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"Functional analysis of *Arabidopsis thaliana* Class I TCP genes *AtTCP7* and its roles in plant development"

Tutors

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I (Wang Bo) confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Table of Contents

Abstract

Plant cell division cycle is a highly complex and tightly controlled process which is regulated by both cell cycle genes conserved across kingdoms and plant-specific regulators. TCP family genes are plant-specific transcription factors reported in recent years that function in many aspects of plant growth, and broadly control the morphology of different organs via positive or negative regulating target genes expression. The initial work in our study was to identify the transcriptional factors regulating *miRNA168a* gene expression. A TCP family gene *AtTCP7* was isolated as a putative regulator of Arabidopsis *miR168a* from yeast one-hybrid screening. Transgenic plants over-expressing *AtTCP7* gene in *Arabidopsis thaliana* showed a decrease of mature *miRNA168a* accumulation, while later crossing analysis co-expressing *35S::AtTCP7* and *pMiR168a::GUS* showed a minor affection on reporter gene *GUS* expression, it indicated that the *AtTCP7* may not directly interact with *miR168a* promoter *in vivo*. In-depth phenotypic characterization of the transgenic lines displayed a variety of growth defects and developmental delay, including shorter roots, delayed flowering and reduced fertility. Analyses at different developmental stages demonstrated that over-expression of *AtTCP7* differentially affected cell size and DNA content in different organs by altering cell cycle genes expression and by restricting the interphase transition from G1 to S phase. Further expression analyses on over-expressing lines uncovered a complex network of genetic interactions between the members of the TCP family, highlighting the functional interaction especially between *TCP7* and *TCP23*.

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Thirdly, as a member of Ecological Genomics (EcoGenomics) group in Fondazione Edmund Mach, I thanks for all the helps from our group members, especially our gentle technician Enrico Barbario. I also would like to thanks to my tutor in University of Trento, Prof. Alberto Inga.

And last, as a son, I apologize to my parents for my capricious and childish, that I was away for four years with a rare time to stay with them. I would like to convey my deepest love and gratitude to them. Thanks them for endue me a wonderful life and encourage me to live a positive life. Therefore, I dedicate my thesis to them.

Chapter I

Screening for transcription factors which regulate *miRNA168* gene expression

1.1 Brief introduction of MicroRNAs and its biogenesis.

MicroRNAs (miRNAs) are endogenous RNAs with the length 21 to 24-nt, which were identified as a small RNA(sRNA) species. MiRNAs was first reported in *Caenorhabditis elegans*[1], it showed the features of miRNA *lin-4* in regulating target gene *lin-14* expression by interaction directly to the 3'UTR of *lin-14* mRNA which inhibited the *lin-14* protein translation. MiRNAs are widely present in animals, plants and diverse eukaryotes, so far there are 30,424 mature miRNAs which are generated from 24,521 miRNA loci in 206 species deposited in MiRBase (http://www.mirbase.org/ , Version 20), and they are conserved in a lineage specific manner. MiRNAs can down-regulate target gene expression by perfect or imperfect sequence complementarity which caused target mRNA cleavage or inhibit translation. Plant miRNAs were identified at early 21st century[2-3], during last decades intensive studies, there are more than three hundred miRNAs identified in model species *Arabidopsis thaliana*, they play important roles in regulating a wide range of plant biological processes such as leaf and floral differentiation and development, root initiation, signal transduction, environmental stresses responses, and even in regulating small RNAs biogenesis pathway. As most protein coding RNAs, the transcription of plant miRNAs is also guided by RNA polymerase II[4], and regulated by many *cis*- and *trans*-factors, such as specific transcription factors(TFs).

After the transcription, single-stranded primary miRNAs (pri-miRNAs) with internal stem-loop structures are first cleaved by Dicer-Like 1 (DCL1) gene in plants which is functionally and homologically associate with Drosha and Dicer in animals[5] to convent pri-miRNAs into the precursor miRNAs (pre-miRNAs), and following with the second cleavage that removed the loop part and cleaved into miRNA/miRNA* duplex[6-7]. Both of two steps are located in nucleus and controlled by DCL1 in plants[7]. After DCL1-mediated cleavage, mature miRNA/miRNA* duplexes are stabilized by the S-adenosyl methionie-dependent methytransferase Hua Enhancer 1 (HEN1), which is an important step that methylates 3' terminal nucleotides of both two strands to protect miRNA or siRNA from 3'-end uridylation

and subsequent degradation[8-9]. Afterwards, the duplex is translocated from nucleus into cytoplasm, this step is facilitated by another protein HASTY, which is homolog of Exportin 5 in animals that exports duplex miRNA into the cytoplasm[10]. After that, miRNAs are dissociated from the duplexes and incorporated into Argonaute (AGO) associated miRNA-induced silencing complexes (miRISCs), by which the target transcripts containing miRNAs complementary recognition sites are sliced through mRNA cleavage or translation inhibition.

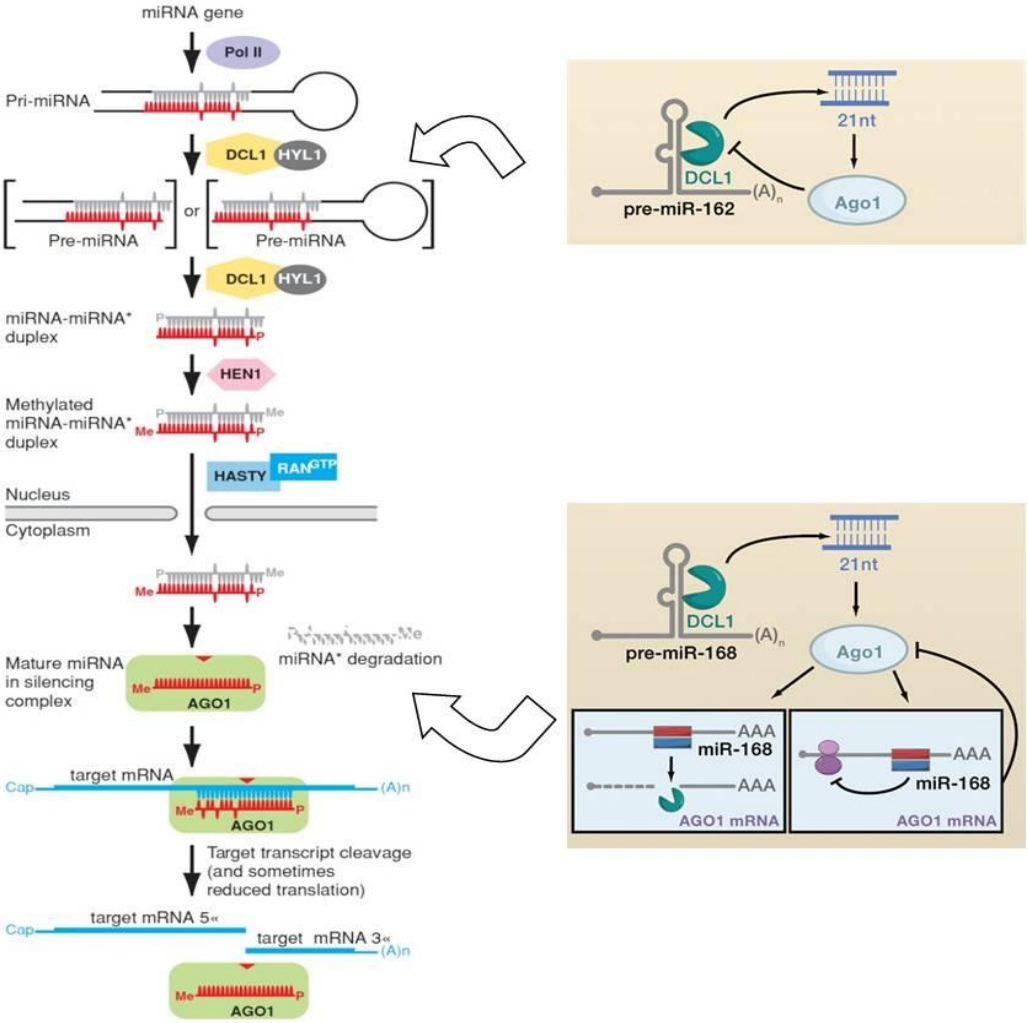


Fig. 1: Plant miRNA biogenesis pathway and the regulation of AGO1 by *miR168*. Figure courtesy of Ref [11-12]

The central of the silencing complexes is a member of the Argonaute (AGO) protein family. In *Arabidopsis*, there are 10 members in AGO family. Among them, AGO1 shows an important role in forming miRNA-induced silencing complexes and siRNA-induced silencing complexes[13]. AGO protein contain two conserved regions, PAZ and Piwi domains. The PAZ domain has the function in RNA binding, which found both in Dicer and Argonaute that essential for RNA-silencing pathways[14-15]. while Piwi domain has a tertiary structure belong to the RNase H enzymes, which cleave single-stranded RNA in an RNA/DNA hybrid[16]. Therefore, through the AGO1 protein mediated the mRNA cleavage, miRNAs regulate their target genes' expression[17].

1.1.1 The importance of *MIR168* in plants.

Previous studies demonstrated that miRNAs control diverse aspects of plant growth and development, in addition to a complex biogenesis pathways, plants conferring a highly dynamic regulation to miRNA activities, such as feedback and buffering system, in order to fine tuning the influences caused by endogenous or exogenous signal fluctuations. Among them, *miRNA168* plays crucial roles as it regulates the expression of AGO1, the slicer of miRISCs assembly, through which most miRNAs are generated and down regulate their targets, and the expression level of *miRNA168* itself is also regulated by AGO1. In addition, *miRNA168* and AGO1 are co-regulated transcriptionally in temporal and spatial manner. By over-expression of *miRNA168a* in *Arabidopsis*, *AGO1* mRNA accumulation was significantly reduced. As the key elements of miRISCs, the decrease of *AGO1* mRNA expression also impacted variety of miRNAs accumulation, such as *miRNA159, miRNA163, miRNA165/166, miRNA390,* etc, and displayed a serrated and adaxialized leaves which were similar to the phenotype of hypomorphic *ago1*

alleles[18-19]. Oppositely, *miRNA168a* and several other miRNAs' accumulation were reduced in *ago1*-null alleles, indicated that AGO1 is important in miRNAs stabilization and the fine-tuned adjustment of *AGO1* and *miRNA168a* levels is important for plant to control the miRNA in regulating their target mRNA expression (Fig. 1), Therefore, In our study, we focus initially on the regulation of miR168 expression, a yeast one-hybrid screening was used to isolate potential candidate transcription factors which may bind and control the expression dynamics of *miRNA168a*.

1.1.2 Yeast one-hybrid assay.

Yeast one-hybrid system is commonly used to identify DNA-protein interaction and isolate novel DNA-binding proteins. It was first used in cloning the mammalian olfactory transcriptional activator Olf-1[20], and later several other transcription factors was obtained by one-hybrid assay. Since it can directly identified the specific DNA-binding protein, the technique is widely used for novel transcription factor identification.

The assay is based on the finding that many eukaryotic transcriptional factors are composed by independent DNA-binding domains and an activation domain (AD) [21], this allow researchers to express a fusion protein with activation domain that can simultaneously bind to specific target DNA sequence and activate down stream report gene transcription. Normally the report genes are HIS3 and lacZ, which allow positive colony growth on minimal medium lacking histidine or verify the DNA-protein interaction by β-galactosidase assay. Thus, by mating report strain which contain specific DNA-binding sequence with AD/library strain that express AD fused transcription factors, the positive combinations which AD fused proteins physically interact with specific DNA-binding sequence can be selected through the activation of report gene expression.. Based on these, researches can identify the potential putative transcriptional factors which bind to a target or cis-acting DNA sequence.

1.2 Materials and Methods

1.2.1 Construction of yeast one-hybrid reporter strain vectors.

The DNA bait regions were amplified with Phusion® High-Fidelity DNA Polymerase, the primers used for this purpose are listed in the *Appendix 1*. The PCR reaction was carried out according to Phusion amplification instruction: for 20µl reaction system, it contained 4µl of 5x Phusion HF buffer, 2µl 2mM dNTPs, 1µl of each 10µM primers, 1µl of diluted *Arabidopsis* cDNA as template, and 0.2µl of Phusion DNA polymerase. The thermocycling conditions for a routine PCR were 98℃ 30 seconds for denaturing, and followed with 35 cycles of 98℃ 10 seconds, 60-62℃ 30 seconds, 72℃ 15 seconds. And the final extension was 72℃ 10 minutes. The PCR products were then purified with DNA clean-up kit, ligated with T4 DNA ligase and cloned into pGEM®-T vector. The ligation products were transformed into *E.Coli* competent cells by electroporation and then transformed product was plated on the LB medium supplemented with Ampicillin. The positive transformation colonies were tested first by colony PCR and later confirmed by sequencing. After this, the positive plasmid in pGEM-T vector containing DNA bait regions were digested with SacI and XbaI and ligased with yeast one-hybrid reporter vector pHISi through multiple clone sites. The ligation product was transformed into *E.Coli* as described above and positive colonies were selected on Ampicillin LB medium plates. The reporter plasmid was then extracted from the positive colony strain and purified for yeast transformation.

1.2.2 Yeast one-hybrid transformation

The LiAc-mediated yeast transformation procedure was based on the CLONTECH Yeast Protocols Handbook, which introduced a simple and highly reproducible lithium acetate (LiAc)-mediated method to integrate linear DNA into the yeast genome.

The YM4271 strain was used for yeast transformation. Yeast colonies were scraped from fresh plates and re-suspended in 1 ml YPDA medium. The resuspension was then transferred into a flask containing 30 ml of YPDA medium and incubated over night at 30℃ with shaking at

250rpm. In the second day, the overnight culture was diluted in 200 ml YPDA medium to reach to starting OD_{600} around 0.2-0.3. The diluted culture was then incubated at 30℃ for 3 hr with shaking at 230rpm till the final OD_{600} reached 0.4-0.6. At this stage, the culture was ready for yeast competent cell preparation. The culture was placed in 50-ml falcon tube and centrifuged at 1,000 x g for 5 min at room temperature to deposit yeast cells. The cell-pellet was then washed by 25 ml 1 x TE buffer once, and resuspended in 1 ml freshly prepared, sterile 1 x TE/1 x LiAc. After this, the cells were ready for transformation.

Before the transformation was started, 1 μg reporter plasmid was linearized by digesting with appropriate restriction enzyme. In our case, Sma I was used for plasmid linearization. 0.1 μg of reporter plasmid DNA and 0.1 mg denatured herring testes carrier DNA were added into a 1.5- ml tube, and then mixed with 0.1 ml of yeast competent cells prepared as mentioned above. Afterwards, the mixture was resuspended with 0.6 ml of freshly prepared 1 x PEG/LiAc solution and vortex at high speed for 10 seconds, later incubated at 30℃ for 30 min with shaking at 200 rpm. After that, 70 μl of DMSO was added into the mixture and heat shock for 15 min at 42℃ water bath. The cells was then chilled on ice for 2 min, and the pellet was collected by centrifugation for 5 seconds at 14,000 rpm at room temperature. The final step was to re-suspend the cells in 0.5 ml 1 x TE buffer and plate the transformed cells on plate containing SD selection medium which was lack of histidine in our experiment. After 2-4 days incubation at 30℃, the positive colonies were grown on the selection medium.

The media recipes referred in this section is present as below:

YPDA medium: 20 g/L peptone, 10 g/L Yeast extract, 20 g/L Agar (only for solid medium) were mixed and pH of the solution was adjusted to 6.5, then autoclaved. Before using, adding 15 ml/L of a filter-sterilized 0.2% adenine hemisulfate solution.

SD selection medium: 6.7 g Yeast nitrogen base without amino acids was dissolved in 900 ml distilled water and the pH was adjusted to 5.8, 20 g Agar for one liter medium (only for solid

medium) was added just before autoclaving. After autoclave, adding 100 ml of appropriate 10 x Dropout solution. For 3-AT analysis, appropriate amount of 1 M 3-AT stock solution was also added into SD selection medium before using.

10 x Dropout solution: Combining the right amount of nutrients listed below at the concentrations indicated to prepare a 10 x Dropout Solution. For different selection propose, the Dropout solution was prepared without specific nutrients. For instance, in our case, *his*$^-$ *leu*$^-$ Dropout solution was prepared without adding L-Histidine HCl monohydrate and L-Leucine. The Dropout solution has to be dissolved in deionized H_2O and autoclaved.

Nutrients	10 x Concentration
L-Adenine hemisulfate salt	200 mg/L
L-Arginine HCl	200 mg/L
L-Histidine HCl monohydrate	200 mg/L
L-Isoleucine	300 mg/L
L-Leucine	1000 mg/L
L-Lysine HCl	300 mg/L
L-Methionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2000 mg/L
L-Tryptophan	200 mg/L
L-Tyrosine	300 mg/L
L-Uracil	200 mg/L
L-Valine	1500 mg/L

10 x TE buffer: Containing 0.1 M Tris-HCl-and 10 mM EDTA, adjusting pH to 7.5. Then autoclaved.

10 x LiAc: Containing 1 M Lithium acetate, using acetic acid to adjust pH to 7.5 and sterilizing by Autoclaving.

50% PEG 4000: preparing with sterile deionized H_2O and autoclaved.

PEG/LiAc solution: For 10 ml of solution, mixing 8 ml of 50% PEG with 1 ml of 10 x TE and 1 ml 10 x LiAc. Freshly prepared before use.

1.3 Results

1.3.1 Preparation of yeast reporter strains

To conduct the yeast one-hybrid screening assay, three yeast reporter strains were prepared according to the promoter sequence of *pre-miR168a*. Previous work in our lab has already identified and isolated several transcriptionally regulated elements on the upstream of *pre-miRNA168a* transcription start site. To meet the requirements for the report strain preparation, three target regions which were 150~250 bp in length were selected and cloned according to the predicted binding domains on promoter of *pre-miRNA168a*: AG box part, F1-5 box part, and F7-12 box part(Fig. 2A). AG box part contains three "A-box" (AGTG), three "G-box" (CACGTG), one "CA-box" (CAGATCA), and one "CC-box" (CGTAATTT), while F1-5 box part includes five newly identified elements, and F7-12 box for the rest of six putative regulatory elements. Afterwards, the target regions were cloned into pHISi vector to generate three individual reporter constructs(Fig. 2B), and integrated into yeast genome by sequential homologous recombination. The positive transformed yeast colonies were then grown on selection medium which lacking histidine and supplemented with 5mM to 60mM of 3-Amino-1,2,4-triazole (3-AT). 3-AT is a competitive inhibitor for the product of *HIS3* gene and thus limit *HIS3* biosynthesis to eliminate background growth of false positives in later screening step. Analysis of colony growth in selection medium showed that tiny colonies were observed on the his^- medium, however, by adding 5mM 3-AT, the growth of all colonies were inhibited, which indicated that the reporter strains were tightly controlled under the selection medium containing 5mM 3-AT.

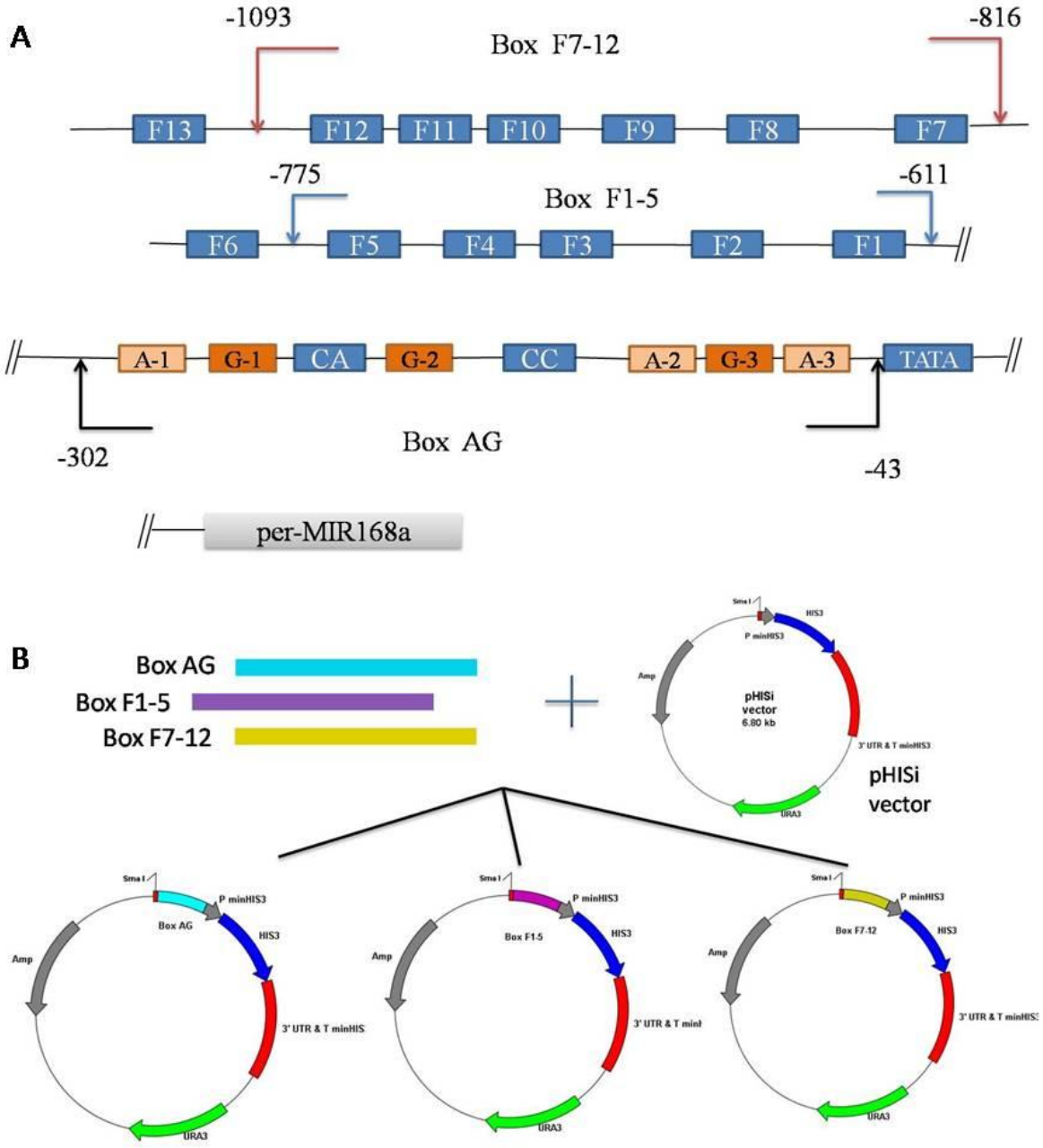


Fig. 2: Yeast one-hybrid report strain preparation. A) Regulation elements on promoter of *miR168a*, which was separated into three regions for yeast one-hybrid reporter vector preparation; B) DNA baits were cloned into pHISi reporter vector, which generated three different yeast one-hybrid reporter constructs.

The reporter construct containing F1-5 box part was first prepared as "F1-6 box part", which contained the first six putative *cis*- elements F1 to F6 boxes. However, in 3-AT assay, we found that the transformed colonies could grow on the His^- selection medium even with 60mM of 3-AT for F1-6 reporter strain. By contrast, the growth of F1-5 box reporter strain was suppressed at lower concentration of 3-AT. Thus, F1-5 box reporter strain in this case was used for later yeast one-hybrid screening as there was a lower background yeast growth compared with F1-6 box reporter strain.

1.3.2 Yeast one-hybrid screening and verification of positive colonies.

In this part of work, we collaborated with Simona Masiero's group in Dipartimento di Bioscienze, Universit à degli Studi di Milano. The AD fusion library constructed with double strand cDNAs derived from different organs of Arabidopsis was used to identify the transcription factors which would interact with the specific regulatory DNA sequence located in different box parts of *miRNA168a* promoter. The library containing at least 1x 10^7 cells in 1ml yeast culture. After screening, the positive colonies were growth on selection medium (SD/-His/-Leu) containing 5 mM 3-AT, and enriched by individual yeast cell culture. cDNA/AD fusion plasmids were then isolated from cell culture and sequenced.

In total 195 colonies had been isolated from three reporter strains after yeast one-hybrid screening, among them 108 colonies were isolated from AG box reporter strain, 85 colonies from F1-5 and 5 colonies from F7-12. After sequencing and blast analysis in NCBI, 94 colonies were found to have annotations *Appendix 2*, Among them, only sequences annotated as transcription factors were selected, through this approach, six candidate genes were obtained for further downstream analysis (Table I).

Table I. Candidate genes isolated from yeast one-hybrid assay.

Gene **Locus**	Annotation
AT5G46910	Transcription factor jumonji (jmj) family protein / zinc finger (C5HC2 type)
AT5G62460	RING/FYVE/PHD zinc finger super family protein
AT5G57660	CONSTANS-like , sequence-specific DNA binding transcription factor activity
AT4G31420	Zinc finger protein
AT5G23280	TCP family transcription factor TCP7
AT2G18090	PHD finger SWIB/MDM2 and GYF domain-containing protein

The positive candidate genes with full-length cDNA/ AD fusion yeast strain were further verified for interaction with its corresponding reporter strain in yeast. After hybridization, the positive colonies were re-streaked on the selection medium (SD/-His/-Leu) containing 5 mM 3-AT, we found that all of them can grown on the selection medium except one gene isolated by F7-12 reporter strain hybridization, a PHD finger family protein (Fig. 3). This indicated that the gene *AT2G18090* may be a false positive, and the rest five candidate genes could be processed for confirming the interaction with regulatory elements of *miRNA168a* promoter *in vivo*.

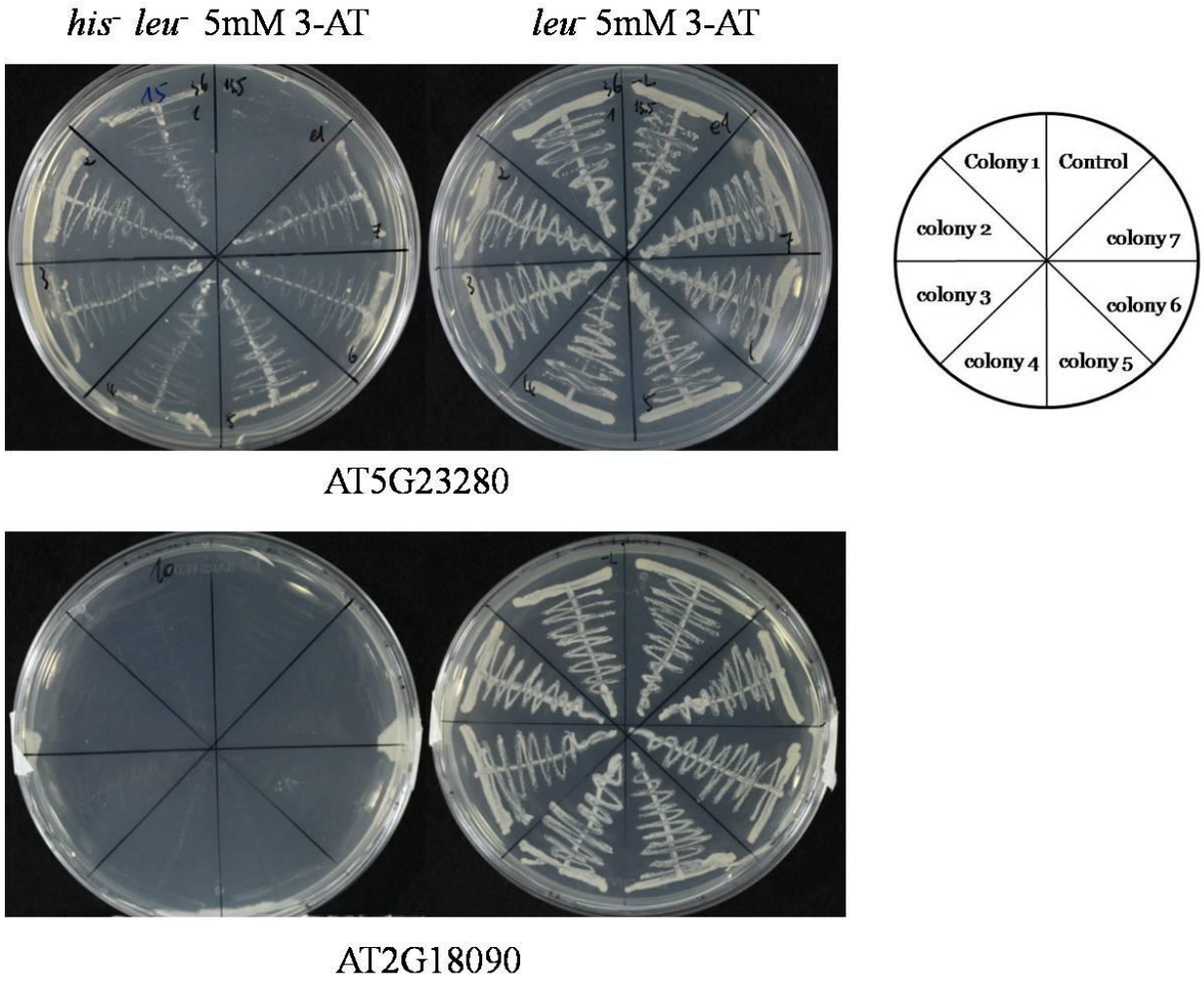


Fig. 3: The yeast one-hybrid verification of candidate genes interact with corresponding reporter strain in yeast. positive colonies were re-streaked on the selection medium (SD/His^-/Leu^-) containing 5 mM 3-AT while SD/Leu^-/5mM 3-AT selection medium also used for yeast growth positive control. Empty library vector was transformed and crossed with candidate gene reporter strain re-streaked on the top right corner as a negative control.

1.4 Discussion

In the first chapter, we focused our work on screening for the potential transcriptional factors regulating *miRNA168a* expression. Yeast one-hybrid assay is a rapid and sufficient method that widely used for screening proteins which bind to a target, *cis*-regulatory DNA sequence upstream of any gene of interest. Through a large scale of yeast one-hybrid screening described above, 195 positive colonies were obtained and five out of them showed DNA-protein binding capability in yeast.

The library we used was a full length cDNA library which contained most of the transcripts in *Arabidopsis*. However, the candidate genes which were fished out were small amount and low hit rate. It could be several reasons: first, even though the library used in this experiment had a sufficient coverage of all genes in *Arabidopsis* according to calculation, the low abundant expression of transcription factors may increase the difficulty of the interaction between candidate AD fusion genes and reporter strains; second, the library might not include the organs of specific developmental stage where the transcription factors are expressed; furthermore, the library was constructed with organs from normal growing condition, the organs treated with different stress were not included in the library, and the endogenous proteins in yeast might interact with the reporter strains used in this study, and all these limitation could lead to a low efficiency on yeast one-hybrid screening and high possibility of false positive binding. Therefore, other researchers intended to use the library enriched only with transcription factors for yeast one-/two-hybrid screening[22]. In which, the researchers used Gate-way system to clone over than 1,500 transcription factors in *Arabidopsis*, which were highly specific for yeast one-/two-hybrid and more efficiency in later screening.

Chapter II

Identification of candidate genes binding with promoter part of *miRNA168a*

2.1 Introduction

To verify the interaction between candidate genes and promoter parts of *miRNA168a*, transgenic over-expression lines of those five candidate genes were generated through *Agrobacterium*-mediated transformation.. In addition, a transgenic line of *miRNA168a* promoter driven by β-glucuronidase (GUS) reporter gene (pMIR168a::GUS) was used for crossing with the over-expression of *AtTCP7* line in order to test the interaction of *AtTCP7* with miRNA168a promoter *in vivo*.

2.1.1 *Agrobacterium*-mediated plant transformation.

Genetic modification mediated by *Agrobacterium* is widely used for higher plant research and biotechnology. *Agrobacterium. tumefaciens* which contain a tumor-inducing (Ti) plasmid can transfer a specific segment (T-DNA region of Ti plasmid) into the genome of an infected plant, the infected cells then over-produce the phytohormones as a consequence of expression of the T-DNA which causes tumourous growth or crown galls on dicotyledonous plants. These phenomena were first reported in *Helianthus annuus* L[23], and later described and established as a plant genetic transformation technique in 1980s[24-25].

The mechanism of *Agrobacterium*-mediated transformation is not fully understood so far. However, several crucial components which take part in the T-DNA transformation procedure have been isolated and characterized. The T-DNA genes on Ti-plasmid are essential for crown gall tumor formation, however, a group of *vir* genes outside of the T-region are also important for the T-DNA transformation. In general, T-regions in Ti-plasmid contain two borders that are 25bp in length and highly homologous in sequence, which delimit the T-DNA. The two borders are the specific target of the VirD1/VirD2 endonuclease which further cleavage T-DNA from the Ti-plasmid and forms a T-strand and VirD2 covalent bonding with the 5’ end of lower strand of the T-DNA[26-27]. Afterwards, VirD4 and VirB proteins make up a type IV secretion system that help T-strand and other Vir proteins transfer into cytoplasm[28]. Once T-strand inside the

cytoplasm, VirD2 protein which contains nuclear localization signal (NLS) sequences may help T-DNA attach to the plant nucleus[29-30]. Meanwhile, VirE2 can alter the random-coil DNA conformation into a elongated shape coil form, which may help T-strand pass through the nuclear pore and also protect T-strand from nucleolytic degradation[31]. Subsequently, the T-DNA associate with plant DNA and integrate into the host genome by synthesis-dependent strand annealing mechanism of non-homologous end-joining (NHEJ) [32-33].

The different methods for *Agrobacterium*-mediated transformation were also developed during the lab practices. So far, a Floral dipping method for *Arabidopsis* is widely used and proved to have advantages of both easy performance and high transformation efficiency[34]. With the help of transgenic engineering technique, researchers are able to perform a variety of gene functional studies in plants, such as over-expression or down regulation of target genes to determine the gene functions, or investigating the gene expression pattern by using the GUS reporter gene systems. In our study, we used the over-expression technique to characterize candidate genes' function, and GUS reporter system to evaluate the interaction between *AtTCP7* gene and promoter of *miR168a*.

2.1.2 GUS reporter system

Agrobacterium-mediated plant transformation provides a powerful tool for the functional study of a gene of interest. In addition, using a reporter gene system which driven by the mutagenized promoter is greatly facilitated the access of gene regulation analyses. The reporter gene which is not natively expressed in the organism being studied is sensitively and quantitatively available for the measurement of reporter gene' s activity, through which the visualized and quantifiable expression level of reporter gene can indicate the activities of fused promoter.

Commonly used reporter genes in plants are jellyfish green fluorescent protein (GFP) which appears to be green under the blue light; the luciferase produces oxyluciferin and light when a small-molecule substrate luciferin is catalyzed; the β-glucuronidase (GUS) enzyme which is

particularly used in plant molecular assay can catalyze different substrates to give different indications.

β-glucuronidase gene (*uidA*) comes from *E.Coli*, it is an acid hydrolase which is catalyzed by two acidic residues to cleave *β*-glucuronides[35]. The normal substrate for GUS analysis which was also used in this thesis is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), the catalytic reaction produces a clear and stable blue color in the position where the GUS protein is expressed, and the procedure is easy to perform. Thus, GUS reporter gene and X-Gluc substrate are commonly used in plant histochemical analysis[36].

2.2 Materials and methods

2.2.1 Construction of over-expression vectors

The full-length cDNAs of candidate genes were amplified with Phusion® High-Fidelity DNA Polymerase, the procedure was the same as mentioned in section **1.2.1**. The purified PCR products were then cloned into pCR™8/GW/TOPO® entry vector and transformed into *E. Coli* DH10B competent cells by electroporation, the recombinant plasmids that purified from positive clones were confirmed by DNA sequencing, and by using the LR Clonase ™ II enzyme, cDNA fragments in pCR8 vectors were then transferred into destination vector pMDC32 through LR reaction. The positive clones were finally transformed into *Agrobacterium* GV3101 (PM90) strain and prepared for next step plant transformation

2.2.2 Plants growth condition and floral dipping methods for *Agrobacterium*-mediated transformation.

Arabidopsis thaliana ecotype Columbia (Col-0) was used for *Agrobacterium*-mediated transformation. Col-0 seeds were stratified at 4 ℃ for two days and transferred into soil, they were grown at 23 ℃ (40-60% humidity) under photoperiod of 16h light and 8h dark (LD) with light intensity approximately 120 to 150 $\mu Em^{-2}s^{-1}$.

The *Agrobacterium* which were transformed with over-expression constructs were grown on YEB plates containing 50 μg/ml of Kanamycin and 100μg/ml Rifampicin antibiotics. Colonies were picked from fresh plates and re-suspended in 50 ml YEB medium supplemented with Kanamycin and Rifampicin, 100μl of 1M Mg_2SO_4 was added into the culture and incubated at 28℃ with shaking at 250rpm over night. Afterwards, the culture was transferred to a 1L flask and mixed with 250 ml fresh YEB medium supplemented with the same antibiotics as above. The culture was then incubated at 28℃ for another 18hr with shaking at 250rpm. After this step, the *Agrobacterium* culture was ready for transformation.

The 300 ml culture was mixed with 300 ml of 5% sucrose solution and 120μl Silwet, and the mixture was continuously stirred by using magnetic bar. During stirring, the flower buds were dipped into the mixture for 15~17 seconds, and in total at least 100 plants were used for transforming one over-expression construct. After transformation, all the plants were covered by black plastic bags in order to keep humidity and avoid light for 22 hr, and then the plants were transferred into GMO green house and grown under the condition as for Col-0 plants.

Transgenic seeds were carefully collected from these plants and stored in GMO labeled container. Positive transgenic plants were selected on the MS plates containing 35μg/ml hygromycin. Only the single copy homozygous lines were used for the phenotypic investigation and gene expression analyses.

2.2.3 Total RNA extraction and qPCR analysis

Total RNA was isolated from whole seedlings or appropriate tissues by TRIzol® reagent (invitrogen), gDNAs were eliminated by treating with Sigma DNase I after RNA extraction. The quality of RNA was controlled by spectrophotometer measurement and agarose gel electrophoresis. 1 μg total RNA per sample was used for individual cDNA synthesis. The routine first strand cDNA synthesis was performed by SuperScript III (invitrogen) reverse transcriptase

with oligo dT(20) primer. For miRNA reverse transcription, NCode™ SYBR® GreenER™ miRNA qRT-PCR Kit was used (invitrogen).

qPCR was performed with Platinum® SYBR® Green qPCR SuperMix-UDG kit (invitrogen), a 10x - 20x diluted cDNA was used as template for analyzing relative genes' expression. The final qPCR reaction volume was 12.5µl, it contained 6.25µl SYBR Green super mix, 0.25µl of each gene specific primer pair (10pmol, shown in *Appendix 1*), 1µl diluted cDNA and 4.75µl DEPC-treated water. Bio-Rad CFX96 Touch™ Deep Well Real-Time PCR Detection System was used to perform qPCR reaction with a standard cycling program: 50 ℃ for 2 minutes hold (UDG incubation), 95 ℃ for 2 minutes hold, following with 40 cycles of 95 ℃ for 15 seconds and 60 ℃ for 30 seconds. Melting curve analysis program was added at the end of the qPCR cycle. The relative transcript levels were calculated by a comparative CT method, and the results were obtained from at least 3 biological replicas .

2.2.4 GUS staining analysis

GUS staining analysis was performed with 7-day old young seedlings, and the procedure was carried out as follows: young seedlings were immersed in freshly prepared Staining Solution and incubated at 37 ℃ incubator over night. Afterwards, the Staining Solution was changed three times with 95% ethanol in 30 min interval by maintaining the reaction at 37 ℃, additional changes were required with 95% ethanol at 37 ℃ with 1-1.5 hr interval until the chlorophyll was eliminated from seedlings. Finally, the stained tissues were stored in 70% ethanol and kept at room temperature over night. After this, stained tissues were ready for histological analysis under the stereomicroscope or long term store in 4 ℃ fridge.

The media recipes referred in this section is present as below:

MS medium: mixing 4,4 g/L Murashige and Skoog Salt Mixture (Powder), 25g/L sucrose, 8g/L phytoagar, adjusting pH to 5.7 and autoclaving.

YEB medium: mixing 5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose and autoclaving.

GUS staining solution: mixing 0.1M $NaPO_4$, pH 7.0, 10.0mM EDTA, 0.5mM $K_3Fe(CN)_6$, 0.5mM $K_4Fe(CN)_6$ and 1.0mM X-Gluc. Making it fresh prior to use.

2.3 Results

2.3.1 Over-expression of candidate genes in *Arabidopsis thaliana*

To over-express five candidate genes, pCR™8/GW/TOPO® entry vector was used for full length cDNA cloning and sequencing verification, the positive recombinant entry plasmids were subsequently used in LR reaction that integrate candidate genes' cDNA with destination vector pMDC32 which contains 2x CaMV 35S promoter. Through *Agrobacterium*-mediated plant transformation and three generations of hygromycin selection, we obtained 6 to 10 independent homozygote transgenic lines for each candidate gene. In addition, transgenic lines obtained from transforming empty vector into col-0 were used as a negative control.

2.3.2 Lower expression level of mature *miR168a* in transgenic over-expressing *AtTCP7* lines

The expression level of *miR168a* in over-expression transgenic lines were examined by real-time quantitative PCR assay. Total RNA was extracted from 14-day old seedlings by using TRIzol® reagent, and the first strand cDNA was synthesized with SuperScript® III reverse transcriptase. The candidate gene expression levels were tested in each corresponding transgenic lines. Semi quantification in agarose gels showed that each candidate gene was highly expressed in tested transgenic lines (Fig. 4A). Therefore, we further analyzed the expression level of mature *miR168a* in each transgenic lines by qPCR. For this assay, NCode™ SYBR® GreenER™ miRNA qRT-PCR Kit was used for miRNA reverse transcription and cDNA synthesis. QPCR data showed that the lower expression level of *miR168a* was detected in the transgenic lines which were highly expressing *AtTCP7*, while the expression level of *miRNA168a* did not show significant changes in the transgenic lines transformed with the other four candidate genes (Fig

4B). These indicated that by over-expressing *AtTCP7* in *Arabidopsis* might affect mature *miRNA168a* expression level, and caused a decrease in *miRNA168a* accumulation, while the other four candidate genes might not correlated with *miR168a* expression.

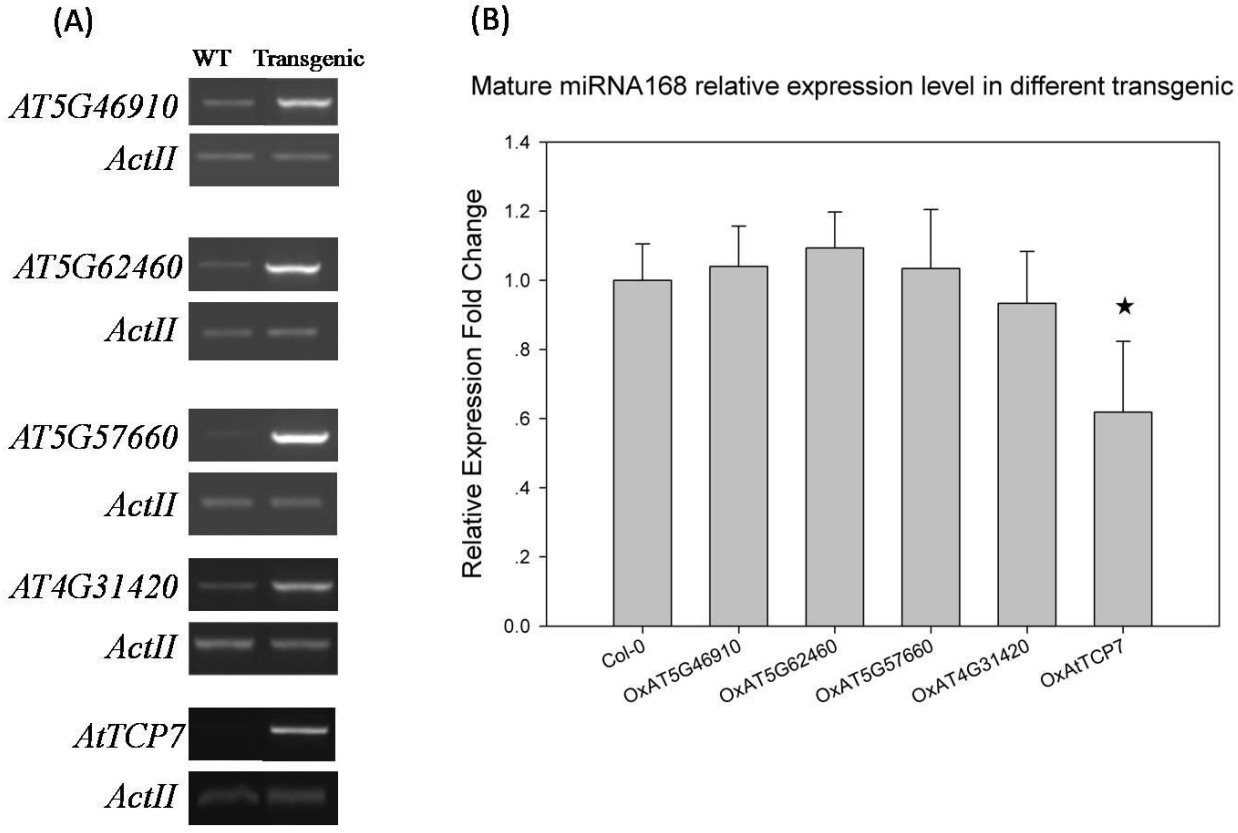


Fig. 4: Relative gene expression analysis. (A) Semi quantification analysis of transgenic lines with corresponding over-expressed candidate genes in agarose gels, *ActII* was amplified as control. (B) QPCR analysis of the mature miRNA168a expression levels among different transgenic over-expression lines. (Asterisk indicate the level of significance compare with WT, $p<0.05$, *t-test*)

2.3.3 *AtTCP7* may not directly interact with promoter of *miR168a*

Although the qPCR showed the difference of *miR168a* expression level in transgenic lines obtained by over-expressing *AtTCP7*, we could not make conclusion that *AtTCP7* directly regulate *miR168a* expression. A further experiment was performed to investigate the interaction between *AtTCP7* and promoter of *miR168a* by crossing over-expression lines of *AtTCP7* (OxTCP7) with pMiR168a::GUS lines. The transgenic lines of pMiR168a::GUS was generated in previous lab work, it was constructed with approximately 1500bp upstream of pre-miRNA168a region to drive *GUS* gene expression. By crossing with OxTCP7 lines, the difference in *GUS* expression pattern would indicate the interaction of *AtTCP7* with promoter of *miR168a.*

We used three independent OxTCP7 lines to cross with pMiR168a::GUS line. After two generation selection, positive lines containing both OxTCP7 and pMiRNA168a::GUS were chosen for GUS staining analysis. The staining results showed that, as control, the *GUS* expression pattern was detected mainly on root tip, shoot apical meristem and vascular tissues in pMiR168a::GUS line , while the major *GUS* expression pattern of crossing lines was shown the same as control(Fig. 5), which indicated that *AtTCP7* may not directly interact with *miR168a* promoter.

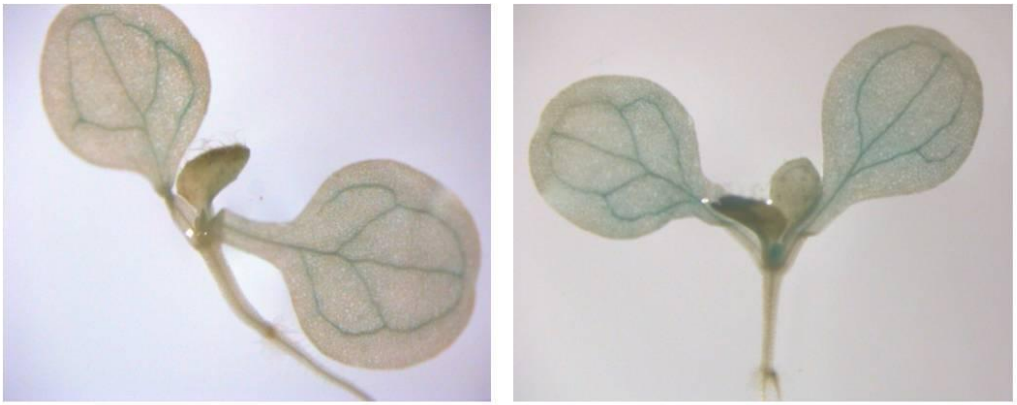


Fig. 5: GUS staining analysis of crossing lines *pMiR168a::GUS* X *2x35S:: AtTCP7* at 7DAG.

2.4 Discussion

In this chapter, we demonstrated that the over-expression of *AtTCP7* caused decreasing in mature *miR168a* accumulation. However, the analysis of *AtTCP7* over-expression lines which were crossed with GUS lines driven by *miR168a* promoter showed a improbable interaction between *AtTCP7* and promoter part of *miR168a*.

The accumulation of mature miRNAs depends on several aspects[37], as described in the section **1.1.2**, the biogenesis of mature miRNAs is coming from complex and tightly controlled pathways, and the process which miRNAs are loaded onto AGO1 protein in cytoplasm can also stabilize miRNAs in order to avoid degradation[17-18]. In our study, among the five candidate genes, beside *AtTCP7*, the over-expression lines of candidate genes didn't show the differences on the accumulation of mature *miR168a*, and no obvious phenotype could be detected in those over-expression lines (data not show). While the over-expressing *AtTCP7* lines displayed a decrease of mature *miRNA168a* level, and a variety of growth defects (described in chapter III). This indicated that *AtTCP7* may affect *miRNA168a* expression, while the other candidate genes have less possibility to involve in the regulation of *miR168a*. Therefore, *AtTCP7* was chosen as a potential candidate gene for further DNA-Protein interaction analysis.

Due to the complexity in maintenance of mature miRNAs, the changes of mature *miRNA168a* levels in our data could not reach a conclusion that *AtTCP7* directly regulates *miRNA168a* expression at transcriptional level. thus, we performed a crossing analysis between *pMiR168a::GUS* reporter line and the *AtTCP7* over-expression lines to demonstrate the DNA-Protein interaction. Regarding the methods in verify DNA-Protein interaction, there are several ways to testify the interaction *in vivo* and *in vitro*, for instance Ch-ip, EMSA, DNase-I footprint, or transactivation of a reporter gene [38]. In our case, it was facilitated for us to perform a crossing experiment between *pMiR168a::GUS* reporter line and the *AtTCP7* over-expression line, since the pMiR168a::GUS reporter line was already generated in previous evolutionary study of *miR168a* in our group[39]. The results of GUS expression pattern in

crossing lines did not show a significant difference compared with *pMiR168a::GUS* control line. As a strong promoter of 2x35S, the expression of *AtTCP7* was extremely up regulated across different tissues and developmental stages, however, the GUS expression pattern in crossing lines didn't affected by this high expression of *AtTCP7*, this indicated that AtTCP7 may not independently and directly interact with promoter part of *miRNA168a*.

With the final verification from the GUS reporter line, also the last of the candidate regulators of *miR168a* resulted to be a false positive. The repetition of the yeast one hybrid screening with a transcription factor-specific library was envisioned as a possibility to reduce the high number of false positives that plagued the first round of screening. Given the extreme workload associated with the creation of such library, therefore, we tried to establish specific collaborations with a German group, which had already constructed a library with more than 1200 transcription factor open reading frames from *Arabidopsis*[40], our work turned out to be either repeat the yeast one hybrid experiment or further characterize the gene function of *AtTCP7*. Before establishment of the collaboration, however, a research group in China reported that *miRNA168a* and *ARGONAUTE1* were involved in abscisic acid homeostasis and response to abiotic stress, and identified the ABF protein in *Arabidopsis* as the transcription factor interacting with the promoter of *miRNA168a* through a highly conserved ABRE motif [41]. This finding significantly decreased the potential of finding novel transcription factors regulating *mir168a* through a second yeast one hybrid screening. Therefore, we decided to focus instead on the in-depth characterization of *AtTCP7* gene function.

The function analysis of *AtTCP7* was barely at primary stage, recent study showed that the available T-DNA insertion lines were all located out side of *AtTCP7* coding sequence, and the RT-PCR also confirmed that the alleles *tcp7* lines did not affect the *AtTCP7* expression. Therefore, the researcher further established a *AtTCP7* fused EAR-repressor domain transgenic line to produce loss-of-function phenotypes of *AtTCP7*, however, only the general phenotype of curved leaves and smaller cells on adaxial and abaxial surfaces were described, which was lack

of in-depth characterization and statistical calculation[42], thus, the over-expression of *AtTCP7* in *Arabidopsis* is essential for its functional characterization and investigating interaction with miRNA168a. In addition, as features of TCP family, most of TCP genes have redundant functions and protein-protein interaction in binding with target genes[43] (described in chapter III), that implies a probability that the interaction between AtTCP7 and promoter of *miRNA168a* may require additional conditions *in vivo*. The most common interaction of TCP genes appeared to be occurred among phylogenicly close related TCP genes, and for *AtTCP7*, it may need a further study to confirm.

Chapter III

Over-expression of AtTCP7 causes a variety of growth defects in *Arabidopsis thaliana*

3.1 introduction of TCP gene family

TCPs are a plant-specific genes family, characterized by the presence of a 59-amino acid basic helix-loop-helix (bHLH) motif and by a DNA-binding domain, which together allow the products they code for to function as dimeric transcription factors. The name TCP derives from the three genes which were first observed to share a bHLH domain: ***T****eosinte branched1*(tb1) in *Zea mays*[44], ***C****YCLOIDEA*(*CYC*) in *Antirrhinum majus*[45], and ***P****ROLIFERATING CELL FACTORS 1* and *2* (*PCF1* and *PCF2*) in *Oryza sativa*[46]. In 1999, Pilar Cubas first described the highly conservation among these genes and also demonstrated the existence in the genome of *Arabidopsis thaliana* of a series of genes not previously characterized that shared the same bHLH motif[47]. In addition to the bHLH motif, which is thought to mediate protein-protein homo- and hetero-dimerization between members of the TCP family, a second region, called the 'Basic' motif is common to all TCP proteins. The TCP genes are present in various plant species, where they act in a multiplicity of pathways mainly related with DNA replication, cell-cycle regulation, hormone responses, and plants development.

The phylogenetic analysis of TCP family genes from higher and lower plants indicate the ancient emergence of TCP genes before the split of the *Zygnemophyta*. During plant evolution, TCP genes continuous expansion through gene duplication and diversification generated progressively larger families across different plant species [48]. In *Arabidopsis*, whole genome homology searches based on the bHLH domain uncovered 24 members of the TCP gene family (Table III) [49]. Based on the difference of the bHLH domain, TCP genes are classified into two main classes: Class I (also called as TCP-P class) and Class II (also called as TCP-C class). Class I TCP genes contain rice *PCF1* and *PCF2* type domain, while class II genes are more similar to *CYC* and *tb1*. Class I and class II TCP proteins differ, respectively, for the absence and presence of four amino acids in the 'Basic' region. The character allowing placement of TCP proteins in class I and class II is a four-amino acid deletion in Class I, it also further classified into three subclasses *TB*-type, *CIN*-type, and *CYC*-type(Fig. 6).

Table III. The TCP transcription factor family in *Arabidopsis*

Locus	Name	Class	Type	Locus	Name	Class	Type
At1g67260	AtTCP1	II	CYC/TB1	At3g02150	AtTCP13	II	CIN
At4g18390	AtTCP2	II	CIN	At3g47620	AtTCP14	I	PCF
At1g53230	AtTCP3	II	CIN	At1g69690	AtTCP15	I	PCF
At3g15030	AtTCP4	II	CIN	At3g45150	AtTCP16	I	PCF
At5g60970	AtTCP5	II	CIN	At5g08070	AtTCP17	II	CIN
At5g41030	AtTCP6	I	PCF	At3g18550	AtTCP18	II	CYC/TB1
At5g23280	AtTCP7	I	PCF	At5g51910	AtTCP19	I	PCF
At1g58100	AtTCP8	I	PCF	At3g27010	AtTCP20	I	PCF
At2g45680	AtTCP9	I	PCF	At5g08330	AtTCP21	I	PCF
At2g31070	AtTCP10	II	CIN	At1g72010	AtTCP22	I	PCF
At2g37000	AtTCP11	I	PCF	At1g35560	AtTCP23	I	PCF
At1g68800	AtTCP12	II	CYC/TB1	At1g30210	AtTCP24	II	CIN

3.1.1 The binding Properties of TCP proteins

Early biochemical studies of TCP domain in rice allowed the functional characterization of the PCF1 and PCF2 proteins, which were demonstrated to be transcription factors. These studies further pinpointed the involvement of PCF1 and PCF2 in the positive regulation of rice cell proliferation by transcriptional activation of the *PCNA* (ACRONYM) gene, master controller of cell cycle in plants. Among the different domains of TCP genes, the 'Basic' domain is highly conserved in all family members, and deletion of this region completely abolishes the binding of the protein to DNA, as demonstrated by yeast-one-hybrid assays using mutant PCF1 [46]. Despite their overall high sequence homology, TCP proteins from class I and II have different DNA-binding specificities. EMSA assays with random binding-site primers in rice PCFs and *Arabidopsis* TCPs, for instance, revealed different consensus binding sites for class I and class II factors: the consensus for class I is GTGGGNCC, while for class II it is GTGGNCCC [50-51].

These studies also demonstrate that the main aminoacidic residues of class I and class II TCPs responsible for target site specificity are the position 11 (Gly) in the basic region of class I TCP domain and the equivalent residue 15(Asp) of class II TCPs. Reciprocal mutations of the basic region of AtTCP16, indeed, display an alternation of preference, especially for a nature class I protein contain an Aspartic acid in position 11, which reveal a preference for class II binding-site sequence [51].

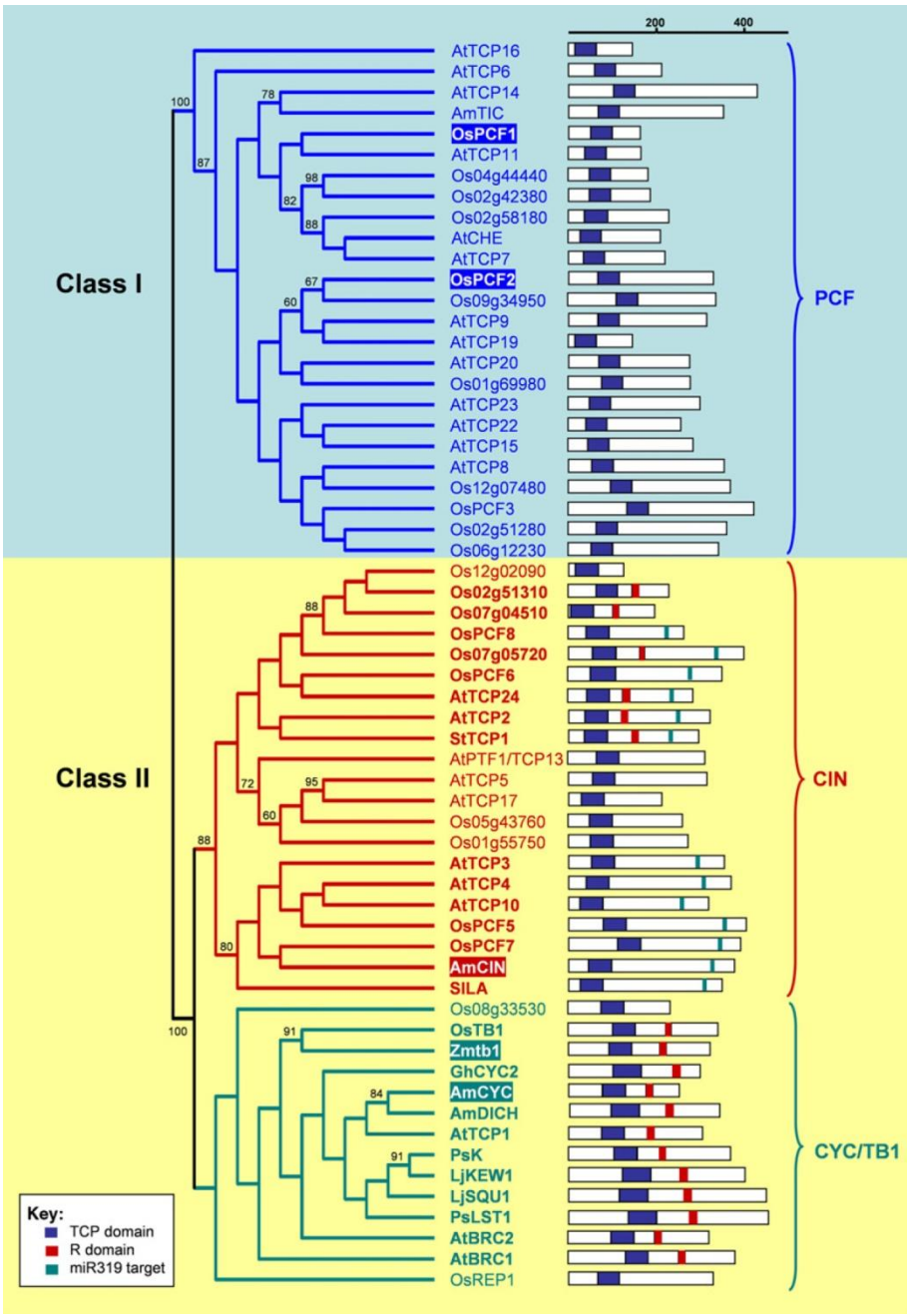


Fig. 6: Phylogenetic tree of TCP gene family among predicted TCP proteins of the eudicot *Arabidopsis thaliana* (At), monocot *Oryzasativa* (Os) and representative members of *Antirrhinum majus* (Am), *Lotus japonicus* (Lj), *Solanum lycopersicum* (Sl), *Gerbera hybrida* (Gh), *Solanum tuberosum* (St),*Pisum sativum* (Ps) and *Zea mays* (Zm). Figure courtesy of Ref[52]

3.1.2 TCP Protein-Protein interaction

Dimerization of TCP proteins and their interaction with target DNA are complex processes, which vary in function of the partners involved: some combinations of TCP monomers can bind to their target DNA only after they form homo- and hetero-dimers , while others can dimerize even in the absence of target DNA. For instance, AtTCP4 requires homodimer formation for DNA binding, as indicated by the fact that aminoacidic mutations at dimer interface can abolish the DNA-protein binding capability [53]. AtTCP11 is able to interact with AtTCP15 in yeast two-hybrid assay, the heterodimers formation of AtTCP11 and AtTCP15 also showed an increasing of DNA-binding efficiency[54].

In addition to form dimers within family members, several reports also show that TCPs are able to interact with a variety of other proteins. AtTCP10, for instance, interacts with histidine-containing phosphotransmitters AHP2 and AHP3[55], which have been implicated in signal transduction of the hormone cytokinin pathways, thus indicating the involvement of AtTCP10 in cell division through cytokinin-dependent pathways. Another TCP protein, AtTCP20, shows physical interaction with AtPurα through yeast two-hybrid assay, which is a DNA- and RNA- binding protein involved in control of transcription, replication and cell cycle regulation. Therefore implicating the function of AtTCP20 related to the control of the cell cycle [56].

The ability of TCP proteins to from homo- or heterodimers with a variety of other TCP and non-TCP transcription factors suggest that the protein-protein interactions may combinatorially

increase the diversity of functions carried out by TCP gene family members and may provide additional specificity to TCP protein target recognitions.

3.1.3 TCP functional redundancy

Like many other gene families where extensive duplication and diversification took place during the radiation of land plants, the TCP genes are expected to have similar binding-site specificities and considerable levels of functional redundance. Analysis of single *TCP* knockout mutants, in fact, confirmed extensive functional redundance among TCP genes, as single knockout TCP mutants display a few distinct phenotypes, which are mainly subtle. On the other hand, double or multiple knockouts have intense phenotypes [57-59]. Due to the high redundance and difficulties in obtaining multiple mutations, the complete elucidation of the role of single TCP genes has remained elusive. The TCP genes subject to post transcriptional gene silencing by endogenous microRNAs represent an exception, as over-expression of the microRNA allowed targeting of several of the genes at once and resulted in a fast system to associate phenotype abnormalities and functions to TCP targets. The *JAGGED AND WAVY* (*JAW-D*) mutation of *Arabidopsis*, for instance, results from over-expression of *MiRNA319a* [60], which leads to the down regulation of multiple class II *TCPs*, namely *TCP2*, *TCP4* and *TCP10*. Later analysis of single knockout mutants of these TCP genes showed a weak phenotype with slightly epinastic cotyledons and subtly enlarged leaves. While *TCP2 TCP4* double mutants displayed stronger phenotypes in leaf size and uncovered a slightly crinkling at the edge of leaf. Finally *TCP2 TCP4 TCP10* triple mutants had obvious defects, close to the ones observed in the *jaw-D* mutant. These observations indicate the *jaw-TCPs* are paralogs with functionally redundant functions [57]. Other examples of functionally redundant TCP gene pairs are *TCP9*/*TCP20* [61], and *TCP14*/*TCP15* [59].

In order to help elucidating the functional redundance among TCP genes, extensive interaction networks among TCP genes have been tested. Both microarray data of TCP gene expression patterns and protein-protein interactions from yeast two-hybrid experiments were used to predict potential interactions among pairs of TCP genes[62]. The results of this

classification efforts point to extensive interaction potentially existing among TCP family members, with some of the genes interaction with as many partners. This work lay an important foundation for future TCP genes' studies, but at same time the high functional redundancy uncovered in this gene family highlights the difficulty of functional characterization of TCPs.

3.1.4 Functions of TCP genes

As transcriptional DNA-binding factors, TCPs mainly regulate target genes expression. With the initial studies on rice PCF1 and PCF2, the general roles of TCPs were claimed to be chiefly the regulation of cell proliferation and plant growth[52]. Later works on more family members, however, uncovered several additional functions, showing that TCP genes participate in multiple processes related to plant growth and through different mechanism and pathways. A certain degree of functional differentiation between class I and class II TCP genes was also uncovered.

Currently, a consensus has been established about the fact that mainly class I TCP genes regulate cell proliferation. In *Arabidopsis*, most class I single mutants show mild or even no phenotype, thus, analysis based on over-expression transgenic lines or TCPs fused to the EAR repressor domain have been mainly used for functional characterization. *AtTCP20::EAR* transgenic lines display a deregulation in the balance between cell proliferation and differentiation. Especially in root, the AtTCP20::EAR protein disrupts root development with an abnormal expansion of epidermal and cortical cells in the rapid elongation zone, which exhibit a maximal cell-expansion phenotype with disorder of cell organization [63]. Further studies of target motifs uncovered that TCP20 protein preferentially bind to the GCCCR element in the promoter of *CYCB1;1* both *in vitro* and *in vivo*, confirm the role of TCP20 in promoting cell growth and division [64]. Also the characterization of another class I TCP gene, AtTCP15, was preformed by means of a fusion protein with the EAR repressor domain. Constitutive expression of TCP15-EAR lead to a lethal phenotype at seedling stage, while the expression under the promoter of *AtTCP15* itself caused several developmental defects, especially in leaf petioles, flower pedicels, and anther filaments. The most remarkable phenotype, however, was the

decrease of leaf cell size both in abaxial and adaxial sides, and loss of the typical puzzle-like cell form in adaxial side, which indicate that converting *AtTCP15* into a repressor results in inhibition of cell growth and differentiation. Further gene expression analysis in the same transgenic lines showed an increase expression level in boundary specific genes *LOB*, *CUC1*, and *CUC2*, and the physical binding of AtTCP15 to the promoter of *IAA3/SHY2*. Taken together, these results suggest that TCP15 is involved in the regulation of boundary-specific genes and that it can affect auxin homeostasis [65]. In a further study, another TCP class I gene, *AtTCP21* (or *CHE*), was found to specifically interact with the *CCA1* promoter. CCA1 is a key regulator of the circadian system that directly binds the promoter and represses the expression of the core regulator TIMING OF CAB EXPRESSION 1 (*TOC1*). Taken together, these results indicate that AtTCP21 is a circadian oscillator of *Arabidopsis* [66].

Different from class I TCPs, the class II TCP genes are mainly preventing cell proliferation and plant growth. Mutant lines of *TB*-type class II TCP genes *AtTCP18* (*BRC1*) and *AtTCP12* (*BRC2*) show an increase in number of rosette branches and in frequency of bud outgrowth compared with wild-type, supporting a repressive role of BRC genes in the proliferation and growth of *Arabidopsis*. In addition, *BRC1* is strongly downregulated in mutants of the *Max* gene, which functions in preventing auxin transport and blocking bud outgrowth, thus suggesting that BRC1 is involved in auxin-induced control of bud growth [67]. Additional evidences of the involvement of class II TCP genes in suppression of cell proliferation and plant growth comes from studies of another subclass of TCP genes, *CIN*-type class II TCPs, which have been characterized in depth through mutant lines. The *CIN*-type subclass of genes include AtTCP2, 3, 4, 5, 10, 13, 17, and 24. Among them, expression of AtTCP2, 3, 4, 10, and 24 is controlled post-transcriptionally through down-regulation of miRNA319. As described in section 'TCP functional redundancy', *CIN*-type TCPs show their function negative regulate cell proliferation [60], especially in leaves.

Other functions of TCPs have been also reported in gametophyte development[68], flower

development[45, 69], hormone pathways[61, 67], and seed germination[70]. More importantly, class I and class II proteins are able to antagonistically co-regulate the same targets, like *PCNA* or *CYCB1;1*[64], which indicate the complexity of TCP family functions and the importance of regulation by TCPs.

In summary, TCP genes are involved in several aspects of plant architectures formation. Through binding to similar but identical consensus target DNA sequences, class I and class II TCP genes control plant growth through different pathways. However, the high redundancy of TCP gene family has till now hindered their functional characterization. Several TCP family members still await in-depth characterization.

3.2 materials and methods

Arabidopsis thaliana was grown under normal conditions as mentioned in section **2.2.2.** Phenotypic analysis were carried out regarding several different aspects of *Arabidopsis* growth and development: root morphology, flowering time, seed production and flower architecture. For root length measurement, wild type Col-0 and OxTCP7-5 lines were grown on vertical MS plates, the length of root and number of lateral roots were calculated at 7 days after germination, and the data were collected from 40 seedlings from each genotype. For flowering time analysis, 30 plants of each genotype were grown in individual pots under the same environmental conditions. Bolting time was counted as the number of days passed after germination until when flower bolts were 1 cm in length, and the flowering time was counted as the date of emergence of the first flower for each plant. The number of rosette leaves was also counted at these two time points, respectively. For seed production, average length of silique and number of seeds per silique were measured and counted from more than 100 fully mature siliques. For the determination of differences in inflorescence architecture, +1 flowering stage floral organs were chosen for analysis. Average length of pistils and stamens were calculated under Leica MZ75 microscope from 30 flowers in 10 individual plants. Two-tails Student’s t-test and Tukey’s test were applied for the statistical comparison of significance of differences among mean values.

3.3 Results

The work described in this section displays the phenotypic differences observed between transgenic lines of over-expressing *AtTCP7* and the corresponding WT lines. In general, taken together, the results show that *AtTCP7* over-expression causes the delay of plant development, and several growth defects on root, leaves, and flowers. which indicate *AtTCP7* function involved in plant development.

3.3.1 Over-expression of *AtTCP7* cause root development defects.

The root development defect is the most obvious phenotype at the seedling stage: transgenic over-expression of *AtTCP7* (OxTCP7) have a clear reduction in root length as compared to wild type (WT) Col-0 and the negative control (NC) plants at 7 days after germination (Fig, 7A). Seven individual transgenic lines resulting from independent transformation events were characterized with respect to root length and statistical analyses were performed to test whether mean root length significantly differs between WT and transgenic lines. Among the seven single copy lines, only one showed no significant difference as compared to wild-type, while the remaining six lines had roots that were significantly shorter than WT (Col-0 ecotype, Fig. 7C). At first glance, the different transgenic lines showed varying degrees of root length reduction, therefore, ANOVA analyses and Tukey's test were used for additional testing of statistical differences: The transgenic lines formed clusters with severely (~9.8mm) and mildly (~13.5mm) reduced mean root length compared with WT (~20.4mm) (Fig, 7C). Moreover, OxTCP7 lines also showed an increase in the number of lateral roots growing at developmental stages comparable to that of WT (Fig, 7D). On day 7 after germination, OxTCP7 lines contained 2 lateral roots on average per seedling in contrast with less than 0.5 lateral roots in wild-type. Expression analyses by qPCR of OxTCP7 lines uncovered an inverse relationship between *AtTCP7* transgenic expression and root length (i.e. the higher the expression, the shorter the root was; Fig. 7B), indicating that *AtTCP7* controls root length in a dose-dependent way.

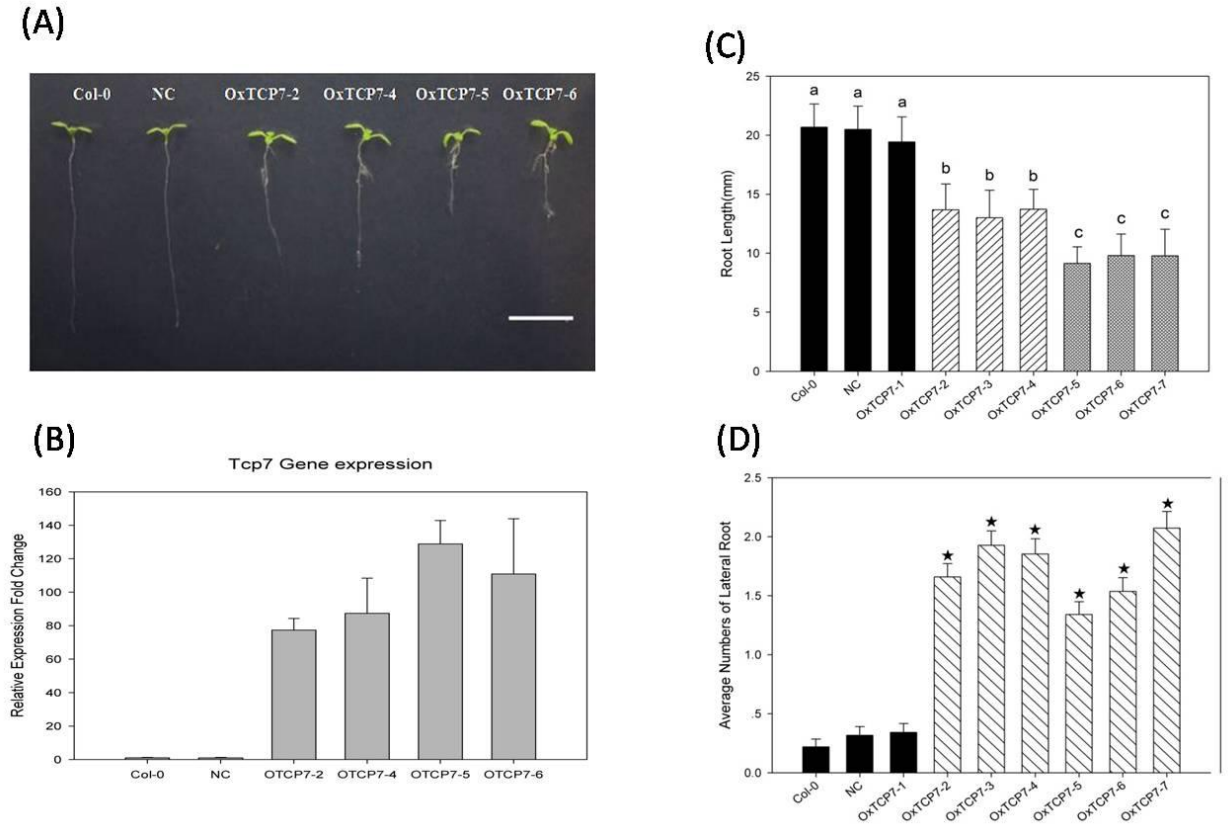


Fig. 7: Over-expression of *AtTCP7* affect root length and architecture. (A) Phenotype of 1 week-old Arabidopsis lines over-expressing *AtTCP7* and wild-type Col-0. Scale bar: 1 cm; (B) Quantitative analysis of *AtTCP7* expression level across different over-expression lines; 7DAG (C) Mean root length of WT and *AtTCP7* over-expressing lines at 7 DAG. Bar colors correspond to the three groups of root lengths identified by lowercase letters. (D) Mean number of lateral roots of transgenic and WT plants at 7 DAG. Col-0: WT Arabidopsis Columbia-0 ecotype; NC: empty vector negative control;. OTCP7-2, OTCP7-4, OTCP7-5, OTCP7-6: transgenic lines over-expressing *AtTCP7* under the control of the 35S viral promoter, each line represent an independent transformation event. Mean values and standard deviations (vertical bars) in (C) and (D) have been calculated for each genotype from a sample (n>40) of randomly selected

individuals. Statistical significance of observed differences has been calculated with a Tukey's test.

3.3.2 Over-expression of *AtTCP7* delay the flowering time.

Mutation or ectopic expression of TCP class I family genes have been previously shown to affect circadian clock, flowering time and pollen development [71-72]. Therefore, two over-expression lines with different dosage effects, severe (OxTCP7-5) and mild (OxTCP7-2), were selected to assess the function of *TCP7* in different organs at later developmental stages during vegetative and reproductive phases.

Under long day conditions(16h/8h of light/dark), both over-expression lines bolted and started to flower significantly later than WT(Fig. 8A), as showed in the box plot in Fig. 8B. Over-expression line OxTCP7-2, which has a mildly short root phenotype, display a delay on flowering time of about 3 days, while in another over-expression line with stronger root phenotype, OxTCP7-5, the delay increased to 6 days. By counting rosette leaf numbers at bolting and flowering stages, the transgenic lines had a significantly higher number of leaves than WT (nearly double), in line with previously reported typical flowering time delay phenotypes. These results indicate that the over-expression of *AtTCP7* strongly affected the normal development of Arabidopsis plants. Moreover, the line OxTCP7-5, which has the highest level of *AtTCP7* gene expression, was dramatically slow in vegetative growth compared with WT and line OxTCP7-2; however, when the plant fully matured, no visible difference in plant height was observed.

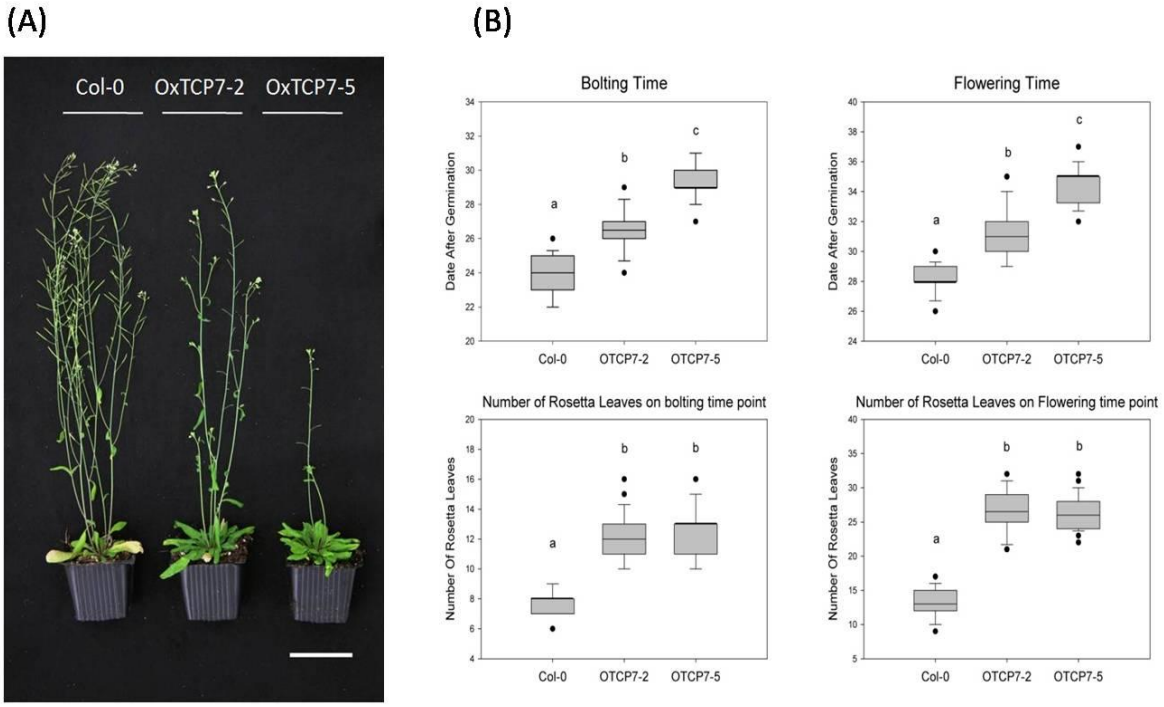
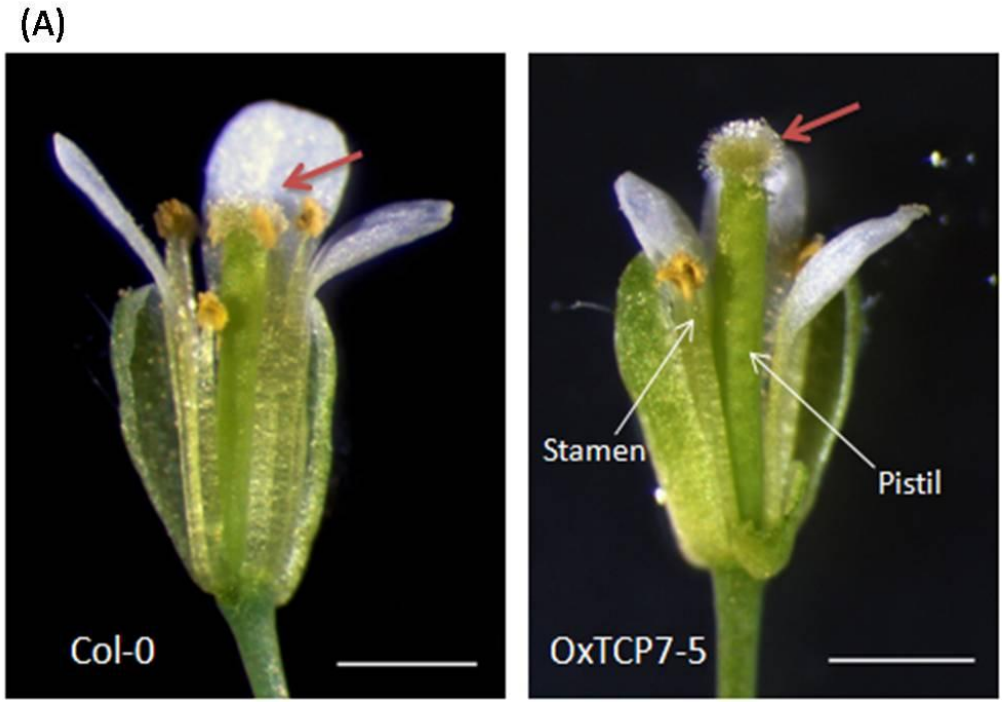


Fig. 8: Over-expression of *AtTCP7* delays growth and flowering time. (A) Phenotype of 5 week-old Arabidopsis lines over-expressing *AtTCP7* compared to WT *Arabidopsis* in long day condition, scale bar= 5 cm. (B) Box-plots show bolting, flowering times(days) and the numbers of Rosetta leaves of WT and TCP7 over-expressing lines. Horizontal lines in each box indicate the median value of each group samples(n=40). Statistical significance of observed differences has been calculated with a Tukey's test, the lowercase letters correspond to significant different groups($p<0.01$). Col-0: WT Arabidopsis Columbia-0 ecotype. OTCP7-2 and OTCP7-5: representative transgenic lines with, respectively, medium and high levels of *AtTCP7* expression.

3.3.3 Over-expression of *AtTCP7* affects the floral architecture

We further analyzed floral architecture and autonomous seed set in wild type Col-0 and OxTCP7-5. In wild type Col-0, anthers were placed at or slightly above the stigma at the +1 flower stage (Fig. 9A). By contrast, in OxTCP7-5, the stigma had stronger exsertion and was more distant from anthers than in WT. Statistical analysis based on the length of pistil and

stamen shows that OxTCP7-5 contains significantly longer pistils and shorter stamens (Fig. 9E), while in wild-type Col-0 no significant difference on the length of these two organs can be detected. In normal growth condition, the distance between anthers and stigma in self-pollinated plants is minute, which allows autonomous deposition of pollen grains on the stigma, thus the exsertion of stigma found in OxTCP7-5 indicates the development of a physical barrier to self-pollination in transgenic plants. In addition, we measured the average length of silique and the autonomous seed production per silique. Statistical analyses uncovered a significant reduction of average silique length (Fig. 9C), associated to a lower seed set as compared with WT (Fig. 9D), indicating that the reduction in seed set could be due to the partial herkogamy (i.e. the separation between anthers and stigma) observed in flowers.



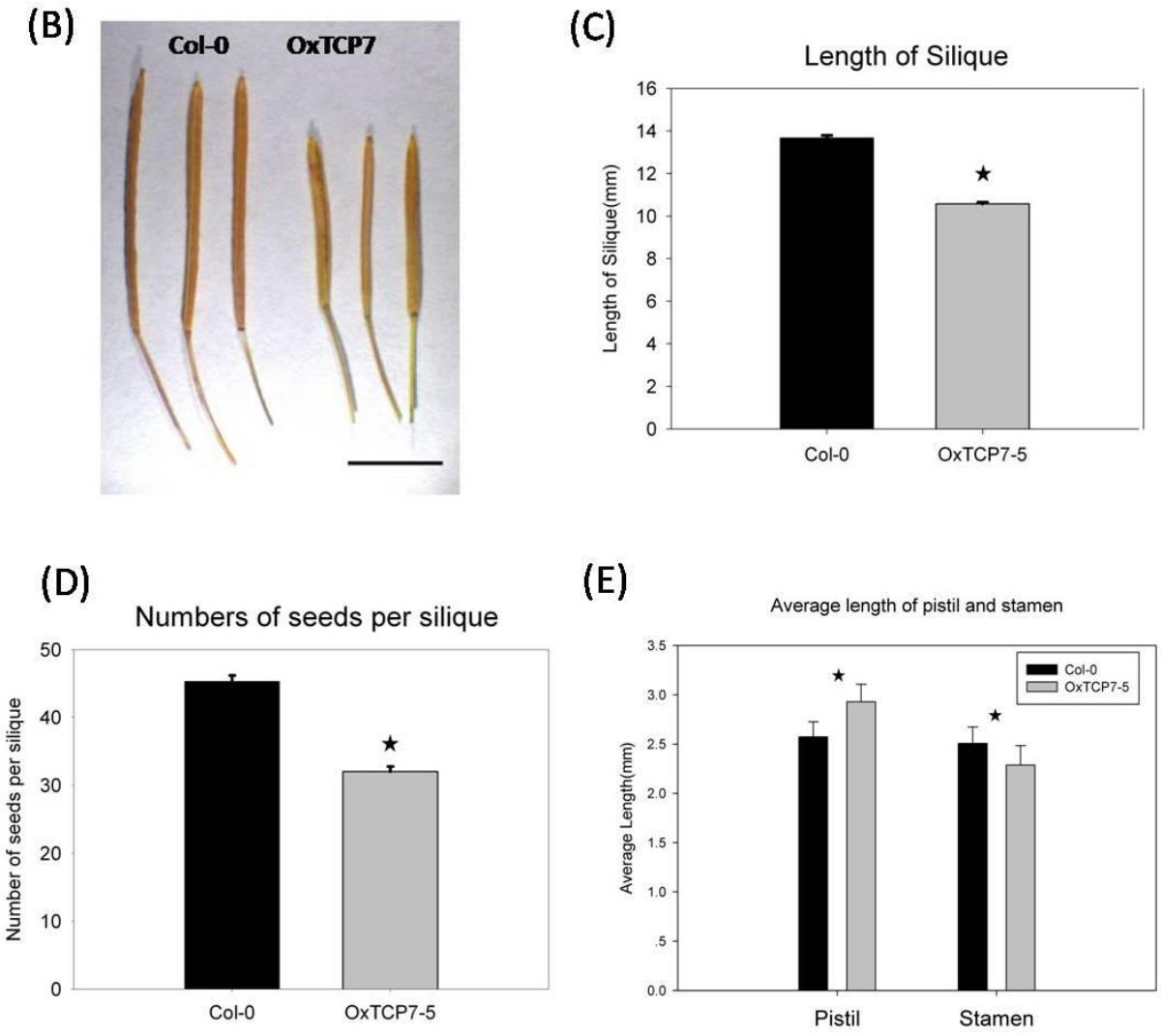


Fig. 9: Over-expression of *AtTCP7* cause a excessive elongation of pistil which lead to a decrease of self-pollination (A) Phenotype of flowers +1 stage on wild type Col-0 and overexpression line OxTCP7-5, scale bar= 1 mm; (B) Phenotype of siliques on WT and OxTCP7-5; scale bar= 1 cm; (C)and (D) Average Length of siliques and numbers of seeds per

siliques on WT and OxTCP7-5. Calculate from sample(n>100), bars show mean values and standard errors (vertical bars); (E)Average length of pistil and stamen on WT and OTCP7-5. Calcuate from samples(N=30), bars show mean values and standard deviations(vertical bars). Asterisk indiate the level of significance compare with WT, $p<0.01$, t-test.

3.4 Discussion

In this chapter, we described a variety of growth defects that resulted from over-expression of *AtTCP7* in *Arabidopsis*, including short root phenotype, flowering time delay, and alternation of floral architecture. In our observation, over-expression of *AtTCP7* present a typical phenotype of short root and flowering time delay. These growth defects correlated to a dosage effect in expression level of *AtTCP7*, which indicate the phenotypes were due to the highly expressed *AtTCP7*. Simultaneously, the defects caused by over-expression of *AtTCP7* appeared in a wide range of tissues from vegetative to reproductive stages, indicating that the *AtTCP7* plays basic and important roles in plant development.

In *Arabidopsis*, several genes when mutated show pleiotropic phenotypes similar to those observed in OxTCP7 lines during roots, leaves and flower development. For instance the *SHORT-ROOT* (*SHR*) and *SCARECROW* (*SCR*) mutants display a disorganization of the quiescent center and loss of stem cell activity, which result in the cessation of root growth[73-74]. *FRIGIDA*(*FRI*) and *FLOWERING LOCUS C* (*FLC*) regulate flowering time through the autonomous floral promotion pathway [75-76]. A mutation in the RNA dependent RNA polymerase RDR6 has been reported to enhance self-incompatibility and cause stigma exsertion [77-78]. Interestingly, most of these genes share the common feature of being involved in the regulation of cell proliferation and differentiation: for instance, *SCR* regulate asymmetric cell division in the root tip, and the *SCR* mutant displays a loss of the radial organization in root, resulting in a mutant cell layer that combines cortex and epidermis [74]. Moreover, *SCR* can also affect cell division during leaf growth, which stimulates S-phase progression by regulating cell-cycle gene expression, as a result of leaf area reduction [79].

The precise placement of TCP with respect to the other genes needs, however, to be investigated in further detail, as currently no genetic evidence of *AtTCP7* control over any of these genes is available. All of these findings, however, indicate that the function of *AtTCP7* may share some common regulation in cell proliferation and differentiation with the above-mentioned genes, possibly resulting in similar growth defects in roots, leaves and flowers. Thus, we further analyzed the cell expansion and division in both roots and leaves in order to understand the function of *AtTCP7* at the cellular level.

Chapter IV

AtTCP7 affects cell expansion and division in root and leaves

4.1 Introduction of plant cell cycle

Cell cycle regulation is of pivotal importance for plant growth and development. It also shows a high conservation of basic mechanisms among all eukaryotes. Most of plant mutant with growth defects phenotypes are highly related to cell cycle control. Thus, it is likely that the developmental defects detected in the *Arabidopsis* OxTCP7 transgenic lines described in the previous chapter could be related at least in part to cell cycle regulation.

4.1.1 *Arabidopsis* Cell Division Cycle

Plant cell division cycle refer to a highly and complexly controlled process through which a single cell undergo division and generating two daughter cells. The regulatory mechanisms involved in plant cell cycle are highly conserved , as indicated both by the high proportion of plant genes homologues to cell cycle genes from other eukaryotes and by the similar phenotypes resulting from their knockouts. In addition to such set of core cell division processes shared with animal cells, however, plants cells have also specific features like, for instance, the presence of a rigid cell wall which is at the basis of the differences existing in cytokinesis. Another difference with respect to animal cells is the ability of mature plant cells to undergo de-differentiation and re-acquire totipotency, which illustrate the importance of unique plant-specific cell cycle regulators [80].

Generally speaking, cell division cycle is composed of two major phases consistently present in most eukaryotes : (1) the duplication of cellular components (interphase) and (2) the cell division (mitosis).

The duplication of cellular components is a phase characterized by active biosynthesis of cellular components, through which the quantity of genetic information and cytoplasmic organelles is doubled. In order to preserve continuity of cellular functions and stability of the whole organism, daughter cells in plants generally have to be genetically identical to their parent cell, which requires the duplication of cell contents in order to prepare for the cell division. For

this reason, this phase is also called cell interphase, i.e. the phase between two subsequent cell divisions. Interphase can be further subdivided in three different cell cycle phases:G1, S and G2. In the G1 phase the cell increases its size and a large synthesizes large amounts of mRNA and proteins necessary to prepare the synthesis of DNA which will take place in the next phase, called the S phase. Duplication of the DNA composing the cell nuclear genome mainly happens in the S phase, after which the cell enters into the G2 phase. During the G2 phase, also called the pre-mitotic phase, the cell continues to enlarge and produce the proteins composing the mitosis-related complexes. Beside these three phases, cells can temporarily or permanently leave the cell cycle and stop dividing by entering the G0 phase, which helps plants to sustain the cell quantities and maintain the functions in certain tissues. G0 cells can also re-enter the G1 phase in certain conditions or upon being subjected to specific stimulation.

The second major phase of cell division is mitosis. Mitosis encompasses five subphases or events: Prophase, Prometaphase, Metaphase, Anaphase and Telophase. Although it is a complex procedure characterized by many steps, the whole mitotic phase lasts only 10% of the whole cell division cycle (Fig, 10). In plants, mitosis starts with chromosome condensation and binding by cohesin proteins at centromere. Duplicated chromosomes then split equally migrating to the opposite sides of the cell by the pull generated by kinetochore microtubules, and form into two sets of identical daughter chromosomes. Afterwards, the new chromosomes are surrounded by the nuclear membrane, and a cell plate formed at the center of cell isolates the two nuclei. At the end, cytokinesis separate the cytoplasm and form into two daughter cells.

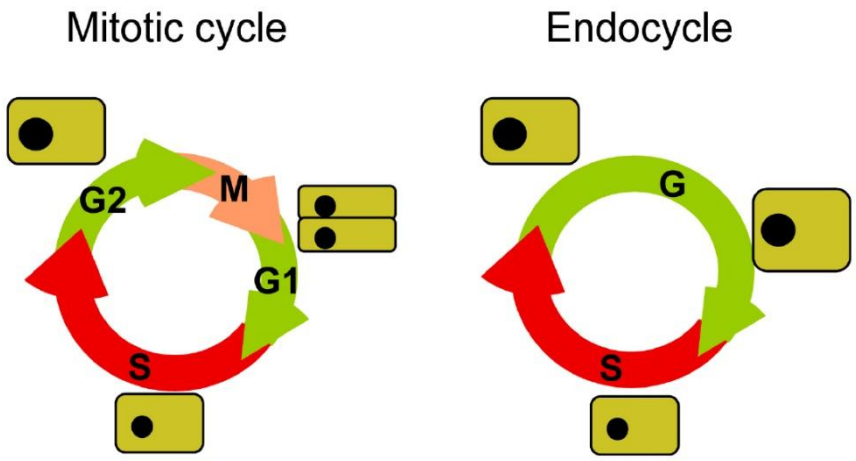


Fig. 10: Plant mitotic cycle and endoreduplication cycle. Figure courtesy of Ref [81]

4.1.2 Endoreduplication Cycle.

The basic cell cycle delineated above can, under the control of endogenous or exogenous stimulations, undergo different modifications to meet the requirements for plant development. One of the most common modifications of the standard cell cycle is endoreduplication, also called endocycling or endoploidization. Endoreduplication is characterized by a total or partial lack of the M phase, with a consequent increase of the cell ploidy level through multiple rounds of DNA replication without cytokinesis (Fig. 10). Endoreduplication has been found in a wide range of organisms, especially in mammals and plants. Megakaryocytes, for instance, are well known mammalian polyploidy cells with DNA contents up to 128n, which are specialized to produce platelets in blood. Interestingly, the ploidy levels of Megakaryocytes are directly correlated with the ability to generate platelets[82], and the higher the ploidy levels are the more platelets are produced. The fetal Megakaryocytes, which are characterized by lower ploidy levels than those in adults' blood, have a quantitative and qualitative abnormal platelets in fetus[83].

In plants, endoreduplication is more common to be found in highly specialized or

metabolically activated cells. Interestingly, endoreduplication is for this reason generally associated to economically important tissues, such as cereal endosperm, tomato fruits and cotton fibers. In *Arabidopsis*, endoreduplication is often observed in trichome growth, hypocotyl elongation and leaf expansion[84]. The determination of cell transition from mitosis cycle to endoreduplication cycle is controlled by variety of cell cycle related genes and environmental physiological conditions, like dark/light stimulations[85], water shortage, and even pathogen interaction[86]. Endoreduplication is believed to play an important role in the differentiation process of plant development, and the onset of endocycle is often regarded as the switch between cell proliferation and differentiation. In general, cell endoreduplication is considered as a process to meet the requirements for the formation of specific tissues and to support the establishment of tissue-specific functions. For instance, unicellular trichomes in *Arabidopsis* differentiate from epidermal cells of developing leaf primordia, protodermal cells committed to trichome cell fate, after cessation of cell division and the onset of endoreduplication. Normally, mature leaf trichomes undergo four endoreduplication cycles, and with the corresponding increase of ploidy levels, trichomes expand out of leaf surface and form into a particularly morphology with three branches[87], which protect plant epidermal cells from potential environment damage.

Besides the functions in cell development and differentiation, endoreduplication also occurs as a response to changes of the physiological conditions. In *Arabidopsis* hypocotyls, it was observed that an extra endoreduplication cycle was triggered by absence of light, which extremely increased the length of hypocotyls and the DNA ploidy level[88]. It is assumed that the extra endoreduplication in dark-grown hypocotyls might act as a strategy to increase the plant's chances to intercept light when it is limiting. Another example of physiological condition affecting endoreduplication is water shortage: in this case, the water deficit condition triggers a decrease in both cell size and DNA content, with the result of reducing the overall leaf area at the end of development. All these phenomena demonstrate that endoreduplication might aid plant and organ growth under stress, and protect plants in extreme environment conditions.

4.2 Materials and Methods

Microscopy and cell morphological analysis

Arabidopsis thaliana was grown under the standard conditions mentioned in section **2.2.2.** Root tissues for microscopy and Cell morphological analysis were collected from young seedlings at 5 days after germination. For tissue fixation, roots were mounted in Carnoy's solution (acetic acid : ethanol=1:3 v/v) over night. After removing the Carnoy's solution, the roots were washed 2 times with 95% ethanol, then stored in 70% ethanol for microscopy observation. The length of mature epidermal cells in differentiation zone was measured at the middle point of a root within 1-mm-region under a Differential Interference Contrast (DIC) microscope (Leica DM 2500) with Leica Application Suite LAS V3.7 software. Means (±SD) were calculated from data collected from 10 roots , and student's t-test was used for statistical analysis.

Kinematic growth analysis of abaxial epidermis cells on cotyledons and the first 1/2 true leaves were carried out with a Leica DMLB Light Microscope from 4 to 22 days after germination, harvesting samples every two days. The whole seedlings were cleaned and mounted in 95% ethanol, then placed on a glass of microscope slide for observation. Average cell numbers and cell size were calculated on transgenic OxTCP7-5 and wild-type Col-0 at each time point, and corresponding cotyledon and true leaf blade areas were also measured. The average cell numbers of 3 random points which were located in the middle of the leaf blade between tip and base were calculated from microscopic images, each point contained at least 30 epidermal cells. Data were collected from 10 seedlings for each time point, and student's t-test was used for statistical analysis. The total number of epidermal cells were estimated by the ratio between the blade size and the average cell size, and finally the cell division rates were calculated from the increased cell number of every neighboring two time points.

4.3 Results

To characterize the phenotype of transgenic plants at the cell level, static measures of cell

structure and proliferation in young seedling root and leaves were carried out.

4.3.1 OxTCP7-5 shows a reduction in root epidermal cell size.

5DAG root of wild-type Col-0 and transgenic OxTCP7-5 were investigated under the microscope. The epidermal cells in the differentiation zone of OxTCP7-5 showed a significant reduction in cell size (Fig, 11A), whereas the root meristem and elongation zone were similar to wild-type. Measurement of epidermal cell length was performed from 30 individual seedling roots, and the average length of wild-type epidermal cell was 135μm, whereas the OxTCP7-5 had 35% shorter cells with an average length of 90μm(Fig. 11B). The difference between mean values is statistically significant, demonstrating that the over-expression of *AtTCP7* affect epidermal cell size in differentiation zone of *Arabidopsis* roots. We further calculated the size of root epidermal cells at earlier developmental stages to test whether a specific developmental stage could be associated to the onset of cell size reduction. The data confirmed that the difference in length was consistently present in root epidermal cells between 2 days after germination to 6 days after germination (Fig. 11C), indicating that *AtTCP7* may affects embryonic or very early stages of post-embryonic cell division in the root.

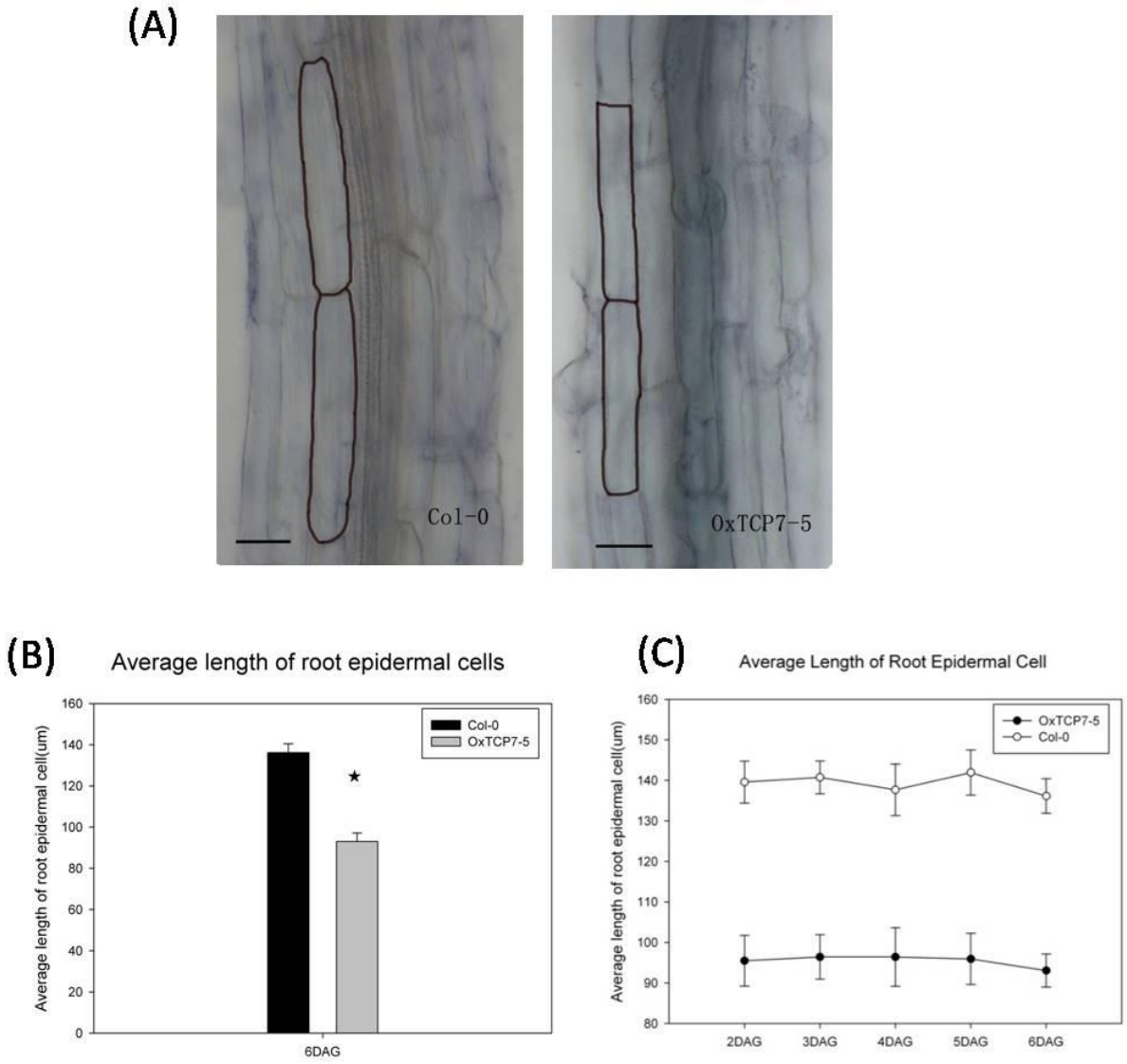


Fig. 11: Root epidermal cell in wild-type Col-0 and OxTCP7-5 (A) Microscope images on mature root epidermal cells; Scale bar= 25μm (B) Average length of root epidermal cells on Col-0 and OxTCP7-5. Calculate from samples(N=30) Asterisk indicate the level of significance compare with Col-0, $p<0.01$, t-test. (C) Time-course study of the length of epidermal cells on Col-0 and OTCP7-5 from 2DAG to 6DAG.

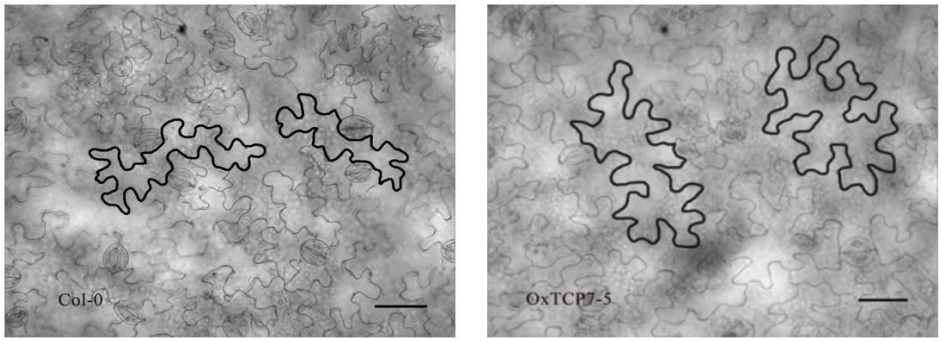
4.3.2 OxTCP7-5 affects cotyledons and true leaves cell size.

Microscopic analyses of plant leaves undergoing cell division and endoreduplication cycle at

different development stages are often used to characterized cell cycle phenotypes, as they can be easily observed. Therefore, the average cell sizes of 12 DAG abaxial epidermal cells of cotyledons and true leaves were investigated.

Figures 12A and 12B show that the average cell size of OxTCP7-5 cotyledons (4000μm^2) was larger than that of wild-type Col-0 (average of 3200μm^2). By contrast, significantly smaller cells were observed on the first pair of true leaves of OxTCP7-5, which were 840μm^2 compared with an average 1300μm^2 in wild-type. These results indicate the existence of marked tissue-specific differences of *AtTCP7* over-expression on cell size. The contrasting phenotypes observed in OxTCP7 could be the result of either a different mode of action of *AtTCP7* in the different tissues or of a different timing in the response to *AtTCP7* expression. Interestingly, while affecting the cell sizes of both cotyledons and true leaves, over-expression of *AtTCP7* didn't cause disruption of the normal cell shape (shaped like a piece of a puzzle) patterns.

(A) abaxial epidermal cells in cotyledons



(B) abaxial epidermal cells in first 1,2 true leaves

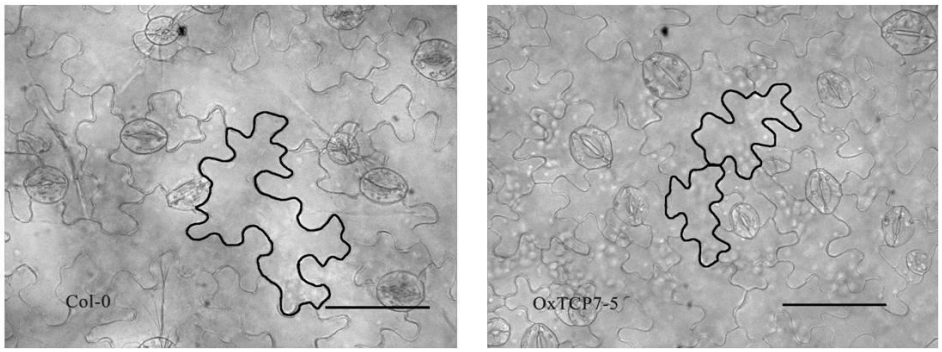


Fig. 12: Over-expression of *AtTCP7* affects cell proliferation and division in cotyledons and true leaves. (A) and (B) Microscope images on abaxial sides of cotyledons and the first 1,2 true leaves at 12DAG. Scale bar= 50μm.

To gain a deeper insight in the underlying mechanisms of the observed phenotypes, therefore, kinematic growth analyses were performed on both cotyledons and true leaves. In cotyledons, consistently with the microscopy observations carried out above, OxTCP7-5 had fewer but larger cells than WT from 10 days until 22 days after germination (Fig. 13B and 13C). Transgenic OxTCP7-5 plants have similar cell size and cell number before 8 DAG compared with wild-type Col-0, which was the stage where cell had sustained expansion with a decreased cell division rate. After day 8 after germination, however, wild-type cell expansion approached saturation, while cell division and expansion kept a balance that total cell number was smoothly increased while cell size steadily around 3500μm^2, cotyledon blade area enlarged until the cotyledons fully expanded around 18 days after germination. By contrast, the cotyledon epidermal cells of OxTCP7-5 kept expanding further expansion even after 8DAG, exhibit a larger cell area than wild-type (Fig, 13B). Simultaneously, cell division rate resulted lower than Col-0 around 10 days to 14 days after germination (Fig, 13D), which caused a significant reduction in the total cell number of OxTCP7-5 compared to wild-type. At 14DAG, OxTCP7-5 cell expansion reached saturation with average cell size around 4500μm^2, and only minor differences were found in total cotyledon area compared to WT. Interestingly, however, a constant difference in cell sizes were observed between WT and OxTCP7-5 during the whole observation, indicating that the bigger size of cells compensated for their lower number in the *AtTCP7* over-expression line. These analyses demonstrate that the major differences caused by *AtTCP7* over-expression took place in cotyledons between 8 days and 10 days after germination, which may indicate the prematurely exit from cell division cycle in OxTCP7-5 transgenic cotyledons, as a consequence of the fewer cells generated and cell over expanded.

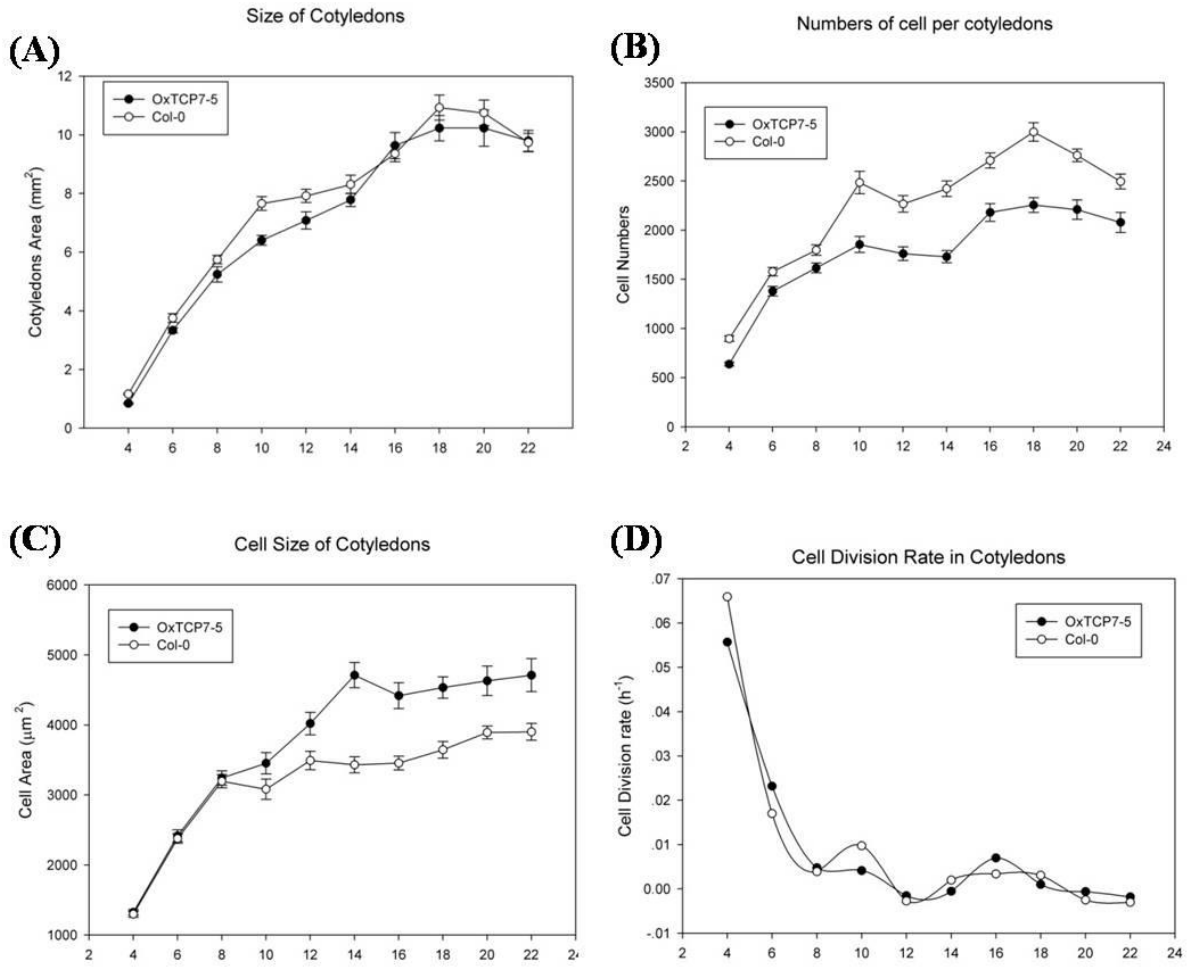


Fig. 13: Kinematic Analysis of Cotyledon Growth. (A) Leaf blade area; (B) Average cell numbers on cotyledons; (C) Average cell size on cotyledons; (D) Cell division rate in cotyledons. Error bars give standard errors (n=10).

In true leaves, the differences between transgenic OxTCP7-5 and wild-type were larger than in cotyledons but of opposite sign: the leaves of OxTCP7-5 had on the average more cells of smaller size than the wild-type. However, the differences in average size and number of leaf cells of both genotypes decreased till nearly disappearing at the end of leaf maturation (22DAG; Fig. 14B and 14C). The rate of cell division in transgenic line over-expressing *AtTCP7* was asynchronous with that of WT (higher at 12-14DAG, lower at 16-18DAG), indicating a

perturbation of normal patterns of cell cycle and/or expansion. Wild-type Col-0 epidermal cell had sustained expansion until 12DAG, with cell area approaching saturation around 1400μm^2, while the cell division rate dropped to lower level. Afterwards, the total cell number continued to grow at a constant but low division rate until 18-20DAG, when cell stopped both expansion and division, becoming fully expanded mature leaves (Fig, 14C and 14D). By contrast, from 10DAG to 14DAG, the growth of OxTCP7-5 true leaves cells showed a higher cell division rate compared with Col-0, and total cell number increased intensively, resulting in significantly more epidermal cells than in wild-type, while the cell size stabilized at around 800μm^2. After 14DAG, the total cell number reached saturation and growth stopped, the correspondingly cell expansion increased instantly. At 16-18DAG, OxTCP7-5 true leaves cells were fully expanded and matured. Taken together, these results indicate that, unlike in cotyledons, over-expression of *AtTCP7* in true leaves promoted cell division and delayed the exit from cell division, anticipating cell division and posticipating their expansion with respect to wild-type.

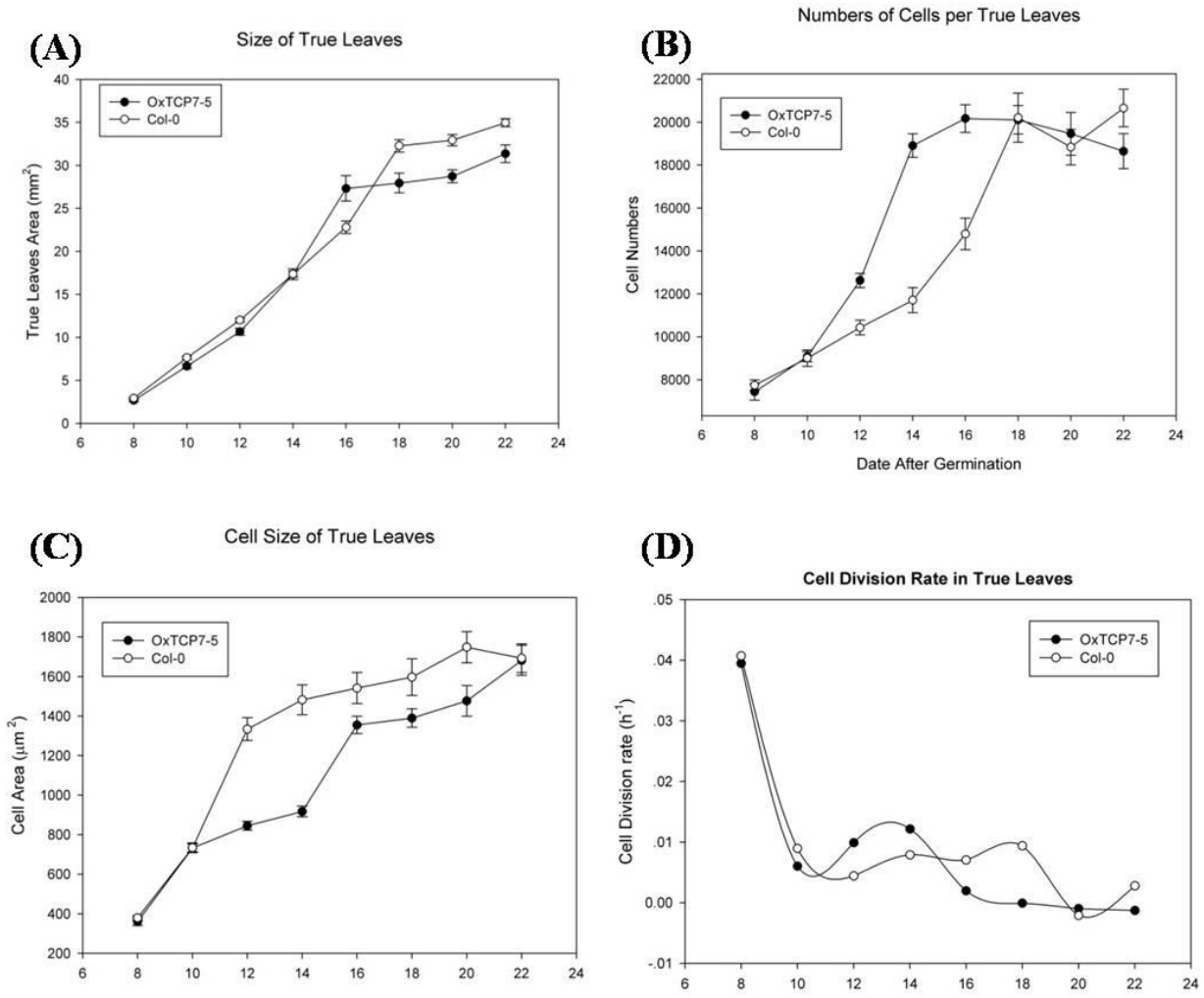


Fig. 14: Kinematic Analysis of True leaves Growth. (A) Leaf blade area; (B) Average cell numbers on true leaves; (C) Average cell size on true leaves; (D) Cell division rate in true leaves. Error bars give standard errors (n=10).

4.4 Discussion

In this chapter, microscope observation was performed to analyze the cell proliferation in OxTCP7-5 over-expression line. The data showed an abnormal in the cell division and expansion in roots, cotyledons, and true leaves, which were also consistent with the phenotype described in the previous chapter. Taken together, these results indicated that *AtTCP7* is involved in the positive regulation of cell proliferation.

The root length is determined by many factors in plants, however, two major control checkpoints exist: (1) cell division in the root apical meristem and (2) cell expansion in the transition zone [89]. As shown in other cell cycle-related mutants like *SHR* and *SCR*, cell loss the proper formative asymmetric division at root tip and generate a mutant cortex/endodermis cell layer, which disrupted the coordination of cell division and led to a cessation of root growth. While in lines over-expressing *AtTCP7*, the formation of root meristem didn't show a obvious difference compared with wild-type Col-0, the epidermal cell length in elongation zone was significantly and consistently reduced, which indicate that the short root phenotype in over-expression lines is mainly caused by a incomplete cell expansion.

For cotyledons and first 1,2 true leaves, kinematic growth analyses were performed to track the cell proliferation during the vegetative stage. The results showed that both cotyledons and true leaves presented an obvious difference in cell proliferation during growth. Intriguingly, however, the phenotypes resulting from these differences in cell formation were opposite in the two tissues analyzed. The epidermal cells of cotyledons in over-expression lines displayed an increase in cell size and reduce in cell numbers, by contrast, in the first true leaves, smaller size but bigger numbers of epidermal cells were observed. These results indicate that the *AtTCP7* is involved in cell proliferation regulation in cotyledons and true leaves, Somehow surprisingly, however, this also reveals that the role of *AtTCP7* can be differentially modulated in different tissues: *AtTCP7* promoted cell division in the first true leaves while in cotyledons, it showed a prematurely exit from cell division cycle, which could be a result of the ectopic expression of *AtTCP7* in cotyledons.

Chapter V

Over-expression of *AtTCP7* restricts the cell G1-S phase transition in true leaves

5.1 Introduction of cell cycle regulation

5.1.1 Molecular mechanisms driving *Arabidopsis* cell cycle

The elucidation of the molecular mechanisms underlying the cell cycle started decades ago and are partly still under way [90-91]. The genome-wide analysis of core cell cycle genes in *Arabidopsis*[92] provided a great impulse to our understanding of this process and uncovered a highly conserved set of homologous cell cycle regulatory layers shared between *Arabidopsis* and other eukaryotes.

5.1.1.1 Cyclin-dependent kinase

Eukaryotic cell division is controlled by a complex system which involves the key regulators Cyclin-Dependent Kinase (CDKs) and other components that trigger DNA replication and mitosis. Plants contain 12 CDKs that can be classified into 6 groups according to their aminoacidic sequences (A to F) [92]. Among these, CDKA has the typical PSTAIRE cyclin binding domain, which has been identified in yeast and plays an important role in cell cycle progression [93]. *Arabidopsis* CDKA;1, a close homologue to mammalian CDK1 and CDK2, has the function of controlling the cell G1-S and G2-M phase transitions. B-type CDKs (CDKB1;1, CDKB1;2, CDKB2;1 and CDKB2;2) are plant-specific CDKs characterized by the PPTALRE motif, expressed mainly during the G2 and mitosis phases, indicating the pivotal role of CDKBs in regulating the G2-M phase transition [94-95]. CDKDs and CDKF are classed as CDK-activating kinases (CAKs): CAK function is that of activating the phosphorylation of CDK, which, in turn, leads to the conformational change that allows CDK to recognize its substrate. CDKD is functionally related to mammalian CAKs, while CDKF is a plant-specific CAK, which phosphorylates CDKDs and RNA polymerase II C-terminal domain in *Arabidopsis thaliana* [96].

5.1.1.2 Cyclins

The kinase activity of CDKs requires the association of cyclins. In *Arabidopsis* a total of 49

different cell cyclin genes were identified, out of which 32 with counterparts in mammals were further classified into 4 groups : A-type (10 cyclins), B-type (11 cyclins), D-type (10 cyclins), and H-type (1 cyclin). The remaining 17 cycle-related genes found in the *Arabidopsis* genome without similarity to any known mammalian cyclin were classified into additional 4 groups called C-type, U-type, L-type and T-type [97].

A-type cyclins have in turn 3 subclasses A1, A2 and A3. Expression analyses indicate that the A-type cyclins appear early during the S phase, and their expression last until the mid of the M phase, which indicate that they are involved in the S to M phase transition. Detailed analyses show that different subclasses have different functions in cell cycle. For instance in case of the A2 cyclins, *CYCA2;3* was demonstrated to be a key regulator in *Arabidopsis* endoreduplication and the mitotic cell cycle [98], which negatively regulates cell endoreduplication and retards the mitotic cell cycle in loss-of-function mutant lines. A3 class cyclin *CYCA3;2* showed different functions in cell division and differentiation: by over-expression of tobacco *CYCA3;2* in *Arabidopsis*, activation of G1/S phase transition was obtained in transgenic over-expression lines with a higher expression level of S phase-specific genes, and the over-production of *CYCA3;2* also caused impairments on shoot and root regeneration in tissue culture[99].

B-type cyclins contain two major subclasses, B1 and B2. In-depth genome-wide analysis of cyclins in *Arabidopsis*, however, uncovered another B-type cyclin, which contains a B-type-like cyclin box with a missing destruction box (D-box), which did not not phylogenetically cluster into either B1 or B2 subclasses. A third subclass with *CYCB3;1* as the only member was therefore created [92]. The expression of B-type cyclins mainly associates with cycling cells: mRNA transcripts are accumulated in G2 and early M phase, but they are cleared from the cell through mitosis. Therefore, B-type cyclin is normally used as an indicator of mitotic activity in plant tissues. Functional analysis of tobacco *CYCB1;1* by microinjecting mRNA into immature *Xenopus* oocytes demonstrated that *CYCB1;1* controls the plant cell G2/M phase transition [100]. Ectopic expression of *CYCB1;2* in *Arabidopsis* trichomes induced the cell G2/M phase transition,

which converts the normal endoreduplication cycles into division cycles, as indicated by the conversion of trichomes from single-celled into multicellular ones [101].

D-type cyclins contain 10 *CYCDs* which are classified into 7 different groups, *CYCD1* to *CYCD7*. D-type cyclins are conserved in both mammalian and plants, which share the common Retinoblastoma Protein(Rb)-interaction motif. The CYCD/Rb pathway regulates E2F is known as the core pathway for cell G1 and S phase transition and cell division [102]. The expression of D-type cyclins in plants is regulated by both growth factors and environmental stimulations. For instance, *CYCD2*, *CYCD3* and *CYCD4* were induced by the presence of sucrose, and the expression levels of *CYCD2* and *CYCD3* were rapidly increased by transfer the young *Arabidopsis* seedlings from non sucrose medium to low concentrations of sucrose-containing medium [103]. *CYCD4* also shows the transcriptional responsiveness in root tips to sucrose stimulations, but in this case also light can trigger upregulation [104]. *CYCDs* also affect hormonal responses in plants. Loss-of-function *CYCD3;1* mutant lines display an impediment in cytokinin responses: despite the *cycd3;1-3* mutant background does not affect the total amount of of cytokinin, it requires exogenous cytokinin to form the callus. In addition it shows a complete absence of shoot development even at higher concentrations exogenous cytokinin, which indicates that in the *cycd3;1-3* mutant cytokinin responses are nearly abolished [105]. By contrast, transgenic lines over-expressing *CYCD3;1* are able to compensate the lack of cytokinin in the culture medium [106]. Thus, *CYCDs* in general play important roles in the mediating the response to the extracellular signals and regulate cell G1-S phase transition [107].

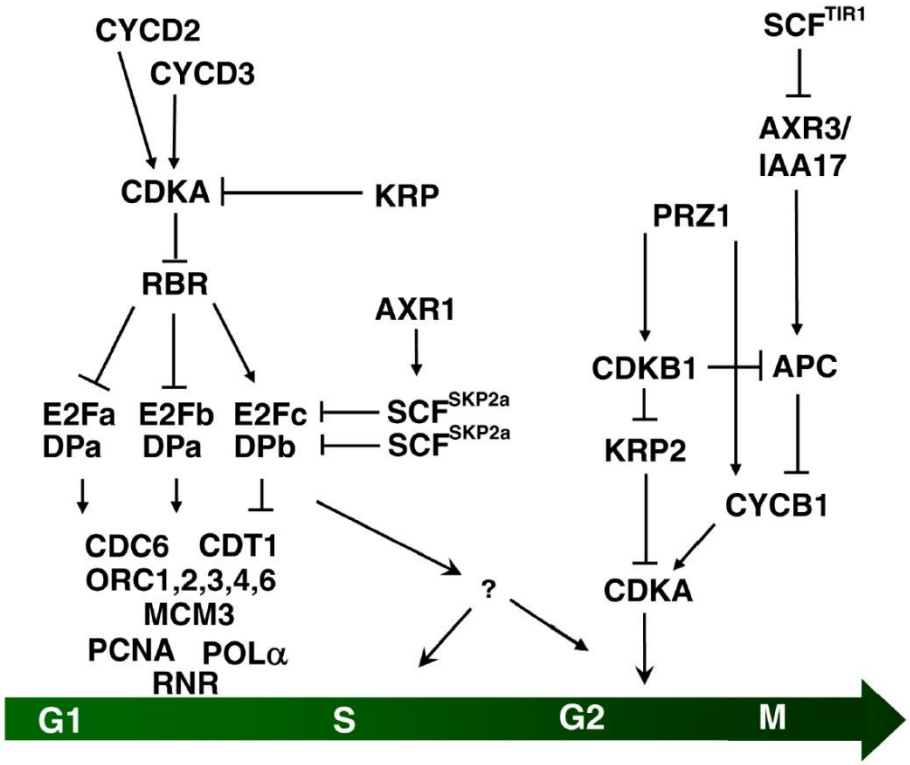


Fig. 15: Basic pathways controlling the different cell phases transition. Figure courtesy of Ref[81]

5.1.2 Cell DNA ploidy level.

Cell ploidy is the number of chromosomal sets present in the nucleus of a cell. In diploid species, like human and *Arabidopsis*, normal cells contain two copies of homologous chromosomes, usually each of the chromosome come from one side of its parenthood. Beside diploid species, the nucleus of a eukaryotic cell could also contain single sets or multiple sets of chromosomes, which are called monoploid or polyploid species, respectively.

All plants can alternate their ploidy level during different developmental stages. Chromosome number is reduced to haploid (*n*) through meiosis as generate gametes, while two

gametes form a diploid (*2n*) zygote that turns back to the normal ploidy level. This cyclic variation of ploidy is also called alternation of generations. By contrast, in the mitotic cycle, the nuclear content of cells is duplicated in the S phase (*4n*), and divided into two identical parts to form daughter cells at the end of cytokinesis (*2n*). In this ways mitosis constitutes the basic mechanism for the proliferation of sporophytic plants cells. During plant development, however, cells can also undergo additional rounds of DNA synthesis without cell proliferation, thus doubling their chromosome numbers by endoreduplication. This process, which has been described in more details in section **4.1.2**, is commonly associated to cell differentiation.

Thus, different cell ploidy levels can reflect the cell state, as the DNA contents changing along with the biological processes. Flow cytometry analyses in this case is widely used for determining ploidy levels to monitor changes in cell states upon different treatments.

5.2 Materials and Methods

For flow cytometry analysis, both OxTCP7-5 and Col-0 young seedlings were grown on MS plates. Cotyledons and first 1,2 true leaves were collected at 8 days and 12 days after germination, respectively. 20 samples for each set were chopped in 300 µl lysis buffer with a razor blade. The supernatant was filtered with 30-µm mesh, and 1µl of DAPI was added for nuclei staining. Analysis was performed at Milano-Bicocca University, Dipartimento di scienze dell'ambiente e del territorio e di scienze della terra, in collaboration with Prof. S. Citterio and Dr. R. Gentili.

For RNA extraction and gene expression qPCR analysis, cotyledons and first 1,2 true leaves were collected at 7 days and 12 days after germination, respectively, the whole root materials were harvested from 2 days young seedlings. The qPCR methods have been described in section **2.2.3.**

5.3 Results

5.3.1 DNA ploidy level changes in OxTCP7-5

Differences in cell size, among other factors, can be caused by changes in DNA ploidy, which in turn can be consequence of differences in cell cycle regulation. Therefore, we analyzed the DNA ploidy distribution of cells from 12 days of first pair of true leaves and 8 days of cotyledons, using flow cytometry assay. Consistently with the putative involvement of TCP transcription factors in cell cycle regulation, DNA ploidy levels are significantly different in transgenic OxTCP7-5 compared to wild-type. In the 12 days of first 1, 2 true leaves, the proportion of 2C of leaves increased by 8%, while 4C decreased by 10%. However, 8C didn't show significant differences among 3 biological replicates (Table IV). We further tested ploidy levels in 8 days cotyledons, an opposite ploidy distribution changes was found: the proportion of 2C decreased by 17%, while 16C significantly increased by 40%, and the 4C and 8C didn't have significant changes in this test (Table V). Taken together, these results indicate that in true leaves over-expression of AtTCP7 did not affect the population undergoing terminal endoreduplication with 8C DNA content at 12 days, while it could block a higher than normal proportion of diploid cells at either the G0 or the G1 phase. However, in cotyledons, over-expression of *AtTCP7* apparently promoted endoreduplication. This is consistent with the observation of contrasting cell-size phenotypes in leaves and cotyledons, which had smaller and bigger cells than WT, respectively.

Table IV Ploidy Levels in 12DAG Col-0 and Transgenic True Leaves

Line	2C(%)	4C(%)	8C(%)
Col-0	29.4 ± 1.5	63.1 ± 1.0	7.3 ± 1.7
OxTCP7-5	37.6 ± 1.3[a]	53.2 ± 3.3[b]	9.1 ± 2.5

Table V Ploidy Levels in 8DAG Col-0 and Transgenic Cotyledons

Line	2C(%)	4C(%)	8C(%)	16C(%)

Col-0	33.3 ± 2.0	26.6 ± 2.5	34.3 ± 2.3	8.6 ± 2.0
OxTCP7-5	27.6 ± 1.5[b]	26.6 ± 1.5	33.6 ± 1.1	12.0 ± 1.0[b]

Data represent Average ± SD(n=3)

[a] represent data P<0.01(Transgenic compare with Col-0, two-tailed Student's t-tests)

[b] represent data P<0.05(Transgenic compare with Col-0, two-tailed Student's t-tests)

5.3.2 Over-expression of AtTCP7 affect cell cycle genes expression.

Expression levels of key cell cycle genes are important markers in presenting progression through the different phases of cell cycle. We, therefore, analyzed the expression of cell cycle genes (*CYCA 2;3, CYCB1;1, CYCD 3;1,* and *E2Fa*) by using quantitative Real-Time PCR. Based on the indications obtained on ploidy from flow cytometry, RT-PCR analyses were carried out at 7 day after germination for cotyledons and 12 DAG for first two true leaves. As no significant differences resulted from day 2 to day 6 from the root cell size time-course study (Fig. 11C), 2DAG roots were used for RT-PCR quantifications.

At 12 days after germination *CYCA2;3* and *CYCB1;1* had increased expression levels, while a significant down regulation of *CYCD3;1* in OxTCP7-5 true leaves compared to wild-type (Fig. 16B). Together with the data from flow cytometry, qPCR results indicated that the cells in transgenic true leaves are strongly restricted the cell transition from G1 to S phase, and may also promoted the cell transition from G2 to M phase.

By contrast, qPCR carried out on 7 DAG cotyledons showed higher expression levels of H4 and slightly lower (although not significantly) expression of *CYCB1;1*. Additionally, The *CYCD3;1* and *E2Fa* were slightly up regulated (but not significantly) at this stage(Fig. 16A), which indicate cells in cotyledons were enriched in S phase, possibly as a result of a restricted cell transition from G2 to M phase.

For root at 2 DAG, G1/S transition and S phase cell cycle marker *E2Fa*, *RBR1* and *H4* were analyzed. As a negative regulator of S-phase entry[108], *RBR1* was found to be up regulated in root (Fig, 16C), while *E2Fa*, which stimulates cell-cycle by triggering the S phase entry, was down regulated. Taken together these results indicated that over-expression of *AtTCP7* caused a likely limitation of cell cycle S-phase entry in roots.

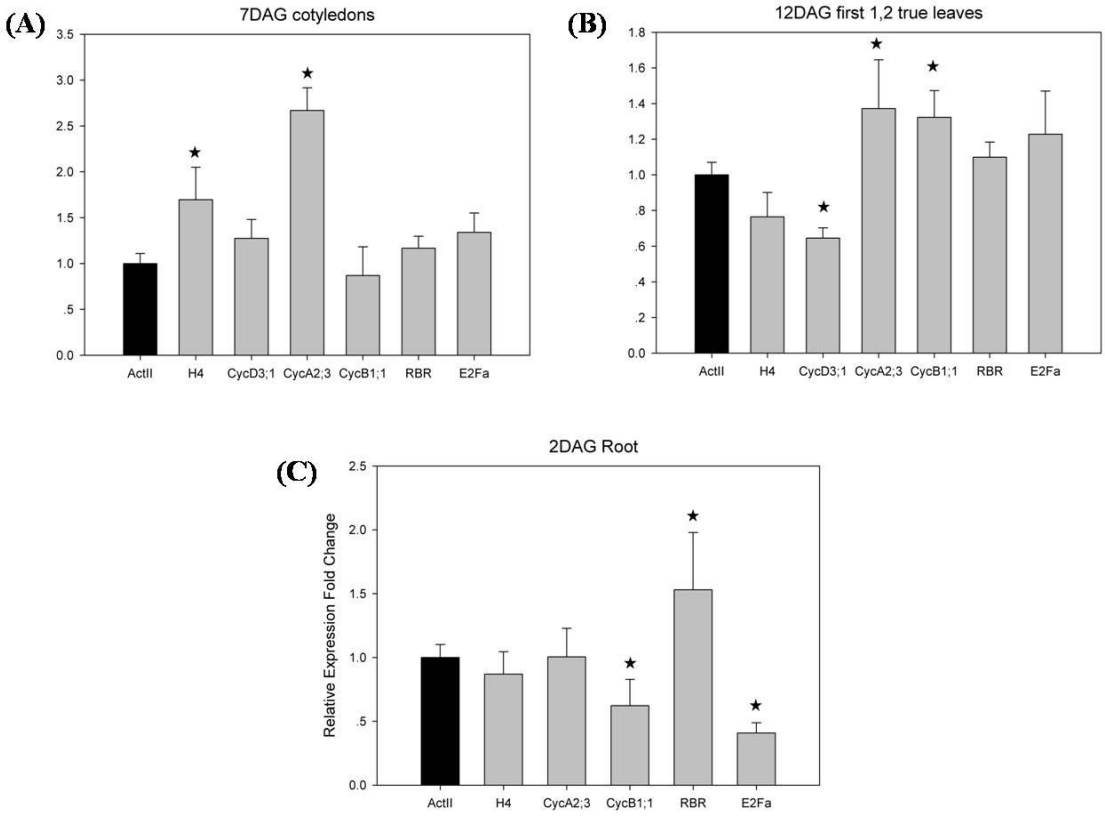


Fig. 16: Quantitative PCR analysis the cell cycle genes on OxTCP7-5 at different organs (cotyledons, first pair of true leaves, roots). Relative expression levels of each gene were compared with their expression in wild-type Col-0 at the same developmental stages. ActII expression level, scaled to 1 in each of the panels above, was used to normalize gene expression using the comparative CT method. Cell cycle gene relative expression fold change represent the gene expression in OxTCP7-5 compared with wild-type Col-0. (A) cell cycle genes' expression

in cotyledons at 7DAG; (B) cell cycle genes' expression in true leaves at 12DAG; (C) cell cycle genes' expression in root at 2DAG. Asterisks indicate differentially expressed genes ($P < 0.05$; $n = 3$), t-test.

5.3.3 Over-expression of *AtTCP7* affects expression of other TCP family genes.

Given the functional redundancy among TCP family genes and the potential protein-protein interactions between evolutionary close TCPs, the over expression of AtTCP7 may affect expression levels of other TCP genes. Therefore, based on the previously published ranking of potential AtTCP7 interactors/co-regulated genes [62], 4 TCP genes, namely AtTCP23, AtTCP22, AtTCP21, and AtTCP14, were selected for comparative expression analyses in the OxTCP7-5 and WT genetic backgrounds. Expression levels at 2DAG in roots, 7DAG and 12 DAG in cotyledons and first 1, 2 true leaves were analyzed through qPCR. Intriguingly, most of these genes' expression level were changed among different tissues and different developmental stages. In particular, AtTCP22 and AtTCP21 had increased levels of expression expression in 7DAG at both cotyledons and true leaves, while at 12 DAG, they were down regulated(Fig. 17A and 17B). In root, AtTCP21 showed a strong down regulation at 2 DAG(Fig. 17C). Beside these, AtTCP23, which is the top-ranking gene among the predicted functional interactors of AtTCP7, showed a continuous down regulation in three different tissues during different developmental stages. Taken together, these results indicate that TCP genes are functionally connected to each other through complex feedback networks and that several of the predicted interactors indeed respond with expression level changes to TCPs over expression.

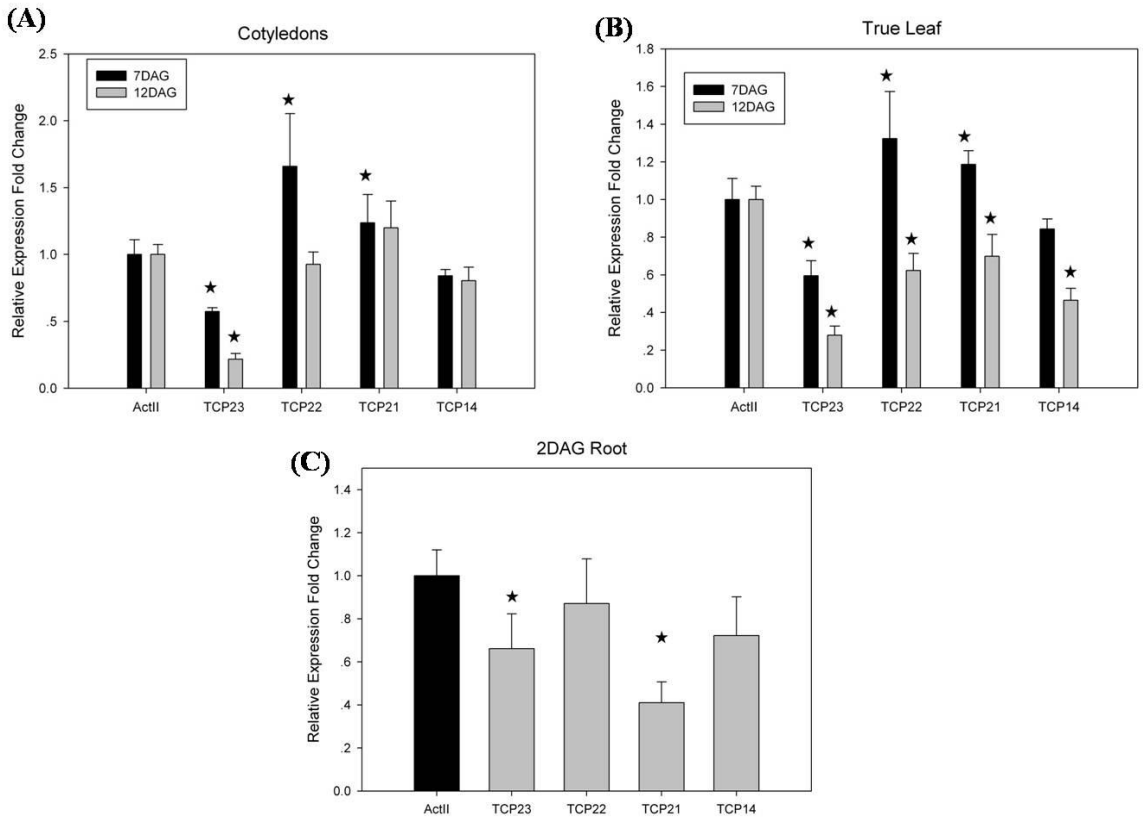


Fig. 17: Quantitative PCR analysis the TCP family genes on OxTCP7-5 at different organs (cotyledons, first pair of true leaves, roots). (A) and (B) Samples harvest at 7DAG and 12DAG, (C) Root materials harvest at 2DAG. Asterisks indicate differentially expressed genes ($P < 0.05$; n =3), t-test.

5.4 Discussion

Ploidy levels measured by flow cytometry coupled to quantification of cell cycle marker gene expression levels by qPCR analysis analyses are a powerful tool to mechanistically dissect the patterns of cell proliferation and differentiation in *AtTCP7* over-expression lines. Based on the results presented in the previous chapters and those previously published on TCP genes, the working hypothesis that the major function of *AtTCP7* is to restrict the cell G1-S phase transition was tested. In addition, the possible functional interactions with other TCP family genes in a

tissue-specific context were investigated.

In first 1,2 true leaves at 12 DAG, the DNA ploidy level was decreased by over-expression of *AtTCP7*, concomitantly to a down regulation of the cell cycle gene *CYCD3;1*. Previous studies about *CYCD3;*1 over-expression and loss of function showed that *CYCD3;1*, as a cell-cycle gene, promotes cell transition from G1 to S phase and also restrains the transition to endocycling [105, 109]. Thus, the down regulation of *CYCD3;1* in OxTCP7-5 line indicates that the cells were limited in G1/S transition, which was consistent with the high 2C ploidy level observed in first 1,2 true leaves. *CYCA2;3* was up regulated in first 1,2 true leaves in OxTCP7-5 line, which may support the assumption that there was a developmental delay in true leaf growth. This is also consistent with the observation that the cell expansion and differentiation initiated at 14 DAG in OxTCP7-5 true leaves instead of 10DAG in wild-type Col-0 (Fig. 14), indicating that the endoreduplication entry was postponed by the high expression of *CYCA2;3*. The up regulation of *CYCB1;1*, an M phase cell cycle marker, is expected lead to an increase of the relative fraction of cells in G2/S phase. Contrary to such prediction, however, the flow cytometry data of DNA ploidy levels showed that there was a lower proportion of G2 cells in true leaves at 12DAG. Currently, the reasons for this are not clear. One likely possibility is that the reduction of G2 cells could be a compensatory effect resulting from the variations of other TCP genes' expression in the *AtTCP7* overexpressor. It was previously reported that TCP family genes are involved in the regulation of expression of cell cycle genes [64], therefore, the abnormal expression of CYCB1;1 may be caused by other TCP genes' regulation without affecting the proportion of G2 cells.

In roots, the RBR/E2Fa pathway was strongly affected in the over-expression line. Especially the down regulation of E2Fa is expected to cause a reduction in the ability of cells to enter the S phase and limit the transition to endoreduplication cycle. It is, therefore, possible that the short length of root epidermal cells may be a consequence of a lower level of endoreduplication due to *AtTCP7* over expression. Unfortunately, the high noise associated with

flow cytometry analyses in *Arabidopsis* roots due to the easy DNA degradation did not allow to obtain precise data of DNA ploidy levels in roots. Therefore, different fixation protocols will be used to obtain accurate ploidy levels in roots and confirm the model above in the future.

The picture emerging from cotyledons is drastically different from those in leaves and roots. According to microscopic observation, cell expansion significantly increased and was accompanied by a lower division rate in cotyledons, while in first 1,2 true leaves most cells were blocked in the G1 phase, and by consequence cells were smaller than in WT. Also the flow cytometry analyses support these opposite trends in cotyledons versus leaves and roots: in cotyledons an increase of DNA ploidy levels were consistent with the observed enlargements of cells, and the qPCR analysis also showed that the S phase maker *H4* was highly expressed. Taken together these results indicate that in cotyledons the population of cells in S phase is over-represented as compared to WT and an indicating increased endocycling. However, the expression levels of cell cycle genes *CYCD3;1*, *E2Fa* and *CYCB1;1* were basically unchanged, indicating that the G1/S and G2/M transitions were only slight affected.

It is worth of note the opposite results obtained from first 1,2 true leaves and roots versus cotyledons: while in leaves and roots the function of *AtTCP7* is most likely that of restraining the cell G1/S transition, the data from cotyledons consistently indicate the opposite function. A possible explanation for these conflicting results may be found in (1) tissue-specific specialization and/or (2) ectopic expression of *AtTCP7*. The effect of AtTCP7 deregulation on the expression of other TCP genes indicates the existence of a complex regulatory network of functional interactions among TCP family genes. Together with the potential protein-protein interactions between *AtTCP7* and other TCPs, it is possible that a different sets of genes modulate the function of TCP7 in the different tissues. For instance, the lack of leaf- and root-specific protein interaction partners in cotyledons may differentially affect the pathways that regulate cell G1/S phase transition. *AtTCP23* showed a constant down regulation across tissues and stages: In light of this observation *AtTCP23* is possibly the strongest candidate as important

interacting partner of TCP7, but whether it could also play a role in tissue specific modulation of *AtTCP7* function remains to be clarified. The fact that normally AtTCP7 is not expressed in cotyledons provides yet another possible explanation for the contradictory inference of TCP function in the different organs: the ectopic expression of TCP in cotyledons may confer novel, non-physiological function to it as a consequence of the different developmental program characterizing this organ. Under this hypothesis, in cotyledons TCP7 may lack its unusual functional partners and/or compete with other TCP paralogs with antagonistic effects. At present, the latter possibility seems to be the most likely one, given the extensive neo-functionalization and differentiation of roles observed in the TCP family.

Chapter VI

Conclusion and future works

6.1 Conclusions

In this thesis, we described a growth defect phenotype caused by over-expression of *AtTCP7* in *Arabidopsis thaliana*, and the preliminary function of *AtTCP7* that affected cell proliferation was also addressed.

Our work started with yeast one-hybrid assay in which we attempted to isolate potential transcription factors that regulate *miRNA168a* gene expression. A total of 6 candidate genes were identified and one of them, *AtTCP7,* showed a decreased accumulation of mature *miRNA168a* by over-expressing *AtTCP7* in *Arabidopsis thaliana*. Therefore, we performed a GUS staining analysis in a transgenic line which expressed both *2x35S::AtTCP7* and *pMiRNA168a::GUS* constructs to verify the interaction between *AtTCP7* and the promoter of *miRNA168a*. The results, however, showed that the GUS expression pattern in crossing lines were similar to the negative control line (*pMiRNA168a::GUS*), thus indicating that in *Arabidopsis*, the expression of *AtTCP7* may affect the accumulation of *miRNA168a,* this effect may not be caused by the direct interaction between AtTCP7 and the promoter of *miRNA168a*. Furthermore, the function of *AtTCP7* still remains elusive since there was no suitable T-DNA insertion line available, and the study of *AtTCP7* fused with EAR-motif repression domain (SRDX) provided only a partial picture. Therefore, the further analysis of *AtTCP7* by over-expression in *Arabidopsis* was deemed essential for precisely pinpointing its function and ascertain the possible interaction with *miRNA168a* . By over-expressing *AtTCP7* in *Arabidopsis*, a variety of growth defects were observed in roots, leaves, and flowers. In lines over-expressing *AtTCP7* the average root length was strongly reduced , and the expression levels of *AtTCP7* in different transgenic lines were found to have a dosage correlation with the severity of root shortening, thus demonstrating that the phenotype we observed was chiefly caused by over-expression of *AtTCP7*. Beside root length, transgenic over-expression lines also showed a delay in flowering time and reduced fertility . Taken together, these results demonstrated that the function of *AtTCP7* in *Arabidopsis* is essential for plant development.

In addition, an in-depth characterization was performed to track the cells growth in first 1,2 true leaves, cotyledons and roots. The results showed that the cell numbers and sizes were significantly different compared with WT Col-0 at same development stages. In first 1,2 true leaves, *AtTCP7* promoted cell division and a higher number of smaller cells was observed. By contrast, cells in cotyledons were over-expanded and fewer in number, which indicated a prematurely transition from cell division cycle to endoreduplication cycle in cotyledons of transgenic lines. Root epidermal cells of differentiation zone were also found to be about 35% shorter in length compared with WT Col-0, which is consistent with the observation of shorter roots and indicated that an insufficient endoreduplication occurred in root epidermal cells. Taken all of these information together, we concluded that *AtTCP7* is involved in cell expansion and division. Therefore, we analyzed DNA ploidy level and a set of key cell cycle genes' expression in a selected transgenic over-expression line. The cell ploidy analysis showed results consistent to those obtained from observation of cell size. In true leaves, there was a higher proportion of 2C cells in transgenic lines compared with Col-0 at 12DAG, which indicated the over-expression of *AtTCP7* was involved in restraining the cell transition from G1 to S phase, and this assumption was also supported by a lower expression level of *CYCD3;1* and a higher expression of *CYCA2;3* However in cotyledons, higher ploidy levels were found together with increased expression of S phase cell maker *H4* gene. Thus, the cells in cotyledons may be over-represented in endocycles, and accordingly the cell size was enlarged. In roots, with the lower expression level of *E2Fa*, the RBR1/E2Fa pathway was stimulated by over-expression of *AtTCP7*, which indicated a possible restriction in cell G1/S transition.

In conclusion, with the kinematic analysis of cell growth and quantitative analysis of cell content and cell cycle genes expression, we conclude that *AtTCP7* functions mainly affecting the cell proliferation by restricting the cell G1/S phase transition, while the different phenotype on cotyledons may be a consequence of of *AtTCP7* ectopic expression.

6.2 Future works

Furthering our understanding of *AtTCP7* function is important from different points of view, as it can provide relevant information about cell cycle regulation in plants and also expand our knowledge of TCP family gene functions. In our work we mainly addressed the phenotype resulting from over-expression of *AtTCP7* and the potential regulatory function that this gene plays in *Arabidopsis thaliana*. However, some points were only partially clarified and deserve further investigation and improvement in future studies.

The first of them is definitely the yeast one-hybrid assay. As discussed in chapter I, the quality of the library is of paramount importance for the results of any yeast one-hybrid screening. One way to possibly circumvent the high number of false positives that plagued our study would be to screen transcript factor-specific libraries. In this way, the specificity of the screening is expected to increase, thus reducing the work-intensive steps necessary for the characterization of candidate genes.

The second improvement that should be carried out concerns the flow cytometry assay: in our study we did not manage to satisfactorily set up a preparation protocol for *Arabidopsis* roots, which are notoriously more difficult to handle than aerial organs. Due to the fragility of root tissues, in fact, we had to fix the cell DNA before staining, but the normal fixation was not successful for our 2 days roots tissues. In the future, therefore, we will need to improve the protocol for root fixation, which will eventually allow us to perform the flow cytometry assay on roots.

The third point to improve in future studies is a deeper understanding of the correlation between *AtTCP7* and *AtTCP23*. Based on previous studies, both *AtTCP7* and *AtTCP23* are involved in cell cycle regulation and have been propose to function in controlling expression of possibly overlapping target genes. According to preliminary results, they probably genetically interact and antagonistically regulate the gene expression of each other. Elucidating the functional interaction between these two genes may therefore provide new insights about TCP

family genes' function and regulation in general and help elucidating the fine regulatory networks controlling *AtTCP7* function.

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Appendix 1: Primers used in this thesis

Primer name	sequence	Note
M168_Y1HAG_SacI	CGAGCTCAACAACCAGGATAACTTTTACATA	Yeast 1 hybrid
M168_Y1HAG_XbaI	GTTCTAGAGGTTACGACCACTCATTTTCC	Yeast 1 hybrid
M168_H1Yf1-5_SacI	CCGAGCTCCAATCTTCTGAATATCCGC	Yeast 1 hybrid
M168_H1Yf1-5_XbaI	GTTCTAGAAGAAAATGGGGGAAGAGAAT	Yeast 1 hybrid
M168_H1Yf7-12_SacI	CCGAGCTCTTGAGATATTCTGACATTTTAGGA	Yeast 1 hybrid
M168_H1Yf7-12_XbaI	GTTCTAGACACGTTGAAATTCTTCTTGAT	Yeast 1 hybrid
AT5G46910_1F	CACCCGGGGTCAACAGAGATAATAGATTACGGAA	Transgenic over-expression
AT5G46910_1R	CCCCCGGGTTACGGCTTTATTAGCCCTTT	Transgenic over-expression
AT5G62460_F	CACCCGGGGAAATTTTGATTTACTTATCTGAATAT	Transgenic over-expression
AT5G62460_R	CGCCCGGGAAATCCAATAGGTGAAGAATCTTT	Transgenic over-expression
AT5G57660_F	CACCCGGGCATCCAAGCAAATCAAATACCC	Transgenic over-expression
AT5G57660_R	CCCCCGGGCAGCCAATAGAAACTATAATTACCAAA	Transgenic over-expression
AT4G31420_F	CACCCGGGGACGCTTTTTTAGAGACAAACACG	Transgenic over-expression
AT4G31420_R	CTCCCGGGTCAGAAGTCAACGTTCGTGTATTAA	Transgenic over-expression
AT5G23280_F	CACCATGTCTATTAACAACAACAACAAC	Transgenic over-expression
AT5G23280_R	TTAACGTGGATCTTCCTCTC	Transgenic over-expression
qPCR_ACTIN2_F	GCACCCTGTTCTTCTTACCG	qPCR
qPCR_ACTIN2_R	AACCCTCGTAGATTGGCACA	qPCR
AT2G28390-qF	AACTCTATGCAGCATTTGATCCACT	qPCR
AT2G28390-qR	TGATTGCATATCTTTATCGCCATC	qPCR
qTCP7f	GTTACTTCGTCAAGCAGAGCCT	qPCR
qTCP7r	GCTGGTGTTGTCGTAAAGGTCTC	qPCR
qTCP21f	GTTTCTCCACTGCTTCTCTCTCCAC	qPCR

qTCP21r	ATTAACGAAGTCCCCATTGTGTGTC	qPCR
qTCP22f	GTAGTAACTGCACCAATGGGGTCA	qPCR
qTCP22r	CCAAACCATCGCCTCTACTGCC	qPCR
qTCP23f	GGGAGGTCAACAGTTAGGGTTAGGT	qPCR
qTCP23r	CACTCACTTGATGTTGAGGCTTTTG	qPCR
qTCP14f	TCGGAGAAGAAAAGAAGAATCCAAA	qPCR
qTCP14r	TGGCTGTAGATCCACTGTTGCTAGA	qPCR
qrtCYCB1:1f	CCTGGTGGAGTGGTTGATTGATG	qPCR
qrtCYCB1:1r	CGACATGAGAAGAGCACTGAGAC	qPCR
qrtH4f	ACCAAATTGCGTGTTTCCATTG	qPCR
qrtH4r	ATGTCTGGTCGTGGAAAGGGAG	qPCR
qrtE2Faf	GCTTTCGCCTCAACAAAACCTC	qPCR
qrtE2far	CCCGATCTACAACACCACAACC	qPCR
qrtCYCA2:3f	CCCAAGCCTTGAAGTCGAGTT	qPCR
qrtCYCA2:3r	AAAACCGCTGAAGCAGCAAC	qPCR
qrtRBRf	CTCATAAGTCGCCTGCTGCTAAG	qPCR
qrtRBRr	TTGCTGTGCTCACTGGTGTTG	qPCR
qrtCYCD3:1f	TCGTTGAACAGTCCAAGCTG	qPCR
qrtCYCD3:1r	TGCAAAATCGGCTTCTTCTT	qPCR

Appendix 2: Yeast one-hybrid screening results

AG_2	pyridoxin (pyrodoxamine) 5'-phosphate oxidase
AG_3	hypothetical protein
AG_4	glutamate synthase 1 [NADH]
AG_5	plasma-membrane associated cation-binding protein 1
AG_6	transcription factor jumonji and C5HC2 type zinc finger
AG_7	protein kinase-like protein
AG_9	transducin/WD40 domain-containing protein
AG_10	60S ribosomal protein L3-1
AG_11	chaperone protein dnaJ 3
AG_12	60S ribosomal protein L3-1
AG_13	embryo defective 2016 protein
AG_14	5'-3' exoribonuclease 3
AG_15	putative peptide/nitrate transporter
AG_16	proton pump interactor 1
AG_18	GPI-anchored glycoprotein membrane precursor
AG_19	protein PROLIFERA
AG_20	delta-aminolevulinic acid dehydratase
AG_21	glycyl-tRNA synthetase 1
AG_21	ribulose bisphosphate carboxylase small chain 1A
AG_22	hypothetical protein
AG_23	Isochorismatase family protein
AG_25	chaperone protein dnaJ 11
AG_26	putative arginase
AG_28	extensin-like protein
AG_29	glycine dehydrogenase [decarboxylating] 1
AG_30_0	dihydrolipoyl dehydrogenase 1
AG_30_1	aquaporin PIP1-2
AG_31	signal recognition particle subunit
AG_32	protein VPS54
AG_33	haloacid dehalogenase-like hydrolase domain-containing protein
AG_34	RING/FYVE/PHD zinc finger-containing protein
AG_35	zinc finger protein CONSTANS-LIKE 5
AG_36	Zinc finger protein 622
AG_37	hypothetical protein
AG_39	aspartyl protease family protein

AG_40	chlorophyll a-b binding protein 4
AG_41	glyceraldehyde 3-phosphate dehydrogenase
AG_42	SAP domain-containing protein
AG_44	photosystem I reaction center subunit II-1
AG_45	phosphoglycerate kinase 1
AG_46	protein phosphatase 2 (formerly 2A), regulatory subunit B
AG_48	60S ribosomal protein L3-1
AG_49	50S ribosomal protein L24
AG_50	PsbP-like protein 1
AG_51	14-3-3-like protein GF14
AG_52	protein N-MYC downregulated-like 2
AG_53	hypothetical protein
AG_58	glucose-1-phosphate adenylyltransferase large subunit 2
AG_66	PsbP-like protein 1
AG_68	SAUR-like auxin-responsive protein
AG_70	hypothetical protein
AG_72	protein disulfide-isomerase A6
AG_74	protein phosphatase 2 (formerly 2A), regulatory subunit B
AG_75	356 bp at 5' side: soluble inorganic pyrophosphatase 1
AG_77	chlorophyll a-b binding protein 1
AG_78	hypothetical protein
AG_79	xyloglucan 6-xylosyltransferase
AG_82	protein N-MYC downregulated-like 2
AG_83	hypothetical protein
AG_84	elongation factor 1-delta 1
AG_85	DNA repair DEAD helicase RAD3/XP-D subfamily protein
AG_86	protein PHLOEM protein 2-LIKE A5
AG_88	DNA repair DEAD helicase RAD3/XP-D subfamily protein
AG_89	DNA repair DEAD helicase RAD3/XP-D subfamily protein
AG_92	hypothetical protein
AG_95	hypothetical protein
F1-5_6	OST3 and OST6 domain-containing protein
F1-5_8b	DNA repair DEAD helicase RAD3/XP-D subfamily protein
F1-5_10	protein phosphatase 2 (formerly 2A), regulatory subunit B
F1-5_10b	ribulose bisphosphate carboxylase small chain 1A
F1-5_14b	protein agamous-like 42
F1-5_14s	putative mannan synthase 3
F1-5_16	SAUR-like auxin-responsive protein

F1-5_17	hypothetical protein
F1-5_18	protein phosphatase 2
F1-5_31	hypothetical protein
F1-5_32	UDP-glucose 4,6-dehydratase
F1-5_33	hypothetical protein
F1-5_34	DNA repair DEAD helicase RAD3/XP-D subfamily protein
F1-5_37	lipoxygenase 6
F1-5_39	protein phosphatase 2
F1-5_46	hypothetical protein
F1-5_49	hypothetical protein
F1-5_50	hypothetical protein
F1-5_65	DNA repair DEAD helicase RAD3/XP-D subfamily protein
F1-5_66	PsbP-like protein 1
F1-5_67	catalase 3
F1-5_68	B12D protein
F1-5_78s	transcription factor TCP7
F1-5_79	putative boron transporter 3
F7-12_F1	PHD finger, SWIB/MDM2 and GYF domain-containing protein
F7-12F2	Luminal-binding protein 2
F7-12-F4	hypothetical protein
F7-12-F5	60S ribosomal protein L3-1

Copyright Acknowledgements

Matthew W. Jones-Rhoades, David P. Bartel, Bonnie Bartel: MicroRNAs and Their Regulatory Roles in Plants. *Annu. Rev. Plant Biol.* 2006, **57:**19-53. **Figure 1**

Olivier Voinnet: Origin, Biogenesis, and Activity of Plant MicroRNAs. *Cell* **136,** 669–687. **Figure 1**

Mart ń-Trillo M, Cubas P: TCP genes: a family snapshot ten years later. *Trends in Plant Science* 2009, **15**:31-39. **Figure 6**

Gutierrez C: The Arabidopsis Cell Division Cycle. *American Society of Plant Biologists* 2009. **Figures 10 and 15**