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STUDIES ON THE MERISTEMATIC AND E2F-DEPENDENT GENE EXPRESSION IN *Arabidopsis thaliana* PLANTS

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ABSTRACT

This thesis focuses on two studies concerning some aspects of cell proliferation and development in plants. The first part describes additional results completing the characterization of the three *AtDRTS* genes of *Arabidopsis thaliana*, which code for bifunctional dihydrofolate reductase/thymidylate synthase enzymes whose activity is fundamental in proliferating cells. These analyses allowed the identification of different *AtDRTS* isoforms, some of which are expected to encode monofunctional dihydrofolate reductases, and revealed common and distinctive patterns of expressions that suggest redundant as well as specific roles of the three genes. The characterization of the AtDRTS promoters revealed distinctive features and an E2F-dependent repression of both *AtDRTS2* and *AtDRTS3*. Moreover, evidence has been obtained that the first intron of *AtDRTS2* and the intragenic region containing the second intron of *AtDRTS1* play crucial roles in the control of promoter activity in the root meristems. Moreover, analyses conducted within this thesis revealed that the first intron of *AtDRTS2* is able to confer strong activity in root apical meristem to a non-meristematic plant promoter. The second part of this thesis describes studies conducted to evaluate the regulation *in planta* of a synthetic promoter, named E2F-Minimal-35S (EM35S), that is expected to be specifically activated by E2F factors, important regulators of cell cycle progression in both plants and animals. Transgenic Arabidopsis plants harboring a construct in which the synthetic promoter drives the expression of the GUS reporter allowed the detection of the E2F-dependent transcriptional activation in different tissues. Moreover, these plants have been used to investigate the cell cycle-dependent regulation of the EM35S promoter activity as well as the effects of epigenetic mechanisms and phosphorylation/dephosphorylation events.

LIST OF ACRONYMS AND ABBREVIATIONS

APH, Aphidicolin
BAM3, β-amylase 3
CDKs, Cyclin-Dependent Kinases
CENH3, Centromeric Histone H3
COL, Colchicine
CYCs, Cyclins
DHFR/TS, Dihydrofolate Reductase/ Thymidylate Synthase (DRTS)
DPs, Dimerisation-Partner proteins
EM35S, E2F-Minimal-35S
eqFP611, Red Fluorescent Protein
GEN, Genistein
GUS, β-glucuronidase
HDACs, Histone Deacetylases
IME, Intron-Mediated Enhancement
OKAD, Okadaic acid
pRB, Retinoblastoma protein
PP1/2A, Protein Phosphatase 1 and 2A
RAM, Root Apical Meristem
RBR, Retinoblastoma-Related protein
SAM, Shoot Apical Meristem
SFH, Sec14-like gene family
STAU, Staurosporine
THF, Tetrahydrofolate
TSA, Trichostatin A

CHAPTER 1.

Studies on the *DRTS* genes of *Arabidopsis thaliana*

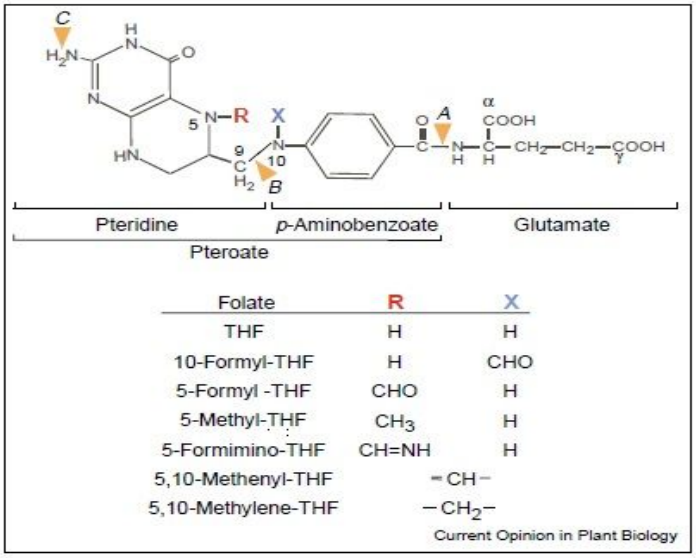
1. INTRODUCTION

1.1 Folates and C1-metabolism in plants

Reactions involving the addition or removal of one-carbon units (C1-metabolism), are essential to all organisms, including plants. The majority of the C1 transfer reactions are mediated by tetrahydrofolate (THF) and its derivatives, commonly named folates or vitamin B9. Folates act as coenzymes in several cellular pathways, including the synthesis of purines and thymidylate, amino acid metabolism, pantothenate synthesis and the synthesis of methionine (Met). Furthermore, because methionine is the direct precursor of *S*-adenosyl-Met (Ado-Met), folates are indirectly required for the synthesis of molecules such as choline, chlorophyll or lignin, as well as ethylene and polyamines [Cossins, 2000; Hanson and Roje 2001]. THF derivatives are also key compunds necessary to support the massive photorespiratory fluxes that occur in green leaves of C3 plants. Photorespiration, in fact, relies on two THF-dependent enzymes present in leaf mitochondria, the glycine decarboxylase complex (GDC) and serine hydroxymethyltransferase (SHMT) [Oliver, 1994; Douce et al., 2001]. These enzymes use up to the 30% of the folate pool for the conversion of glycine to serine [Gambonnet et al., 2001]. Moreover, folates are also involved in the synthesis of pantothenate (vitamin B5) because the first enzyme of this pathway, ketopantoate hydroxymethyltransferase, uses 5,10-methylene tetrahydrofolate as a cofactor [Smith et al., 2007].

1.2 THF structure and biosynthesis

THF (figure 1) is a tripartite molecule, composed of pterin, *p*-aminobenzoate (*p*ABA) and glutamate moieties. The one-carbon units at various oxidation levels are attached at N5 of the pteridine ring, N10 of the PABA unit, or bridged between the two nitrogens. Folates are synthesized *de novo* by plants, fungi, most bacteria and protozoa, whereas in animals folate supply is ensured by the diet. As for humans, plant-derived food products are the most relevant source of folates [Scott et al., 2000].



Folate	R	X
THF	H	H
10-Formyl-THF	H	CHO
5-Formyl -THF	CHO	H
5-Methyl-THF	CH_3	H
5-Formimino-THF	CH=NH	H
5,10-Methenyl-THF	$-CH-$	
5,10-Methylene-THF	$-CH_2-$	

Fig. 1 Chemical structures of tetrahydrofolate and its C1-substituted derivatives [From: Hanson and Gregory, 2002].

In all organisms folates occur predominantly as polyglutamylated molecules, with a short γ-linked chain of glutamyl residues attached to the first glutamate. These polyglutamylated folates are the preferred substrates for most folate-dependent enzymes [Cossins, 2000; Scott et al., 2000]. In plants, the biosynthesis of THF depends on the activity of enzymes that are localized in the cytosol, plastids and mitochondria (figure 2) [Neuburger et al., 1996; Rebeillé et al., 1997].

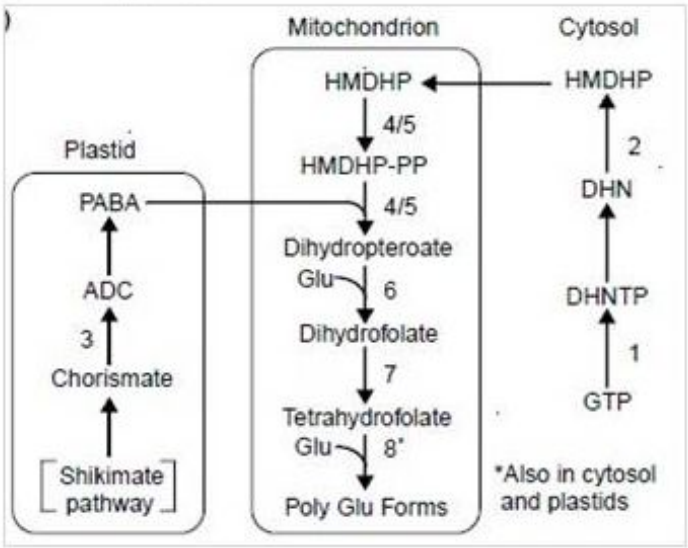


Fig. 2 Folate synthesis pathway in plant cells, enzymes are:1, GTP cyclohydrolase I; 2, dihydrofolate aldolase;3, ADC synthase; 4/5, HPPK/DHPS; 6, DHFS; 7, dihydrofolate reductase/thymidylate synthase; 8, folylpolyglutamate synthetase [From: Hanson and Gregory, 2002].

Cytosolic steps: synthesis of the pterin branch.

The initial reactions of the THF *de novo* synthesis in plants occur in the cytosol, where dihydropterin (or hydroxymethyldihydropterin, HMDHP) is synthesized from GTP in three steps. In the first reaction, GTP-cyclohydrolase I (GTPCHI) catalyzes the formation of dihydroneopterin triphosphate (DHNTP). Then the triphosphate side chain of DHNTP is removed to produce dihydroneopterin (DHN) in two reactions. First, the pyrophosphate group is detached by a specific nudix hydrolase [Klaus et al., 2005], then a non-specific phosphatase [Suzuki and Brown, 1974] cleaves the remaining phosphate. In the last step, dihydroneopterin aldolase (DHNA) removes the lateral three-carbon side chain of dihydroneopterin to release dihydropterin.

Plastidic steps: synthesis of the pABA branch.

The synthesis of *p*ABA occurs in the plastids and requires the conversion of chorismate to aminodeoxychorismate, mediated by the aminodeoxychorismate (ADC) synthase. ADC is subsequently aromatized to *p*ABA by ADC lyase [Basset et al., 2004].

Mitochondrial steps: synthesis of THF from pterin, pABA, and glutamate moieties.

The final reactions necessary for THF synthesis occur in mitochondria, which contain all the required enzymes. In mitochondria, dihydropterin is firstly activated into its pyrophosphorilated form which is then combined with *p*ABA, resulting in dihydropteroate. These two reactions are catalysed in plants by a single mitochondrial bifunctional enzyme, which possesses the two activity necessary for these two steps: HPPK (hydroxymethyldihydropterin pyrophospho-kinase) and DHPS (dihydropteroate synthase) [Neuburger et al., 1996; Rebeillé et al., 1997]. In plants, DHPS is feedback inhibited by its own product, dihydropteroate [Prabhu et al., 1997]. The next step for THF synthesis is the ATP-dependent glutamylation of dihydropteroate to form dihydrofolate (DHFR) in a reaction catalyzed by dihydrofolate synthase (DHFS), which allows the attachment of the first glutamate to the carboxyl moiety of *p*ABA. The presence of HPPK/DHPS and DHFS enzymes in plant mitochondria, but not in mammal cells, allow plants to be autotrophic for folates. DHFR is finally reduced to THF by dihydrofolate reductase (DHFR) using NADPH as a cofactor. As for the polyglutamylated derivatives of THF (polyglutamyl-THF), they are formed through a sequential addition of γ-linked glutamate residues by the folyl-polyglutamate synthase (FPGS) enzyme. The glutamylation increases the negative charge of

the folates and appears to be necessary for a correct compartmentation within the cell [Appling, 1991].

1.3 Roles of DHFR and TS enzymes in the synthesis of nucleotides

In all organisms, the enzymes thymidylate synthase (TS) and dihydrofolate reductase (DHFR) are crucial for the synthesis of DNA precursors. In fact, the synthesis of thymidylate (figure 3), catalyzed by thymidylate synthase, requires N5,N10-methylene tetrahydrofolate to methylate and reduce deoxyuridine monophosphate (dUMP) to dTMP, yielding 7,8-dihydrofolate (DHF) as a secondary product. To enable efficient recycling of the resulting DHF, the activity of TS must be linked to the activity of dihydrofolate reductase (DHFR), the last enzyme of the biosynthetic pathway. The role of the DHFR and TS enzymes in the synthesis of DNA precursors highlights the importance of a coordinated regulation of both activities.

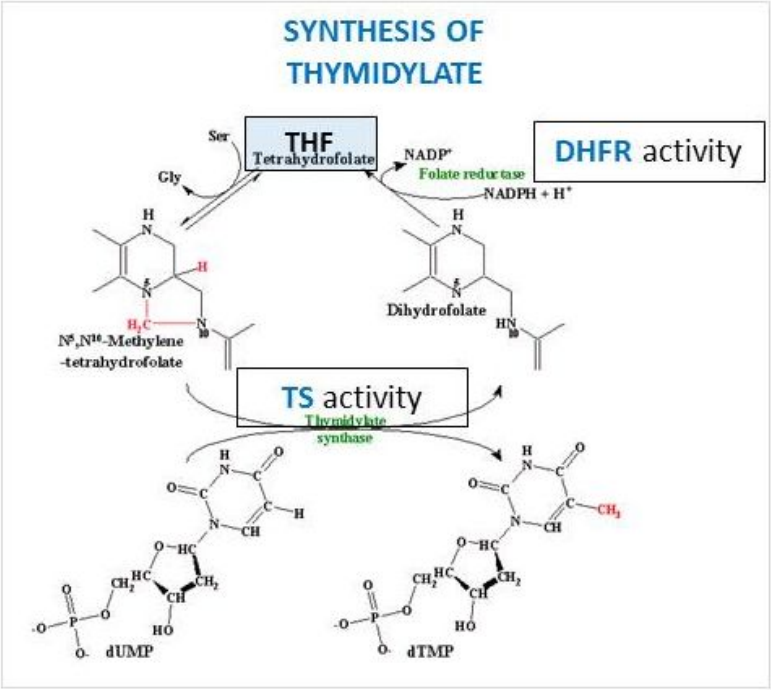


Fig. 3 Biosynthesis of thymidylate and role of the bifunctional DHFR/TS enzyme.

TS and DHFR have been described as distinct monofunctional proteins in animals, fungi, metazoa and bacteria, but plants and protozoa possess a bifunctional DHFR/TS enzyme (DRTS). The DHFR domain of the bifunctional enzyme maintains the tetrahydrofolate level by reducting the DHF originating from either the *de novo* synthesis pathway (monoglutamate form) or the oxidation of THF by the TS activity (polyglutamate form). 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]. Nevertheless, also monofunctional DHFR enzymes have been identified in plants [Toth et al., 1987].

1.4 DRTS genes in higher plants

Plant *DRTS* genes have been described in *Arabidopsis*, carrot, soybean and maize [Lazar et al., 1993; Luo et al., 1993; Wang et al., 1995; Cox et al., 1999] but additional *DRTS* sequences of other species are available through genomic and EST databases, including sequences from several primitive plants and algal species. All the DRTS proteins possess a conserved N-terminal DHFR region separated from the conserved C-terminal TS domain by a junctional region of variable sequence which, according to studies in *Plasmodium falciparum*, has been shown to be essential for TS activity and domain-domain interaction of the bifunctional enzyme [Chaianantakul et al., 2013]. Furthermore, plant DHFR/TS bifunctional enzymes share conserved regions which specifically bind compounds such as metotrexate, dUMP and folates.

Studies on the different species, revealed peculiarities of the *DRTS* plant genes. Analyses of the 5'-ends of the carrot gene have demonstrated the presence of DRTS isoforms which are expected to have a specific subcellular localization (Luo et al., 1997). These isoforms are encoded from two distinct transcript species with differing lengths. Because the DHFR and TS activity are essential for the biosynthesis of nucleotides, analyses have focused on their importance in proliferating tissues or in tissues that are characterized by endoreduplication events. *In situ* hybridization analyses carried out in *Daucus carota* revealed that *DcDRTS* transcripts are particularly abundant in dividing cells of somatic embryos. In addition, Northern blot hybridization experiments revealed a stronger accumulation of *DcDRTS* transcripts in proliferating suspension cells compared to cells in stationary phase or cells blocked with propyzamide [Albani et al., 2005]. In *Zea mays*, high expression of *ZmDRTS* was detected during early stages of kernel formation, exhibiting developmentally controlled endoreduplication, as well as in root tips, where cell division occurs, whereas low expression

was found in the root elongation zone and leaves [Cox et al., 1999]. Also a recent investigation of the expression of the four *ZmDRTS* genes found in the maize genome revealed that all of them are maximally expressed at the beginning of kernel formation [Lian et al., 2015]

1.5 DRTS genes in Arabidopsis thaliana

All the information on the *DRTS* genes of *Arabidopsis thaliana* is reported in the TAIR (The *Arabidopsis* Information Resource) database at the website *https://www.Arabidopsis.org/*. Arabidopsis possesses three *DRTS* genes, called *AtDRTS1*, *AtDRTS2* and *AtDRTS*3 (or, alternatively *THY1*, *THY2* and *THY3)*, that show a similar genomic organization and are located downstream of three members of the sec14-like (*SFH*) gene family, which suggests their origin from evolutionary genome duplications [Jiao et al., 2012]. The SFHs are proteins with distinct subcellular localizations and varied physiological functions related to lipid metabolism, phosphoinositide mediated signalling and membrane trafficking.

The *AtDRTS1* and *AtDRTS2* genomic sequences have been described previously [Lazar et al., 1993] and a gene model has been proposed for *AtDRTS*3, but information concerning the expression and the regulation of the *AtDRTS* genes has not been reported so far. The *AtDRTS1* gene, annotated as At2g16370 in the TAIR database, is located on the minus strand of chromosome 2. According to the proposed gene model, the gene extends 2774 bp, from position 7088865 to 7091639, and is divided into 10 exons that give rise to a transcript of 1924 bp. The predicted ATG start codon is located in the second exon and the resulting coding region translates into a protein of 519 aa with a MW of 58.1 KDa. The *AtDRTS2* gene, annotated as At4g34570, based on the gene model spans 3310 bp on the minus strand of chromosome 4, from position 16511006 to 16514316 and contains 12 exons resulting in a 1926 bp transcript. The ATG start codon is found at the end of the second exon and the predicted coding region translates into a protein of 565 aa with a MW of 63.2 KDa. Finally, the *AtDRTS3* gene, with annotation At2g21550, is located on the plus strand of chromosome 2 and the proposed gene model is divided into 10 exons and extends 2980 bp, from the ATG triplet at position 9234289 to the TAA stop codon at 9237269. The predicted transcript includes an open reading frame of 1476 bp that is expected to code for a protein of 492 aa with a MW of 55,3 KDa.

2. STATE OF THE ART AND AIMS OF RESEARCH

This part of my thesis describes additional data concerning the molecular characterization of the three *DRTS* genes of *Arabidopsis thaliana*, named *AtDRTS1*, *AtDRTS2* and *AtDRTS3*. These genes play a major role in the biosynthesis of DNA precursors and, as a consequence, are expressed in dividing cells. Previous analyses conducted by our research group [Ghisaura, 2010; Marche, 2013] have demonstrated that the three DRTS promoters present differential profiles of activity. The results obtained, in fact, showed that the *AtDRTS1* promoter was highly active in vascular tissues but, unexpectedly, not in root meristems. As for the *AtDRTS2* promoter, a strong activity in both root and shoot apical meristems has been observed, whereas the *AtDRTS3* promoter presented a meristematic expression in the shoot apex but not in the root, were strong activity has been detected only in the columella and in the central cylinder and not in the apical meristem. Furthermore, *in silico* analyses, allowed to identify several regulatory *cis* elements which have been reported to be involved in gene regulation in proliferating cells. Studies had been focusing in particular on the E2F sites contained in the *AtDRTS2* and *AtDRTS3* promoters and on the HEXAMER site of the *AtDRTS2* promoter. Mutation of the E2F *cis*-elements increased considerably the activity of both promoters, suggesting that the E2F transcription factors act as repressors of *AtDRTS2* and *AtDRTS3*, whereas mutation of the HEXAMER site determined a decrease of the *AtDRTS2* promoter activity, suggesting an activating role of this *cis* element. Moreover, other analyses revealed also the importance of the first intron of *AtDRTS2* for the expression in proliferating cells and it was shown that this intron is able to confer activity in root meristems not only to *AtDRTS2*, but also to the *AtDRTS1* promoter [Marche, 2013].

In this respect, one of the aims of this part of my research project was to expand the molecular characterization of the *AtDRTS* genes, analysing the presence of different isoforms and extending the analysis *in silico* of the promoters. Experiments explaining contradictory aspects concerning the meristematic activity of the *AtDRTS1* promoter were also carried out. Moreover, the suggested E2F-dependent repression of the *AtDRTS2* and *AtDRTS3* promoters was further investigated. Finally, additional studies concerning the first intron of *AtDRTS2* were performed to better define its capability to confer gene expression in proliferating cells.

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3. MATERIALS AND METHODS

3.1 Plant material and plant transformation

For germination and growth in aseptic conditions, wild type or transgenic *Arabidopsis thaliana* ecotype Columbia seeds were surface sterilized for 8/10 hours in 2% v/v PPM® (Plant Preservative Mixture, Plant Cell Technology) supplemented with 50 mg/L magnesium salts ($MgSO_4$). Seeds were imbibed for 2 days in 0,1 % agarose at 4°C in the dark and then germinated on petri plates containing MS salts (Duchefa Biochemie), supplemented with Sucrose (10g/l) and Phyto agar (8g/l) (Duchefa Biochemie) and incubated in a growth cabinet at 22°C under long day conditions of 16 h of light and 8 h of dark .

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].

For transformation, a colony of Agrobacterium containing the recombinant plasmid has been picked up to inoculate 4 ml of YEP medium(Bactotryptone 10 g/L; Yeast extract 10 g/L; NaCl 10 g/L adjusted at pH 7 with NaOH) containing the selection agents Kanamycin 40 mg/l and Rifampicin 50 mg/l , which are specific for the plasmid and for the Agrobacterium strain respectively. The culture was incubated O/N at 28°C with gentle shaking and then used to inoculate 400 ml YEP medium. After a further incubation at 28°C O/N, the culture was ready to transform plants. Each culture was transferred into 50 ml conical tubes, centrifuged at 4000 rcf for 7 minutes, at 4°C and the liquid poured away leaving a pellet. Infiltration media was prepared as following 50 g/l sucrose, 400 µl/l silwet L-77, and kept cold. A small amount of infiltration media was first added to the tubes to resuspend the Agrobacterium cells and then the remaining was added up to 500 ml to perform the floral dipping. Plants were dipped into infiltration media for 45 secs, placed on their side in a plastic bucket for 24 hours and left at RT. The transformed plants have then been transferred in a growth chamber to grow to maturity.

Transformed T1 and progeny plants were selected on MS plates containing the resistance antibiotic (Hygromycin, 10 mg/l or kanamycyn, 40 mg/l). At two weeks of age, the resistant plants were transferred to recovery plates and grown for one more week in aseptic conditions without the selection agent.

3.2 Generation of promoter constructs

All the primer sequences used for the production of the recombinant constructs are detailed in Table 1.

Table 1 List of the primers used for vector construction. The restriction sites are highlighted in red.

NAME	SEQUENCE 5'-3'	RESTR. SITE
F16F4	GTC**TCTAGA**GGTTTAGACTTTTGATGAAAC	*Xba*I
F16F5	GGC**GGATCC**AATGCTTCCCTACACAAAT	*Bam*HI
T4L20	GTG**GGATCC**AGTCGCCGTCGTCTCCCGCC	*Bam*HI
T4L21	AAA**TCTAGACCATGG**TCAGAGTGAATCTACGCA	*Xba*I; *Nco*I
221.9 Rev	TGG**ACTAGTAGATCT**CCCCCGTGTTCTCTCCA	*Spe*I; *Bgl*II
M13RV	GGAAACAGCTATGACCATG	
BAM3PRH	AAA**AAGCTT**GCAGCATTCAGGCAGTCCA	*Hind*III
BAM3PRB	GAA**GGATCC**TTTGTGTTTGAGAGAAAGA	*Bam*HI

SFH7/DRTS1i2 construct

For the production of the SFH7/DRTS1i2 dual reporter construct the region spanning from the start ATG codon of the AtSFH7 gene to the beginning of the third exon of the *AtDRTS1*gene was amplified by PCR from *Arabidopsis thaliana* genome, using high fidelity Pfx Taq polymerase (Invitrogen). This amplification was performed using the primers F16F4 (which anneals next to the AtSFH7 start codon, at position -1420 relative to *AtDRTS1* transcription start, and creates a terminal *Xba*I site) and F16F5 (which anneals at the beginning of the *AtDRTS1* third exon, at position +760 relative to *AtDRTS1* transcription start, and creates a *Bam*HI site). The resulting DNA fragment was *Xba*I/*Bam*HI digested and cloned into pBlueScript-KS plasmid (also digested with *Xba*I/*Bam*HI), giving rise to the F16F45 plasmid, which has been sequenced to verify the fidelity. The F16F45 plasmid was then digested with *Xba*I/*Bam*HI and the resulting fragment cloned into the SFH7/DRTS1 plasmid (digested with *Xba*I/*Bgl*II) [Ghisaura, 2010], replacing the fragment which comprises the intergenic region spanning from the start codon of the AtSFH7 gene to the ATG codon of *AtDRTS1* (located at the beginning of the second *AtDRTS1* exon). In this dual reporter construct, named SFH7/DRTS1i2, the AtSFH7 promoter directs the expression of the *eqFP*611 reporter gene, whereas a large portion of the amino-terminal DHFR domain of the *AtDRTS1* protein is fused in frame with the GUS reporter coding sequence. The backbone of this plasmid is the pCambia 1301 binary vector, suitable for *Agrobacterium*-mediated plant transformation.

BAM3/DRTS2i1 construct

The β-amylase 3 (*BAM3*) promoter (TAIR accession number: At4g17090) was isolated by PCR from *A. thaliana* genome with high fidelity Pfx Taq polymerase (Invitrogen), using the BAM3PRH primer, which includes a *Hind*III restriction site and pairs at position -794 respective to the *BAM3* transcriptional start, together with the BAM3PRB primer, which anneals at position +9 respective to the *BAM3* transcription start and contains a *Bam*HI site. The resulting fragment was *Hind*III/ *Bam*HI digested and cloned into the *Hind*III and *Bam*HI sites of the TL2021 plasmid, positioning the *BAM3* promoter upstream of the *AtDRTS2* 5'-UTR, giving rise to the B3TL2021 plasmid. The *BAM3* promoter/*AtDRTS2* 5'-UTR region was then isolated digesting the B3TL2021 plasmid with *Hind*III and *Nco*I and cloned upstream of the GUS reporter gene into the pBI221.9 plasmid (cut *Hind*III/*Nco*I) producing the pBI221.9/B3TL2021. Finally, the pBI221.9/B3TL2021 was digested with *Hind*III and *Eco*RI to isolate the *BAM3* promoter/*AtDRTS2* 5'-UTR/GUS fragment which was cloned into the *Hind*III and *Eco*RI sites of pBI121 binary vector, thus producing the BAM3/DRTS2i1 reporter construct.

BAM3 construct

For the production of the *BAM3* reporter construct, firstly the B3TL2021 plasmid was digested with *Hind*III and *Bam*HI and the resulting *Hind*III/*Bam*HI fragment was cloned into the polylinker of a pCambia 1301 binary vector cut *Hind*III/*Bam*HI, producing the pC13/*BAM3* construct. The pC13/*BAM3* plasmid was then digested with *Hind*III and *Nco*I to obtain a *BAM3* promoter fragment suitable for cloning into the *Hind*III and *Nco*I sites of the pBI221.9 vector, upstream of the GUS reporter gene, to give rise to the pBI221.9/B3 plasmid. Finally, the *BAM3* promoter/GUS region of pBI221.9/B3 was isolated as a *Hind*III/*Sac*I fragment and cloned into the *Hind*III/*Sac*I sites of the pBI121 binary vector, thus giving rise to the BAM3 reporter construct.

DRTS2i1/M35S construct

For the production of the DRTS2i1/M35S reporter construct the plasmid pGemT4L20 [Ghisaura, 2010] was digested with *Pst*I and *Bam*HI and the resulting fragment was cloned into the *Pst*I/*Bam*HI sites of the pBI221.9 vector, inserting the 5'-UTR containing the first intron of *AtDRTS2* upstream of the -60 CaMV35S minimal promoter/GUS/ Nos PolyA region, thus creating the pBI221.9-DRTS2i1/M35S plasmid. This vector was then digested

with *Hind*III and *Eco*RI and the resulting fragment cloned into the pBI121 binary vector (digested *Hind* III/ *Eco* RI), suitable for *Agrobacterium*-mediated plant transformation, giving rise to the DRTS2i1/M35S reporter construct

M35S/DRTS2i1 construct

For the production of the M35S/DRTS2i1 reporter construct the 5'-UTR region of *AtDRTS2*, containing the first intron, was amplified by PCR (using high fidelity Pfx Taq polymerase-Invitrogen) from *Arabidopsis* genome using the T4L20 primer, which anneals at the beginning of the *AtDRTS2* 5'-UTR (at position +5 relative to the transcription start) and introduces a *Bam*HI restriction site, together with the T4L21 primer, which pairs at the beginning of the first non-coding exon of *AtDRTS2* (at position +222 relative to the transcription start) and contains a *Nco*I site overlapping a *Xba*I site. The resulting fragment was digested with *Bam*HI/*Xba*I and cloned into pBlueScript-KS plasmid (also cut with *Bam*HI/*Xba*I), giving rise to the TL2021 plasmid. Subsequently, the minimal -60 CaMV35S promoter was amplified from the pBI221.9/E2F plasmid [Albani et al., 2000], using the M13-REV universal primer and the 221.9 REV primer (which introduces a *Spe*I site and a *Bgl*II site). The fragment obtained was *Hind*III/*Bgl*II digested and cloned into the TL2021 plasmid (cut with *Hind*III and *Bam*HI), upstream of the *AtDRTS2* 5'-UTR. The resulting plasmid, named M35S/TL2021, was then cut with *Hind*III/*Nco*I to isolate the minimal -60 CaMV35S/*AtDRTS2* 5'-UTR fragment, which was cloned into the pBI221.9 vector digested with *Hind*III/*Nco*I, upstream of the GUS reporter gene, to give rise to the pBI221.9-M35S/TL2021 construct. Finally, the minimal -60 CaMV35S/ *AtDRTS2* 5'-UTR/ GUS fragment was isolated by digestion with *Xba*I/*Sac*I and cloned into the *Xba*I and *Sac*I sites of pBI121 binary vector, suitable for *Agrobacterium*-mediated plant transformation, thus producing the M35S/DRTS2i1 reporter construct.

3.3 Nucleic acids extraction and qPCR analyses

Total RNA extractions were performed using the Qiagen RNeasy mini-kit. The RNA samples were digested with DNase I during the extraction using the Qiagen RNase-free DNase set, to efficiently remove also small amounts of DNA during the on-column purification, as recommended by the manufacturer. RNA concentration and quality have been evaluated by spectrophotometry using A260/A280 ratio and by electrophoresis on denaturing formaldehyde gel. For qPCR analyses, 1µg of RNA has been reverse transcribed using the Invitrogen

SuperScript® III Reverse Transcriptase with a combination of hexamers and oligo dT primers. Quantitative real-time PCR was performed on the BioRad iCycler iQ ™, using the Qiagen QuantiTect SYBR® Green PCR Kit. Triplicate PCR reactions have been performed, following the manufacturer's recommended amplification conditions. For all the analyses the amplification 18S RNA has been used as a reference for normalization. Quantification was calculated following the $\Delta\Delta^{Ct}$ method. The PCR primers were designed using the Primer3 online software (http://primer3.ut.ee/) and all their sequences are detailed in Table 2.

Table 2 List of the primers used for qRT-PCR analyses.

NAME	SEQUENCE 5'-3'
RT-DRTS1-F	AAGTGTCGCCATTGAAATCC
RT-DRTS1-R	GCGAGTTTTCTGGAGAGGTG
RT-DRTS2-F	GAACAAGATCGCAGACGTGA
RT-DRTS2-R	ATGCCACATGTTTGCACAGT
RT-DRTS3-F	CACATGGCACGCTTATATCG
RT-DRTS3-F	TCTAGCTGCCACAACATTGC
RT-18S-F	CCTGCGGCTTAATTTGACTC
RT-18S-R	TTAGCAGGCTGAGGTCTCGT

3.4 GUS assays

Histochemical detection of GUS activity was performed on transgenic plants using 5-bromo-4-chloro-3-indolyl- β-D-glucuronide (X-Gluc) [Jefferson et al., 1987]. Plants at different developmental stages were incubated overnight at 37 °C in the GUS solution (50 mM pH 7 phosphate buffer, 1 mg/mL X-Gluc, 1 mM potassium ferricyanide). After staining, chlorophyll interference was removed treating the samples in 70% ethanol.

For quantitative analyses, the level of GUS activity was detected fluorimetrically using the fluorogenic substrate MUG (4-methyl umbelliferil–glucuronide). Seedlings of the same developmental stage were ground in GUS extraction buffer (50 mM $NaPO_4$ pH 7, 10 mM EDTA, 0.1% Triton, 0.1% Sodium Lauryl Sarcosine, 10 mM β-Mercaptoethanol). An aliquot of 44 µl of the extracts was added to 396 µl of assay buffer (50 mM $NaPO_4$ pH 7, 10 mM EDTA, 0.1% Triton, 0.1% Sodium Lauryl Sarcosine, 10 mM b-Mercaptoethanol, 1mM MUG) and the reactions were incubated at 37 °C. At four different time points, 100 µl of the reaction mix were added to 900 µl of stop buffer (0.2 M Na_2CO_3) and the amount of 4MU produced was measured using a fluorimeter (BioRad). The protein concentration of each extract was assayed using the Bradford method [Bradford, 1976] to allow calculation of the specific GUS activities.

3.5 Treatments with cell cycle inhibitors

To perform the treatments with cell cycle inhibitors, 30 seeds of selected homozygous transgenic lines harbouring the SFH7/DRTS1i2 construct were imbibed in sterile water alone (as control) or in water containing 5 µg/ml aphidicolin (Fisher Scientific) or 5 mg/ml colchicine (Apollo Scientific). After 72 h of imbibition in growth chamber at 22 °C under a regimen of 16 h of light at and 8 h of dark, proteins were extracted and fluorimetric assays of GUS activity were performed.

4. RESULTS

4.1 Molecular characterization of the AtDRTS genes

Database sequences and experimental analyses of the transcripts revealed the existence of at least two isoforms of each *AtDRTS* gene, some of which are potentially coding for truncated proteins lacking most of the TS domain (Fig. 4A and B). In this respect, although the genomic structure of *AtDRTS1* and *AtDRTS2* was supported by cDNA sequences, cDNA clones confirming entirely the predicted gene model of *AtDRTS3* have not been reported. The only *AtDRTS3* cDNA sequence available in databases (accession number BX820604) confirms the predicted position of the first three exons but extends the fourth exon into part of the following intron, that contains a transposon-like element in which an in frame stop codon interrupts the coding sequence (Fig. 4A). Thus, it appears that the presence of the transposon-like element in the fourth intron of *AtDRTS3* can cause premature termination of the primary transcripts and yields a mRNA that retains part of the fourth intron and codes for a protein of 311 aa, with a predicted mass of 35 kDa, that is expected to possess only DHFR activity. However, although *AtDRTS3* cDNA sequences including all the TS coding region have not been reported, microarray analyses (ATH1 Probe Set 263546_at) suggested the expression of transcripts spanning over the 3' end of the putative *AtDRTS3* gene model. To verify whether full length *AtDRTS3* transcripts corresponding the proposed gene model can be actually produced, RT-PCR reactions were performed using a forward primer that overlaps the ATG start codon in the first exon and a reverse primer that overlaps the predicted TAA terminating triplet, which is located in the tenth exon of the gene. These RT-PCR reactions were performed with high fidelity Taq polymerase, using retrotranscribed RNA isolated from *Arabidopsis* seedlings, and allowed the amplification of a cDNA containing the entire predicted coding region of the *AtDRTS3* gene model. Although the resulting sequence did not show any nucleotide change compared to the exonic sequences reported in the TAIR database, the 5' splicing site of the sixth intron appears to occur 9 bp upstream of the predicted one and yields a mRNA that is coding for an AtDRTS3 protein of 489 aa, with a predicted MW of 54.9 kDa that is slightly smaller than the protein proposed by the gene model. Thus, in spite of the transposon element in the fourth intron, it appears that the *AtDRTS3* gene can give rise to a full length transcript encoding a bifunctional DHFR/TS protein (Fig. 4B). Based on these results, the large isoform, corresponding nearly exactly to the gene model, has been called *AtDRTS3.1*, whereas *AtDRTS3.2* is the name of the smaller one, terminating at the fourth

intron and coding for a protein that lacks most of the C-terminal TS domain. Interestingly, a similarly truncated protein appears to be encoded also by an alternatively spliced transcript of *AtDRTS1* corresponding to the cDNA clone reported in databases with the accession number BX820156. This isoform, called *AtDRTS1.2*, retains the third intron, containing an in frame stop codon, and the interrupted open reading frame is predicted to code for a protein of 270 aa, with a predicted MW of 30 kDa. Compared to the 519 aa long AtDRTS1.1 protein of 58,1 kDa, the *AtDRTS1.2* isoform lacks most of the TS domain and, similarly to *AtDRTS3.2*, is expected to display only DHFR activity (Fig. 4B). Also concerning the *AtDRTS2* gene two isoforms have been detected but both are coding for bifunctional DHFR/TS proteins (Fig. 4A). In this respect, 5'RACE analyses previously performed in our laboratory have revealed the existence of alternatively spliced *AtDRTS2* transcripts lacking the second exon that contains the proposed ATG start codon of the gene. Its absence in the alternative transcripts results in the translation of a smaller isoform, named AtDRTS2.2, that begins from the in-frame ATG codon located in the fourth exon originally proposed as a start codon by Lazar et al. (1993).

As described in table 3, a comparison of the larger isoforms of the *AtDRTS* proteins reveal a close homology between *AtDRTS1* and *AtDRTS2*, showing over 86 % amino acid identity, whereas *AtDRTS3* appears to have partially diverged, with 56,8 and 57,4 % identity to *AtDRTS1* and *AtDRTS2*, respectively. This divergence is further highlighted by comparison of the highly variable hinge region separating the two functional domains, that shows as much as 64,7 % identity between *AtDRTS1* and *AtDRTS2* whereas for *AtDRTS3* shows only 33,3 and 38,7 % identity compared to *AtDRTS1* and *AtDRTS2*. Remarkably, as shown in figure 5, compared with the DRTSs of other angiosperms described in literature and databases, *AtDRTS3* groups together with a subset of the plant DRTS sequences. Moreover, although the cysteine corresponding to the active site in the TS domain is conserved in all three *AtDRTS* large isoforms, nearly all the substrate binding sites are perfectly conserved between *AtDRTS1* and *AtDRTS2* but, as shown in figure 4B, several amino acid substitutions characterize most of the substrate binding sites of AtDRTS3 and could reflect functional peculiarities of this protein.

Compared to *AtDRTS2.1*, the *AtDRTS2.2* isoform lacks the first 47 aa and could potentially lack a signal peptide or could possess an amino-terminal region allowing a different organellar targeting of the enzyme. To investigate this possibility, predictions of the subcellular localization of the *AtDRTS* isoforms were performed with 10 different platforms

available online. As shown in Table 4, *AtDRTS1.1* was predicted to be cytosolic by 8 of the platforms while the truncated *AtDRTS1.2* isoform was predicted to be cytosolic by only 5 of the softwares and additional predictions, including cell membrane, chloroplast, and extracellular locations, were proposed by some of the programs. Interestingly, the localization of *AtDRTS2.1*, which possesses a N-terminal extension that is absent in the other AtDRTSs, was predicted mostly as plastidial and/or mitochondrial whereas the smaller AtDRTS2,2 isoform was largely predicted as cytosolic. For *AtDRTS3.1* a prevalence of cytosolic over plastidial localization was reported while the truncated *AtDRTS3.2* isoform was predicted more as plastidial or membrane bound rather than cytosolic. Thus, different subcellular localizations of the *AtDRTS* proteins and of some of their isoforms are likely to occur.

Table 3 Percent Identity Matrix of the *AtDRTS* large isoforms and of their Hinge region (H).

	AtDRTS1	AtDRTS2	AtDRTS3	AtDRTS1/H	AtDRTS2/H	AtDRTS3/H
AtDRTS1	100.00					
AtDRTS2	86.85	100.00				
AtDRTS3	56.82	57.41	100.00			
AtDRTS1/H	-	-	-	100.00		
AtDRTS2/H	-	-	-	64.71	100.00	
AtDRTS3/H	-	-	-	33.33	38.71	100.00

Table 4 Predicted subcellular localization of the *AtDRTS* isoforms. The consensus indicates the most common predicted localization(s).

Platform	AtDRTS1.1	AtDRTS1.2	AtDRTS2.1	AtDRTS2.2	AtDRTS3.1	AtDRTS3.2
iPSORT	PL	PL	M	PL	PL	PL
SubLoc	CY	EX	CY	CY	CY	NU
WoLFPSORT	CY	NU/CY	M/PL	M/PL	PL	PL
CELLO	CY	PM	M/PL	CY	PM	PM
EuLoc	CY	PM	CY	CY	CY	NU/PM
iLoc-Plant	NU	CY	NU	NU	CY	CY
PSI predictor	CY	CY/PL	M/PL	CY	CY/PL	PM/PL
PProwler	CY	CY	M	CY	CY	CY
YLoc	CY	EX	PL	CY	NU	CY
Plant-mPLoc	CY	PM/PL/CY/M/PX	CY/PL	CY	CY	PM/PL
CONSENSUS	**CY**	**CY**	**PL/M**	**CY**	**CY**	**PL/PM**

Legend: CY = cytosol PL = plastid M = mitochondrion NU = nucleus PX = peroxisome
PM = plasma membrane EX = extracellular

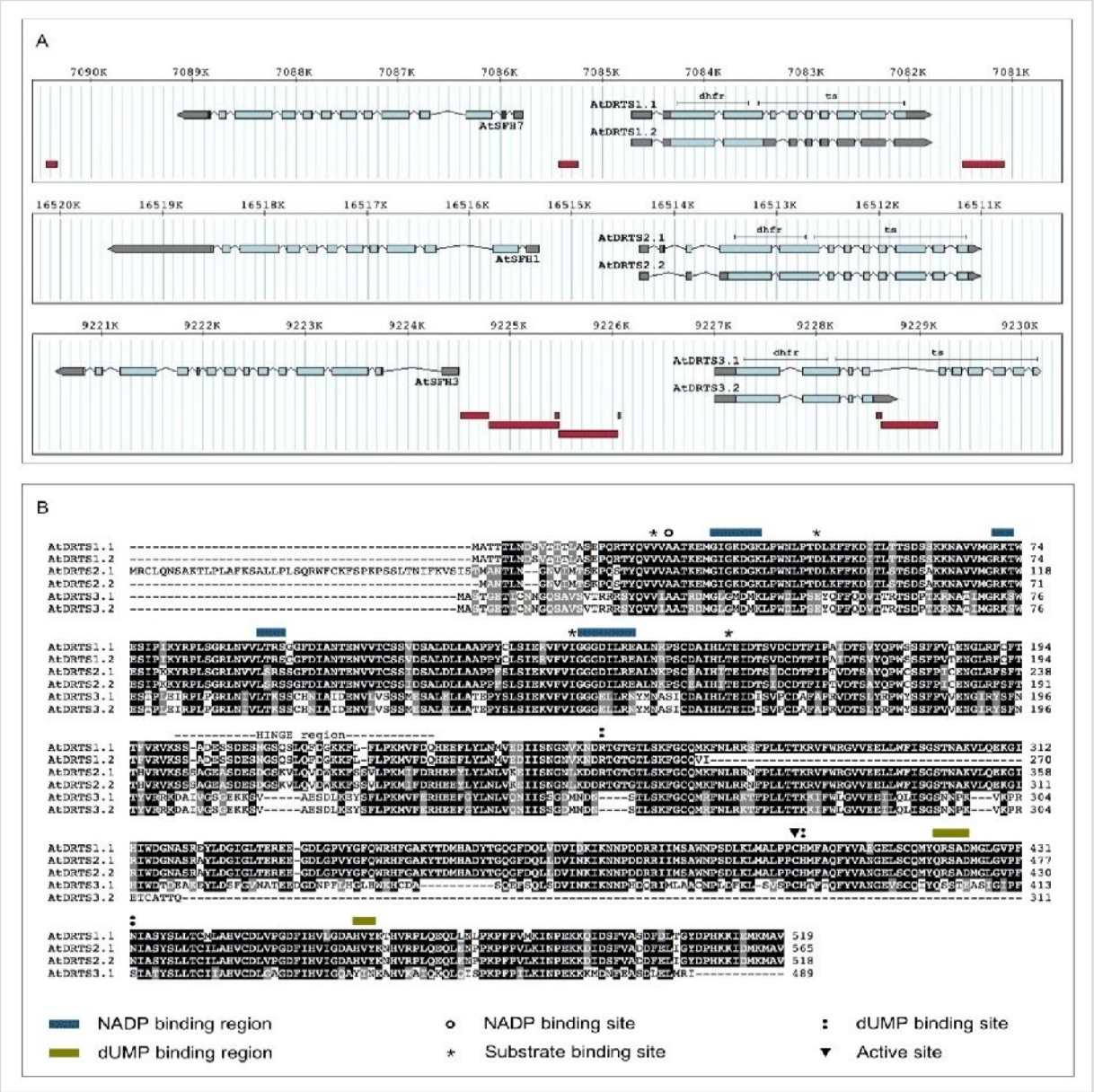

Fig. 4 *AtDRTSs* gene structure and protein isoforms**.** (**A**) Genomic organization of the *AtDRTS* gene paralogs and of the upstream *AtSFH* members. The exons are indicated as boxes with the UTR regions shown in gray and the coding portions in light blue. The portions corresponding to the DHFR and TS domains are indicated above the structure of the longest isoforms. The position of transposable elements is shown as dark red boxes below the gene structures. (**B**) Amino acid sequence comparison of the AtDRTS isoforms. The functional sites are indicated as described in the legend.
The Matrix was created by Clustal2.1 and the sequence alignments were performed using the T-Coffee program (http://www.ebi.ac.uk/Tools/msa/tcoffee/).

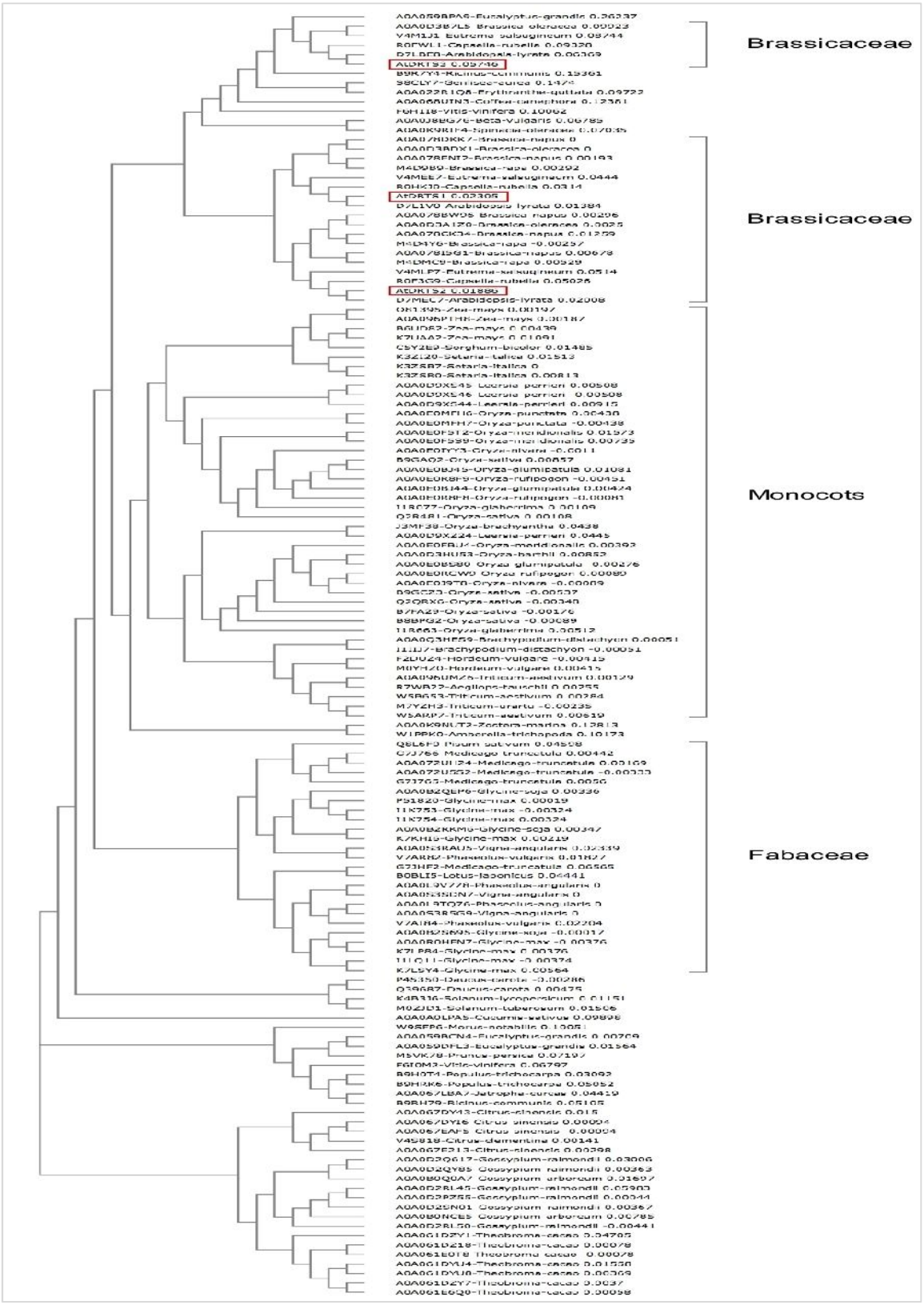

Fig. 5 Evolutionary relationships of angiosperm DRTS proteins. The Phylogenetic Tree was created aligning the aminoacid sequences with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The branches including monocots, fabaceae and brassicaceae are pointed out. The AtDRTSs are indicated with red boxes.

4.2 Patterns of activity of the three AtDRTS promoters

Previous studies, conducted by our research group, were carried out to define the patterns of expression of the *AtDRTS* genes by analysing by the activity of their promoters in transgenic *Arabidopsis* plants. Considering that the intergenic region upstream of the *AtDRTSs* contains also the promoter of the divergent *AtSFH* genes, dual reporter constructs had been assembled in which the DRTS promoter was controlling the expression of the GUS reporter gene while a gene coding for the red fluorescent protein eqFP611 [Wiedenmann et al., 2002] was placed under the control of the SFH promoter (figure 6A). The genomic fragments of each intergenic region, spanning from the start codon of the *AtSFH* gene to the ATG codon of each *AtDRTS* gene, were amplified by PCR using high fidelity Taq polymerase and used for the production of the dual reporter constructs. All these dual promoter constructs, called SFH7/DRTS1, SFH1/DRTS2 and SFH3/DRTS3, contained the 5' untranslated region of the genes, which in several cases has been shown to be important for the correct activity of the promoters. Concerning the histochemical GUS analyses of the transgenic lines, the most consistent patterns of GUS staining observed with each construct revealed remarkable differences in the activity of the three *AtDRTS* promoters (figures 6, B to D).

Interestingly, the *AtDRTS1* promoter activity was very strong in both leaf and root vascular tissues but was not detectable in any of the root tips (fig. 6B). As for the *AtDRTS2* promoter, it showed a strong activity in the proliferating cells of both root and shoot apical meristems, whereas its activity in differentiated tissues was practically undetectable (fig. 6C). Finally, concerning the *AtDRTS3* promoter, it showed activity in the shoot apical meristem but not in the root apical meristems, and its activity in the root was confined to the root columella and the central cylinder (fig. 6D) [Ghisaura, 2010].

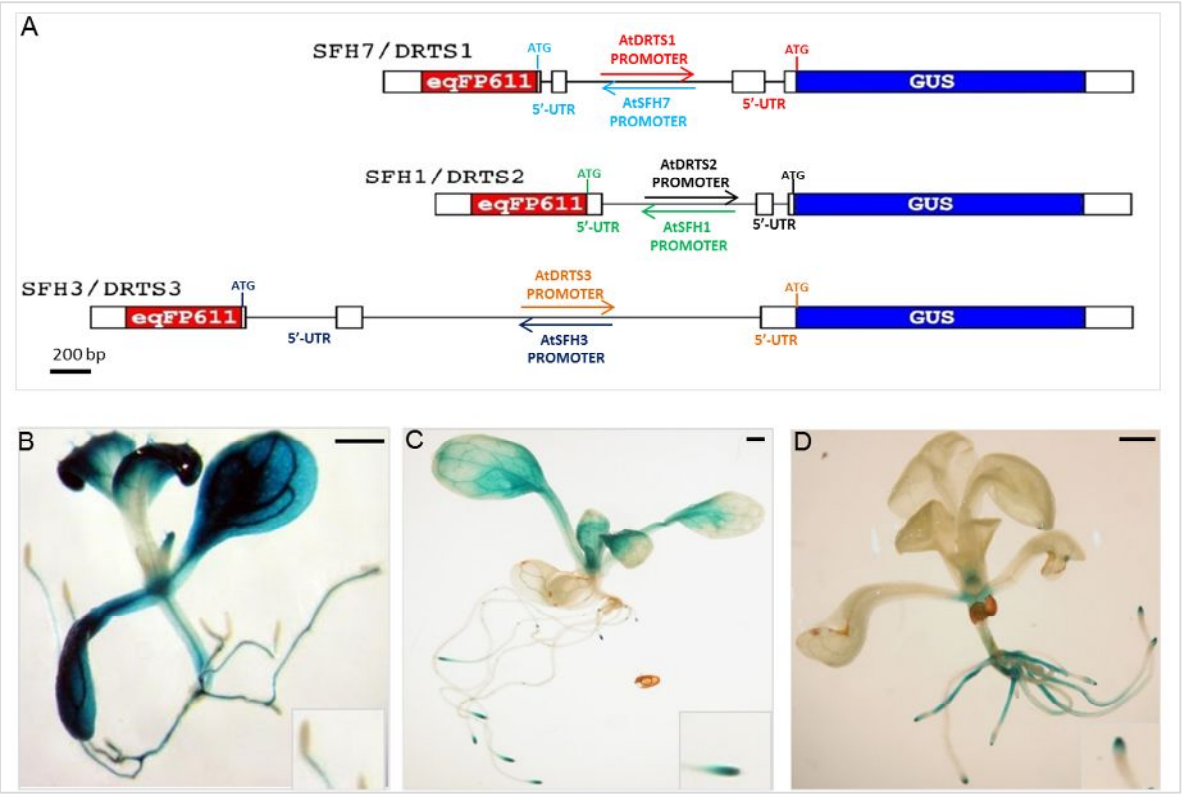

Fig. 6 (**A**) Schematic representation of the dual reporter constructs used to test the activity of the divergent *AtDRTS* and *AtSFH* promoters; The gene coding for the GUS protein is under the control of the *AtDRTS* promoters whereas the gene encoding the red fluorescent protein eqFP611 is controlled by the *AtSFH* promoters. Patterns of activity of the (**B**) *AtDRTS1*, (**C**) *AtDRTS2* and (**D**) *AtDRTS3* promoter, in two weeks-old seedlings. In the insets is highlighted the pattern of GUS expression in the root apices. Scale bars: 1 mm.

4.3 The AtDRTS genes are differentially expressed in both meristematic and differentiated tissues

The surprising result that emerged from the previous analyses was the absence of activity of the *AtDRTS1* promoter in the root apices. This feature could not be verified analysing microarray data which are reported at the *Arabidopsis* eFP browser of the Bio-Array Resource (BAR) website (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al., 2007) and at the Genevestigator V3 web tool (https://www.genevestigator.ethz.ch/gv/index.jsp; Hruz et al., 2008). These expression data, in fact, are related to experiments performed using the Affimetrix ATH1 array in which *AtDRTS3* is represented by a specific probe set (263546_at) whereas *AtDRTS1* and *AtDRTS2* transcripts are hybridizing to a unique probe set (263601_s_at) and their individual patterns of expression is therefore not distinguishable. Nevertheless, as described in figure 7 and 8A, the expression of the *AtDRTS1*/*AtDRTS2* couple and of the *AtDRTS3* gene appear to be very distinctive. More specifically, the strongest signal for the *AtDRTS1*/*AtDRTS2* probe set was detected in the shoot apex and in seeds 24 hours after imbibition, whereas the *AtDRTS3* expression level is reported to be very strong in columella and lateral root cap (figure 7), confirming the results obtained from analyses on the transgenic plants, carrying the *AtDRTS3* promoter reporter constructs. To further investigate the expression of the *AtDRTS* genes and to verify whether, in addition to *AtDRTS3*, also *AtDRTS1* and *AtDRTS2* can show distinctive patterns of expression, qRT-PCR analyses were performed on *Arabidopsis* seedlings and organs, using pairs of primers which specifically amplify the three *AtDRTS* cDNAs, as confirmed by sequencing the PCR fragments obtained. To discern the expression of the *AtDRTSs* in meristematic versus differentiated cells, the analyses were conducted with RNA isolated from root and shoot apexes, as well as leaves, hypocotyls and cotyledons. The relative level of expression of the *AtDRTSs* in the various organs compared to the leaves was calculated by the $\Delta\Delta^{Ct}$ method and is reported in figure 8B. These results reveal a remarkably higher expression of *AtDRTS3* in the root apex compared to the other organs, which agrees with the high level of expression detected in root caps by microarray analyses. A slight upregulation of *AtDRTS3* occurs also in hypocotyls, whereas similar levels of expression compared to the leaves are detected in seedlings, cotyledons and shoot apices. Concerning the expression of *AtDRTS1* and *AtDRTS2*, distinctive patterns were detected which, in agreement with the microarray analyses, reveal an upregulation of both genes in shoot apices compared to the leaves. In particular, the expression of *AtDRTS2* appears to be maximal in shoot apices and is clearly upregulated also in cotyledons and

hypocotils, as well as in the root apices to a lower extent. Conversely, *AtDRTS1* shows the strongest expression in hypocotyls and is clearly upregulated also in shoot apices and cotyledons but shows similar levels of expression in the root apices and in leaves. The expression of all the *AtDRTS* genes in the shoot apex is likely to correlate to different extents with cell proliferation and, at least for *AtDRTS1* and *AtDRTS2*, this correlation probably occurs in the root apex as well. The strong and variable expression of all three *AtDRTS* genes observed in differentiated tissues could be linked in part to cellular endoreduplication, but is likely to reflect also the involvement of the AtDRTS proteins in additional cellular processes.

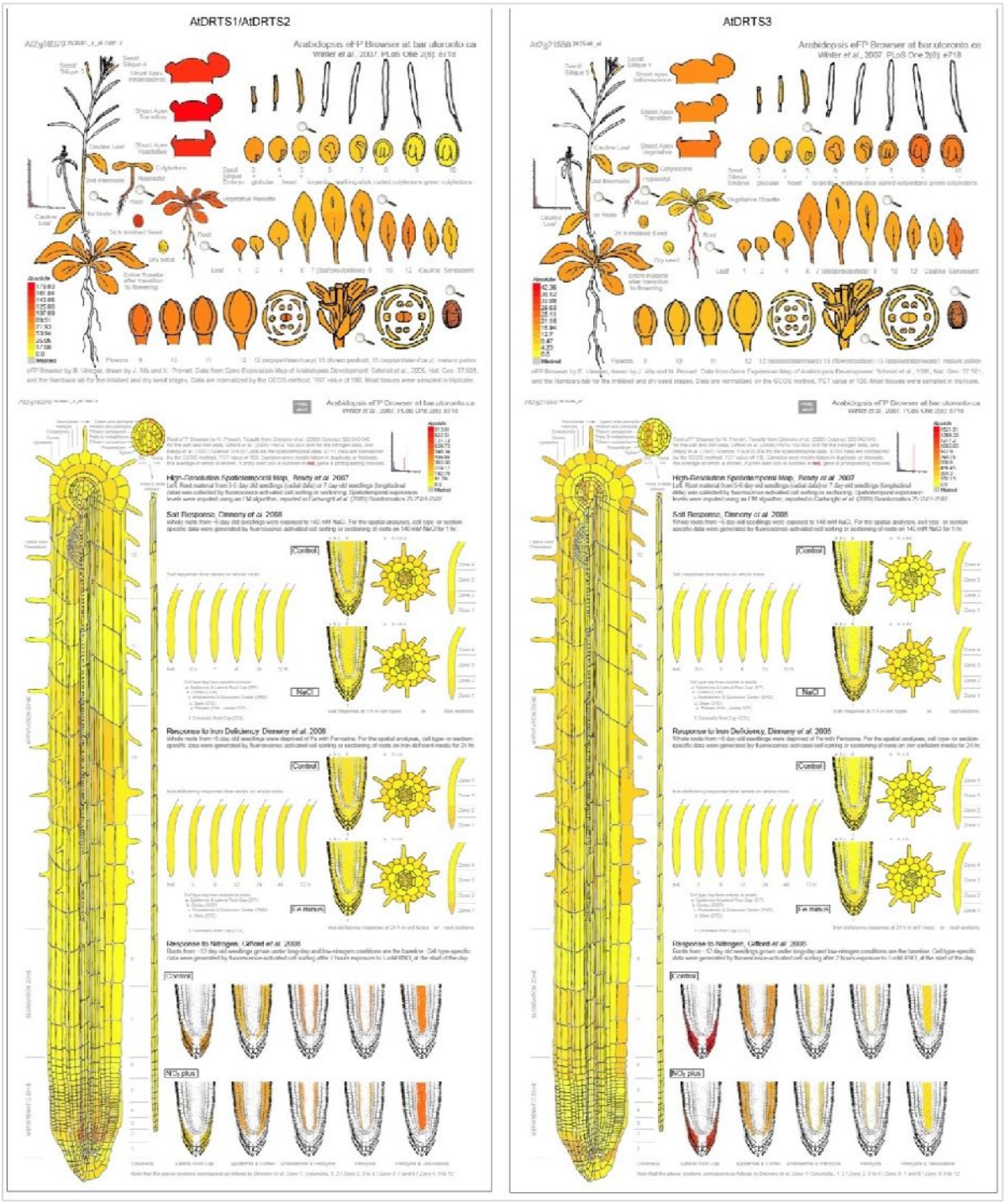

Fig. 7 Spatial patterns of accumulation of the *AtDRTS1/2* and *AtDRTS3* transcripts according to Microarray analyses.
Data shown are reported at the Botany Array Resource (BAR) Browser (http://bar.utoronto.ca/).

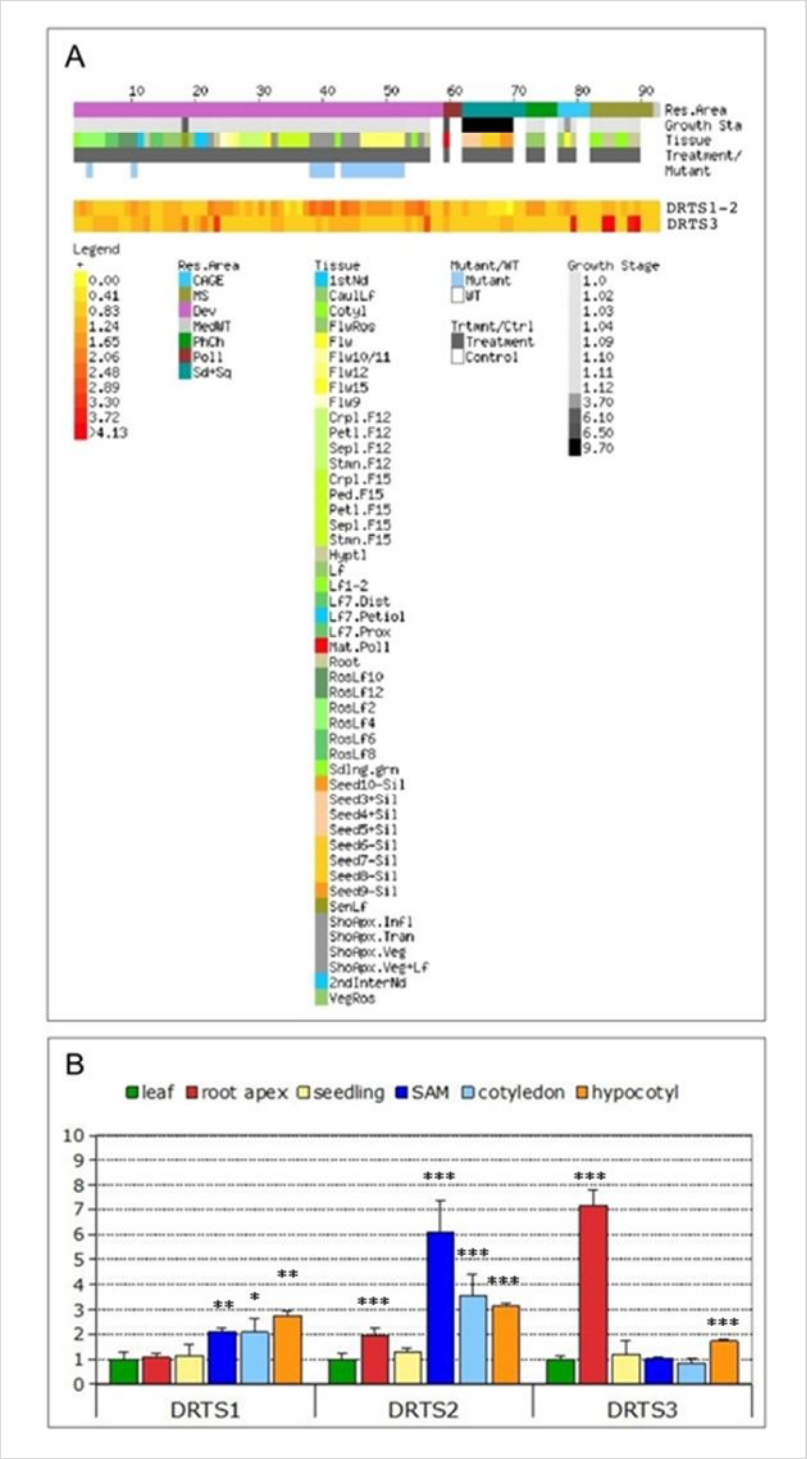

Fig. 8 Analysis of the expression of the *AtDRTS* genes. (**A**) E-Northern analysis of the expression of the *AtDRTS* transcripts revealed by microarray data. Heat maps showing the expression levels of the *AtDRTS1/AtDRTS2* common gene set and of *AtDRTS3* across different samples were generated using the Expression Browser tool of the Botany Array Resource (BAR) (http://bar.utoronto.ca/). (**B**) qRT-PCR analysis of the relative expression levels of the *AtDRTSs* in representative organs compared to leaves. The qRT-PCR analyses were repeated three times using independent biological replicates and quantification was normalized to 18S RNA levels. The bars show standard errors. *p<0.05, **p<0.01, ***p<0.001.

4.4 Intragenic regions regulate the activity of AtDRTS1 in root meristems

The qRT-PCR analyses suggested that regions required for the correct *AtDRTS1* promoter activity in the root apex could be missing in the SFH7/DRTS1 construct. Because all the intergenic region upstream *AtDRTS1* was included in the first promoter construct, experiments have been performed as part of this thesis to verify whether intragenic regions could be involved in the regulation of the *AtDRTS1* promoter. Several studies have previously reported that some of the introns of various genes are able to strongly influence their expression, an effect known as intron mediated enhancement (IME) [Rose, 2008]. This IME is usually associated to the first intron that could be located in the 5'-untranslated region of the gene, close to the transcription start, but examples of the influence of additional introns, located in the coding regions, have also been described. A software programme, called IMEter (http://korflab.ucdavis.edu/cgi-bin/IMEter_2014/web-imeter2.1.pl), has been used. This software scores the probability of introns to act as IME elements [Parra et al., 2011]. As described in figure 9A, according to the IMEter analysis of *AtDRTS1* both the first intron of the gene, located in the 5'-UTR and included in the SFH7/DRTS1 construct, as well as the second intron, located 420 bp downstream of the ATG codon and past the middle of the DHFR coding region, show remarkably high scores. Considering the lack of GUS activity in root apices of the SFH7/DRTS1 transformants and the high IMEter score of the second intron of *AtDRTS1*, an additional promoter construct was prepared that extends to the beginning of the third exon of the gene. In this construct, called SFH7/DRTS1i2, the GUS coding sequence is fused in frame with a large portion of the amino-terminal DHFR domain of the *AtDRTS1* protein (figure 9B). Remarkably, the transgenic plants transformed with this construct revealed a strong GUS activity in the root apices indicating that the *AtDRTS1* promoter can drive expression in the root apical meristem only when the intragenic region that includes the second intron of the gene is present downstream of the promoter (Fig. 10A to 10E). Interestingly a similar situation has been described for the *CENH3* gene of *Arabidopsis*, whose expression in root meristems, but not in other meristematic tissues, requires the intragenic region containing the second intron of the gene [Heckmann et al., 2011].

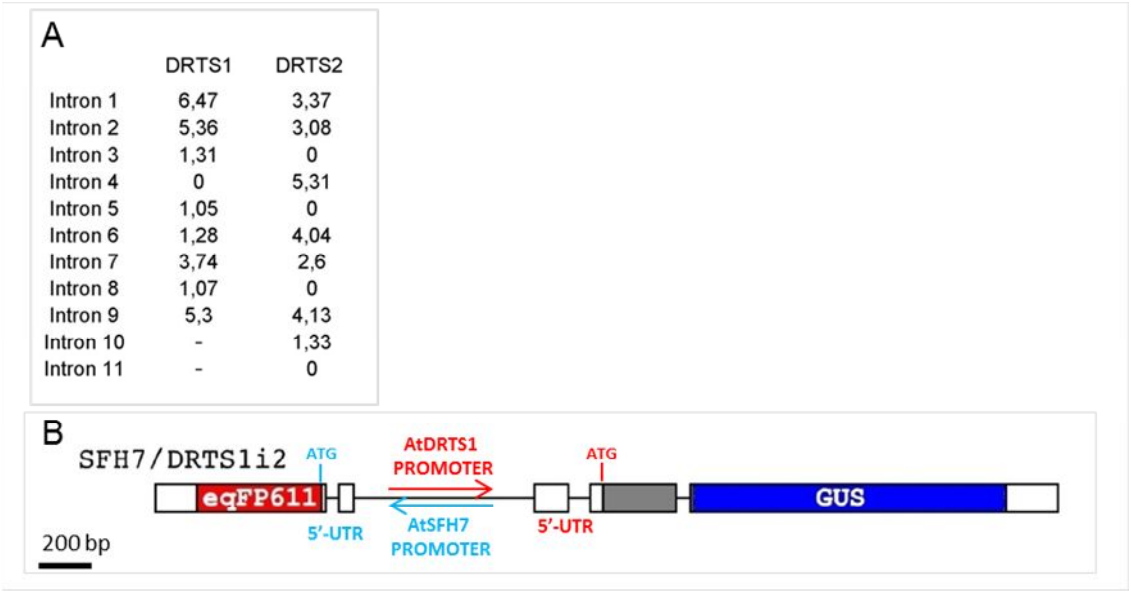

Fig. 9 (**A**) IMEter analysis of the introns of the *AtDRTS1* and *AtDRTS2* genes. (**B**) Schematic representation of the new SFH7/DRTS1i2 dual reporter construct.

Thus, with the inclusion of the intragenic region, the *AtDRTS1* promoter becomes able to drive GUS expression in both apical meristems but is also broadly active in differentiated tissues of the roots, hypocotyls and cotyledons, which show particularly strong GUS staining of the vascular tissues (Fig. 10, D and E). The meristematic activity is already detectable in lateral root primordia. For some of the lines, *AtDRTS1* promoter activity can be detected also in trichomes and in hydathodes (Fig. 10, inset B1). In mature flowers, the GUS staining can be detected in the style and ovary as well as in the vascular tissues of stamen filaments, whereas in developing flowers the promoter appears to be strongly active also in the stigmas (Fig. 10C). Moreover, GUS activity is clearly detected also in maturing seeds and in embryos (Fig. 10 D and E). Thus, the *AtDRTS1* gene appears to be highly expressed in meristematic tissues but is also very active in various differentiated tissues, in agreement with the pattern of expression detected by qRT-PCR.

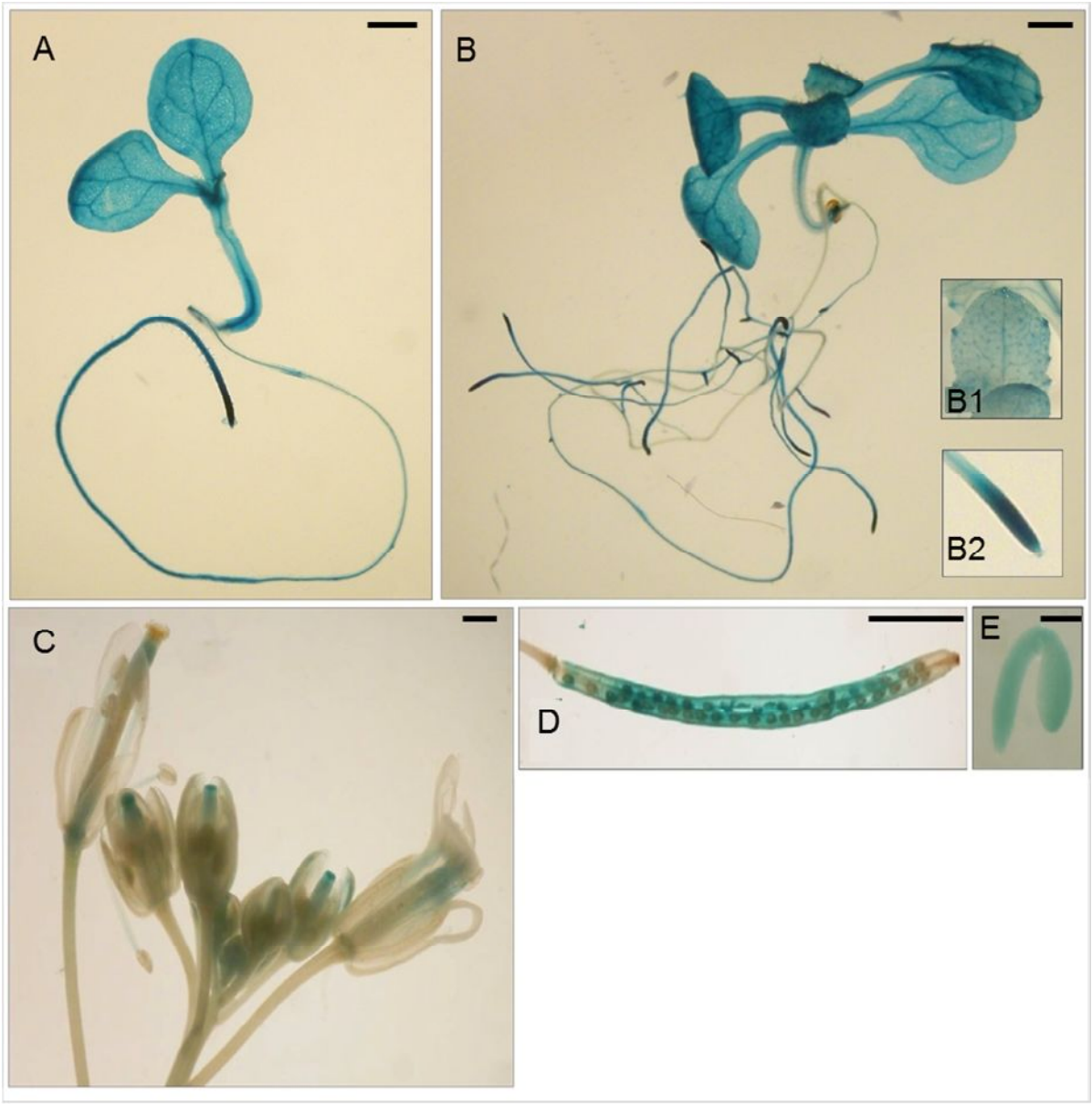

Fig. 10 Localization of GUS activity in lines carrying the SFH7/DRTS1i2 construct in: one-week-old (**A**) and two-week-old (**B**) seedlings, which show a strong activity in hydatodes (inset **B1**) and RAM (inset **B2**); inflorescence; siliques (**D**); mature embryos (**E**). Scale bars: 1 mm in **A**, **B** and **C**; 3 mm in **D**; 250 μm in **E**.

4.5 In silico analyses of the AtDRTS promoters reveal distinctive promoter architectures

The *AtDRTS* genes, although with variable strength, appear to be all expressed in the shoot apical meristem and common regulatory circuits could be involved in their control in this specific context. However, the different patterns of expression observed in root apical meristems and in other plant organs clearly suggest a distinctive regulation of the *AtDRTS* promoters. To verify the presence of common as well as specific regulatory elements in the *AtDRTS* promoters, *in silico* analyses were performed searching against the PLACE (http://www.dna.affrc.go.jp/PLACE/) and PlantPAN (http://plantpan2.itps.ncku.edu.tw/) databases, as well as using the RSAT (Regulatory Sequence Analysis Tools) (http://rsat.ulb.ac.be/rsat/) and JASPAR (http://jaspar.genereg.net/) web platforms. Because the 5'UTR of many genes have been shown to contain functional *cis*-elements, the analyses were carried out including all the DNA sequences upstream of the ATG start codons. Moreover, the intergenic region upstream of the *AtDRTS* genes contains also the promoters of *AtSFH* genes. Only 1311 bp separate the coding regions of *AtSFH1* and *AtDRTS2*, whereas the intergenic region upstream of the *AtDRTS1* ATG start codon is 1638 bp long and the *AtSFH3* and *AtDRTS3* ATGs are separated by 3471 bp. Considering the presence of the two promoters in the intergenic region, it is not possible to exclude that distant *cis* elements that are involved in the regulation of the *AtSFH* genes could be influencing also the activity of the *AtDRTS* promoters. Thus, the promoter analyses were performed on the entire intergenic regions separating the *AtSFH* and *AtDRTS* ATG start codons, although it is plausible that putative *cis* elements that are closer to the *AtDRTS* genes are more likely to regulate specifically their expression. The outputs of these analyses revealed a distinctive organization of the three *AtDRTS* promoters, with differences concerning the presence and the distribution of several putative *cis* elements. Overall, 93 different putative regulatory elements, varying in number and location, could be identified in at least one of the three intergenic regions (Table 5). Interestingly, only 17 of these 93 *cis* elements are found in all three intergenic regions and only 9 of them are also invariably located, in one or more copies, at positions that are suggesting their possible involvement in the regulation of the *AtDRTS* genes. 32 of the remaining putative regulatory elements are shared by only two of the intergenic regions: 18 are found in the intergenic regions upstream of both *AtDRTS1* and *AtDRTS3* and 13 are shared by the intergenic regions upstream of *AtDRTS2* and *AtDRTS3,* whereas only one *cis* elements is found specifically in the *AtDRTS1* and *AtDRTS2* upstream regions and in both cases is located closer to the *AtDRTS* genes. Interestingly, almost half of the putative regulatory

elements (44) are found, in single or multiple copies, in only one of the three intergenic regions. In this respect, 16 *cis* elements are found specifically in the region upstream of *AtDRTS1*, with 11 of them closer to *AtDRTS1*, and 5 *cis* elements are found only in the *AtSFH1/AtDRTS2* intergenic region, with 3 of them closer to *AtDRTS2*, whereas 23 putative elements are found specifically in the *AtSFH3/AtDRTS3* intergenic region, 9 of which are also located in positions that are closer to *AtDRTS2* (Table 5). Moreover, considering that an intragenic region containing the second intron of *AtDRTS1* appears to be required for full activity of the *AtDRTS1* promoter in the root apical meristem, additional *in silico* analyses were performed to verify whether the proposed intron mediated enhancement could be linked to the presence of particular *cis* elements in the second intron of the gene. 11 different putative regulatory sites are found in this intron, three of which are not found in the intergenic region upstream of the *AtDRTS1* coding region (Table 6). Overall, the requirement of intragenic regions for full activity of the *AtDRTS1* promoter in root apical meristems and the diversity of the various putative regulatory elements found closer to the *AtDRTS* genes in the upstream intergenic regions suggest very different architectures of the three *AtDRTS* promoters, which are likely to be controlled by distinctive transcriptional circuits.

Because all the *AtDRTS* promoters are able to drive expression in meristematic tissues, I focused my attention on the presence of promoter elements that have been reported to be involved in the control of gene expression in proliferating cells. Moreover, the balance of auxins and cytokinins plays important roles in the control of cell proliferation and *cis* elements linked to auxin and cytokinin regulation of gene expression were also taken in consideration. The presence and the location of these putative regulatory sites in the intergenic regions upstream of the *AtDRTSs* is described in figure 15. Because the E2F transcription factors have been reported to regulate genes involved in DNA synthesis and cell proliferation in both plants and animals [Berckmans and De Veylder, 2009], the presence of putative E2F binding sites was investigated in detail. The E2F factors are known to bind specifically a consensus sequence TTTSSCGSS (where S can be C or G) and an E2FAT *cis* element (TYTCCCGCC) has been reported in the promoters of many potential plant E2F target genes [Ramirez-Parra et al., 2003]. One copy of this element is actually found 199 nucleotides upstream of the *AtDRTS2* coding region but is not found upstream of *AtDRTS1* and *AtDRTS3* coding regions. However, according to chromatin immunoprecipitation ChIP-exo and ChIP-seq experiments, a shorter consensus element (TCCCGCC) is recognized *in vivo* by E2F factors [Yan et al., 2013; Morgunova et al., 2015]. A search for this sequence in the intergenic

regions upstream of the *AtDRTSs* revealed the presence of a putative E2F binding site also upstream of *AtDRTS3* but not in the promoter of *AtDRTS1*. Remarkably, also using less stringent criteria to detect E2F-like elements (TSSCGSS) no additional putative E2F sites could be found in any of the intergenic regions upstream of the *AtDRTS* genes. Interestingly, the E2F-like *cis* element of *AtDRTS3* is located 1591 nucleotides upstream of the ATG start codon, in the middle of a transposon-like element, and a recent study has reported that E2F sites are relatively common in plant transposable elements [Hénaff et al., 2014].

Other *cis* elements linked to cell proliferation that are found upstream of some of the *AtDRTS* genes include UP1ATMSD (GGCCCAWWW), which corresponds to the UP1 motif shown to be over-represented in the promoter of several genes that are up-regulated after main stem decapitation in *Arabidopsis* [Tatematsu et al., 2005]. This site contains the SORLIP2AT motif (GGGCC), an element over-represented in light-induced promoters of *Arabidopsis*, and is also overlapping with the SITEIIATCYTC element (TGGGCY), a site involved in the regulation of the *Arabidopsis Cytc-1* promoter that is strongly active in root and shoot meristems [Welchen and Gonzalez, 2005]. Remarkably, combined UP1ATMSD/SITEIIATCYTC *cis* elements are located upstream of both *AtDRTS2* and *AtDRTS3*, 283 bp upstream of the *AtDRTS2* ATG and 1040 bp upstream of the *AtDRTS3* coding region. As in the case of the E2F-like elements, these putative regulatory sites are much closer to the *AtDRTS* sequences than to the *AtSFH* genes and could be involved in the regulation of *AtDRTS2* and *AtDRTS3*. Moreover, two additional SITEIIATCYTC elements are found 1054 and 1116 bp upstream of the *AtDRTS3* coding region and could also regulate its expression. Interestingly, the intergenic region upstream of *AtDRTS1* contains only one SITEIIATCYTC element that is very close to the *AtSFH7* coding region (position -1238 bp) and is less likely to be involved in the control of *AtDRTS1* expression. Therefore, the activity of the *AtDRTS1* promoter in apical meristems is likely to be regulated differently than *AtDRTS2* and *AtDRTS3*. This is also stressed by the fact that the intragenic sequence including the second intron of *AtDRTS1* and required for promoter activity in root apical meristems does not contain any of the putative *cis* elements reported to be involved in gene regulation in proliferating cells. Moreover, specific regulatory circuits could control *AtDRTS2* because additional *cis* elements that are known to be particularly relevant for the control of genes expressed in proliferating cells are found only in its upstream region. One of these *cis* elements corresponds to UP2ATMSD (AAACCCTA), another motif found in several genes up-regulated after main stem decapitation in *Arabidopsis* [Tatematsu et al., 2005], that is located at position -130 with respect to the ATG codon and

next to the splice donor site in the first intron of *AtDRTS2*. The second site is HEXAMERATH4 (CCGTCG), the hexamer motif of *Arabidopsis* histone H4 promoter [Chaubet et al., 1996], that is located 208 nucleotides upstream of the *AtDRTS2* coding region. Finally, a MYBCOREATCYCB1 site (AACGG), known to control the M-phase-specific expression of the *Arabidopsis cyclin B1:1* gene [Planchais et al., 2002], is found at position -196 in the *AtDRTS2* gene. This putative *cis* element is also seen twice, although closer to the *AtSFH3* gene, in the intergenic region upstream of *AtDRTS3* but is not detectable upstream of *AtDRTS1* (figure 15).

Concerning the distribution of putative *cis* elements linked to auxin and cytokinin control of gene expression, sequences of four elements associated to auxin-dependent promoter regulation and of one element associated to cytokinin-dependent expression are found in the intergenic regions upstream of some of the *AtDRTS* genes. Interestingly, auxin-related *cis* elements can be detected upstream of *AtDRTS1* and *AtDRTS3* but none of them is found upstream of *AtDRTS2*. In particular, upstream and close to *AtDRTS1* coding region there is one NTBBF1ARROLB site (ACTTTA), at position -377, one ARFAT site (TGTCTC), at position -392, and one CATATGGMSAUR site (CATATG), 108 bp upstream of the ATG codon. Upstream of *AtDRTS3*, four of the five NTBBF1ARROLB sites and two of the three CATATGGMSAUR sites are close to the *AtDRTS* gene and could be influencing its expression whereas a D1GMAUX28 site is close to the *AtSFH3* gene and is less likely to be involved in *AtDRTS3* regulation. Moreover, the *cis* element CPBCSPOR (TATTAG), corresponding to a sequence critical for the cytokinin-dependent binding of a nuclear protein to the *CsPOR* promoter of cucumber [Fusada et al., 2005], is found twice upstream of *AtDRTS2* and three times upstream of *AtDRTS3* but is absent upstream of *AtDRTS1*. Nevertheless, the three CPBCSPOR sites in the *AtSFH3/AtDRTS3* intergenic region are very close to the *AtSFH3* coding region and are less likely to be involved in *AtDRTS3* regulation whereas one of the two sites found upstream of *AtDRTS2* is very close to the *AtDRTS* coding region, at position -388, and could be involved in the control of its expression.

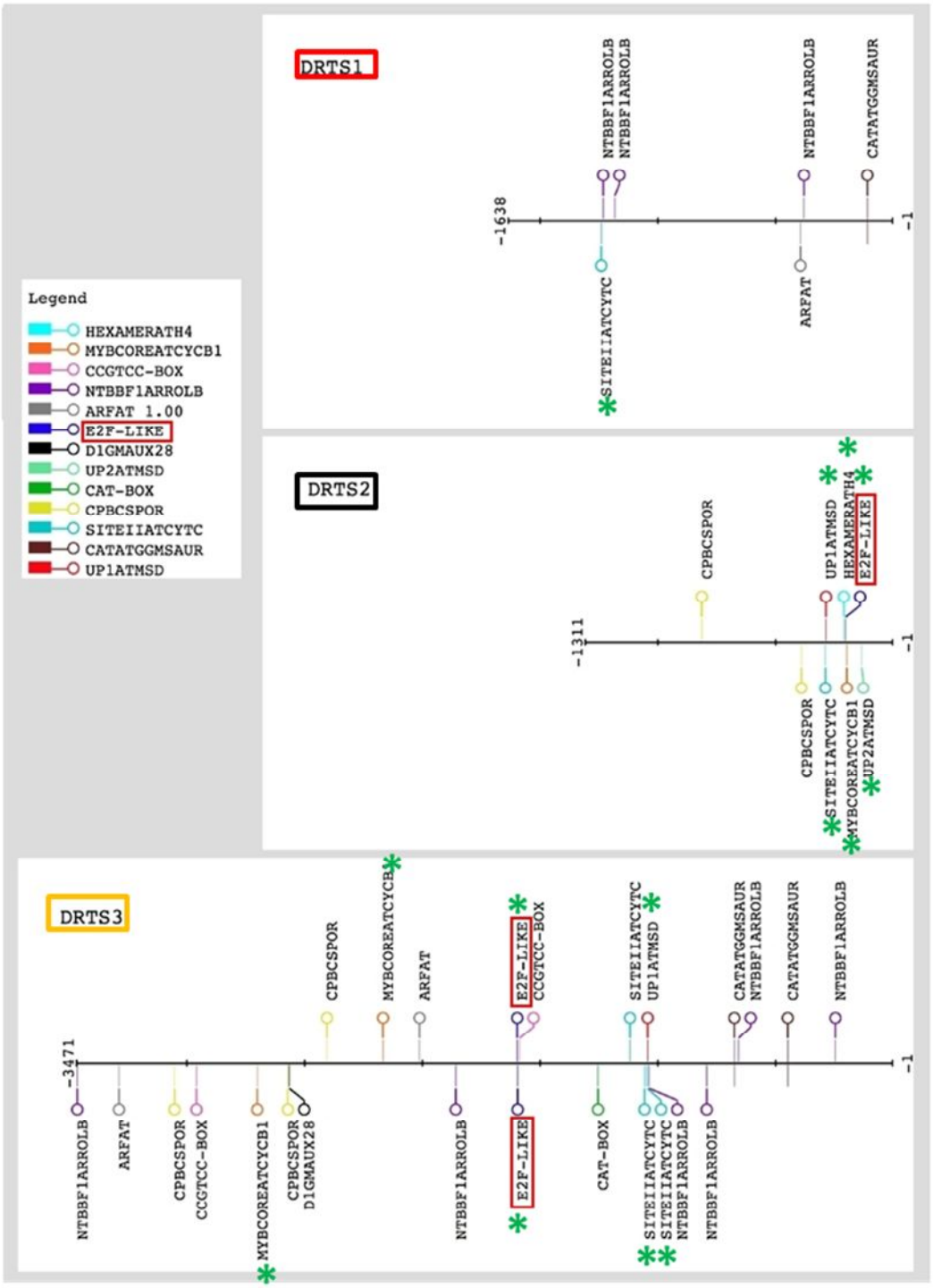

Fig. 11 Map of the most relevant *cis* elements identified in the intergenic regions separating the diverging *AtDRTS* and *AtSFH* coding sequences. The E2F sites found upstream of *AtDRTS2* and *AtDRTS3* are indicated with red boxes. The map was created using the drawing tool of the RSAT (Regulatory Sequence Analysis Tools) platform **(**http://www.rsat.eu/**).** The green asterisks show proliferation-related *cis* elements.

Table 5 Presence and location of *cis* elements in the SFH/DRTS intergenic region. The distance from DRTSs ATG codon is reported.
Sites which are closer to *AtDRTS* than to *AtSFH* coding regions are shown in red.
Sites associated to cell proliferation, endosperm expression or hormonal responses are highlighted in different colours as reported in the legend.

CIS element	Sequence	DRTS1	DRTS2	DRTS3
OSE1ROOTNODULE	AAAGAT	-1607, -1497, -1046, -959, -520, -216	-1244, -1141	-2395, -384, -89
MYBST1	GGATA	-1061, -974, -718, -465, -454, -122	-543	-2891, -912
REALPHALGLHCB21	AACCAA	-1372, -925, -837, -800, -730	-633, -592	-2985, -1022, -634, -570, -20
PYRIMIDINEBOXOSRAMY1A	CCTTTT	-903, -314, -163	-313	-3350, -2961, -2879, -2819, -2801, -645
DPBFCOREDCDC3	ACACNNG	-1554, -1342, -92	-372	-2709, -865, -666, -270
MYBCORE	CNGTTR	-810, -773	-293	-3442, -2562, -2358, -2096, -1006, -121, -55
RAV1AAT	CAACA	-559	-1254, -784	-3442, -2059, -1771, -1556, -1002, -52
SURECOREATSULTR11	GAGAC	-393, -75	-204, -143	-3287, -2010, -1232
CCA1ATLHCB1	AAMAATCT	-1594, -631	-1075, -979	-1320, -1290, -512
SITEIIATCYTC	TGGGCY	-1238	-286	-1116, -1054, -1043
CCA1-B	AGATAYR	-1302	-492	-2392, -238
MYCATERD1	CATGTG	-1343, -688	-524, -372	-667
-300CORE	TGTAAAG	-128, -179	-1246	-386
PREATPRODH	ACTCAT	-1524	-1300, -1175	-823
ACGTATERD1	ACGT	-389	-156	-799, -213
SEF1MOTIF	ATATTTAWW	-591	-835, -625	-1384, -1302
SEF3MOTIFGM	AACCCA	-257	-747	-3204
LTRE1HVBLT49	CCGAAA	-900, -713, -663	-301	
EECCRCAH1	GANTTNC	-868, -582, -83, -44, -4		-3327, -2857, -2524, -2500, -715, -452
NTBBF1ARROLB	ACTTTA	-1230, -1181, -377		-3463, -1856, -1035, -790, -655, -244

SP8BFIBSP8BIB	TACTATT	-625, -603		-2566, -2452, -1242, -840
GT1CORE	GGTTAA	-802		-3466, -3413, -1791, -1720, -1134, -632
CIACADIANLELHC	CAANNNNATC	-126		-3223, -1408, -1025, -396
PYRIMIDINEBOXHVEPB1	TTTTTTCC	-1544		-2803, -878, -831
AACACOREOSGLUB1	AACAAAC	-1368		-2336, -1865, -1268
LTRECOREATCOR15	CCGAC	-430		-2182, -1984, -347
ARFAT	TGTCTC	-392		-3286, -2010
GAREAT	TAACAAR	-1428		-3192, -1864
CATATGGMSAUR	CATATG	-108		-673, -446
LECPLEACS2	TAAAATAT	-1162		-2588, -1640
IBOX	GATAAG	-1417, -332		-1910
WBOXNTCHN48	CTGACY	-189, -99		-718
CANBNNAPA	CNAACAC	-89		-543
S1FBOXSORPS1L21	ATGGTA	-1493		-2622
SV40COREENHAN	GTGGWWHG	-464		-435
MYBPLANT	MACCWAMC	-728		-3110
CBFHV	RYCGAC		-774, -268, -69	-1984, -959, -891, -347
CPBCSPOR	TATTAG		-811, -388	-3050, -2569, -2403
MYB2CONSENSUSAT	YAACKG		-293	-2562, -2096, -121, -55
SORLIP2AT	GGGCC		-310, -287	-1116, -1055, -1044
NAPINMOTIFBN	TACACAT		-672, -373	-665
RBCSCONSENSUS	AATCCAA		-915	-3385, -2854, -805, -27
MYBPZM	CCWACC		-1263	-3152, -3112, -1590
TBOXATGAPB	ACTTTG		-330	-2212, -1737, -1684
BOXLCOREDCPAL	ACCWWCC		-1263	-3111, -333
MYBCOREATCYCB1	AACGG		-196	-2700, -2165
-10PEHVPSBD	TATTCT		-345	-1873

UP1ATMSD	GGCCCAWWW		-283	-1040
E2F-LIKE	NNTSSCGSS		-199	-1591
TATCCAOSAMY	TATCCA	-1061, -974, -465, -122		
ASF1MOTIFCAMV	TGACG	-1262, -1108		
EMHVCHORD	TGTAAAGT	-1228, -1179		
BOXIINTPATPB	ATAGAA	-1274, -410		
SREATMSD	TTATCC	-1060, -973		
AMMORESIIUDCRNIA1	GGWAGGGT	-1504		
SURE1STPAT21	AATAGAAAA	-1272		
MYC2 ELEMENT	TCACATG	-688		
TATCCACHVAL21	TATCCAC	-465		
REBETALGLHCB21	CGGATA	-454		
CAREOSREP1	CAACTC	-308		
ABRE-LIKE	BACGTGKM	-388		
GADOWNAT	ACGTGTC	-389		
BS1EGCCR	AGCGGG	-336		
NRRBNEXTA	TAGTGGAT	-466		
2SSEEDPROTBANAPA	CAAACAC	-89		
BOXIINTPATPB	ATAGAA		-937	
MYB1LEPR	GTTAGTT		-852	
GCN4OSGLUB1	TGAGTCA		-218	
HEXAMERATH4	CCGTCG		-208	
UP2ATMSD TELOBOXATEEF1AA1	AAACCCTA		-130	
CCA1-A	AATATCY			-2889, -2684, -1478
SEBFCONSSTPR10A	YTGTCWC			-3438, -2010
AMYBOX1	TAACARA			-3192, -1864
CTRMCAMV35S	TCTCTCTCT			-2906, -2845
ELRECOREPCRP1	TTGACC			-2794, -1773
ERELEE4	AWTTCAAA			-2208, -1890
MARABOX1	AATAAAYAAA			-3353, -2128
MYBGAHV	TAACAAA			-3192, -1864

P1BS	GNATATNC			-3135, -2997
QELEMENTZMZM13	AGGTCA			-2973, -1774
PALBOXAPC	CCGTCC			-2957, -1585
CGCGBOXAT	VCGCGB			-2711, -1715
HDZIP2ATATHB2	TAATMATTA			-2238, -845
ANAERO2CONSENSUS	AGCAGC			-943, -760
WUSATAg	TTAATGG			-2823
WBBOXPCWRKY1	TTTGACY			-2794
ABRERATCAL	MACGYGB			-2710
D1GMAUX28	ACAGTTACTA			-2561
L1BOXATPDF1	TAAATGYA			-2364
SORLIP1AT	GCCAC			-1252
SORLIP5AT	GAGTGAG			-825
RHERPATEXPA7	KCACGW			-797
RYREPEATLEGUMINBOX	CATGCAY			-669

AUXIN
CYTOCHININ
GIBBERELLIN
JASMONATE
ABA
ETHYLENE
ENDOSPERM
CELL PROLIFERATION

Table 6 Presence and location of *cis* elements in the intragenic 5' region of *AtDRTS1*. The distance from DRTSs ATG codon is reported.
Position from ATG is the bp distance downstream from the ATG codon.
Sites located within the second intron of the *AtDRTS1* gene are shown in red.

CIS element	Sequence	Position from ATG
DPBFCOREDCDC3	ACACNNG	34
TBOXATGAPB	ACTTTG	160
ELRECOREPCRP1	TTGACC	163
GT1GMSCAM4	GAAAAA	186
LTRE1HVBLT49	CCGAAA	248
LTRECOREATCOR15	CCGAC	254
ACGTATERD1	ACGT	263
SEF3MOTIFGM	AACCCA	285
GAREAT	TAACAAR	348, 391
SREATMSD	TTATCC	444
MYBST1	GGATA	445
REALPHALGLHCB21	AACCAA	475, 492, 496
MYBPLANT	MACCWAMC	494
BOXLCOREDCPAL	ACCWWCC	494

4.6 The meristematic expression of AtDRTS1 in germinating seeds is cell cycle-regulated

Considering the crucial role played by the DRTS enzymes in DNA synthesis, the expression of the *AtDRTS* genes in proliferating cells is expected to be preferentially linked to the G1/S phase of the cell cycle. As shown from my previous results, *AtDRTS1* is strongly expressed in the proliferating cells of embryos and also in root apical meristems. In this respect, I carried out experiments using cell cycle inhibitors in germinating seeds which are characterized by synchronous cell cycle progression during the early stages of germination [Barroco et al., 2005]. In dormant dry seeds most of the cells of the embryo are known to be blocked at the G1 phase. Upon seed imbibition, cells in the radicle progress into S phase and start the synthesis of DNA, which terminates approximately 42 hours after imbibition (HAI), when the radicle starts to protrude. Then the cells complete the cell cycle, passing through the G2 and M phase, which occurs 48 HAI. The imbibition and germination of the seeds in the presence of aphidicolin appears to block the cells in S phase, while the germination in the presence of colchicine allows the completion of the first S phase and blocks the cells at the M phase [Varadarajan et al, 2010]. To analyse the activity of the *AtDRTS1* promoter during seed germination, seeds of a representative SFH7/DRTS1i2 line that show a clear GUS activity from the beginning of germination, were imbibed for 72 hours in the dark at room temperature with or without cell cycle inhibitors. In the absence of inhibitors, this length of time would allow the meristematic cells to complete two divisions. The extracts from the germinated seeds were then analyses fuorimetrically to quantify the level of GUS activity. As shown in figure 12, germination in the presence of colchicine decreased the GUS activity in the seeds of the transgenic lines, whereas incubation with aphidicolin did not change significantly the level of GUS activity compared to control seeds germinated without the inhibitors. Although it is known that the GUS protein is relatively stable and can persist during cell cycle progression in proliferating cells [Adachi et al., 2006], these results suggest that the activity of the *AtDRTS1* promoter in germinating seeds is cell cycle-regulated, being high in G1/S but low or absent in G2/M.

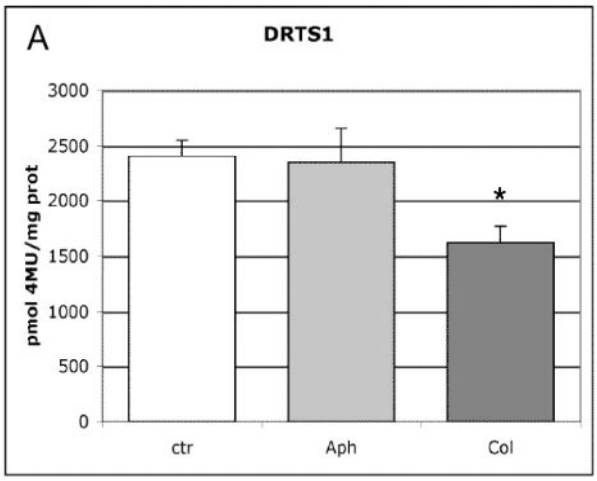

Fig. 12 Results of the fluorimetric analysis of GUS activity, carried out on extracts obtained from germinating seeds of the transgenic line harbouring the *AtDRTS1* construct. The extracts were incubated 72 h without (ctr) or with cell cycle inhibitors (Aph, Col). The bars show standard errors. *$p<0.05$.

4.7 E2F transcription factors are able to repress the activity of the AtDRTS2 and AtDRTS3 promoters

Previous analyses conducted by our research team have demonstrated that the mutation of E2F *cis* elements contained within the *AtDRTS2* and *AtDRTS3* promoters increased the activity of both promoters, suggesting a likely repressive role of the E2F sites [Ghisaura, 2010]. To better evaluate the influence of E2F factors on *AtDRTS* gene expression, Arabidopsis plants overexpressing the AtE2Fa factor were produced and the expression of the three *AtDRTS* genes was analysed by qRT-PCR in two lines showing strong overexpression of the *AtE2Fa* transcripts (Table 7). These lines, in agreement with previous reports concerning the overexpression of AtE2Fa or AtE2Fb [De Veylder et al., 2002; Sozzani et al., 2006], display a significant increase in cotyledonary epidermal cell number compared to wild type plants (Table 7). Interestingly, increased expression of *AtDRTS1* can be detected in both AtE2Fa^{OE} lines whereas the expression of *AtDRTS2* and *AtDRTS3* clearly diminished (figure 13). These results confirm an E2F-dependent repression of the *AtDRTS2* and *AtDRTS3* promoters, which could be direct targets of E2F factors, but reveal a positive influence of AtE2Fa overexpression on the expression of *AtDRTS1* which, however, is not necessarily reflecting a direct regulation but could be linked to the increased cell proliferation observed in the AtE2Fa^{OE} lines.

Table 7. Features of the two Arabidopsis lines overexpressing the AtE2Fa factor.

	AtE2Fa^{OE} #1	AtE2Fa^{OE} #5	Wild Type
Fold overexpression	113,74 ± 11,83	158,37 ± 12,73	-
Cotyledon size (mm^2)	4,4 ± 0,5	5,4 ± 0,3	3,1 ± 0,2
Cotyledon cell size (µm^2)	2976,19 ± 106,3	3215,43 ± 175,8	5494,51 ± 211,3
Cotyledon cell number	1478,4 ± 168,0	1679,4 ± 93,3	564,2 ± 36,4

The accumulation of the *AtE2Fa* mRNA in one week-old seedlings was quantified by qPCR following the $\Delta\Delta^{Ct}$ method using the18S RNA as a reference for normalization. The qPCR analysis was carried out using three biological replicates. The mean level of expression with the SE is reported. The phenotypic analysis of the cotyledons was carried out on 12 day-old plants using 8 to 12 samples. The size of the adaxial epidermal cells was calculated counting the number of cells contained in an area of 100,000 µm^2 . The total epidermal cell number was estimated dividing the cotyledon size by the cell size. The mean values with the SE are reported.

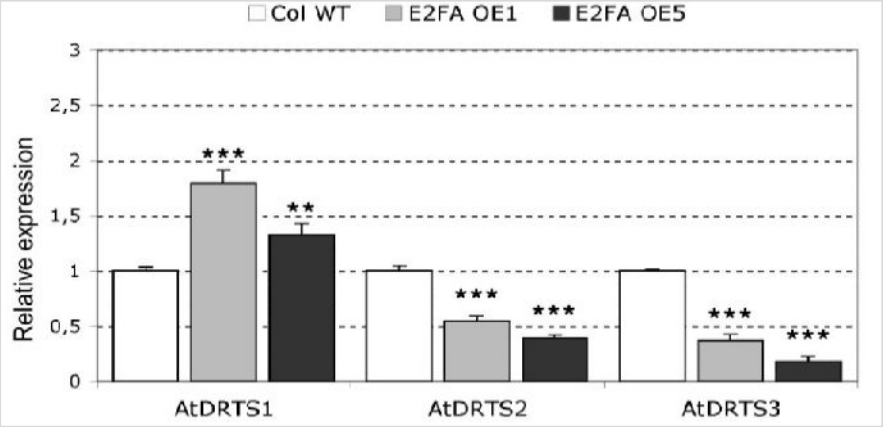

Fig. 13 qRT-PCR analyses performed on transgenic lines overexpressing the AtE2Fa activating factor revealed that *AtDRTS2* and *AtDRTS3* are downregulated, whereas *AtDRTS1* shows an increased expression.
The qRT-PCR analyses were repeated three times using independent biological replicates and quantification was normalized to 18S RNA levels. The bars show standard errors. ** $p<0.01$, ***$p<0.001$.

4.8 The first intron of AtDRTS2 can confer meristematic activity to a promoter that is not active in plant meristems

As for the *AtDRTS2* promoter, previous functional analyses conducted in our laboratory have demonstrated that the mutation of several *cis* elements, connected to cell proliferation, such as the UP1ATMSD, UP2ATMSD, Hexamer and E2F elements, did not alter the pattern of activity in the meristematic root apices [Ghisaura, 2010; Marche, 2013]. Moreover, experiments demonstrated the importance of the first *AtDRTS2* intron, contained in the 5'-UTR of the gene, for the activity of the *AtDRTS2* promoter in root meristems. In *Arabidopsis* plants transformed with a *AtDRTS2* promoter construct lacking the region containing the first intron, in fact, the promoter lost its activity in root apical meristems (figure 14), whereas in seedlings harboring a reporter construct which includes the *AtDRTS2* promoter and the 5'-UTR, up to the beginning of the first non-coding exon of the gene, a strong GUS activity in the root apices was observed (figure 15) [Ghisaura, 2010].

Importantly, additional analyses also revealed that this intron, when placed within the 5'-UTR of *AtDRTS1*, is able to activate the *AtDRTS1* promoter in root meristems [Marche, 2013]. As previously described in this chapter, it is clear that the second intron of *AtDRTS1* is necessary for its meristematic expression in root apices and, therefore, the previous data indicate that the first intron of *AtDRTS2* is able to functionally substitute the second intron of *AtDRTS1* when placed downstream of the *AtDRTS1* promoter.

Fig. 14 Activity of the *AtDRTS2* promoter construct lacking the first intron of the gene, in seedlings of one, two and three weeks of age and the enlarged view of a root apex [from: Ghisaura, 2010].

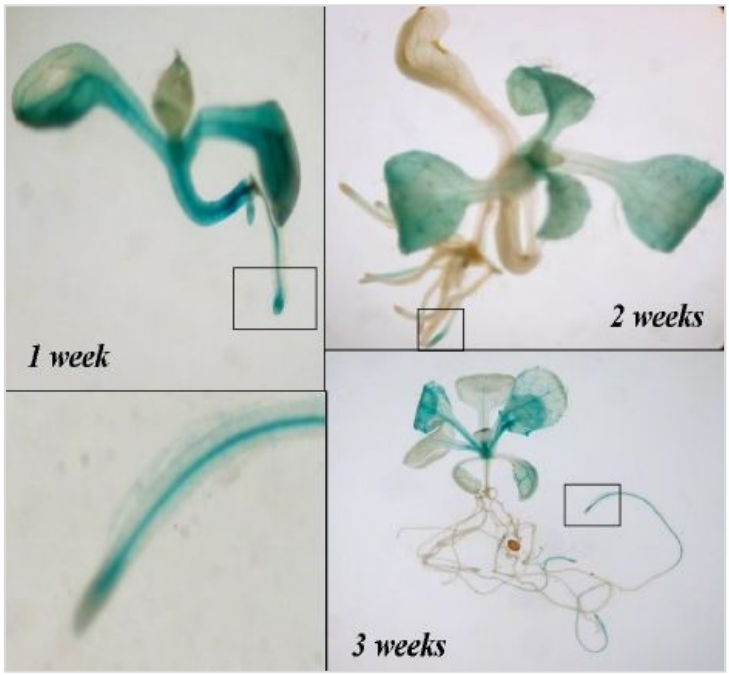

Fig. 15 Activity of the *AtDRTS2* promoter construct which includes the first intron of the gene, in seedlings of one, two and three weeks of age and the enlarged view of a root apex [from: Ghisaura, 2010].

Considering these experimental evidences, further investigations were conducted with this thesis to verify whether the activation in root meristems conferred by the first *AtDRTS2* intron, could be obtained also using the promoter of a gene which, contrarily to *AtDRTS1*, is not expressed in root meristems. To this purpose, I engineered a new GUS reporter construct, called BAM3/DRTS2i1 (figure 16A), in which the 5'-UTR containing the first intron of *AtDRTS2* (from position +5 to +222, respective to the *AtDRTS2* transcription start), is placed downstream of a non-meristematic promoter, such as the β-amylase 3 (*BAM3*) promoter, which is strongly active in leaves but not in meristems [Francisco et al., 2010]. As control, plants have also been transformed with a reporter construct in which only the *BAM3* promoter controls the GUS gene expression (figure 17A). Histochemical analyses of these plants revealed strong GUS staining limited to the vascular tissue of the leaves (figure 17B), confirming the expected pattern of activity of the *BAM3* promoter. Remarkably, the histochemical GUS assays performed on 14 T2 transgenic lines obtained by transformation with the BAM3/DRTS2i1 reporter construct showed that the presence of the *AtDRTS2* 5'-UTR altered drastically the pattern of activity of the *BAM3* promoter, which, instead of being confined only in leaves, spread out in the root, including also the root apical meristems (figure 16B). These results clearly indicate that the first intron of *AtDRTS2* is necessary and sufficient to confer a strong activity in root meristems to a non-meristematic promoter.

In consideration of these remarkable results, additional experiments have been carried out to evaluate whether the first *AtDRTS2* intron by itself, and not in combination with the additional *cis* elements of a complete promoter, is able to determine the activation in root apical meristems also of a minimal promoter, which contains only the TATA box as a regulatory element. For this investigation, two new GUS recombinant constructs, named DRTS2i1/M35S and M35S/DRTS2i1, have been produced. In these constructs, the region containing the first intron of *AtDRTS2* is inserted respectively upstream and downstream of the minimal -60 CaMV35S promoter (figure 18A and B). After transforming *Arabidopsis thaliana* plants with the constructs, several transgenic lines have been selected and histochemical analyses of the GUS reporter activity have been performed on the T2 progeny plants. According to this analysis, however, GUS activity could not be detected in any of the plant lines (figure 18C and D). These results indicate that the first intron of *AtDRTS2* is not sufficient to activate a minimal promoter in root meristems and its ability to confer meristematic activity thus requires the presence of the additional regulatory regions that constitute the architecture of a complex promoter.

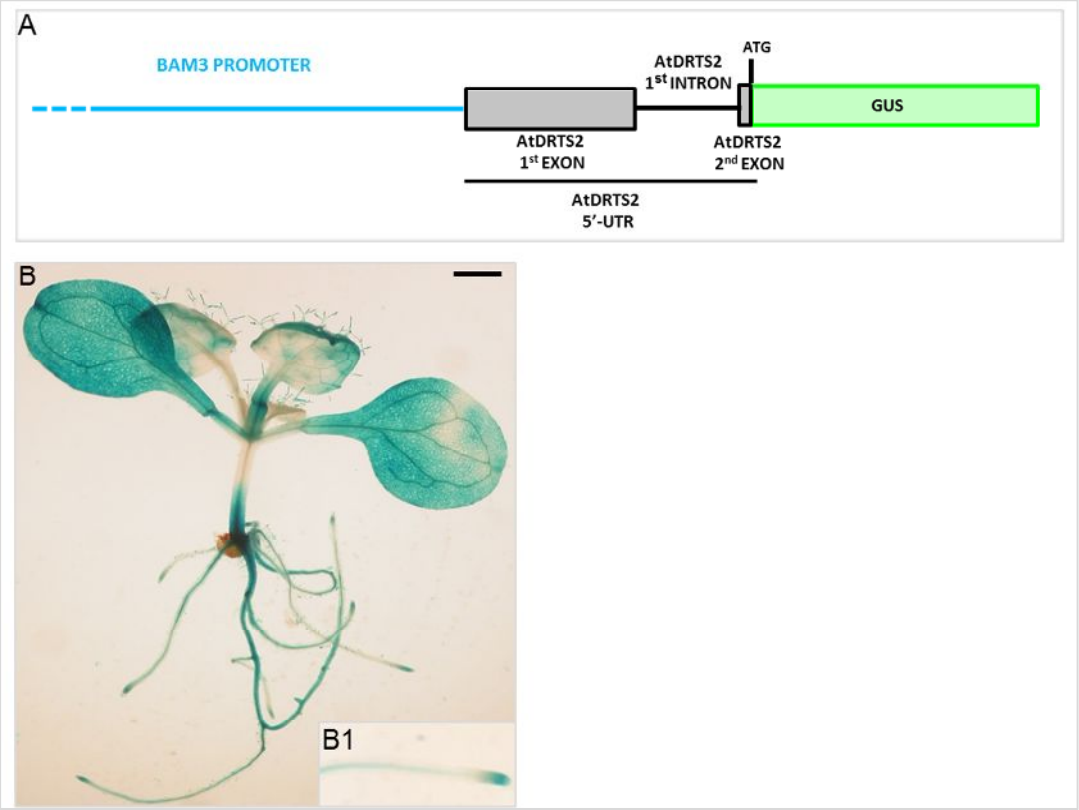

Fig. 16 (**A**) Schematic representation of the BAM3/DRTS2i1 construct. (**B**) Localization of GUS activity in two-week-old seedlings carrying the BAM3/DRTS2i1 construct; the inset **B1** shows the strong GUS expression in the root apex, which includes the meristematic cells of the RAM.
Scale bar: 1 mm.

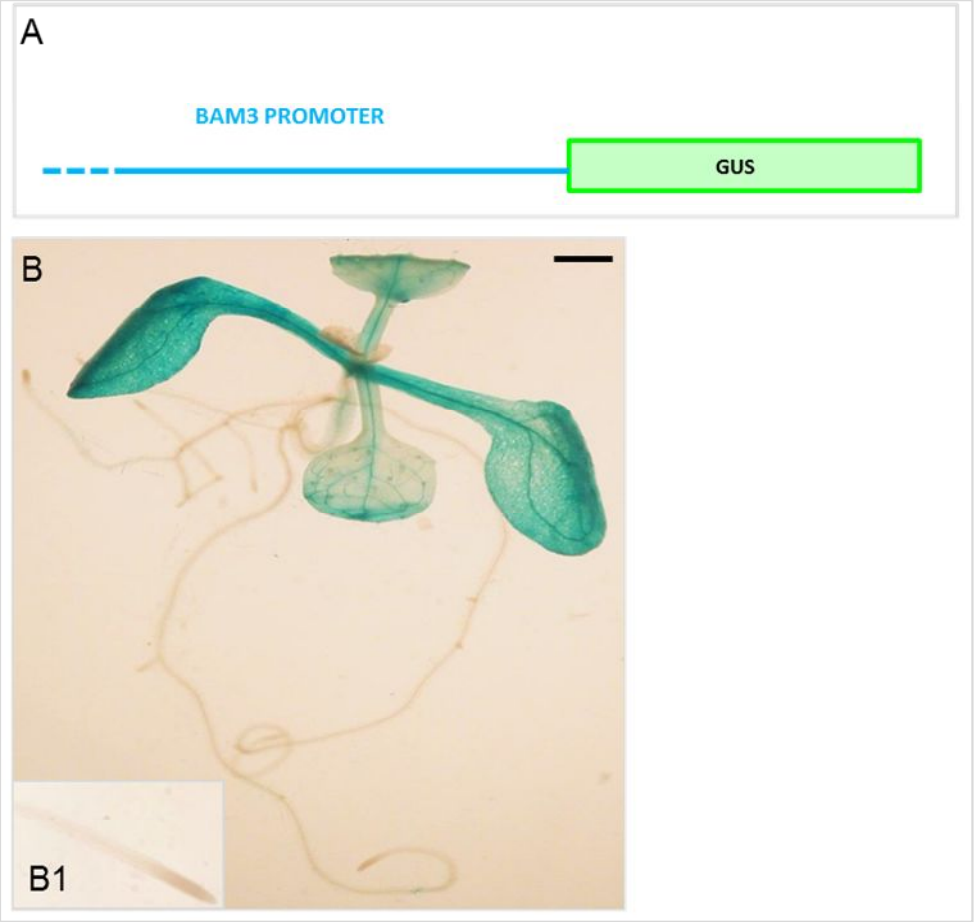

Fig. 17 (**A**) Schematic representation of the *BAM3* promoter construct. (**B**) The *BAM3* promoter is strongly active in leaves whereas no GUS activity is detected in the root apices, as highlighted in the inset **B1.** Scale bar: 1 mm.

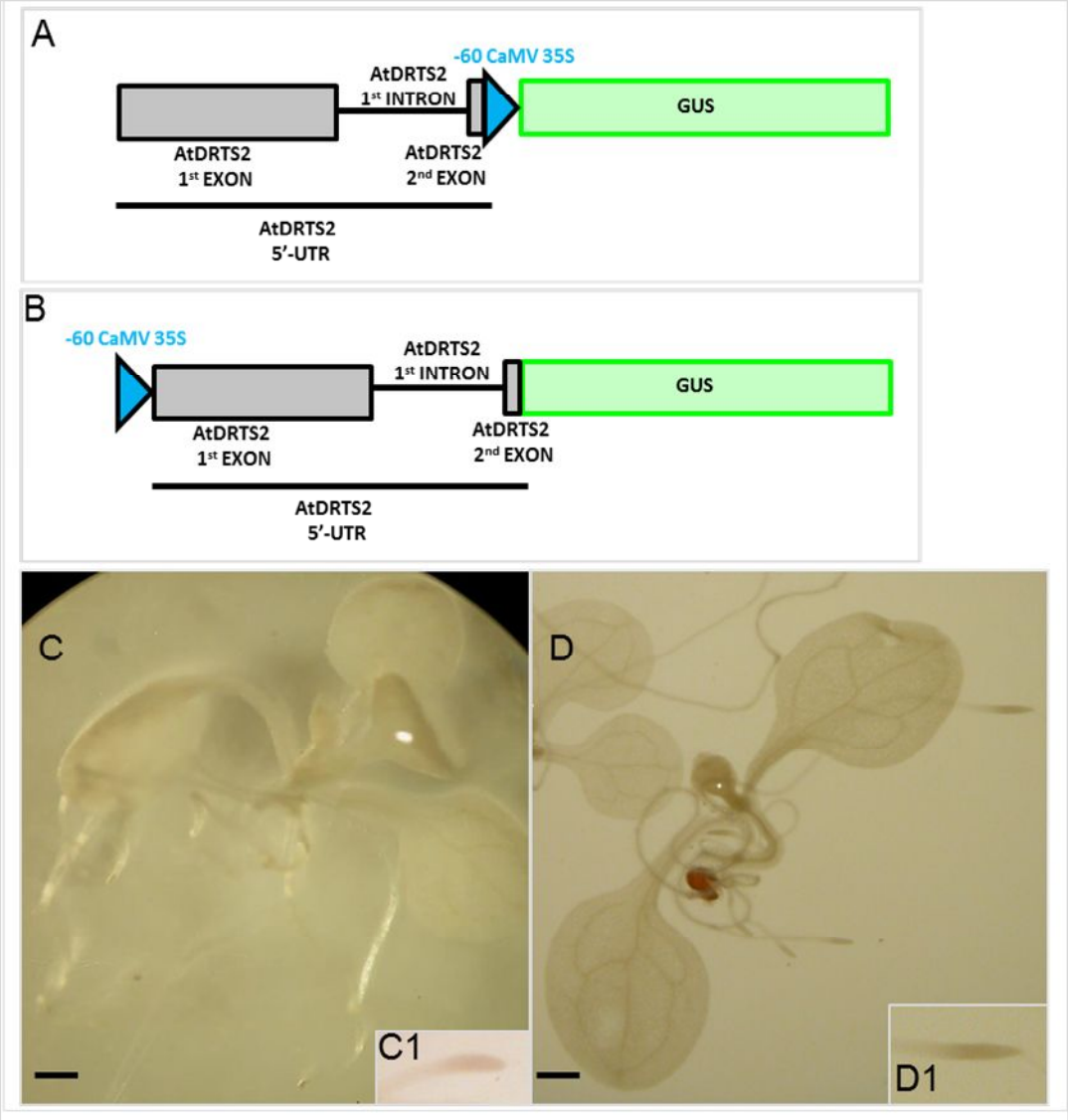

Fig. 18 Schematic representation of the (**A**) DRTS2i1/M35S and (**B**) M35S/DRTS2i constructs. No GUS activity has been detected in two-week-old seedlings carrying the DRTS2i1/M35S (**C**) and M35S/DRTS2i1 (**D**) constructs. The insets **C1** and **D1** highlight the lack of GUS staining in the root meristematic apex. Scale bars: 1 mm.

5. DISCUSSION

This chapter of the thesis expands the molecular characterization of the three members of the *DRTS* gene family of *Arabidopsis thaliana*, revealing the existence of remarkable isoforms and of distinctive promoter features that reflect differential patterns of expression. The *DRTS* genes are peculiar to plants and protists and code for bifunctional proteins characterized by the union in a single molecule of the domains specifying two enzymatic activities, dihydrofolate reductase (DHFR) and thymidylate synthase (TS), which in animals, fungi and bacteria are encoded by separate genes. DHFR catalyses the last reaction in the synthesis of tetrahydrofolate (THF), whereas TS uses N5,N10-methylene THF to reduce and methylate deoxyuridine monophosphate (dUMP) to dTMP, yielding 7,8-dihydrofolate (DHF) as a secondary product. Because DHFR activity is needed to recycle the resulting DHF, TS relies on DHFR activity and the presence of both enzymes in the same polypeptide, known as metabolic channelling, clearly increases the efficiency of thymidylate synthesis. Being involved in the synthesis of nucleotide precursors, the DHFR/TS bifunctional enzyme is essential in proliferating cells.

All three *DRTS* genes of *Arabidopsis* are downstream of divergently oriented members of the *AtSFH* gene family and could derive from successive genome duplications that occurred during Brassicaceae evolution. The *AtDRTS1* and *AtDRTS2* proteins are more similar to each other than to *AtDRTS3* and form a clade together with other Brassicaceae DRTSs. On the contrary, *AtDRTS3* groups with a subset of DRTSs conserved also in other eudicots. It appears, therefore, that *AtDRTS1* and *AtDRTS2* could derive from a recent duplication event that occurred after the separation of Brassicaceae from other plant families and before the divergence of the Arabidopsis and Brassica lineages [Bowers et al., 2003]. In all the plant and protist DRTS proteins the amino-terminal DHFR domain and the carboxy-terminal TS domain are separated by a linker region whose variable structure reflects evolutionary changes and has been used as marker for phylogenetic classification [O'Neil et al., 2003]. Although length and sequence of the linker region have been shown to be critical for TS activity and domain-domain interaction of the bifunctional enzyme [Chaianantakul et al., 2013], the two enzymatic activities appear to be largely autonomous and inhibition of each one with specific drugs does not affect the other activity [Neuburger et al., 1996]. In this respect, because the synthesis of THF is not needed only for TS activity but is necessary for a myriad of other metabolic pathways, it is not surprising that DHFR activity in plant cells has been reported to be at least

20 to 30 folds higher than TS activity. Moreover, the domain responsible for TS activity appears to be much more sensitive to protease action than the DHFR domain [Neuburger et al., 1996]. Interestingly, earlier studies have suggested the existence in plants of monofunctional DHFRs, associated with TS in a large multimeric enzyme complex [Toth et al., 1987].

The analysis of the *Arabidopsis DRTS* gene family reveals that alternatively spliced isoforms of *AtDRTS1* and *AtDRTS3* are potentially coding for truncated proteins that are expected to possess only DHFR activity. The differential splicing of *AtDRTS3* transcripts is likely to be associated to the presence of a transposon-like element in the fourth intron of the gene, causing a termination of the primary transcripts before reaching the regular 3' splicing acceptor site of the intron. Alternative splicing has been detected also for the *AtDRTS2* transcripts and is expected to results in the use of two different ATG codons, giving rise to protein isoforms possessing different amino-terminal regions. According to various targeting predictions, these *AtDRTS2* isoforms could be localized to different sub-cellular compartments and the larger one is mostly expected to be targeted to mitochondria and/or plastids, whereas the smaller one is mainly predicted to be cytosolic. A similar scenario has been reported also for a carrot *DRTS* gene showing alternative transcription starts that give rise to two isoforms, one of which possesses a N-terminal region with the features of a transit peptide that could target the protein to the plastids [Luo et al., 1997]. Mitochondrial localization of plant DRTSs is very likely because a huge pool of THF is needed for the photorespiratory process in leaf mitochondria of C3 plants and folate and thymidylate synthesis in plants have been shown to occur predominantly in mitochondria [Neuburger et al., 1996]. Nevertheless, compartmentalization of plant DRTSs is still an open question and, as predicted for the smaller *AtDRTS2* isoform, a cytosolic localization is mostly proposed also for the *AtDRTS1* and *AtDRTS3* proteins. Thus, it is possible that in particular cellular or developmental contexts some of the AtDRTSs could be localized, to various extents, not only in mitochondria but also in plastids and in the cytosol as well.

Expression analyses, conducted by qRT-PCR and evaluating the activity of the *AtDRTS* promoters in transgenic plants, revealed that the *DRTS* genes of *Arabidopsis* are variously expressed in meristematic and differentiated cells. Interestingly, previous data indicated that the first intron of *AtDRTS2* is required for meristematic expression of the gene and experiments performed with this thesis revealed that the presence of the intragenic region that includes the second intron of *AtDRTS1* is necessary for the activation of its promoter in the

root meristematic apex, similarly to what described also for the *CENH3* gene of *Arabidopsis* [Heckmann et al., 2011]. Moreover, additional investigations of the role played by the first intron of *AtDRTS2*, conducted during this work of thesis, revealed that this intron, when placed downstream to a plant promoter, is able to confer strong meristematic activity also to the promoter of a gene that is highly expressed in leaves and not in plant meristems. This feature does not appear to be autonomous but requires other functional promoter elements because is not observed when the intron is placed either downstream or upstream to a minimal 35S promoter.

The distinctive patterns of expression of the three *AtDRTS* genes in differentiated tissues suggest specific roles not necessarily linked to cell proliferation or endoreduplication. In this respect, *AtDRTS1* appears to be the most widely expressed gene and its promoter is strongly active in the vascular tissues, whereas *AtDRTS2* and *AtDRTS3* show narrower and more specific patterns of expression. The strong expression of *AtDRTS1* in vascular tissues emphasizes the important roles played by folates in the synthesis of lignin and of other cell wall components [Srivastava et al., 2015]. Of all three genes, *AtDRTS2* is the only one that is predominantly expressed in meristematic tissues. Meristematic expression is clearly linked to the need of thymidylate for DNA synthesis in proliferating cells, whereas the expression in many differentiated cells could be associated to DNA endoreduplication or to the synthesis of the folate cofactors required for various biochemical reactions. With respect to the meristematic expression, all three genes appear to be expressed in shoot apices, with *AtDRTS1* and *AtDRTS2* showing considerably higher expression compared to *AtDRTS3*. Also developing ovaries, in which cell proliferation occurs, show weak activity of all three *AtDRTS* promoters, whereas the expression of the *AtDRTSs* in other tissues and organs appears to be shared by only two of the genes or is rather specific for *AtDRTS1* or *AtDRTS3*. Interestingly, root apical meristems exhibit strong expression of both *AtDRTS1* and *AtDRTS2* but there is no evidence of the expression of *AtDRTS3*, which in the root apex is strongly and specifically expressed only in the columella and in the lateral root cap. Thus, although strong meristematic expression of *AtDRTS1* and *AtDRTS2* is evident in both shoot and root apical meristems, meristematic expression of *AtDRTS3* appears to be restricted to the shoot apex only. Moreover, according to their promoter activities, *AtDRTS1* and *AtDRTS2* are expressed widely in developing embryos, whereas the expression of *AtDRTS3* appears to be confined to a narrow region at the very tip of the embryonic root. These results suggest that *AtDRTS1* and *AtDRTS2* are consistently expressed in all the cells that undergo proliferation, whereas the

expression of *AtDRTS3* in proliferating cells occurs but is restricted to particular developmental or spatial contexts. In addition, the effects of cell cycle inhibitors on the activity of the *AtDRTS1* and *AtDRTS2* promoters in germinating seeds revealed that both genes are cell cycle-regulated in root meristems, showing higher expression at the G1/S phase in accordance with their importance for DNA synthesis.

Although the activities of the *AtDRTS1* and *AtDRTS3* promoters are partially overlapping in hydathodes, the *AtDRTS1* and *AtDRTS3* promoters show specific patterns of activity in other parts of the plant. The promoter of *AtDRTS1* appears to be active in trichomes and in the developing stigmatic papillae of flower buds, but not in mature stigmas of open flowers. The expression of *AtDRTS1* in these cell types could be linked to endoreduplication, which has been shown to occur during both trichomes and stigmatic papillae development [Martin and Glover, 2007]. Conversely, the *AtDRTS3* promoter appears to be specifically active in the stipules and in few cells at the adaxial base of inflorescence branches. This expression recalls the activity of two *PECTATE LYASE-LIKE (PLL)* promoters, *PLL15* and *PLL24*, that have been shown to drive *GUS* gene expression within a restricted region on the adaxial side of the base of pedicels and, as most other *PLL* promoters, are active also in the stipules of *Arabidopsis* plants [Sun and van Nocker, 2010] The function of stipules is still unclear but, together with hydathodes, they are believed to be primary sites for the synthesis of *indole-3-acetic acid (IAA)* associated with vascular differentiation and leaf morphogenesis [Aloni et al., 2003]. The transcription of *AtDRTS1* and *AtDRTS3* genes at sites of IAA synthesis could reflect an auxin-dependent upregulation of their promoters, also supported by the presence of several auxin-responsive *cis* elements. However, the expression of *AtDRTS* genes in stipules, hydathodes and root caps could also imply links between folates and IAA biosynthesis. Auxin distribution and signaling have been recently shown to be modulated by interactions between folate biosynthesis and Sucrose signaling [Zolman et al., 2008]. Moreover, it is known that 5,10-CH2-THF is a methyl donor for the synthesis of CoA molecules that, in addition to their engagement in various metabolisms, are also necessary for the β-oxidation of the auxin precursor *indole-3-butyric acid* (IBA) [Stokes et al., 2013]. Interestingly, expression in lateral root cap cells of the *IBR3* gene, encoding a protein involved in the conversion of IBA into IAA, has been shown to create a local auxin source that stimulates LR formation [Xuan et al., 2015].

The strong and specific expression of *AtDRTS3* in the columella and lateral root cap could support also other functions. Root caps protect the RAM and play important roles in root

growth allowing gravity perception. High metabolic activity is known to occur in root cap cells, which secrete mucilage to facilitate root penetration in the soil and release diverse secondary metabolites that influence rhizosphere microbiota composition [Bais et al., 2006]. Root border cells also produce antimicrobial phenolic compounds important in the defence against fungal pathogens and DHFR activity in root cap cells could be involved in the production of these defensive molecules. A recent study of *Arabidopsis* root exudates has revealed the release of a large array of compounds, including thymidine and degradation products of methionine-derived glucosinolates [Strehmel et al., 2014]. Moreover, DNA synthesis has been reported to occur in root cap border cells [Clowes, 1968; Phillips and Torrey, 1971] and the release of large amounts of extracellular DNA (exDNA) by root tips has been shown to play important defensive roles [Wen et al., 2009]. Together with secreted proteins, the released exDNA forms traps that are able to block pathogens and protect growing root tips from invasion. The defensive role of the exDNA is fully demonstrated by the loss of resistance upon treatments with DNaseI and by the capacity of bacterial strains to release nucleases to increase their virulence. Although the release of exDNA by root border cells has not been investigated in *Arabidopsis*, this feature has been described in different plant species and is likely to be widespread. Thus, it is possible that the strong expression of *AtDRTS3* in root caps is associated to the occurrence of this phenomenon also in *Arabidopsis*. Interestingly, important involvements of folate metabolism with plant defence have been already suggested. Folate content in rice seeds is associated with the induction of defence-related genes [Blancquaert et al., 2013] and folic acid has been shown to induce local and systemic SA-mediated defence in *Arabidopsis* [Wittek et al., 2015]. Moreover, in a study carried out in maize, two QTL that relate to brown plant-hopper (BPH) resistance have been shown to be associated with *ZmDRTS* genes [Ramalingam et al., 2003]. Although redundancy of the *AtDRTS* genes could support vital functions related to general metabolism and cell proliferation, functional analyses of the individual *AtDRTS* genes will be useful to assess whether *AtDRTS1* or *AtDRTS3* can be involved in auxin distribution and signaling and to verify whether *AtDRTS3* can play important roles in plant defense from pathogens.

According to the distinctive patterns of expression observed, the three *AtDRTS* genes appear to be differentially regulated to a large extent. In agreement with this finding, *in silico* analyses of the *AtDRTS* promoters revealed remarkably different distributions of several putative *cis* elements. Focusing my attention on *cis* elements reported to be involved in the regulation of gene expression in proliferating cells and in response to auxin and cytokinin, I

found clear differences among the *AtDRTS* promoters. Several proliferation-related elements are found upstream and closer to the *AtDRTS2* and *AtDRTS3* coding regions whereas the only putative site found upstream of *AtDRTS1* is much closer to the *AtSFH* gene than to the *AtDRTS* coding region and is less likely to be involved in *AtDRTS1* regulation.

The *AtDRTS2* promoter, which shows mostly meristematic activity, is particularly enriched in proliferation-related regulatory sites and contains six different *cis* elements that are associated to expression in proliferating cell and are all grouped closely upstream of the ATG codon. In comparison, the *AtDRTS3* promoter contains four diverse proliferation-related *cis* elements that are more dispersed and distant form the *AtDRTS* coding region. Considering the presence of auxin- or cytokinin-responsive *cis* elements, it is notable that various sites related to auxin regulation are found only in the *AtDRTS1* and *AtDRTS3* promoters, that are strongly active at sites of auxin production, and are absent in the *AtDRTS2* promoter, whereas two and three copies of a *cis* element responsive to cytokinin are seen upstream of *AtDRTS2* and *AtDRTS3*, respectively, but are absent in the *AtDRTS1* promoter. Thus, the expression of *AtDRTS1* and *AtDRTS3*, but not *AtDRTS2*, is likely to be regulated by auxin whereas cytokinin could control *AtDRTS2* and *AtDRTS3* expression. Although overlapping patterns of expression are seen in some meristematic tissues, the remarkably distinctive promoter architectures of the three *AtDRTS* genes suggest that their expression in proliferating cells is likely to be controlled to a large extent by different regulatory circuits. Moreover, as seen already with the *AtCENH3* promoter [Heckmann et al., 2011], results obtained with this work of thesis revealed that the meristematic expression of *AtDRTS1* is controlled differently in the RAM compared to the shoot apical meristem.

Among proliferation-related *cis* elements, the E2F sites are believed to play particularly important roles and have been shown to be required for meristematic expression of some plant genes. However, functional analyses of the E2F-like sites in the *AtDRTS2* and *AtDRTS3* promoters suggested previously that both genes are negatively regulated by endogenous E2F factors. An E2F-dependent regulation of *DRTS* genes has never been described before and the control of mammalian *DHFR* and *TS* genes by E2F factors appears to be controversial because various studies have shown contrasting results and strong regulation at the post-transcriptional level [Abali ET AL., 2008; Le Francois et al., 2007]. The E2F-mediated repression of *AtDRTS* genes was confirmed also in this thesis by analysing plants overexpressing AtE2Fa, in which *AtDRTS2* and *AtDRTS3* are downregulated whereas *AtDRTS1*, which lacks putative E2F sites, appears to be upregulated. However, *AtDRTS1* is

not expected to be an E2F target but is strongly expressed in proliferating cells and its upregulation in AtE2Fa^{OE} plants could simply reflect the increased cell proliferation caused by AtE2Fa overexpression.

The activity of typical E2Fs is finely controlled by post-translational modifications and through their association with Retinoblastoma-Related (RBR) proteins. Free typical E2Fs can function as activators, but the interaction with RBRs confers them repressive roles [Balck and Azizkhan-Clifford, 1999]. Among the typical AtE2Fs, AtE2Fa and AtE2Fb have been proposed to act mainly as transcriptional activators that are able to upregulate the expression of several cell cycle genes. On the contrary, AtE2Fc, the third typical E2F of Arabidopsis, as well as the three atypical E2Fs, AtE2Fd to AtE2Ff, are believed to act mainly as repressors of E2F-regulated genes [Ramirez-Parra et al., 2007]. However, repressive roles of AtE2Fa and AtE2Fb have been also reported. In apical meristems AtE2Fa was shown to be mostly associated with AtRBR1, to repress genes involved in endoreduplication and cell expansion, whereas AtE2Fb is believed to interact with RBR1 only in elongating and differentiating cell, repressing cell cycle genes in cells leaving the meristems [Magyar et al., 2012]. The downregulation of *AtDRTS2* and *AtDRTS3* in AtE2Fa^{OE} plants could be linked to a repressive role exerted by AtE2Fa on genes involved in DNA synthesis in proliferating cell. Nevertheless, the overexpression of AtE2Fa has been shown to upregulate AtRBR1 and remarkable interplays are known to occur among E2F genes, many of which appear to be E2F-regulated [De Veylder et al., 2002]. Thus, we cannot rule out the possibility that the downregulation of *AtDRTS2* and *AtDRTS3* in AtE2Fa^{OE} plants is not exerted directly by AtE2Fa, but could result from an upregulation of repressive E2Fs caused by AtE2Fa overexpression. In any case, our results clearly show that *AtDRTS2* and *AtDRTS3*, but not *AtDRTS1*, are negatively controlled by E2F factors. Remarkably, also the *AtCNH3* gene has been proposed to be downregulated by E2F factors in Arabidopsis protoplasts, even if its expression appeared to increase in plants overexpressing AtE2Fa or AtE2Fb [Heckmann et al., 2011].

CHAPTER 2.

Visualization of the E2F-dependent transcriptional activation *in planta*

1. INTRODUCTION

1.1 Plant development

In all organisms a fine coordination between cell proliferation, growth and differentiation is fundamental for the normal development. The main feature of plants is that the process of organogenesis occurs during the entire life of the organism, whereas in animals the formation of new organs takes place during embryogenesis. This peculiarity of plants is the reason of their indefinite growth via cell division and elongation, which can be maintained thanks to two niches of stem cells, that constitute the root and the shoot apical meristems named RAM and SAM, respectively (figure 1). These tissues are localized at the end of the main embryonic body axis and provide a pool of undifferentiated, pluripotent and highly proliferating cells that, after their exit from the meristem and differentiation, allow the formation of the adult organs and tissues. Each meristem, in fact, has a distinct organization and cell types that arise from it. The SAM is responsible for the formation of stem, leaves and buds, whereas the RAM provides cells that will originate the complex system of radical tissues

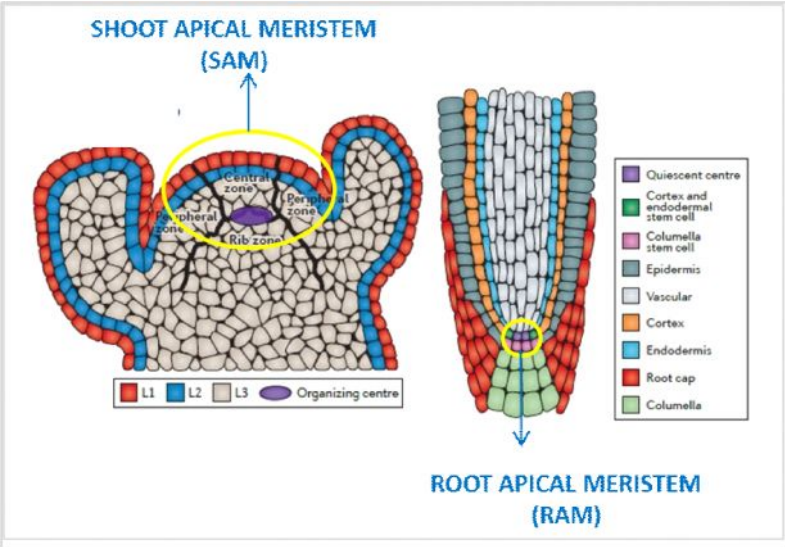

Fig. 1 Simplified representation of the structure of shoot and root apical meristems [Adapted from: Sparks et al., 2013].

1.2 The plant cell cycle and its regulators

Growth and development of multicellular organisms are based on cell proliferation which occurs following a minutely regulated process, called cell cycle (figure 2). The cell cycle is divided in four sequential phases, which end up in the formation of two daughter cells. The first phase is G1 (Gap phase 1), which begins when cells commit for a new division, G1 is followed by the S phase, where the DNA synthesis occurs. The G2 phase (Gap-phase 2) separates the S phase from the M phase, which embraces the karyo- and cytokinesis, where the duplicated genome and cellular components, are divided into the newborn cells. The principal mechanisms that regulate the progression trough the cell cycle are highly conserved across evolution. In eukaryotes, the cell cycle is coordinated, at multiple points, by a class of serine/threonine protein kinases, called cyclin-dependent kinases (CDKs). The CDKs activity is regulated by different proteins, called cyclins (CYCs), like as the D-type cyclins (CYCD), which are essential during the G1 to S phase transition, and the A-type (CYCA) and B-type (CYCB) cyclins, which control the progression through the S phase and the G2 to M phase transition, respectively [De Veylder et al., 2007]. Plants contain different types of CDK, involved in the control of the cell cycle, that have been initially identified in *Arabidopsis thaliana.* The two major CDKs, which control the cell cycle in higher plants, are the CDKAs and CDKBs. The A-type CDKs all contain the conserved amino acid sequence PSTAIRE in their cyclin-binding domain, whereas plant CDKBs could show either a PPTALRE or a PPTTLRE sequence [Joubès et al., 2000]. The CDKBs are present in higher plants in two sub-types called CDKB1 and CDKB2. *A. thaliana* genome includes two genes, which encode for each CDKB sub-type: CDKB1;1, CDKB1;2, CDKB2;1, and CDKB2;2, whereas the CDKAs are encoded by a single gene called CDKA;1 [Boudolf et al., 2001]. As mentioned before, the complexes CDKs-cyclins are essential for the progression through the cell cycle, in fact, the CDKs have no activity if not associated with the cyclins. *Arabidopsis* genome encodes 10 A-type cyclins, 11 B-type cyclins, 10 D-type cyclins and 1 H-type cyclin. Phylogenetic analyses of A- and B-type plant cyclins shows that cyclin classes are conserved between animals and plants[Renaudin et al., 1996].

The levels of cyclins are regulated during transcription, as well as by specific protein-turnover mechanisms. A- and B-type cyclins possess “destruction box” sequences, which have been reported to mediate protein degradation by an anaphase-promoting complex (APC) during M phase [Glotzer et al., 1991]. D-type cyclins are conjugated to ubiquitin by an SCF complex and then degraded by the proteasome pathway [Dewitte and Murray, 2003].

The activity of CDKs is positively regulated by phosphorylation, carried out by a CDK-activating kinase (CAK). In plants, the phosphorylation of a conserved threonine residue, equivalent to Thr-160 of human CDK2, which is within a loop of the protein named T-loop, induces a conformational change that enables substrate binding by the CDKs catalytic site [Dewitte and Murray, 2003] . Mechanisms of negative regulation of CDK activity have also been described. A family of proteins, named CDK inhibitors (CKIs), inhibits CDKs activity by tight association with the cyclin/CDK complexes. In *Arabidopsis*, seven genes have been identified, which encode for Kip-related proteins (KRPs), which present an homology with the KIP proteins, a class of mammal CKIs [De Veylder et al., 2001].

CDKs activity is additionally controlled by inhibitory phosphorylation. To ensure an appropriate continuance of mitosis in eukaryotic cells, CDKs are negatively regulated by the inhibiting WEE1 and MYT1 kinases, which phosphorylate the sites equivalent to human Thr-14 and Tyr-15 of CDK2. WEE1 and MYT1 activity is balanced by the dual-specificity phosphatase CDC25, which dephosphorylates both the Thr-14 and Tyr-15 residues [Kumagai and Dunphy, 1991]. WEE1 and CDC25 analogues have been identified in *Arabidopsis* [Landrieu et al., 2004; Sorrell et al., 2002].

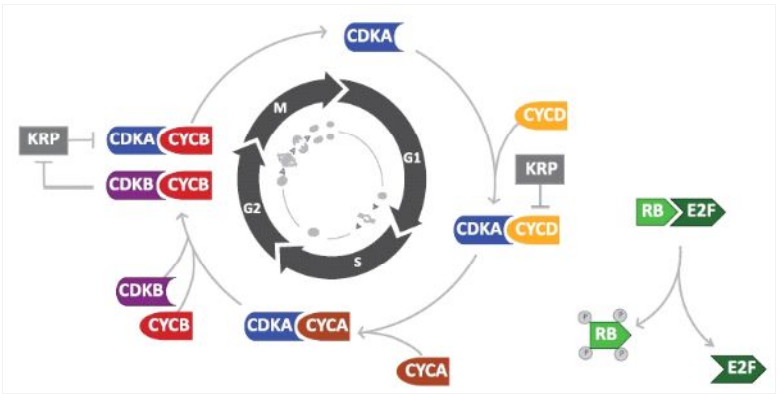

Fig. 2 Simplified view of the plant cell cycle, which requires several regulators [From: Scofield et al., 2014].

1.3 Cell cycle and E2F/RB pathway

During G1 to S transition, at a point called START in yeast and restriction point in mammals, cells either continue through the cell cycle or stop to differentiate. The response to various hormones, like as abscisic acid and auxin, is able to lead cells to a new division cycle [Gutierrez et al., 2002]. The regulatory pathways that control cell cycle progression are conserved in animal and higher plants and include the CycD/RB/E2F pathway, a major regulator of cell proliferation that plays a key role in the G1 to S transition. The D-type cyclins, associated with CDKs, interact with a tumour-suppressing protein, called retinoblastoma protein (RB), through a short LxCxE sequence (x = any aminoacid) contained near the CycD N-terminal region. Phosphorylation of RB is essential for regulating the activity of the E2F transcription factors, which are necessary for the transcriptional activation of a wide range of genes that are essential for the G1 and S phase progression of the cell cycle. These activating E2F factors act as heterodimers together with the Dimerisation Partner proteins (DPs). Beside their role in cell cycle progression, the E2Fs have been involved also in the control of other cellular processes.

1.4 The Retinoblastoma protein

The human RB, is a nuclear phosphoprotein of approximately 100 kDa and shares about 40% sequence identity with its counterpart in plants, called Retinoblastoma-related (RBR). Its structure shows three protease-resistant domains that are the N-terminal domain and the A- and B-domains, which form the "pocket domain" responsible for the interaction with the E2Fs [Hensey et al., 1994]. In plants, the RBRs have been first identified in maize [Grafi et al., 1996; Ach et al., 1997] and, subsequently, in many plant phyla. Arabidopsis contains only a single retinoblastoma-related gene (RBR1).

RB activity is regulated by phosphorylation/dephosphorylation events (figure 3). At the end of mitosis, the Ser/Thr Protein Phosphatases PP1 and PP2A are responsible for the activation of RBs by dephosphorylation. When hypophosphorylated, RBs bind the E2Fs through a so called "pocket domain" and block their activity. Subsequently to mitogenic stimuli, phosphorylation by the CDK-CYCD complexes dissociates RBs from the E2F-DP heterodimers, which are thus activated. Because the lack of RBR function in Arabidopsis is gametophytic lethal [Ebel et al., 2004; Johnston et al., 2008; Johnston and Gruissem, 2009], functional studies on the Retinoblastoma Related protein have been performed through virus-induced gene silencing [Park et al., 2005; Jordan et al., 2007] or RNAi experiments

[Wildwater et al., 2005; Borghi et al., 2010]. RBR is not involved only in the control of cell cycle progression but it acts also as a transcription regulator via epigenetic mechanisms [Gutzat et al., 2012]. Moreover, it has been reported that a local reduction of RBR expression in Arabidopsis roots, is connected to an increase of the stem cells population by preventing their differentiation in columella cells and in lateral root cap tissues [Wildwater et al., 2005]. On other hand, RBR overexpression in tobacco reduced the population of stem cells pool in shoot apical meristems [Wyrzykowska et al., 2006].

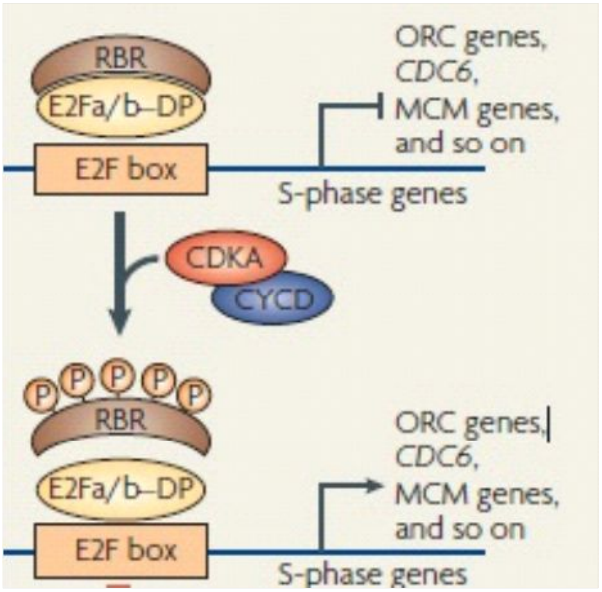

Fig. 3 Simplified view of the CyclinD/Retinoblastoma/E2F pathway [Adapted from: De Veylder et al., 2007].

1.5 The E2F transcription factors in plants

The E2Fs have been first described as transcription factors able to bind and activate the E2 promoter in Adenovirus [Kovesdi et al., 1986]. This family of transcription factors can activate a wide range of genes by binding a specific consensus sequence TTTSSCGSS (where S can be C or G).

Most of the information on E2F factors derives from studies on animal E2Fs since they have been identified first in mammals and only later in higher plants. As for plants, studies of promoters containing E2F consensus sites concerned the RNR1, RNR2 and PCNA promoters of tobacco and the CDC6 and MCM3 promoters of *Arabidopsis thaliana* [Chaboutè et al., 2000; Kosugi and Ohashi, 2002; De Jager et al., 2001].

E2F factors have been identified in several plant species like tobacco, wheat, carrot and Arabidopsis [Ramirez-Parra et al., 1999; Sekine et al., 1999; Albani et al., 2000; Magyar et al., 2000]. In particular, the Arabidopsis genome encodes six E2Fs (named *AtE2F*s) that can

be classified into two classes: typical and atypical E2Fs. The first group includes the AtE2Fa, AtE2Fb and AtE2Fc factors that possess all the canonical regions conserved in other typical E2Fs, organized similarly to the mammalian E2F1 to E2F5 factors [Shen, 2002]. All the typical E2Fs possess a DNA-binding domain closed to the N-terminus that is followed by a DP heterodimerization domain, a marked box and a transactivation domain, in the C-terminal region, that includes the retinoblastoma (RB) binding region. In Arabidopsis, the typical AtE2Fs are able to dimerize with two Dimerisation Partner proteins, called AtDPa and AtDPb. It has been shown that both AtE2Fa and AtE2Fb factors are able to transactivate a synthetic E2F-responsive promoter, confirming their ability to act as positive transcriptional regulators, whereas AtE2Fc has been shown to function as a transcriptional repressor, in association with the RBR1 protein [Mariconti et al., 2002; Del Pozo et al., 2002]. On other hand, the three atypical E2Fs of Arabidopsis, which are called also DP-E2F-Like (DEL), are AtE2Fd/DEL2, AtE2Fe/DEL1 and AtE2Ff/DEL3, and contain duplicated DNA-binding domains that enable these proteins to bind their *cis* elements without the formation of heterodimers with AtDP proteins [Lammens et al., 2009]. Moreover, the atypical E2F factors have no transactivating ability and are believed to be transcriptional repressors which are able to compete with the typical E2Fs for binding to the same consensus sites [Mariconti et al., 2002]. This feature is confirmed by studies in which the knockout of the atypical *E2F* genes leads to an overexpression of genes activated by typical E2Fs [Li et al., 2008; Ramirez-Parra et al., 2004]. Nevertheless, the atypical E2Fs lack the RBR binding domain, suggesting that they repress transcription independently from the RBR protein [Lammens et al., 2009].

Both AtE2Fa and AtE2Fb are regulated by CYCD3;1. It has been demonstrated that the CYCD3;1 overexpression results in an increase of cell proliferation, whereas its knockout compromises cell division [Dewitte et al., 2003; Dewitte et al., 2007]. When released from RBR upon phosphorylation of the retinoblastoma protein by CYCD3;1/CDKA;1 complex, the AtE2Fb factor is able to stimulate cell proliferation by activating genes involved in cell cycle progression.

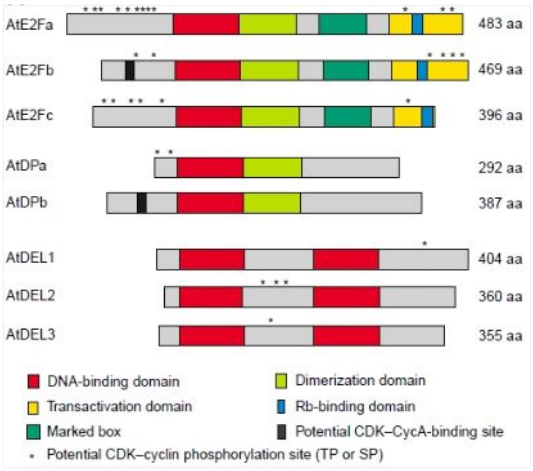

Fig. 4 Structural organization and DNA-binding properties of the Arabidopsis E2F factors. [From: Shen, 2002].

Further studies in Arabidopsis showed that the overexpression of the AtE2Fa factor is able to induce cell proliferation in normally differentiated tissues, such as in hypocotyls and cotyledons [De Veylder et al., 2002], and the AtE2Fa/AtDPa heterodimer is able to stimulate cell divisions, inducing protoplasts from mature leaves to re-enter S phase [Rossignol et al., 2002]. Arabidopsis plants overexpressing AtE2Fb showed a deeply modified morphological structure such as shorter primary roots, larger cotyledons and absence of trichomes in leaves [Sozzani et al., 2006].

The plant E2Fs are also controlling the endoreduplication (alternatively named endocycle), which is considered a modified cell cycle in which the chromosomes replicate several times without mitosis, giving rise to polyploid cells. Transgenic Arabidopsis plants overexpressing the AtE2Fa-DPa complex show either augmented ploidy levels, or ectopic cell division. This result indicates a likely involvement of AtE2Fa in the regulation of the endoreduplication [De Veylder et al., 2002]. It has been proposed a dual role of AtE2Fa which allows to control, separately, cell proliferation and endoreduplication. In proliferating cells, in fact, AtE2Fa forms a stable repressor complex with RBR1, necessary to inactivate the expression of genes responsible for endocycle onset. Upon dissociation from RBR1, through an unclear mechanism, AtE2Fa activates the endocycle in cells committed for differentiation [Magyar et al., 2012]. A role in the regulation of endocycle have been described also for AtE2Fc whose overexpression forces cells to endoreduplicate [del Pozo et al., 2006].

Recent studies showed how the AtE2Fs are involved in other processes as well, such as the Effector-Triggered Immunity (ETI), the first mechanism of defense from pathogens carried out by plants. During ETI, hyperphosphoryation of AtRBR through an unknown kinase results in over-activation of the typical AtE2Fs and effector-triggered PCD and disease resistance [Wang et al., 2014]. Furthermore, it has been demonstrated the importance of the plant E2Fs

in secondary wall synthesis. In *Arabidopsis*, in fact, AtE2Fc appears to be a regulator of VND7 (VASCULAR-RELATED NAC DOMAIN 7), which is involved in xylem vessels formation [Taylor-Teeples et al., 2015]. In addition, the atypical AtE2Fe/DEL1 of Arabidopsis has been reported as a regulator of the production of salicylic acid (SA), which is an activator of the immune response in plants [Chandran et al., 2014].

1.6 Synthetic promoters in plant biology

Synthetic promoters are nowadays widely used in scientific research. Plant promoters, thanks to their modular structure, can be easily manipulated so as to modify the architecture of their *cis*-acting elements. This *cis*-engineering allows to create unique combinations of regulatory promoter elements that can be positioned, in any order, upstream to the TATA box, the element to which the TATA box-Binding Protein (TBP) of the TFIID complex can associate to initiate the transcription in eukaryotes [Rushton et al., 2002]. The principal feature of the synthetic promoters is that they can provide patterns of expression that do not exist in nature. In the scientific literature, numerous strategies to create synthetic promoters have been reported and, thanks to bioinformatic tools, the design of the different combinations of *cis* elements has recently become more simple. One widely used approach is the intramolecular hybridization used to join important regulatory elements of a promoter to the core element of another one, thus giving rise to a chimeric synthetic promoter [Ranjan et al., 2011]. This strategy could be used also to ligate two different promoters, oriented in different direction, to create a bidirectional promoter. Further common approaches are used to mutate a promoter by adding and/or deleting repetitions of a regulatory element. For example, the DR5 promoter is a highly active synthetic promoter that contains repeats of the auxin responsive element and has been used to study auxin response in plants [Ulmasov et al., 1997]. Similarly, the CaMV35S promoter, which is commonly used to increase gene expression in transgenic plants, possesses a duplicated enhancer within a synthetic sequence [Guerineau et al., 1992].

A considerable number of synthetic promoters has now been devised and described in studies *in planta*, and may be classified considering their specific functional features. Several synthetic promoters have been used to evaluate the plant response to biotic and abiotic stresses, allowing the characterization of different *cis* elements involved in the activation of anti-pathogen genes [Pastuglia et al., 1997; Matton et al., 1993], or of genes required for adaptation to stress conditions such as temperature variations or altered accessibility to water. Other synthetic promoters have been essential in understanding hormone-responsive

processes [Ulmasov et al., 1997; Muller and Sheen, 2008; Zheng et al., 2011], whereas tissue-specific synthetic promoters have been designed to drive the expression of foreign genes in different tissues [Lam and Chua, 1991; Van der Meer et al., 1992].

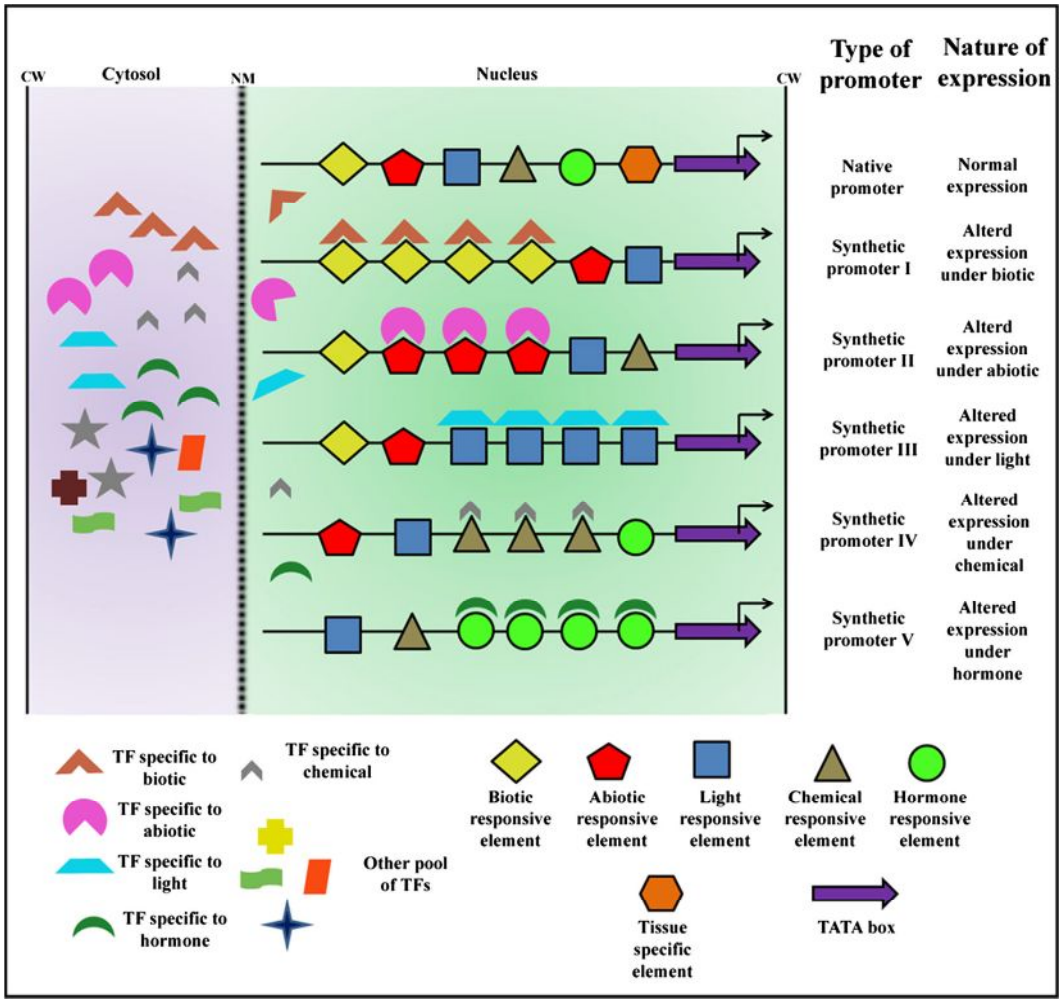

Fig. 5 Simplified representation of different plant synthetic promoter which are able to respond to different biotic and/or abiotic stimuli [From: Dey et al., 2015].

2. STATE OF THE ART AND AIMS OF RESEARCH

The purpose of this part of my research work was setting up a system which is expected to enable the visualization of the E2F-dependent transcriptional activation in *Arabidopsis thaliana* plants. Through the so called Cyclin D/E2F/Retinoblastoma pathway, the E2F transcription factors play a key role in the regulation of the expression of a wide range of genes that are essential for the G1 and S phase progression through the cell cycle. For this reason the E2F factors are fundamental in controlling cell proliferation as well as endoreduplication. However, in both plants and animals, the E2Fs have been shown to regulate other processes as well. The three typical E2Fs of Arabidopsis, named AtE2Fa, AtE2Fb, AtE2Fc, bind their *cis* elements together with the Dimerisation Partner proteins AtDPa and AtDPb, whereas three atypical E2Fs, called AtE2d, AtE2Fe and AtE2Ff, possess duplicated DNA-binding domains which allows DNA binding independently of an AtDP protein. Several studies have demonstrated that both AtE2Fa and AtE2Fb factors act as positive transcriptional regulators, whereas AtE2Fc has been shown to function as a transcriptional repressor in association with the RBR1 Retinoblstoma-related protein. The atypical E2Fs have no transactivating ability but are believed to act as repressors by competing with the activating E2Fs for the binding to the same *cis* elements. Moreover, the atypical E2F lack the RBR-binding domain, suggesting that they repress transcription independently from the RBR protein [Lammens et al., 2009].

Previous experiments of transient expression have demonstrated that a synthetic E2F-regulated promoter can be *trans*-activated by the typical AtE2Fs in plant protoplasts [Mariconti et al., 2002]. The aim of this part of my thesis was to evaluate *in planta* the activity and the regulation of this synthetic promoter, named E2F-Minimal-35S (EM35S), that is expected to be specifically activated by E2F factors also when stably integrated in the plant genome. The analysis of Arabidopsis plants transformed with a construct in which the EM35S promoter drives the GUS reporter gene can easily allow the identification of tissues in which strong E2F-dependent transcriptional activation is likely to occur. Moreover, these plants can be used to verify the cell cycle-dependent regulation of the EM35S promoter activity as well as its dependence on epigenetic mechanisms of regulation such as DNA methylation, histone acetylation or phosphorylation/dephosphorylation events.

3. MATERIALS AND METHODS

3.1 Plant material and plant transformation

For germination and growth in aseptic conditions, wild type or transgenic *Arabidopsis thaliana* ecotype Columbia seeds were surface sterilized for 8/10 hours in 2% v/v PPM® (Plant Preservative Mixture, Plant Cell Technology) supplemented with 50 mg/L magnesium salts ($MgSO_4$). Seeds were imbibed for 2 days in 0,1 % agarose at 4°C in the dark and then germinated on petri plates containing MS salts (Duchefa Biochemie), supplemented with Sucrose (10g/l) and Phyto agar (8g/l) (Duchefa Biochemie) and incubated in a growth cabinet at 22°C under long day conditions of 16 h of light and 8 h of dark .

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]. For transformation, a colony of *Agrobacterium* containing the recombinant plasmid has been picked up to inoculate 4 ml of YEP medium(Bactotryptone 10 g/L; Yeast extract 10 g/L; NaCl 10 g/L adjusted at pH 7 with NaOH) containing the selection agents Kanamycin 40 mg/l and Rifampicin 50 mg/l , which are specific for the plasmid and for the Agrobacterium strain respectively. The culture was incubated O/N at 28°C with gentle shaking and then used to inoculate 400 ml YEP medium. After a further incubation at 28°C O/N, the culture was ready to transform plants. Each culture was transferred into 50 ml conical tubes, centrifuged at 4000 rcf for 7 minutes, at 4°C and the liquid poured away leaving a pellet. Infiltration media was prepared as following 50 g/l sucrose, 400 µl/l silwet L-77, and kept cold. A small amount of infiltration media was first added to the tubes to resuspend the Agrobacterium cells and then the remaining was added up to 500 ml to perform the floral dipping. Plants were dipped into infiltration media for 45 secs, placed on their side in a plastic bucket for 24 hours and left at RT. The transformed plants have then been transferred in a growth chamber to grow to maturity.

Transformed T1 and progeny plants were selected on MS plates containing the resistance antibiotic (Kanamycin, 40 mg/l). At two weeks of age, the resistant plants were transferred to recovery plates and grown for one more week in aseptic conditions without the selection agent before transferring them to soil. Plants were grown to maturity in growth cabinets set at long day conditions of 16 h of light (22±3°C) and 8 h of dark (22±3°C), with 70% relative humidity.

3.2 Generation of EM35S construct

For the production of the E2F/Minimal -60 CaMV35S (EM35S) reporter construct, the plasmid pBI221.9/E2F [Albani et al., 2000] was first digested with *Nhe*I/*Eco*RI and the fragment cloned into the pBI221.9 vector digested with *Xba*I/*Eco*RI, replacing the -60 CaMV35S promoter/GUS/Nos Poly A region with the 10xE2F/-60 CaMV35S promoter/ GUS/ Nos Poly A fragment, thus giving rise to the pBI221.9/EM35S construct.

Secondly the pBI221.9/EM35S plasmid was digested with *Hind*III/*Eco*RI and the fragment (10xE2F/-60 CaMV35S promoter/ GUS/ Nos Poly A) was cloned into the pBI121 binary vector (digested with *Hind*III/*Eco*RI), suitable for *Agrobacterium*-mediated plant transformation, giving rise to the EM35S binary construct.

3.3 GUS assays

Histochemical detection of GUS activity was performed on transgenic plants using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) [Jefferson et al., 1987]. Plants at different developmental stages, or specific tissues, were incubated overnight at 37 °C in the GUS solution (50 mM pH 7 phosphate buffer, 1 mg/mL X-Gluc, 1 mM potassium ferricyanide). After staining, chlorophyll interference was removed treating the samples in 70% ethanol. For quantitative analyses, the level of GUS activity was detected fluorimetrically using the fluorogenic substrate MUG (4-methyl umbelliferil–glucuronide). Seedlings of the same developmental stage were ground in GUS extraction buffer (50 mM $NaPO_4$ pH 7, 10 mM EDTA, 0.1% Triton, 0.1% Sodium Lauryl Sarcosine, 10 mM β-Mercaptoethanol). An aliquot of 44 µl of the extracts was added to 396 µl of assay buffer (50 mM $NaPO_4$ pH 7, 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 four different time points, 100 µl of the reaction mix were added to 900 µl of stop buffer (0.2 M Na_2CO_3) and the amount of 4MU produced was measured using a fluorimeter (BioRad). The protein concentration of each extract was assayed using the Bradford method [Bradford, 1976] to allow calculation of the specific GUS activities.

3.4 Treatments with inhibitors of: DNA methylation, HDACs, phosphatases and kinases

The following inhibitors have been used for these experiments:

- Genistein (Sigma-Aldrich), which inhibits the DNA-methyltransferases
- Trichostatin A (Sigma-Aldrich), a histone deacetylases (HDACs) inhibitor
- Okadaic acid (Santa Cruz Biotechnology), a phosphatases inhibitor
- Staurosporine (Santa Cruz Biotechnology), a kinases inhibitor

The treatments have been performed with seedlings grown on MS/sucrose/Phyto agar plates for 10 days in growth chamber at 22 °C under a regimen of 16 h of light at and 8 h of dark. Three seedlings per treatment were then transferred, in a 24-multi well plate, to 1 ml MS/sucrose liquid medium supplemented with the inhibitors at final concentrations of 50μM Genistein, 1μg/ml Trichostatin A, 100 nM Okadaic acid or 50 μM Staurosporine. As untreated control, seedlings have been transferred to liquid MS/sucrose medium addictioned with DMSO as the inhibitors are solubilized in this solvent. The treatments have been performed in growth chamber set at the same conditions. After 24 h of treatment protein were separately extracted from aerial parts and roots and fluorimetric assays of GUS activity were performed. Histochemical GUS assays have been performed on 10 days-old treated and untreated seedlings. All the treatments have been performed in triplicate.

3.5 Treatments with cell cycle inhibitors

To perform the treatments with cell cycle inhibitors, 30 seeds of selected homozygous transgenic type A lines were imbibed in sterile water alone (as control) or in water containing 5 μg/ml aphidicolin (Fisher Scientific) or 5 mg/ml colchicine (Apollo Scientific). After 72 h of imbibition in growth chamber at 22 °C under a regimen of 16 h of light at and 8 h of dark, proteins were extracted and fluorimetric assays of GUS activity were performed. The treatments have been carried out in a biological triplicate.

3.6 qRT-PCR analyses on seedlings treated with Trichostatin A

WT Arabidopsis seedlings were grown for 10 days on MS/sucrose/Phyto agar plates in growth chamber set a at 22 °C under a regimen of 16 h of light at and 8 h of dark. Thereafter, 20 seedlings were transferred for 24 hours on MS/sucrose/Phyto agar plates supplemented with 1μg/ml Trichostatin A. As untreated control 20 seedlings were transferred for 24 hours on MS/sucrose/Phyto agar plates supplemented with DMSO. Total RNA was extracted from the

root and shoot apical regions using the Qiagen RNeasy mini-kit. The RNA samples were digested with DNase I during the extraction using the Qiagen RNase-free DNase set.

RNA concentration and quality were evaluated by spectrophotometry using A260/A280 ratio and by electrophoresis on denaturing formaldehyde gel. For qPCR analyses, 1µg of RNA has been reverse transcribed using the Qiagen QuantiTect Reverse Transcription Kit with a combination of hexamers and oligo dT primers. Quantitative real-time PCR was performed on the Qiagen Rotor-Gene® Q, using the BioRad iTaq™ Universal SYBR® Green Supermix kit. Triplicate PCR reactions were performed following the manufacturer's recommended amplification conditions. For all the analyses, the amplification of Actin transcripts has been used as a reference for normalization. Quantification was calculated by comparative quantitation using the Rotor-Gene® Q analysis software. The PCR primers were designed using the Primer3 online software (http://primer3.ut.ee/) and all their sequences are detailed in Table 1.

Table 1 List of the primers used for qRT-PCR analyses.

Name	5'-3'Sequence
AtE2FA-F	TGATAGCCGTCAAAGCTCCT
AtE2FA-R	TCGATGTCATGGTGTCCTGT
AtE2FB-F	AAGCACCGAAAGAAACATGG
AtE2FB-R	GTTTGTGGCTGCTCCAAGAT
AtE2FC-F	GAGTCTCCCACGGTTTCAGA
AtE2FC-R	CTTGTTTCCGCACTGTCTCC
AtE2FD-F	CTCACCATCTCCCAGACCTG
AtE2FD-R	GCAATGTCGTAAAGGCGTCT
AtE2FE-F	CACACTGAGCAGCGATTTGT
AtE2FE-R	CCTGGTGCAAAAGGTCCAAA
AtE2FF-F	GGAATCGAAACCAGCTGCAA
AtE2FF-R	CCATTTCTTCCATGCCTCCG
GUS-F	AAGCGTGGTGATGTGGAGTA
GUS-R	GTTCAGGCACAGCACATCAA
Actin-F	ACATTGTGCTCAGTGGTGGA
Actin-R	CTGAGGGAAGCAAGAATGGA

4. RESULTS

4.1 The EM35S promoter shows two distinct patterns of activity

In the second part of this thesis, Arabidopsis plants have been stably transformed with a GUS reporter construct controlled by a E2F-regulated synthetic promoter, named E2F Minimal-35S (EM35S), in which repetitions of a canonical E2F binding site are placed upstream to a minimal –60 CaMV35S promoter. The same promoter construct has been already tested in transient-expression experiments in plant protoplasts but its sequence had not been determined. Sequencing was performed before assembling the binary vector and revealed that the EM35S promoter contains 10 repetitions of the canonical E2F binding sites TTTCGCGC. The sequence of the 10XE2F fragment is reported in figure 6, which shows the schematic representation of the construct. The transient-expression experiments have already demonstrated that this promoter can be *trans*-activated by the typical E2F transcription factors [Mariconti et al., 2002].

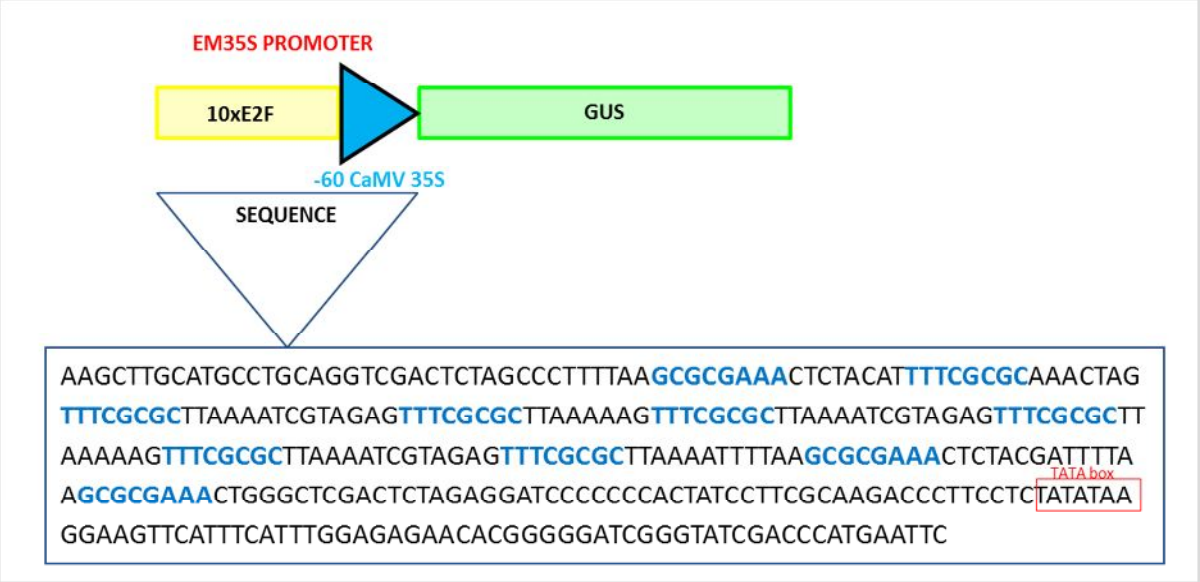

Fig. 6 Schematic representation of the EM35S reporter construct and sequence of the synthetic promoter. The ten repetitions of the E2F consensus elements are highlighted in blue, whereas the TATA box of the minimal -60 CaMV35S promoter is boxed in red.

Following *Agrobacterium*-mediated floral transformation, 18 transgenic EM35S Arabidopsis T1 lines were selected and grown to maturity. Surprisingly, histochemical analyses of the GUS reporter activity performed on one- and two-week-old seedlings of the T2 progeny revealed two distinct patterns of activity of the synthetic promoter. In 9 of the transgenic lines (50%), named type A lines, strong GUS activity was localized in the root apical meristems

(figure 7, A and B), a pattern predictable for an E2F-activated promoter, and weak staining could be seen in the root vascular cylinder. However, no evidence could be found of promoter activity in aerial parts, including the shoot apical meristem. The strong GUS staining detected in the type A lines could be observed also in the lateral root primordia of two-week-old seedlings (figure 7B, inset B1). The pattern of activity of the EM35S promoter, in type A lines, is consistent with an E2F-dependent transcriptional activation in proliferating cells, presumably driven by the typical AtE2Fa and AtE2Fb factors [Mariconti et al., 2002; De Veylder et al., 2007].

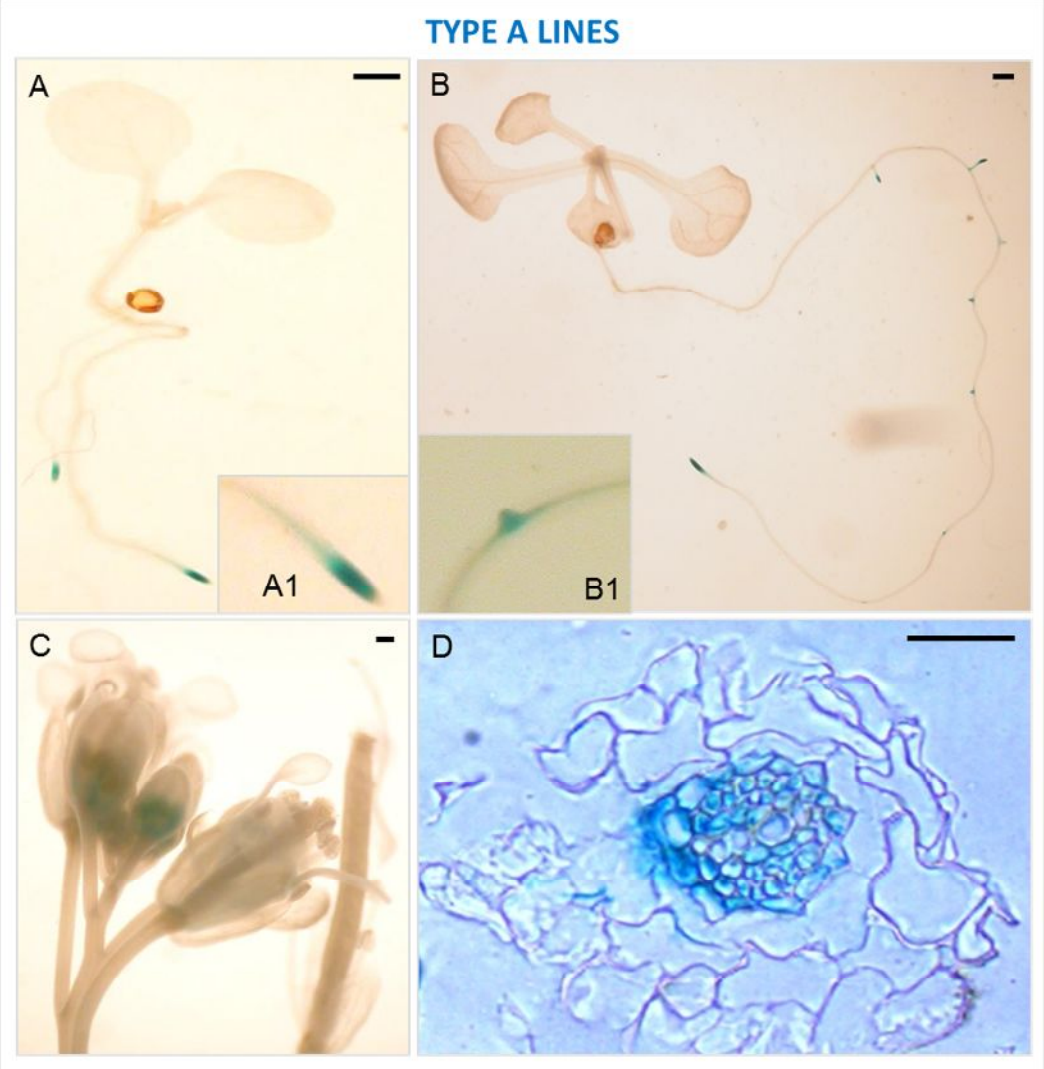

Fig. 7 Localization of GUS activity in type A lines: one-week-old (**A**) and two-week-old (**B**) seedlings, which show a strong activity in the root apical meristems (inset **A1**) as well as in the lateral root primordia (inset **B1**). GUS staining has been observed also in the developing stamens of young floral buds (**C**). As for the activity detected in the root vascular tissue, transverse root sections highlighted GUS localization also in the pericycle cells (**D**). Scale bars: 1 mm in **A**, **B** and **C**; 250 µm in **D**.

On the other hand, in the remaining 50% of the transgenic lines, named type B lines, the EM35S promoter does not appear to be active in the meristematic cells. The GUS activity, instead, was detected strongly in cotyledons (figure 8, A and B), in which the early growth mainly involves cell enlargement rather than cell proliferation.

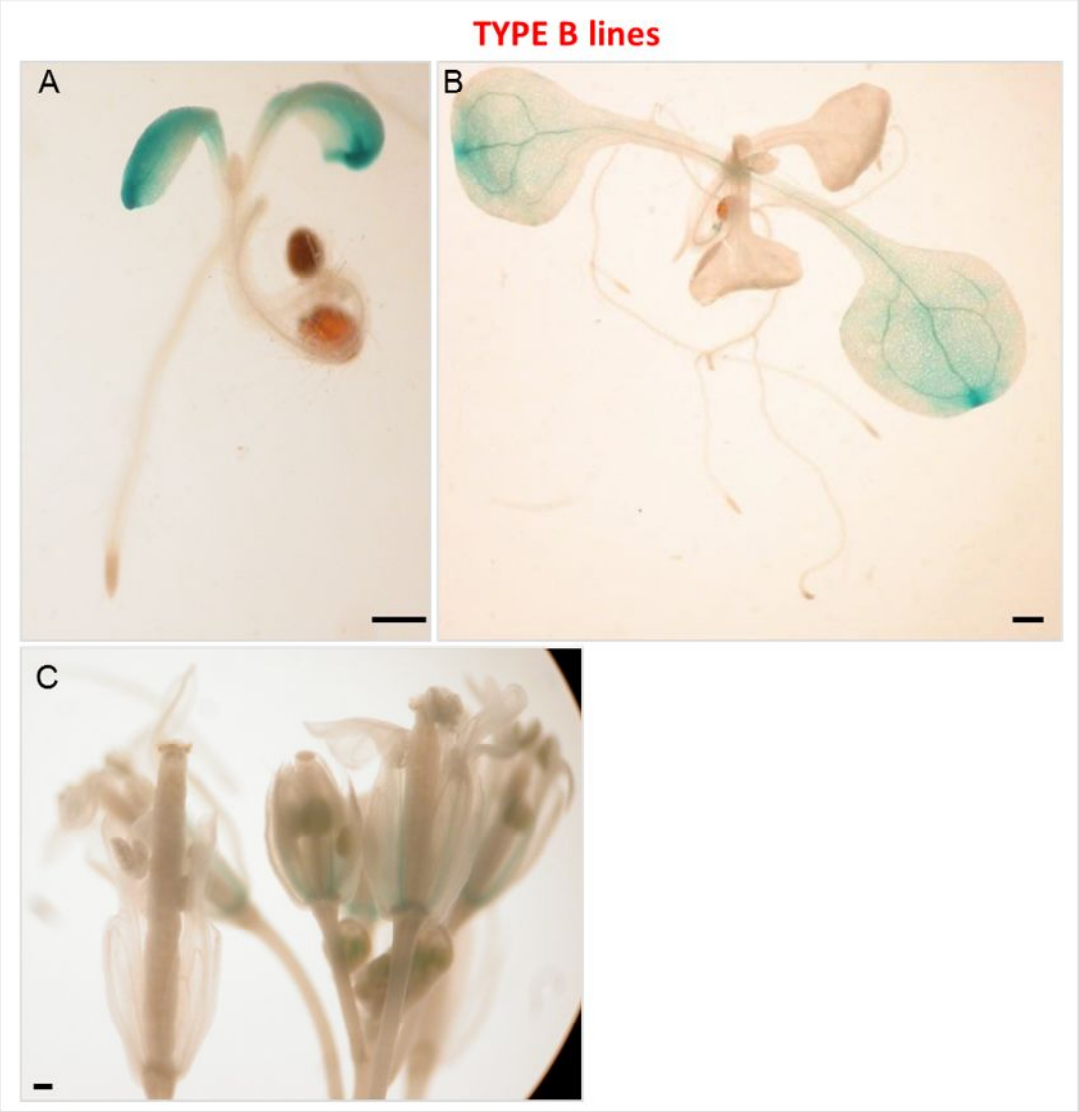

Fig. 8 Localization of GUS activity in type B lines: one-week-old (**A**) and two-week-old (**B**) seedlings, in which GUS expression is detected only in the vascular tissue of cotyledons, with a stronger accumulation of GUS staining in the cotyledons tips. In the inflorescence (**C**), a weak activity has been observed in the vascular tissue of petals. Scale bars: 1 mm.

Additional histochemical analyses, performed on the inflorescences of mature T2 plants, allowed the detection of GUS activity in the developing stamens of immature flowers of type A lines (figure 7D), whereas a weak GUS staining could be observed in the vascular tissue of the petals in type B lines (figure 8C). Further analyses on root sections of type A lines,

allowed to better describe the profile of activity of the EM35S promoter in the root vascular tissue. As shown in figure 7D, GUS staining appears to be localized in the floematic sieve elements and in pericycle cells as well. To verify whether the two patterns described are linked to the presence of multiple copies of the transgene in the lines analyzed, qPCR analyses have been performed to determinate the transgene copy number in each line. As shown in figure 9, the type A lines possess predominantly less copies of the transgene, whereas the type B are mostly characterized by a high number of copies. Nevertheless, exceptions are observed in the type A line #22, showing the highest copy number, as well as in the type B lines #12 and #18, which suggest that the correlation between the transgene copy number and the two patterns observed is only partial.

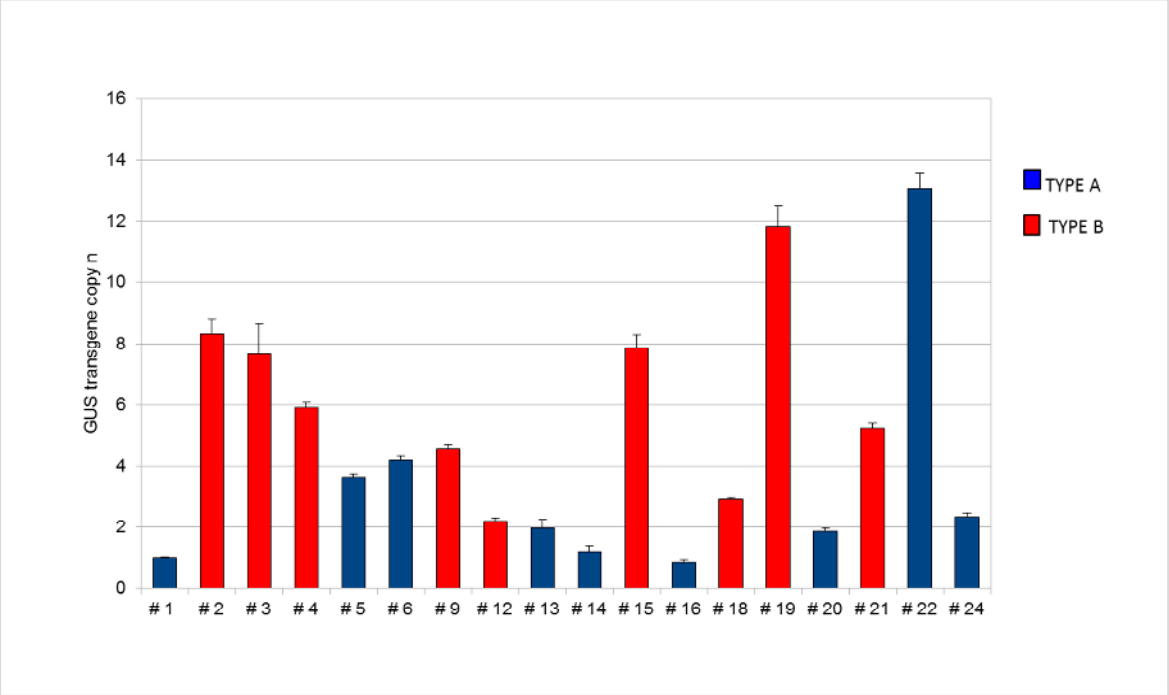

Fig. 9 Quantification by qPCR of the GUS transgene copy number performed on gDNA extracts, obtained from the 18 EM35S lines studied. The quantification was normalized to the *AtE2Fe* gDNA amount. Bars show standard errors.

4.2 Role of epigenetic mechanisms on the control of EM35S promoter activity

Considering that only 50% of the lines showed a clear activity of the EM35S promoter in root meristems, predictable for a E2F-activated promoter, analyses were carried out to verify whether the unexpected pattern observed in type B lines, could reflect an inactivation of the EM35S promoter in the cells of the RAM of these transgenic lines. In this respect, it was possible that the synthetic promoter could be epigenetically silenced. In fact, the E2F *cis*-element (TTTCGCGC) contains CpG dinucleotides and it is possible that the 10 repetitions of this element could be a *hot spot* for DNA methylation, which is known to be associated to transcriptional repression. Moreover, several studies in mammalian cells have implicated a role of histone deacetylases (HDACs) in the repression of E2F-regulated promoters [Brehm et al., 1998; Ferreira et al., 1998]. Thus, to verify the influence of epigenetic mechanisms, the role of DNA methylation as well as the possible effects of histone acetylation on the activation of the EM35S promoter were investigated.

To verify the effects of DNA methylation, 10 days-old seedlings of two homozygous type A and B lines were treated with Genistein (GEN), an inhibitor of cytosine methylation that is known to hinder the activity of the methyl-transferase MET1, the enzyme responsible for the maintenance of DNA-methylation in plants [Arase et al., 2012]. Moreover, to analyze the role of histone acetylation, the same transgenic lines were treated with Trichostatin A (TSA), a widely used inhibitor of histone deacetylases (HDACs) whose effects are rapid and position-independent [Yoshida et al., 1990; Xu et al., 2005]. After 24 hours of treatment, GUS histochemical assays performed on whole treated and untreated seedlings showed that, in both type A and B lines, inhibition of DNA methylation or histone deacetylation did not alter the spatial pattern of activity of the EM35S promoter (figure 10, A to C and figure 11, A to C). These results suggest that epigenetic mechanisms are not responsible for the dual pattern of activity of the promoter but do not exclude possible effects of DNA-methylation or histone deacetylation on its activity in different plant organs. To better verify this possibility, GUS fluorimetric analyses of the lines treated for 24 hours with the two inhibitors were carried out to quantify the level of activity of the EM35S promoter. Following the treatments, proteins were separately extracted from the roots and from the aerial parts (which include the shoot apical meristem, the cotyledons, the first couple of leaves and the hypocotyl) and GUS fluorimetric analyses, comparing treated and untreated seedlings, were carried out. As revealed in figures 10 and 11, the activity of the EM35S promoter appears to be partly under epigenetic control.

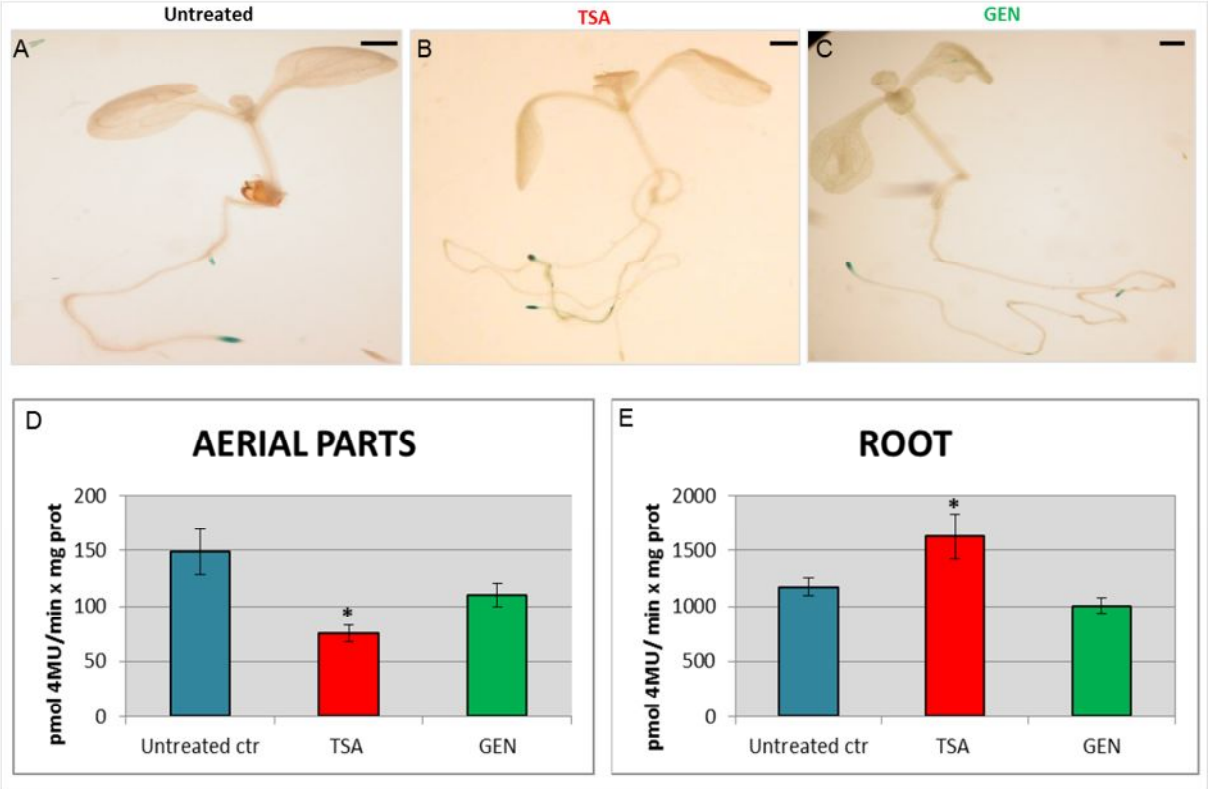

Fig. 10 GUS histochemical analyses performed on ten days old type A seedlings, untreated (**A**), treated with Trichostatin A (**B**) and geinistein (**C**). Scale bars: 1 mm.
Results of the fluorimetric analysis of GUS activity, carried out on extracts obtained from the aerial parts (**D**) and root (**E**) of the ten days old type A seedlings, untreated and treated with the two inhibitors. The bars show standard deviations. *p<0.05.

The experiment carried out with the type A line revealed that the TSA treatment decreased the activity of the EM35S promoter in the aerial parts (figure 9D), whereas increased activity was observed in the roots (figure 9E). The Genistein treatment, instead, had no significant effects. The variation of GUS activity observed in the type A line after TSA treatment suggests that histone hyperacetylation of the EM35S promoter in the meristematic cells of the root may directly affect its activity. However, indirect affects cannot be excluded because it is also possible that the TSA treatment can increase the expression of activating E2Fs in the root apical meristems. In the aerial parts the results were different. Here the EM35S promoter confers very low levels of GUS expression but its activity is further decreased by the TSA treatment (figure 9D). Because HDACs inhibition can lead to a transcriptional activation of silenced deacetylated promoters, the decreased activity of the EM35S promoter in the aerial parts of the A type line observed after TSA treatment suggests that histone hyperacetylation cannot have direct effects on the activity of the promoter, but is rather increasing, possibly in

the shoot apical meristem, the expression of repressing E2F factors that can downregulate the EM35S promoter.

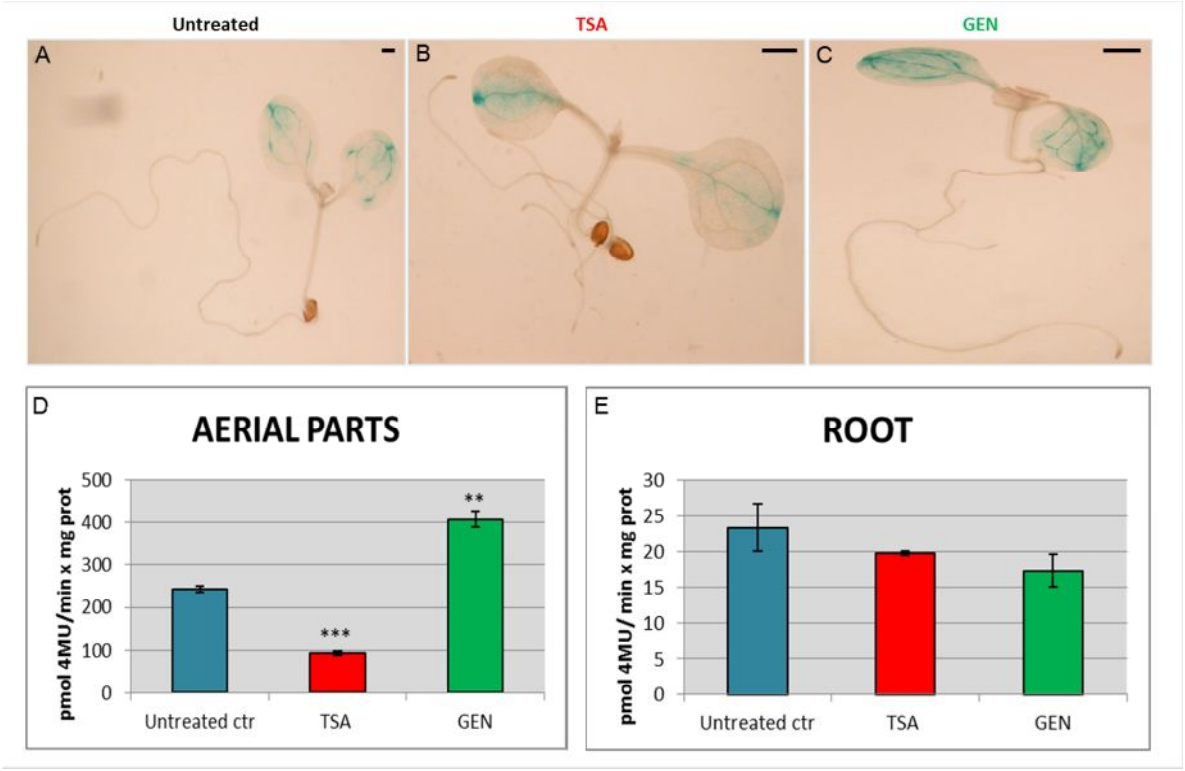

Fig. 11 GUS histochemical analyses performed on ten days old type B seedlings, untreated (**A**), treated with Trichostatin A (**B**) and geinistein (**C**). Scale bars: 1 mm.
Results of the fluorimetric analysis of GUS activity, carried out on extracts obtained from the aerial parts (**D**) and root (**E**) of the ten days old type B seedlings, untreated and treated with the two inhibitors. The bars show standard deviations. **p<0.01, ***p<0.001.

Concerning the type B line, which show strong activity of the EM35S promoter in the cotyledons, significant variations of the promoter activity after the treatment with either Genistein and TSA were observed only in the aerial parts of the plants (figure 11D), whereas the background activity of the EM35S promoter in the roots was not affected (figure 11E). In the aerial parts, Genistein treatment produced a relevant increase of the GUS activity but, as in the type A line, decreased promoter activity could be detected after treatment with TSA (figure 11D). The effects of Genistein suggests that, in the aerial parts of type B line, the EM35S promoter may be downregulated by DNA methylation and, similarly to what observed also in the type A plants, its activity is clearly not affected directly by histone acetylation. In fact, also in the aerial parts of the type B line, the histone hyperacetylation caused by TSA treatment may activate the expression of repressing factors, underlining an indirect control of the EM35S promoter.

To verify whether the TSA treatments can actually alter the transcription of *AtE2F* genes in

Arabidopsis plants, possibly increasing the expression of repressing *AtE2Fs* in the aerial parts, qRT-PCR analyses were performed with RNA samples isolated from both the shoot and root apical regions of seedlings treated for 24 hours with the HDACs inhibitor. As shown in figure 12, compared to the untreated samples, the expression of all the three atypical *AtE2Fs* increased significantly in the shoot apices of TSA treated plants, whereas in the root apical regions only *AtE2Fb* appears to be upregulated after the TSA treatment. These results confirm that the downregulation of the EM35S promoter associated to histone hyperacetylation in the aerial parts, including the shoot apical meristems, is likely linked to the upregulation of repressing atypical AtE2Fs.

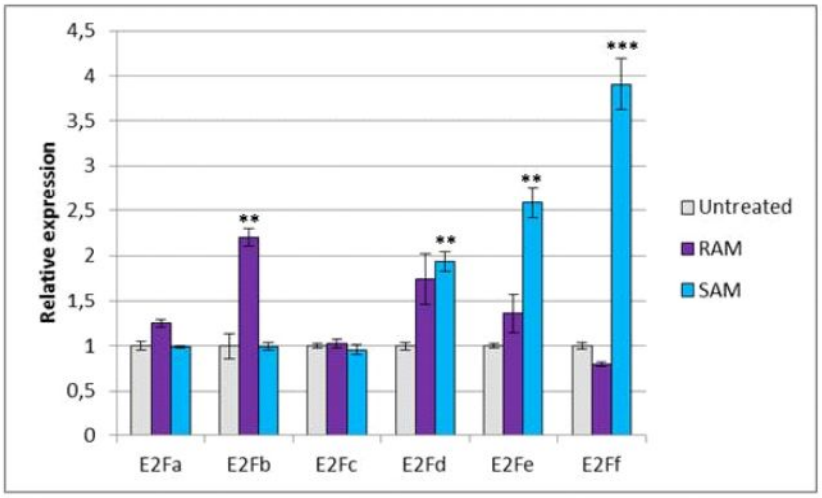

Fig. 12 qRT-PCR analysis of the *AtE2F* genes expression, in response to the TSA treatment.
The analysis has been carried out on RNA samples obtained from RAM and SAM of WT seedlings treated with TSA for 24 h, comparing the expression levels of the *AtE2F* genes to untreated controls. The quantification was normalized to Actin RNA levels. The bars show standard errors. ** $p<0.01$, ***$p<0.001$.

4.3 Phosphorylation/dephosphorylation events affect differently the EM35S promoter activity in the two types of lines

The activity of the typical E2Fs, which are expected to activate the EM35S promoter, is controlled by their interaction with the RBR protein. In the RB/E2F pathway the phosphorylation and dephosphorylation of the RBR protein are key events involved in the regulation of the activating E2Fs. Thus, to confirm that the EM35S promoter activity can be activated by AtE2Fa or AtE2Fb, analyses of the effects of phosphatase or kinase inhibitors have been performed. To this purpose, ten-day-old seedlings of two selected homozygous type A and B lines were treated with Okadaic acid (OKAD), which is known to inhibits phosphatases involved in RBR regulation, and with Staurosporine (STAU), which is a kinase inhibitor commonly used in cell cycle studies. After 24 hours of treatment with the two inhibitors, the EM35S promoter activity in the root of the type A line and in the aerial parts of type B line, the organs in which the EM35S promoter shows a strong activation, was quantified by fluorimetric GUS analyses, comparing treated and untreated seedlings. Also histochemical GUS assay were carried out on both treated and untreated seedlings. As shown in figures 13 (A to C) and 14 (A to C), the treatment with either inhibitors did not alter the spatial pattern of the EM35S promoter, whose activity remained confined in the root apices of the type A line and in the cotyledons of the type B line. Interestingly, however, the fluorimetric analysis shown in figure 12D revealed that treatment with either Okadaic acid or Staurosporine increased dramatically the activity of the EM35S promoter in the root of the type A line compared to untreated controls. The increased activity associated to Okadaic acid agrees with an E2F-dependent regulation of the EM35S promoter because the inhibition of phosphatases is expected to lead to a hyperphosphorylated state of the RBR protein, which allows the release of activating E2Fs and upregulation of the synthetic promoter.

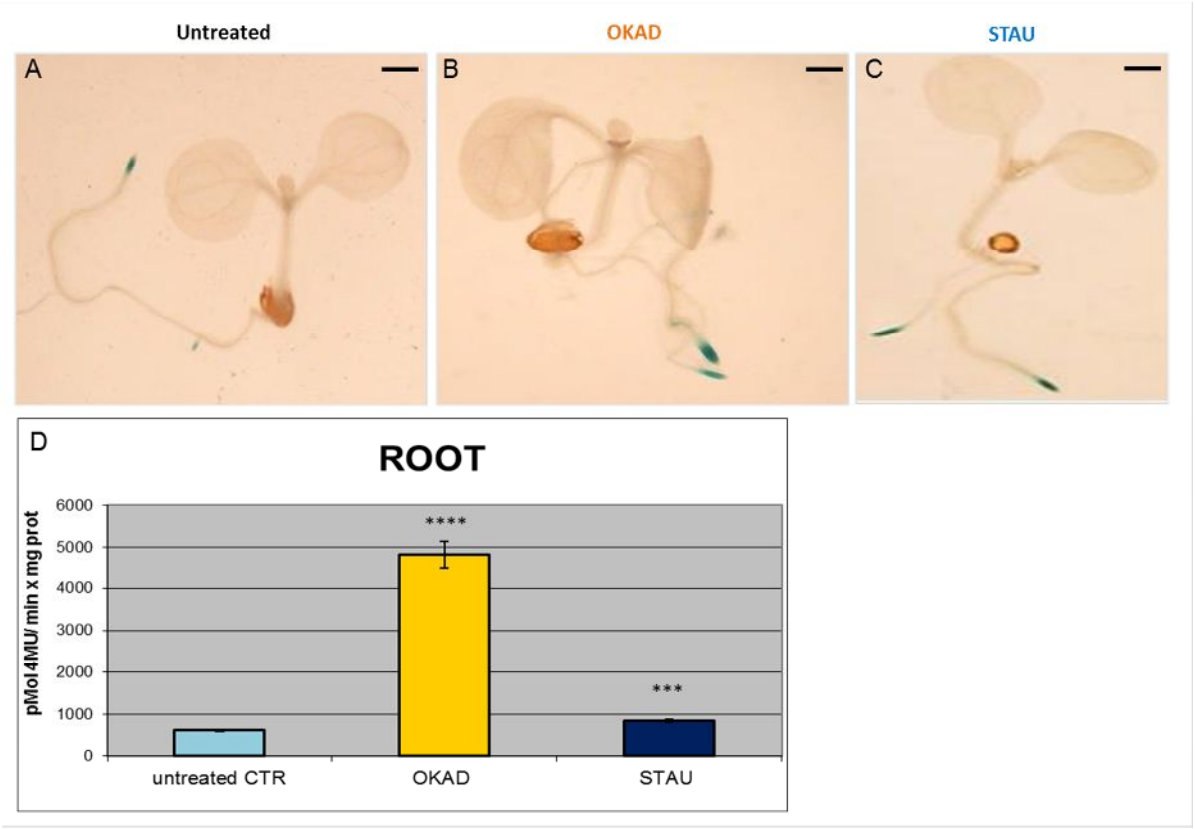

Fig. 13 GUS histochemical analyses performed on ten days old type A seedlings, untreated (**A**), treated with Okadaic acid (**B**) and Staurosporine (**C**). Scale bars: 1 mm.
Results of the fluorimetric analysis of GUS activity, carried out on extracts obtained from root of the ten days old type A seedlings (**D**), untreated and treated with the two inhibitors. The bars show standard deviations. ***p<0.001, ****p<0.0001.

On other hand, an opposite effect on the E2F-activated promoter should be obtained upon treatment with Staurosporine which, by blocking the kinases, should maintain RBR in an hypophosphorylated form that can repress the E2F factors. This results is not clear but a possible explanation of this discrepancy could be a direct and dominant negative regulation of the activating E2Fs by phosphorylation. Hampering this inhibitory phosphorylation with the kinase inhibitor would increase the activity of at least some of the activating E2Fs even if the RBR protein remains in its hypophopshorylated form. Therefore, the possible inhibitory phosphorylation of the activating E2F factors may have a stronger effect on the EM35S promoter activity than the RBR-dependent negative regulation.

A partially different situation was observed in the aerial parts of the type B line studied (figure 14D). In this case, the EM35S promoter activity appears to be affected only by the treatment with Staurosporine which, as in the case of the line A roots, yielded increased activity whereas the Okadaic acid treatment did not determine any effect. The latter result is of considerable

importance because it suggests a possible explanation for the EM35S promoter behavior in the type B lines. In fact, if also in these lines the EM35S promoter were effectively regulated by activating E2Fs, treating the seedlings with a phosphatases inhibitor leading to hyperphosporylation of the RBR protein would be expected to increase its activity, similarly to what observed in the type A line roots. Thus, the absence of a relevant effect of Okadaic acid on the activity of the synthetic promoter in the type B lines suggests that an RBR-dependent regulation is unlikely to occur in these plants. In this respect, it is plausible that the activation of the EM35S promoter in the type B lines may not be caused by E2Fs but could be mediated by a different class of transcription factors.

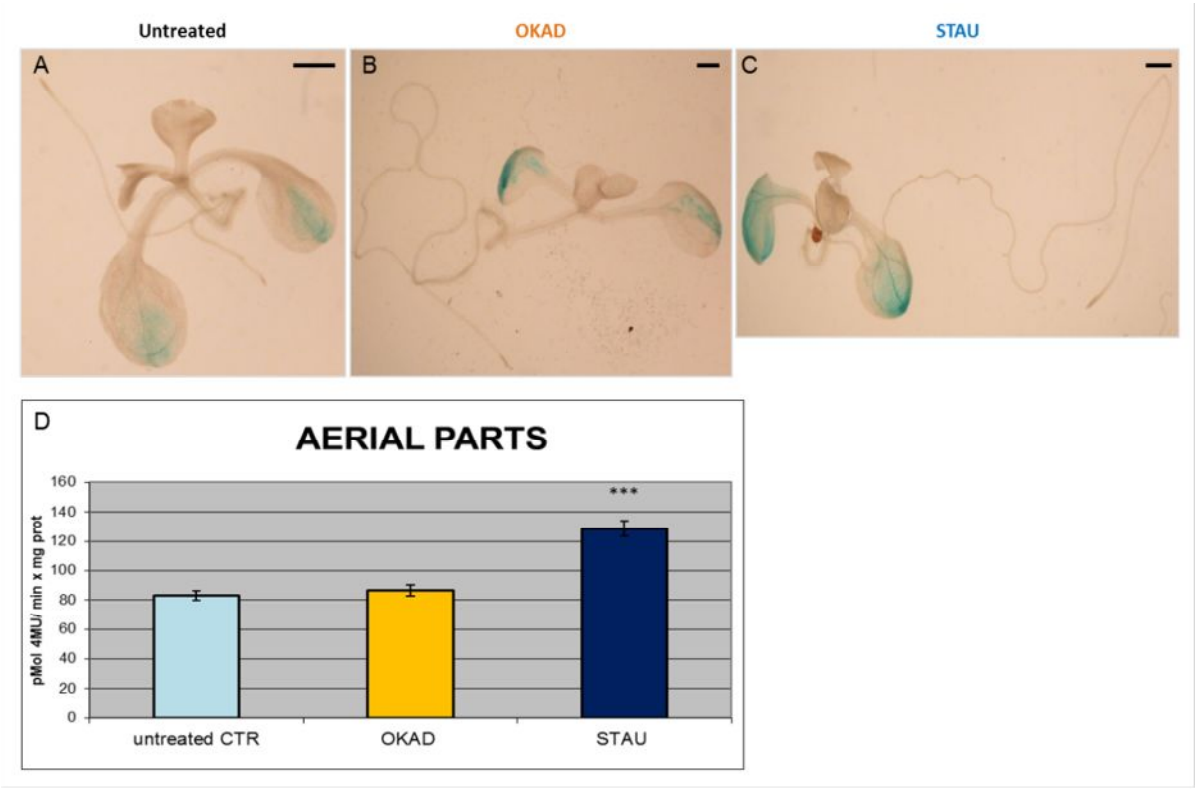

Fig. 14 GUS histochemical analyses performed on ten days old type B seedlings, untreated (**A**), treated with Okadaic acid (**B**) and Staurosporine (**C**). Scale bars: 1 mm.
Results of the fluorimetric analysis of GUS activity, carried out on extracts obtained from aerial parts of the ten days old type B seedlings (**D**), untreated and treated with the two inhibitors. The bars show standard deviations. ***$p<0.001$.

4.4 The constitutive expression of an exogenous activating E2F reveals that only type A lines are E2F-responsive

Because the experiments with phosphatase and kinase inhibitors revealed that the EM35S promoter could be under the control of activating E2Fs only in the type A lines, studies have been carried out to verify whether the constitutive expression of an exogenous activating E2F, such as the carrot DcE2F factor which has been reported as a strong *trans* activator [Albani et al., 2000], can increase the GUS activity in these lines but not in the type B ones. For this analysis, homozygous type A and type B lines were stably transformed with a recombinant construct carrying the carrot *DcE2F* cDNA sequence under the control of the double 35S promoter [Perrotta, 2012], which is able to give rise to a constitutive expression of the DcE2F transcripts. Histochemical GUS analyses of the double transformants derived from the type A line revealed a remarkable change in the pattern of the EM35S promoter activity in 16 of the 18 double transgenic lines. As shown in figures 15A and 15B, in these plants strong GUS activity was not anymore confined to the root apex, as in the original type A line, but extends to the shoot apical meristem and to the vascular tissues of roots, leaves and cotyledons, with a particularly strong staining in the cotyledonary veins.

The extended spatial pattern observed after transformation with the DcE2F construct confirms that the activity of the synthetic promoter is regulated by E2F factors in the type A lines and demonstrates that, in vascular cells as well as in the proliferating cells of the SAM, the EM35S promoter is fully responsive to an exogenous activating E2F factors.

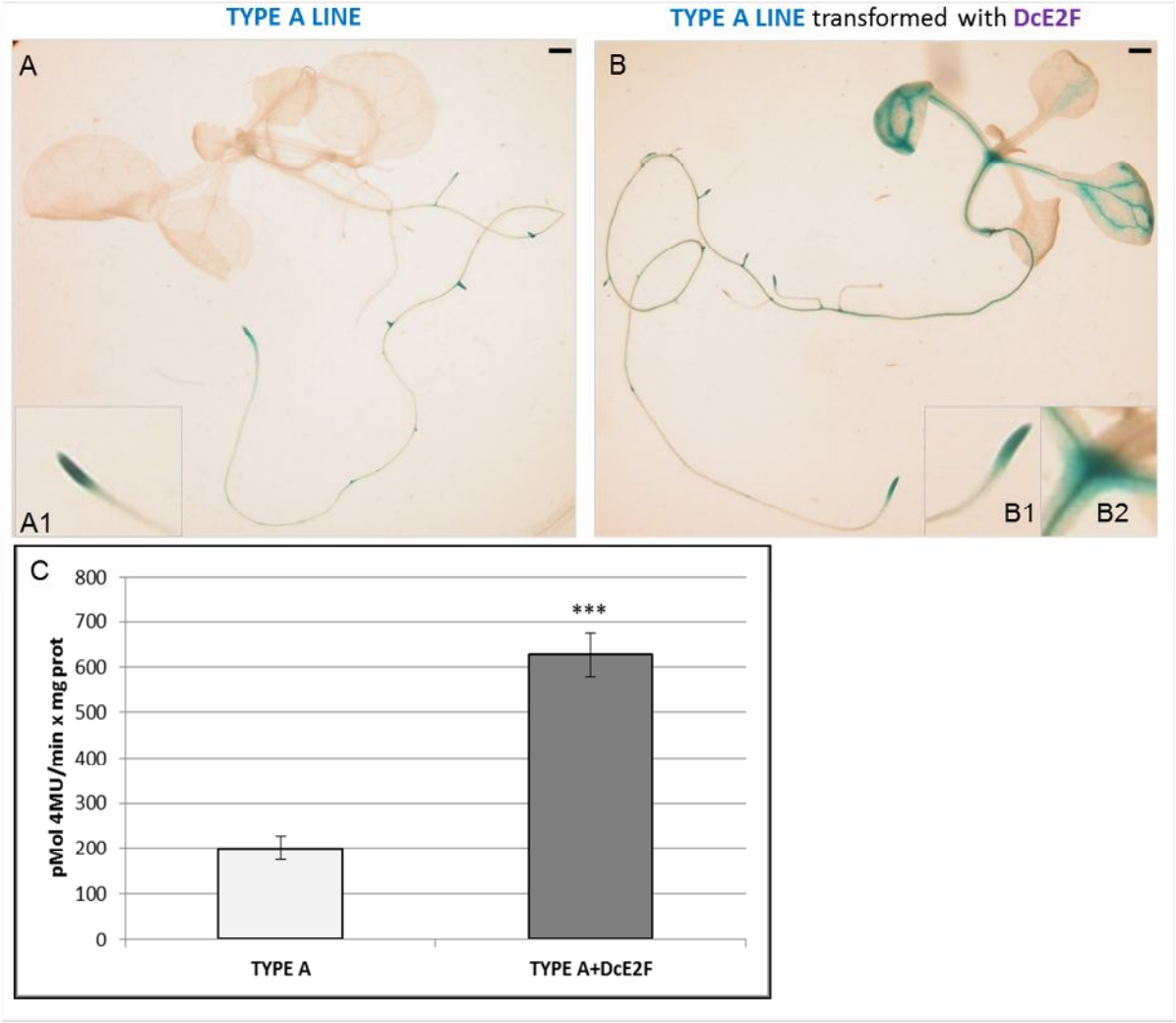

Fig. 15 Localization of GUS activity in two-week-old seedlings of: (**A**) type A lines; (**B**) type A re-transformed with the DcE2F. The constitutive expression of the DcE2F drastically alters the spatial pattern of activity of the EM35S promoter, which is not anymore confined only to the root apex, as in the original line (inset **A1**), but it is strongly active in both root and shoot meristematic cells (insets **B1** and **B2**) and in the vascular tissue of root and cotyledons. Scale bars: 1 mm.
(**C**) Results of the fluorimetric analysis of GUS activity, carried out on extracts obtained from two distinct pools of type A lines and type A lines constitutively expressing the DcE2F factor. The bars show standard deviations. ***$p<0.001$.

On the contrary, as shown in figures 16A and 16B, none of the 14 double transformants derived from the homozygous type B line revealed significant changes in the pattern of GUS staining. These results further indicate that in the type B lines the synthetic promoter is unlikely to be regulated by E2F factors and other unknown transcription factors could be involved in its activation in the aerial parts of the plants.

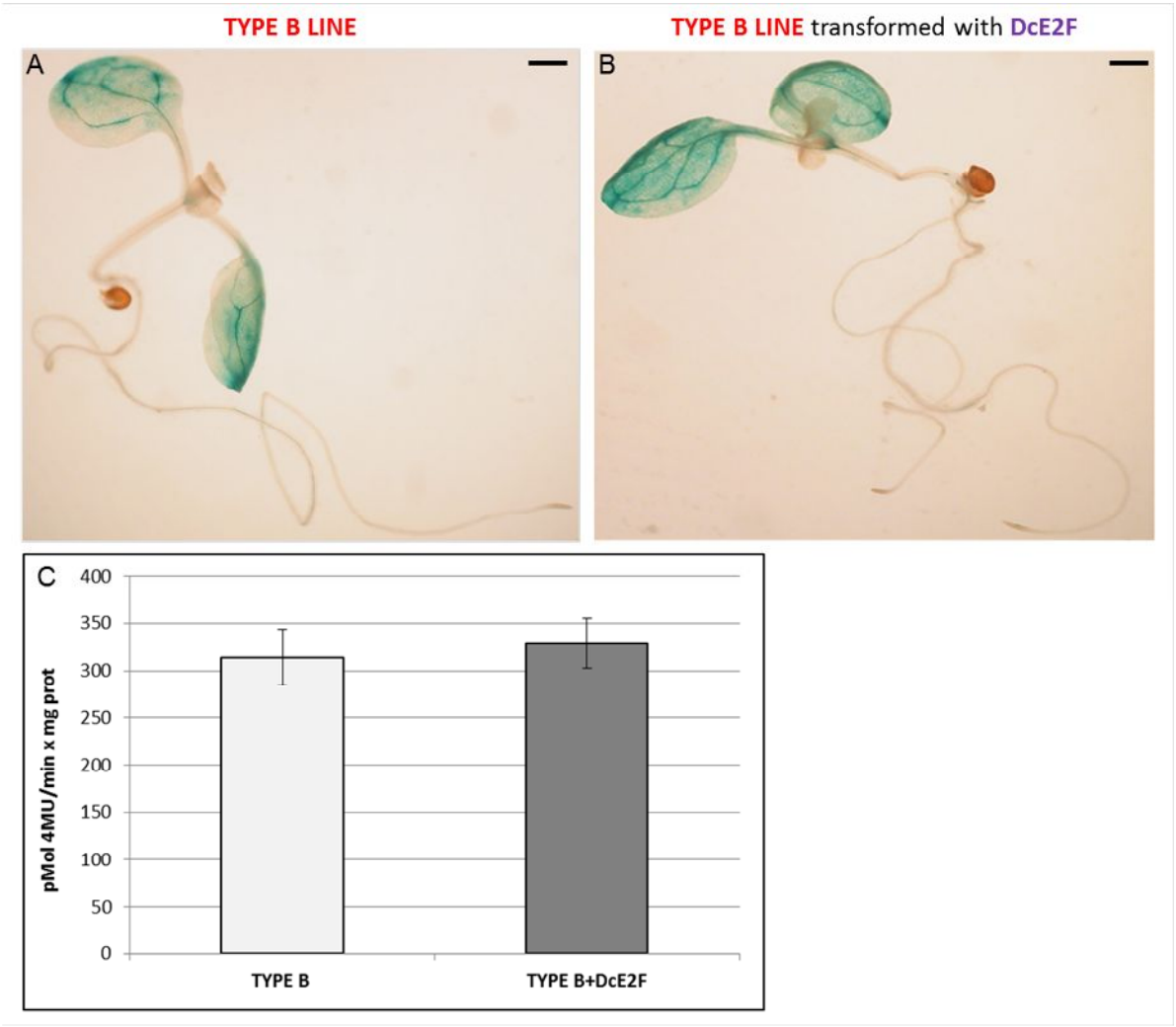

Fig. 16 Localization of GUS activity in two-week-old seedlings of: (**A**) type B lines; **(B**) type B lines re-transformed with the *DcE2F*. The constitutive expression of the *DcE2F* is not able to alter the pattern of activity of the EM35S promoter, which remains inactive in proliferating cells. Scale bars: 1 mm.
(**C**) Results of the fluorimetric analysis of GUS activity, carried out on extracts obtained from two distinct pools of type B lines and type B lines constitutively expressing the *DcE2F* factor. The bars show standard deviations.

To fully confirm that the EM35S promoter is responsive to the carrot factor in the A type line but is not activated by DcE2F in the type B line, fluorimetric GUS assays have been performed and the level of GUS activity measurable in pools of the double transformants were compared to the activities observed in the original type A and type B lines. As shown in figure 15C, the constitutive expression of DcE2F increased significantly the activity of the EM35S promoter in the type A line, whereas in the type B line the promoter activity was not affected (figure 16C). These results confirm the hypothesis that in type A lines E2F-dependent activation of the synthetic promoter occurs and is particularly efficient in the root apical meristem, whereas in the proliferating cells of the shoot apical meristem the activation of the EM35S promoter could be limited by low levels of endogenous activating E2Fs. On the contrary, in the type B lines the EM35S promoter is clearly not E2F-responsive even if the seedlings are ectopically expressing a strong activating E2F factor, and the pattern of activity observed is likely associated to an activation of the synthetic promoter by unknown endogenous transcription factors that are preferentially active in the vascular tissue of the cotyledons.

4.5 The E2F-dependent activation of the EM35S promoter in proliferating cells of type A lines occurs mainly during the G1/S transition

Further analyses have been carried out to investigate the likely cell cycle-dependent regulation of EM35S synthetic promoter, in the type A lines. To this purpose, experiments using the cell cycle inhibitors aphidicolin and colchicine were performed in germinating seeds. In dormant dry seeds most of the cells of the embryo are quiescent at the G1 phase [Barroco et al., 2005] but, upon seed imbibition, cells in the radicle enter the cell cycle and progress into S phase, which terminates approximately 42 hours after imbibition (HAI), when the radicle starts to protrude. Then the cells pass through the G2 and M phase that occurs approximately 48 HAI and is followed by a new cell cycle in the daughter cells. The imbibition and germination of dry seeds in the presence of aphidicolin blocks the cells in S phase whereas the germination in the presence of colchicine allows the completion of the first S phase and blocks the cells in the M phase [Varadarajan et al., 2010]. For this experiment, dry seeds of a selected homozygous type A line were imbibed with or without cell cycle inhibitors for 72 hours, a length of time which, in the absence of the cell cycle inhibitors, can allow cells to complete two divisions. After the treatment, the proteins extracted from treated and untreated germinated seeds have been used for the GUS fluorimetric analyses, necessary to evaluate variations of the activity of the EM35S promoter in response to the cell cycle inhibitors.

As shown in figure 17, compared to the untreated controls, the activity of the synthetic promoter increased significantly in seeds treated with aphidicolin and decreased upon colchicine treatment. This result clearly indicated that in type A lines the EM35S promoter is subjected to a cell cycle-dependent regulation and is mainly activated by the E2F factors during the G1/S transition, whereas its activity is lower or absent during the G2 and M phases of the cell cycle.

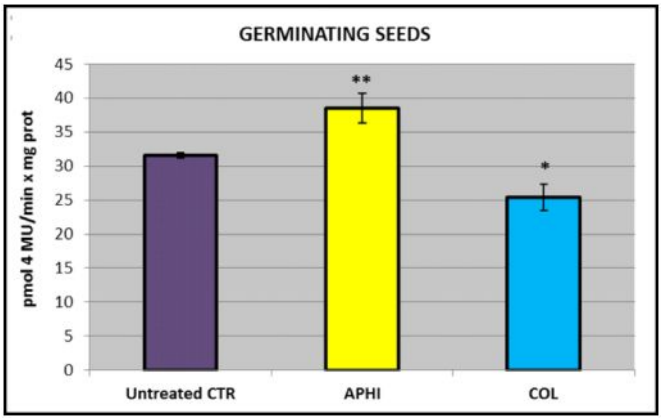

Fig. 17 Results of the fluorimetric analysis of GUS activity, carried out on extracts obtained from germinating seeds of a representative type A line.
The extracts were incubated 72 h without (ctr) or with cell cycle inhibitors (Aphidicolin, Colchicine). The bars show standard deviations. *p<0.05, **p<0.01.

5. DISCUSSION

This section of the thesis is focused on the setup of a system that, using a synthetic E2F-responsive promoter, can enable the visualization *in planta* of the transcriptional activity of the plant E2F factors. This system can be a valuable tool for the analysis of the roles of E2F factors in plant development and physiology.

The regulatory pathways that control cell cycle progression are conserved in animal and higher plants and include the cyclin D/Retinoblastoma /E2F pathway, which is fundamental in controlling cell proliferation. In this pathway, the D-type cyclins interact with the retinoblastoma protein, whose counterpart in plants has been called Retinoblastoma-Related (RBR), which controls the activity of the E2F transcription factors. The E2Fs are responsible for the transcriptional activation of a wide range of genes whose expression is required during the transition through the G1 and S phase of the cell cycle.

The genome of *Arabidopsis thaliana* encodes three typical E2Fs, named AtE2Fa, AtE2Fb, AtE2Fc, that dimerize with the DP proteins AtDPa and AtDPb, and three atypical E2Fs called AtE2Fd, AtE2Fe, AtE2Ff. AtE2Fa and AtE2Fb have been reported as activators of transcription, whereas AtE2Fc has been shown to function as a transcriptional repressor in association with the RBR1 protein. Conversely, the atypical E2Fs are not able to transactivate and are believed to be competitive inhibitors of the activating E2Fs. These factors possess a duplicated DNA-binding domain which allows to bind the E2F *cis* elements independently of an AtDP protein. Moreover, the atypical E2Fs lack the RBR-binding domain, suggesting a repressive role independently from the RBR protein [Lammens et al., 2009]. The activity of the RB proteins, is regulated by phosphorylation/dephosphorylation events. In its hypophosphorylated form RBR binds the E2F-DP dimers, hindering their activity, whereas, in response to mitogenic stimuli, phosphorylation by the CDK–CYCD complex dissociates RBR from the E2F-DP heterodimers, which are thus activated and are able to induce the transcription of genes required for cell cycle progression and DNA replication. As for the typical E2Fa and E2Fc proteins, they are also regulating the expression of endocycle-related genes [De Veylder et al., 2002; Magyar et al., 2012; del Pozo et al., 2006].

With the work carried out for this thesis, a construct containing a synthetic E2F-responsive promoter, named EM35S (E2F Minimal-35S), driving the expression of the reporter GUS, was introduced in a suitable vector and tested in stably transformed Arabidopsis plants. This promoter is composed of ten repetitions of the E2F consensus sites (TTTCGCGC) placed

upstream to the -60 CaMV35S minimal promoter. Transient expression experiments have already demonstrated that this synthetic promoter can be *trans*-activated by the activating E2F factors in plant protoplasts [Mariconti et al., 2002]. Thus, the EM35S promoter was expected to be E2F-responsive also when stably integrated in transformed Arabidopsis plants. Surprisingly, the histochemical analyses of GUS activity carried out on 18 transgenic Arabidopsis lines harboring the EM35S construct allowed us to detect two distinct and alternative patterns of expression of the reporter gene. In 50% of the transgenic lines, named type A lines, a strong GUS staining was mainly localized in the highly proliferating cells of the root apical meristem and in lateral root primordia. In the remaining 50% of the transgenic lines, called type B lines, the GUS accumulation was mainly detected in cotyledons, but never in the meristematic tissues. The presence of the two distinctive patterns of promoter activity is only partly correlated to the copy number of the transgene, which is generally lower in the type A lines.

As for the type A lines, the spatial pattern of activity is consistent with an E2F-dependent transcriptional activation of the EM35S promoter in cells that undergo proliferation. In fact, considering their role as S-phase inducers, the activating E2Fs are expected to be highly active in dividing cells [Mariconti et al., 2002; De Veylder et al., 2007]. Confirming a cell proliferation-related activation of the EM35S promoter, in the type A lines GUS activity can be observed in pericycle cells as well. The pericycle, in fact, contains a population of stem cells that contribute to the formation of the lateral root primordia at the xylem poles [Inzè, 2008; Dubrovsky et al., 2000; Beeckman and De Smet, 2014]. Weak GUS staining was observed also in the inflorescence, in particular in the developing stamens, and could be due to the activation of the EM35S promoter by AtE2Fb which, according to the microarray data reported at the Arabidopsis eFP browser of the Bio-Array Resource (BAR) website (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; [Winter et al., 2007]), is more expressed than AtE2Fa in the stamens of immature flowers. Concerning the type B lines, the activity of the EM35S promoter, observed in cotyledons, appears unlikely to be related to cell proliferation. The cotyledons of light-grown seedlings, in fact, increase their dimensions mostly through cell expansion rather than cell divisions [Stoynova-Bacalova et al., 2004]. Moreover, additional analyses conducted on the seedlings of the two types of lines and the effects caused by the constitutive expression of the carrot DcE2F transcriptional activator confirmed that the EM35S promoter is E2F-regulated in the type A lines, but its activity is likely to be under the control of other transcription factors in the type B lines.

Considering that most of the type B lines are characterized by high copy number of the transgene, initial analyses were performed to understand whether epigenetic mechanisms, such as DNA methylation or histone acetylation, could affect the activity of the EM35S promoter. These two epigenetic modifications may be responsible for the loss of transcriptional activity of transgenes and could explain, at least in part, the dual pattern of activity. Moreover, being rich in CpG dinucleotides, the ten repetitions of the E2F *cis*-element of the synthetic promoter could be subjected to DNA methylation, leading to gene silencing. In fact, in both plant and in mammalian genomes, cytosine methylation in the context of CG dinucleotides is the most frequent DNA modification and is strongly associated to transcriptional repression. In addition, the acetylation of the lysine residues of histones is necessary to switch the chromatin to a permissive state accessible to transcription factors. This covalent modification of histones is a reversible process and it is carried out by the histone acetylases (HATs) and histone deacetylases (HDACs). In both mammals and plants, the histone deacetylases are recruited by the Rb/E2F complex through the Rb-associated protein RbAp48 so that the transcription of E2F-target genes is repressed [Shen, 2002; Brehm et al., 1998; Ferreira et al., 1998]. In this respect, type A and B lines have been treated with Genistein, an inhibitor of the MET1 DNA-methyltransferases, and with Trichostatin A (TSA), which inhibits the HDACs. The two inhibitors are expected to re-activate the expression of genes that are epigenetically silenced. The results obtained with these treatments revealed that epigenetic mechanisms are not responsible for the two distinct patterns of activity of the EM35S promoter, which remained unaltered in both type A and B lines after treatment with the two inhibitors. Nevertheless, alterations of DNA methylation and histone acetylation appeared to affect the activity of the synthetic promoter in both lines. Inhibition of DNA-methylation produced a significant effect in the aerial parts of the type B line investigated, in which decreased activity of the EM35S promoter indicates that the methylation of the CpG dinucleotides may influence directly its activation. Concerning the TSA treatment, decreased activity of the EM35S promoter was observed in the aerial parts of both type A and type B lines, but a significant increase of the promoter activity was observed in the root of type A lines, in which strong GUS activity is restricted to the root meristematic apices. These results indicate that acetylation of the histones may play a direct role in the regulation of the EM35S promoter activity in the proliferating cells of the type A line roots, but is not affecting directly the activity of the synthetic promoter in the aerial parts of the transgenic seedling analyzed. In this respect, qRT-PCR analyses have revealed that the hyperacetylation of the histones due to

the TSA treatment can rather activate the transcription in the shoots apical regions of atypical E2Fs that can negatively regulate the synthetic promoter.

Additional information concerning the regulation of the EM35S promoter in the two lines was obtained evaluating the effects of phosphatase and kinase inhibitors. These treatments, in fact, indicate a possible involvement of RBR in the control of the EM35S promoter activity in type A lines only. The phosphorylation and dephosphorylation of RBR are expected to be able control the activity of the E2Fs regulating the EM35S promoter. Thus, experiments using inhibitors of phosphatases and kinases, such as Okadaic acid and Staurosporine, have been carried out. In mammalian cells, Okadaic acid can hinder the activity of the PP1 and PP2A phosphatases, which are the principal activators of the Retinoblastoma protein at the end of mitosis [Yan and Mumby, 1999]. On other hand, Staurosporine is able to block the activity of the CDKs during the G1/S transition [Orr et al., 1998], so that the hypophosphorylated Retinoblastoma protein is able to bind and repress the E2Fs. Although the histochemical GUS analyses on the EM35S lines revealed that both Okadaic acid and Staurosporine could not alter significantly the spatial pattern of the EM35S promoter, whose activity remained confined in the root apices of the type A lines and in the cotyledons of type B lines, a drastic increase of the activity of the EM35S promoter in the roots of type A seedlings treated with Okadaic acid indicates that the activity of the EM35S promoter in these lines is likely to be regulated by the RBR/E2F pathway. In fact, the inhibition of phosphatases should lead to the hyperphosphorylation of RBR that releasing activating AtE2Fa or AtE2Fb allows them to bind and upregulate the EM35S promoter. Interestingly, although an opposite result should have been observed blocking the kinases, an increase of the EM35S promoter activity was detected after treatment with Staurosporine in the type A line roots as well as in the type B line aerial parts. A possible explanation of this result could be linked to a direct control of the activity of the E2Fs by phosphorylation. In human cell models, in fact, a repressive role of the Cyclin A/CDK2 complex has been described, which by direct phosphorylation inhibits the activity of the E2F1 factor [Xu et al., 1994; Krek et al., 1994; Dynlacht et al., 1994]. Therefore, a similar scenario could be hypothesized in the type A seedlings studied, in which the removal of this inhibitory phosphorylation on the E2Fs could have a predominant effect respect to the RBR-dependent regulation, thus increasing the EM35S promoter activity.

The constitutive expression of an exogenous activating E2F factor, such as the DcE2F of *Daucus carota* [Albani et al., 2000], fully confirmed that the EM35S promoter is thoroughly subjected to an E2F-dependent regulation only in the type A lines. Conversely, a constitutive

expression of DcE2F in type B lines did not produced significant modifications, supporting the hypothesis that the synthetic promoter is not E2F-regulated in these lines. Moreover, the increased activity of the EM35 promoter in the aerial parts of the type A line constitutively expressing the DcE2F factor indicates that the E2F-dependent activation of the synthetic promoter in the shoot apical meristem could be limited by the amount of activating E2Fs. Moreover, as emerged from analyses performed using inhibitors of cell cycle progression, the E2F-mediated activation in proliferating cells of the A lines occurs primarily at the G1/S phase of the cell cycle, confirming the important roles played by the E2Fs in the control of cell cycle progression.

It is not clear why in half of the lines transformed with the EM35S promoter construct the synthetic promoter does not appear to be E2F-regulated, but is likely to be under the control of another class of transcriptional activators. Because DcE2F cannot overcome their activity, these unknown transcription factors are unlikely to compete with the E2Fs for binding to the same *cis* elements and, possibly, can recognize other sequences inadvertently contained, singularly or in multiple copies, in the synthetic promoter fragment. The transgenic lines were obtained by Agrobacterium-mediated transformation and the integration of the T-DNA in different locations can give rise to unexpected patterns of expression, reflecting what is known as the "position effect". However, this effect is expected to give rise, at lower frequency, to multiple misregulated patterns of expression of the transgene and the possibility that two distinct but equally frequent patterns of expression can occur has never been reported. Thus, although a position effect could justify the lack of the E2F-dependent regulation in half of the lines, the misregulated expression in the remaining lines would not be expected to be uniform and, instead, should be highly variable, if not absent, in many of the transgenic lines. Moreover, although it is possible that mutations in the repetitive sequence of the promoter could have occurred in the bacterial culture or in the plant cells during DNA integration, it is not reasonable that this could yield with the same frequency two distinctive patterns of expression in the transformed lines. Although the dual regulation of the EM35S promoter remains enigmatic, the equal frequency of E2F-regulated and E2F-unresponsive lines and the stability of this feature in their progeny suggests that two different and mutually exclusive states of the promoter may exist and could have been imprinted with the same frequency in the transgenic lines. It is not clear whether this could reflect an acquisition of these two states during the process of plant transformation or could reflect alternative conformational features of the promoter structure associated to differential integration in the

genome. In any case, the isolation of type A lines, which have been clearly demonstrated to be regulated by E2F factors, will open the way to further studies concerning the RB/E2F pathway in Arabidopsis that will increase our comprehension of its roles in the control of plant development and of important physiological activities.

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