard et al., 2010). The MRN complex is necessary to recognize the DSB and acts to recruit ATM to broken DNA molecules, resulting in activation of its kinase activity and the consequent phosphorylation of downstream targets that initiate cell cycle arrest, DNA repair or programmed cell death.

MRE11 has nuclease and helicase activities, necessary to process broken ends before rejoining, while RAD50 has coiled-coil domains that interact with each other to hold and connect the broken ends together (Hopfner et al., 2002). The role of NSB1 is related to signalling event, as it is necessary for ATM recruitment to DSB site. In 2007, Akutsu et al. identified the plant homolog of *Nbs1* in *Oryza sativa* (*OsNbs1*) and in *Arabidopsis thaliana* (*AtNbs1*). Phenotypic analysis in Arabidopsis has revealed an involvement of NBS1 in HR-mediated DSB repair in both somatic and meiotic cells. Moreover, it has been demonstrated that it can mediate ATM-independent signalling events that might occur in early meiosis (Waterworth et al., 2001). Null mutants of this gene are lethal in mammals and *Nbs1* mutation has been known as responsible of the human Nijmegen breakage syndrome, a disease

associated with chromosomal instability (Waterworth et al., 2001). Interestingly, null mutations of the other MRN components cause lethality in mammals (Stracker et al., 2004), whereas in Arabidopsis it has been found that *mre11* and *rad50* mutants are still viable but hypersensitive to the alkylating agent methyl methanosulfonate (Gallego et al., 2001; Bundock and Hooykaas, 2002).

As mentioned above, the DNA damage that takes place at the replication fork involves the activity of ATR. When DNA polymerase stall occurs, the MCM replicative helicases continue to unwind the DNA ahead of the replication fork, causing the generation of DNA single strands. The ssDNA are detected by the single-strand binding protein complex RPA (Replication Protein A) that recruits signalling component as Rad17 and the Rad9-Hus1-Rad1 and the ATR to the site of replication stress (Harper and Elledge, 2007).

In mammals it has been shown that the kinase activity of ATM and ATR determine the phosphorylation of several targets, including the histone 2A isoform H2AX, NBS1, and the checkpoint associated protein kinases Chk1 and Chk2 (Matsuoka, 2007). The phosphorylated H2AX is necessary to signal the presence of DSB and facilitates the recruitment of DNA damage mediator to the site of repair (Lukas et al., 2004). In plants H2AX phosphorylation occurs rapidly upon DSB induction and although it is predominantly ATM-dependent an ATR involvement has been also demonstrated, responsible for a subset of *c*. 10% of foci (Friesner et al., 2005).

Upon DNA damage, the action of transcription factors is required in order to modulate gene expression and achieve a response. In Arabidopsis it has been found that the transcription factor SOG1 (suppressor of gamma response 1) is responsible for transcriptional changes in response to DNA damage, as it has been reported that the *sog1-1* mutant is defective in the induction of transcripts necessary in the response to ionizing radiation (IR). SOG1 belongs to a very large, plant-specific family of putative transcription factors, known as NAC domain proteins (based on NAM, ATAF1 and CUC2), and is believed to act downstream of ATM and ATR. It has been also demonstrated that SOG1 drives the expression of several other transcription factors, inducing therefore a transcriptional regulators cascade upon IR damage (Yoshiyama et al., 2009).

1.2. Cell cycle regulation in response to DNA damage

In response to DNA damage the cell cycle can undergo a delay, allowing the cell to activate the DNA repair machinery, or, if it is not enough, the cell cycle progression can be arrested in order to permit the cell to repair the damage before proceeding into mitosis. In eukaryotes this response results in the activation of some cell cycle checkpoints: the G1/M checkpoint regulates entry into the S phase, the intra-S checkpoint prevents further progression through the S phase and the G2/M checkpoint delays mitosis. Moreover, cells can also exit the cell cycle and enter a quiescent state, known as G0, or undergo endoreduplication, in which DNA replication is not followed by cell division, resulting in a increase of ploidy state.

In mammalian cells the checkpoint effector kinases Chk1 and Chk2 are involved in the regulation of cell cycle arrest and are activated by ATM/ATR phosphorylation, through distinct mechanisms. Chk1 is activated by ATR in response to SSBs at the replication fork, DNA crosslink, ultraviolet (UV) radiation damage and, to lesser extent, ionizing radiation (IR), whereas Chk2 is activated by ATM in response to DSBs. Studies on human and mouse cells have revealed that Chk1 is the primary effector of the intra-S and G2/M phase checkpoints while Chk2 seems to play a partial role on the same checkpoints (Stracker et al., 2009). The cell cycle progression is controlled by the phosphorylation state of CDKs, mediated by the interplay between the CDC25 phosphatase and WEE1 kinase. In animals it has been observed that upon DNA damage, ATM and ATR phosphorylate the checkpoint effector kinases that act on the protein phosphatase CDC25, allowing its degradation by the proteasome or sequestration by 14-3-3 proteins. As a result of this pathway, CDKs cannot be activated and the cell cycle progression is arrested (Cools and De Veylder, 2009).

In high plants, a CDC25-like protein with the conserved C-terminal domain has been identified but it lacks the N-terminal regulatory domain characterizing the yeast and animal counterpart. Analysis on plants overexpressing or lacking this protein have revealed that it does not play any role in cell cycle arrest upon DNA damage, suggesting that in plants the DNA damage checkpoint can operate independently of the inactivaction of CDC25 (Dissmeyer et al., 2009; Spadafora et al., 2010).

On the other hand, the protein kinase WEE1, that controls the phosphorylation state of CDKA;1 and inhibits cell cycle progression, is involved in cell cycle arrest during DNA replication stress. In fact it has been observed that, upon treatment of replication inhibitor drug, *wee1* knockout plants and *atr* mutants displayed similar effects on growth, suggesting that the arrest or at least the delay in cell cycle progression is necessary to activate the DNA repair machinery (De Schutter et al., 2007; Culligan et al., 2004). These plants, when exposed to hydroxyurea, were more sensitive to the genotoxic stress, while the overexpression of *AtWEE1* led to a permanent activation of cell cycle arrest in Arabidopsis(De Schutter et al., 2007; Ricaud et al., 2007). Moreover, a nonfunctional WEE1 kinase compromised the accumulation of phosphorylated CDKs, leading the cells to enter mitosis with damaged DNA (De Schutter et al., 2007).

In plants there are Kip-related proteins (KRPs), related to the animal Cip/Kip CDK inhibitors (CKIs), but their involvement in DNA damage response has not been demonstrated yet. However, a new class of these kinases has been discovered and indicated as related to the SIAMESE (SIM) protein that acts as cell cycle inhibitor in trichomes by its association with CDKs (Churchman et al., 2006). It has been reported that upon DNA damage some *SIM*-related genes can be induced in an ATM-dependent manner, unlike the other Kip-related proteins (Culligan et al., 2006).

A recent study on suspension culture cells from root tip has revealed that the expression of some cell cycle regulatory genes changes considerably upon exposure to UV-B radiation (Jiang et al., 2011). These authors focused on the expression of *CYCD3;1*, that is necessary for the G1/S transition (Menges et al., 2006), and on the expression of *KRP2,* that regulates the G1/S transition through the inactivation of CDK/cyclin complexes. In particular, it has been found a postponement of *CYCD3;1* expression that suggest a delay in G1/S transition caused by UV-B radiation. This hypothesis is also supported by the results found for *KRP2*, that upon UV-B radiation treatment becomes highly expressed (Jiang et al., 2011).

As demonstrated by Sakamoto et al. (2009), the exposition to DNA damaging agents or replication inhibitors affects root growth in Arabidopsis plants. In these plants the abundance of cyclin B and the related mitotic activity was monitored through the

expression of *CYCB1;1:GUS* that encodes a labile cyclin:GUS fusion protein. These system allowed to observe an abundant accumulation of CYCB1;1:GUS upon γ-irradiation or aphidicolin treatment, suggesting a temporal arrest of cell cycle by checkpoint responses. Moreover, the GUS activity monitored in a UV-B sensitive mutant, known as *suv2*, and in *atr* plants was weaker than in wild-type plants, suggesting that these mutants are defective in cell cycle arrest in response to DNA damage (Sakamoto et al., 2009).

1.3. Programmed cell death and E2F transcription factors role in animal cells

Programmed cell death (PCD) is a mechanism that occurs in all eukaryotes to remove damaged cells affected by mutations or infections. In animal systems, different PCD mechanisms have been discerned based on morphological characteristics and are classified as apoptosis, autophagy and necrosis. Apoptosis is characterized by chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies, occurring through the activation of caspase family proteases and the consequent degradation in lysosomes of phagocytic cells. Conversely, autophagy takes place in the absence of chromatin condensation and is characterized by the accumulation of autophagy vacuoles, whereas necrosis occurs as plasma membrane rupture and the release of cell content around the area affected (Lord and Gunawardena, 2012).

Both in animals and in plants, the response to DNA damage aims to avoid damage transmission to the daughter cells, as this can compromise the development and the function of tissues or organs and, in case of DNA damage to the germline, avoids its transmission to the next generation. Although both DNA repair and cell death eliminate the deleterious effects of DNA damage to the organism, the response performed by the cell death machinery result to be more stringent and might explain the tendency to suicide of animal stem cells (Rich et al., 2000).

In animal cells, it is known that DNA damage sensors are able to act on the transcription factor E2F1, increasing its stability and affecting its interaction with the retinoblastoma protein (pRB), or its binding to promoters of specific E2F-regulated genes. (Engelmann and Pützer, 2010). The upregulation of E2F1 in response to DNA

damage suggests that the role of the E2F transcription factors is not only restricted to the regulation of cell cycle progression but is extended to other aspect of cell fate, such as DNA repair or programmed cell death. E2F1 belongs to a family of transcription factors that include eight members (E2F1-E2F8) and is divided in two groups based on their transcriptional regulatory activities: E2F1-E2F3a are known as activators of gene expression while E2F3b-E2F8 act as repressors (Engelmann and Pützer, 2010). It has been demonstrated that, upon DNA damage, E2F1 and E2F2 are transcriptionally induced in an E2F3-dependent manner, suggesting that all three E2F transcription factors are involved in the DNA damage response (Martinez et al., 2010).

From different studies, it is clear that E2F1 induces both cell proliferation and cell death, although it is notcompletely understood how this transcription factor can decide which process is necessary to be activated. The absence of DNA damage and the presence of external growth signals might trigger the repression of E2F1 apoptotic action, suggesting that E2F1 can behave differently depending on the cell context (Polanger and Ginsberg, 2008; Chong et al., 2009).

As mentioned above, the DNA damage response in mammalian cells is described as a linear process of phosphorylation events. The first sensors of the DNA damage are the two phosphatidylinositol 3-kinases ATM and ATR that transduce signals downstream to the checkpoint kinases Chk1 and Chk2 (Engelmann and Pützer, 2010) which, in turn, phosphorylate E2F1 at Serine 31 and 364 (Lin et al., 2001, Stevens et al., 2003, Urist et al., 2004). Experiments using small interfering RNA (siRNA) have demonstrated that knockout of both Chk1 and Chk2 prevents the induction of E2F1 activity upon DNA damage, confirming that E2F1 is a downstream target of these kinases (Urist et al., 2004). However, phophorylation alone is not enough to increase E2F1 stability and the action of some proteins able to block ubiquitination is also necessary (Wang et al., 2004).

Another aspect that has been investigated concerns the retinoblastoma protein and how its binding to E2F1 can influence the DNA damage response. In the first model proposed, pRB acts as an indirect regulator of the E2F1-dependent apoptosis during DNA damage response through its phosphrylation by Chk1/2 in an ATM-dependent

manner and through its acetylation by p300. The phosphorylation of pRB at Ser612 does not disrupt the complex pRB/E2F1 but results in the repression of cell death genes like p73, causing an antiapoptotic response that lead to cell cycle arrest (Inoue et al., 2007). On the contrary, the acetylation of pRB in the C-terminal E2F1 binding domain causes the release of E2F1 that induce an apoptotic response through the activaction of cell death genes (Engelmann and Pützer, 2010). In the second model, DNA damage induces the formation of a complex containing pRB, E2F1 and the histone acetylase P/CAF that activates the expression of proapoptotic genes like p73 and caspase 7 (Ianari et al., 2009).

Apoptosis induced by E2F1 is basically classified as p53-dependent or p53-independent. The p53 tumor suppressor plays an important role in different cellular stresses. Although E2F1 does not regulate the *p53* promoter, the effect is mediated by *Arf*, a gene that is transcriptionally activated by E2F and encodes a protein that stabilizes and activates p53 (Polanger and Ginsberg, 2008). It has also been demonstrated that E2F1 can regulate the expression of apoptotic cofactors that enhance the interaction of p53 with pro-apoptotic targets or promote p53 phosphorylation (Hershko et al., 2005). In addition, E2F1 causes the stabilization of p53 protein also through the transcriptional activation of ATM, Chk1 and Chk2 (Berkovich and Ginsberg, 2003; Yang et al., 2008). This suggests that, upon DNA damage, E2F1 has a double role in triggering cell death: it can act downstream ATM, Chk1 and Chk2, being a target of them, as well as upstream of these kinases, controlling their expression.

On the other hand, the p53-independent apoptosis is characterized by the up-regulation of various pro-apoptotic genes: Apaf1 (apoptotic protease activating factor 1), caspases, Bcl-2 homology 3 (BH3)-only proteins, and the p53 family member p73 (Polanger and Ginsberg, 2008).

1.4. Programmed cell death in plant

Concerning PCD, plants cells have unique features that differ them from animal cells, like the inability to form apoptotic bodies and the presence of a cell wall that prevent the phagocitosis by other cells. For this reason, plant PCD has been identified as a vacuolar cell death or as necrosis. The former is an autophagy-like process, achieved by hydrolases released from collapsed vacuoles, whereas the latter is characterized by plasma membrane rupture and the consequent contraction of the protoplast (Lord and Gunawardena, 2012). Besides, plants are sessile organisms that evolved a specific DNA damage response. This might explain why, although the DNA damage response machinery is conserved among eukaryotes, some targets of ATM and ATR, like p53, Chk1 or Chk2, are missing in plants. (Cools and De Veylder, 2009). Studies on Arabidopsis have demonstrated that ATM and ATR are involved in different response to DNA damage, as cell cycle checkpoint activation or DNA repair (Cullingan et al., 2004, 2006; Ricaud et al., 2007), and more recent studies, focused on stem cell niches, have proposed an ATM/ATR role in inducing programmed cell death upon DNA damage (Fulcher and Sablowski, 2009; Furukawa et al., 2010).

Stem cell niches are a pool of undifferentiated cells, localized at the shoot apical meristem (SAM) and root apical meristem (RAM), that provide cell precursors and are necessary to assure the formation of new tissues throughout the entire plant life, as well as the formation of the reproductive organs after the transition from the vegetative to the reproductive phase. In order to avoid the onset of mutated cell populations, the organism must control and repair any DNA damage, especially in the SAM where the somatic mutation can be fixed and transmitted to the next generation after transition to the reproductive phase (Klekowski et al., 2003). Fulcher and Sablowski demostrated that upon treatment with radiomimetic drugs (bleomycin and zeocin) or x-rays the cell death observed in root initials was due to a genetically programmed response to DNA damage, mediated by ATM/ATR activity. They observed that the cell death was strongly inhibited in the *atm-1, atm-2* and *atr-2* mutants, that being loss of function alleles still showed a small amount of cell death, attributable to residual ATM/ATR activity (Fulcher and Sablowski, 2009).

The role of ATR, ATM and SOG1 in cell death induction has been investigated in root stem cells also in another study, following UV-B and ionizing radiation (IR) treatments that are able to generate roughly 30.000 potential replication-blocking photoadducts and 24 double-strand breaks respectively (Furukawa et al., 2010). This study, in agreement with the results reported by Fulcher and Sablowski (2009), revealed that both UV-B and IR treatments induce programmed cell death at the stem cell niches in almost every root tip and the action of ATM, ATR and the SOG1 transcription factor was required. However, analyses on the *atr* and *atm* mutants revealed a high amount of cell death upon UV-B treatment, which suggests that both ATM and ATR may activate PCD. Moreover, in root tips of the double mutant *atm atr* the PCD after UV-B induction was lower than in wild-type but cell death was not totally inhibited (Furukawa et al., 2010).

The effect of DNA damage has been investigated also in the shoot meristems, where the stem cells are localized in the central zone of both the inflorescence and floral meristem. DNA damage induced by zeocin treatments caused a preferential cell death in the central zone of the inflorescence and floral meristems. Unlike in the RAM, it has been observed that cell death in the SAM was inhibited in the *atm-1* mutant but not in the *atr-2*, suggesting a different signal transduction pathway in shoot and root meristems (Fulcher and Sablowski, 2009).

Recently, it has been reported by Smentana et al. that BY-2 cells have been induced to activate PCD after bleomycin (BLM) treatment (Smetana et al., 2012). The observed cell death features resembled paraptotic-like PCD rather than apoptotic-like PCD or autophagic PCD. Paraptosis involves cytoplasmatic vacuolization and mitochondrial swelling, in the absence of apoptotic markers such as caspase activation, oligonucleosomal DNA cleavage or nuclear fragmentation and so far it had been identified only in animals and protists (Sperandio et al., 2000, 2004; Jimenez et al., 2009). To understand if the ATM/ATR sensors were involved in PCD induction in BY-2 cells, the ATM/ATR inhibitor caffeine was used. Caffeine intake during BLM treatment led to a significant decrease of PCD, chromatin condensation and vacuolar disintegration at a high BLM concentration, suggesting that the paraptotic-like PCD is partially dependent on ATM/ATR kinases. The involvement

of ATM/ATR in PCD activation was supported also by studies on Arabidopsis root meristematic cells, in which caffeine inhibited the PCD induced by the ATM/ATR pathway (Smetana et al., 2012). This study also reported that the minor decrease in cell viability observed upon BLM treatment at low concentration was not affected by caffeine. In this case the small amount of PCD might be induced by residual ATM/ATR activity or by another pathway independent of ATM and ATR (Smetana et al., 2012).

1.5 Role of plant E2F transcription factors in DNA damage response

In plants, E2F transcription factors act as activators or repressors of gene transcription and share some features with their animal counterpart. *Arabidopsis thaliana* possesses six E2F transcription factors that have been subdivided in two groups, known as typical and atypical.

The typical E2Fs (AtE2Fa-AtE2Fc) have a DNA binding domain followed by a dimerization domain that is necessary to bind the DP partner, a related protein with a similar DNA binding domain. The formation of the E2F/DP complex is required to bind with high affinity the E2F sites in the promoters of target genes. On the other hand, atypical E2Fs (AtE2Fd-AtE2Ff), that are believed to be transcriptional repressors, lack a dimerization domain but possess a duplicated DNA-binding domain that allows DNA binding without interaction with DPs (Shen, 2002). As already mentioned above, it is known that E2Fs, in addition to their role in controlling cell cycle, are player in the DNA damage response. Studies on animal cells have revealed that the E2F transcription factors can be involved in the response to DNA damage in different ways, like the transcriptional control of genes directly involved in DNA damage repair or acting along a pathway that lead to programmed cell death.

Concerning studies in plants, microarray analyses have revealed that *AtE2Fa* is induced after BLM plus MMC treatment (Chen et al., 2003) and the overexpression of AtE2Fa/AtDPa up-regulates several genes involved in DNA damage response (Vandepoele et al., 2005; Naouar et al., 2009). Among these, genes coding for ribonucleotide reductase (RNR) have been shown to be up-regulated upon DNA damage, since RNR plays an important role in providing the dNTPs needed for DNA

replication and DNA repair. The tobacco *NtRNR1a* gene possesses two E2F elements in its promoter and the NtE2F factor has been shown to mediate transcriptional induction of this gene upon UV-C irradiation (Lincker et al., 2004). Arabidopsis contains tree *RNR2* genes (*AtTSO2*, *AtRNR2A* and *AtRNR2B*) coding the R2 catalytic subunits. Roa et al. (2009) have demonstrated that AtE2Fa is upregulated in response to BLM treatment and mediates the induction of *AtTSO2* by binding a consensus E2F *cis*-element present in the promoter. Besides, it has been found that the upregulation of *AtE2Fa* after BLM treatment was lost in an *atm* mutant while an increased expression was observed in an *atr* mutant, suggesting an ATM-mediated transcriptional activation of *AtE2Fa* in response to DSBs (Roa et al., 2009).

An involvement of E2Fs in regulating DNA damage response upon UV-B radiation has been proposed as well. The mismatch repair system (MMR) is known to recognise and correct the mispaired and unpaired bases (Modrich and Lahue, 1996) and is involved in the response to UV-B radiation. This has been demonstrated also for Arabidopsis and maize in which the genes *MSH2* and *MSH6* are up-regulated after UV-B exposure (Lario et al., 2011). The *MSH6* gene of Arabidopsis contains three consensus E2F-binding sites in its promoter and its expression increases considerably in plants that overexpress AtE2Fa or AtE2Fb. Surprisingly, this study reported that *MSH6* expression increases also in plant overexpressing any one of the atypical AtE2Fs (Lario et al., 2011).

Recently, it has been demonstrated that the AtE2Fe transcription factor is also involved in DNA damage response because a down-regulation of its transcription has been observed upon UV-B treatment (Radziejwoski et al., 2011). Specifically, AtE2Fe has been proposed to act as transcriptional repressor of the type-II cyclobutane pyrimidine dimerphotolyase DNA repair gene *PHR1* since knockout plants for *AtE2Fe* displayed increase tolerance towards UV-B (Radziejwoski et al., 2011). Moreover, it was reported that AtE2Fe represses the endocycle onset, acting on the *CCS52A2* gene encoding a CDH1-related activator of the anaphase-promoting complex (APC) ubiquitin ligase. In the presence of AtE2Fe, the APC activity is limited, causing the accumulation of G2/M-specific cyclins necessary for mitosis. On the other hand, the loss of AtE2Fe stimulates the endoreduplication onset because

APC activated by CCS52A2 destroys the mitotic CDK/cyclin complexes. (Vlieghe et al, 2005; Lammens et al, 2008). Endoreduplication has been proposed to protect cells against DNA damage because of the presence of multiple copy genes, as indicated by the analyses of the *uv4* mutant that displayed tolerance to UV-B radiation associated to an increased ploidy level (Kondorosi and Kondorosi, 2004; Hase et al 2006). Therefore, it has been proposed that UV-B radiation resistance showed by the E2Fe knockout plants might be due to the resumption of endocycle and high basal level of PHR1 (Radziejwoski et al., 2011).

Another aspect that has been investigated in DNA damage response concerns the formation of the phosphorylated histones H2AX (γH2AX) repair foci, a large proteic complex forming at the DNA damage site that appears to involve the activity of E2F transcription factors (Lang et al., 2011). In animals, it has been reported that the γH2AX repair foci occurs at the site affected by DSB and its formation is dependent on the ATM activity and, on a less extent, on the ATR activity (Burma et al., 2001; Ward and Chen, 2001). It has also been demonstrated that loss of H2AX compromises genomic stability and γH2AX-deficient mice are radiation-sensitive and growth-retarded (Celeste et al., 2003a; Franco et al., 2006). In Arabidopsis, the γH2AX protein amount increased upon BLM treatment and the formation of γH2AX foci was detected in root tip, suggesting a role in DSB repair (Lang et al., 2011). An H2AX knockout line (miH2AX) for both gene *H2AXa* and *H2AXb* was created by RNAi approach and used to demonstrate that DSB induction of the *AtTSO2* gene disappeared in miH2AX. In addition it has been proposed a direct link between Arabidopsis H2AX and AtE2Fa protein that influence the transactivaction of *AtTSO2*, already characterized as an E2F-regulated gene. This aspect has been confirmed by observation of tobacco E2F factor fused to GFP that, upon BLM treatment, relocalized to nuclear foci in both *Arabidopsis* root tip cells and in BY-2 cells (Lang et al., 2011).

As shown by these various studies, plants E2F are clearly involved in the response to DNA damage and it has been demonstrated that PCD occurs in plant meristems in response to the DNA damage (Fulcher and Sablowski, 2009; Furukawa et al 2010). According to microarray analyses, the overexpression of AtE2Fa/AtDPa does not up-

regulate PCD-related genes (Vandepoele et al., 2005; Naouar et al., 2009). Nevertheless, it is possible that the transcription of PCD-related genes in Arabidopsis could be mediated by other E2Fs. Recently, to understand whether the plant E2Fs could be actually involved in a cell death program, Smentana et al. (2012) focused their studies on the tobacco NtE2F, a transcriptional activator that had been demonstrated to be up-regulated in response to UV-C radiation (Lincker et al., 2004). PCD was analysed on a BY-2 cell line overexpressing a *GFP:NtE2F* fusion under the control of the constitutive 35S promoter. Upon BLM treatment, a decrease in PCD was observed in the NtE2F overexpressing lines compared to the wild-type cells. Even at high BLM concentration, cellular features of PCD, as chromatin condensation and vacuolar collapse, were not found (Smetana et al., 2012). Because PCD induction in BY-2 cells has been shown to be differentially regulated throughout the cell cycle (Herbert et al., 2001; Kuthanova et al., 2008a), Smentana et al. counted dead cells during cell cycle progression in synchronized culture subjected to 10^{-5} M BLM treatment. When BLM was applied in G2, the mortality was significantly reduced in NtE2F-expressing cells compared to wild-type cells, whereas BLM treatment during S or M phases revealed only a partial inhibition of cell death in NtE2F-expressing cells. Also after a 10^{-4} M BLM treatment applied in G2 phase the induction of cell death was down-regulated by NtE2F overexpression but the entry into mitosis was not affected. Moreover, most of the cells had a 4C DNA content in both the NtE2F and wild type lines, suggesting that the G2/M checkpoint activation was not altered by NtE2F overexpression. Since NtE2F overexpression appeared to prevent cell death induction, it has been proposed that this transcription factor can take part in the DNA damage response mediating the up-regulation of DNA repair genes such as *NtRNR1b* (Smetana et al., 2012).

2. AIM OF THE PROJECT

Plant postembryonic development and growth are assured by the activity of meristems at the root tip and the shoot apex. In fact, meristematic stem cells constantly divide and provide cell precursors that differentiate to form new tissues and organs. As the DNA damage affecting this pool of undifferentiated cells might be deleterious and compromise plant growth and viability, different mechanisms have been evolved to protect genome integrity. Recently it has been discovered that in response to DNA damage plant stem cells can achieve a programmed cell death (PCD) involving the activity of the ataxia telangiectasia mutated (ATM) and the ATM and Rad3-related (ATR) kinases (Fulcher and Sablowski, 2009; Furukawa et al., 2010).

In plant, little is known about the contribution of E2F transcription factors to DNA damage response. Some studies have shown that they are involved in the transcriptional control of genes directly implicated in DNA damage repair (Modrich and Lahue, 1996; Lario et al., 2011; Radziejwoski et al., 2011), although it is not completely understood whether they can also act along a pathway that lead to PCD, as documented for animals (Berkovich and Ginsberg, 2003; Hershko et al., 2005; Polanger and Ginsberg 2008; Yang et al., 2008). Here, I analysed Arabidopsis lines overexpressing AtE2Fa or AtE2Fb, as well as the corresponding insertional mutants, to investigate the role of E2F trascription factors in the PCD pathway that is induced in root apical meristem upon DNA damage.

3. MATERIAL AND METHODS

3.1. Plant Material

Arabidopsis thaliana ecotype Columbia was used throughout this research.

The insertional mutants used are showed below:

Mutants	Insertion ID
e2fa	GK-348E09.01
e2fb	SALK_103138C

The AtE2Fa and AtE2Fb overexpressing (OE-E2Fa1; OE-E2Fa5; OE-E2Fb7; OE-E2Fb13) lines have been created previously in our lab (Perrotta, 2011).

3.1.1 Seed sterilization

Approximately 20-30 µl of seeds were put in labelled 1.5 ml Eppendorf tubes and placed in a desiccator jar, under a chemical flow hood. In the jar, 3 ml hydrochloric acid ~ 36 % was added in a beacker with 100 ml sodium hypochlorite ~ 10% and left for 3 hours with the open tubes to allow seeds to sterilize in chlorine gas.

3.1.2. Growth conditions

Seeds were sown directly onto germination media (GM): 4.4 g/l Murashige and Skoog salts, 1% glucose, 0.5 g/mL 4-morpholineethanesulfonic acid, 0.8% agar, pH 5.7. Media was autoclaved and left to cool before pouring into 90 mm petri dishes. Plates were sealed with surgical tape 3M and placed for 48 hrs at 4°C in the dark. After stratification, plates were transferred to long day growth chambers at 21°C (16 hrs light 8 hrs dark). For plants grown to maturity, seedlings were transferred to soil after formation of the first two leaves. Plants were grown in JIC Arabidopsis soil mix consisting of Levingtons F2 compost (with Intercept) and grit at a ratio of 6:1. Seedlings were then placed into CER 51-10 under long day growth conditions (16 hrs light, day 18°C and night 20°C, humidity 80%).

3.1.3. Plant crosses

Crosses were carried out on flowers from primary or secondary inflorescences, using fine-pointed forceps and a binocular headband magnifier. To isolate female pollen acceptors, mature flowers and the shoot apex were removed from inflorescence meristems. The unopened floral buds were then emasculated leaving the immature carpel; this was performed on approximately 5 to 10 buds. Emasculated plants were then labelled and left for 2 days to allow carpel to mature. The carpel was then pollinated from mature flowers by dabbing anthers onto the stigma and left to mature into siliques; just before desiccation, siliques were bagged into 88mm x 120mm cellophane bags to collect seeds.

3.2. Genotyping

3.2.1. DNA extraction

To extract DNA from transgenic lines, 3 cauline leaves were collected from each plant and placed into a labelled Eppendorf tube. Tubes were dipped into liquid nitrogen and tissue was ground within the Eppendorf using a micro-pestle. 600 µl of DNA CTAB extraction buffer (2% CTAB, 1.42M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0) were added to the tubes. After vortexing, tubes were incubated at 65°C for 20 mins and 600 µl of Chloroform:Iso Amyl Alcohol (24:1) were added and mixed by vortex. Samples were centrifuged at full speed for 10 mins and the aqueous phase was transferred to fresh tubes. A volume of isopropanol was pipetted and, after mixing by invertion, samples were transferred at -20°C for at least 15 mins, to allow DNA precipitation. Tubes were then centrifuged at full speed for 15 mins and the liquid was poured away. The pellet was washed by adding 1 ml ethanol 70%, followed by 10 mins of centrifugation. After pipetting off the ethanol, tubes were left to dry for ~ 30 mins and pellet were resuspended in 200 µl of dH_2O.

3.2.2 PCR genotyping

After completing DNA extractions, PCR reactions were performed in order to confirm T-DNA insertional mutants. PCR reaction was prepared as following: 1 µl Genomic DNA, 1 µl Forward oligo 10 µM, 1 µl Reverse oligo 10 µM, 0.5 µl dNTPs 10 mM (Roche), 2,5 µl 10x Taq Buffer (Roche), 0.1 µl homemade Taq and 18.9 µl dH_2O. The following PCR programme was used for genotyping: 94°C for 5 mins then 30 cycles of 94°C for 30 secs, specific primer temperature for 30 secs, 72°C for 30 secs, followed by final 10 mins at 72°C. On completion of PCR, samples were run on 2 % agarose gel.

Below are reported the primers (SIGMA) used:

LPAtE2Fa	tcctggtggtggagtactcac
RPAtE2Fa	ggtttccaggtctgtctttcc
AtE2Fa	ttccaggtctgtctttcctatttc
TDNAa	atattgaccatcatactcattgc
LPAtE2Fb	cgtagcagtggattcttctgg
RPAtE2Fb	tcttctcactggggaaacatg
LBb1.3	attttgccgatttcggaac

For e2fa genotyping, the pair LPAtE2Fa/RPAtE2Fa was used to amplify the wild type fragment and the pair AtE2Fa/TDNAa for the T-DNA fragment. For e2fb genotyping, the pair LPAtE2Fb/RPAtE2Fb was used for the wild type fragment and RP E2Fb/LBb1.3 for the T-DNA fragment.

3.3. Root DNA damaging experiments

3.3.1 Seed sterilization

Approximately 10 µl of seeds were put in labelled 1.5 ml Eppendorf tubes and placed in a desiccator jar, under a chemical flow hood. In the jar, 3 ml of hydrochloric acid ~36% was added in a becker containing 100 ml of sodium hypochlorite ~10% solution and left for 3 hours with the open tubes of seeds.

3.3.2. Growth conditions and zeocin treatment

Seeds were sown directly onto germination media (GM): 4.4 g/l Murashige and Skoog salts (Sigma), 1% glucose, 0.5 g/mL 4-morpholineethanesulfonic acid (Sigma), 0.7% agar, pH 5.7. Media was autoclaved and left to cool before pouring into 90 mm petri dishes. Plates were sealed with surgical tape 3M and placed for 48 hrs at 4°C in the dark. After stratification, they were placed vertically in growth chambers at 21°C, in continuous light, for 72 hrs. 3old-day seedlings were moved in ½ MS medium (2.2 g/l Murashige and Skoog salts, 0.7% agar, pH 7.5) supplemented with 35 μg/ml zeocin (Invitrogen); plates were protected from light to prevent degradation of zeocin and placed back into continuous growth chamber for 24 hrs, before imaging.

3.3.3. Imaging

For imaging, propidium iodide was pipetted directly into the slide and seedlings were lain down. A cover slip of 22 x 50 mm was added to cover the roots, leaving cotyledons out. Before imaging, slides were incubated for 5 mins at room temperature (RT). Confocal imaging was performed using a Leica SP1 equipped with an argon krypton laser. PI was excited using the 488-nm argon ion laser and emission was collected between 600 - 656 nm (PI). A 40X objective was used and each image was averaged four times.

4. RESULTS

4.1. Cell death induction in root meristem of plants overexpressing AtE2Fa or AtE2Fb

The programmed cell death (PCD) is a DNA damage response that occurs in plant meristems to eliminate cells that accumulate mutations. As the stem cells in the meristem divide constantly and provide the cell precursors for the formation of new tissues and organs, the activation of PCD is necessary to avoid the transmission of the mutations to the daughter cells and to assure plant development.

To understand whether the E2F transcription factors are involved in the pathway leading to PCD upon DNA damage, experiments of cell death induction have been performed in the root apical meristems of Arabidopsis seedlings overexpressing AtE2Fa or AtE2Fb as well as in the root meristems of *e2fa* and *e2fb* insertional mutants. In these experiments, cell death was induced by zeocin, a compound that causes DNA double-strand breaks, and the presence of dead cells was detected by propidium iodide staining and confocal microscope imaging.

Firstly, we investigated the cell death pattern in 3 days-old seedlings constitutively overexpressing the transcription factors AtE2Fa or AtE2Fb, obtained previously in our laboratory (Perrotta, 2011). Since it is well documented that the overexpression of these transcription factors can trigger cell proliferation, we investigated whether, upon DNA damage, overexpression of AtE2Fa or AtE2Fb could modify the cell death profile as well. The cell death pattern observed was compared with the one obtained in wild type seedlings and in the *atm* mutant. Because ATM is involved in the activation of PCD, when the root apical meristem of a *atm* mutant is treated with zeocin cell death is not detected or very few dead cells can be found. On the other hand, the meristem of wild type root is dramatically affected by cell death upon zeocin treatment.

The investigation of cell death patterns was performed in two independent lines overexpressing AtE2Fa, as well as in two lines overexpressing AtE2Fb. Figure 1 shows the results obtained for the two AtE2Fa lines (OE-E2Fa1, OE-E2Fa5). In both lines, after zeocin treatment, meritematic cells showed a pattern cell death that was

similar to the one observed in wild type seedlings. Regarding the two lines overexpressing the transcription factor AtE2Fb (OE-E2Fb7; OE-E2Fb13), the results are reported in Figure 2. As observed in the AtE2Fa lines, also the two AtE2Fb lines showed a cell death profile similar to the one observed for the wild type seedlings.
In conclusion, the overexpression of either AtE2Fa or AtE2Fb did not modify the cell death profile in root apical meristem.

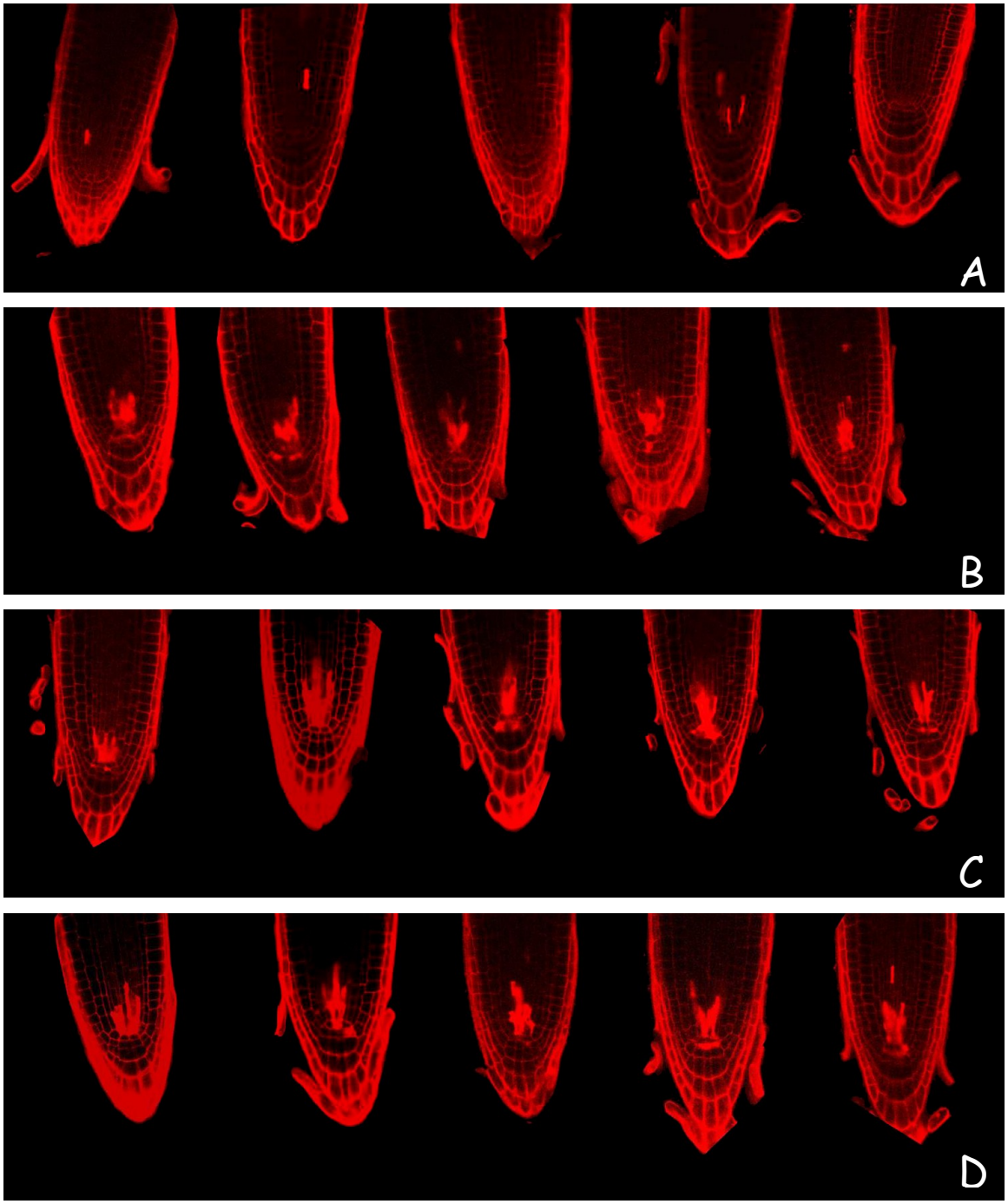


Figure 1: Rapresentative confocal images of root tips stained with PI: (A) *atm*; (B) wild type; (C) OE-E2Fa1; (D) OE-E2Fa5.

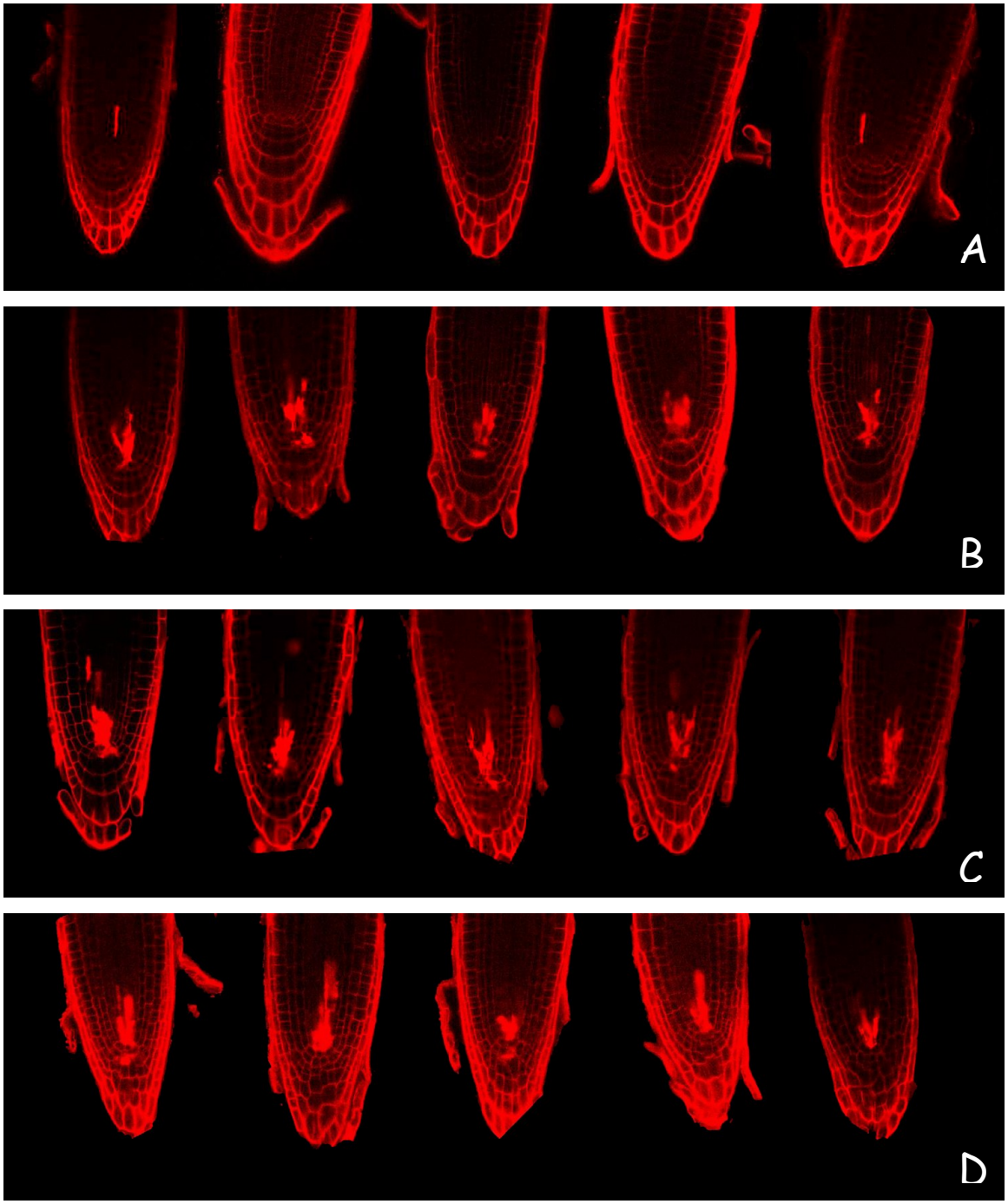


Figure 2: Rapresentative confocal images of root tips stained with PI: (A) *atm*; (B) wild type; (C) OE-E2Fb7; (D) OE-E2Fb13.

4.2. Cell death induction in root meristem of *e2fa* and *e2fb* insertional mutants

In animal cells it has been demonstrated that, upon DNA damage, some of the E2F transcription factors are involved in the activation of apoptotic genes, in a linear process of phosphorylation events that involves the activity of ATM. Considering the evidence in animal systems, we addressed whether the absence of AtE2Fa or AtE2Fb could abolish the DNA damage-induced cell death in root apical meristem. To verify this point, cell death experiments were performed on *e2fa* and *e2fb* insertional mutants. The *e2fa* line GK-348E09.01 and the *e2fb* line SALK_103138C, which were reported to be homozygous, were obtained from the Nottingham Arabidopsis Stock Centre and their seedlings were genotyped to check their homozygosity. These mutants are expected to be loss of function due to the insertion of the T-DNA in exon 10 (*e2fa*) or exon 9 (*e2fb*), that could lead to the production of a protein lacking the transactivaction domain. However, both mutants are fully fertile and did not reveal any obvious phenotype in normal growth conditions. For the analysis of cell death induction, experiments were performed as described above for the E2F overexpressing lines.

Figure 3 shows the cell death profiles obtained in *e2fa* and *e2fb* that were compared with the patterns observed in the *atm* mutant and wild type seedlings. In both cases, the insertional mutants displayed a cell death profile very similar to the one detected in the wild type and in the overexpressing lines. These results clearly indicate that either AtE2Fa or AtE2Fb are dispensable for the triggering of programmed cell death in the root meristem. However, this evidence cannot rule out the possibility that these E2Fs could be redundant in the activation of the PCD. To answer this question, the double mutant *e2fa-e2fb* was generated. Thus, the homozygous seedlings for *e2fa* and *e2fb* mutants were crossed and the resulting F1 plants were self crossed to segregate out the wild-type alleles. PCR genotyping was then carried out on the F2 seedlings to select plants with homozygous T-DNA insertions in both the *AtE2Fa* and *AtE2Fb* genes as shown in Figure 4. Surprisingly, despite the lack of both AtE2Fa and AtE2Fb the double mutant appears to be viable and no obvious phenotype was seen in 3 weeks-old seedlings as shown in Figure 5. A characterization of the *e2fa-e2fb*

double mutant is currently underway and experiments of cell death induction in the root meristem will be performed to investigate whether these E2F genes could be involved in the activation of plant PCD.

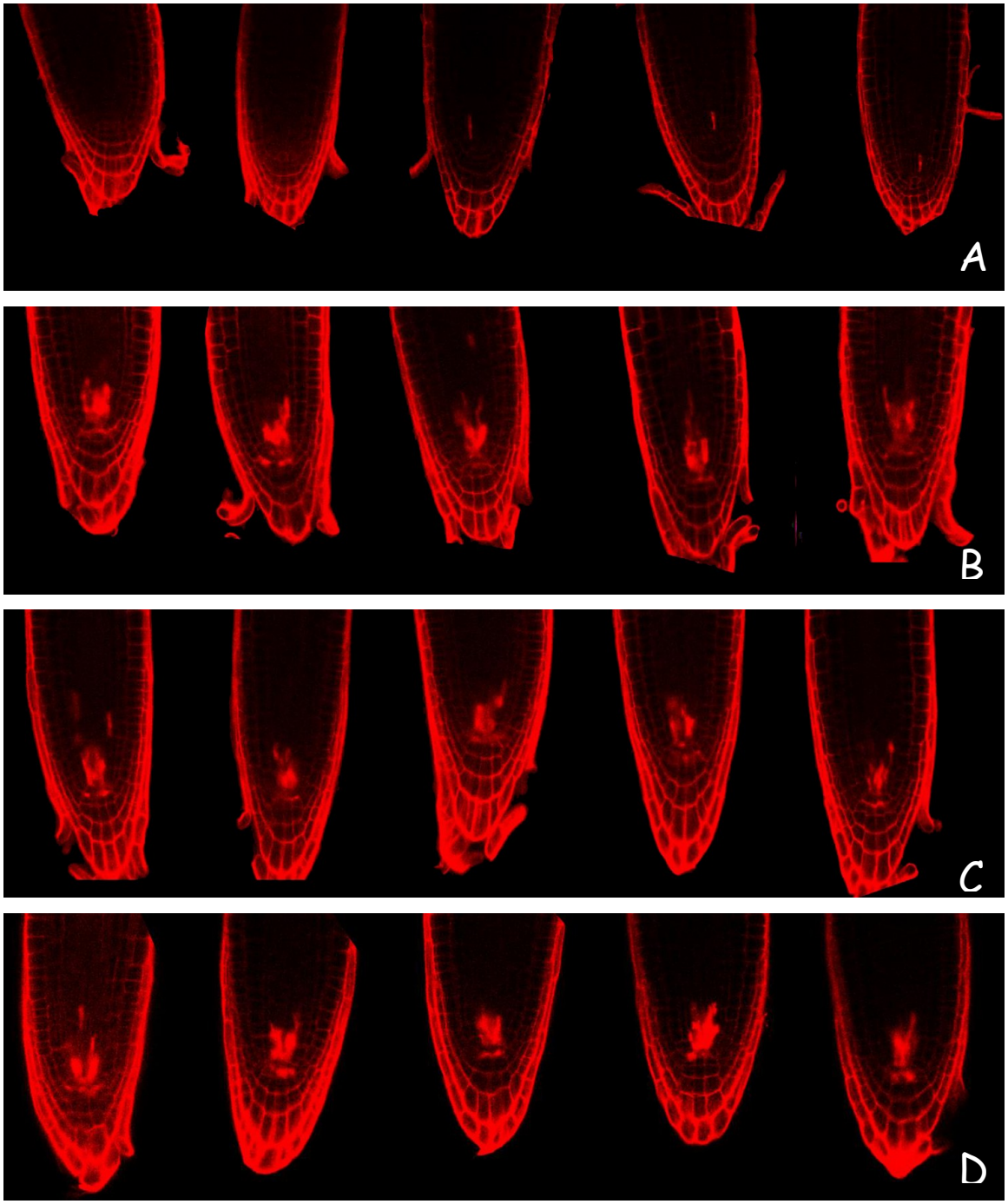


Figure 3: Representative confocal images of root tips stained with PI : (A) *atm*; (B) wild type; (C) *e2fa*; (D) *e2fb*.

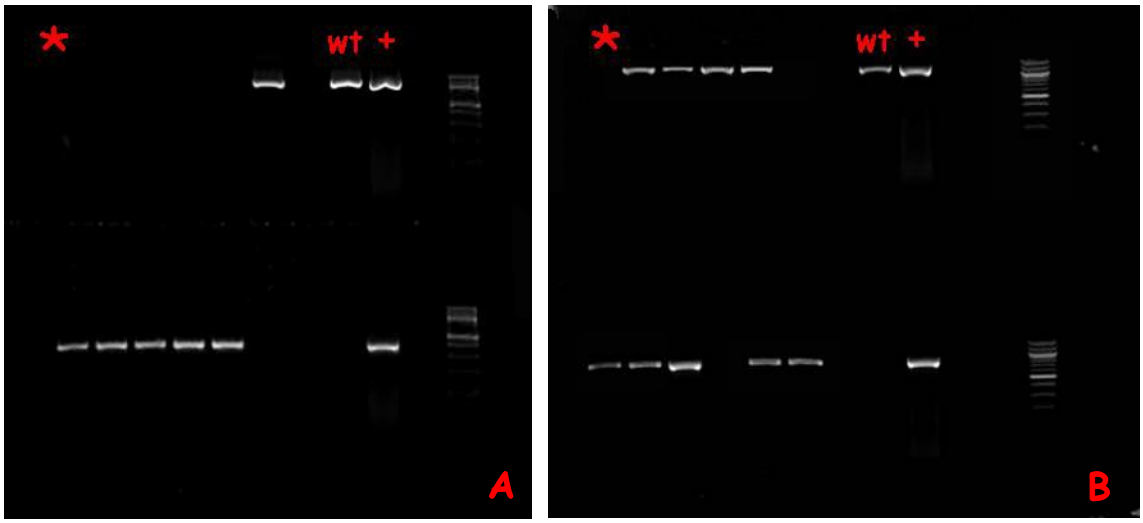


Figure 4: Electrophoresis gels of genotyping PCR for T-DNA insertion in the *AtE2Fa* (A) and *AtE2Fb* (B) genes: wt, wild-type; *, double mutant; +, heterozygous control.

Figure 5: 3 weeks-old *e2fa-e2fb* (double mutant) and wild-type seedlings.

5. DISCUSSION

The study of the response to the DNA damage in meristems is important to understand how plants control growth and development. Plants are sessile organisms, constantly exposed to environmental factors that can damage the DNA. To avoid the deleterious effects of accumulating mutations, cells have evolved different mechanisms such as the activation of a DNA repair machinery and the delay or even the arrest of the cell cycle. The response to DNA damage can be different depending on the cell features: DSBs cause cell cycle arrest at G1/S in meristem cells, but the same response has not been observed in differentiating cells that are endoreduplicating (Hefner et al., 2006). Recently it has been shown that plant stem cells and their early descendents trigger a programmed cell death (PCD) in response to DNA damage (Fulcher and Sablowski, 2009). This "general suicidal tendency" was firstly described as a more stringent response to DNA damage in animal cells (Rich et al., 2000), because of the necessity to eliminate damaged cells to avoid their uncontrollable proliferation and the consequent onset of tumours. Unlike plant cells, animal cells do not possess a wall that prevents damaged somatic cells to move and trigger metastasis. On the other hand, PCD in plants is an important mechanism to assure the proper development, rather than to avoid the onset of metastasis. In fact, plant development relies on the activity of stem cells, localized at the meristems, that constantly divide to provide a pool of undifferentiated and pluripotent cells. Upon hormonal and external signals, the descendents of these cells can turn to a differentiated status, allowing the formation of new organs. In this context, the programmed cell death is necessary to eliminate cells with DNA defects, avoiding the formation of clonal populations of mutant cells that can compromise plant growth and viability (Waterworth et al., 2001). To confirm this, it has been demonstrated that the preferential death of initials in the meristem, upon induction of DSBs, is a downstream response to the perception of DNA damage mediated by ATM and ATR, rather than a consequence of the damage itself (Fulcher and Sablowski, 2009).

All the mechanisms of DNA damage response are tight connected to the control of the cell cycle. Upon DNA damage, the progression and modulation of the cell cycle undergo a genetic regulation and the study of the E2F/RB pathway is therefore

important in understanding the mechanisms behind the genotoxic stress response. In plants, E2F transcription factors can act as activators or repressor and share some features with the animal counterparts. It has been demonstrated that plant E2Fs play important roles in regulating genes involved in cell cycle control as well as genes implied in DNA repair (Chaubotè et al., 2000; Lincker et al., 2004; Vandepoele et al., 2005; Roa et al., 2009; Radziejwoski et al., 2011).

Many studies in animals have focused on the understanding of E2Fs activities and their function in *vivo* either as an oncogene or as a tumor suppressor. These investigations concerned in particular E2F1 and its ability to induce either cell proliferation or apoptosis, upon mitogenic signals and DNA damage respectively (Polanger and Ginsberg, 2008). Under genotoxic stress conditions, it has been reported a double role for E2F1 in the triggering of apoptosis. It can act downstream the ATM, Chk1 and Chk2 pathway where, after phosphorylation and acetilation, activates its apoptotic target genes. In addition, it can act also upstream of these kinases, controlling their expression and inducing apoptosis through the stabilization and activation of p53 or through direct up-regulation of apoptotic genes. In light of the role played by animal E2F1 in cell death, we asked whether a similar scenario could be proposed for plant E2Fs.

Thus, part of the work of this thesis addressed the role that the plant E2F transcription factors could play in the control of PCD. This research has focused on the AtE2Fa and AtE2Fb transcription factors of Arabidopsis, analyzing overexpressing plants and insertional mutants in experiments of DNA damage induction. In Arabidopis it has been well documented that the overexpression of AtE2Fa with its patner AtDPa can induce ectopic cell division as well as endoreduplication (De Veylder et al., 2002), but any involvement of this transcription factor in PCD has not been documented yet. Moreover, microarray data concerning the expression of PCD-related gene did not show any change in expression in plants overexpressing AtE2Fa-AtDPa. (Vandepoele et al., 2005; Naouar et al., 2009). However, the microarray analyses performed on these plants were not carried out under DNA damage conditions and it is not known whether the upregulation of AtE2Fa activity can affect the PCD induced by genotoxic stress.

The aim of our experiments was to understand whether the overexpression of the activating E2Fs could stimulate cell death upon genotoxic stress condition. The results obtained in this thesis show that the overexpression of either AtE2Fa or AtE2Fb did not cause a dramatic change of the PCD induced by DNA damage because, upon genotoxic treatment, the meristems of the overexpressing plants preserve the same cell death pattern observed in wild type seedlings. These results retract our initial hypothesis that, based on a possible involvement of these transcription factors in the activation of PCD, forecasted an increased propensity to cell death in the seedlings constitutively overexpressing AtE2Fa or AtE2Fb. Therefore, it can be suggested that the contribution of these E2Fs to the DNA damage response could simply concern the activation of genes that are involved in DNA repair mechanisms, as it has been reported for the *MSH6* gene, belonging to the mismatch repair system (MMR), which is upregulated in plants overexpressing AtE2Fa or AtE2Fb (Lario et al., 2011). Evidence supporting this hypothesis comes also from studies concerning the formation of the phosphorylated histones H2AX (γH2AX) repair foci, a large proteic complex forming at the DNA damage site, that seems to involve the activity of E2F transcription factors (Lang et al., 2011). On the other hand, since it has been shown that the phenotype observed in plants constitutively overexpressing AtE2Fa was strongly enhanced by co-expression with AtDPa, it is possible that the overexpression an activating E2F/DP complex might be necessary to promote PCD in root meristem.

Also the experiments performed with the *e2fa* and *e2fb* insertional mutants suggest that upon DNA damage the activity of these E2F transcription factors might be restricted to the control of genes directly involved in the repair machinery and not involved in the activation of PCD. In fact, a positive role of these E2Fs in the triggering of PCD would be expected to result in the abolition, or at least an evident decrease, of cell death in these mutants. On the contrary, the results obtained revealed that the homozygous seedlings of both mutants show a cell death profile similar to the one seen in wild type seedlings at the same development stage. However, these results cannot rule out any gene redundancy for *AtE2Fa* and *AtE2Fb* that could explain why both mutants were still affected by normal levels of cell death. It is clear

that the next passage of this investigation will concern the understanding whether the unchanged cell death pattern observed in the insertional mutants is due to gene redundancy or whether PCD in root apical meristem is triggered by a mechanism that is not dependent by these E2F transcription factors. To address this point, the last part of the work has focused on the isolation of the double mutant *e2fa-e2fb*, which will be analysed to verify its cell death profile (while writing this thesis the seedlings of the *e2fa-e2fb* mutant were not yet available to perform the experiments).

The *e2fa* and *e2fb* mutants are expected to be loss of function or could be potentially dominant negative because the T-DNA insertion is just upstream of the transactivation domain, a condition that might give origin to truncated E2Fs which could still bind DNA but should be unable to interact with the basal component of the transcriptional apparatus. We supposed that this condition could be sufficient to compromise plant development because AtE2Fa and AtE2Fb are important players in the control of genes involved in the G1/S transition and are believed to be activators of cell division. Moreover, it has been shown that these transcription factors interplay to assure plant development by balancing cell division, endoreduplication and differentiation (Sozzani et al., 2006). Based on these evidences, we expected that, although the single mutants did not show major defects, the double mutant should be unable to develop properly. Surprisingly, however, the *e2fa-e2fb* plants appear to be viable and no phenotype was detected so far. Therefore, further investigations characterizing the *e2fa-e2fb* double mutant will be necessary to understand how plant development can still occur despite the potential production of truncated AtE2Fa and AtE2Fb proteins lacking the transactivation domain.

In conclusion, the role of the E2F transcription factors in PCD is far to be entirely clear. The main question is to understand whether the activity of E2Fs during DNA damage response in plants is restricted to the regulation of DNA repair genes or is extended to the control of PCD and whether it requires the DPs partner. The identification of the transcription factors controlling the expression of PCD-related genes will allow us to understand whether the induction of PCD is a process dependent or not by the activity of the E2F transcription factors.

CHAPTER 3. Investigations on the regulatory elements of the *AtDRTS2* gene of *Arabidopsis thaliana*

1. INTRODUCTION

1.1. Folates and their importance in plant development

The metabolism of plants and all other organisms involves the activity of cofactors derivated by the tetrahydrofolate (THF) and collectively named folates or vitamin B9. Folates are essential for the generation, interconvertion and donation of one carbon (C1) units. These reactions are necessary to perform the synthesis of a multitude of compounds involved in major cellular processes. THF coenzymes are required for the synthesis of purines and thymidylate, in the amino acid metabolism, for the pantothenate synthesis and for mitochondrial and chloroplastic protein biogenesis. Since the folates mediate Met synthesis, as a direct precursor of *S*-adenosyl-Met (Ado-Met) they are also necessary for the synthesis of molecules such as choline, chlorophyll, or lignin, that need a source of methyl units from the Ado-Met (Cossins, 2000; Scott et al., 2000; Hanson and Roje, 2001).

THF is a tripartite molecule, comprising pteridine, *p*-aminobenzoate (PABA) and glutamate moieties, to which C1 units at various oxidation levels are attached at N5 of the pteridine ring, or at N10 of the PABA unit, or bridged between the two. Folates exist mainly as polyglutamylated forms in which a γ-linked chain of up to about eight residues is added to the glutamate moiety (Fig. 1).

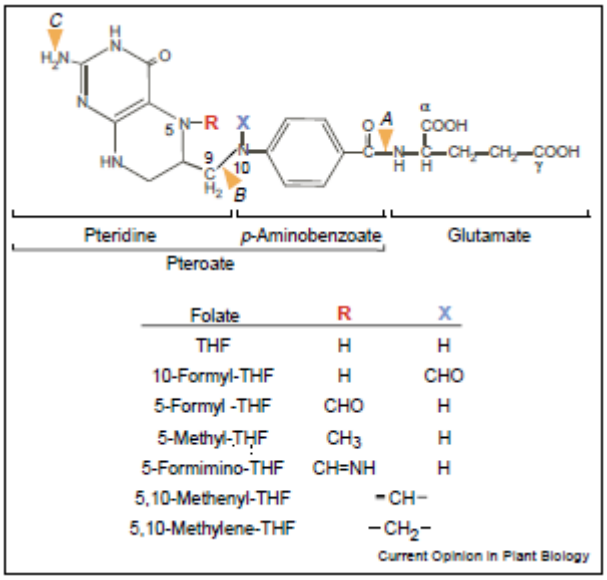


Figure 1: Chemical structures of tetrahydrofolate and its C1-substituted derivatives. In orange are labelled the attack sites of various catabolic enzymes (Hanson and Gregory, 2002).

In plants folates are present at trace concentrations (typically ≤5 nmol g^{-1} fresh weight) and are all unstable, particularly prone to the oxidative cleavage into pteridine and PABA-glutamyl fragments promoted by light. However, they are stabilized *in vivo* when are bound to proteins (Cossins, 1984; Scott et al., 2000). The THF is present in various compartments of the plant cell: the cytosol contains the largest pool of folates, predominantly in the form of polyglutamylated methyl derivatives, but it has been shown in pea leaves that mitochondria and chloroplasts also contain folates, principally (>90%) in polyglutamylated forms. Moreover it has been estimated that the distribution of folates among the organelles is unequal, since their concentration in mitochondria is 100–150-fold higher than in chloroplasts.

Despite their low concentration, folates are very important during seedling development due to the house-keeping functions mediated by folate coenzymes. As demostrated for *Pisum sativum*, the pool of folates increased in cotyledons during germination and the inhibition of their synthesis, using folate analogs, blocked seedling development (Roos and Cossins, 1971; Gambonnet et al., 2001). In fact, it has been shown that the initial folate content in the embryo can support root elongation during the first 1–2 days but further growth of the seedling was not

possible without *de novo* synthesis of folates. In proliferating cells there is a high request of nucleotide synthesis that is associated to THF activity. Therefore folate turnover could be a potential limiting factor in tissues where cells are dividing.

It has been reported that during organogenesis, the folate content of young roots and shoots was similar to that in the initial embryo. On the other hand, the folate content of young leaves increased rapidly to reach a value two to three times higher than in other tissues after 7 days of growth and remained roughly constant thereafter (Gambonnet et al., 2001). It has been reported that folates are actively synthesized and accumulated in photosynthetic leaves where a huge demand for folate, and thus for C1-transfer reactions, is associated with leaf development in the light (Oliver et al., 1994). The high concentration in leaves is also linked to the fact that folates support large metabolic fluxes, as it has been demonstrated in photorespiring C3 leaf where the 30% of the total folates participates in the THF-mediated glycine→serine reaction in mitochondria (Gambonnet et al., 2001).

1.2. THF biosynthesis

Folates are synthesized *de novo* by plants, fungi, most bacteria and protozoa. On the contrary, humans and metazoa lack a complete pathway for folate synthesis and therefore, folates are assimilated by the diet.

The biosynthesis of THF ($H_4F\text{-}Glu_n$) in plants and microorganisms needs dihydropterin and *p*-aminobenzoate (pABA) and requires the sequential operation of five reactions that take place in the mitocondria (Fig. 2). The first three reactions are absent in animal cells (Scott et al., 2000; Hanson and Gregory, 2002).

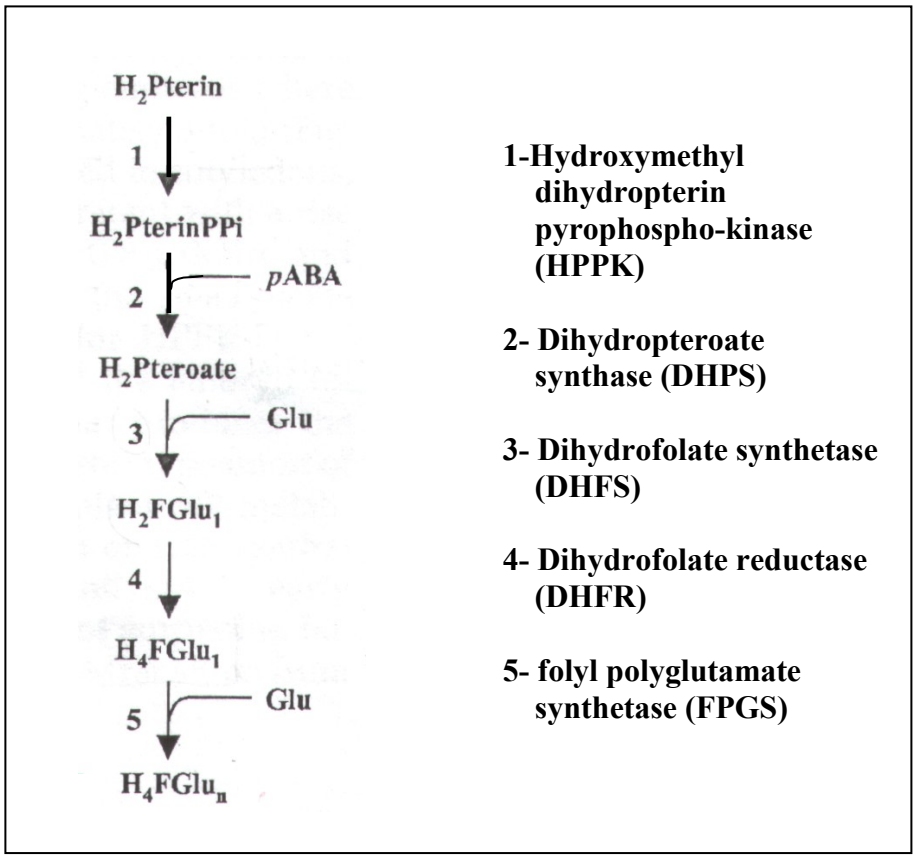


Figure 2: Biosynthesis of $H_4F\text{-}Glu_n$: the sequential operation of five reactions in the mitochondria (modified from Jabrien et al., 2003).

H_4F-Glu_n biosynthesis in plants involves also the activity of enzymes that are localized in plastids and mitochondria (Neuburger et al., 1996; Rebeillé et al., 1997; Ravanel et al., 2001).

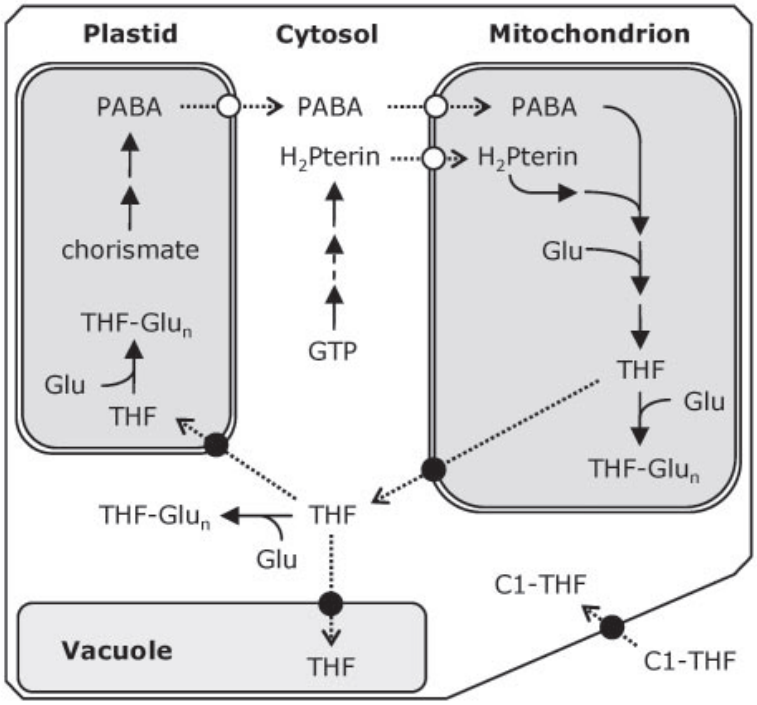


Figure 3: Subcellular compartmentation of THF synthesis in plant cells.
PABA, *para*-aminobenzoate; *H2Pterin*, hydromethyldihydropterin; *C1-THF*, C1 derivative of THF. Solid arrows indicate enzymes involved in THF synthesis; dotted arrows indicate transport steps (Bedhome et al.,2005)

1.2.1. Cytosolic steps

In the cytosol dihydropterin (or hydroxymethyldihydropterin) is synthesized from GTP in three steps. None of the enzymes required for this synthesis have predicted targeting peptides, suggesting that they are located only in the cytosol. The first reaction is catalysed by GTP-cyclohydrolase I (GTPCHI) to form dihydroneopterin triphosphate. GTPCHI is present in organisms synthesizing H_4F de novo and in mammalian cells where it is involved in the synthesis of pteridines such as tetrahydrobiopterin, a coenzyme participating in redox reactions (Werner-Felmayer et al., 2002). The triphosphate side chain of dihydroneopterin triphosphate is further

removed to produce dihydroneopterin. Two steps are required for this process, in fact the pyrophosphate group is removed by a specific nudix hydrolase (Klaus et al., 2005), followed by the action of a non-specific phosphatase (Suzuki and Brown, 1974). Lastly, dihydroneopterin aldolase (DHNA) cleaves the lateral side chain of dihydroneopterin to release dihydropterin.

1.2.2. Plastidic steps

p-ABA is synthesized in plastids. There are evidences that p-ABA is a potential limiting step for folate accumulation in plants. The first enzyme responsible for this synthesis in plant, which is a protein fusion of the PabA and PabB domains, possesses an ADC (aminodeoxychorismate) synthase activity and exhibits a transit peptide that is typical of chloroplast targeting. The last step in the synthesis of p-ABA is catalysed by an ADC lyase, a protein also located in plastids (Basset et al., 2004).

1.2.3. Mitochondrial steps

The final steps of H_4F synthesis occur in mitochondria. In fact, leaf mitochondria contain all the required enzymes and the first three steps are presumably exclusively localized in this compartment. Into mitochondria, dihydropterin and p-ABA are combined together with glutamate to produce H_2F. Four reactions are required to produce $H_4F\text{-}Glu_1$ (the monoglutamate form of H_4F). Dihydropterin is activated into its pyrophosphorilated form to be combined with p-ABA in a second step, resulting in dihydropteroate. These two reactions are catalysed in plants by a single bifunctional enzyme, Hydroxymethyldihydropterin pyrophospho-kinase/Dihydropteroate synthase (HPPK/DHPS) that was only detected in mitochondria (Neuburger et al., 1996; Rebeillé, 1997). A detailed analysis of kinetic properties of the mitochondrial HPPK-DHPS isoform indicates that the DHPS reaction is feedback inhibited by dihydropteroate, H_2F and $H_4F\text{-}Glu_1$. The third step is the ATP-dependent attachment of glutamate to the carboxyl moiety of p-ABA to form $H_2F\text{-}Glu_1$. It is catalysed by dihydrofolate synthetase (DHFS). This enzyme, together with the preceding reaction catalysed by HPPK-DHPS is absent in animals, rendering these organisms

autotrophic for folates. $H_2F\text{-}Glu_1$ is then reduced to $H_4F\text{-}Glu_1$ by dihydrofolate reductase (DHFR) using NADPH.

Higher plants and protozoa possess a bifunctional enzyme bearing DHFR and thimidilate synthase (TS) activities. TS catalyses the methylation of deoxyuridine-monophosphate into deoxythimidine-monophosphate in the presence of $CH_2\text{-}H_4F\text{-}Glu_n$ (where $H_4F\text{-}Glu_n$ stands for tetrahydro-pteroylpolyglutamate or tetrahydrofolate) (Ivanetich, 1990; Cella and Parisi, 1993). In this reaction $CH_2\text{-}H_4F\text{-}Glu_n$ acts both as C1-unit donor and as reducing agent, producing $H_2F\text{-}Glu_n$ (dihydropteroyl-polyglutamate or dihydrofolate). Thus, the DHFR domain of the bifunctional enzyme is involved in the reduction of H_2F originating from either the *de novo* synthesis pathway (monoglutamate form) or the oxidation of $H_4F\text{-}Glu_n$ by TS activity (polyglutamate form).

In all organisms, the polyglutamate tail of $H_4F\text{-}Glu_n$ is formed by the sequential addition of γ-linked glutamate residues to $H_4F\text{-}Glu_1$, a reaction catalysed by folyl-polyglutamate synthetase (FPGS). Polyglutamylation is essential to retain folate in a given compartment of the cell by increasing the anionic nature of folate coenzymes, thus impairing their diffusion through hydrophobic barriers (Appling, 1991). $H_4F\text{-}Glu_1$, once synthesized in the mitochondria, is exported to the other cell compartments before the final glutamylation step. In plant cells, the cytosol, mitochondria and chloroplasts contain folates predominantly in the form of polyglutamylates derivatives (Cossins, 2000).

1.3. The DHFR and TS enzymes and the synthesis of nucleotides

The synthesis of DNA precursor requires the involvement of THF that is a critical intermediate for thymidylate and purine biosynthesis. Either in prokaryotic or eukaryotic cells this process involves the activity of two enzymes, known as Thymidilate synthase (TS) and Dihydrofolate reductase (DHFR). These enzymes can occur as distinct monofunctional proteins or as a bifunctional polypeptide. The former have been identified in most organisms such as bacteria, fungi and all metazoa (Luo et al., 1997) while the latter has been found only in plants and protozoa, even if

in plants the monofunctional enzymes have been reported as well (Toth et al., 1987; Cella and Parisi, 1988, 1993).

It is not still clear whether bifunctionality arose independently during plant evolution or derived from a common ancestor shared with the protozoa (Philippe et al., 2000). The evidence that protozoa and plants possess the two enzymes DHFR and TS combined together in a single polypeptide highlights their correlated functionality. In fact, thymidylate synthase is exclusively involved in *de novo* dTMP biosynthesis while DHFR can have two roles depending on the source of dihydrofolate: *de novo* synthesis of tetrahydrofolate or recycling of the dihydrofolate released as one of the end products of the TS catalysed reaction. As show in Figure 4, 5,10-methylenetetrahydrofolate acts both as donor of the methyl group and as a reducing agent, giving rise to DHF. (Luo et al., 1993). In a subsequent reaction, dihydrofolate reductase (DHFR) catalyzes the reduction of 7,8-dihydrofolate to tetrahydrofolate (Luo et al., 1993). Therefore, TS is dependent on DHFR for regeneration of tetrahydrofolate, which is in turn necessary for the formation of 5,10-methylene-tetrahydrofolate.

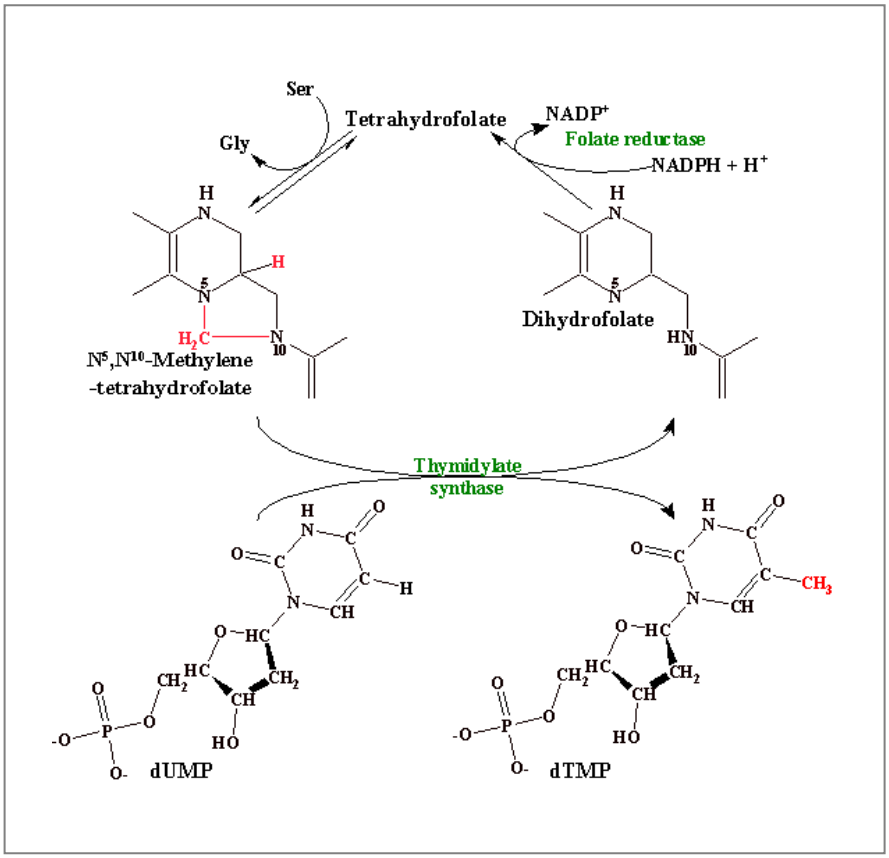


Figure 4: Biosynthetic pathway of dTMP.

1.4. *dhfr-ts* genes in high plants

Plant *dhfr-ts* genes have been first discovered in *Arabidopsis thaliana* (Lazar et al., 1993) and in *Daucus carota* (Luo et al., 1993), followed by their identification in *Glycine max* (Wang et al., 1995) and *Zea mays* (Cox et al., 1999). In all these cases, sequencing and cloning of the cDNA confirmed that the coding sequences of the DHFR and TS domains reside on single transcripts, encoding bifunctional gene products, and the DHFR-specific sequences are upstream of the TS region coding. Moreover, the comparison of the deduced amino acid sequences revealed over 75% similarity and the conservation of motifs typical of DHFR and TS proteins, for example the metotrexate, folate and dUMP binding sites. In *Daucus carota* the mapping of the 5' end of the *dhfr-ts* gene by primer extension and by rapid amplification of 5' cDNA ends (RACE) revealed the production of two major classes of transcripts derived from alternative promoters and coding for isoforms with

potentially different subcellular localization (Luo et al., 1997). The sequencing of the 5' flanking genomic region confirmed the presence of two promoters with well defined TATA box sequences located 25 and 27 bp upstream of the most proximal transcription start points (Luo and Cella, 1998).

As the DHFR and TS activity are essential for the biosynthesis of nucleotides, various analysis have focused on their biochemical importance in proliferating tissues, such as the meristems, or in tissues that are characterized by endoreduplication events. In situ hybridization analyses carried on *Daucus carota* confirmed that *DHFR-TS* transcripts are highly accumulated in meristematic tissues (Albani et al., 2005). The major amounts of these transcripts were found in pro-embryogenic masses as well as in shoot and root meristems of somatic embryos. In addition, a well defined expression was detected in expanding cotyledons at the torpedo/plantlet stage. Besides, northern hybridization experiments confirmed a higher accumulation of carrot *DHFR-TS* transcripts in proliferating suspension cells compared to cells in stationary phase or cells blocked with propyzamide, and revealed a low expression of the *DHFR-TS* gene in carrot leaves (Albani et al., 2005).

In *Zea mays* the investigation was focused on the endosperm tissue of maize kernels that exhibits developmentally controlled endoreduplication (Kowles et al., 1988, 1997). In fact DNA synthesis in developing kernels was reported to be highest between 10 and 16 DAP (days after pollination), and still detectable at 28 DAP (Kowles et al., 1988, 1997; Grafi and Larkins, 1995). Thus, *ZmDHFR-TS* gene expression was investigated in kernels at different stages of development from 8 to 35 DAP. RNA gel blot hybridizations of total RNA from each set of kernels in the time course showed high levels of *ZmDHFR-TS* expression from 8 to 20 DAP, followed by a decrease at 24 DAP. *ZmDHFR-TS* expression was maintained in both embryo and endosperm tissues at 35 DAP. Although the relative amount of *ZmDHFR-TS* mRNAs appeared to be similar in both 35 DAP endosperm and embryo tissues, in these samples *ZmDHFR-TS* mRNA levels were lower than in the 12 and 18 DAP kernels. At 35 DAP the embryo is still developing (Abbe and Stein, 1954) and requires DNA synthesis whereas cell division and endoreduplication are generally completed in the endosperm tissue by this stage (Kowles and Phillips, 1988). The

persistence of *ZmDHFR-TS* mRNA in endosperm at 35 DAP may be due to a requirement of nucleotides by mitochondria or to the onset of desiccation in the endosperm.

1.5. *dhfr-ts* genes in *Arabidopsis thaliana*

Arabidopsis thaliana possesses three *DHFR-TS* genes called *AtDRTS1, AtDRTS2* and *AtDRTS3*. All three genes show the same genomic organization suggesting their origin from evolutionary genome triplication (Jiao Y et al., 2012), with three members of the sec14-like *SFH* gene family found upstream of the *DHFR-TS* genes and oriented in opposite direction. SFHs (Sec Fourteen Homologues) are a diverse group of proteins with distinct subcellular localizations and varied physiological functions related to lipid metabolism, phosphoinositide mediated signalling and membrane trafficking (Fig. 5).

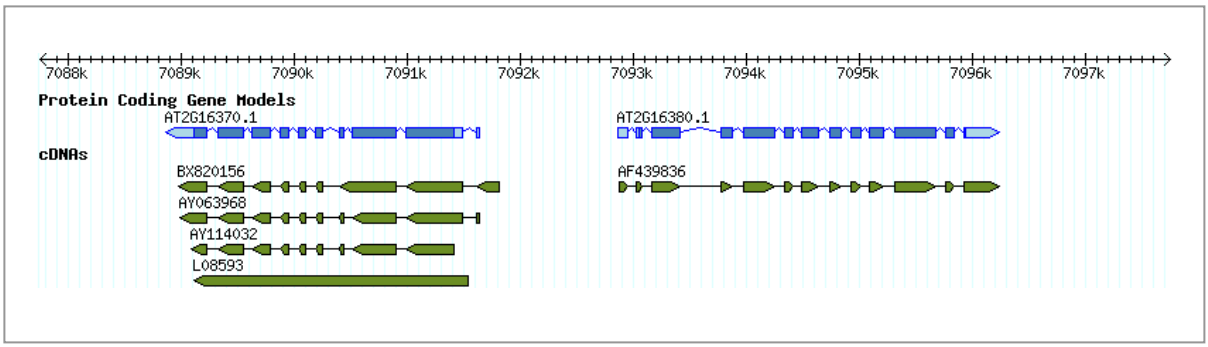
All the information following and regarding gene position and structure are reported in the TAIR (The Arabidopsis Information Resource) database at the site *www.arabidopsis.org.*

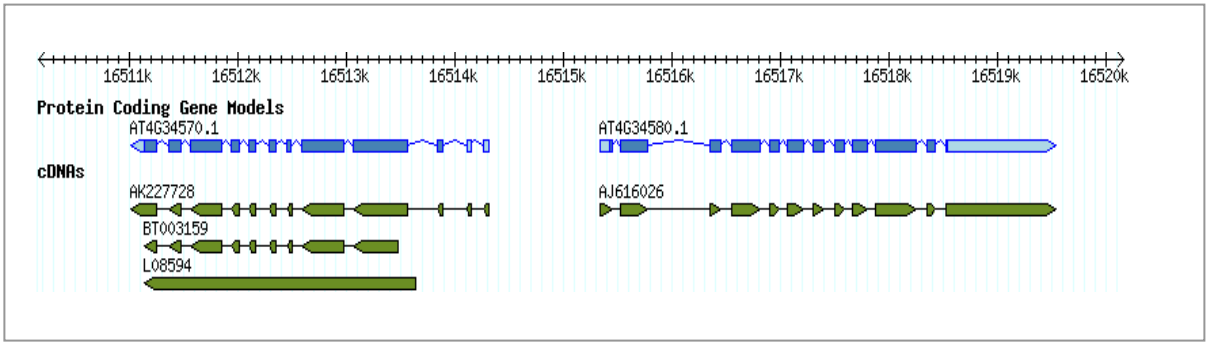
AtDRTS1, annotated with the sequential number At2g16370, is located in chromosome 2 and extends 2654 bp in length, from position 7088985 to 7091639. The gene contains 10 exons and the resulting cDNA is 1660 bp long. The ATG start codon is located in the second exon at position 225 and the coding region translates into a protein of 519 aa with a MW of 58KDa.

AtDRTS2, annotated with the sequential number of At4g34570, is located in chromosome 4, extending 3303 bp in length from position 16511012 to 16514316. This gene contains 12 exons and the resulting cDNA is 1683 bps long. The ATG start codon is located in the second exon at position 201 and the coding region translates into a 565 aa protein with MW of 63 KDa. In our laboratory 5'RACE analyses have revealed that alternative splicing can give rise to transcripts lacking the second exon, wich contains the first ATG codon of the gene. As a consequence, the alternative transcripts code for a smaller isoform of the AtDRTS2 protein that starts from the ATG located in the fourth exon of the gene (unpublished results). As suggested for the carrot *DHFR-TS* gene, alternative isoforms of the AtDRTS2 protein could

potentially lack or possess different signal peptides, necessary for subcellular localization.

AtDRTS3, with the sequential number At2g21550, is located in chromosome 2 and extends 2980 bp, from position 9234289 to 9237269. According to the coding capability, this gene is proposed to contain 10 exons, yielding a cDNA of 1469 bp from the ATG codon and expected to translate into a 492 aa protein with a MW of 54 KDa. However, the genomic organization just described corresponds to the gene model reported in the TAIR databases and the corresponding cDNA sequence has not been described in scientific publication and/or databases. Instead, the cDNA sequence for *AtDRST3* described in databases appears to contain only the first four exons. This cDNA sequence terminates in the center of the fourth intron, which contains a transposon-like element and is much larger than the corresponding introns in the *AtDRTS1* and *AtDRTS2* genes. It appears, therefore, that the insertion of the transposon-like element abolishes the splicing of the fourth intron that causes a premature termination of the transcripts and introduces a spurious TAG stop codon in the reading frame of the gene. Therefore, this *AtDRTS3* cDNA codes for a partial DHFR-TS protein that is truncated in the TS domain at the C-terminus. However, it is not known yet whether the transcripts corresponding to this *AtDRTS3* cDNA are actually translated into a product and whether the resulting protein possesses only DHFR activity or is having also partial TS activity.





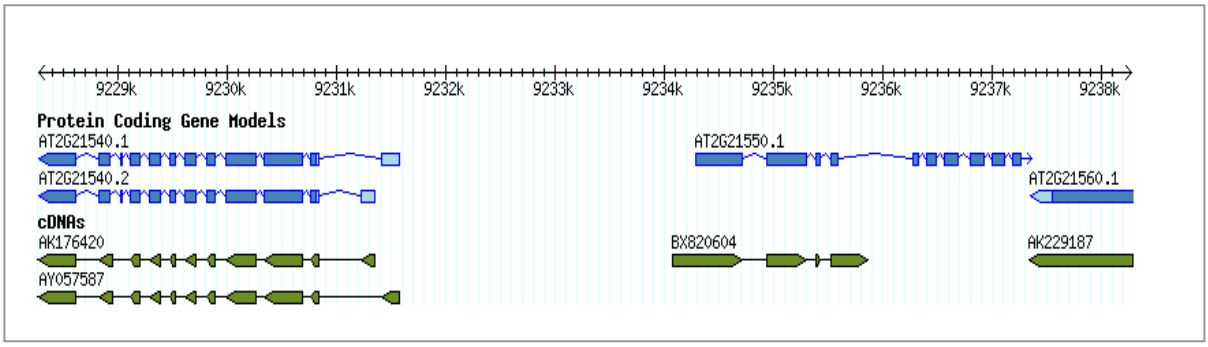


Figure 5: Organization of the AtDRTS and AtSFH genes and cDNAs.
From up to down AtDRTS1-AtSFH7; AtDRTS2-AtSFH1 and AtDRTS3-AtSFH3.

1.6. Role of *cis*-elements in transcriptional gene regulation

Transcriptional regulatory network need to be coordinated in order to assure organ and cell specific pattern of gene expression. In eukaryotes, the transcriptional regulation of gene expression is assured by the activity of transcription factors (TFs) that are recruited to *cis*-regulatory elements and interact with other TFs and the basal transcriptional machinery. This network is essential to regulate gene transcription spatially and temporally. It has been estimated that in plants more than 1500 TFs are involved in the control of gene expression and each of them can regulate from ten to thousands of genes (Reichmann et al., 2009; Guo et al., 2008). In addition, since it is well know that in all eukaryotes only a small fraction of the genome is occupied by protein coding sequences, analyses of non-coding sequences and the identification of regulatory elements are essential to understand how gene expression is regulated and how the transcription factor network is coordinated. Moreover, a highly specific pattern of gene expression involves the activity of *cis*-regulatory modules (CRMs) that are short DNA regions of a few hundred bp containing multiple transcription factor binding sites (TFBSs). In high eukaryotes there are at least 5 times more CRMs than genes (Priest et al., 2009). Therefore, the transcriptional regulation is a wide network that involves various *cis*-elements, different in sequence and location, and TFs that can be dissimilar in structure and function, since they can act as activactors or repressors of gene transcription. The task of this regulatory network is to coordinate the expression of the genes that undergo different fates. For example many genes have a cell cycle-regulated expression profile, suggesting that the activity of specific transcription factors and precise mechanisms of transcriptional regulation are important for the control of cell cycle progression. Moreover, during development and cell specification, it is necessary to coordinate the expression of genes in order to initiate the correct differentiation programmes and to suppress earlier or inappropriate programmes (Kaufmann et al., 2010).

A pivotal role in transcriptional gene regulation during cell cycle and development is played by the E2F transcription factors that are involved in the expression of cell cycle genes. They act in the retinoblastoma/E2F pathway that, although absent in fungi, is well conserved among animals and plants and is crucial to control the

transition from G1 to S phase. The typical E2F transcription factors bind the DNA at the canonical sequence TTTSSCGS (where S is either C or G) in a complex with dimerization partners DP. In the general model proposed, the Retinoblastoma protein (pRB) is associated with E2F, repressing E2F-regulated gene transcription in G1 cells. Upon mitogenic stimuli, the cyclin-CDK complexes phoshorylate pRB, triggering the release of E2F that is now able to act as a transactivator, allowing the expression of genes involved in DNA replication and cell cycle progression. In addition, the E2Fs are known to regulate genes that are necessary for DNA repair, defence response and signalling (Menges et al., 2002; Ramirez-Parra et al., 2003).

The study of *RNR1*, *RNR2* and *PCNA* promoters of tobacco and the *CDC6* and *MCM3* promoters of *Arabidopsis thaliana* revealed the presence of functional E2F consensus sites. The *RNR2* promoter analysis demonstrated the presence of two E2F consensus sites that caused an up-regulation of gene transcription at the G1/S transition. More precisely, it has been shown that one of the sites caused repression of gene expression outside the S phase (Chaboutè, et al., 2000). In *Nicotiana benthamiana*, the promoter of the *PCNA* gene is characterized by two E2F binding sites as well. In this case, it has been demonstrated that one of these sites activates the transcription in proliferating cells whereas the other acts as a repressor in differentiated tissues. Moreover, in the rice and tobacco *PCNA* promoters, the E2F consensus sequences are involved in the activation of a reporter gene in both cultured cells and whole plants (Kosugi and Ohashi, 2002).

The study of the transcriptional regulation of histone genes is important in plants since histone-mediated changes in chromatin structure and DNA organization affect various processes such as root growth, flowering time, as well as gametophyte or embryo formation (Berr et al., 2011). The identification of important *cis*-elements involved in cell cycle-dependent gene expression comes from studies on histone promoters, where negative and positive elements have been discovered. It has been reported a preferential activity of histone gene promoters in dividing cells that identified transcriptional regulation as the predominant control for plant histone gene expression (Atanassova et al., 1992; Brignon and Chaubet, 1993; Terada et al., 1995; Chaubet et al., 1996; Atanassova et al., 1998; Taoka et al., 1999; Minami et al.,

2000). The nonameric motif (NON, CATCCAACG) and the CCGTC (CG) element are two positive cis-element that are common to all known replication-dependent plant histone genes and are reported to regulate interactively histone gene transcription in meristems (Brignon and Chaubet, 1993; Chaubet et al., 1996). The nonameric motif was first identified in wheat *H3* and maize *H4* genes (Nakayama et al., 1992; Lepetit et al., 1993) whereas the CG element was first described as a *trans*-factor-binding site of Arabidopsis *H4*, as well as of maize *H3* and *H4* gene promoters (Brignon and Chaubet, 1993). Another nonameric motif, the AGATCGACG stretch of the Arabidopsis histone *H4A748* gene promoter, is a positive regulator of histone gene expression in meristematic tissues (Brignon and Chaubet,1993; Chaubet et al., 1996; Shen and Gigot, 1997; Meshi et al., 2000). In addition, a highly conserved *cis*-element, the octamer CGCGGATC (Oct), is common to most plant histone gene promoters. Analyses in transient and stable expression assays indicate it as a proliferation-coupled and S phase-specific *cis*-element (Chaubet et al., 1996; Ohtsubo et al., 1997; Taoka et al., 1999). Besides, octamers often act in combination with another module, thereby determining three types (types I, II, and III) of Oct-containing composite elements (OCEs), which all function as separable S phase-specific elements (Yang et al., 1995; Ohtsubo et al., 1997; Taoka et al., 1998, 1999; Meshi et al., 2000). The type I element (CCACGTCANCGATCCGCG) consists of a reverse-oriented Oct paired with another conserved histone promoter motif, the hexameric ACGTCA sequence (HEX). Nevertheless, the HEX element of type I OCEs has also been identified as a separate motif in wheat *H3* and *H4* promoters (Tabata et al., 1989, 1991; Minami et al., 2000) and it has also been shown that mutational knockout or deletion of this hexamer sequence reduced promoter strength and led to a loss of cell-cycle-specific expression (Terada et al., 1995; Taoka et al., 1999).

Besides the *cis*-elements in gene promoters, also introns can regulate gene expression and can promote the transcription of gene that harbour them. Since their discovery in 1977, many studies have focused on understanding their role in eukaryotic genes and the regulatory mechanism in which they are involved. In fact, the high cost of maintaining exons and the existence of the elaborate splicing machinery to remove

them suggest that introns play a more fundamental and evolutionarily conserved role in eukaryotic cells (Moabbi et al., 2012). It has been reported that introns significantly enhance gene transcription: the expression level of intronless transgene in mammalian cells is often 10–100 times lower than the one of their intron-containing counterparts. On the other hand, the inclusion of just one intron near the 5′ end of the gene can increase its transcription many folds (Le et al., 2003). In yeasts, it has been shown that although less than 5% of genes contain introns, the intron-containing genes produce approximately 27% of total cellular mRNA (Ares et al., 1999; Spingola et al., 1999). Introns can mediate transcriptional regulation through two different mechanisms: by acting as transcriptional ehancers/promoters or by a process known as intron-mediates enhancement (IME).

Although the mechanism of IME has not been completely understood, it has been described as a splicing-dependent regulation that requires a functional, splicing-competent intron, within the body the gene. In fact, these introns cannot affect transcription if their splicing is compromised by a mutation in the conserved sequences at the 5′ or 3′ splice site, and if they are inserted in an antisense orientation (Charron et al., 2007; Furger et al., 2002). Moreover IME need the presence of an intron near the 5′ end of the gene. In fact it has been proposed that a promoter proximal 5′ splice site facilitates recruitment of the transcription machinery to the promoter and, therefore, helps in initiation of transcription (Kwek KY, et al., 2002). Recently, in budding yeast, the intron-mediated transcriptional activation has been proposed as a mechanism that needs the formation of a gene loop, as a consequence of the interaction between the promoter and its terminator in the presence of the intron. In fact, in strains looping-defective the intron-mediated transcriptional activation was abolished (Moabbi et al., 2012).

The function of introns in the enhancement the gene transcription has been investigated also in *Arabidopsis thaliana* and several studies have demonstrated that this enhancement can be minor or very large, depending on the gene specificity. For example it has been reported that an intronless *ERECTA* gene produced about 500–900 times less protein compared with the gene containing introns, and was unable to rescue the phenotype of *erecta* mutant plants (Karve et al., 2011). Akua et al. showed

that the leader intron at the 5'untraslated region (5'UTR) of the *MHX* gene enhanced gene expression. Although almost no expression was seen in the absence of this intron, it did not act as a transcriptional enhancer and was unable to support expression in the absence of the enhancer elements of the promoter (Akua et al., 2010). Analyses carried out on the first intron of the gene PAT1 have demonstrated that when its splicing was prevented by mutation of 5' and 3' splice sites, or when its U-richness was reduced, the intron retained some ability to increase mRNA accumulation. This study also demonstrated that the splicing was not required to elevate mRNA accumulation because when the same intron was moved to the 3'UTR and spliced it lost the ability to enhance transcription (Rose, 2008).

2. STATE OF THE ART AND AIMS OF THE PROJECT

The *DHFR-TS* genes play a central role in DNA precursors biosynthesis and are expected to be expressed in the G1/S phase of the cell cycle. Previous studies have demonstrated that the *DHFR-TS* transcripts accumulate in highly dividing meristematic tissues of *Daucus carota* and in proliferating suspension cells compared to cells in stationary phase (Albani et al., 2005). As the *DHFR-TS* genes code for enzymes that are important for S phase progression, the study of their promoter is important to understand how gene expression is regulated during plant cell proliferation.

The pattern of expression of the three *DHFR-TS* genes of *Arabidopsis thaliana*, called *AtDRTS1*, *AtDRTS2* and *AtDRTS3* has been investigated previously in our laboratory analysing the activity of the *AtDRTS* promoters (Ghisaura, 2010). These analyses showed that the *AtDRTS1* promoter was not active in the meristems but was highly expressed in the vascular tissues. On the contrary, the *AtDRTS2* promoter drove a very strong cell proliferation-specific expression in root and shoot apical meristems, whereas the *AtDRTS3* promoter showed strong activity in the shoot meristem but not in the meristem of the root, where the expression was confined to the root columella and the central cylinder. In addition, *in silico* analyses revealed that *AtDRTS2* and *AtDRTS3* promoters possess putative *cis*-acting elements conserved in the promoters of several cell cycle-dependent genes. Among these sites, the investigation focused on the E2F site of both *AtDRTS2* and *AtDRTS3* promoters and ChIP analysis revealed that, in both cases, the E2F site was recognised *in vivo* by AtE2Fa or AtE2Fb, which have been characterized as activating AtE2F factors. Moreover, analyses performed to evaluate the activity of the promoters with mutated E2F sites revealed that the inactivation of the E2F *cis*-elements increased considerably the strength of both promoters, indicating that the E2F factors recognizing these sites are able to repress *AtDRTS2* and *AtDRTS3* expression. Concerning *AtDRTS2*, the role of the first intron, located in the 5' untranslated region, was also investigated since it has been reported that introns in this region can regulate gene expression in both monocotyledonous (Callis et al., 1987; McElroy et al., 1990; Christensen et al., 1992; Xu et al., 1994; Jeon et al., 2000; Morello et al., 2002) and

dicotyledonous plants (Norris et al., 1993; Gidekel et al., 1996; Rose and Last, 1997; Plesse et al., 2001; Mun et al., 2002). In the case of *AtDRTS2*, the analysis showed that a promoter construct lacking the first intron was not able to drive expression in meristematic tissues.

In summary, the results obtained previously in our laboratory indicated that, among the Arabidopsis *DHFR-TS* genes, *AtDRTS2* is certainly expressed in both shoot and root meristems, revealing its importance for the synthesis of DNA precursors in cells that are actively proliferating. In this thesis, the expression patterns of the three Arabidopsis *DHFR-TS* genes have been further analysed to confirm the patterns of promoter activity seen previously. Moreover, since it has been shown that the E2F site in the *AtDRTS2* promoter plays a repressive role and activating sites have not been described, one of the aims of this thesis was to carry out additional analyses of the *AtDRTS2* promoter in order to identify *cis*-elements involved in the activaction of this promoter. Finally, because the meristematic expression of this gene was shown to require the presence of the first intron in the 5' UTR, part of the work of this thesis concerned also experiments of gain of function to confirm the importance of this intron for meristematic expression.

3. MATERIAL AND METHODS

3.1. Plant Material

Wild type or transgenic *Arabidopsis thaliana* ecotype Columbia seeds were surface sterilized for 8/12 hours in 2% v/v PPM® (Plant Preservative Mixture, Plant Cell Technology) supplemented with 50 mg/L magnesium salts (magnesium sulphate). Seeds were imbibed over-night in 0,1% agarose at 4°C in the dark and then germinated in a growth cabinet at 22°C under long day conditions (16 hrs of light and 8 hrs of dark) on petri plates with MS salts (Duchefa Biochemie) and Phyto Agar (8 g/l), supplemented with Sucrose (10 g/L). The transgenic Arabidopsis lines used in this study were generated by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* EHA105 strain (Koncz and Schell, 1986). Progeny plants were selected on MS plates containing the resistence antibiotic (Hygromicin, 30 mg/L) and PPM®. After two weeks on selection, the transformed plants were transferred to recovery plates without the selection agent and at four weeks of age transferred to soil and grown to maturity in growth cabinets set at long day conditions of 16 hrs of light (22±3°C) and 8 hrs of dark (22±3°C) with 70% relative humidity.

3.2. Cloning

All the enzymes and buffers used for cloning were supplied by Fermentas; Taq and dNTPs were supplied by GeneSpin; a high fidelity Taq (Platinum Pfx Polymerase) was supplied by Invitrogen. To purify the DNA the QIAquick PCR Purification Kit (Qiagen) and the QIAquick gel extraction kit (Qiagen) were used. Minipreps were performed with QIAprep Spin Miniprep kit (Qiagen).

3.2.1 Mutation of the HEX site in the *AtDRTS2* promoter

To mutate the HEX site in the *AtDRTS2* promoter, PCR reactions were performed on the plasmid pGEM-T4L20 (Ghisaura, 2010), containing the *AtDRTS2/AtSFH1* intergenic region. Portions of the cloned genomic fragments flanking both sides of the HEX site were amplified using primers (T4L16/T4L17) pairing over the HEX site to introduce the *Xba*I restriction site in the sequence. These primers were used in

combination with the universal primers M13FW and M13RV, pairing next to the polylinker in the vector. Below are the sequences of the primers used:

M13FW	acgttgtaaaacgacggc
M13RV	ggaaacagctatgaccatg

T4L16	tctagatctcccgccgtttatacctct
T4L17	tctagacgactgagtcacactgagtcg

The PCR fragments were then subcloned into the pBS-KS vector. The portion toward the *DRTS2* gene, named T4L16 because of the primer used, was cloned as a *Xba*I/*Apa*I fragment whereas the portion toward the *SFH* gene, named T4L17, was cloned as an *Xba*I/*Sac*I fragment. Thus, the pBS-T4L16 and pBS-T4L17 were obtained. To reconstruct the mutated *AtDRTS2/AtSFH1* intergenic region, the pBS-T4L16 and pBS-T4L17 were both digested with *Xba*Iand *Apa*I. The T4L16 fragment was then gel purified and cloned into the pBS-T4L17 plasmid, giving rise to the pBS-T4ΔHEX plasmid. The ΔHEX intergenic region was then cut as a *Bam*HI-*Nco*I fragment and used to replace the E2F mutated sequence in the pCAMBIAΔE2F plasmid (Ghisaura, 2010) to obtain pCAMBIAΔHEX, in which the mutated promoter is placed upstream to the GUS reporter gene.

3.2.2.Insertion of the *AtDRTS2* first intron in the 5'UTR of *AtDRTS1*.

To create a construct containing the first intron of *AtDRTS2* introduced into the 5'UTR of *AtDRTS1* gene, three PCR reactions were performed on suitable plasmids to amplify the *AtDRTS1* promoter fragment, including also the transcription start point (TSP) and the initial portion of the 5'UTR, to amplify the remaining portion of the 5'UTR of *AtDRTS1* and to amplify the *AtDRTS2* first intron. Below are the sequences of the primers used:

***AtDRTS1* promoter**

F16F3	gagaagcttctgaaactaattaagacatg
F16F5	ttactgcagacggacgtgtctctatca

***AtDRTS1* 5'UTR**

F16F2	gttggatccattgtggaaatcaaaaccttg
F16F4	ttactgcagatctactttaagcgttttacga

***AtDRTS2* first intron**

T4L18	gtgctgcagtcgccgtcgtctcccgcc
T4L19	attggatccccttttcagagtgaatctacgcat

After isolation and purification from gel, the PCR products were digested and cloned into the pBS-KS vector. The *AtDRTS1* promoter and TSP region was cloned as a *Hind*III/*Pst*I fragment, whereas the *AtDRTS1* 5'UTR and the *AtDRTS2* intron were cloned as *Pst*I/*Bam*HI fragments to give rise to the three plasmids pBS-*AtDRTS**1-P***, pBS-*AtDRTS**1-5'*** and pBS-*AtDRTS**2-I***.

To join the *AtDRTS2* intron upstream of the 5' end of *AtDRTS1*, pBS-*AtDRTS**1-5'*** and pBS-*AtDRTS**2-I*** were double digested with *Bgl*II/*Xba*I and *Bam*HI/*Xba*I, respectively, and the *AtDRTS**1-5'*** fragment was ligated into pBS-*AtDRTS**2I*** to give rise to the construct named pBS-*AtDRTS**2-I/ 1-5'***.

Then the pBS-*AtDRTS**1-P*** plasmid was cut with *Pst*I/*Cla*I and the excised fragment was introduced into pBS-*AtDRTS**2-I/ 1-5'*** to obtain the plamid pBS-*AtDRTS**1-P/2-I/1-5'***,** in which the *AtDRTS2* intron is placed in the 5'UTR of *AtDRTS1*. This sequence was then isolated as a *Kpn*I/*BamH*I fragment and cloned upstream of the GUS reporter gene into a modified p1305.1 binary vector (p1305.1/Nos).

3.3. Phenotypic analyses of transgenic plants

Histochemical detection of GUS activity on transgenic Arabidopsis plants was performed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) (Jefferson et al., 1987). Plants were incubated in the GUS solution (50 mM phosphate buffer pH 7.0, 1 mg/mL X-Gluc, 1 mM potassium ferricyanide) for at least 1 h at 37°C. After staining, chlorophyll interference was removed treating the samples in 70% ethanol.

For quantitative analyses, the GUS activity was detected fluorimetrically using the fluorogenic substrate MUG (4-metil umbelliferil–glucuronide). For this analysis, plantlets of *Arabidopsis thaliana* at the same developmental stage (emergence of the third pair of leaves) were ground in 200 µl of extraction buffer (50 mM $NaPO_4$ pH 7.0, 10 mM EDTA, 0.1% Triton, 0.1% Sodium Lauryl Sarcosine, 10 mM β-Mercaptoethanol). The homogenate was then centrifuged in a microfuge at 15000 rpm for 10 min at 4°C and 100 µl of the surnatant were recovered and stored at -80°C. To perform the fluorimetric assays, 30 µl of extracts were added to 270 µl of assay buffer (50 mM $NaPO_4$ pH 7.0, 10 mM EDTA, 0.1% Triton, 0.1% Sodium Lauryl Sarcosine, 10 mM β-Mercaptoethanol, 1mM MUG) and the reactions were incubated at 37 °C. At three different time points, 100 µl aliquots of the reaction were transferred to 900 µl of stop buffer (0.2 M Na_2CO_3) and the amounts of 4MU produced was measured using a fluorimeter.

3.4. Gene expression analysis

3.4.1 RNA extraction

RNA was extracted from two week-old seedlings and from root apices and leaves of seedlings at the same devepoment stage (emergence of the third pair of leaves). The material was collected, placed in 1.5 ml Eppendorf tubes and transferred to liquid nitrogen. RNA was extracted using the RNeasy plant mini kit (QIAGEN) and treated with DNaseI (QIAGEN), according to the manufacturer instructions. The RNA concentration was measured using SmartSpec Plus (BIO-RAD). To confirm the concentration and to check the RNA quality, the RNA samples were analysed by electrophoresis on 1,3% formaldehyde agarose gel prepared combining 0,43 g

agarose, 3,3 ml MOPS 10X, 29 ml H_2O DEPC; after boiling to dissolve the agarose, the solution was cooled down and 1,7 ml formaldehyde 37% was added under chemical fume hood.

For the running, 3,5 µl of RNA Loading Buffer (20 µl MOPS 10X; 100 µl formamide; 10 µl formaldehyde 37%, 11,25 µl H_2O DEPC, 2 µl ethidium bromide 10 mg/ml, 20 µl GBBE buffer) were added to each µl of RNA sample. The GBBE buffer was prepared as following: 2,5 ml glycerol, 2,5 mg bromophenol blue, 50 µl EDTA 0,5 M, H_2O DEPC up to 5 ml. The RNA samples in loading buffer were incubated at 65°C for 20 min and then moved to ice before loading on gel. Electrophoresis was performed in MOPS 1X at 100 V, until bromophenol blue reached 2/3 of the gel.

3.4.2. Reverse transcription RT-PCR

1 µg of DNAse treated RNA was reverse transcribed using 1 µl of random hexamers 50 µM (Invitrogen), 1 µl dNTPs 10 mM (Roche) and H_2O DEPC to 10 µl final volume. Reaction was denatured at 65°C for 5 mins and placed back on ice. Then the following was added: 2 µl 10X RT buffer (Invitrogen), 4 µl $MgCl_2$ 25 mM (Invitrogen), 2 µl DTT 0.1 M (Invitrogen), 1 µl RNasin RNase inhibitor (Promega), 1 µl Superscript III Reverse Transcriptase (Invitrogen). Samples were incubated at 25°C for 10 mins, then at 45°C for 1 hour and 5 mins at 85°C.

A semi-quantitative PCR was performed to target the 18S ribosomal gene and cDNA quality was checked on 1,5% agarose gel. PCR parameters were programmed as shown in the table:

Denaturation	1 cycle	94°C	3 mins
Amplification	10 cycles	94°C	1 min
		55°C	1 min
		72°C	30 secs
Final elongation	1 cycle	72°C	10 mins

3.4.3. Real Time PCR

Real time PCR was performed with the BIO-Rad iCycler thermal cycler using the QuantiTect SYBR Green I PCR kit (Qiagen). The cDNA was diluted 1:20 and added to the primer mix 1,2 μM. The data were analysed according to Livak et al., 2001. PCR on 18S ribosomal RNA was used to normalize the data.

Below are the sequences of the primers (Sigma-Aldrich) for *AtDRTS1, AtDRTS2, AtDRTS3, AtBAM3 AtRNR* and 18S ribosomal RNA.

AtDRTS1 FW	aagtgtcgccattgaaatcc
AtDRTS1 RV	gcgagttttctggagaggtg
AtDRTS2 FW	gaacaagatcgcagacgtga
AtDRTS2 RV	atgccacatgtttgcacagt
AtDRTS3 FW	cacatggcacgcttatatcg
AtDRTS3 RV	tctagctgccacaacattgc
AtBAM3 FW	gacctttgcgaagatgaagc
AtBAM3 RV	tttgttcaaatgccctgaca
AtRNR FW	gggcttagcagtgaccattgtg
AtRNR RV	tacacgcagcataggaacaata
18S FW	ttccatgctaatgtattcagag
18S RV	atggtggtgacgggtgacgc

PCR parameters were programmed as shown in the table:

Denaturation	1 cycle	94°C	15 mins
Amplification	45 cycles	94°C	30 secs
		55°C	30 secs
		72°C	30 secs

4. RESULTS

4.1. Study of the pattern of expression of *DHFR-TS* genes.

The activity of the promoters of three *DHFR-TS* genes of *Arabidopsis thaliana* has been previously investigated in our laboratory. Histochemical GUS assays of plants transformed with the three *AtDRTS* promoter constructs suggested different patterns of expression (Fig. 6). Surprisingly, the *AtDRTS1* promoter was highly active in the vascular tissues of the transformed seedlings but was not active in the meristems. Instead, as expected, the *AtDRTS2* promoter was able to drive a very strong cell proliferation-specific expression in root and shoot apical meristems, whereas the *AtDRTS3* promoter showed strong activity in the shoot meristem but not in the apical meristems of the root, where the expression was confined to the root columella and the central cylinder (Ghisaura, 2010).

Figure 6: Activity of the DHFR-TS promoters in two weeks-old seedlings: (a) DRTS1, (b) DRTS2, (c) DRTS3.

As part of the work of this thesis, analyses of the levels of expression of the *AtDRTS* genes were carried out by qRT-PCR, to confirm whether the patterns of activity of the promoters reflected the actual patterns of expression of the *AtDRTS* genes. Thus, the expression levels of these genes were analysed and compared in leaves, root apices and two week-old seedlings. The qRT-PCR was performed also for the *AtRNR1* and *AtBAM3* genes that were chosen as controls. The *AtRNR1* gene, coding for a

ribonucleotide-diphosphate reductase, is known to be expressed strongly in proliferating tissues, whereas *AtBAM3*, which encodes a beta-amylase targeted to the chloroplast, is expressed only in differentiated leaf cells.

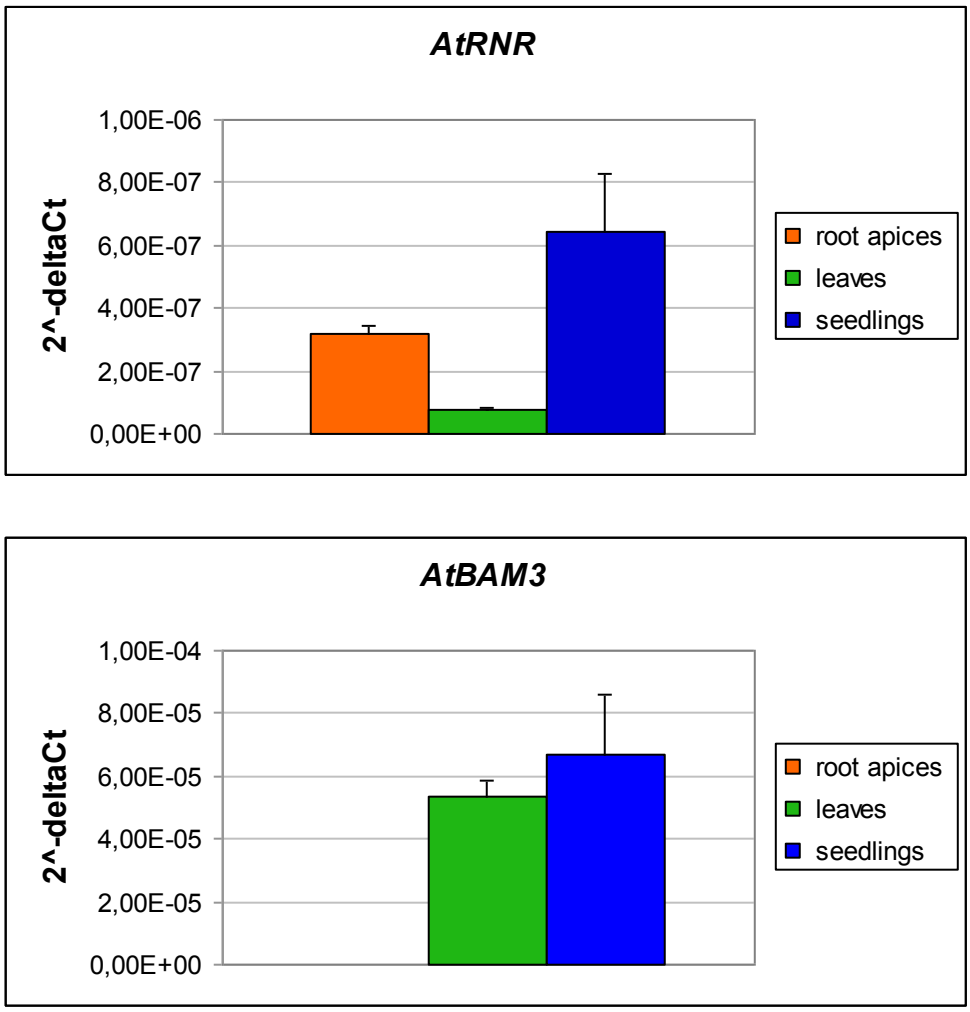


Figure 7: Expression levels of *AtRNR1 and AtBAM3* in root apices, leaves and young seedlings

As expected, the results of this analysis revealed that *AtRNR1* is highly expressed in young seedlings and in root apices, which both contain proliferating cells, whereas its expression in leaves appears to be considerably lower. Conversely, *AtBAM3* is highly expressed in leaves and in seedlings but its expression is not detectable in root apices, as shown in Figure 7.

The qRT-PCR analysis of the *AtDRTS1* transcripts revealed that the gene is highly expressed in leaves and in seedlings, as predicted by the pattern of promoter activity seen previously, but a remarkably stronger expression was detected also in the root apices, a place in which the *AtDRTS1* promoter was not reported to be active (Fig. 8). Concerning *AtDRTS2*, this gene appears to be strongly expressed in root apices but shows lower expression in seedlings, and even less expression in leaves, a scenario that mirrors the pattern revealed by the *AtDRTS2* promoter activity. Finally, regarding *AtDRTS3*, the qRT-PCR analysis of its RNA accumulation shows that the gene is highly expressed in the root apices, confirming the strong activity of the promoter in the root columella, and shows much lower expression in seedlings and leaves (Fig. 8).

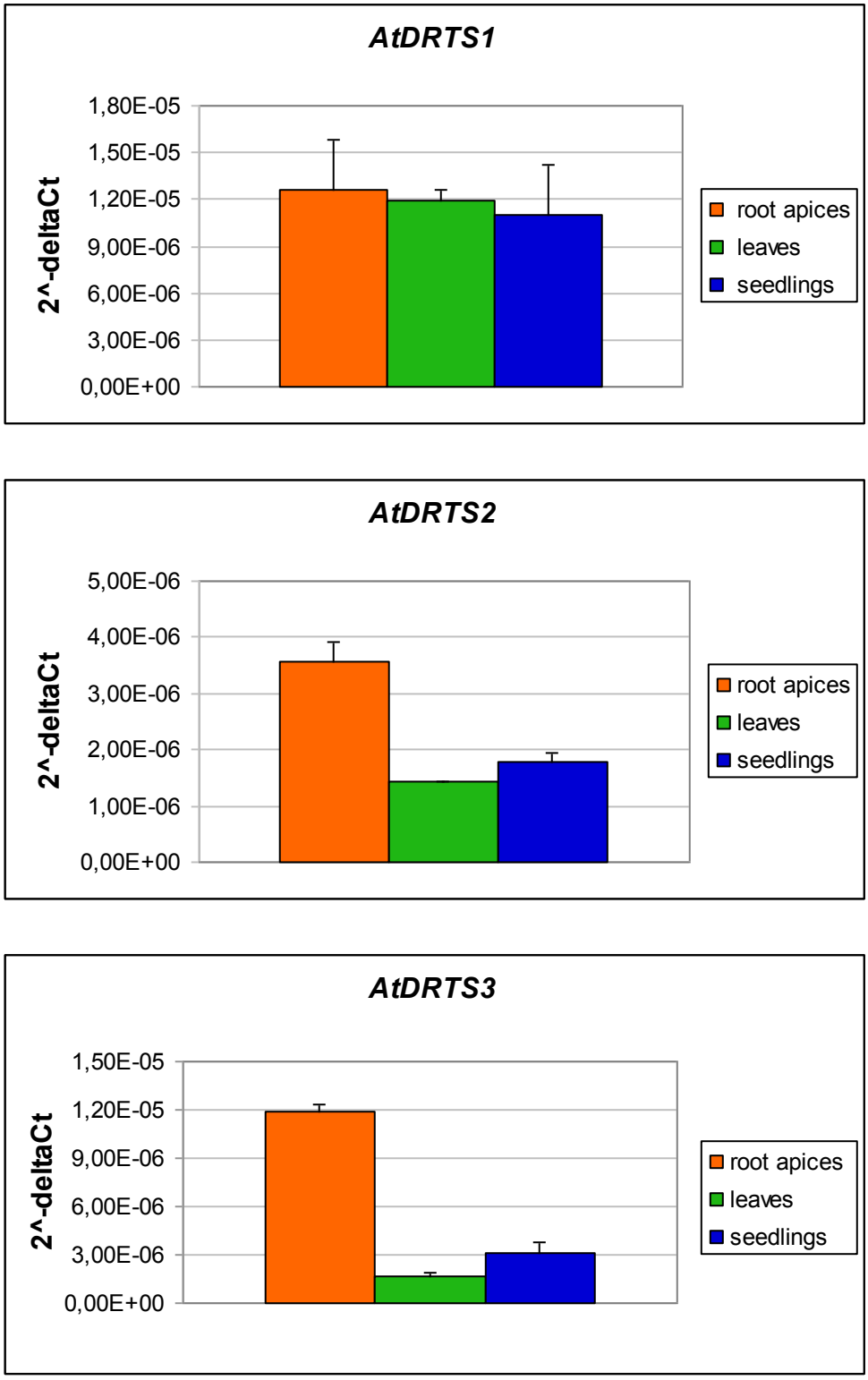


Figure 8: Expression levels of *AtDRTS1, AtDRTS2 and AtDRTS3* in root apices, leaves and young seedlings

Based on these results, the expression levels of *AtDRTS2* and *AtDRTS3* measured in the three samples are in agreement with the pattern of expression revealed by the promoter constructs. On the contrary, the results seen for *AtDRTS1* appear to be partially in contrast with the pattern of activity revealed by the promoter, which showed expression of the GUS gene in leaves and vascular tissues but not in the root meristem. In fact, although the root apices used for the RNA extraction might include a small portion of differentiated root vascular tissues, in which the *AtDRTS1* promoter is known to be active, the RAM and the columella are clearly preponderant and it is unlikely that the eventual presence of a small amount of vascular tissues in this sample could justify a stronger expression of *AtDRTS1* in the root tips rather than in the leaf sample. Thus, the results of the qRT-PCR analysis indicate that *AtDRTS1* is likely to be strongly expressed in the root apical meristems (and possibly also in shoot apical meristems) as well as in the vascular tissues of roots and leaves. Moreover, an expression of *AtDRTS1* in proliferating cells is also suggested by the isolation of *AtDRTS* insertional mutants. Of the three genes, only *AtDRTS1* and *AtDRTS2* are coding for bifunctional DHFR-TS enzymes, whereas *AtDRTS3* is believed to code for a truncated protein that is unlikely to possess TS activity. Considering that TS activity is necessary and essential for DNA synthesis, the mutation of genes responsible for the production of TS in plant meristems is expected to affect strongly plant development and could even cause a lethal phenotype. Thus, if *AtDRTS2* were actually the only gene coding for a TS enzyme expressed in the meristems its loss of function would be expected to affect strongly plant development or to be lethal. However, homozygous insertional mutants that are assumed to be loss of function of *AtDRTS1* (SALK_054193) and *AtDRTS2* (SALK_016377) could be isolated and both are viable and do not show any striking phenotype. This evidence suggests that both genes could compensate for each other, in agreement with the fact that both *AtDRTS1* and *AtDRTS2* are likely to be expressed in meristematic cells.

4.2. Investigations on the regulatory elements involved in the meristematic activity of the *AtDTRS2* promoter

The promoter of *AtDRTS2* has been investigated to indentify the *cis*-elements that can confer meristematic gene expression. The *in silico* analysis performed on the intergenic region spanning between the coding sequences of *AtDRTS2* and *AtSFH1* showed that only four of the putative sites are found in the vicinity of the *AtSFH1* sequence, whereas all the remaining *cis*-elements are closely grouped at the 5'end of the *AtDRTS2* gene (Fig. 9) (Ghisaura, 2010).

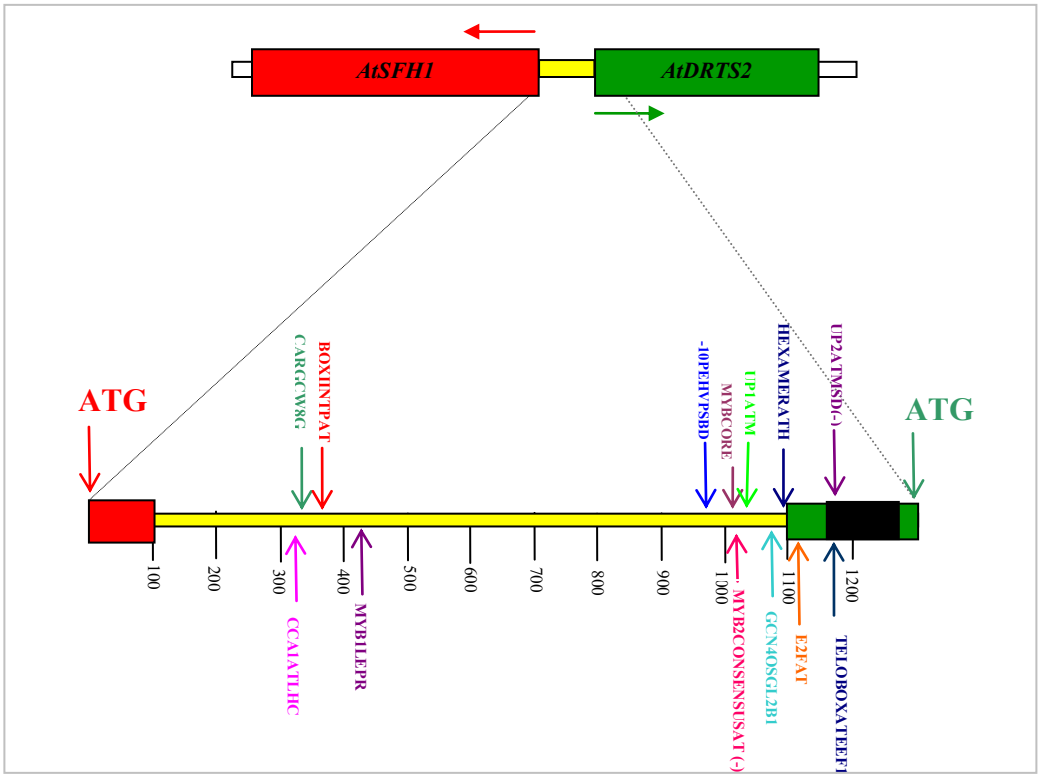


Figure 9: Rapresentation of the intergenic region (in yellow) between the *AtSFH1* and *AtDRTS2* genes. The putative cis-elements are shown in different colours.

In this region there are several *cis*-elements that have been reported to regulate gene expression in proliferating cells. Notably, an E2F binding site, overlapping the predicted transcription start point (TSP), is found adjacent and downstream to a Hexamer motif. Moreover, an UP1 site is located in the upstream region, whereas an UP2 site can be found in the intron interrupting the 5' untranslated region. Previous

studies in our laboratory have focused on the E2F site and revealed that this site is functional and plays a repressive role in the control of *AtDRTS2* promoter activity. This evidence suggested that other *cis*-elements must be involved in the activation of the *AtDRTS2* promoter in meristematic cells. The analysis of UP2 revealed that this site was not necessary to confer meristematic expression, whereas the role of the HEX site was not investigated yet. Therefore, as a part of the work of this thesis, an analysis of the role of the HEX site was undertaken. For this purpose, the HEX sequence in the *AtDRTS2* promoter was substituted with the sequence of an *Xba*I site, and the resulting promoter was assembled into a GFP/GUS reporter construct (pAtDRTS2ΔHEX).

HEX site	5'- ccgtcg -3'
*Xba*I site	5'- tctaga -3'

Figure 10: Structure of the AtDRTS2ΔHEX promoter. In the table are reported the sequences of the HEX and XbaI sites

Transgenic plants containing the *AtDRTS2*ΔHEX promoter construct were then obtained and the histochemical GUS assay on the roots of T1 lines revealed that the mutated promoter is still able to drive expression of the GUS reporter gene in the meristems, as show in Figure 11. However, although it is known that the GUS activity in different transgenic lines is very variable, the histochemical assay already suggested a lower activity of the mutated promoter compared to the wild-type promoter.

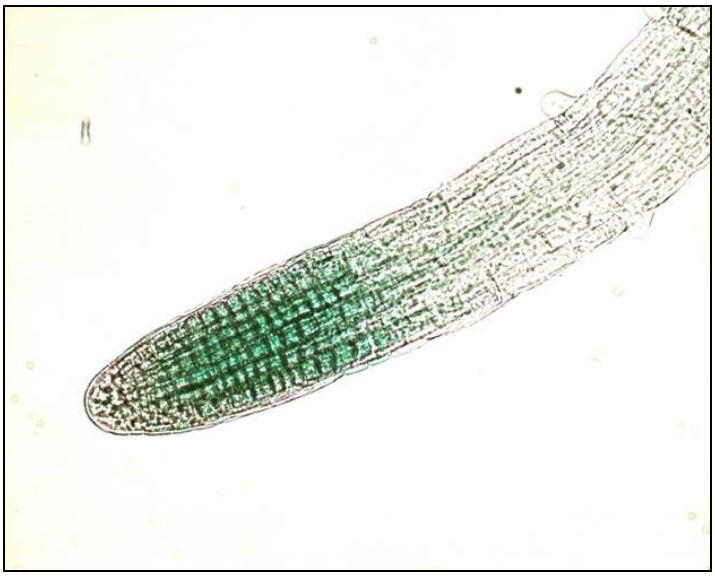


Figure 11: Histochemical GUS assay on root of AtDRTS2 ΔHEX seedlings

To quantify the change in the level of activity, fluorimetric assays were performed on two weeks seedlings of all the transgenic lines. As shown in Figure 12, the GUS activity conferred by the mutated promoter appears to be considerably lower compared to the activity conferred by the wild type promoter, and is thus indicative of a positive role for the Hex site in the control of *AtDRTS2* meristematic expression.

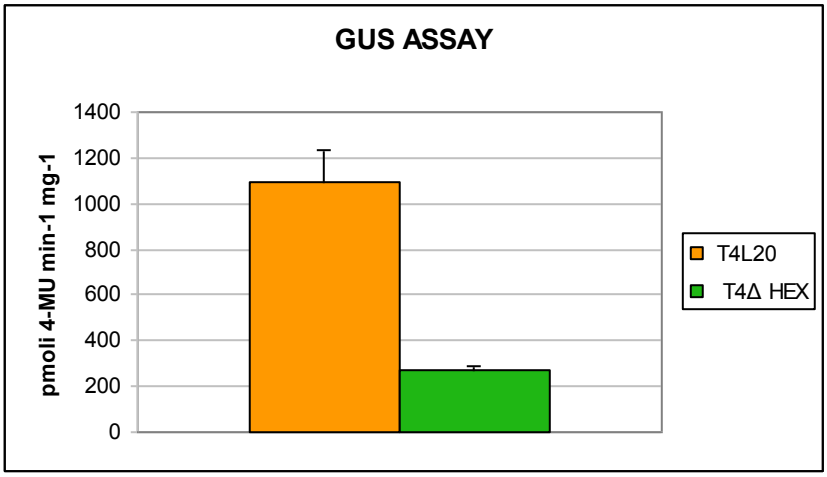


Figure 12: Activity of AtDRTS2 ΔHex promoter compared to the wild type

4.3. The *AtDRTS2* first intron can confer meristematic expression.

Additional studies on the 5' regulatory region *AtDRTS2*, performed previously in our laboratory, revealed that the removal of the first intron, located in the 5'UTR, nearly abolished the activity of the *AtDRTS2* promoter in the root meristems (Ghisaura, 2010). Thus, as part of this thesis, the role of the leader intron in *AtDRTS2* expression was further investigated. To verify whether the first intron of *AtDRTS2* 5'UTR is sufficient to confer meristematic expression, a experiment of gain of function was performed. As the pattern of activity of the three *AtDRTS* promoters revealed that the *AtDRTS1* promoter was not able to express the GUS reporter gene in the root meristems, we decided to verify whether the insertion of the first *AtDRTS2* intron in the 5'UTR of *AtDRTS1* promoter construct could modify its pattern of activity, rendering it active also in the RAM.

Therefore, using a PCR approach that creates suitable restriction sites at the desired locations, the sequence of the first intron of *AtDRTS2* was inserted in the 5'UTR of *AtDRTS1*, placing it at the same distance from the transcription start seen in *AtDRTS2*, to give rise to the construct named AtDRTS**1-P/2-I/1-5'** shown in Figure 13.

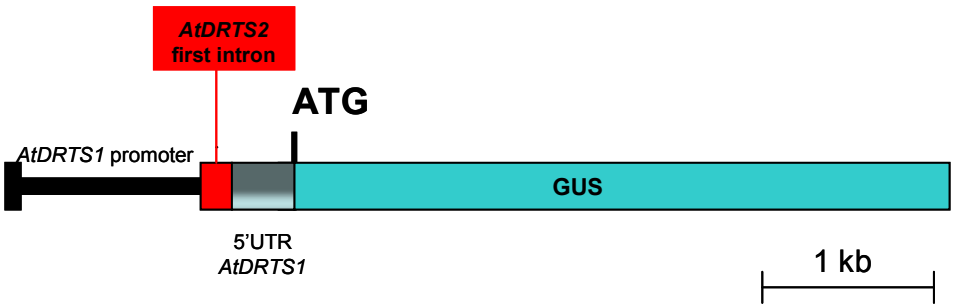


Figure 13: Structure of the AtDRTS1-P/2-I/1-5' promoter construct

Following transformation of Arabidopsis plants, histochemical GUS assays were performed on roots of the transgenic seedlings (Fig. 14). The analysis revelead that the pattern of expression of the *AtDRTS1* promoter was modified by the presence of the *AtDRTS2* intron, since a strong GUS activity was detected also in the root meristem. Thus, these data indicate that the first intron of *AtDRTS2* is necessary and can be sufficient for meristematic gene expression.

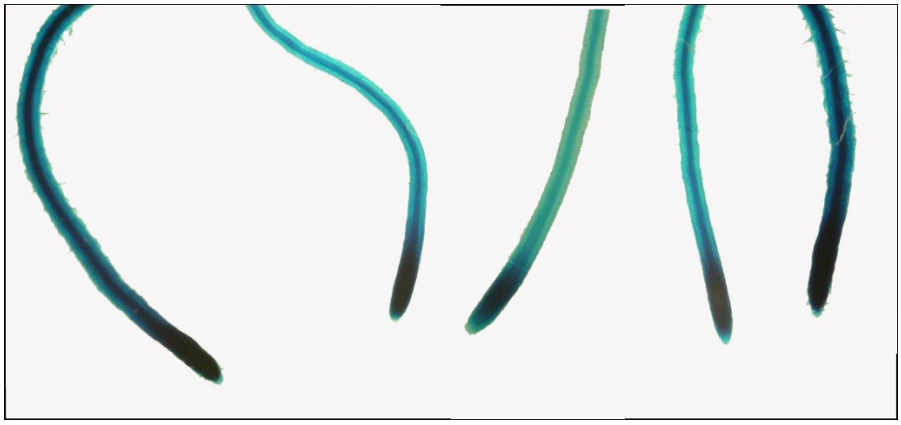


Figure 14: Histochemical GUS assay on roots of AtDRTS1-P/2-I/1-5' seedlings

5. DISCUSSION

Plant growth is a continuous and flexible process that is not restricted to the embryonic phase but occur throughout the entire life of the organism. This feature is linked to the activity of the apical meristems, localized at the end of the main embryonic body axis, where undifferentiated and pluripotent cells constantly divide. Under environmental and endogenous signals, the cells produced in the meristem turn to a differentiated status, allowing the emergence of new organs. The development transition and the cell fate specification imply changes in the expression of many genes involved in the differentiation program as well as in the regulation of the cell cycle. Therefore, the transcriptional regulation of genes expressed in proliferating cells and the coordination of the developmental gene regulatory network is essential for the activity of the meristems and the consequent plant development. Here, to better understand the transcriptional regulation that occurs in plant proliferating cells, we have focused on the *DHFR-TS* genes of *Arabidopsis thaliana* that encode a bifunctional enzyme possessing dihydrofolate reductase (DHFR) and thymidilate synthase (TS) activities. This enzyme plays a central roles in the biosynthesis of DNA precursors and is expected to be active during the G1/S transition. As reported by Albani et al. (2005), studies on *Daucus carota* revealed that the *DHFR-TS* transcripts accumulate in highly dividing meristematic tissues and a high accumulation has been detected also in proliferating suspension cells compared to cells in stationary phase. In *Arabidopsis thaliana* three *DHFR-TS* genes, called *AtDRTS1*, *AtDRTS2* and *AtDRTS3*, have been identified. The first two genes appear to code for bifunctional DHFR-TS enzymes whereas *ATDRTS3* is believed to encode an isoform truncated in the C-terminal TS domain. In fact, this gene shows the insertion of an ATREP transposable element in its third intronthat appears to impair the correct splicing leading to a premature termination of the messenger RNA and of the *AtDRTS*3 open reading frame.

Previous investigations in our laboratory focused on the activity of the *AtDHFR-TS* promoters. The patterns observed suggested that *AtDRTS1* was not expressed in the meristems but was highly expressed in the vascular tissues. On the contrary, the *AtDRTS2* promoter drove a very strong cell proliferation-specific gene expression in

root and shoot apical meristems, whereas the *AtDRTS3* promoter showed strong activity in the shoot meristem but not in the meristem of the root where the expression was confined to the root columella and the central cylinder (Ghisaura, 2010). Because these results were obtained analysing histochemically the expression of the GUS reporter gene driven by the promoter constructs, here we investigated the expression levels of the *AtDRTS* genes by qRT-PCR. These analyses were performed with RNA samples extracted from root apices, leaves and two week-old seedlings. The *AtDRTS1* gene was found to be expressed strongly in leaves, as expected by the activity of the promoter construct, but a particularly strong expression was found also in root apices, where only the expression of *AtDRTS2* was expected to be high. Concerning the expression of the *AtDRTS2* and *AtDRTS3* genes, the results were consistent with the patterns of activity shown by the promoter constructs. The accumulation of *AtDRTS1* transcripts detected in root apices could partly result from the possible presence of a portion of differentiated root vascular tissue, where a strong activity of the promoter was observed. However, the high expression in root tips revealed by qRT-PCR analysis, which is similar to what found in leaves, clearly suggest that the *AtDRTS1* gene is highly expressed in both meristematic and vascular tissues. Moreover, phenotypic analysis of *AtDRTS1* and *AtDRTS2* homozygous insertional mutants seedlings, which appeared to be viable and didn't show any striking phenotype, suggests that these genes can complement each other, and are thus both expected to be active in the meristems. Interestingly, analyses *in silico* of the *cis*-elements found in the two promoters revealed little conservation of the putative regulatory regions (Ghisaura, 2010).

Considering the clear meristematic activity shown by the *AtDRTS2* promoter, previous studies in our laboratory focused on the functional identification of *cis*-elements in *AtDRTS2* promoter responsible for its meristematic expression. Surprisingly, the functional inactivation of an E2F-binding site, which is overlapping the transcription start point (TSP) and was shown to be recognised *in vivo* by activating E2Fs, was shown previously to increase the promoter activity in the vascular and meristematic tissues. Interestingly, until now, a repressive role of E2F-binding sites was demonstrated only in plant promoters that contain at least two E2F

cis-elements, one of which upregulates gene expression whereas the other represses it (Egelkrout et al., 2002). Nevertheless, the repressive role of the single E2F site found in the *AtDRTS2* promoter suggests that other *cis*-elements must act as positive regulators of *AtDRTS2* gene transcription.

The *in-silico* analysis of the *AtDRTS2* promoter revealed, upstream and adjacent to the E2F site, the presence of a HEX site (CCGTCG) that has been identified as an important regulatory element involved in the cell cycle-dependent activation of the wheat *H3* and *H4* promoters (Tabata et al., 1989, 1991; Minami et al., 2000). It has been shown that mutational inactivation or deletion of this site reduced promoter strength and lead to a loss of cell-cycle-specific expression (Terada et al., 1995; Taoka et al., 1999), suggesting that this *cis*-element could be important also for the regulation of the *AtDRTS2* promoter. Thus, transgenic plants harbouring a *β-glucuronidase* gene placed under the control of the *AtDRTS2* promoter mutated in its HEX site (AtDRTS2ΔHEX) were produced and GUS assays were performed. The expression pattern of the mutated promoter was not changed, since GUS activity was still detected mainly at the root apical meristems, but the level of activity, quantified by fluorimetric assays, was considerably lower in the AtDRTS2ΔHEX plants compared with the plants transformed with the wild type promoter construct. This result indicates that the HEX site acts as a positive regulator but is not essential for *AtDRTS2* gene expression and other regulatory elements must be responsible of the meristematic expression of this *DHFR-TS* gene. In this respect, the expression of *AtDRTS2* was found previously to rely on the presence of the first intron, located in the 5'UTR of the gene. In fact, analyses carried out in our in our lab showed that the pattern of activity of the *AtDRTS2* promoter drastically changed when this intron was removed, since its expression in root and shoot meristems was completely abolished. Analyses *in silico* of the sequence of the *AtDRTS2* first intron identified a regulatory *cis*-element, named Up2, that is known to regulate the transcription of genes expressed in proliferating cells (Trémousaygue et al., 2003; Tatematsu et al., 2005). However, a functional analysis of the Up2 site in the *AtDRTS2* intron was conducted previously in our laboratory and did not reveal any positive role for this site on

AtDRTS2 transcription because its absence didn't abolish the meristematic expression of the gene (Ghisaura, 2010).

In this thesis, an experiment of gain of function was performed to verify whether the first intron of *AtDRTS2* could be able to confer high level of meristematic expression to a gene construct not expressed in the meristem, . For this purpose the intron was placed in the 5'UTR of the *AtDRTS1* promoter construct, which was previously shown to be inactive in the meristems. Moreover, it is interesting to notice that the *AtDRTS1* promoter might require the presence of additional regulatory regions, possibly intronic ones, to drive gene expression in the meristems since additional analyses described in this thesis revealed that *AtDRTS1* is actually expressed in apical root meristems. The histochemical GUS assays performed on root apices of the plants transformed with the gain of function construct revealed that the insertion of the *AtDRTS2* intron in the 5'UTR of the *AtDRTS1* promoter construct changes considerably its pattern of activity and was sufficient to confer high activity in root meristems.

Concluding, the experiments carried out in this thesis identified the HEX site as a positive regulator of At*DRTS2* in proliferating cells, although its presence is not essential to confer meristematic expression. Moreover, the gain of function analysis on the *AtDRTS2* first intron confirmed that it is sufficient and necessary to confer high level of gene expression in proliferating cells. Further studies will be needed to characterize the role of the *AtDRTS2* intron, understanding whether it acts as a promoter enhancer, therefore at the DNA level, or acts instead post-transcriptionally, through a process known as intron-mediates enhancement (IME) that requires a splicing-dependent regulation (Charron et al., 2007; Furger et al., 2002).

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