CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL

INSTITUTO POLITÉCNICO NACIONAL

UNIDAD DE GENÓMICA AVANZADA

“Characterization of the *MED12* subunit of Mediator in low phosphate responses in *Arabidopsis thaliana*”

Thesis presented by

L. C. G. Carol Martínez Camacho

To obtain the degree of

Master in Science (M. Sc.)

Specializing in Integrative Biology

Thesis Directors:

Dr. Stewart Gillmor

Dr. Ruairidh Sawers

Committee members:

Dra. June Simpson

Dr. Luis Alfredo Cruz Ramírez

August, 2015

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“Caracterización de la subunidad *MED12* de Mediador en respuesta a la carencia de fosfato en *Arabidopsis thaliana*”

Tesis que presenta:

L. C. G. Carol Martínez Camacho

Para obtener el grado de

Maestra en Ciencias

En la Especialidad de Biología Integrativa

Directores de Tesis:

Dr. Stewart Gillmor

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Miembros del comité:

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The experimental work of the present thesis was carried out at the Unidad de Genómica Avanzada (UGA) in CINVESTAV-Irapuato. Specifically in the laboratories 6, 10 and 11, led by PhD Luis Herrera, PhD Stewart Gillmor and PhD Ruairidh Sawers, respectively. Maize field work was done in Puerto Vallarta Experimental Maize Field and in CINVESTAV-Irapuato Experimental Maize Field.

Dedication

To my family, to my sweet lab-friends, to my super BI friends, to Stewart and Ruairidh, to myself.

Acknowledgments

Thank you to CONACYT for the grant to accomplish a Master degree.

Thank you to my advisors, Ruairidh and Stewart, who showed me patience and confidence. I am sure I couldn't have better advisor's example as yours. Thank you to the Plant Development and Morphogenesis Lab: Marce, Alma, Dani, Quio, Joel, Manuel, Gera, Dani Lepe. At some moment I wished to be forever at the lab with you all. Thank you to the Maize Genetics and Genomics Lab: Eric, Chío, Nancy, Eliécer, Darío, Odette, Felipe, Anna, Karina, Chayo, Erasmo. You know what you did for me to be glad to you. Jessy Carcaño, I have to thank you twice. Thank you to Alex, Beto, Jonhy, Joey, Felipe, Nelly and Fabricio for every laugh, talk, cheer, company, thank you very much. Thank you to my friends outside the labs-inside Langebio: Alex Morales, Chío Martínez, Lupita y Fabi, Ana, Ahiko, Rigel, Javi Carpinteiro, Eri y Uri, Jessi, Abraham, Colli, Roberto Álvarez, Rocío y Fred. Thank you to Javier Mora who helped me all the time with the experiments. Thank you to Luis Valle who motivated me to measure many roots with Root Reader 2D. Thank you to June Simpson and Alfredo Cruz for the comments and corrections along this master thesis.

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Abstract

Mediator is a transcriptional regulatory complex conserved across eukaryotic kingdom. The Mediator complex is composed of four modules: the head, middle, and tail, as well as an additional detachable kinase module, known as the CDK8 module. MED12 is a subunit of the CDK8 module of the Mediator complex. *MED12/CCT* has been shown to be important for proper control of developmental programs in Arabidopsis (Gillmor et al., 2010). In other organisms like Drosophila, amoeba and yeast, it is also involved in cellular adaptations to environmental cues (Loncle et al., 2007; reviewed in Huber, 2013; Raithatha et al., 2012; Lindsay et al., 2014; Khakhina et al., 2014).

RNAseq analysis showed that in *med12/cct* microRNAs involved in phosphate (Pi) starvation response are upregulated (*i.e.* miR827; Joel Medina's Master Science Thesis, 2015). We show that in sufficient Pi conditions, *med12/cct* Root System Architecture (RSA) resembles to a Pi starved plant, with shorter primary root and longer root hairs than wt. Interestingly, in low Pi *med12/cct* RSA is less altered than wt. *pCCT::GUS* is expressed primarily in vascular tissues of the root and shoot, and responds to low Pi conditions. The results obtained in this thesis suggest *MED12/CCT* could be acting as an environmental Pi status integrator to Arabidopsis RSA.

Resumen

El Mediador es un complejo proteico regulador de la transcripción, que se encuentra conservado en todos los eucariotes. Mediador está formado por cuatro módulos: la cabeza, el medio, y la cola, así como un módulo desacoplable conocido como módulo CDK8. MED12 es una subunidad del módulo CDK8. En Arabidopsis, *MED12/CCT* participa en el control adecuado de programas del desarrollo como la embriogénesis (Gillmor et al., 2010). En otros organismos tales como Drosophila, amiba y la levadura, *MED12* también está involucrado en la adaptación a estímulos ambientales (Loncle et al., 2007; reviewed in Huber, 2013; Raithatha et al., 2012; Lindsay et al., 2014; Khakhina et al., 2014).

Un análisis de secuenciación masiva de RNAs pequeños mostró que en *med12/cct* se encuentran sobre-expresados microRNAs involucrados en la respuesta a carencia de fosfato (*i.e.* miR827; Tesis de Maestría en Ciencias de Joel Medina, 2015). En este trabajo mostramos que en condiciones de suficiente fosfato, la Arquitectura del Sistema de Raíces (RSA) de *med12/cct* se parece al de una planta silvestre en respuesta a carencia de fosfato, mostrando una raíz primaria más corta y pelos radicales más largos que la planta silvestre. De manera interesante, en bajo fósforo el RSA de *med12/cct* está menos afectado que la planta silvestre. *pCCT::GUS* se expresa principalmente en los tejidos vasculares de la raíz y la parte aérea de Arabidopsis, y responde a condiciones de bajo fósforo. Los resultados obtenidos en esta tesis sugieren que *MED12/CCT* podría estar actuando como un integrador del estado ambiental del fósforo con el desarrollo del RSA de Arabidopsis.

Introduction

Root development

Land plants present a wide diversity of root system architectures (RSA), or spatial configurations of the root. RSA development is guided by a genetically controlled embryonic and post-embryonic root developmental program that, besides anchorage, permits high phenotypic plasticity in response to stressful environmental conditions, allowing the plant to efficiently explore the soil in search of water and nutrients (reviewed in Sánchez-Calderón *et al*., 2013). Thus, plant survival depends on the appropriate development, growth and function of the RSA.

Use of the model organism *Arabidopsis thaliana* has allowed great advances in the study of root development. Roots of Arabidopsis have a very simple cellular organization and can be grown in Petri dishes, which permits observation of RSA in different environments. The root system (RS) consists of all roots that a plant has. Its classification based on development is the most typical and useful way to analyze RS growth. According to this, the RS is composed by three types of roots: primary root (PR), lateral roots (LR) and adventitious roots (AR; Fig. 1). The PR is the first root to emerge, during germination, and is derived from the proliferation and growth of cells at the root apical meristem (RAM), a tissue in the root tip that is a source of undifferentiated cells for root growth and development. LRs are formed post-embryonically from reprogrammed pericycle cells along the PR. ARs are nodal roots, emerging from the shoot and sharing all characteristic features found in PR and LRs (reviewed in Sánchez-Calderón *et al*., 2013).

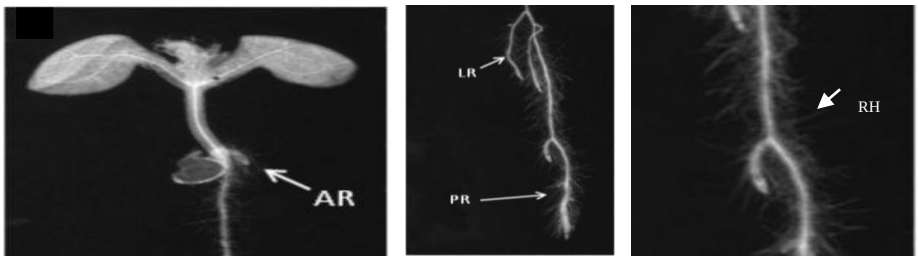

Figure 1. The root system. AR: adventitious roots; LR: lateral roots; PR: primary root; RH: root hairs. (Modified from Sánchez-Calderón *et al*., 2013).

The spatial arrangement of the RS is determined by the number, position and growth position of the PR, LRs and ARs. RSA can be modulated in several ways: through promotion or inhibition of primary root growth, elongation or increasing number of lateral roots, formation of adventitious roots and through an increase in the number and length of root hairs (reviewed in Osmont *et al*. 2007). During the lifetime of a plant, RSA undergoes alterations determined by a plastic genetic program which is modulated by environmental cues. Phenotypic plasticity is the ability of a given genotype to generate a range of phenotypes under different environmental conditions (reviewed in Grossman & Rice, 2012). Root plasticity allows plants to optimize RSA in a heterogeneous and constantly changing environment (reviewed in Giehl *et al.*, 2014).

Morphology of RSA and the underlying biological processes

Primary root length

In Arabidopsis, root growth is indeterminate, which means roots can grow indefinitely in length. As it is continuously growing, all the developmental stages are present all the time. Four types of stem cells (or initial cells) at the tip of the root generate all of the cell files that create the radial symmetry of the root: 1) the epidermal/lateral root cap initials; 2) the columella set of initials; 3) The cortex/endodermal initials; and 4) the vascular tissue and pericycle set of initials. Internal and contacting all the initials is the quiescent center (QC), a small number of central mitotically less-active stem cells (Fig. 2; Scheres *et al*., 2002). The initials and the QC together form the root stem cell niche or RSCN (reviewed in Heidstra and Sabatini, 2014).

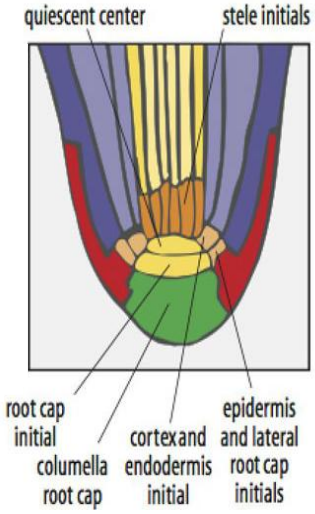

Figure 2. The four sets of initials of the Quiescent Center. Root tissues are produced from root apical meristems by a highly stereotypical pattern of cell divisions and give rise to concentric rings of tissue layers (Modified from Staveley BE, 2010).

The determination of the length

of a root is affected by a combination of three major cell biological processes: the rate of cell division, the extent of expansion and elongation of cells, and the rate of cell differentiation (Giehl *et al*., 2014). Cell division occurs in the Meristematic Zone (MZ), which is composed by the RSCN and the transition zone (TZ). When cells stop dividing, they enter the Elongation Zone (EZ). Differentiation occurs in the Differentiation Zone (DZ), after cells have left EZ (Fig. 3). Perturbations in any of these four zones will ultimately affect primary root length. The MZ is the reservoir of all cells that will constitute the primary root, so if cells differentiate in this zone the supply of new cells, for elongation and differentiation, is over. The extent of elongation or its rate, as well as the time when cells start terminal differentiation, also regulate root length (Dello-Ioio *et al.*, 2012).

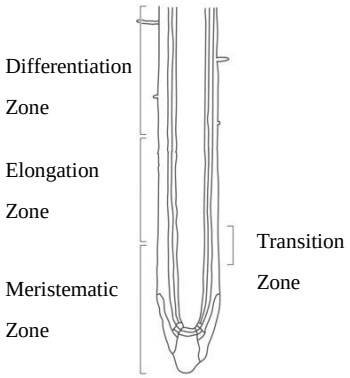

Figure 3. Root anatomy. The meristematic or division zone (MZ), where cell division occurs; the elongation zone (EZ); the differentiation zone (DZ); and the transition zone (TZ).

Root growth is regulated by signaling pathways involving hormones and developmental genes (Fig. 4). Coordination of the overlapping activity between auxin and cytokinin is pivotal to the regulation of root development. Exogenous application of cytokinins or overexpression of a gene involved in cytokinin biosynthesis reduces root meristem size and root growth without interfering with QC specification and stem cell function (Dello-Ioio *et al*., 2007). Exogenous application of cytokinin reduces PIN1 auxin efflux carrier transcription in root tip, and diminishes the level of auxins in root meristem, which determines the length of the meristem (making it smaller) by acting on the rate of cell differentiation, and consequently affects the position of the TZ and the root length (Dello-Ioio *et al*., 2007; Ruzicka *et al*., 2009).

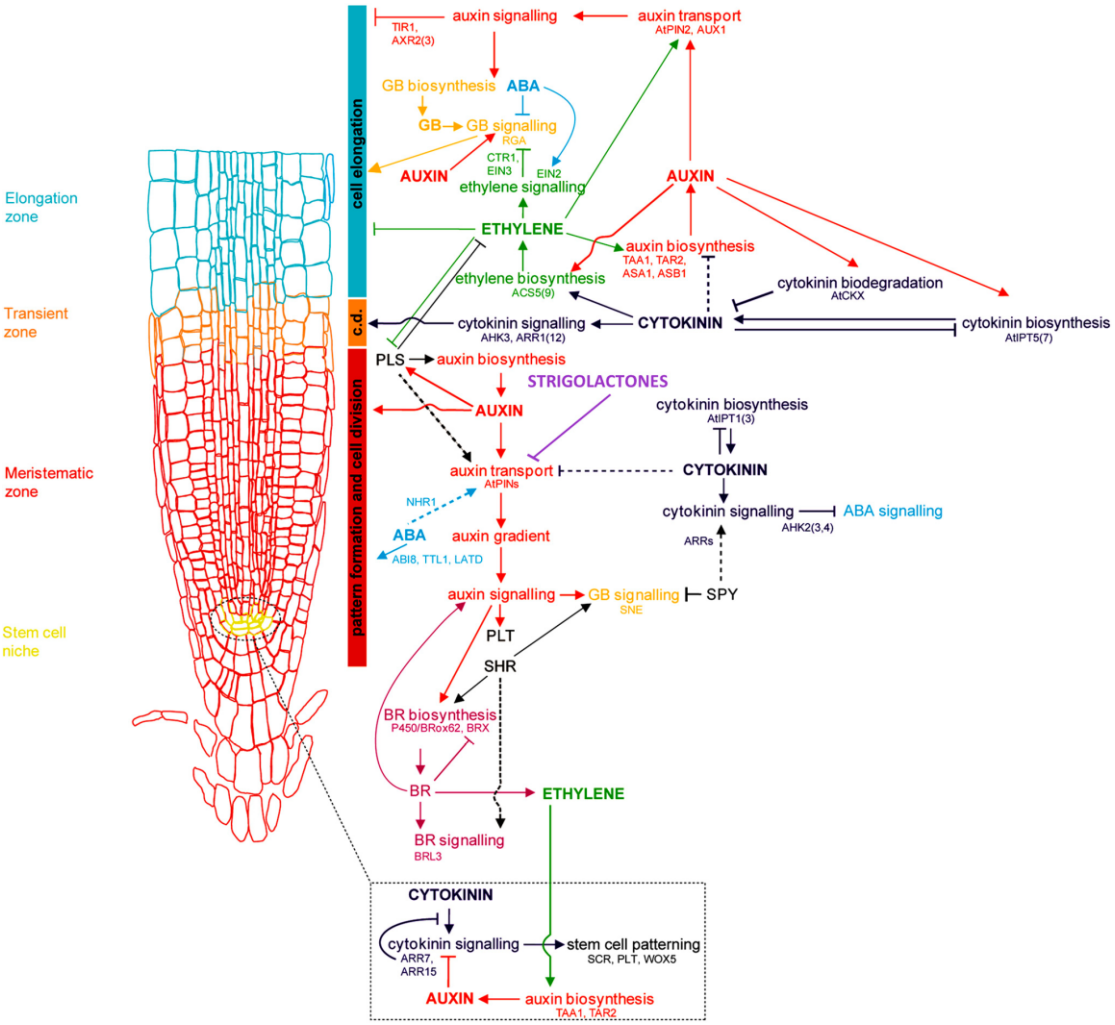

Figure 4. Complex interactions between hormones and developmental genes regulate root development and Root System Architecture (Taken from Jung & McCough, 2013).

Lateral roots

The number and length of LRs represent a dominant feature of RSA (reviewed in Giehl *et al*., 2014). PR and LRs are structurally very similar. The main difference between them is the developmental time and the source from which they are originated. LRs are developed post-embryonically. LR initiation is dependent on auxin-cytokinin antagonism, through priming xylem pole pericycle cells by auxin activation (Péret *et al.*, 2009). These primed cells become pericycle founder cells and undergo the first anticlinal and assymetric division, and will continue to divide and create a dome-shaped primordium, that will eventually give birth to a

new meristem. Then, the patterning common to primary roots will emerge. The degree of root length and branching impacts the efficiency of water uptake, nutrient acquisition and anchorage by plants (reviewed in Péret *et al*., 2009).

Root hairs

RHs are tubular growing extensions from root epidermal cells, named trichoblasts, in the differentiation zone. Root hairs increase the root surface area, the root diameter and subsequently the interface between the plant and the soil. In Arabidopsis, root hairs are approximately 10 µm in diameter and grow to be 1 mm or more in length (reviewed in Grierson *et al*., 2014). RH grow in a distinct position-dependent pattern of root hair cells and non-hair cells. Root hair cells are located over two cortical cells, whereas non-hair cells are located over a single cortical cell (reviewed in Grierson *et al*., 2014). There is an exception to this pattern only in a region called the collet (near the root-hypocotyl junction). Genes that specify non-hair fate are inactive in the collet, so root-hair like extensions are formed in all cells (reviewed in Parsons, 2009).

The hormones auxin and ethylene promote root hair cell differentiation in Arabidopsis, and control the positioning of the hair. Mutations that cause a hairless root phenotype can be suppressed by the addition of auxin and ethylene to the growth media. For root hair elongation ion movement, the cytoskeleton and cell wall modifications act together in a coordinated manner (reviewed in Datta *et al*., 2011).

Plant phosphate homeostasis, the systemic pathway and the role of microRNAs

Inorganic phosphate (P_i) is one of the essential macronutrients for all organisms; it is required in numerous biological processes including the biosynthesis of nucleic acids and phospholipids, energy transfer, and the regulation of enzyme reactions and metabolic pathways (reviewed in Secco *et al*., 2012; Theodorou and Plaxton, 1993; Auesukaree *et al*.,

2003). Low P_i availability is a major constraint on plant growth and agricultural productivity worldwide (Barber *et al.*, 1963). For plants, P_i is generally in short supply as it is difficult to assimilate. As such, it is very important to use scarce P_i effectively (Chiou *et al*., 2006; reviewed in Doerner, 2008). This challenge required plants to evolve a series of adaptive responses for the integration of information about the intra- and extra-cellular availability of this nutrient, to maintain P_i homeostasis and regulate cell growth, cell division and plant development. Plant strategies to cope with and to improve P_i acquisition during P_i-limitation include RSA modifications such as increased lateral root density and inhibition of primary root growth (Williamson *et al*., 2001; López-Bucio *et al*., 2002), proliferation of long root hairs to increase the competitiveness of the plant in the low-phosphate environment (Bates and Lynch, 2001), increasing high-affinity P_i uptake capacities through establishing symbiotic interactions with mycorrhizal fungi (Tian *et al*., 2013), and induction of enzymes and organic acids that enable phosphate-use efficiency (reviewed in Zhang *et al*., 2014). The sensing and response mechanisms to P_i status involve global changes in gene expression (Misson *et al*., 2005), that trigger pleiotropic effects in phenotype and metabolism. Although many P-responsive genes have been reported, a global coordinator of the molecular mechanisms regulating these P_i-starvation-responsive genes and effects remain to be determined.

P_i itself, the phytohormones auxin, ethylene, cytokinins (CKs), abscisic acid (ABA), gibberellins (GA) and the strigolactones (Sls), along with sugars, miRNAs, and $Ca2^+$ have all been implicated in local and systemic P_i sensing and signaling pathways (Fig. 6; reviewed in Zhang *et al*., 2014). In addition, the microRNA miR399 and its target *PHO2*, are a subcomponent of the P_i-signaling network that regulates a subset of P_i-dependent responses involved in the systemic control of P_i allocation (Bari *et al*., 2006; reviewed in Doerner *et al*., 2008). *PHO2* encodes an E2 ubiquitin-conjugating enzyme, and is a major component for the maintenance of P_i homeostasis in Arabidopsis (Bari *et al*., 2006). Under high Pi conditions, the ubiquitin conjugase activity of PHO2 promotes PHO1 degradation, a protein localized in endo-membranes implicated in P_i loading to the xylem in low P_i conditions (Liu *et al.*, 2012). The *pho2* mutant shows continuous induction of a subset of phosphate starvation-induced (PSI) genes, including some encoding phosphate transporters, for example Pht1;8 and Pht1;9,

even in P_i-replete conditions (Bari *et al*., 2006; Datt Pant *et al*., 2008). The mutant also accumulates P_i to high levels (three- to five-fold increase) in shoots but not in roots (Bari *et al*., 2006; Datt Pant *et al*., 2008).

PHO2 expression is post-transcriptionally regulated by microRNA399 (miR399), which acts by inducing cleavage of its transcript, recognizing the complementary sequences at the long 5' UTR, and probably also mediates PHO2 translational repression (Bari *et al*., 2006). *MIR399* transcription induced by phosphate deficiency is among the best studied nutrient-dependent miRNAs. *MIR399* is induced by perceived P_i limitation, and its expression is detected in vascular tissues, specifically in companion cells and phloem (Aung *et al*., 2006). miR399 functions in systemic signaling, as a phloem-mobile long-distance signal, and its expression in the shoot is enough to repress *PHO2* in roots, for regulation of P_i homeostasis in Arabidopsis (Datt Pant *et al*., 2008). *MIR399* overexpression phenocopies the *pho2* loss-of-function phenotype of elevated shoot P_i accumulation, suggesting that *PHO2* is the primary target of the miR399 family (Doerner, 2008). *PHO2* homologs have been identified in monocots and eudicots (Bari *et al*., 2006), and miR399 has been clearly identified in core and basal eudicots and monocots, but there is no evidence for miR399 in bryophytes or lycophytes (reviewed in Doerner *et al*., 2008). It would appear that the miR399-*PHO2* pathway is an evolutionarily conserved mechanism for P_i homeostasis among angiosperms (reviewed in Zhang *et al*., 2014).

The loss-of-function of long non-coding RNAs (lncRNAs) has shown the participation of these lncRNAs in the phosphate response pathway. *At4* loss-of-function, a lncRNA in Arabidopsis, resulted in altered shoot to root P_i ratios (Shin *et al*., 2006). It was shown that *At4* genes conserve a 22-23 base sequence with incomplete central complementarity that act by sequestering miR399, so the miRNA cannot act on *PHO2* transcripts. This mechanism is

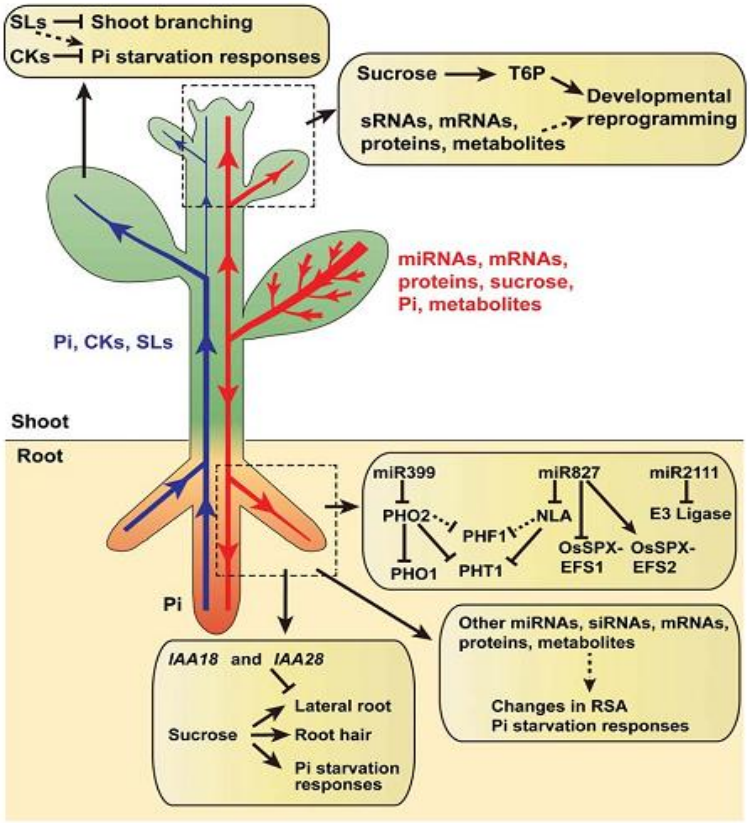

Figure 6. Components of P_i systemic sensing and signaling in plants. There is a fine-tuned communication from shoot to root in P_i sensing and signaling for a proper response to P_i status in the plant. Hormones, sugars, mRNAs, miRNAs and other non-coding RNAs act in an elegant way for P_i homeostasis regulation (taken from Zhang *et al.*, 2014).

known as target mimicry (Franco-Zorrilla *et al*., 2007; reviewed in Doerner *et al*., 2008). The MYB factor PHR1 has been shown to be required for the expression of *At4* genes and *MIR399* under P_i starvation (Bari *et al.*, 2006). In addition, in the *pho2* mutant *AT4* is up-regulated in P_i-replete conditions, and PHR1 *cis*-elements are usually found 160 to 270 nt upstream of the *MIR399 genes*, indicating that PHR1 acts upstream of *At4, PHO2*, and *MIR399* (Bari *et al.*, 2006).

MIR399 primary transcripts are among the most P_i-responsive transcripts in Arabidopsis, and their expression is highly specific (Bari *et al*., 2006). In addition, *MIR827* and *MIR2111* are induced specifically in response to P_i deprivation, and the abundance of miR156, miR778,

miR828, miR169, miR395, miR398, miR866, miR391, and miR163 is also altered in Arabidopsis during phosphate deprivation, which suggests a role for other miRNAs in phosphate homeostasis (Hsieh *et al.*, 2009). In a study where microRNAs were associated with low phosphate tolerance in *Zea mays*, it was found that there are some of the same Arabidopsis miRNA families associated with low P_i stress that are conserved in maize and other plant species (i. e. miR399, miR169, miR395, miR398, miR408, and miR827), which suggested that they may be involved in conserved low P_i response pathways (Pei *et al*., 2013). But there is evidence that some miRNA families may be involved in species-specific low P_i response pathways (Pei *et al*., 2013).

NLA, the target of miR827, was reported to be the E3 ligase that act together with PHO2, an E2 conjugase, to repress PT2 (Phosphate Transporter 2) by protein degradation to maintain Pi homeostasis in Arabidopsis (Park *et al.*, 2014).

Root plasticity and Phosphate stress

In the context of morphology, plasticity can be defined as the developmental changes that follow the perception and integration of environmental information (reviewed in Novoplansky, 2002). In natural environments, plasticity is an important strategy plants use to cope with insufficient nutrient supply, which significantly impairs growth and development. Through metabolic, physiological, and morphological alterations root plasticity allows plant roots adapt to nutrient stress environments, showing a particularly high variation in their morphological response to different nutrient deficiencies (Fig. 5) (reviewed in Kehr, 2012).

The genetic component of the plant determines the fundamental morphology and blueprint of the RS, whereas environmental cues like nutrient availability shape root architecture by modifying the intrinsic genetic program (reviewed in Kehr, 2012). How nutrient signals are integrated into root developmental processes is still poorly understood (reviewed in Giehl *et al.*, 2014). There are many ongoing efforts to better understand nutrient signaling and corresponding morphological and biochemical changes.

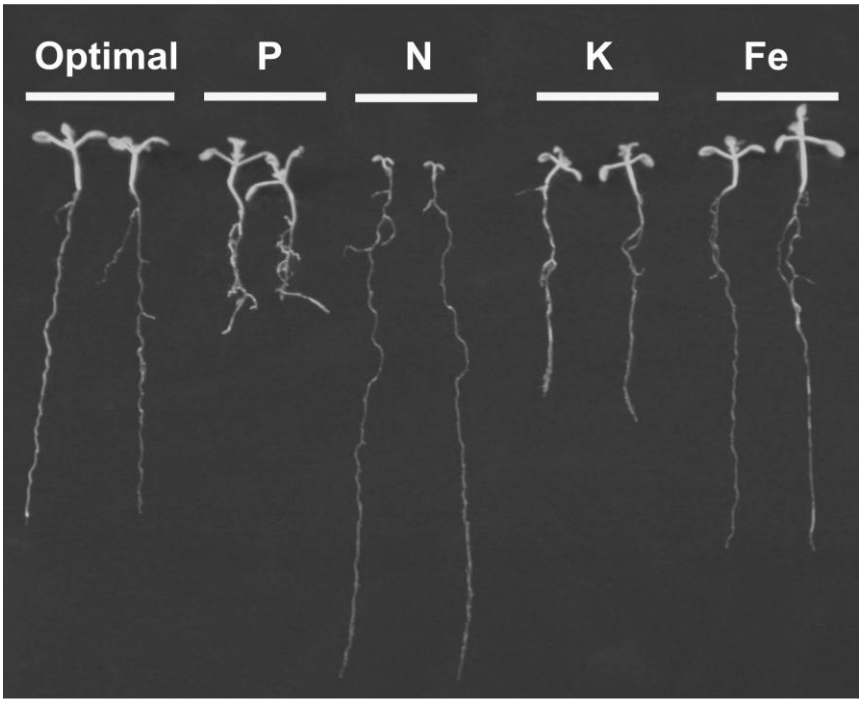

Figure 5. Changes in root system architecture of *Arabidopsis* seedlings grown on media depleted of different nutrients. Phosphorous (P), nitrogen (N), potassium (K) and iron (Fe) (taken from Sánchez-Calderón *et al*., 2013).

When Arabidopsis is grown under phosphate (Pi) limiting conditions, RSA changes consist of reduction in PR length, and increased formation of LRs and root hairs. Other characteristics of plants in Pi starvation are a gradual reduction in cell division in the root meristem, premature differentiation leading to total inhibition of cell elongation, and the loss of the meristematic activity caused by the full differentiation of the RSCN, and irreversible phenomena also known as meristem exhaustion, which directly affect root growth and morphology (reviewed in Sánchez-Calderón *et al.*, 2013).

The MEDIATOR complex

Transcriptional control of gene expression is an essential process in life. In eukaryotes, the major proteins involved in transcription are RNA polymerase II, the general transcription factors (GTFs), a variety of trans-acting activators and repressors, and the Mediator complex (reviewed in Kidd *et al.*, 2011). Mediator is an evolutionarily conserved transcriptional regulatory complex, and its structure is shown to be conserved across eukaryotic kingdoms (Cai *et al.*, 2009).

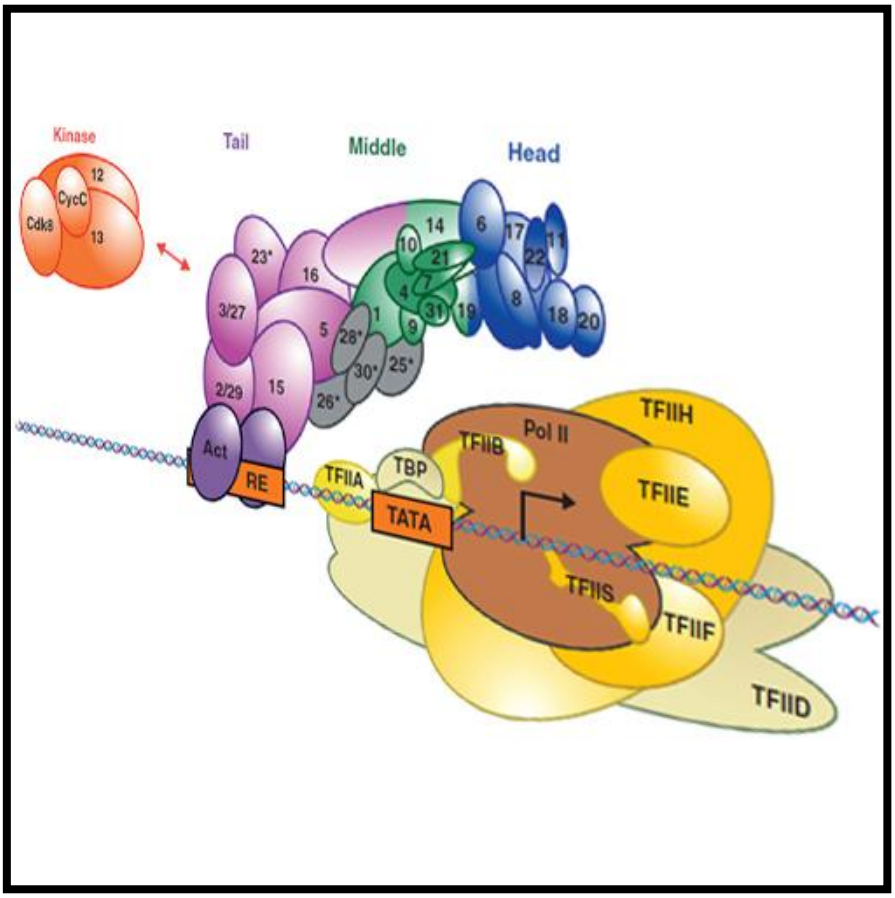

Figure 7. Mediator complex. Its modular structure consists of a Head (blue), a Middle part (green), a Tail (purple), and a detachable module (CDK8 module, in orange). In gray are subunits not assigned to any module. Mediator acts as a bridge between activators and repressors of gene transcription and the general RNA Pol II (Taken from Larivière *et al*., 2012).

The Mediator complex is composed of four modules: the head, middle, and tail, as well as an additional detachable kinase module, known as the CDK8 module (Fig. 7). Mediator acts as a general transcription factor that integrates regulatory signals from transcriptional regulators binding at gene promoters with changes in Pol II activation, thus effecting positive and negative control of transcription (Hemsley *et al*., 2014).

In *Arabidopsis thaliana*, several Mediator subunits have been functionally characterized, and have been shown to be required for correct growth and development, and environmental interactions (reviewed in Kidd *et al.*, 2011). The plant Mediator is composed of 21 conserved and 6 plant-specific subunits (Bäckström *et al.*, 2007). It was first described in 2007, and as a

result, studies on plant Mediator and its role in plant physiological and cellular functions are scarce. Until now, Mediator subunits are reported to be implicated in hormone and plant defense signaling, and embryo, vegetative and reproductive development (Lai *et al.*, 2014; Gillmor *et al.*, 2010; Imura *et al.*, 2012; Gillmor *et al*., 2014).

Mediator as a signal integrator: external signals regulating developmental programs

The large size of Mediator facilitates diverse functional interactions between RNA Pol II and other general and gene-specific transcription regulatory factors, as well as between DNA topological domains (reviewed in Allen and Taatjes, 2015). Through the integration of diverse signaling inputs and the interaction with different transcription factors and other modulators of gene expression, Mediator influences transcription of multiple genes (Hemsely *et al*., 2014; Lai *et al*., 2014; Dhawan *et al*., 2009).

MED12, MED13, CDK8 and CYC are the subunits of the detachable CDK8 module. MED12 and MED13 have been shown to be important for proper control of developmental programs in eukaryotes such as yeast (Samuelsen *et al*., 2003), *Drosophila* (Janody *et al*., 2003), *C. elegans* (Yoda *et al*., 2005), and Arabidopsis (Gillmor *et al*., 2010; Imura *et al*., 2012). MED12 and MED13 mutants also show overlapping phenotypes, and physically interact in vivo (Janody *et al*., 2003). In Arabidopsis, *MED12/CCT* and *MED13/GCT* are required for correct embryo development, regulating the timing of embryo patterning (Gillmor *et al.*, 2010). They are also flowering regulators under long-day conditions (Imura *et al.*, 2012), and have been described as global regulators of developmental transitions in Arabidopsis (Gillmor *et al.*, 2014).

The role of Mediator in microRNA biogenesis

Mediator is required for microRNA (miRNA) biogenesis by recruiting Pol II to promoters of

miRNA genes, and for the production of long noncoding scaffold RNAs (Kim *et al.*, 2011). Now, it is well recognized that microRNAs play a crucial role in regulation of gene expression. So regulation of microRNA transcription represents another level in the cascade of regulation of gene expression. Recently, it was also shown that *MED12/CCT* and *MED13/GCT* subunits of Mediator act upstream of a miRNA-TF cascade that regulates vegetative growth, controlling levels of miR156, a microRNA expressed during the juvenile stage of Arabidopsis (Gillmor *et al.*, 2014).

Sequencing of small RNAs (sRNA) in *med12/cct*, *med13/gct* and *cdk8/hen3* mutants has revealed further sRNAs to be regulated by the CDK8 module. RNA extraction and library preparation experiments were performed by the Dr. Stewart Gillmor laboratory. Aerial tissue of 18 day old *Arabidopsis thaliana,* Col-0 plants was collected and two biological replicates were used for this study. The bioinformatic approach to analyze the results obtained was performed in collaboration with Dr. Cei Abreu's laboratory (Joel Rodríguez Medina, Master's thesis, 2015). It was found that *CCT*, *GCT*, and *CDK8* regulate aproximately 150 miRNAs (123 upregulated and 24 downregulated). These miRNAs participate in processes such as cell division, drought response, leaf traits that change during vegetative development, and nutrient homeostasis (Joel Rodríguez Medina, Master's thesis, 2015).

My analyses were focused on the results shed by the *med12*/*cct* mutant, because the experiments performed for this thesis were carried out using the *cct* mutant background. One of the most affected miRNAs in the *med12*/*cct* mutant backgrounds is miR827. miR827 is one of the most studied microRNAs that respond to phosphate starvation (Hsieh *et al*., 2009; Lundmark *et al*., 2010; Kant *et al*., 2011; Wang *et al*., 2012; Lin *et al*., 2013; Park *et al*., 2014), and its response is conserved across other plant species such as rice, barley and maize (Lin *et al*., 2010; Hackenberg *et al*., 2013; Pei *et al*., 2013). We looked for other microRNAs that respond to phosphate starvation in the list of microRNAs affected by the *cct* mutant, and we found four more (Fig. 8).

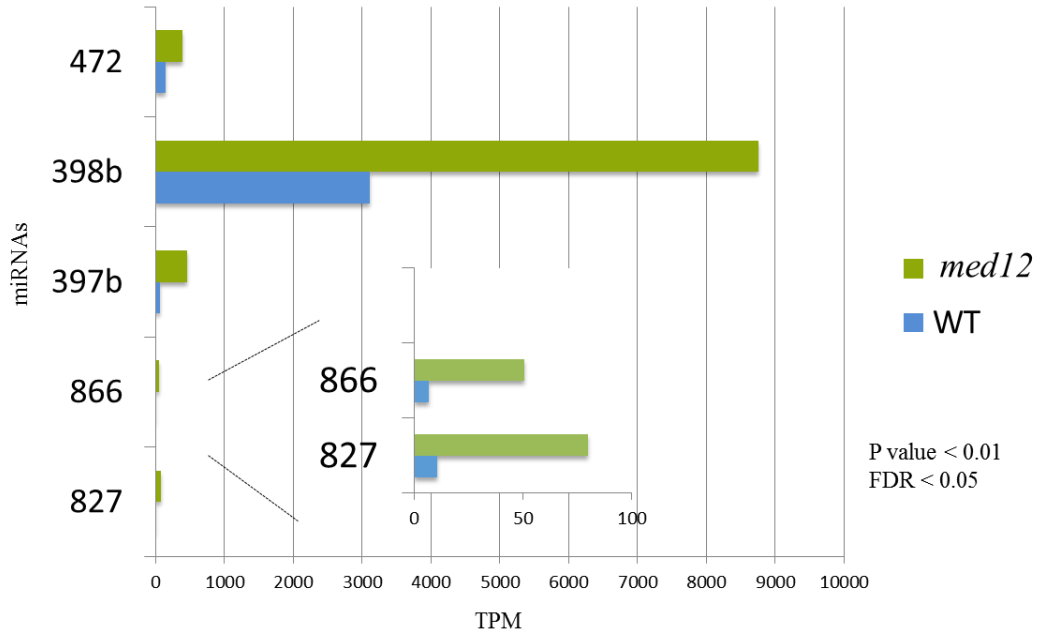

Figure 8. miRNAs that respond to phosphate starvation are up regulated in Arabidopsis *med12* mutants. Data obtained from RNA sequencing of Arabidopsis 18 day old seedlings (shoot only). Raw normalized data is employed. The expression value equals the mean of two replicates of the experiment. TPM: transcripts per million.

Justification

Since phosphate is a macronutrient that is very relevant not only for plant growth, development, and reproduction, but also because P_i availability is a constraint on plant productivity in many natural and agricultural ecosystems, I want to gain insight into the physiological, cellular, developmental and genetic programs where the CDK8 module and the responses that plants use to adapt to limiting P_i growth conditions are interrelated.

Hypothesis

MED12 participates in the control of phosphate homeostasis in plants

General objective

Characterization of *MED12* in low phosphate in *Arabidopsis thaliana*

Specific objectives

1) Analysis of the *CCT/MED12* gene of Arabidopsis in root development and phosphate responses.

a) Analysis of *CCT* gene expression in response to normal and low phosphate concentrations, using the *pCCT::GUS* reporter.

b) Characterization of root morphology in wt and *cct* mutants in normal and low phosphate conditions.

c) Microscopic characterization of wt and the *cct* mutant in normal and low phosphate conditions.

Results

MED12/CCT is a subunit of the CDK8 module of the Mediator complex. It has been reported that the Arabidopsis *cct* mutant shows aberrant phenotypes in embryo development (Gillmor et al., 2010) and vegetative to reproductive phase-transition (Gillmor et al., 2014), as well as seed yield. In other organisms like Drosophila, amoeba and yeast, it is involved in cellular adaptations to environmental cues (Loncle *et al*., 2007; reviewed in Huber, 2013; Raithatha *et al*., 2012; Lindsay *et al*., 2014; Khakhina *et al*., 2014). We have previously shown that microRNAs involved in phosphate starvation responses are missregulated in *cct* mutants. To gain insight of the role of *cct* in linking environmental cues to developmental genetic programs, we characterized the RSA of Arabidopsis *cct* mutants in phosphate-sufficient and phosphate-deficient conditions.

pCCT::GUS is expressed primarily in vascular tissues of the root and shoot

To determine the spatio-temporal transcriptional activity of *CCT* in response to phosphate availability, first we analyzed Arabidopsis seedlings carrying the transcriptional fusion *pCCT::GUS* (Del Toro-De León *et al*., 2014) that were germinated and grown in MS medium, until 7 days after germination (dag), with sufficient phosphate concentration (1000 µM) and stained for *GUS* activity.

pCCT::GUS was expressed throughout vascular tissue in Arabidopsis seedlings at 7 days after germination (dag) (Fig. 9, Fig. 10). In the shoot, high *GUS* expression was observed in cotyledon tips (Fig. 9A), the hypocotyl (Fig. 9B) and stipules (Fig. 9C), as well as vasculature tissue (Fig. 9D-F). In the roots, *pCCT::GUS* was expressed also in the vasculature of PR (Fig. 9D-E) and LR (Fig. 9F), although it was not expressed in the initial stages of LR primordia formation, only when the vasculature was differentiated (Fig. 9D-F).

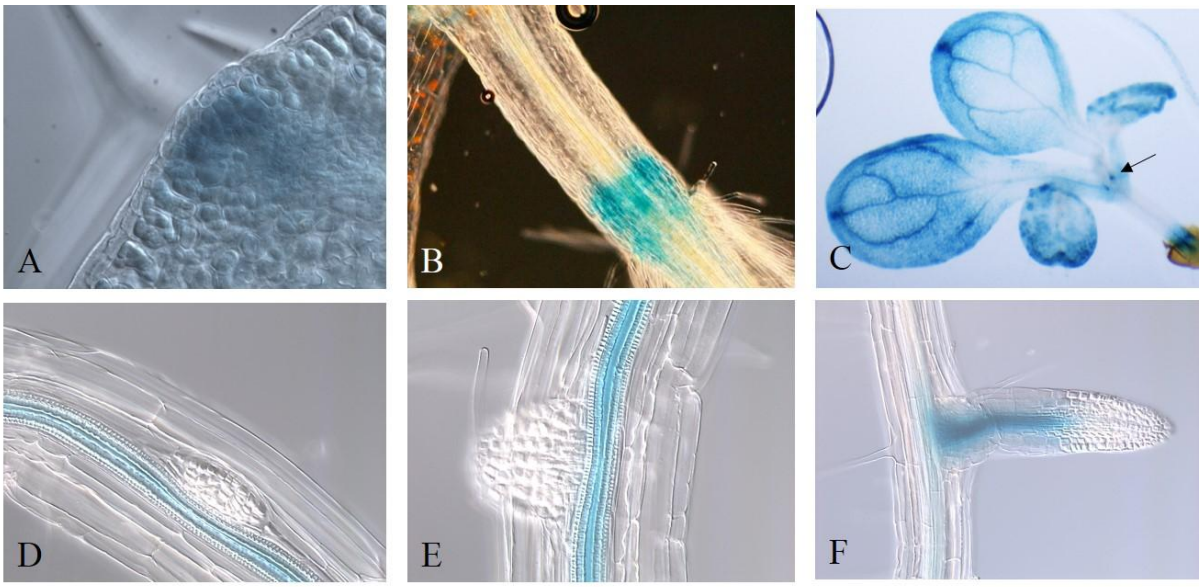

Figure 9. *GUS* expression driven by the 5Kb upstream sequences of *MED12/CCT*. Arabidopsis seedling at 7 days after germination in 1000 µM Pi. A. *pCCT::GUS* expression is observed at cotyledon tips. B. *pCCT::GUS* expression at hypocotyl. C. *pCCT::GUS* expression is shown in vasculature of cotyledons and first leaves. The arrow shows *pCCT::GUS* expression at stipules. D-F. *pCCT::GUS* expression in roots, at the vasculature. GUS signal is not seen in first stages of LR primordia formation, until vasculature is differentiated.

pCCT::GUS expression responds to low phosphate conditions

To determine whether expression of the *CCT* gene responds to low phosphate, Arabidopsis seedlings carrying a *pCCT::GUS* reporter were germinated and grown in Petri dishes containing MS medium with low phosphate concentrations (5 µM), until 7 dag, and stained for GUS activity.

In low phosphate conditions (5 µM Pi; Fig. 10B), GUS staining increased in the root, while it decreased in the shoot, in comparison with the sufficient phosphate conditions (1000 µM Pi; n = 10; Fig.10A). Looking at the shoot, it seems that low phosphate conditions decrease the transcriptional activity of the *CCT* promoter, revealed by GUS expression, or make it more specific.

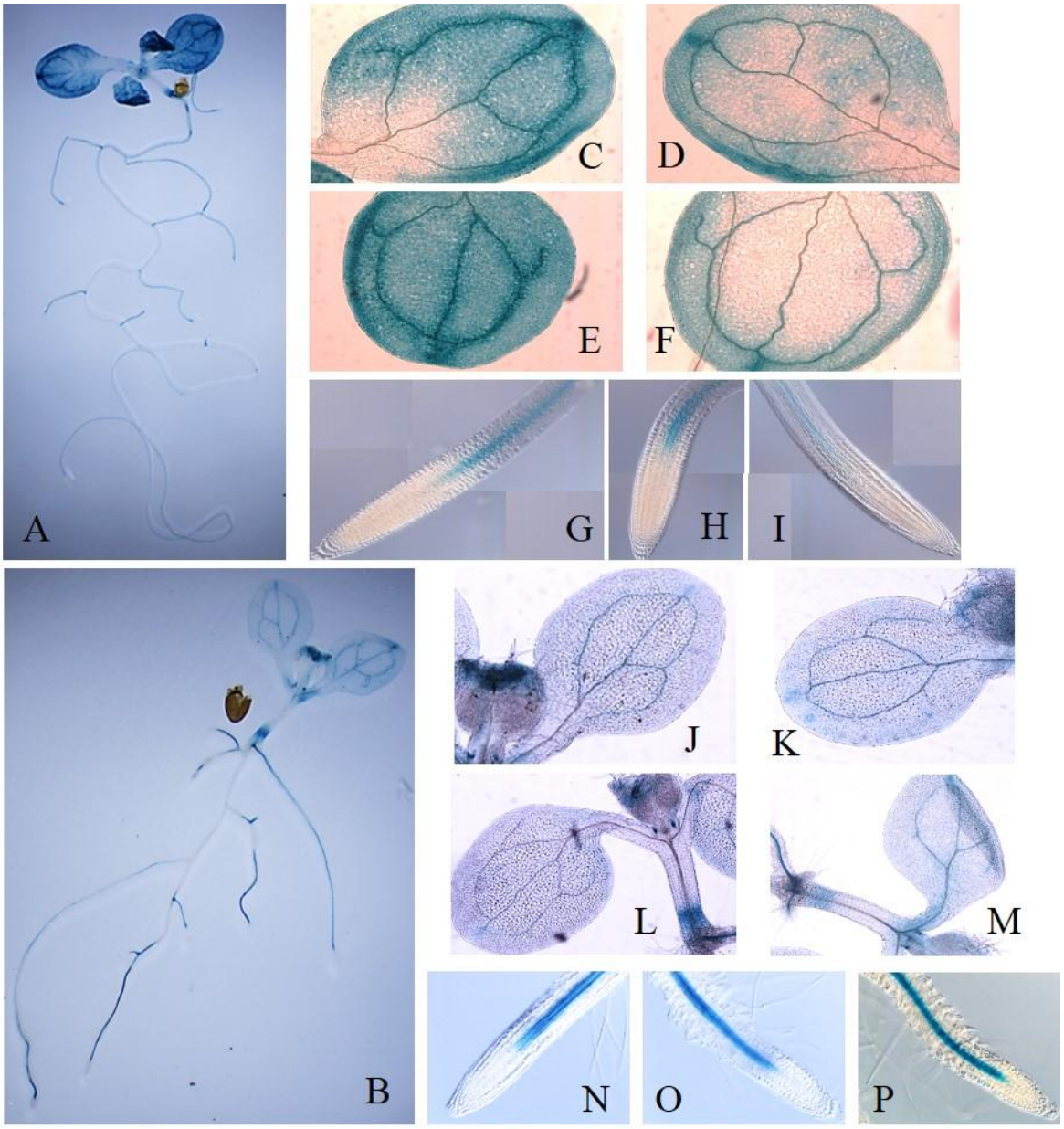

Figure 10. Comparison of *pCCT::GUS* expression in sufficient Pi and low Pi conditions, in 7 dag Arabidopsis seedlings. A. Whole Arabidopsis seedling in sufficient Pi (1000 µM) treatment. B. Whole Arabidopsis seedling in low Pi (5 µM) treatment. C-F. Cotyledon in sufficient Pi. G-I. PR tip in sufficient Pi. J-M. Cotyledon in low Pi. N-P. PR tip in low Pi.

cct mutants have altered root morphology in both normal and phosphate limiting conditions

Upregulation of phosphate-responsive miRNAs in *cct* mutants, as well as a different expression pattern of *CCT* in response to contrasting phosphate levels suggest that the *CCT* gene is involved in low phosphate responses. With this hypothesis in mind, and since RSA modifications are one of the most immediate phenotypic responses to low phosphate, I further characterized the RSA of wt and *cct* mutant seedlings in response to phosphate availability.

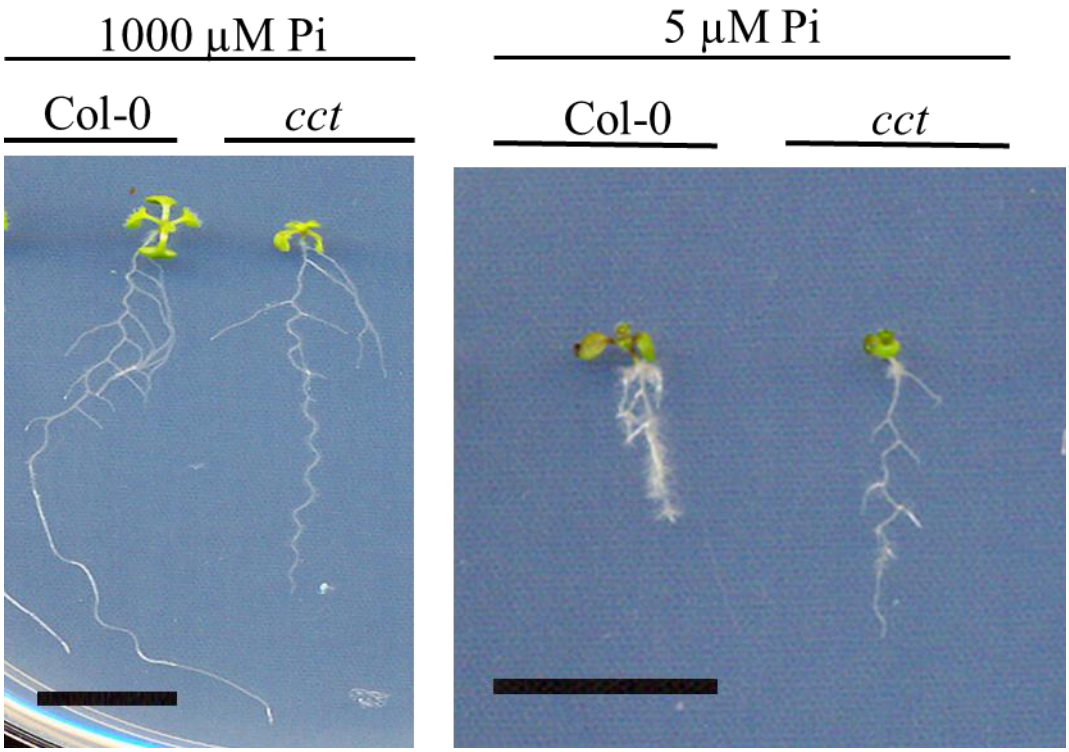

Figure 11. Root System Architecture of Columbia-0 wt and *cct* in sufficient or low Pi concentrations. 7 dag wt and *cct* seedlings germinated and grown in MS medium with sufficient or low Pi concentrations. A striking difference in RSA is evident when *cct* mutant root is compared to wt root in low phosphate as well as in sufficient phosphate conditions. The primary root is shorter in *cct* when they are grown in sufficient Pi concentration, but it is longer in low Pi. The scale bar is 1 cm.

When the *cct* mutant seeds were grown in sufficient Pi medium, it was observed that the primary root was significantly shorter at 7dag compared to wt (Fig. 11, Fig. 14B; wt PR mean length = 5.58 cm vs. *cct* PR mean length = 3.18 cm, $p < 0.001$). During the first 3 dag, this

difference was not evident (data not shown), but from day 5 it became evident. Other differences were in the length of the longest lateral root (Fig. 14D; wt longest LR mean = 1.27 cm vs. *cct* longest LR mean = 0.91 cm, $p < 0.05$), and the root hairs along the primary root (Fig. 12). The *cct* seedlings showed a primary root covered by longer root hairs than the wt (Fig. 12; n=8). There was also a significant difference in the length from the root tip upward where root hairs started growing in wt and *cct* (wt mean = 2.21 mm vs. *cct* mean = 1.60 mm; $p < 0.001$)

Another significant difference was in the diameter of the primary root, measuring where the root hairs started to appear. The mean of the diameter for wt and *cct* was 0.22 and 0.18 mm, respectively ($p < 0.05$). This data, plus the previous result that *CCT* is expressed in vasculature, could indicate that there is a difference in cell layers in the vasculature. To test this possibility, it will be necessary to analyze the roots by cross-sections.

Interestingly, when wt and *cct* seedlings were grown in low Pi conditions, the differences observed in PR length and RH growth were the opposite than in sufficient-phosphate (Fig. 11, Fig. 14B; wt PR length mean = 0.63 vs. *cct* PR length mean = 1.48, $p < 0.001$). PR length was longer in *cct* than in wt in the low-phosphate treatment. The striking difference was obvious not only in the primary root length, but also at the level of complete root system architecture (RSA). There were fewer root hairs in primary and lateral roots of *cct* (Fig. 13), and also the shoot accumulated less red color (presumably less anthocyanin). Mean length of the longest *cct* lateral roots was 0.45 cm, while for wt it was 0.46 cm, in low Pi, an insignificant difference (Fig. 14D).

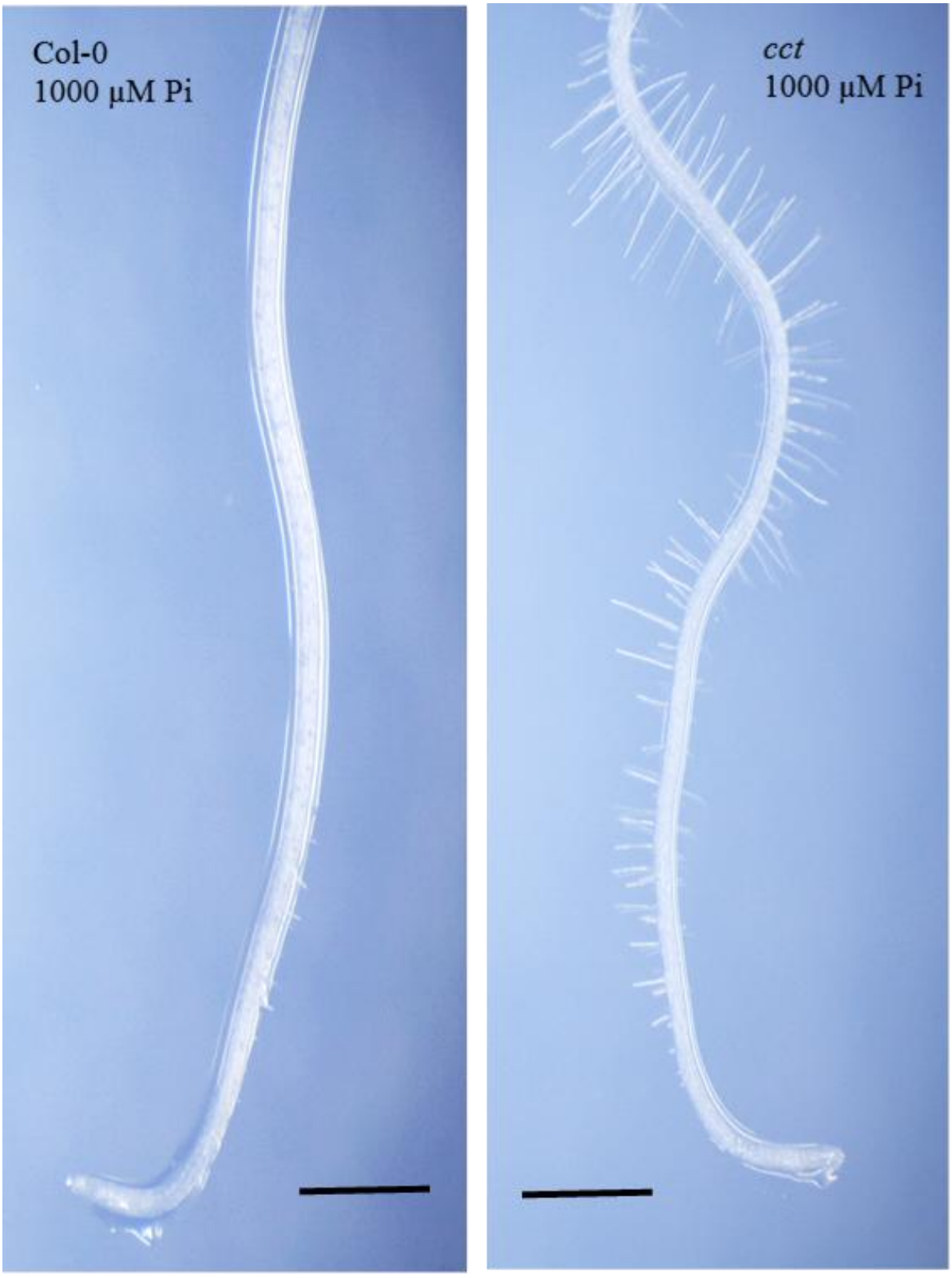

Figure 12. Primary root and root hairs in sufficient Pi (1000 µM) in Columbia-0 wt and *cct*. 7 days old after germination wt and *cct* seedlings. Along the primary root of wt there are shorter root hairs, as well as they start to grow farther from root tip, in comparison to *cct* mutant. The plants were grown in MS medium with sufficient (1000 µM) Pi concentration. Scale: 1 mm.

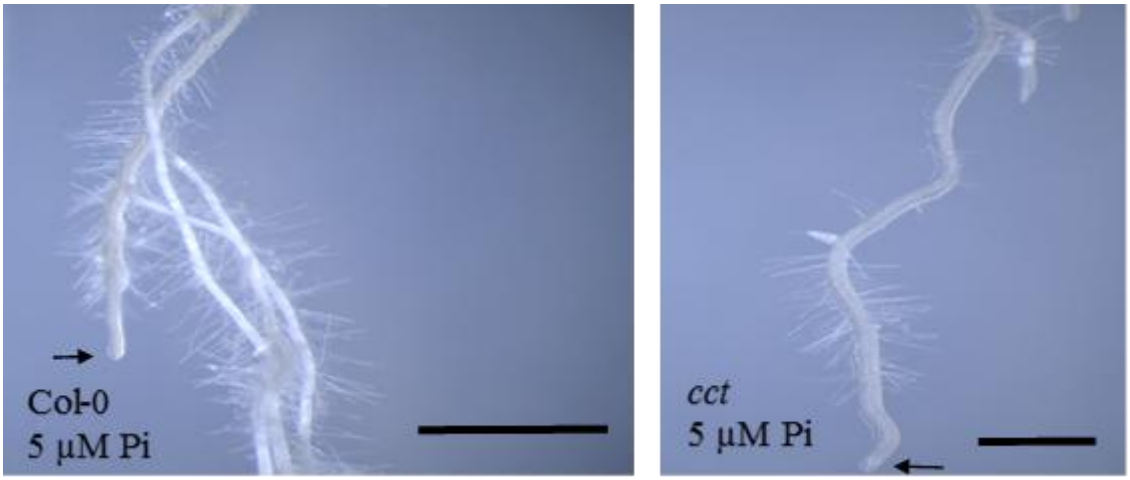

Figure 13. Primary root and root hairs in low Pi (5 µM) in Columbia-0 wt and *cct*. RSA in low phosphate for wt and *cct* mutant is shown. The root hair density and the length of lateral roots are the most evident differences. The arrows point to the root tip of the primary root. The scale is 1mm.

The distribution of the data of primary root length measurements of wt and *cct*, in sufficient and low Pi, shows that wt and *cct* present inverse patterns. That is, when in sufficient Pi (Fig. 14B, yellow) primary root length in general is longer in wt than *cct*. When in low Pi (Fig. 14B, blue), primary root length is longer in *cct* than in wt. Also, the difference in primary root length within genetic backgrounds is obvious. In wt, the low Pi treatment severely affects whole RSA, while in *cct* the RSA is not drastically affected by low Pi treatment. There are differences in other aspects of RSA, like the total root length (TRL; Fig. 14A). TRL distribution is similar to PRL distribution.

Lateral Root Density [LRD; (number of lateral roots of first order + number of lateral roots of second order)/primary root length; Fig. 14C] distribution between wt and *cct* in sufficient Pi conditions, is not significantly different. In low Pi, wt and *cct* show a significant difference, since the wt shows a higher LRD than *cct*. This may be due to the apparent lack of plasticity of *cct*, or the reduction in phenotypic change to low Pi conditions. In the same way than in other RSA characteristics, LRD within genetic backgrounds is less affected in *cct* than in wt.

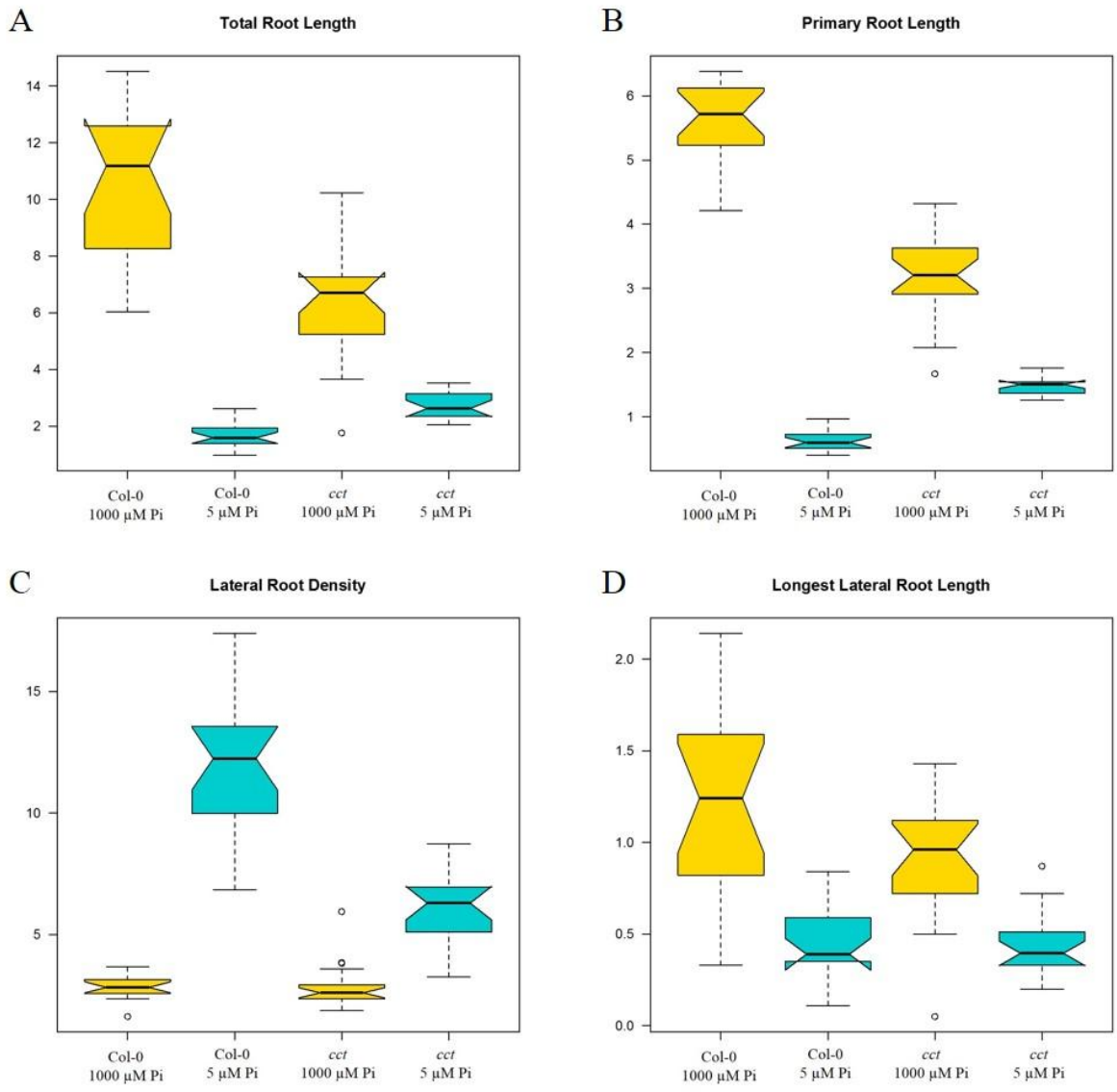

Figure 14. Distribution of Primary Root Length, Total Root Length, Lateral Root Density and Longest Lateral Root Length in Columbia-0 wt and *cct* in sufficient phosphate (1000 µM) and low phosphate (5 µM) treatments. Y axis is in cm.

Microscopic observations of wild type and cct root tips

Low-phosphate conditions alter RSA by arresting primary root growth, and increasing lateral root and root hair density (Williamson et al., 2001; López-Bucio et al., 2002). The most studied mechanism that controls inhibition of primary root growth by low phosphate is the

shift from an indeterminate to a determinate root growth program, in which the root meristem gets exhausted (Sánchez-Calderón et al., 2005).

Using Nomarski microscopy I was not able to notice any differences in the Quiescent Center (QC) of wt and *cct* in either phosphate-sufficient or in phosphate-low treatments. A noticeable difference between root tips of wt and *cct* in low Pi treatment, however, was observed in the integrity of the columella, where 10 of 11 *cct* plants conserved the columella, while 2 of 8 wt plants conserved the columella in the same treatment (Fig. 15).

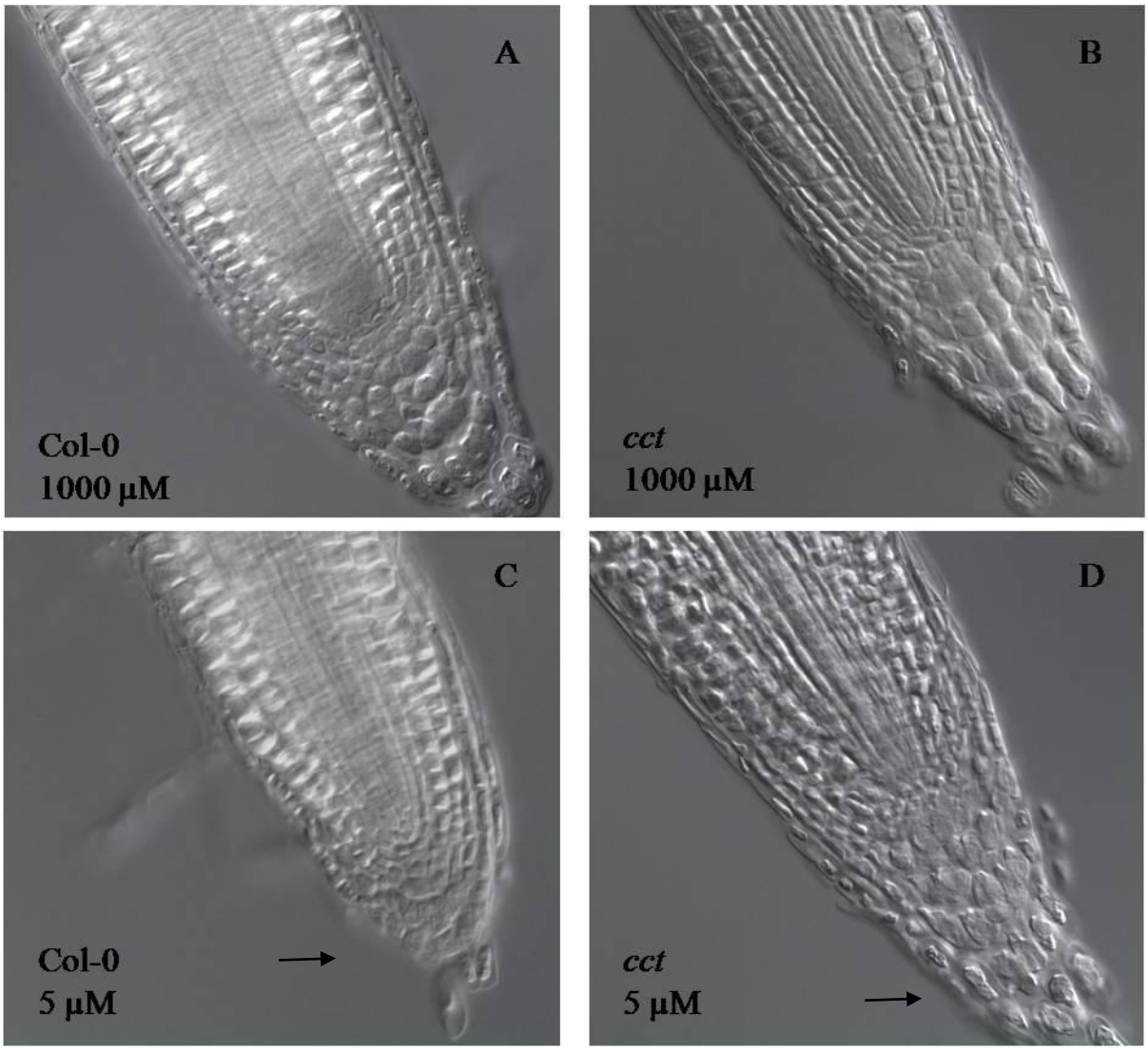

Figure 15. Primary root (PR) tips of Col-0 and *cct* in low and sufficient Pi, using a Nomarski microscopy 40X. 7 day after germination Arabidopsis seedlings, cleared according to the protocol described in Methods. A and B. PR tip of Col-0 (A) and *cct* (B) in sufficient Pi conditions. No noticeable difference is observed between wt and *cct* columella integrity. C and D. PR tip of Col-0 (C) and *cct* (D) in low Pi conditions. PR tip in wt in low-phosphate is almost depleted of columella cells, while *cct* in the same treatment keeps a longer columella.

Discussion

CCT/MED12 expression in the vascular system is affected by phosphate levels

The vascular system is a continuous multicellular network within the plant body that transports water and nutrients, photosynthates, as well as signaling molecules like microRNAs and hormones (Endo *et al.*, 2008). *CCT* transcriptional activity revealed in the *pCCT*::*GUS* transgenic line is observed mainly in the vasculature of the shoot and the root. It was also observed in hypocotyl, stipules and cotyledon tips. The hypocotyl is a region of cell elongation (Wang et al., 2015). It has been demonstrated that stipules are primary sites for the accumulation of auxin associated with vascular differentiation and leaf morphogenesis (Sun and Nocker, 2010). The cotyledon tip is the place where hydathodes are developed, and also presents an auxin maximum, although hydathode development is not necessary for the high auxin levels (Pillitteri et al., 2008). Hydathode pores are oversized stomatal structures, incapable of regulating aperture, and therefore remain open to release water and dissolved solutes of the xylem (Esau 1977; Pillitteri et al., 2008).

In the roots, in low Pi GUS expression appears increased compared to sufficient Pi conditions, maybe because of the earlier differentiation of the cells (Fig. 10G-I, Fig. 10N-P). This expression has to be explored more by combining *CCT* expression with markers in the MZ, EZ and DZ.

Taking into account that some microRNAs that participate in the phosphate starvation response are overexpressed in the shoot of the *cct* mutant (Joel Rodríguez, Master Thesis, 2015), and that *pCCT::GUS* expression was increased in seedlings grown in sufficient phosphate, I hypothesize that in sufficient Pi conditions, *CCT* directly or indirectly represses the transcription of microRNAs such as miR399 and miR827. miR827 expression, as with other phosphate starvation induced (PSI) miRNAs, is increased in the *cct* mutant in sufficient Pi conditions, which leads to the hypothesis that *CCT* represses *miR827* transcription in the

shoot when no stress is detected. The observation of lower *pCCT::GUS* expression in the shoot in low Pi is in agreement with miR827 increased expression in response to low Pi in the shoot. This leads to the question: how might low phosphate levels act to control transcription of *CCT*? One possibility is that microRNAs responding to low-phosphate could target *CCT* transcript, or another component of Mediator. And when low-phosphate is detected the microRNA that will target the *CCT* transcript is expressed, and then *CCT* transcript levels are reduced. Also, low-phosphate could induce the activity of a transcription factor that represses *CCT* transcription. It is also possible that *CCT* expression is mediated by a hormone responding to phosphate levels. Hormones play critical roles in growth and differentiation of plants. It has been shown that *MED12* slightly increases its expression in auxin treatment, but a higher increase in expression was observed in brassinosteroid treatment, almost at the level of *SAUR9*, a brassinosteroid-responsive gene (Pasrija and Thakur, 2012). Also, in this work they showed that high-light increases *CCT* expression more than 2-fold (Pasrija and Thakur, 2012). As *CCT* could be acting as an environmental integrator, it is very possible that multiple cues are regulating its expression.

Although *pCCT::GUS* expression in low and sufficient Pi shows differences in expression, it is necessary to do appropriate measurements in order to accurately determine the differences in expression in the shoot and in the root by Quantitative-PCR. Also, it will be very interesting to determine with greater resolution the expression of *CCT* among the xylem, phloem or cambium, by cross-section analysis of the root.

PR length is reduced in *cct* mutant background in sufficient Pi

In sufficient Pi conditions, the *cct* mutant shows severely reduced primary root growth. Shortening of PR length is characteristic of Arabidopsis plants growing in low Pi treatments. In *cct*, this doesn't seem to be related to meristem integrity, because no evident meristem defect is noted. To analyze meristem integrity in Arabidopsis seedlings older than 7 dag will allow having a complete picture of this process in the *cct* mutant. The use of meristem

molecular markers will help to validate this observation. Longer RHs are also characteristic of Pi starved plants, and interestingly *cct* in sufficient Pi shows this phenotype. The distribution, density and length of root hairs are very plastic in response to phosphate availability. In Arabidopsis, the elongation of root hairs is regulated by phosphorus availability, and not by other nutrients (Bates and Fynch, 2001).

As *CCT* expression is observed in the vascular system, it will be necessary to trace vascular system integrity longitudinally with fuchsin, in cleared roots, in order to look for developmental aberrations in tissue specification. Cross-sections will be also very informative to look for the reason PR diameter is lower in *cct* mutant than wt.

The *cct* mutant root in sufficient Pi resembles a Pi-starved Arabidopsis root. In accordance with this phenotype, transcriptomic data showed that microRNAs that respond to Pi starvation (i.e. miR827, miR399) are upregulated in *cct* mutant shoots in sufficient Pi conditions. To get insight into the role of *cct* in RSA or phosphate starvation response, it will be important to determine if *cct* is acting directly to repress these microRNAs in sufficient Pi conditions, or if it is acting upstream this regulation.

RSA is not drastically affected in *cct* in low Pi treatment

Despite a reduction in PR growth in low Pi compared to sufficient Pi in *cct* seedlings, this reduction was not as drastic as in the wt. One possible interpretation for the *cct* phenotype in low Pi treatment is that *cct* has less plasticity to respond dramatically to low Pi environment. This change in plasticity can represent an advantage because it is evident that the less severe phenotypic response of *cct* mutants to low phosphate does not perturb development so drastically, at least until 7 days after germination, the stage at which I analyzed the phenotype. The *cct* mutant seedlings showed less damaged shoots and roots in low Pi than wt, which means that if we affect the plant plasticity it can survive better (at least just considering some phenotypic traits) to low Pi. It will be very informative to analyze *cct*

mutant phenotype in low phosphate until other developmental stages like flowering and senescence.

Low Pi treatment was represented by 5 µM, and in this treatment we observed that the comparison in RSA characteristics between wt and *cct* was the inverse to what we observed in sufficient Pi. In sufficient Pi, the mean PRL of *cct* was less than wt, while in low Pi, the mean PRL of *cct* was longer than wt. Also, in low Pi *cct* showed less RH than wt, when in sufficient Pi we noted the opposite. It will be very interesting to test different Pi concentrations, in order to seek for gradual phenotypic differences.

The columella is the apical part of the root cap, an organ essential for root meristem maintenance (Tsugeki and Fedoroff, 1999) and auxin fluxes (Kramer and Bennet, 2006). In 2007 Svistoonoff *et al*., demonstrated that root growth arrest is triggered when the root tip senses the low phosphate concentration of the medium (Svistoonoff et al., 2007). Maybe, that the columella guards its integrity in *cct* in low phosphate conditions could be due to not sensing correctly the low phosphate environment, therefore primary root growth is not drastically inhibited in *cct* as in wt.

CCT/MED12 as an integrator of environmental Pi to plant RSA development

RSA shows developmental plasticity when exposed to stress environments. How *CCT* can mediate changes in RSA is an open question. One possibility would be through regulation of the abundance of miRNAs such as miR156 (Gillmor *et al*., 2014), that can have dual activities in development and stress responses, acting as signal integrators (Stief *et al*., 2014). In this sense, CCT as a subunit of the CDK8 module, could be repressing microRNAs that integrate responses to low phosphate and root development. A very interesting approach could be to test the possible dual role of the microRNAs involved in phosphate starvation responses, which are induced in the *cct* mutant in phosphate-sufficient conditions. It will be a

great contribution if we can establish how *CCT* is acting in modifying RSA in response to environmental cues, which other elements are regulating its expression, and which other genes are being regulated by *MED12/CCT* (Fig. 16).

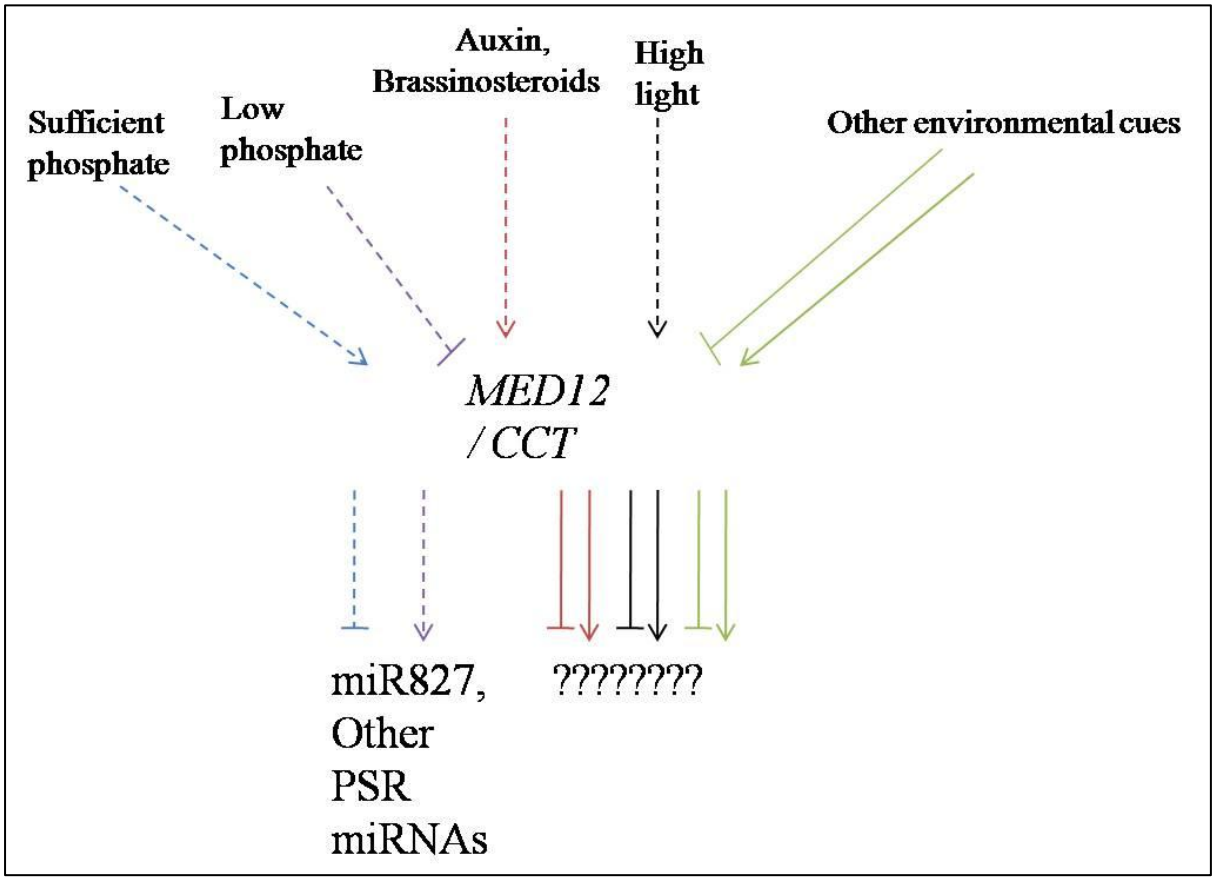

Figure 16. Diagram of *MED12/CCT* regulation in the shoot, through multiple environmental cues.

As the transcription expression results of the microRNAs involved in phosphate-starvation responses (PSR) affected in the *cct* mutant, come from shoot tissue, the diagram representing the *CCT* regulation of*miR827* and other phosphate starvation responsive microRNAs (Fig. 16,blue & purple lines) is only exemplifying what could be happening in the shoot.

Conclusions

CCT/MED12 subunit of Mediator is expressed throughout the plant vascular system. The results obtained in this thesis suggest it is acting as an environmental Pi status integrator to

Arabidopsis RSA. Primary root and root hair growth are the most affected morphological characteristics. The *cct* mutant shows a less dramatic response to low Pi in comparison to wt, which implies that phenotypic plasticity is compromised in *cct* mutant.

Perspectives

To determine more precisely the physiological role of *CCT*, a cross-section of a plant with the GUS reporter system will allow to study *CCT* specific expression in the vascular system. Confocal analysis using fluorescent markers of root tip in *cct* mutant background, like WOX5 (QC marker)), Cyclin B (Meristematic zone marker), root cap, and elongation and differentiation zone markers will help us elucidate which zone is principally being affected in the root tip of *cct*. To extend the study to 10, 12 and 14 days after germination, and to compare QC integrity in low phosphate and sufficient phosphate with wild type. And to measure quantitatively GUS expression in sufficient and low phosphate conditions by Real Time PCR.

Materials and Methods

Seed

Seeds used in this work were of the *Arabidopsis thaliana* wild type Columbia (Col-0) ecotype (Arabidopsis ABRC stock center under accession CS1092), the *cct-1* mutant (Col-0 ecotype) (Arabidopsis ABRC stock center under accession CS65890), and the *pCCT::GUS* reporter line (Col-0 ecotype) (available from the Gillmor laboratory, described in detail in Del Toro-De León et al., 2014). The *pCCT::GUS* reporter line consists of 5kb of DNA upstream of the *CCT/MED12* (At4g00450) start codon, fused to the GUS + gene. Arabidopsis seeds were surface sterilized for 1 min in EtOH 100%, then 5 min in 20% (v/v) bleach, and rinsed four times in sterile miliQ water. Seeds were individually pipetted out in a single row at the top of the Petri dishes, and then given a cold treatment for 4 days at 4°C, in the dark. All plants were grown at 22°C under a 16h light/8h dark regime and a light intensity of approximately 85 µmol m^{-2} s^{-1}.

Plant growth

Arabidopsis seeds were germinated and grown on Petri dishes containing +Pi (1000 µM) or –Pi (5 µM) MS medium, until 7 days after germination (dag). The basic MS medium contained the following: 2.0 mM NH_4NO_3, 1.9 mM KNO_3, 0.3 mM $CaCl_2$ $2H_2O$, 0.15 mM $MgSO_4$ $7H_2O$, 1mM Na_2MoO_4 $2H_2O$, 0.1 mM $CuSO_4$ $5H_2O$, 0.1mM $CoCl_2$ $6H_2O$, 0.1 mM $FeSO_4$ $7H_2O$, 0.1 mM Na_2EDTA $2H_2O$, 10 mg/L inositol, 0.2 mg/L glycine, 0.05 mg/L pyridoxine chlorhydrate, 0.05 mg/L nicotinic acid and 0.01 mg/L thiamine hydrochloride, in addition to the P source, Pi (KH_2PO_4) which was added as required. pH 5.7, 1% (w/v) Sucrose, 1% (w/v) TC agar and 2.5 mM MES

Histology and histochemistry

7 dag seedlings were stained for GUS activity for 2 hours in the following solution: EDTA 1mM pH 8, Potassium ferricyanide 5mM, Potassium ferrocyanide 5mM, Sodium phosphate 100 mM pH 7, Triton-X-100 1%, X-Gluc 1 mg/ml predissolved in a drop of *N,N*-dimethylformamide. Stained and unstained roots were cleared for 60 min at 62°C in Clearing Solution I (20% methanol, 0.8% HCl), and then replaced with Clearing solution II (7% NaOH, 60% Absolute Ethanol) for 20 min at room temperature. Roots were rehydrated overnight at room temperature in 40% EtOH. Then, for 20 minutes each in 20% and 10% EtOH, and 10% EtOH / 50% glycerol. Roots were mounted in 50% glycerol on glass microscope slides. All samples were observed using a Nomarski microscope and a stereoscope.

Determination of Primary Root Length, Total Lateral Root Length, Primary Root Diameter, and Root Hair Growth Zone

Digital images of plants were traced by hand using RootReader2D (http://www.plantmineralnutrition.net/rr2d.php). Pixels were converted to the appropriate metric equivalent using a ruler as a parameter. The primary root was measured by tracing from the root-shoot junction to the root tip. The total lateral root length equals the sum of the lengths of all individual lateral roots on an individual plant. The primary root diameter was measured at the location on the root where the first root hair was visible at the macroscopic

level. The length from the root tip to the location of the macroscopic emergence of the first root hair was also measured.

Statistical tests

T-student tests were performed on root measurements data. P-value ≤ 0.05 was considered for a statistically significant difference between unpaired samples.

Notched box plots where created with R software. In the notched boxplot, if two boxes do not overlap this is "strong evidence" their medians differ (Chambers *et al*., 1983, p. 62).

Bibliography

Allen BL, Taatjes DJ. (2015) The Mediator complex: a central integrator of transcription. Nat Rev Mol Cell Biol. 16 (3): 155-66

Auesukaree C, Homma T, Kaneko Y, Harashima S. (2003) Transcriptional regulation of phosphate-responsive genes in low-affinity phosphate-transporter-defective mutants in *Saccharomyces cerevisiae*. BBRC. 306: 843-850

Aung K, Lin SI, Wu CC, Huang YT, Su CL, Chiou TJ. (2006) pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol. 141(3): 1000-11

Bäckström S, Elfving N, Nilsson R, Wingsle G, Björklund S. (2007) Purification of a plant mediator from *Arabidopsis thaliana* identifies PTF1 as the Med25 subunit. Mol Cell. 26(5): 717-29

Bari R, Datt Pant B, Stitt M, Scheible WR. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol. 141: 988-999

Bates TR, Fynch JP. (2001) Root hairs confer a competitive advantage under low phosphorus

availability. Plant and Soil. 236 (2): 243-250

Cai G, Imasaki T, Takagi Y, Asturias FJ. (2009) Mediator structural conservation and implications for the regulation mechanism. Structure 17:559–567

Carlsbecker A, Lee JY, Roberts CJ, Dettmer J, Lehesranta S, Zhou J, Lindgren O, Moreno-Risueno MA, Vatén A, Thitamadee S, Campilho A, Sebastian J, Bowman JL, Helariutta Y, Benfey PN. (2010) Cell signaling by microRNA165/6 directs gene dose-dependent root cell fate. Nature. 465 (7296): 316-321

Chambers JM, Cleveland WS, Kleiner B, Tukey PA. (1983) Graphical methods for data analysis (Statistics).

Chiou TJ, Aung K, Lin SI, Wu CC, Chiang SF, Su C. (2006) Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. The Plant Cell 18: 412-421

Datt Pant B, Buhtz A, Kehr J, Scheible WR. (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. The Plant J. 53: 731-738

Datta S, Kim CM, Pernas M, Pires ND, Proust H, Tam T, Vijayakumar P, Dolan L. (2011) Root hairs: development, growth and evolution at the plant-soil interface. Plant soil. 346: 1-14

Del Toro-De León G, García-Aguilar M, Gillmor CS. (2014) Non-equivalent contributions of maternal and paternal genomes to early plant embryogenesis. Nature. 514 (7524): 624-627

Dello Ioio R, Scaglia Linhares F, Scacchi E, Casamitjana-Martínez E, Heidstra R, Costantino P, Sabatini S. (2007) Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation. Current Biology. 17: 678-682

Dello Ioio R, Galinha C, Fletcher AG, Grigg SP, Molnar A, Willemsen V, Scheres B, Sabatini S, Baulcombe D, Maini PK, Tsiantis M. (2012) A PHABULOSA/Cytokinin feedback loop controls root growth in *Arabidopsis*. Current Biology. 22: 1699-1704

Dhawan R, Luo H, Foerster AM, Abugamar S, Du HN, Briggs SD, Mittelsten SO, Menciste T. (2009) HISTONE MONOUBIQUITINATION1 interacts with a subunit of the mediator complex and regulates defense against necrotrophic fungal pathogens in Arabidopsis. Plant Cell. 21 (3): 1000-19

Doerner P. (2008) Phosphate starvation signaling: a threesome controls systemic P_i homeostasis. Curr Op in Plant Biol. 11: 536-540

Dolan L, Janmaat K, Willemsen V, Linstead P, Poething S, Roberts K, Scheres B. (1993) Cellular organization of the *Arabidopsis thaliana* root. Development. 119: 71-84

Endo A, Koshiba T, Kamiya Y, Nambara E. (2008) Vascular system is a node of systemic stress response. Plant Signal Behav. 3(12): 1138-1140

Giehl R, Gruber BD, von Wirén N. (2014) It's time to make changes: modulation of root system architecture by nutrient signals. Journal of Experimental Botany. 65 (3): 769-778.

Gillmor CS, Park MY, Smith MR, Pepitone R, Kerstetter RA, Poethig RS. (2010) The MED12–MED13 module of Mediator regulates the timing of embryo patterning in *Arabidopsis*. Development 137: 113–122

Gillmor CS, Silva-Ortega CO, Willmann MR, Buendía-Monreal M, Poething RS. (2014) The *Arabidopsis* Mediator CDK8 module genes *CCT* (*MED12*) and *GCT* (*MED13*) are global regulators of developmental phase transitions. Development. (141): 4580-4589

Grierson C, Nielsen E, Ketelaar T, Schiefelbein J. (2014) Root hairs. The *Arabidopsis* book.

Grossman JD, Rice KJ. (2012) Evolution of root plasticity responses to variation in soil nutrient distribution and concentration. Evolutionary Applications. 850-857

Hackenberg M, Shi BJ, Gustafson P, Langridge P. (2013) Characterization of phosphorus-regulated miR399and miR827 and their isomirs in barley under phosphorus-sufficient and phosphorus-deficient conditions. EMC Plant Biol. 13: 214 doi: 10.1186/1471-2229-13-214.

Heidstra R, Sabatini S. (2014) Plant and animal stem cells: similar yet different. Nature Rev. Mol. Cell Biol. 15: 301-312

Hemsley PA, Hurst CH, Kaliyadasa E, Lamb R, Knight MR, De Cothi EA, Steele JF, Knight H. (2014) Plant Cell. 26 (1): 465-484.

Hsieh LC, Lin SI, Shih ACC, Chen JW, Lin WY, Tseng CY, Li WH, Chiou TJ. (2009) Phosphate deficiency in Arabidopsis by deep sequencing. Plant Phys. 151 (4): 2120-2132

Huber RJ. (2013) The cyclin-dependent kinase family in the social amoebozoan *Dictyostelium discoideum*. Cel Mol Life Sci. 71 (4): 629-639

Janody F, Marlirosyan Z, Benlali A, Treismar JE. (2003) Two subunits of the Drosophila mediator complex act together to control cell affinity. Development. 130 (16): 3691-701

Imura Y, Kobayashi Y, Yamamoto S, Furutani M, Tasaka M, Abe M, Araki T. (2012) CRYPTIC PRECOCIOUS/MED12 is a novel flowering regulator with multiple target steps in Arabidopsis.Plant Cell Physiol. 53(2): 287-303

Jung JKH, McCouch S. (2013) Getting to the roots of it: genetic and hormonal control of root architecture. Frontiers in plant science. 4 (186). doi: 10.3389/fpls.2013.00186

Kant S, Peng M, Rothstein SJ. (2011) Genetic regulation by NLA and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in Arabidopsis. Plos Genet. 7 (3): e1002021

Kehr J. (2012) Roles of miRNAs in nutrient signaling and homeostasis. Signaling and Communication in Plants. 15: 197-216

Khakhina S, Cooper KF, Strich R. (2014) Med13p prevents mitochondrial fission and programmed cell death in yeast through nuclear retention of cyclin C. Mol Biol Cell. 25 (18): 2807-2816

Kidd BN, Cahill DM, Manners JM, Schenk PM, Kazan K. (2011) Diverse roles of the Mediator complex in plants. Seminars in Cell & Developmental Biology 22(7): 741-748

Kim YJ, Zheng B, Yu Y, Won SY, Mo B, Chen X. (2011) The role of Mediator in small and long noncoding RNA production in *Arabidopsis thaliana*. EMBO J. 30(5): 814-22

Lai Z, Schluttenhofer CM, Bhide K, Shreve J, Thimmapuram J, Lee SY, Yun DJ, Mengiste T. (2014) MED18 interaction with distinct transcription factors regulates multiple plant functions. Nat Commun. 5: 3064 doi:10.1038/ncomms4064

Lenburg ME, O'Shea EK. (1996) Signaling phosphate starvation. TIBS. 21: 383-387

Lin SI, Santi C, Jobet E, Lacut E, El Kholti N, Karlowski WM, Verdeil JL, Breitler JC, Périn C, Ko SS, Guiderdoni E, Chiou TJ, Echeverria M. (2010) Complex regulation of two target genes encoding SPX-MFS proteins by rice miR827 in response to phosphate starvation. Plant Cell Physiol. 51 (12): 2119-31

Lin WY, Huang TK, Chiou TJ. (2013) Nitrogen limitation adaptation, a target of microRNA827, mediates degradation of plasma membrane-localized phosphate transporters to maintain phosphate homeostasis in Arabidopsis. Plant Cell. 25 (10): 4061-74

Lindsay AK, Morales DK, Liu Z, Grahl N, Zhang A, Willger SD, Myers LC, Hogan DA. (2014) Analysis of *Candida albicans* mutants defective in the Cdk8 module of Mediator reveal links between metabolism and biofilm. Plos Genet. DOI: 10.1371/journal.pgen.1004567

Liu TY, Huang TK, Tseng CY, Lai YS, Lin SI, Lin WY, Chen JW, Chiou TJ. (2012) PHO2-dependent degradation of PHO1 modulates phosphate homeostasis in *Arabidopsis*. The Plant Cell. 24(5): 2168-83

Loncle N, Boube M, Boschiero C, Werner M, Cribbs DL, Bourbon HM. (2007) Distinct roles for Mediator Cdk8 module subunits in Drosophila development. EMBO J. 26 (4): 1045-54

López-Bucio J, Hernández-Abreu E, Sánchez-Calderón L, Nieto-Jacobo MF, Simpson J, Herrera-Estrella L. (2002) Phosphate availability alters architecture and causes changes in hormone sensitivity in the Arabidopsis root system. Plant Physiol. 129 (1): 244-256

Lundmark M, Komer CJ, Nielsen TH. (2010) Global analysis of microRNA in Arabidopsis in response to phosphate starvation as studied by locked nucleic acid-based microarrays. Physiol Plant. 140 (1): 57-68

Novoplansky A. (2002) Developmental plasticity in plants: implications of non-cognitive behavior. Evolutionary Ecology. 16: 177-188

Osmont KS, Sibout R, Hardtke CS. (2007) Hidden branches: Developments in root system architecture. Annu. Rev. Plant Biol. 58: 93-113

Park BS, Seo JS, Chua NH. (2014) NITROGEN LIMITATION ADAPTATION recruits PHOSPHATE2 to target the phosphate transporter PT2 for degradation during the regulation of Arabidopsis phosphate homeostasis. 26 (1): 454-64

Parsons R.F. (2009) Hypocotyl hairs: an historical perspective. Aust. J. Bot. 57:106–108

Pasrija R, Thakur JK. (2012) Analysis of differential expression of Mediator subunit genes in Arabidopsis. Plant Signal Behav. 7 (12): 1676-1686

Pei L, Jin Z, Li K, Yin H, Wang J, Yang A. (2013) Identification and comparative analysis of low phosphate tolerance-associated microRNAs in two maize genotypes. Plant Phys. Bioch. 70: 221-234

Péret B, De Rybel B, Casimiro I, Benková E, Swarup R, Laplaze L, Beeckman T, Bennet MJ. (2009) *Arabidopsis* lateral root development: an emerging story. Trends Plant Science. 14 (7): 399-408.

Raithatha S, Su TC, Lourenco P, Goto S, Sadowski I. (2012) Cdk8 regulates stability of the transcription factor Phd1 to control pseudohyphal differentiation of *Saccharomyces*

cerevisiae. Mol Cel Biol. 32 (3): 664-674

Ruzicka K, Simaskova M, Duclercq J, Petrasek J, Zazimalova E, Simon S, Friml J, Van Montagu M, Benkova E. (2009) Cytokinin regulates root meristem activity via modulation of the polar auxin transport. PNAS. 106 (11): 4284-4289

Samuelsen CO, Baraznenpk V, Khorosjutina O, Spahr H, Kieselbach T, Holmberg S, Gustafsson CM. (2003) TRAP230/ARC240 and TRAP240/ARC250 Mediator subunits are functionally conserved through evolution. Proc Natl Acad Sci USA. 100 (1): 6422-6427

Sánchez-Calderón L, López-Bucio J, Chacón-López A, Cruz-Ramírez A, Nieto-Jacobo F, Dubrovsky JG, Herrera-Estrella L. (2005) Phosphate starvation induces a determinate developmental program in the roots of *Arabidopsis thaliana*. Plant Cell Physiol. 46 (1): 174-184

Sánchez-Calderón L, Ibarra-Cortés ME, Zepeda-Jazo I. (2013) Root development and abiotic stress adaptation. Plant Responses and Applications in Agriculture. Chapter 5.

Scheres B, Benfey P, Dolan L. (2002) Root development. The Arabidopsis book.

Sebastian J, Ryu KH, Zhou J, Tarkowská D, Tarkowski P, Cho YH, Yoo SD, Kim ES, Lee JY. (2015) PHABULOSA controls the Quiescent Center-independent root meristem activities in *Arabidopsis thaliana*. PLOS genetics. 11 (3): e1004973.

Secco D, Wang C, Shou H, Whelan J. (2012) Phosphate homeostasis in the yeast *Saccharomyces cerevisiae*, the key role of the SPX domain-containing proteins. FEBS Letter 586: 289-295

Shin H, Shin HS, Chen R, Harrison MJ. (2006) Loss of *At4* function impacts phosphate distribution between roots and the shoots during phosphate starvation. Plant J. 45(5): 712-26

Theodorou ME and Plaxton WC. (1993) Metabolic adaptations of plant respiration to nutritional phosphate deprivation. Plant Physiol. 101: 339-344

Tian H, Drijber RA, Li X, Miller DN, Wienhold BJ. (2013) Arbuscular mycorrhizal fungi differ in their ability to regulate the expression of phosphate transporters in maize (*Zea mays L.*). Mycorrhiza. 23 (6): 507-14

Wang C, Huang W, Ying Y, Li S, Secco D, Tyerman S, Whelan J, Shou H. (2012) Functional characterization of the rice SPX-MFS family reveals a key role of OsSPX-MFS1 in controlling phosphate homeostasis in leaves. New Phytol. 196 (1): 139-48

Wang CQ, Sarmast MK, Jiang J, Dehesh K. (2015) The transcriptional regulator BBX19 promotes hypocotyl growth by facilitating COP1-mediated EARLY FLOWERING3 degradation in *Arabidopsis*. The Plant Cell. www.plantcell.org/cgi/doi/10.1105/tpc.15.00044

Williamson L, Ribrioux S, Fitter A, Leyser O. (2001) Phosphate availability regulates root system architecture in Arabidopsis. Plant Physiol. 126: 1-8

Yoda A, Kouike H, Okano H, Sawa H. (2005) Components of the transcriptional Mediator complex are required for asymmetric cell division in *C. elegans*. Development. 132 (8): 1885-93

Zhang Z, Liao H, Lucas WJ. (2014) Molecular mechanisms underlying phosphate sensing, signaling, and adaptation in plants. Journal of Integrative Plant Biology. 56 (3): 192-220

Appendix: *Med12* in Maize

The CDK8 module in maize is composed of 5 subunits: 1 Cyclin C, 1 Cyclin Dependent Kinase 8, 1 Med13 and 2 Med12 subunits. The Med12 subunits were named Med12a and Med12b.

As part of Tania Nuñez Master Thesis (Tania Nuñez Master Thesis, 2012), she confirmed that the two *Med12* genes are transcribed and expressed in shoot and root. She also generated and corroborated two different Ds insertions in *Med12a*. One insertion was into the 10th exon, and was called C2.7 (Fig. 1). The other insertion was called A5.12, and was inserted approximately 900 base pairs before the translation start site (Fig. 1). My contribution was to check the mRNA integrity of these two Ds insertions, and to generate more seeds of the homozygous lines.

For *Med12b*, there was a Mu-insertion available in the Mu database. The insertion mu1058248, was into the first exon. From the stock, 5 seeds were grown and self-pollinated. I confirmed they have the Mu insertion, checked the mRNA transcript integrity, and generated more seeds of the families containing the Mu-insertion.

Molecular analysis of the mutant alleles of *ZmMed12a*

After genotyping progeny of the existing alleles and identifying homozygous mutants, it was necessary to examine the gene transcripts. Figure 1 shows the molecular characterization of the mutant allele A5.12, where the Ds insertion is located 918bp upstream of the translation start site; and the mutant allele C2.7, inserted into de 10th exon of *Med12a*. The A5.12 allele showed no perturbation of mRNA stability, so I didn't work more with it. However it could be interesting to check if the mRNA levels are affected because of the insertion in the possible 5'UTR.

Because the oligo pairs used covered from the 11th exon to the 3' UTR it was possible to

notice the C2.7 homozygous mutant mRNA was disrupted after the Ds insertion site. During the maize winter field 2013-2014 in Puerto Vallarta, the seeds of C2.7 were amplified in number (homozygous and heterozygous lines). From the homozygous lines, I did a mix of 6 seeds of 5 siblings, and they were randomly sowed in the summer field 2014 in Irapuato. Some of the plants grew and were genotyped to confirm the C2.7 Ds insertion (Fig. 2).

The whole *Med12a* coding region in C2.7 homozygous mutant allele was analyzed by Reverse-Transcriptase PCR. I corroborated that the mRNA integrity was affected after the Ds insertion, generating a truncated allele (Fig. 3).

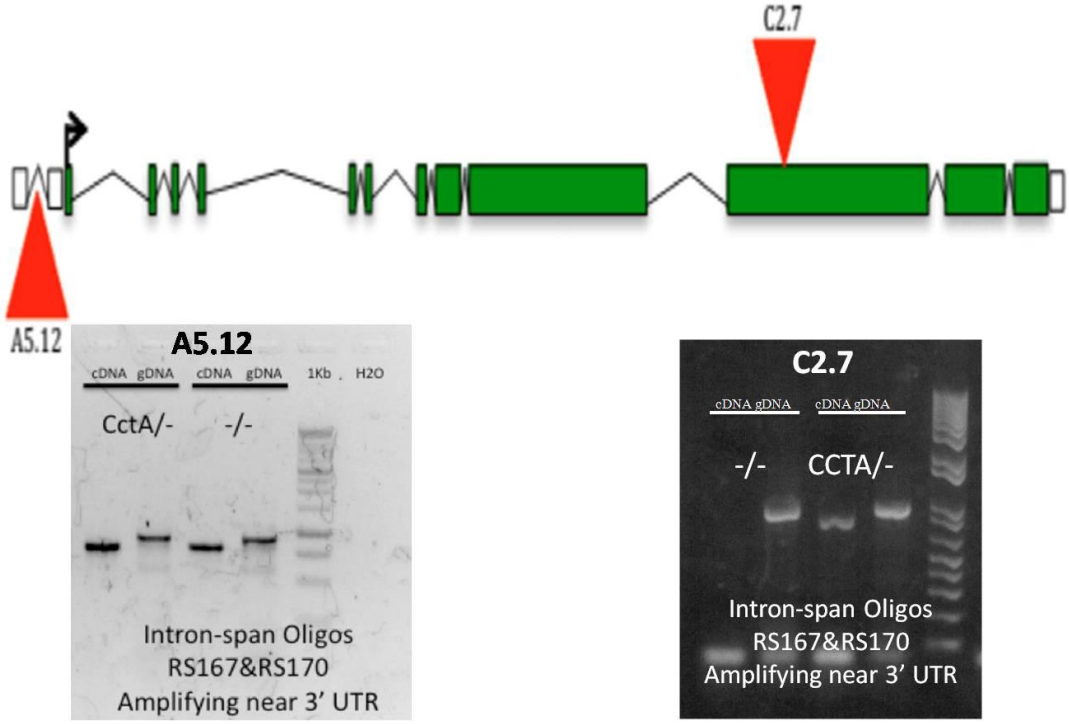

Figure 1. Transcript accumulation in the two Ds mutant alleles of *Med12a*. The same oligo pairs were used to check the mRNA integrity, from the 11th exon to the beginning of 3'UTR. CCTA/- represents a heterozygous *Med12a*/- line used. -/- represents a homozygous line. Genomic DNA (gDNA) was used as a control to differentiate the length of the band for cDNA and gDNA. For A5.12 we could amplify a band at the end of the transcript, which implies the mRNA integrity is not affected. For C2.7, no band was observed for the amplification at the end of the transcript.

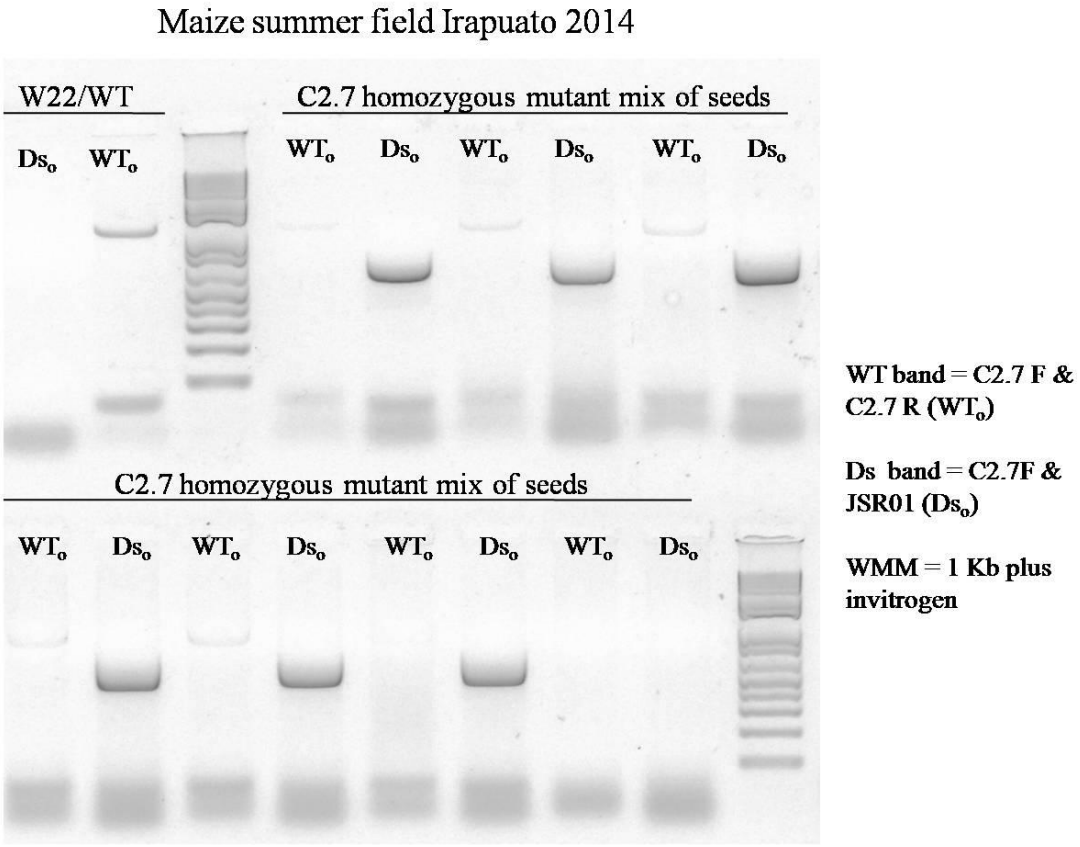

Figure 2. PCR of genomic DNA to verify C2.7 Ds insertion in *Med12a*. Maize plants grown in the field were randomly chosen to confirm the Ds insertion in these families. They indeed have the C2.7 Ds insertion and are homozygous mutant lines.

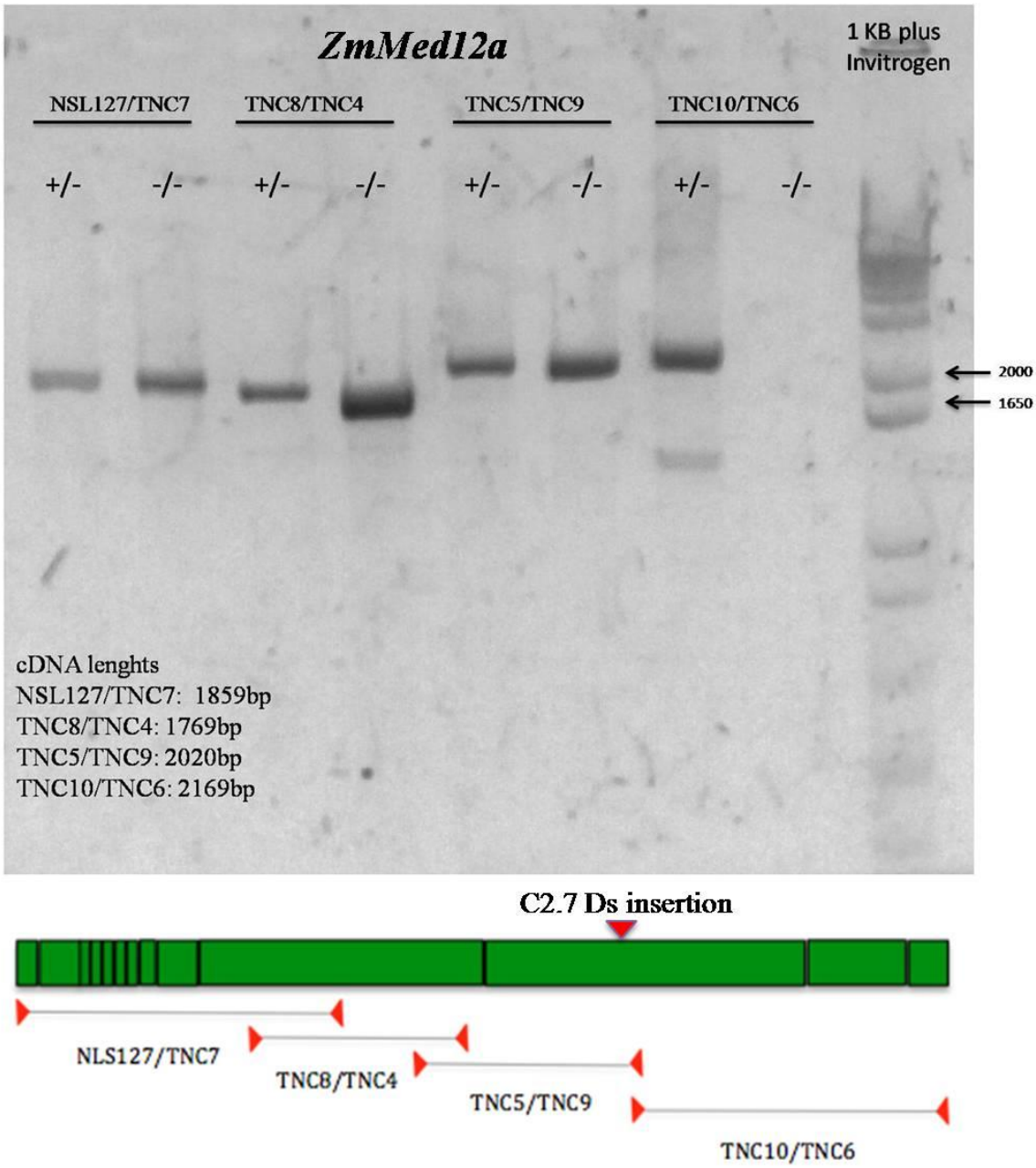

Figure 3. RT-PCR amplification of *ZmMed12a* C2.7 allele using the 4 primer sets used when the gene was cloned. +/- represents a heterozygous line used. -/- represents a homozygous line. The insertion is in the 10th exon, from a total of 12 exons. In the heterozygous line, there is amplification of the transcript after the Ds insertion, while in the homozygous line there is no amplification, suggesting a truncated transcript was generated.

Molecular analysis of the mutant allele of *ZmMed12b*

Five families descending from self-pollinated mu-1058248 original stock were verified (294.6, 294.8, 294.9, 294.10, and 294.11). Just three of them contained the Mu insertion: 294.6, 294.8 and 294.9. A homozygous mutant was used to check the mRNA integrity in this mutant line (Fig. 4). The mRNA was indeed affected. In the Maize summer field 2014 in Irapuato, 15 seeds of each of these 3 families were sowed. Some of them grew, and were genotyped to confirm the insertion (Fig. 5).The whole *Med12b* coding region in the mu-1058248 homozygous mutant allele was analyzed by Reverse-Transcriptase PCR. I corroborated that the mRNA integrity was affected after the mu insertion, generating a longer transcript (Fig. 6).

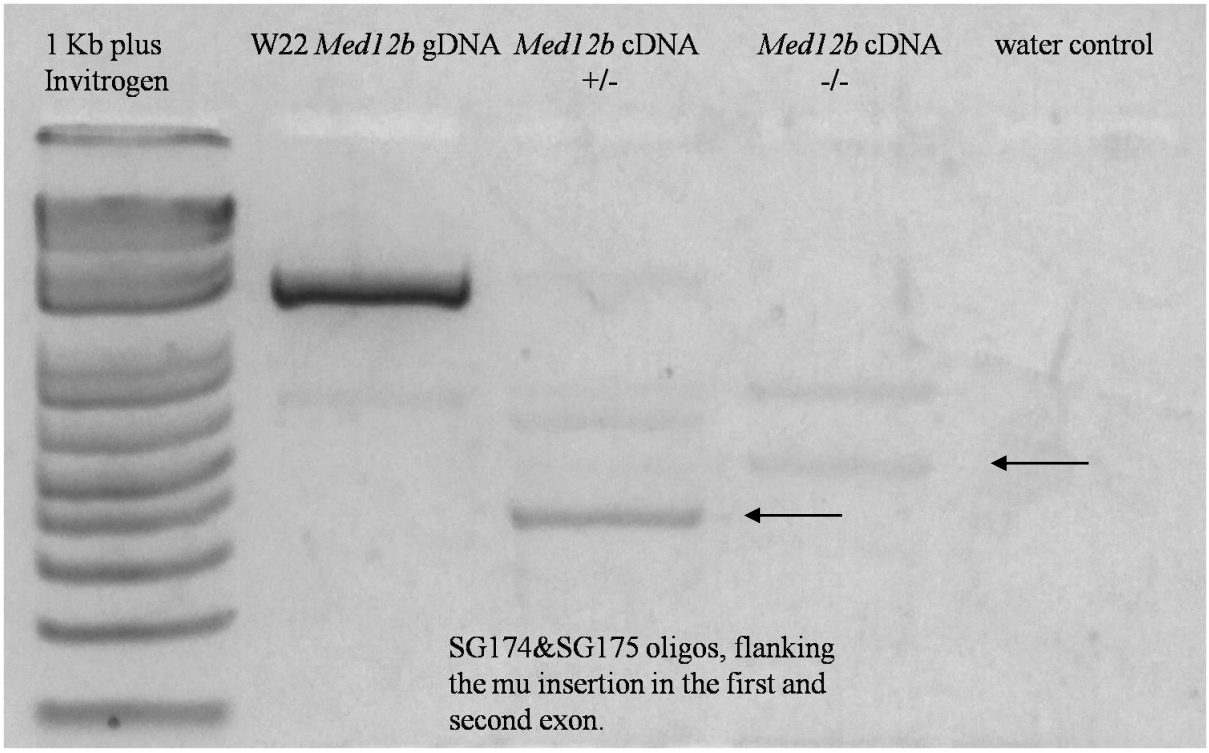

Figure 4. *Med12b* mutant line mu1058248 generates a longer allele. The same oligo pairs were used to check the mRNA integrity, from the 1th exon to the beginning of the 2nd, flanking the mu insertion. +/- represents a heterozygous *Med12b/-* line used. -/- represents a homozygous line. Genomic DNA (gDNA) was used as a control to differentiate the length of the band for cDNA and gDNA.

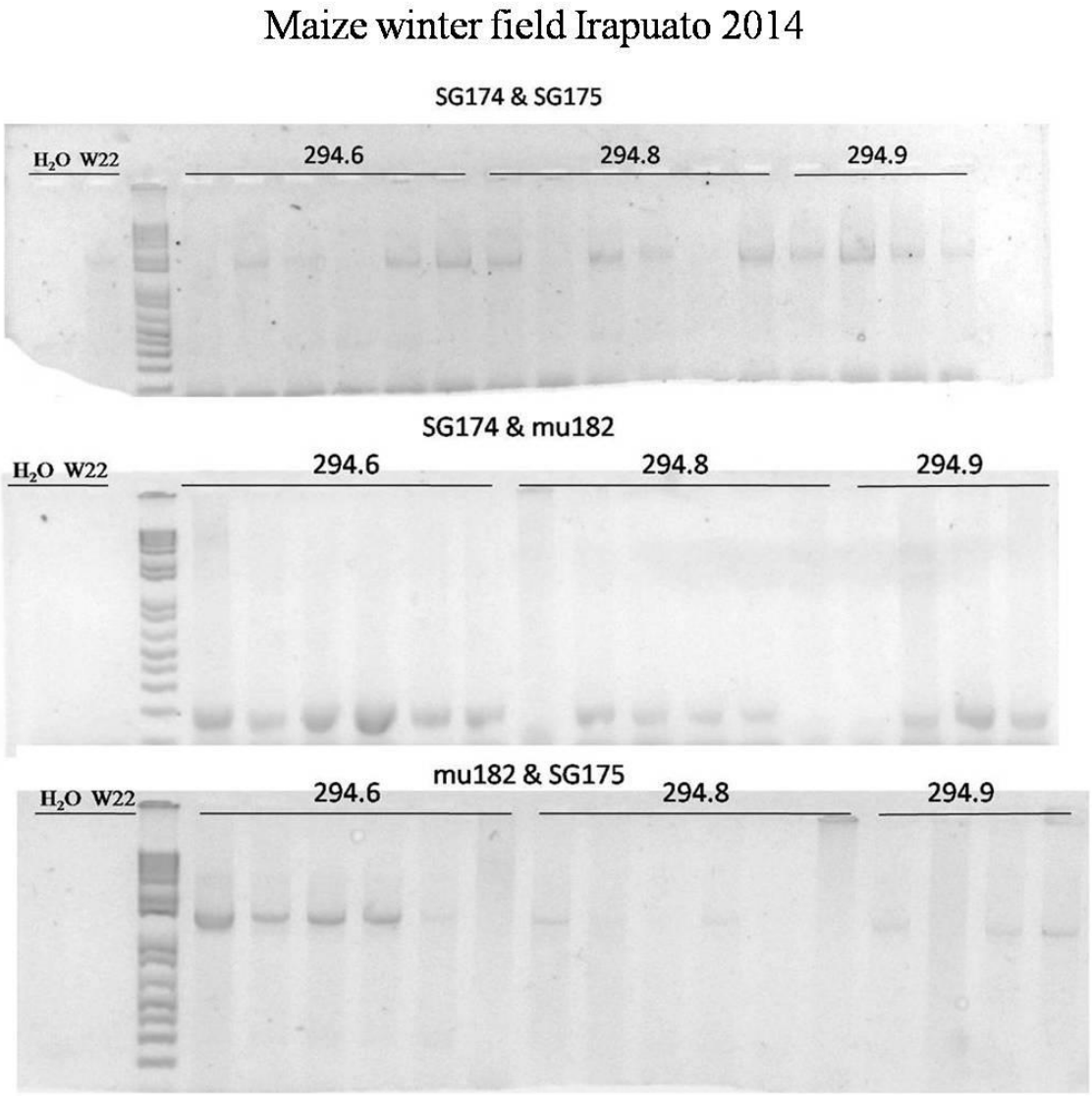

Figure 5. PCR of genomic DNA to verify mu-1058248 insertion in *Med12b*. Maize plants grown in the field were randomly chosen to confirm the mu insertion in these families. They indeed have the mu-1058248 insertion and are segregant mutant lines.

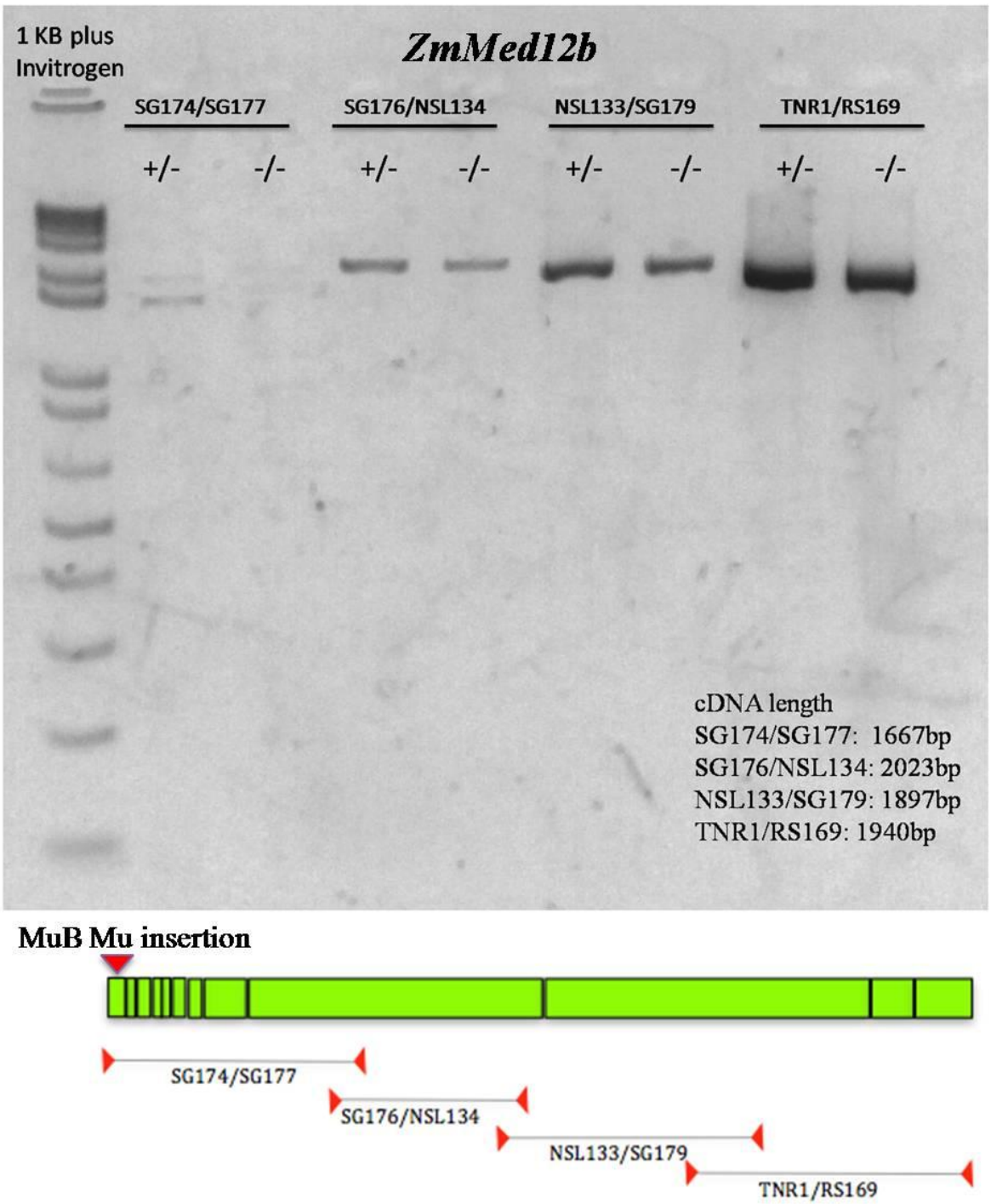

Figure 6. RT-PCR amplification of *ZmMed12b* mu-1058248 allele using the 4 primer sets used when the gene was cloned. +/- represents a heterozygous line used. -/- represents a homozygous line. The insertion is in the 1th exon, from a total of 12 exons. In both heterozygous and homozygous lines, there is amplification of the transcript flanking the Mu insertion, and all along the transcript.

CDK8 module phylogeny

In order to reconstruct the evolutionary history of CDK8 module subunits, *Arabidopsis thaliana* proteins were used to obtain the sequences of the orthologous candidates in *Zea mays* (Zm), *Brassica rapa* (Br), *Sorghum bicolor* (Sb), and *Amborella trichopoda* as outgroup, representing the most ancient flowering plant. For maize, *Med12b* and *Med13* are not fully annotated in the maize database, and are reported as two different genes each. *ZmMed12b* is already cloned (Tania Nuñez Master Thesis, 2012), so the full sequence is available at Ruairidh Sawers and Stewart Gillmor laboratories. *ZmMED13* sequence employed in this analysis is not the complete sequence.

MEGA version 6 was employed to construct Maximum Likelihood trees. The program found the best protein model,

General information of the ML trees:

- Statistical method: Maximum likelihood
- 10000 bootstrap replications
- Model/Method: Jones-Taylor-Thornton (JTT) FOUND by MEGA
- Rates among Sites: Gamma Distributed (G)
- No of discrete Gamma Categories: 5
- Protein sequences

Gaps/Missing Data Treatment: Use all sites

As expected, in all trees *Arabidopsis thaliana* and *Brassica rapa* are grouped together as they are more closely related to each other. The same was expected and obtained for *Zea mays* and *Sorghum bicolor* (Fig. 7-10). For Med12, the tree indicatesthat the common ancestor of each of these two groups had at least two *Med12* copies, and that Sorghum and Arabidopsis lost the other copies and just retained one (Fig. 7).

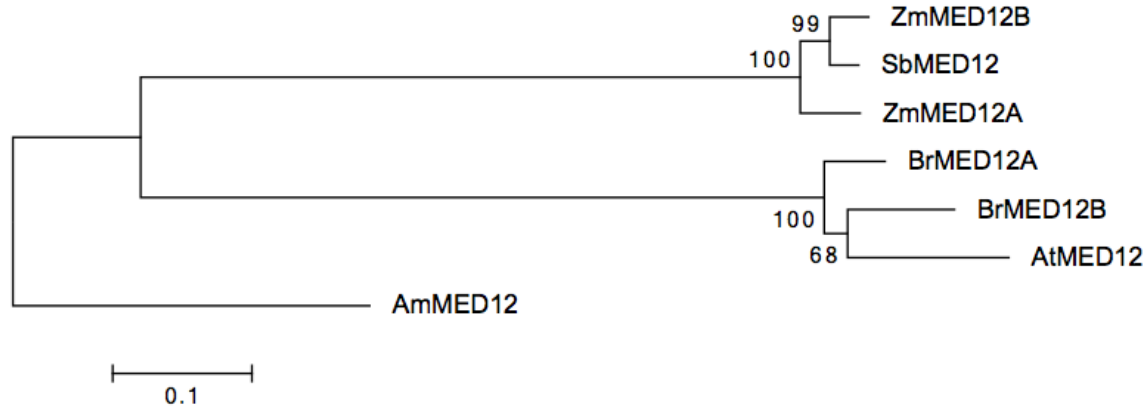

Figure 7. MED12 phylogenetic reconstruction using MEGA6.

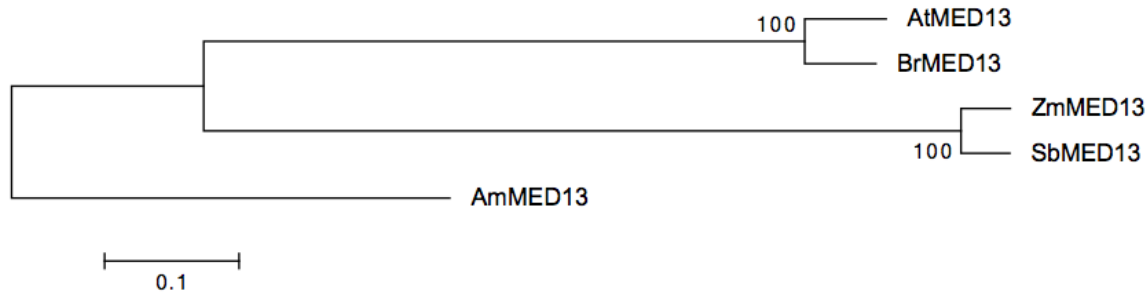

Figure 8. MED13 phylogenetic reconstruction using MEGA6.

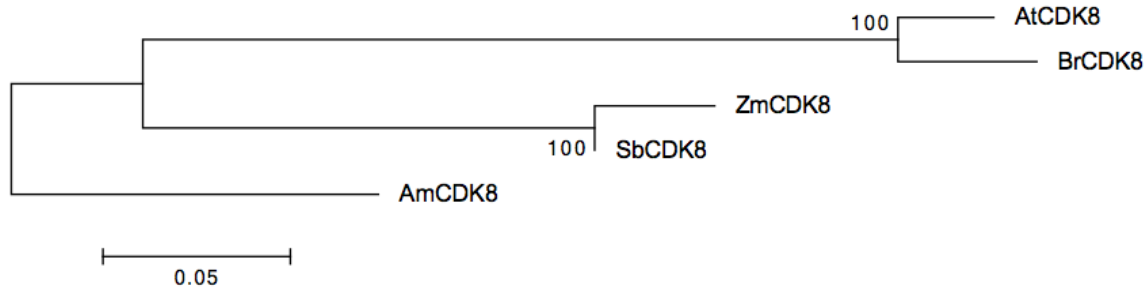

Figure 9. CDK8 phylogenetic reconstruction using MEGA6.

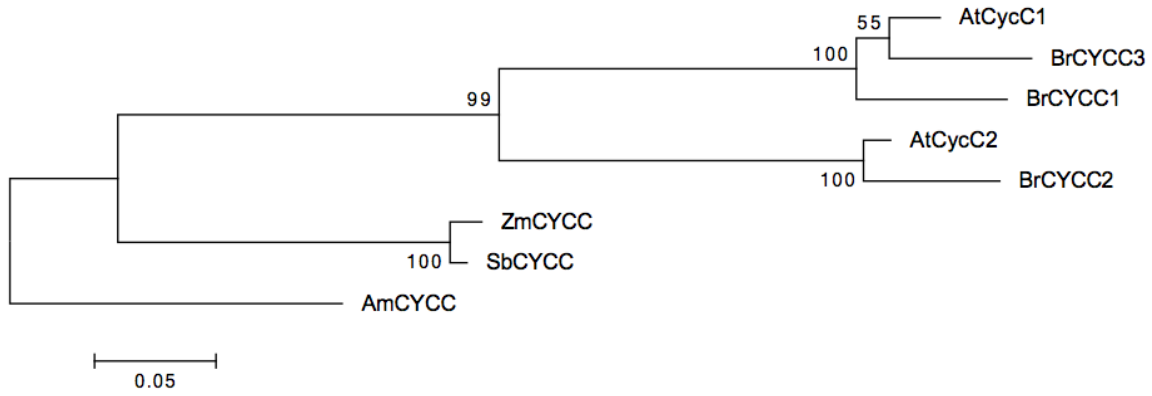

Figure 10. CYCLIN C phylogenetic reconstruction using MEGA6.