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The role of the CDK8 module of Mediator in vegetative phase change of *Arabidopsis thaliana*

Thesis presented by

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La función del módulo CDK8 de Mediador en el cambio de fase vegetativo de *Arabidopsis thaliana*

Tesis que presenta:

M. en C. Manuel Buendía Monreal

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1. ABSTRACT

In plants, the transition from the juvenile to the adult vegetative phase is regulated by a genetic pathway involving the microRNAs miR156 and miR172, and the miR156-targeted *SPL* transcription factors. Despite the extended knowledge about this pathway, very little is known about what regulates these microRNAs. It has been recently reported that sugar promotes vegetative phase change by repressing miR156. In our laboratory we have demonstrated that *CCT/MED12* and *GCT/MED13,* members of the CDK8 module of Mediator, also act upstream of the miR156-*SPL*-miR172 pathway. One important question is whether the CDK8 module and sugar regulate miR156 separately or together. I found that sugar and the CDK8 module regulate the juvenile to adult transition in a convergent manner, as double mutants of *ch1* (a photosynthetic gene) and CDK8 module subunits show an additive effect on heteroblasty traits, and they synergistically repress *MIR156* expression; furthermore, sugar treatment repressed miR156 expression in a CDK8 module-independent manner.

For my thesis, I was also interested in exploring further whether other genes of the CDK8 module of Mediator regulate vegetative phase change, and in determining which specific genes of the miR156-SPL pathway are under CDK8 module control. My results show that *hen3/cdk8* mutants exhibit a delay in flowering time and an extended juvenile vegetative phase, although less severe than *cct* and *gct* mutants. In addition, *hen3 cct* double mutants have an additive effect in delaying flowering time, indicating that *HEN3/CDK8* plays a role in the regulation of the reproductive transition that could be independent of *CCT*. The extended juvenile phenotype of *hen3* mutants was correlated with higher expression of both *MIR156A* and *MIR156C,* as observed in qPCR and GUS-assays. *SPL* genes were differentially regulated in *hen3* mutants, with *SPL3* upregulated and *SPL9* downregulated, possibly explaining the less severe phenotype of *hen3* plants compared to *cct* and *gct*. Interestingly, *SPL9* was regulated by *HEN3* in a miR156- independent manner. Finally, *hen3 mir156a mir156c* triple mutants show an intermediate phenotype between *hen3* and *mir156a mir156c*, indicating that the *hen3* phenotype is partially due to overexpression of miR156. In summary, my results demonstate that *HEN3/CDK8* regulates vegetative phase change by transcriptional regulation of *SPL* genes, as well as indirect regulation of *SPL* genes via regulation of miR156.

RESUMEN

En plantas, la transición de la fase vegetativa juvenil a la fase adulta está regulada por una ruta genética que involucra los microRNAs miR156 y miR172, así como los factores de transcripción SPL que son marcados por miR156. A pesar del basto conocimiento acerca de esta ruta, se conoce muy poco acerca de qué es lo que regula a estos microRNAs. Recientemente se reportó que el azúcar promueve el cambio de fase vegetativo a través de reprimir a miR156. En nuestro laboratorio hemos demostrado que *CCT/MED12* y *GCT/MED13*, miembros del módulo CDK8 de Mediador, también actúan arriba de la ruta de miR156-SPL-miR172. Resulta importante saber si el módulo CDK8 y el azúcar regulan miR156 juntos o por separado. En este trabajo, muestro que el azúcar y el módulo CDK8 regulan la transición juvenil-adulto de manera convergente, puesto que mutantes dobles de *ch1* (un gen fotosintético) y subunidades del módulo CDK8 muestran un efecto aditivo en rasgos de heteroblastia, y reprimen de manera sinergista la expresión de *MIR156*; además, la adición de azúcar fue capaz de reprimir la expresión de *MIR156* independientemente del módulo CDK8.

Para esta tesis, quise además explorar si otros genes del módulo CDK8 de Mediador regulan el cambio de fase vegetativo, y determinar específicamente qué genes de la ruta miR156-SPL están controlados por el módulo CDK8. Mis resultados muestran que las mutantes *hen3/cdk8* también exhiben un retraso en el tiempo de floración y una fase juvenil extendida, aunque el fenotipo es menos severo que el de las mutantes *cct* y *gct*; por otra parte, las dobles mutantes *hen3 cct* tienen un efecto aditivo en retrasar el tiempo de floración, lo que indica que *HEN3/CDK8* cumple una función importante en la regulación de la transición reproductiva, que podría ser independiente de *CCT*. El fenotipo juvenil extendido de la mutantes *hen3* correlaciona con la expresión incrementada de *MIR156A* y *MIR156C* observada en ensayos qPCR y GUS. Por otra parte, algunos genes *SPL* fueron regulados diferencialmente en mutantes *hen3*, contrastando la expresión inducida de *SPL3* y la expresión reprimida de *SPL9*, lo cual también podría explicar el fenotipo menos severo de las plantas *hen3*. De forma interesante, la expresión de *SPL9* mostró ser regulada por *HEN3* independientemente de miR156. Finalmente, las triples mutantes *hen3 mir156a mir156c* mostraron un fenotipo intermedio entre *hen3* y *mir156a mir156c*, lo que indica que el fenotipo de *hen3* se debe parcialmente a la sobreexpresión de miR156. En resumen, mis resultados demuestran que *HEN3/CDK8* regula el cambio de fase vegetativa mediante regulación transcripcional de genes *SPL*, así como regulación indirecta vía miR156.

2. INTRODUCTION

2.1. Vegetative development and the juvenile-to-adult transition in plants

The life cycle of a flowering plant is an ordered series of developmental phases, with the transitions between these phases controlled by both environmental and endogenous signals (Figure 1). After seed germination, the plant begins its vegetative development program by increasing photosynthetic activity, and its size and mass by the production of leaves. Eventually, the plant acquires reproductive competence: the shoot apical meristem (SAM) turns into an inflorescence meristem (IM), which in turns converts into a floral meristem (FM) or produces lateral meristems. This floral transition is tightly controlled by a complex genetic network which integrates environmental signals like temperature with endogenous signals like hormones and sugar concentration (reviewed in Amasino, 2010). Within the flower, male and female haploid gametes are produced (gametophytic phase) before fusing to form a new diploid zygote (sporophytic phase). Finally, embryogenesis begins when the zygote is fertilized and then the stem cells are established at the opposite poles of the developing seed, forming the root apical meristem (RAM) and the shoot apical meristem (SAM) (reviewed in Huijser and Schmid, 2011).

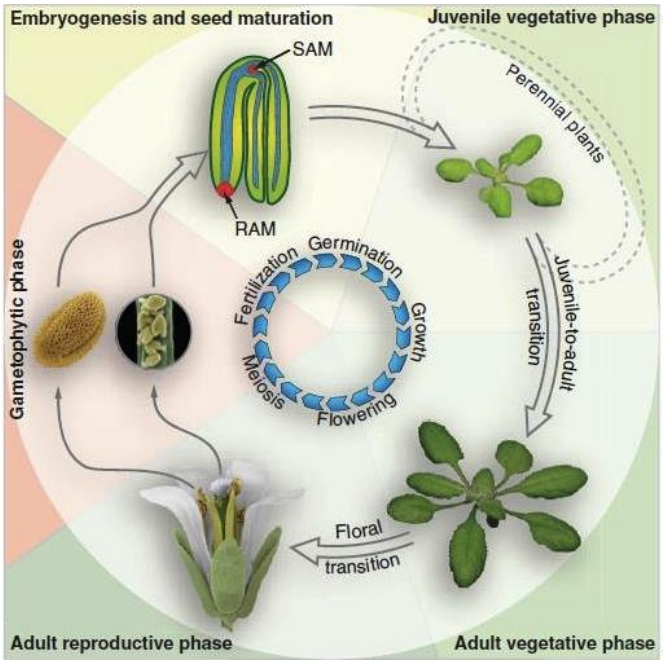

Figure 1. The major phase transitions of plant development (Huijser and Schmid, 2011)**.**

During vegetative growth, plants produce organs around a stem, resulting in both juvenile and adult leaves being present at the same time in the same organism, a condition known as heteroblasty. The transition from juvenile to adult is often evident in

terms of leaf traits such as leaf size and shape, margin shape, petiole length and trichome distribution, and sometimes is associated with other traits like phyllotaxy, production of adventitious roots, the presence or absence of phytochemicals such as anthocyanins, and disease- or insect-resistance (Poethig, 2013). In Australian *Acacia* species, the transition is accompanied by dramatic changes in leaf morphology (Figure 2): they produce horizontally-oriented, bipinnately compound leaves in the juvenile stage, whereas in the adult phase they produce vertically-oriented, simple leaves; indeed, plants in the transition stage produce leaves in which both leaf types are present in a single leaf. Similar changes in leaf morphology occur in other woody plants like *Eucalyptus globulus*, English ivy (*Hedera Helix*) and sawtooth oak (*Quercus acutissima*), although the changes are less dramatic (Wang et al., 2011). In maize, juvenile leaves lack trichomes but possess epicuticular wax, whereas adult leaves have the opposite traits (Poethig, 2003). In Arabidopsis thaliana, the most studied model plant species, the transition from juvenile to the adult stage is evident in some leaf traits: juvenile leaves show a small and round blade, smooth margins and absence of trichomes on the abaxial side, whereas leaves produced in the adult stage have a larger and elongated blade, serrated margins and trichomes on both adaxial and abaxial sides (Figure 2).

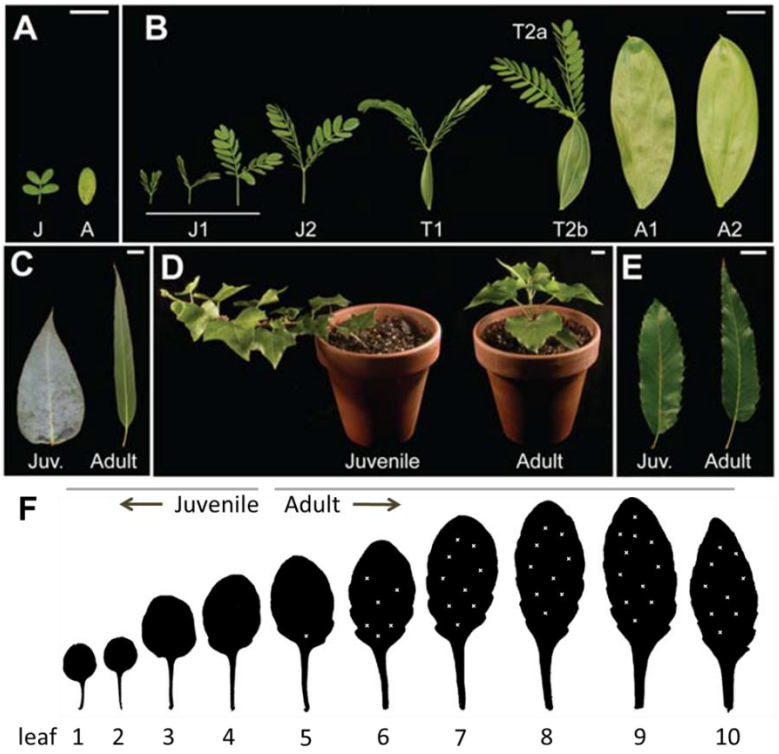

Figure 2. Juvenile-to-adult transition shown in terms of leaf heteroblasty. Morphological changes between juvenile and adult leaves in woody plants (A-E) and Arabidopsis thaliana (F). (A) Morphology of first two leaves of *Acacia confusa*. (B) Morphology of the first 8 leaves of *Acacia colei*. J = juvenile, T = transition, A = adult. (C) Juvenile and adult leaves from a single tree of *E. globulus*. (D) Juvenile and adult clones of *H. helix* (English ivy). (E) Juvenile and adult leaves of *Q. acutissima*. (F) Morphology of first ten leaves of Arabidopsis thaliana. Abaxial trichomes are represented like white spots. Figures A-E from Wang et al., 2011.

The proper timing of developmental transitions is controlled by both endogenous and exogenous signals. Signaling pathways involving microRNAs (miRNAs), as well as nutritional status, have been pointed out as major regulators of temporal coordination of development in *Caenorhabditis elegans*, plants and humans (Poethig, 2013; Rougvie, 2005; Tolson and Chappell, 2012). In plants, the juvenile-to-adult phase transition and the reproductive phase transition share some major regulators: the miRNAs miR156 and miR172, and their respective targets.

miR156 is the most conserved and one of the most abundant miRNAs in plants (Axtell and Bartel, 2005; Cuperus et al., 2011). In Arabidopsis, there are 8 genes (*MIR156A-H*) encoding the mature microRNA miR156; additionally, microRNA miR157 has an almost identical mature sequence to miR156 and is encoded by 4 genes (*MIR157A-D*). It has been reported that the genes *MIR156A* and *MIR56C*, as well as *MIR157A* and *MIR157C*, are those which contribute the most to their respective miRNA and are the most important for vegetative development (Yang et al., 2013; Yu et al., 2013). Plants overexpressing miR156 show a prolonged juvenile phase, increased branching, accelerated leaf production and delayed flowering (Shikata et al., 2009; Wang et al., 2009; Wu et al., 2009; Wu and Poethig, 2006) whereas loss-of-function mutants of *MIR156A* and *MIR156C* and plants with reduced function of miR156 by a target site mimic (35S::MIM156) produce the opposite phenotype: early acquisition of adult leaf traits, reduced leaf production and early flowering (Figure 3; Franco-Zorrilla et al., 2007; Wu et al., 2009; Yang et al., 2013; Yu et al., 2013). Thus, miR156 is both necessary and sufficient for the expression of the juvenile phase. Consistently, the expression of miR156 is high in young seedlings and decreases during the juvenile-to-adult transition (Wahl et al., 2013; Wang et al., 2009; Wu et al., 2009; Wu and Poethig, 2006).

The miR156/miR157 family targets 10 out of the 16 *SPL* (*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*) genes in Arabidopsis. miR156 represses the expression of *SPL* genes by directing the cleavage of their transcripts and by translational repression (Addo-Quaye et al., 2008; Gandikota et al., 2007). The SPL proteins constitute a family of transcription factors that is conserved in all green plants (Preston and Hileman, 2013; Riese et al., 2007), and play diverse functions in plant growth and development, including vegetative phase change, flowering, branching, plastochron, plant architecture and responses to stresses (Wang and Wang, 2015). Nearly all the miR156-targeted *SPL* genes promote vegetative and reproductive transitions by direct activation of *LEAFY (LFY), FRUITFULL (FUL), APETALA1 (AP1)* and miR172, which further promote flowering (Wang et al., 2009; Wu et al., 2009; Yamaguchi et al., 2009). The phenotype of plants with versions of *SPL* genes resistant to miR156 function (*rSPL*), and plants overexpressing miR172, is similar to that of plants with reduced function of miR156 (35S::MIM156) (Figure 3; Aukerman and Sakai, 2003; Cardon et al., 1997; Wang et al., 2009; Wu et al., 2009; Wu and Poethig, 2006), and is consistent with the regulatory pathway described in Figure 3E; *SPL* genes and miR172 have the opposite expression pattern to miR156: they are expressed at low levels in juvenile stages, and increase during the transition to adult.

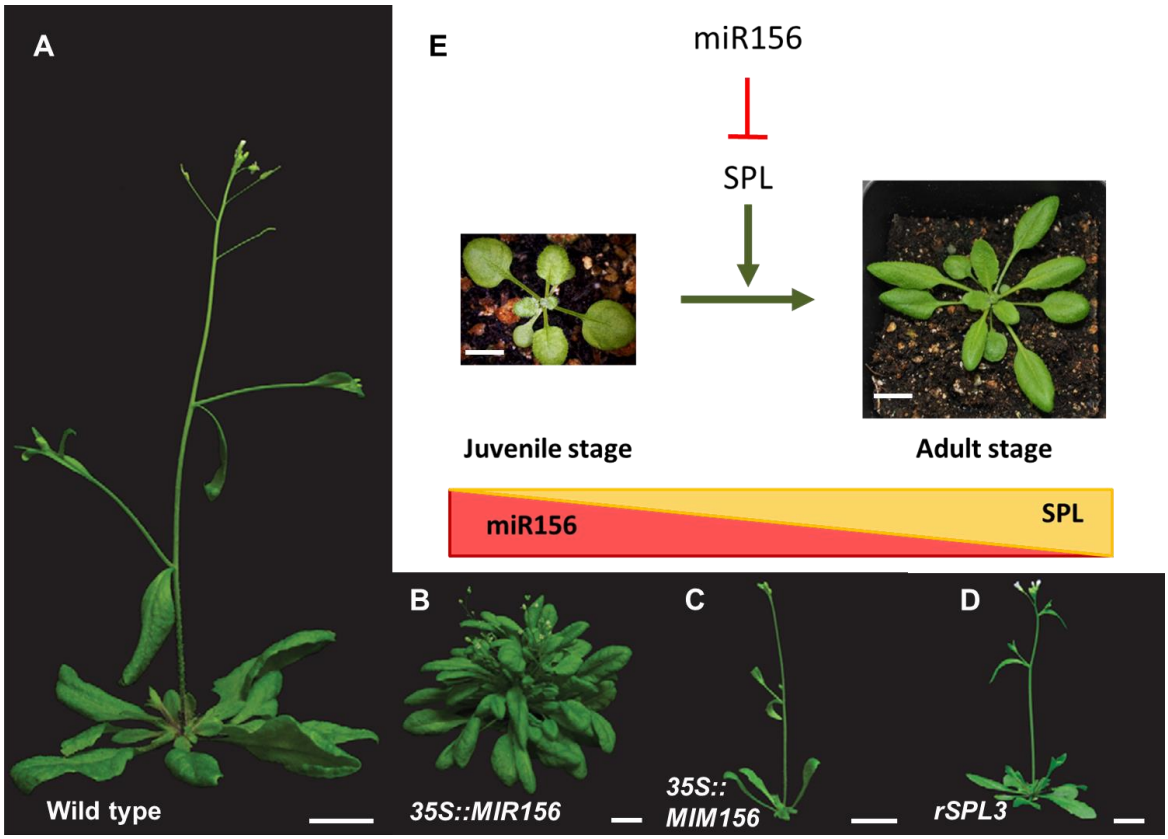

Figure 3. The miR156/SPL pathway controls vegetative phase change. (A) Col-0 (wild type) control. (B) Plants overexpressing miR156 (35S::MIR156) show a prolonged vegetative phase (increased number of juvenile leaves). (C) Constitutive expression of the target mimic (35S::MIM156), which reduces functional levels of mature miR156, promotes the transition to adult and flowering. (D) Overexpression of a miRNA-resistant version of the miR156 target SPL3 (rSPL3) also promotes the transition to adult and flowering. (E) The juvenile-to-adult transition is promoted by the SPL factors, which are repressed by the microRNA miR156. The expression of miR156 declines and that of SPL factors increase during vegetative development. Figures A-D taken from Huijser and Schmid, 2011.

Although there is some knowledge of the roles of miR156 and its targets, very little is known about how the expression of this miRNA is regulated, information which is essential to understand the timing of vegetative and reproductive development. Some recent findings have demonstrated that sugar functions as a mobile signal, derived from leaves, that represses miR156 in the shoot (Yang et al., 2013, 2011; Yu et al., 2013; see the section "4.1. Background"). Genetic studies from our group (Gillmor et al., 2014) suggest that *CENTER CITY (CCT)* and *GRAND CENTRAL (GCT)*, genes belonging to the CDK8 module of Mediator, control several developmental transitions by regulating the temporal expression of miR156. However, the mechanisms by which sugar, *CCT* and *GCT*, regulate miR156 expression are poorly understood. It has been reported that downregulation of *MIR156A* and *MIR156C* during vegetative phase change is associated with an increase in Histone 3 Lysine 27 trimethylation (H3K27me3) and the binding of Polycomb Repressive Complex (PRC2) to these genes (Xu et al., 2016a). Whether sugar

and/or the CDK8 module repress miR156 by recruiting PRC2 and promoting the establishment of such negative epigenetic mark remains to be studied. Besides endogenous factors, some environmental conditions also induce the expression of miR156, including cold stress (Lee et al., 2010; Zhou et al., 2008) and phosphate starvation (Lei et al., 2016), whereas other conditions like high concentrations of CO_2 (May et al., 2013) and salt stress (Ding et al., 2009) downregulate its expression; but the physiological meaning and the mechanism of these regulations have not been well defined.

2.2. The Mediator complex as a signal integrator for transcriptional control

Mediator is a large protein complex that serves as a molecular bridge between gene-specific transcription factors bound at enhancers, and RNA polymerase II (RNA pol II). In yeast, Mediator consists of 25 subunits; in mammals approximately 31 subunits; and in plants, approximately 34 subunits (reviewed in Allen and Taatjes, 2015; Samanta and Thakur, 2015). Mediator was first discovered in yeast as a large protein complex that was required for transcription (Kelleher et al., 1990; Flanagan et al., 1991), and was subsequently purified from human cells (Fondell et al., 1996), and from plant cells (Bäckström et al., 2007). Because of the low sequence conservation between Mediator subunits from different species (typically as low as 20% amino acid identity), many initial studies of Mediator in yeast and animals did not recognize that proteins that had been isolated based on their differing effects on transcription, were indeed Mediator components, and in some cases, the same Mediator subunit from different organisms (Kornberg, 2005; Sato et al., 2004). This discovery led to a unified nomenclature for Mediator subunits in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces. pombe*, and the animals *C. elegans*, *Drosophila melanogaster*, and human (Bourbon et al., 2004), which was also used for the Arabidopsis Mediator (Bäckström et al., 2007). Shortly thereafter, Mediator components were identified from genomic sequences of many eukaryotes, indicating that Mediator has been widely conserved in evolution (Bourbon, 2008).

Structural studies of Mediator complexes have classified Mediator as having four different modules, referred to as the Head, Middle, Tail, and Cyclin Dependent Kinase 8 (CDK8) modules (reviewed in Chadick and Asturias, 2005; Conaway et al., 2005) (Figure 4). The Head module is thought to have the most important initial interactions with RNA pol II, while the Middle module serves a structural function as well as interacting with RNA pol II once Mediator's conformation changes after its initial interaction with RNA pol II. The Tail module plays an especially important role in interacting with gene-specific transcription factors (Robinson et al., 2015; Tsai et al., 2014). In yeast, animals, and plants, Mediator has been purified in two forms: as a complex of the Head, Middle and Tail modules (commonly referred to as Core Mediator), and as a larger complex containing Core Mediator and the CDK8 module. Core Mediator preparations support transcription in vitro, while Core Mediator preparations containing the CDK8 module do not (reviewed in Björklund and Gustafsson, 2005). The CDK8 module consists of 4 proteins: MED12, MED13, Cyclin C (CycC), and Cyclin Dependent Kinase 8 (CDK8). The MED12 and MED13 subunits are both about 2000 AA, much larger than most other Mediator subunits (Buendía-Monreal and Gillmor, 2016). The large size of MED12 and MED13 may be related to their role as signal integrators, allowing large surface areas for protein interactions, as well as protein modifications that can affect their stability.

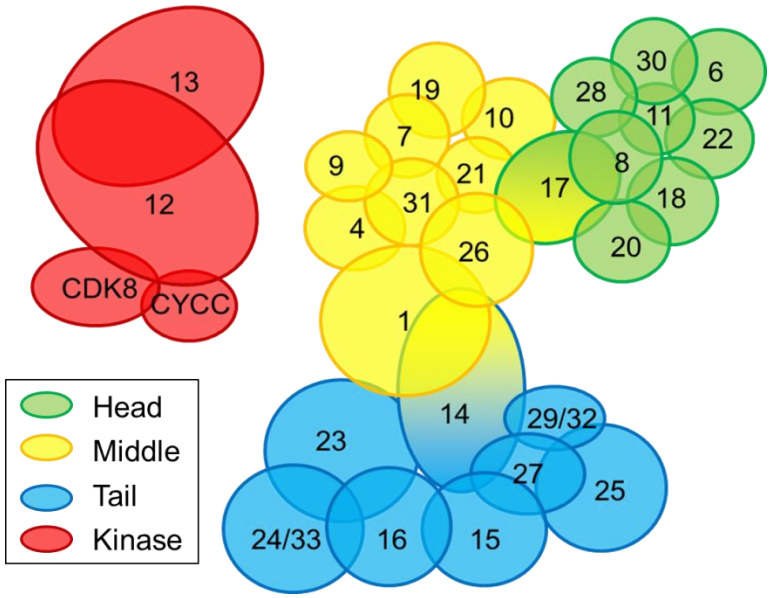

Figure 4. Submodular structure of the plant Mediator complex. Structure is depicted on the basis of tridimensional reported structures of yeast Mediator and human Mediator (Robinson et al., 2015; Tsai et al., 2014). Subunit sizes are according to predicted protein length. Note that Med14 and Med17 are represented in split color since the Med14 C-terminal domain (CTD) belongs to the Tail module, and the Med14 N-terminal domain (NTD) belongs to the Middle module. The Med17-NTD belongs to the Middle module and Med17-CTD to the Head. Med1 is absent in plants, although it has been suggested that CBP1 could act as a tetramer to play the role of Med1 in plants (Li et al., 2015). Figure published in Buendía-Monreal and Gillmor, 2016.

The size of MED12 and MED13 is also almost certainly related to their mechanism of action. Initial studies of the CDK8 module of Mediator reported that its effect was to prevent transcription by steric hindrance of interactions between Core Mediator and RNA pol II (Elmlund et al., 2006). A recent report expanded on earlier work by demonstrating that the yeast CDK8 module interacts with certain Head and Middle module Mediator subunits, in order to occupy the RNA pol II binding cleft of Core Mediator, preventing the initial association of RNA pol II and Core Mediator that leads to activation of transcription (Tsai et al., 2013) (Figure 5). The MED13 protein plays the most important role in this interaction (Knuesel et al., 2009). The other CDK8 module components can repress gene expression through alternate methods, recruiting histone methylation marks that repress transcription, as well as decreasing histone marks that promote transcription. The identification of Med12 as a novel Polycomb Group gene in Drosophila indicated that Med12 could be acting by an epigenetic mechanism (Gaytán de Ayala et al., 2007). This hypothesis was confirmed by Ding et al. (2008), who demonstrated that Med12 is required for an extraneuronal epigenetic silencing network, in which both the RE1 silencing transcription factor (REST) and Med12 interact with G9a histone methyltransferase to silence REST target genes through imposing transcriptionally repressive histone H3K9 dimethylation. Meanwhile, the CDK8-CYCC complex represses transcription by at least two phosphorylation events: (1) by phosphorylating the Cyclin H subunit of TFIIH, leading to the repression of the ability of the TFIIH to activate transcription and to the inhibition of

the CTD kinase activity of the CDK7, partner of Cyclin H (Akoulitchev et al., 2000); (2) by phosphorylating the CTD of the RNA pol II prior to the formation of the preinitiation complex (Liao et al., 1995; Rickert et al., 1996; Wang and Chen, 2004). In *S. cerevisiae*, the homologs of CDK8/CYCC promote pseudohyphal growth by inhibiting H3 Lys4 trimethylation at the *FLO11* locus, activating the expression of *FLO11* (Law and Ciccaglione, 2015). In human cell cultures, the CDK8 module can repress transcription by interacting, through CDK8 and CDK19, with the histone arginine methyltransferase PRMT5 (Tsutsui et al., 2013). In the absence of the CDK8 module, RNA pol II is able to interact with the Head and Middle domains in the RNA pol II binding pocket. Through mechanisms that are still poorly understood, the conformation of the Middle and Tail domains changes until RNA pol II occupies a site at the Middle domain, adjacent to the Tail domain (Robinson et al., 2015; Tsai et al., 2013, 2014).

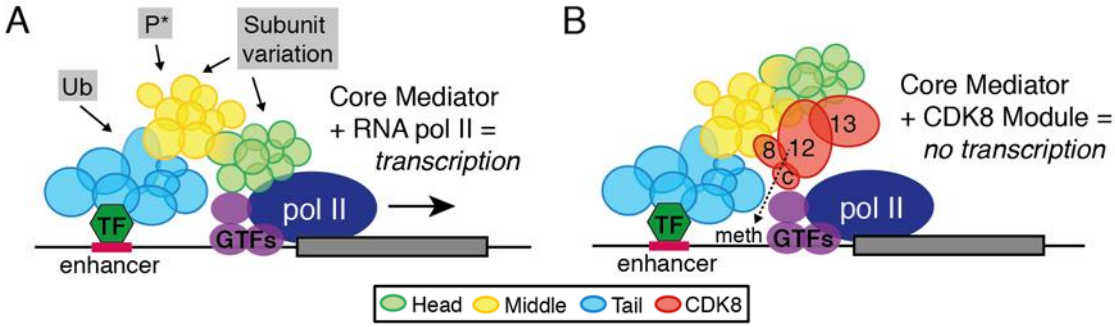

Figure 5. Regulation of transcription by Core Mediator and the Cyclin Dependent Kinase 8 (CDK8) module of Mediator. A simplified representation of the role of Core Mediator and the CDK8 module of Mediator in regulation of transcription, based on literature cited in this review. (A) Core Mediator (composed of Head, Middle and Tail modules) serves as a molecular bridge between transcription factors (TF) bound at enhancers, and RNA polymerase II (pol II) and general transcription factors (GTFs) at the transcription start site. Individual subunits of each module are represented by colored circles. The composition of Core Mediator is dynamic, varying between different target genes (Subunit variation). Stability and activity of Mediator subunits can be regulated by ubiquitination (Ub), and by phosphorylation (P*). (B) The CDK8 module (composed of CDK8 (8), CyclinC (C), MED12 (12) and MED13 (13)) often acts to prevent transcription, either by steric inhibition of interactions between Core Mediator and RNA pol II, or through increasing epigenetic marks that inhibit transcription (such as H3K9me^2), or reducing epigenetic marks that promote transcription (such as H3K4me^3). Figure published in Buendía-Monreal and Gillmor, 2016.

Although the CDK8 module is widely considered as a negative regulator of transcription, there is also growing evidence for involvement of this kinase module in transcriptional activation. In particular, CDK8 is a positive corregulator of transcription of several p53 target genes and of a thyroid receptor-regulator gene (Belakavadi and Fondell, 2010; Donner et al., 2007); CDK8 is also a positive regulator of transcription of several serum response genes that are required for transcription elongation (Donner et al., 2010). The kinase module has also a positive role in β-catenin-dependent transcription by directly phosphorylating E2F1, an inhibitor of β-catenin (Morris et al., 2008), and indirectly by recruitment of Mediator via interaction with Med12 and/or Med13 (Carrera et al., 2008). Interestingly, Med12 and Med13 are also required for transcriptional activation by other

transcription factors, like Nanog and members of the GATA and RUNX families, in a CDK-independent manner (Gobert et al., 2010; Tutter et al., 2009).

In addition to RNA pol II complex assembly, Core Mediator also participates in multiple steps of transcription, such as RNA pol II initiation, pausing and elongation, and reinitiation. Core Mediator can also alter genome architecture by looping DNA to put distant enhancers (with bound TFs) in close proximity to promoters (a mechanism that includes non-coding RNAs), as well as promote the formation of super enhancers (Allen and Taatjes, 2015; Kagey et al., 2010; Pelish et al., 2015; Whyte et al., 2013). In addition, Core Mediator has been shown to be required for transcription of miRNA precursors, as well as some siRNA precursors (Kim et al., 2011).

Since the discovery of Mediator about 25 years ago, the vast majority of research has focused on biochemical and structural studies of Mediator preparations purified from yeast or human cells (reviewed in Poss et al., 2013). These studies have focused primarily on the activities of the whole Core Mediator complex as a transcriptional co-activator, or in the case of CDK8 module, as a repressor. Meanwhile, developmental biology studies, particularly genetic screens for mutants affecting a particular process of interest, have discovered discrete roles for animal Mediator subunits from all three modules of Core Mediator, and in particular for the Kinase (CDK8) module (reviewed in Grants et al., 2015; Yin and Wang, 2014). This research has demonstrated an essential role for Mediator as a signal integrator and specificity factor, with discrete Mediator subunits specific to certain developmental pathways. Mediator has been discovered to play an essential role in some of the most important signaling pathways in animals, including Wnt-β-catenin (Carrera et al., 2008; Rocha et al., 2010; Yoda et al., 2005), Hedgehog (Janody, 2003; Mao et al., 2014; Zhou et al., 2012), RAS-MAPK (Balamotis et al., 2009; Grants et al., 2016; Pandey et al., 2005), and TGFβ-SMAD signaling (Alarcón et al., 2009; Huang et al., 2012; Kato et al., 2002; Zhao et al., 2013). Mediator components have also been found to interact with several Sox transcription factors, which in turn bind to β-catenin and GLI, downstream components of the Wnt-β-Catenin and Hedgehog signaling pathways (Hong et al., 2005; Kamachi and Kondoh, 2013; Nakamura et al., 2011; Rau et al., 2006). Thus, Mediator serves as a transcriptional activator or repressor in a pathway-dependent manner, and can interact with components of signaling pathways like β-catenin (Kim et al., 2006), as well as cofactors of signaling pathway effectors such as Pygopus (Carrera et al., 2008), and Sox transcription factors (Zhou et al., 2002).

In plants, Mediator has been shown to regulate basic cellular processes such as cell proliferation, cell growth, and organ growth; as well as developmental timing, and hormone responses. In particular, the Med25 subunit and the CDK8 module have shown to be involved in many cellular and developmental processes. MED25 restricts cell expansion and cell proliferation, as plants overexpressing *MED25* show smaller organs and *med25* mutants produce larger organs due to an increased period of cell expansion and cell proliferation (Raya-González et al., 2014; Xu and Li, 2011); on the other hand, mutations in the subunits CDK8, MED12, MED13, MED8 and MED14 result in smaller organs as a result of reduced cell expansion and/or cell proliferation (Autran et al., 2002;

Gillmor et al., 2014, 2010; Wang and Chen, 2004; Xu and Li, 2012). MED25 also participates in regulation of Reactive Oxygen Species (ROS) (Foreman et al., 2003; Sundaravelpandian et al., 2013), and along with MED5, MED8 and MED16, regulates cell wall composition (Bonawitz et al., 2014, 2012; Seguela-Arnaud et al., 2015; Sorek et al., 2015).

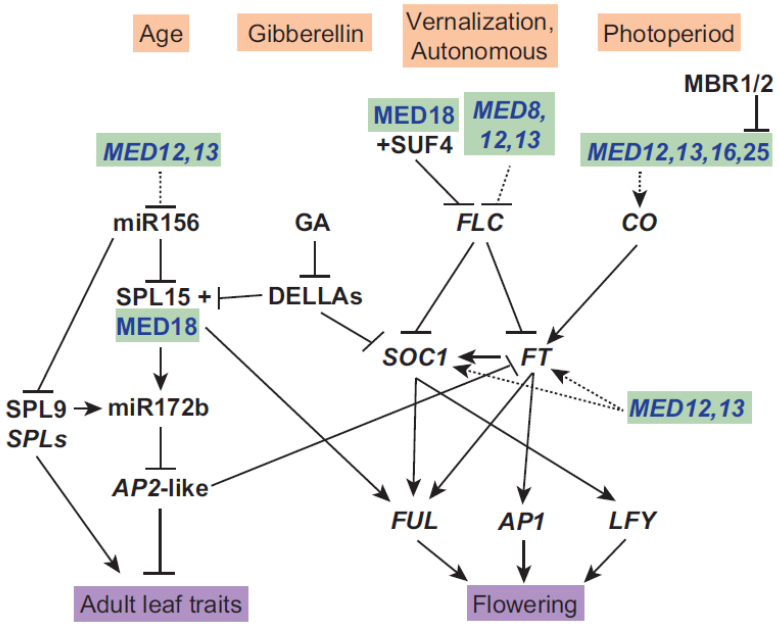

Figure 6. Mediator regulation of vegetative and reproductive transitions. A simplified model of the genetic network regulating vegetative phase change and the transi-tion to flowering, showing Mediator regulation of components of the network discussed in this review. Mediator regulation of transcription that has not been determined to be direct or indirect is shown with dotted lines. Direct regulation of transcription or protein stability is shown with solid lines. Protein-protein interactions are denoted with '+'. The different pathways controlling vegetative and reproductive transitions are shown with an orange background, Mediator components are shown with a green background, and phenotypic outputs are shown with a purple background. Figure published in Buendía-Monreal and Gillmor, 2016.

The CDK8 module subunits MED12 and MED13, as well as MED18 and MED25, participate in the regulation of several phase transitions during plant development (see Figure 6). MED12 and MED13 control the timing of pattern formation during early embryogenesis (Gillmor et al., 2010) and promote the seed to seedling transition by repressing seed specific genes (Gillmor et al., 2014). MED18 and MED25 regulate ABA responses during germination (Chen et al., 2012; Lai et al., 2014). MED12 and MED13 regulate the juvenile to adult vegetative transition by fine tuning the levels of miR156 and promote flowering through downregulation of *FLC*, which is a repressor of vernalization and autonomous flowering pathways (Gillmor et al., 2014; Imura et al., 2012); similarly, MED8 and MED18 promote flowering by repressing *FLC*, and MED18 directly induces the expression of the floral integrator gene *FRUITFULL* (*FUL*) (Hyun et al., 2016; Kidd et al., 2009; Lai et al., 2014; Zheng et al., 2013). MED25 promotes flowering by enhancing light sensitivity in the photoperiod pathway (Cerdán and Chory, 2003; Iñigo et al., 2012; Klose

et al., 2012), whereas MED16 acts upstream of the circadian clock (Knight et al., 2009, 2008).

Single mutants in the Med12 and Med13 homologs show the same phenotypic characteristics throughout different organisms. In *S. pombe*, both mutants are highly flocculent and both regulate, in the same direction, a common small set of genes: only 4 genes are repressed and 10 genes are induced in these mutants (Samuelsen et al., 2003); in *C. elegans*, mutations in *let-19* or *dpy-22* (the Med12 and Med13 homologs) cause similar defects in asymmetric division of vulval precursor cells (Yoda et al., 2005); whereas in *D. melanogaster*, loss of either *kohtalo* or *skuld* (the Med12 and Med13 homologs), distort the anterior-posterior and the dorsal-ventral boundaries, and their corresponding proteins interact with each other, suggesting both Med12 and Med13 act as a single unit (Janody, 2003; Treisman, 2001). In *D. melanogaster,* CDK8-CYCC and Med12–Med13 act as pairs, sharing some functions like their role in external sensory organ development, but also having distinct functions, e.g. Med12-Med13 act independently of CDK8-CYCC during early eye development (Loncle et al., 2007).

In my thesis, I contribute to the knowledge of the CDK8 module functions in plant development by addressing two related questions: 1) how does the CDK8 module coordinate with sugar signaling to regulate miR156?, and 2) what is the role of HEN3/CDK8 in the regulation of miR156?

3. GENERAL OBJECTIVE

- To contribute to the understanding of how the CDK8 module of Mediator regulates the juvenile to adult transition via miR156-SPL.

4. HOW IS REGULATION OF miR156 BY THE CDK8 MODULE COORDINATED WITH SUGAR SIGNALING?

4.1. Background

In the early 20th century, Karl Goebel hypothesized that vegetative phase change is driven by changes in the nutritional status of the shoot (Goebel, 1908). Subsequently, Allsopp demonstrated that sugar is required and sufficient to produce adult leaves (Allsopp, 1952; Allsopp, 1953), and Röbbelen reported that the products of photosynthesis promote the transition to the adult phase (Röbbelen, 1957). Recent studies have shown that leaves are the source of the signal that represses miR156 (Yang et al., 2011), which is the major regulator of vegetative phase change (see Introduction). Later on, two independent groups demonstrated that this signal is sugar: *ch1* mutants, which are impaired in photosynthesis, show increased expression of miR156, and the addition of glucose is able to repress miR156 (Yang et al., 2013; Yu et al., 2013; Figure 7). How sugar represses miR156 remains to be completely understood, though *HEXOKINASE1 (HXK1)* and Trehalose-6-phosphate (T6P) likely play important roles in this developmental transition, since they have an effect on miR156 expression (Wahl et al., 2013; Yang et al., 2013).

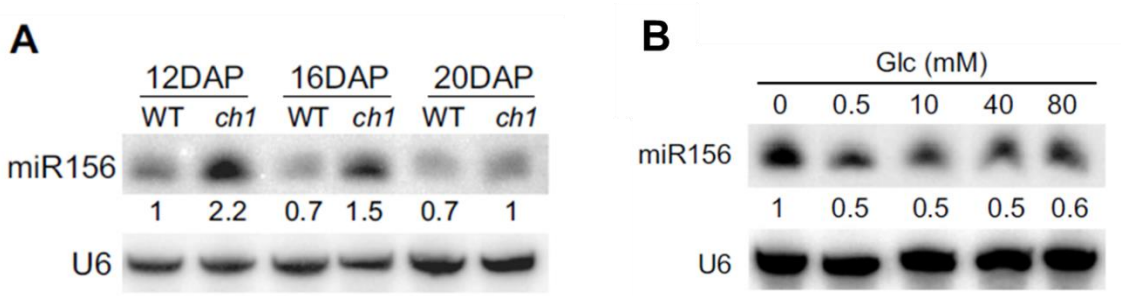

Figure 7. miR156 expression is negatively regulated by photosynthesis and sugar. (A) Northern blot of mature miR156 in *ch1-4* and Col (WT) reveals that miR156 is elevated in *ch1-4* and declines at a slower rate in this mutant. Hybridization intensities are compared to the value in WT 12DAP. DAP means days after planting. (B) Northern blot of miR156 in 12-day-old *ch1-4* plants treated with different amounts of glucose. 0.5 mM produced a 50% reduction in miR156, and higher amounts of glucose did not produce a further reduction. U6 was used as a loading control for both experiments. Figure from Yang et al., 2013.

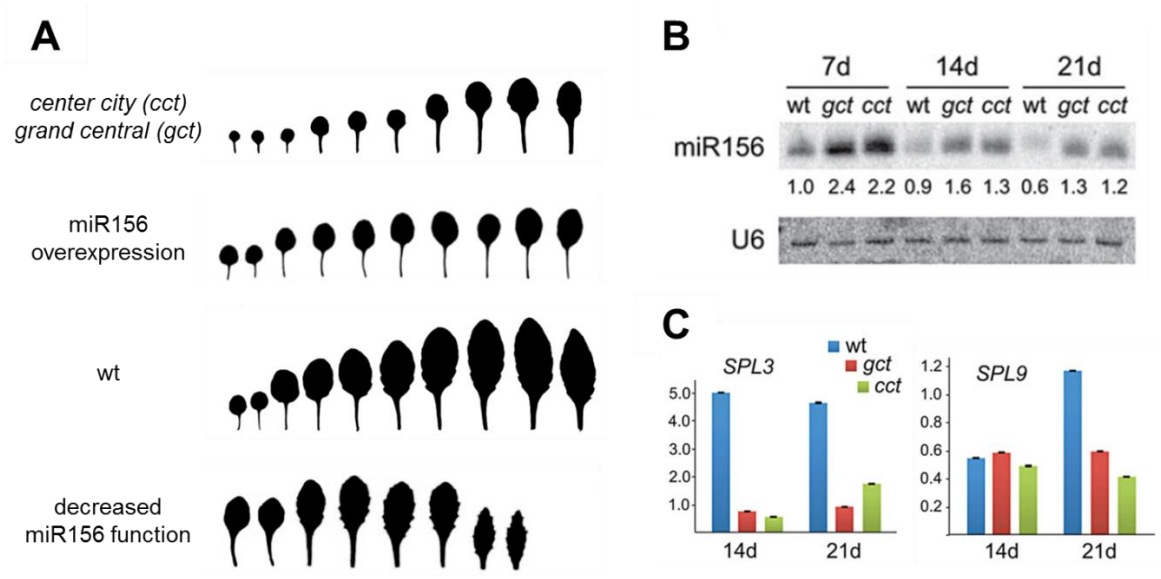

Figure 8. *CCT* and *GCT* regulate the expression of miR156. (A) The first successive rosette leaves from *cct* and *gct* mutants, plants transformed with 35S::MIR156A (Wu and Poethig, 2006), Col-0 wild type plants, and *sqn* mutants, which show decreased miR156 function (Smith et al., 2009). (B) Northern blot detection of miR156 in 7 d, 14 d and 21 d wt, *gct* and *cct* plants grown in long-day conditions. (C) qRT-PCR analysis of *SPL3* and *SPL9* transcript levels, normalized to *EIF4A*. Figures B & C from Gillmor et al., 2014.

The Arabidopsis *CENTER CITY (CCT)* and *GRAND CENTRAL* (*GCT)* genes also repress miR156 during vegetative development. Due to an increase in miR156 levels and the consequent decrease in the expression of some *SPL* genes, *cct* and *gct* mutants show a delay in the juvenile to adult vegetative transition (Gillmor et al., 2014; Figure 8). *CCT* and *GCT* encode the Arabidopsis homologs of MED12 and MED13, components of the CDK8 module of Mediator that regulate transcription by modulating the association of Core Mediator with RNA polymerase II (Allen and Taatjes, 2015; Ding et al., 2008; Gillmor et al., 2010; Tsai et al., 2013). Thus, both sugar and the CDK8 module of Mediator control the timing of vegetative development by modulating miR156 levels. Whether this regulation is independent or part of the same genetic pathway is unknown.

I used functional genetic and gene expression analyses to test whether sugar and the CDK8 module of Mediator regulate miR156 expression in a linear pathway, or independently.

4.2. General objective

❖ Determine whether the CDK8 module and sugar signaling regulate miR156 in the same or in separate genetic pathways

4.3. Specific goals

- Compare the effect on vegetative development of combining mutations in the CDK8 module with one that alters photosynthesis.
- Compare the individual and combined effects of mutations in photosynthetic and CDK8 module genes on *MIR156* expression.
- Test if sugar affects *MIR156* expression in the absence of CDK8 module genes.
- Test if sugar can rescue the vegetative phenotype of CDK8 module mutants.
- Test if the expression of CDK8 module genes is regulated by sugar.

4.4. Materials and Methods

4.4.1. Genetic stocks and growth conditions

All seed stocks were in the Columbia ecotype, with the exception of *gin2-1* which was in the *Landsberg erecta* (*Ler*) background. The CDK8 module mutant lines used in this chapter were *gct-2* (ABRC stock #CS65889) and *cct-1* (ABRC stock #CS65890), which are EMS-induced alleles located in the genes *GCT* (At1g55325) and *CCT* (At4g00450) respectively (described in Gillmor et al., 2010), and *hen3-675*, which is a T-DNA insertion line located in the *HEN3/CDK8* gene (At5g63610, described in detail in the next chapter). *ch1-4* and *gin2-1* are mutant alleles for the *CHLOROPHYLL A OXYGENASE* gene (*AtCAO*, At1g44446) and *HEXOKINASE1* gene (At4g29130) respectively, and were provided by Scott Poethig. *cct/+, gct/+* and *hen3/+* plants were crossed to either *ch1/-* and *gin2/-* plants to obtain the double homozygous mutants in the F3 generation; *cct, gct* and g*in2* mutations were genotyped using dCAPS markers; *hen3* mutations were genotyped with SALK LBa1 primer (named here as *hen3-R mut*) and gene specific *HEN3* primers (Table 1; Gillmor et al., 2014). Seeds expressing a transcriptional fusion with the *CCT* promoter (4.9 Kb from the *CCT* translational start site up to the previous gene) driving the expression of GUS followed by the *CCT* 3' UTR (pCCT::GUS), and a translational fusion with the *GCT* promoter (900 bp from the *GCT* translational start site up to the previous gene) driving the expression of GUS and the entire genomic *GCT* region, including the *GCT* 3'UTR, cloned in frame downstream of GUS (gGCT-GUS) were generated by Stewart Gillmor (Del Toro - De León et al., 2014).

Seeds were sown on a mixture of vermiculite (GRACE MAN-FIN), perlite (AGROL125) and sunshine mix (PREMEZ FWSS3) (1:1:3 v/v/v); or ½ MS plates; and placed at 4°C for 3 days, before moving flats or plates to Percival growth chambers. Plants were grown either under long days (LD) (16 hr light) or short days (SD) conditions (10 hr light) at a constant 22°C under a 3:1 ratio of standard Philips F17T8/TL741 lamps and Osram Lumilux Deluxe Daylight 18W/954 fluorescent lamps (170 – 180 µmol/m2/s).

For measuring *MIR156* expression in sugar, seedlings were grown on plates with MS medium with no sugar and plates with MS medium containing 10 mM Glucose under LD conditions.

Wild type plants and CDK8 module mutants were grown on plates with MS medium with no sugar and plates with MS medium containing 4% Sucrose, under LD conditions, in order to test if sugar can rescue the phenotype of CDK8 module mutants.

For analyzing the effect of sugar on the expression of *CCT* and *GCT*, pCCT::GUS and gGCT-GUS seedlings were grown on plates with MS medium with no sugar (NS) for 12 or 21 days (LD), then transferred to a 10mM Glucose-containing medium or to MS NS medium (mock), and stained with GUS (overnight at 37C) after one day.

4.4.2. Morphological analysis

Heteroblasty traits such as number of leaves and the presence of abaxial trichomes were measured at flowering time in order to allow plants completely develop rosettes and leaves reach their final shape. Flowering time was counted from the day seeds were placed in the growth chamber until the day plants opened the first flower. The presence of abaxial trichomes was scored using a dissecting microscope.

4.4.3. Expression analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and reverse transcribed into cDNA using Super Script II Reverse Transcriptase (Invitrogen). *MIR156A* and *MIR156C* expression was tested by real-time PCR using SYBR Green I in a Light Cycler 480 instrument II from Roche following the manual instructions. Transcript levels were normalized against *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A* (*EIF4A*). Relative quantification was analyzed by Pfaffl method (Pfaffl, 2001). Student's t test, and ANOVA test followed by Bonferroni correction, were applied to distinguish significant differences between samples. Sequences of the primers are listed in the Table 1.

For the histochemical analysis of the pCCT::GUS and gGCT-GUS expression marker lines, whole seedlings were immersed in permissive GUS staining solution (50 mM Sodium Phosphate Buffer pH 7.2, 2 mM X-Gluc dissolved in dimethylformamide, 2 mM $K_4[Fe(CN)_6]$, 2 mM $K_3[Fe(CN)_6]$, 0.2% Triton X-100) and kept 16 hours at 37C in darkness. Then, GUS-stained seedlings were washed with serial dilutions of ethanol: 30%, 50%, 75% and 96%.

Table 1. Sequences of primers used in chapter 4.

Name	Sequence (5' to 3')	Purpose	Notes
MIR156A-F	CAAGAGAAACGCAAAGAAACTGACAG	qRT-PCR	
MIR156A-R	AAAGAGATCAGCACCGGAATCTGACAG	qRT-PCR	
MIR156C-F	AAGAGAAACGCATAGAAACTGACAG	qRT-PCR	
MIR156C-R	GGGACCGAATCGGAGCCGGAATCTGAC	qRT-PCR	
EIF4a-F	AAACTCAATGAAGTACTTGAGGGAC	qRT-PCR	
EIF4a-R	TCTCAAAACCATAAGCATAAATACCC	qRT-PCR	
cct-1-F	agtccagcatcaacaagcc	Genotyping by dCAPS	Described in Gillmor et al., 2014
cct-1-R	actgtagaagacgcaccagata		
gct-2-F	actggagatggcttgtaagcatccg	Genotyping by dCAPS	Described in
gct-2-R	tcgaagaaattcccaatgcg		

			Gillmor et al., 2014
hen3-F	ATGGGAGATGGGAGTTCCAGTAGATCC	Genotyping *hen3* mutants	***hen3-F*** and ***hen3-R WT*** produce a 1186 bp amplicon only in WT and *hen3*/+ plants, whereas ***hen3-F*** and ***hen3-R mut*** produce a 790 bp amplicon only in *hen3*/- and *hen3*/+ plants
hen3-R WT	GCCCATTCCATGAGCTCCTGCC		
hen3-R mut	TGGTTCAGGTAGTGGGCCATCG		
gin2-F	CTACTAAAGACGAGGAGCTG	Genotyping by dCAPS	Amplicon is 150 nt. FW primer has a C instead of a G to create a PstI site that can be cut only in wild type
gin2-R	TGGAGTGAGTGACTTCAACG		

4.5. Results and discussion

4.5.1. *ch1 cct* and *ch1 hen3* double mutants show an increased delay in vegetative and reproductive transitions compared with single mutants

In *Arabidopsis thaliana*, juvenile vegetative leaves are round with smooth margins and lack abaxial trichomes (leaf hairs), while adult leaves are elongated with abaxial trichomes and serrated margins (Telfer et al., 1997; Tsukaya et al., 2000). Perturbations in photosynthesis affect sugar production and consequently delay the juvenile to adult transition (Yang et al., 2013). *chlorina1* (*ch1*) plants have a mutation in the *CHLOROPHYLL A OXYGENASE* gene (*AtCAO*, At1g44446), which encodes the key enzyme for chlorophyll b biosynthesis (Espineda et al., 1999). Under long day (LD) growth conditions (16 hour light), *ch1-4* mutants are yellow, grow more slowly, produce abaxial trichomes 1.5 leaves later, and flower 7 days (d) later compared to wild type (wt) plants (Figure 9A&B and Table 2). *cct/med12* and *hen3/cdk8* plants are also delayed in the juvenile-to-adult and flowering transitions: *cct* mutants produce 7 more leaves without abaxial trichomes and flower 22 d later than wt plants whereas *hen3* mutants produce 1.5 more leaves without abaxial trichomes and flower 10 d later than wt plants (Figure 9A&B and Table 2). The number of both rosette and cauline leaves in *cct* and *hen3* mutants is significantly higher compared to wt plants, consistent with the delayed flowering transition (Table 2). By contrast, *ch1* mutants produce less rosette leaves and slightly fewer cauline leaves, yet flower later than wt (Table 2). Decreased photosynthesis in *ch1* mutants may constitute a physiological stress, inducing the switch to a reproductive meristerm earlier than in wt (leading to fewer rosette leaves), while the slow growth rate of the *ch1* inflorescence may delay flowering (as measured by the first open flower).

In order to test the genetic interaction between sugar and the CDK8 module, I constructed *ch1 cct* and *ch1 hen3* double mutants, and assayed their effect on vegetative and reproductive transitions in LD and short day (SD, 10 hour light) conditions. The interaction between sugar and *gct/med13* was not analyzed, because I was unable to recover *ch1 gct* double mutant seedlings, perhaps due to a strong effect of the double mutant on germination or growth. Compared to *ch1* and *cct* single mutants, the effect of the *ch1 cct* double mutant on vegetative and reproductive transitions was increased; similarly, the effect of the *ch1 hen3* double mutant was higher compared to the single *ch1* and *hen3* mutants (Figure 9 and Table 2). When compared to wt in LD conditions, abaxial trichomes were delayed 1.5 leaves in *ch1*, 7.0 leaves in *cct*, and 9.7 leaves in *ch1 cct*, while flowering was delayed 6.9 days in *ch1*, 21.5 days in *cct*, and 28.9 days in *ch1 cct*. The phenotype of the *ch1 hen3* double mutants makes the additive effect on this heteroblasty traits more evident: abaxial trichomes were delayed 1.5 leaves in *ch1*, 1.5 leaves in *hen3*, and 2.9 leaves in *ch1 hen3*, while flowering was delayed 6.9 days in *ch1*, 9.8 days in *hen3*, and 17.1 days in *ch1 hen3.* The number of both rosette and cauline leaves in *ch1 cct* and *ch1 hen3* double mutants was lower than in *cct* and *hen3* plants, respectively, which indicates an additive interaction, since *ch1* mutants produce less

rosette and cauline leaves than wt plants (Table 2). SD conditions allow evaluation of effects on vegetative phase change in the absence of flowering. Similar to LD conditions, in SD *ch1 cct* plants had an additive effect on the number of leaves without abaxial trichomes, compared to *ch1* and *cct* single mutants (Figure 9C and Table 2). The delay in the acquisition of abaxial trichomes in SD conditions in *ch1 hen3* was slight, not statistically significantly higher than the delay in *ch1* plants, perhaps because of the small size of the samples (Table 2). The additive interactions observed between *ch1* and *cct/hen3* mutants suggest that sugar and the CDK8 module act separately to promote the timing of vegetative and reproductive morphological traits.

Table 2. Vegetative and flowering traits of single *ch1*, *cct* and *hen3* mutants, and double *ch1 cct* and *ch1 hen3* mutants.

Standard deviation is shown in brackets. Every genotype showed significantly different traits (Student's t test $p<0.05$), with the exception of those sharing the same superscript letter.

	1st leaf with abaxial trichomes	n	Flowering day	n	# of rosette leaves at flowering	n	# of cauline leaves at flowering	n
Long Days (LD)								
wt Col	7.1 (0.7)	22	30.5 (1.3)	11	15 (0.9)	12	3.7 (0.5) [a]	12
ch1	8.6 (0.8)	21	37.4 (1.7)	11	12.6 (1.2)	10	3.0 (0.5) [a]	10
cct	14.1 (1.2)	16	52.0 (3.2)	13	33.0 (0.6)	7	7.6 (1.2)	12
ch1 cct	16.8 (1.9)	11	59.4 (3.1)	10	26.5 (1.7)	8	5.8 (0.7)	8
hen3	8.6 (0.8)	24	40.3 (1.7)	12	22.2 (1.2)	6	6.2 (0.4)	6
ch1 hen3	10.0 (1.2)	24	47.6 (1.9)	11	17.8 (0.7)	9	4.8 (0.4)	9
Short Days (SD)								
wt Col	11 (0.9)	6	73.2 (3.2)	6				
ch1	19.9 (1.6) [b]	9	>90	9				
cct	24 (1.1)	6	>90	6				
ch1 cct	30 (3.6)	6	>90	6				
hen3	12.6 (0.5)	5	82.8 (1.3)	5				
ch1 hen3	20.4 (0.7) [b]	8	>90	8				

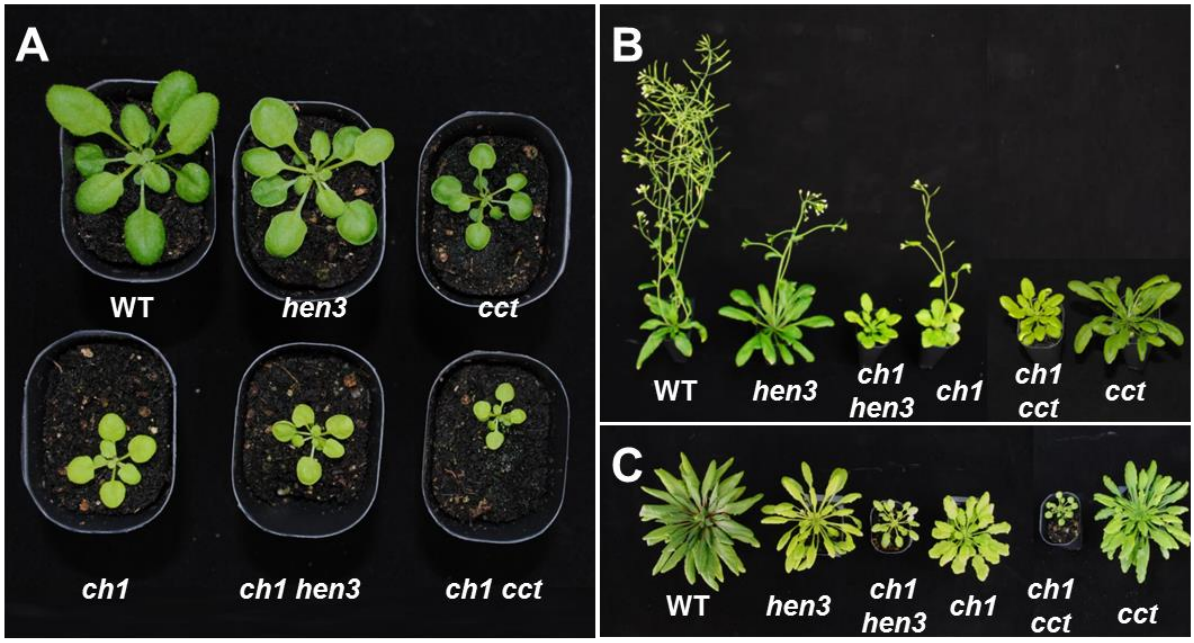

Figure 9. *ch1* shows an additive delay with *hen3* and *cct* on vegetative and reproductive transitions. Phenotype of wt Col, *ch1*, *cct*, *hen3*, *ch1 cct* and *ch1 hen3* plants grown in long days (LD) (A and B) or short days (SD) (C) for 20 days (A), 60 days (B) and 80 days (C). Part of this Figure published in Buendía-Monreal and Gillmor, 2017.

Besides its enzymatic function in the first step of glycolysis, *HEXOKINASE1 (HXK1)* functions as a sugar sensor and can regulate transcription in response to changes in glucose concentration; this latter signaling function is independent of its catalytic activity (Moore et al., 2003) and it is carried out by a HXK1-containing nuclear complex, which binds to the promoter of specific genes when glucose levels are high (Cho et al., 2006). Under conditions of low sugar availability, *HXK1* promotes the transcription of *MIR156*, in such a way that in early vegetative development, *gin2-1* (a null allele for the *HXK1* gene) plants have less *MIR156* expression than wild type plants; consequently, *gin2-1* mutants have a small but statistically significant advance in the acquisition of abaxial trichomes (Figure 10A, Yang et al., 2013).

In order to study the genetic interaction between *HEXOKINASE1* and the CDK8 module, I crossed *cct*/+, *gct*/+ and *hen3*/+ plants to *gin2-1,* to generate double mutants. If the phenotype of either *gin2-1* or *cct/gct/hen3* mutants is epistatic to the other, then both *gin2-1* and the particular CDK8 module gene should regulate vegetative development in the same pathway. Conversely, if the phenotype of the double mutants would be intermediate, i.e. a rescue of the wild type phenotype, then *gin2-1* and the CDK8 module would be interpreted to regulate miR156 and vegetative development independently. However, under our both LD and SD conditions, *gin2-1* plants showed a delay in the acquisition of abaxial trichomes and flowering time, an opposite phenotype to that reported in (Yang et al., 2013), and which may be due to differences in growth conditions between

our lab and Scott Poethig's lab (Figure 10). Because of this, I did not continue with the selection of double homozygous mutants.

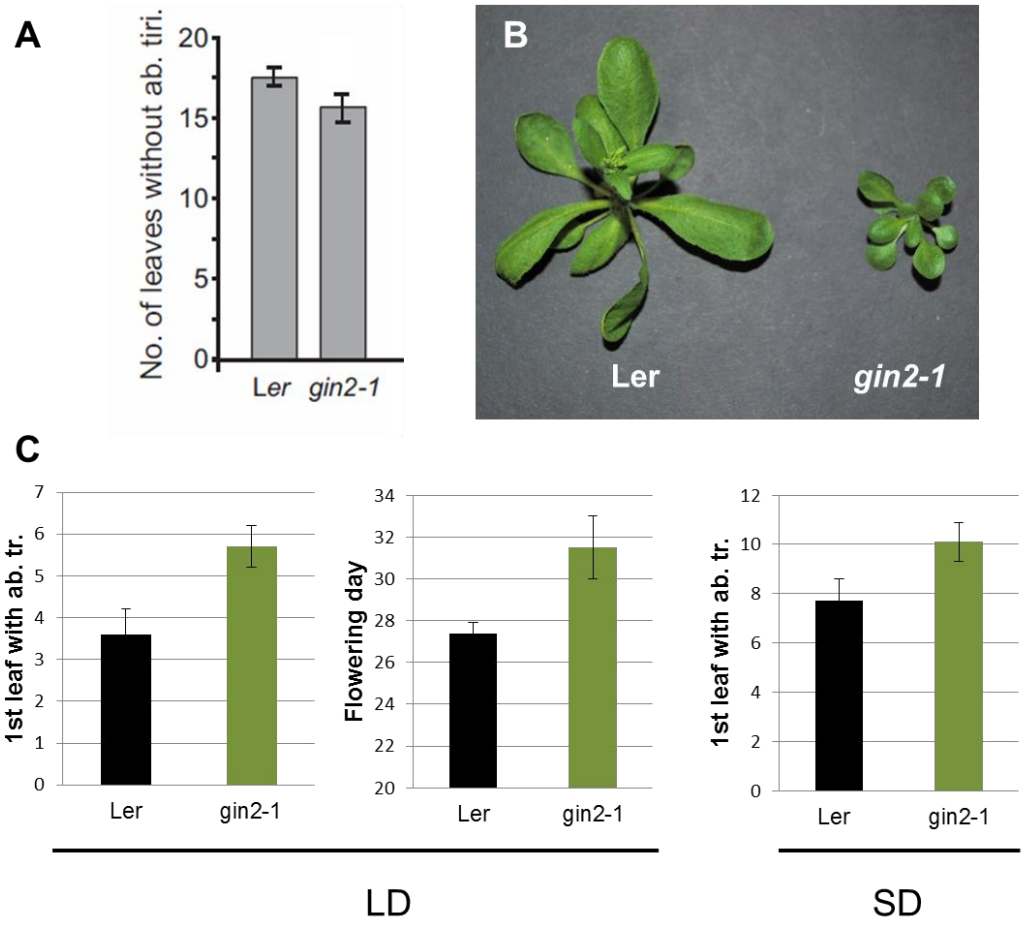

Figure 10. Vegetative phenotype of *gin2-1*. (A) Number of leaves without abaxial trichomes in Ler and *gin2-1* plants, under SD conditions, as reported by Yang et al., (2013), (B) 27 day-old Ler and *gin2-1* plants grown under our LD conditions, (C) Number of the first leaf with abaxial trichomes and flowering day for Ler and *gin2-1* plants grown under our LD and SD conditions.

4.5.2. *ch1 cct* double mutants have increased *MIR156A* and *MIR156C* transcript levels compared to *ch1* or *cct* single mutants

The transition from the juvenile to the adult vegetative phase is controlled by miR156 (reviewed in Huijser and Schmid, 2011). *MIR156A* and *MIR156C* play dominant roles within the miR156 gene family: they are the only miR156 genes that are developmentally regulated, and *mir156a mir156c* double mutants shorten the juvenile phase of development (Yang et al., 2013; Yu et al., 2013). To test if the delayed vegetative transitions seen above correlate with higher miR156 levels, we measured pri-*MIR156A* and pri-*MIR156C* transcript levels in wt, *ch1*, *cct* and *ch1 cct* mutants grown in LD (Figure 11A-D). In these conditions, *MIR156A* and *MIR156C* show a steady decrease from 12 to 16 to 20 d in both wt and *ch1* plants, whereas in *cct*, the decrease is much slower, and in

ch1 cct, *MIR156* levels remain steady from 12 to 20 d (Figure 11 A&B). At 12 days, *MIR156A* and *MIR156C* show similar expression levels among all genotypes tested; at 16 days, the expression is significantly higher in *cct* and *ch1 cct* compared to wt and *ch1* plants. At 20 days, *MIR156* levels are increased in *ch1* and *cct* single mutants compared to wt, and are even higher in *ch1 cct* double mutants (Figure 11 C&D).

To better quantify the interaction between *ch1* and *cct* on *MIR156* expression, I measured *MIR156A* and *MIR156C* levels in SD (to avoid the effect of flowering), and over a longer time period (10 to 40d), so that the relationship between *ch1* and *cct* would be more clear (Figure 11E-H). Wt and *ch1* showed a gradual decrease in *MIR156A* and *MIR156C* levels over the period examined, with slightly elevated *MIR156* expression in *ch1* compared to wt (Figure 11E&F). *MIR156A* levels at 10d and 20d were 8 and 16 times higher in *cct* compared to *ch1* and wt (Figure 11G), while *MIR156C* was more than twice as high (Figure 11H). Thus, the effect of loss of *CCT* on *MIR156* expression is much greater in SD than in LD, in agreement with the greater effect of *cct* on abaxial trichomes in SD compared to LD (Table 2). The larger effect of *cct* on *MIR156A* than *MIR156C* suggests that *MIR156A* contributes more than *MIR156C* to the *cct* vegetative phenotype.

The *ch1 cct* double mutant showed a dramatic effect on *MIR156A* expression. At 10d, *MIR156A* levels were twice as high in *ch1 cct* as in *cct*; at 30d, *MIR156A* levels are about 3 times higher; and at 40d, *MIR156A* levels were more than 6 times higher (Figure 11G). The *ch1 cct* double mutant showed a twofold increase in *MIR156C* compared to *cct* at 20d, and a slight increase at 40d (Figure 11H). The increase in *MIR156* expression in *ch1*, *cct* and *ch1 cct* is consistent with the effects of *ch1, cct,* and *ch1 cct* on morphological traits of vegetative phase change (Figure 9 and Table 2). The increase in *MIR156A* expression in *ch1 cct* double mutants is much greater than the additive interaction that would be expected if *CH1* and *CCT* regulated *MIR156A* strictly independently, suggesting that *CH1* and *CCT* interact synergistically in their regulation of *MIR156A*.

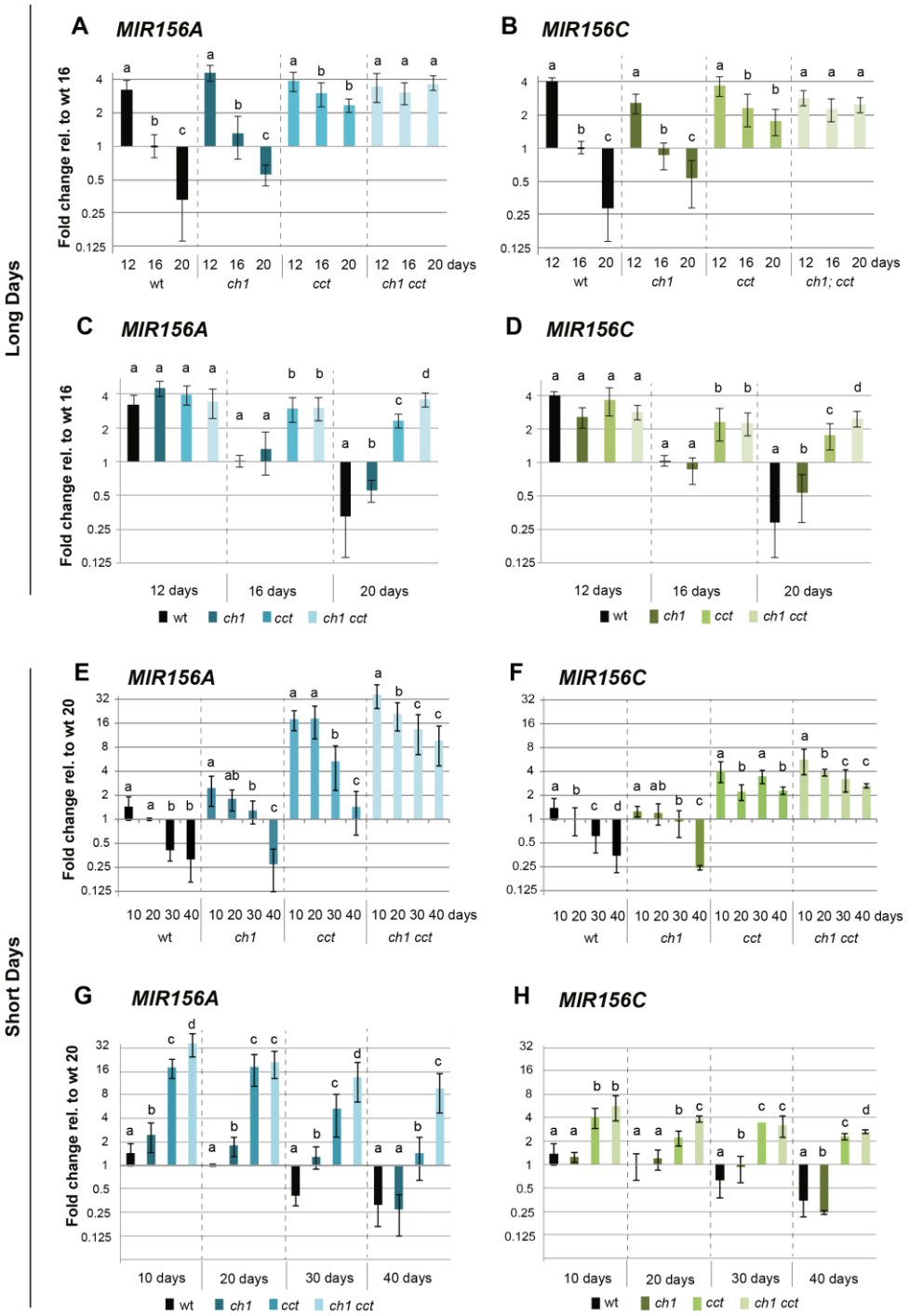

Figure 11. *ch1* and *cct* interact in their regulation of *MIR156.* Transcript levels of *pri-miR156A* (A, C, E, G) and *pri-miR156C* (B, D, F, H) in wt, *ch1*, *cct* and *ch1 cct* plants at 12, 16 and 20 long days (A-D) and at 10, 20, 30 and 40 short days (E-H). Fold change is shown relative to expression of wt at 16 long days (A-D) or wt at 20 short days (E-H). Expression values were first normalized against *EIF4A* as a reference gene. Values shown are the mean of three technical replicates for three biological replicates. Standard deviation represented by bars. Data are grouped by genotype (A, B, E, F) and by time point (C, D, F, H). Samples that are not significantly different ($p>0.05$, Student's t test) share the same letter. Figure published in Buendía-Monreal and Gillmor, 2017.

4.5.3. Sugar can repress miR156 in the absence of *CCT/MED12* and *GCT/MED13*

Both glucose and fructose have previously been demonstrated to reduce miR156 levels in 12d seedlings of Arabidopsis (Yang et al., 2013). To test whether glucose can repress miR156 levels in the absence of *CCT/MED12* or *GCT/MED13* function, I measured *MIR156A* and *MIR156C* transcripts by qPCR in 12d wt, *cct,* and *gct* seedlings grown on MS medium with no sugar (MS NS) and on MS medium with 10 mM glucose (MS GLU), in LD conditions (Figure 12). In the absence of glucose, *MIR156C* levels were elevated 3-4 fold in both *cct* and *gct* seedlings compared to wt, while *MIR156A* increased ~1.5 fold in *cct* compared to wt. Interestingly, *MIR156A* levels were not significantly different in *gct* compared to wt, suggesting that *GCT* does not play an important role in regulating *MIR156A* (Figure 12). Growth of seedlings on glucose caused a significant decrease of *MIR156A* in wt, although no significant decrease was observed for *MIR156C*. The lack of effect of glucose on *MIR156C* in wt may be attributable to rapid processing of pri-*MIR156C* transcripts: a previous study of the effect of glucose on *MIR156C* was conducted in *serrate-1* mutants, in order to slow the processing of pri-*MIR156C* transcripts, so they are better substrates for qPCR (Yang et al., 2013). Importantly, glucose did cause a significant decrease in steady-state levels of *MIR156C* in *cct* and *gct* mutants, and of *MIR156A* in *cct* mutants, demonstrating that glucose repression of these genes does not require *CCT* (*MIR156A* and *MIR156C*) or *GCT* (*MIR156C*) (Figure 12).

The expression of *MIR156A* and *MIR156C* in *hen3* mutants at 12 days did not change in the presence of 10 mM glucose (data not shown), which may indicate that *HEN3/CDK8* is necessary for the negative regulation of miR156 by sugar. However, this is a preliminary result since only two biological replicates were tested.

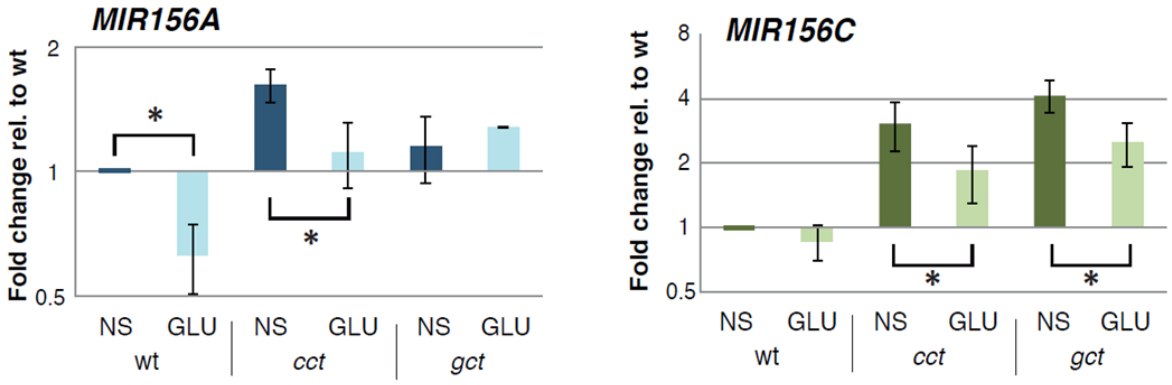

Figure 12. Sugar can repress *MIR156* in the absence of *CCT* and *GCT*. Transcript levels of *pri-miR156A* and *pri-miR156C* in WT Col, *cct* and *gct* plants at 12 days grown in long days in the absence of sugar (No Sugar, NS) or in the presence of 10mM Glucose (GLU). Expression values were first normalized against the reference gene *EIF4A.* Values shown are the mean of three technical replicates for three biological replicates. Standard deviation represented by bars. Asterisks indicate significant difference ($p<0.05$, Student's t test) between samples. Figure published in Buendía-Monreal and Gillmor, 2017.

4.5.4. Sugar can promote growth in mutants of the CDK8 module

In addition to the negative effect on *MIR156* expression, Yang et al. reported that growing plants in the presence of 4% Sucrose, under SD conditions, results in precocious acquisition of abaxial trichomes, compared to plants grown on 0% Sucrose medium. They used sucrose for this experiment because sucrose causes less deleterious effects on plant growth in a long-term experiment than glucose (Yang et al., 2013).

In order to test whether sugar can rescue the wild type phenotype in mutants of the CDK8 module, I grew wild type, *hen3*, *cct* and *gct* plants on plates with either MS medium with no sugar or MS medium with 4% sucrose, under SD conditions. After 52 days, all plants grown in MS medium with no sugar were small and produced few leaves, whereas plants grown in MS medium with 4% sucrose were larger, and wt and *hen3* plants indeed flowered, but no genotype showed leaves with abaxial trichomes (Figure 13). Thus, our SD growing conditions did not allow us to know whether sugar can rescue specific heteroblasty traits in mutants of the CDK8 module, but do suggest that *hen3*, *cct* and *gct* can respond to growth on sugar, similar to wt. Because the point of this experiment was to look at the heteroblasty trait abaxial trichomes, and trichomes were not even present on wt plants, I did not continue further with this experiment.

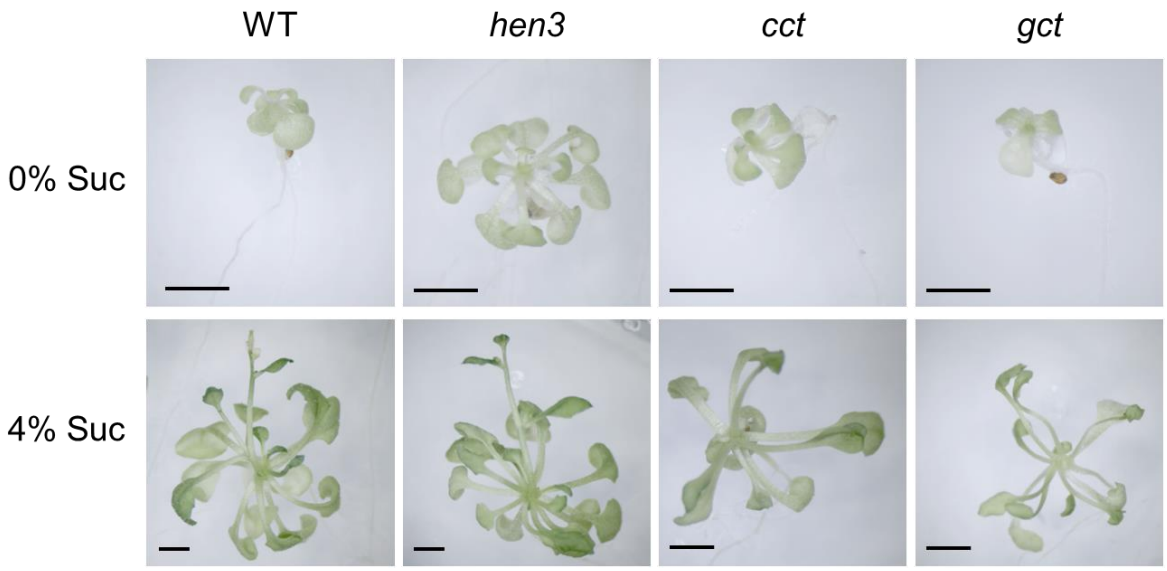

Figure 13. Phenotype of wild type, *hen3*, *cct* and *gct* plants grown for 52 short days in MS medium with either 0% or 4% sucrose. No abaxial trichomes were produced in any genotype.

4.5.5. Sugar downregulates the expression of *CCT/MED12* and *GCT/MED13*

Since I demonstrated that both sugar and the CDK8 module promote vegetative phase change by repressing the expression of miR156, and that they can act in a synergistic manner, I wanted to understand whether sugar and the CDK8 module regulate each other.

In order to know whether sugar influences the expression of *CCT/MED12* and/or *GCT/MED13*, I took advantage of two GUS-marker lines previously generated by Stewart Gillmor. The pCCT::GUS line contains a transcriptional construct where the whole promoter of *CCT* drives the expression of β-glucuronidase (GUS), whereas the gGCT-GUS line contains a translational fusion where the whole *GCT* gene is fused in frame next to the β-glucuronidase (GUS) coding region (see Methods).

The expression of both *CCT* and *GCT* was analyzed in 12 and 21 day-old seedlings grown on plates with MS medium with no sugar, and then transferred to MS medium with 10 mM glucose, so that the observed expression changes would be due to the presence of glucose. Seedlings transferred to MS medium with no sugar were used as a control experiment. In 12 d seedlings transferred to glucose for 1 day, the expression of *GCT* in the presence of glucose was similar to that in the absence of glucose; however, the expression of *CCT* in 12 d seedlings appeared lower after 1 day in the presence of glucose (Figure 14). In 21 day-old seedlings, the negative effect of glucose on the *CCT* and *GCT* expression is qualitatively evident (Figure 14). Glucose almost completely restricted the expression of *CCT* and *GCT* to the newest leaves and the shoot apical meristem, whereas plants growing in the absence of glucose show higher *CCT* and *GCT* expression in all tissues. These preliminary results are interesting since both sugar and the CDK8 module repress miR156, and one easy explanation could be that sugar promotes the expression of the CDK8 module and thereby repress miR156; nevertheless, the regulation seems to be more complicated. A more definitive result as to the effect of glucose on CCT and GCT expression will require quantitation of *CCT* and *GCT* gene expression, for example by qPCR.

On the other hand, whether the CDK8 module regulates sugar sensing or sugar signaling is currently unknown. RNA-Seq data, obtained from 18 day-old seedlings in our lab, do not show a statistical significant change of *CH1* in *cct*, *gct* or *hen3* mutants, whereas *GIN2* (*HXK1*) expression is significantly increased (FDR: 0.03347) only 1.4 times in *cct* mutants, compared to wt plants (unpublished data from Gillmor and Abreu labs). These results suggests that photosynthesis is not regulated by the CDK8 module, but the expression of the main sugar sensor could be repressed by the CDK8 module in wild type plants, thereby contributing to the negative regulation of miR156.

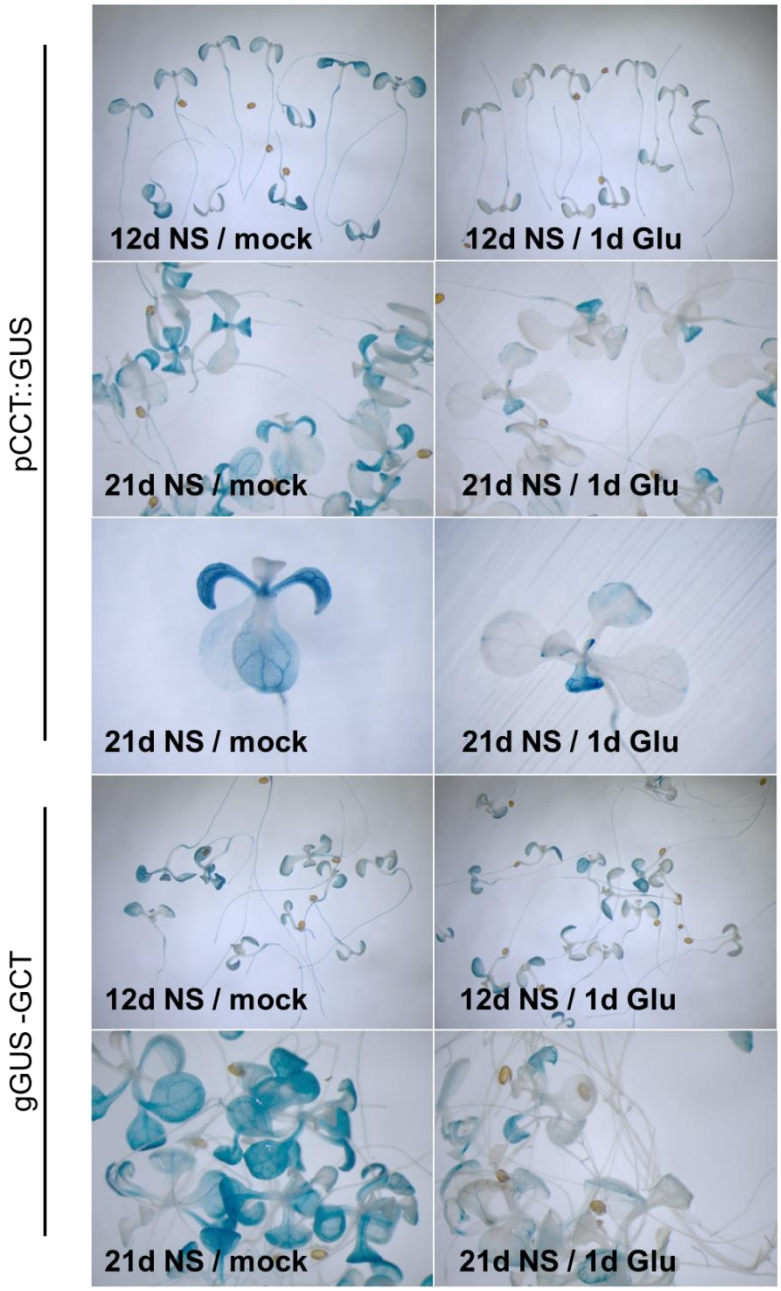

Figure 14. Glucose downregulates the expression of *CCT* and *GCT*. Seedlings expressing a pCCT::GUS fusion or a gGCT-GUS fusion were grown for 12 days or 21 days in MS medium with No Sugar (NS), then transferred to MS medium NS (mock) or MS medium with 10 mM glucose (Glu), and GUS-assayed after 1 day.

5. CDK8/HEN3 PROMOTES VEGETATIVE AND REPRODUCTIVE TRANSITIONS

5.1. Background

The MED12 and MED13 subunits of Mediator participate in the control of the proper timing of several developmental transitions in Arabidopsis. They promote the seed to seedling transition by repressing seed specific genes, then they repress miR156 during vegetative development allowing the transition to the adult phase, and then they promote the reproductive transition by repressing the flowering repressor *FLC* (Gillmor et al., 2014).

In almost all eukaryotes, MED12 and MED13 form, with Cyclin C (CYCC) and Cyclin Dependent Kinase 8 (CDK8), a tetra-protein complex called "CDK8 module" which can bind and regulate the function of the Core Mediator complex (see Introduction). In Arabidopsis, *MED12* has been identified as *CENTER CITY* (*CCT*; Gillmor et al., 2010) or *CRYPTIC PRECOCIUS* (*CRP*; Imura et al., 2012), and *MED13* has been identified as *GRAND CENTRAL* (*GCT*; Gillmor et al., 2010) or *MACCHI-BOU 2* (*MAB2*; Ito et al., 2011). The homologs of mammalian CDK8 are named CDKE in plants; in Arabidopsis it was identified as *HUA ENHANCER 3* (*HEN3*; Wang and Chen, 2004).

The specific functions of HEN3/CDK8 (referred hereafter as HEN3) in plant development are still poorly studied. It was first demonstrated that HEN3 is required for cell specification of floral organs and cell expansion in leaves (Wang and Chen, 2004). It also contributes to plant immunity to fungal pathogens by controlling jasmonate-mediated defense and promoting defense-active secondary metabolites (Zhu et al., 2014). The Arabidopsis genome contains two genes encoding Cyclin C: *CYCCA* (At5g48630) and *CYCCB* (At5g48640); both Cyclin C proteins interact with HEN3. Since both *CYCC* genes are linked, it is very difficult to generate double mutants in order to elucidate the function of those genes; however, there is a T-DNA line, whose insertion is located in the small intergenic region between the two genes, in which both genes are downregulated and show increased susceptibility to the necrotrophic pathogen *Alternaria brassicicola* compared to the single mutants or wild type plants, which could be useful for future research (Zhu et al., 2014).

Here I focused on revealing the function of *HEN3* in vegetative development. Previously, Claudia Silva (a former postdoc in the Gillmor Lab) tested, by Northern blot, the mRNA levels of *HEN3* in four available T-DNA lines to look for null alleles for this gene. The SALK T-DNA line 138675 (referred hereafter as *hen3-675*) showed no detection of mRNA with the corresponding probe, demonstrating it to be a null allele (Figure 15). This same allele was also shown to have undetectable levels of *HEN3* mRNA by qPCR (Zhu et al., 2014).

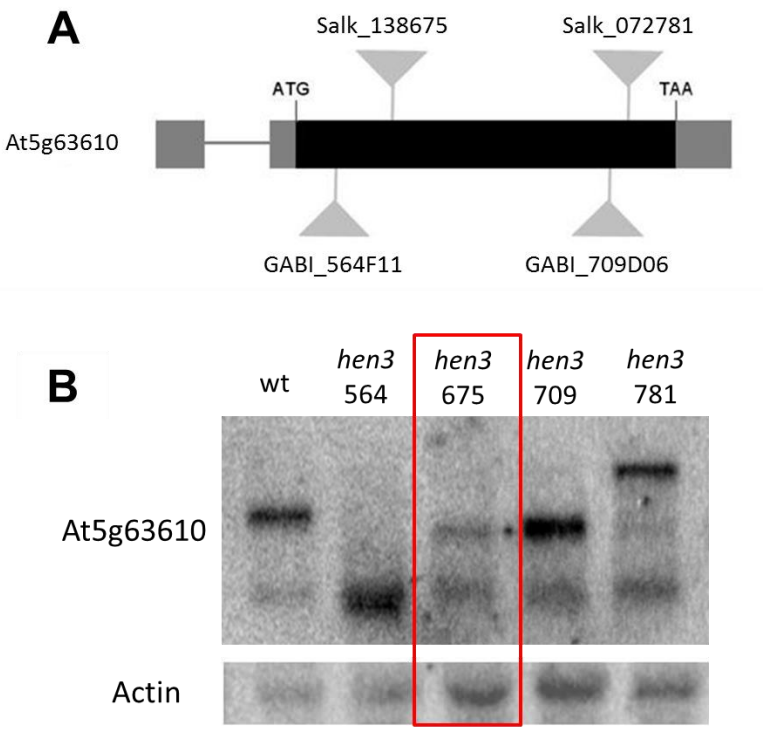

Figure 15. *hen3-675* is a null allele for the *HEN3* gene. (A) Location of four T-DNA insertions available for the *HEN3* gene (At5g63610). Bars represent exons and the line represents the only intron; coding sequence is shown in black. (B) Detection of *HEN3* mRNA by northern blot in wild type (wt) plants and mutants for the four different *hen3* alleles shown in (A). In red is remarked the absence of *HEN3* mRNA in *hen3-675* mutants. Northern blot performed by Claudia Silva-Ortega.

5.2. General objective

❖ Characterize the function of *HEN3* in the regulation of miR156/SPL pathway during vegetative development

5.3. Specific goals

- Characterize the vegetative phenotype of *hen3* mutants.
- Characterize the spatio-temporal expression of *HEN3* during vegetative development.
- Test whether the expression of *MIR156* and *SPL* genes is affected in *hen3* mutants.
- Characterize the spatio-temporal regulation of *MIR156* and *SPL* gene expression by *HEN3*.
- Test whether reduced function of miR156 rescues the wild type phenotype in *hen3* plants.

5.4. Materials and methods

5.4.1. Genetic stocks and growth conditions

All seed stocks were in the Columbia ecotype. The four T-DNA insertion lines used to characterize the loss of function of the *HEN3* gene (At5g63610) were GABI_564F11, SALK_138675, GABI_709D06 and SALK_072781. The mutants for the *CCT/MED12* and *GCT/MED13* genes were the same used in Chapter 4: *cct-1* (ABRC stock #CS65890) and *gct-2* (ABRC stock #CS65889). For generating *hen3 cct* double mutants, *cct*/+ plants were crossed to *hen3*/+ plants to obtain the double homozygous mutants in the F3 generation.

gHEN3-GUS and gHEN3-GFP transgenic lines were generated by transforming either wild type and *hen3* plants with a translational fusion of *HEN3* tagged with either β-glucuronidadase (GUS) or Green Fluorescent Protein (GFP); the procedure to obtain these lines is described in detail in the next section.

MIR156A-GUS and MIR156C-GUS were generated and provided by Scott Poethig's lab; these transgenic lines were obtained by replacing the miR156 hairpin region by GUS. The reporter lines for the expression of *SPL3* and *SPL9* were also generated and provided by Scott Poethig's lab, and were obtained by inserting GUS at the 3' end of a genomic fragment comprising the whole *SPL* locus from the end of the upstream gene to the end of the coding region. Given that the miR156 binding site of *SPL3* is located at 3'UTR, the resistant version rSPL3 was obtained by simply deleting this region. The resistant version rSPL9 was obtained by introducing mutations in the miR156 binding site of the *SPL9* gene. For visualizing the expression of these *MIR156* and *SPL* genes in the mutants of the CDK8 module, *cct*/+ and *hen3*/+ plants were crossed to the GUS reporter lines and double homozygous (for the mutation and the GUS transgene) were obtained in the F3 generation. *cct* and *hen3* mutants were selected using the primers described in Table 3; homozygous plants for the GUS transgene were selected by choosing different F2 plants positive in GUS-assays and selecting F3 plants by progeny test.

mir156a mir156c and *mir157a mir157c* were generated and provided by Scott Poethig's lab. *mir156a mir156c* double mutants were obtained by crossing *mir156a-2* (SALK_131562) to *mir156c-1* (GT22288). *mir156a-2* has an insertion in the first intron of *MIR156A*, whereas *mir156c-1* has an insertion in the first exon of *MIR156C*; both insertions are located upstream of the miR156 hairpin region. *mir157a mir157c* double mutants were obtained by crossing *mir157a* (FLAG375C03) to *mir157c* (GABI_369D05). To obtain the triple *hen3 mir156a mir156c* and *hen3 mir157a mir157c mutants, hen3*/+ plants were crossed to either *mir156a mir156c* and *mir157a mir157c,* and triple mutants were selected until the F3 generation using the primers described in Table 3.

Growth conditions were the same used in Chapter 4. Seeds were sown on a mixture of vermiculite (GRACE MAN-FIN), perlite (AGROL125) and sunshine mix (PREMEZ FWSS3) (1:1:3 v/v/v); or ½ MS plates; and placed at 4°C for 3 days, before moving flats or plates to Percival growth chambers. Plants were grown either under long days (LD) (16 hr light) or short days (SD) conditions (10 hr light) at a constant 22°C under a 3:1 ratio of standard Philips F17T8/TL741 lamps and Osram Lumilux Deluxe Daylight 18W/954 fluorescent lamps (170 – 180 µmol/m2/s).

5.4.2. Generation of gHEN3-GFP and gHEN3-GUS lines

For generating plants expressing a transgene of *HEN3* tagged with GFP or GUS, we first cloned a 2.7 Kb genomic fragment of *HEN3* including the whole 5' region (from the end of the previous gene upstream) and the coding region into pGEM-TEasy (Promega), using a FW primer with an additional XmaI site and a RV primer with an additional sequence encoding a NAAIRS linker and a NcoI site (Table 3). This XmaI-5'HEN3-HEN3CDS-NAAIRS-NcoI cassette was subsequently cloned in frame into the XmaI and NcoI sites of either pCAMBIA-GUS+ or pCAMBIA-GFP, thus obtaining transformation vectors with translational fusions gHEN3-GUS and gHEN3-GFP. Then, we transformed wild type and

hen3 mutants with each construct, by the floral dip method. Transgenic plants were identified using Basta resistance, and their T2 progeny were screened to identify lines expressing GUS or GFP, and that rescued the wild type phenotype (in the case of transformed *hen3* mutants). Since gHEN3-GUS did not rescue the wild type phenotype in *hen3* mutants, the expression of HEN3-GUS was characterized in wild type plants transformed with gHEN3-GUS. The T2 progeny of both wild type and *hen3* mutants transformed with gHEN3-GFP showed a wild type phenotype.

5.4.3. Morphological analysis

Heteroblasty traits such as number of leaves and the presence of abaxial trichomes were measured at flowering time, in order to allow plants to completely develop rosettes and leaves to reach their final shape. Flowering time was counted from the day seeds were placed in the growth chamber, until the day plants opened their first flower. The presence of abaxial trichomes was scored using a dissecting microscope. For obtaining images of the whole dissected rosettes, measuring the length/width ratio and counting the number of serrations, plants were collected at flowering time and their leaves were pasted consecutively onto double tape and scanned.

5.4.4. Expression analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and reverse transcribed into cDNAs using Super Script II Reverse Transcriptase (Invitrogen). The expression of *MIR156A, MIR156C, SPL3*, *SPL9*, *SPL13* and *SPL15* was tested by real-time PCR using SYBR Green I in a Light Cycler 480 instrument II from Roche following the manual instructions. Transcript levels were normalized against *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A* (*EIF4A*). Relative quantification was analyzed by Pfaffl method (Pfaffl, 2001). Sequences of the primers are listed in Table 3.

The expression of HEN3-GUS, MIR156A-GUS, MIR156C-GUS, SPL3-GUS, rSPL3-GUS, SPL9-GUS and rSPL9-GUS was analyzed after immersing whole seedlings in permissive GUS staining solution (50 mM Sodium Phosphate Buffer pH 7.2, 2 mM X-Gluc dissolved in dimethylformamide, 2 mM $K_4[Fe(CN)_6]$, 2 mM $K_3[Fe(CN)_6]$, 0.2% Triton X-100) and keeping them for 16 hours at 37C in darkness. Then, GUS-stained seedlings were washed with serial dilutions of ethanol: 30%, 50%, 75% and 96%.

Table 3. Sequences of primers used in chapter 5.

Name	Sequence (5' to 3')	Purpose	Notes
EIF4a-F	AAACTCAATGAAGTACTTGAGGGAC	qRT-PCR	
EIF4a-R	TCTCAAAACCATAAGCATAAATACCC		
MIR156A-F	CAAGAGAAACGCAAAGAAACTGACAG	qRT-PCR	
MIR156A-R	AAAGAGATCAGCACCGGAATCTGACAG		
MIR156C-F	AAGAGAAACGCATAGAAACTGACAG	qRT-PCR	
MIR156C-R	GGGACCGAATCGGAGCCGGAATCTGAC		
SPL3-F	CTTAGCTGGACACAACGAGAGAAGGC	qRT-PCR	
SPL3-R	GAGAAACAGACAGAGACACAGAGGA		
SPL9-F	CAAGGTTCAGTTGGTGGAGGA	qRT-PCR	
SPL9-R	TGAAGAAGCTCGCCATGTATTG		
SPL13-F	GAAGCAAATGAGGGACTGACGACG	qRT-PCR	
SPL13-R	CCAATCTCTTCTTCTCCAAACAGTACCAGAAGC		
SPL15-F	GAATGTTTTATCACATGGAAGCTC	qRT-PCR	
SPL15-R	TCATCGAGTCGAAACCAGAAGATG		
hen3-F	ATGGGAGATGGGAGTTCCAGTAGATCC	Genotyping *hen3* mutants	***hen3-F*** and ***hen3-R WT*** produce a 1186 bp amplicon only in WT and *hen3*/+ plants, whereas ***hen3-F*** and ***hen3-R mut*** produce a 790 bp amplicon only in *hen3*/- and *hen3*/+ plants
hen3-R WT	GCCCATTCCATGAGCTCCTGCC		
hen3-R mut	TGGTTCAGGTAGTGGGCCATCG		
cct-1-F	agtccagcatcaacaagcc	Genotyping *cct* mutants by dCAPS	Described in Gillmor et al., 2014
cct-1-R	actgtagaagacgcaccagata		
XmaI-5'HEN3-F	CCCGGGCCAATATAGCCTTTTGATTC	Make gHEN3-GUS and gHEN3-GFP constructs	Produce a 2760 bp amplicon with XmaI and NcoI sites at its ends
NcoI-NAAIRS-HEN3-R	**CCATGG***ACCTGATAGCGGCGTT*-GAGGCGTCTGGATTTGTTAG		
mir156a-F WT	AAAGGCTAAAGGTCTCCTCCC	Genotyping *mir156a*	***mir156a-F WT*** and

		mutants	***mir156a-R*** produce a 1163 bp amplicon only in WT and *mir156a*/+ plants, whereas ***mir156a-F mut*** and ***mir156a-R*** produce a ~800 bp amplicon only in *mir156a*/- and *mir156a*/+ plants
mir156a-F mut	TGGTTCAGGTAGTGGGCCATCG		
mir156a-R	CGCGCTTCACTTAAAATTACG		
mir156c-F	Acagtactttgcaagatccatgac	Genotyping *mir156c* mutants	***mir156c-F*** and ***mir156c-R WT*** produce a 1278 bp amplicon only in WT and *mir156c*/+ plants, whereas ***mir156c-F*** and ***mir156c-R mut*** produce a ~800 bp amplicon only in *mir156c*/- and *mir156c*/+ plants
mir156c-R WT	taccactcccatcgtgaaagacca		
mir156c-R mut	TCCGTTCCGTTTTCGTTTTTTAC		
mir157a-F	agcaggccatattgtacaaggtc	Genotyping *mir157a* mutants	***mir157a-F*** and ***mir157a-R WT*** produce a 1264 bp amplicon only in WT and *mir157a*/+ plants, whereas ***mir157a-F*** and ***mir157a-R mut*** produce a ~800 bp amplicon only in *mir157a*/- and *mir157a*/+ plants
mir157a-R WT	caagttcgtgatgttcatagaggt		
mir157a-R mut	CGTGTGCCAGGTGCCCACGGAATAGT		
mir157c-F WT	gctcaaaggctgaatctcagtgga	Genotyping *mir157c* mutants	***mir157c-F WT*** and ***mir157a-R*** produce

mir157c-F mut	atattgaccatcatactcattgc	a 1242 bp amplicon only in WT and *mir157c*/+ plants, whereas ***mir157c-F mut*** and ***mir157c-R*** produce a ~800 bp amplicon only in *mir157c*/- and *mir157c*/+ plants
mir157c-R	tgctatctactggctggatgctga	

5.5. Results and discussion

5.5.1. *hen3* mutants show an extended juvenile phenotype

In order to confirm that *hen3-675*, the null allele (see "5.1. Background" section), shows the most severe phenotype among the different T-DNA lines available, I characterized the vegetative phenotype of the four different mutant alleles. All *hen3* mutant alleles are delayed by at least one leaf in the acquisition of abaxial trichomes compared to wild type plants, which is indicative of a delayed juvenile-to-adult transition (Figure 16). The alleles *-709*, *-564* and *-675* are also delayed in the flowering transition, with the *hen3-675* allele the one with the largest effect (36 days to first open flower) compared to wild type plants (26 days) (Figure 16). Furthermore, only the *hen3-675* allele produces more rosette leaves than wt plants, whereas all mutant alleles show more cauline leaves than wt plants, again with the *hen3-675* allele the one with the largest effect, doubling the number of cauline leaves of wt plants (Figure 16). The fact that different alleles show a juvenile phenotype confirms that *HEN3* gene has a role in the vegetative phase change.

Since the *hen3-675* allele is a null allele and shows the most severe phenotype, I characterized in more detail the vegetative phenotype of this allele, and I used this mutant line for further experiments.

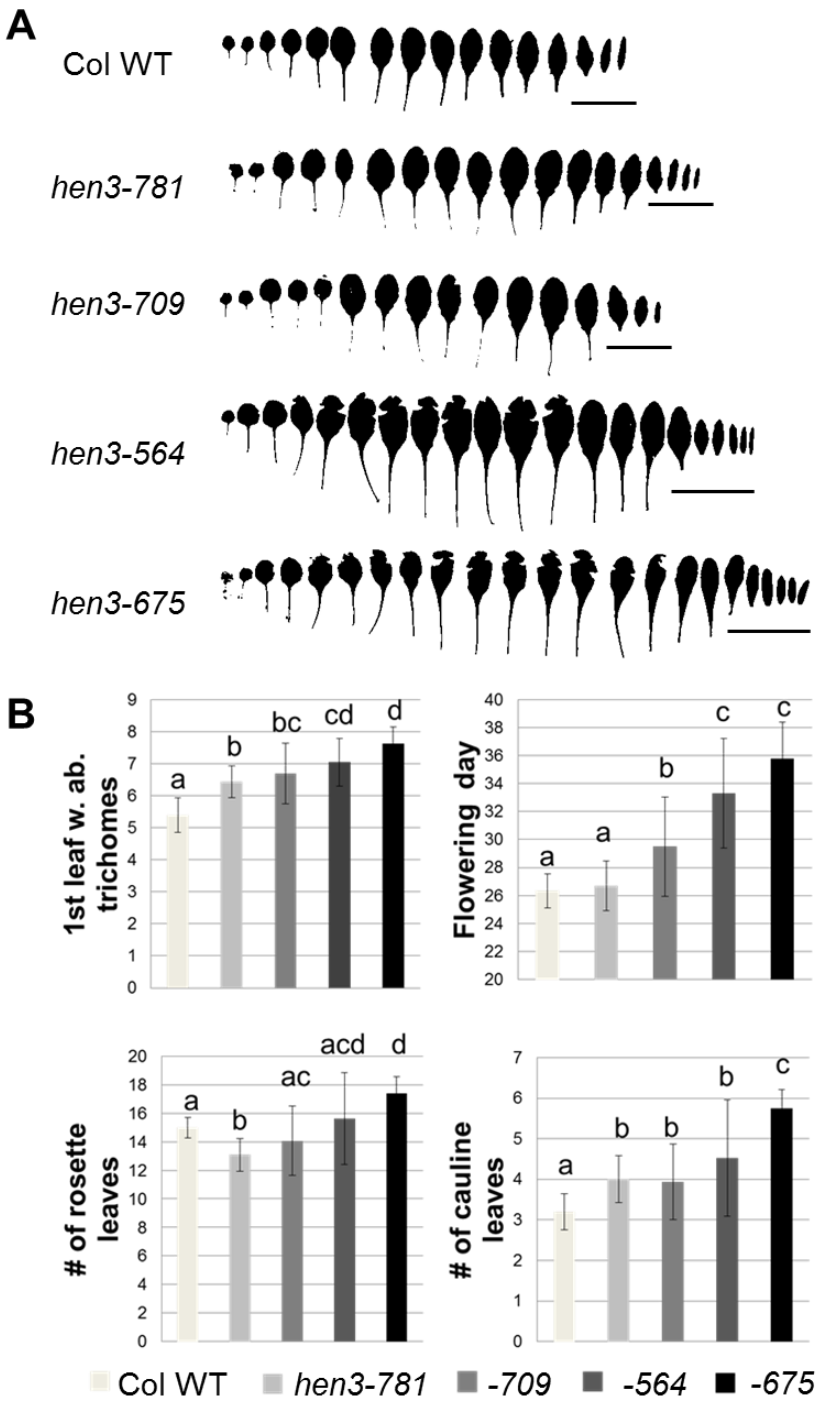

Figure 16. The mutant allele *hen3-675* shows the most severe phenotype. (A) The complete rosette of a representative plant from each Columbia wild type and the four mutant alleles *hen3-781*, *hen3-709*, *hen3-564* and *hen3-675*, dissected by consecutive leaves. Some leaves show incisions made to facilitate adhering to paper to scan them (due to their bent over nature); cauline leaves are underlined. (B) The number of the first leaf with abaxial trichomes, flowering day, the number of rosette leaves and the number of cauline leaves of Columbia wild type and the four *hen3* mutant alleles.

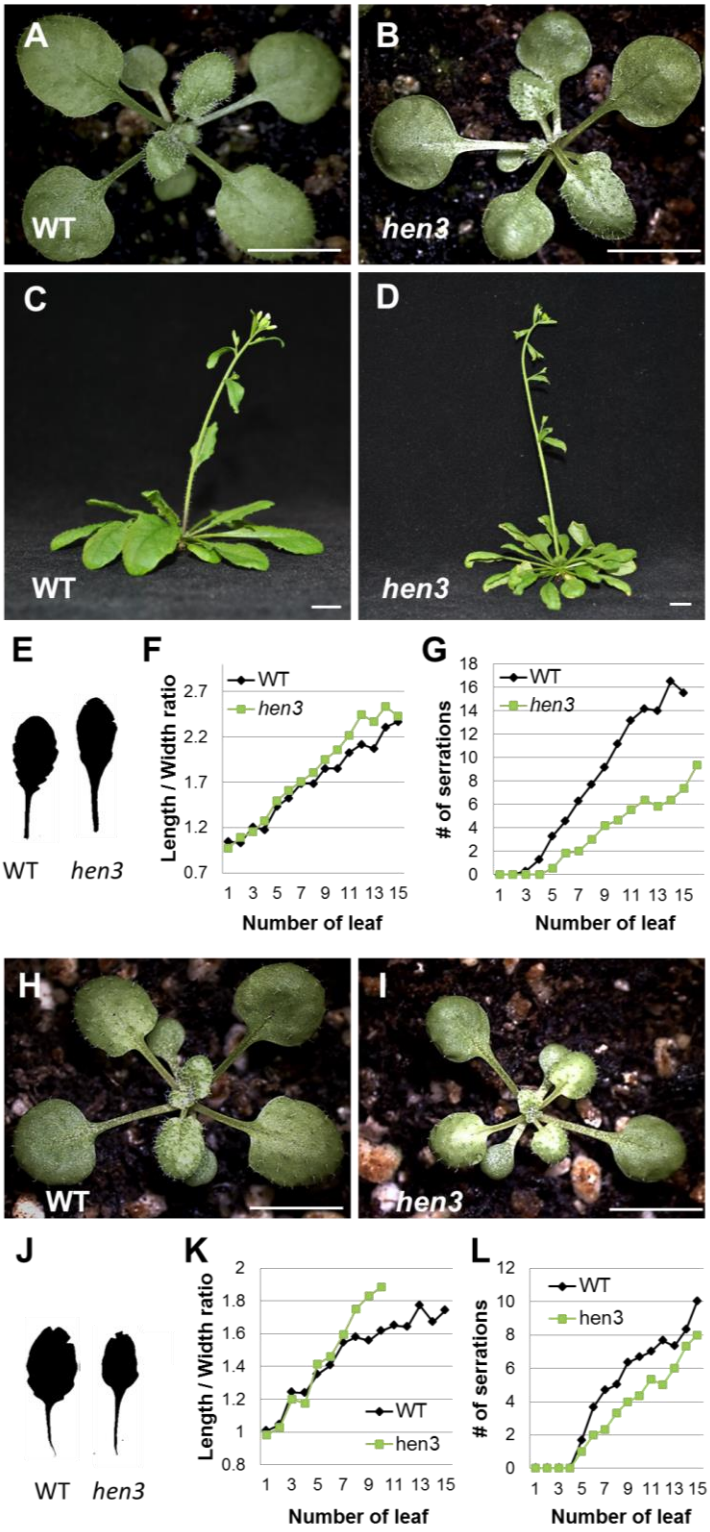

Figure 17. *hen3* mutants show extended juvenile traits in both LD (A-G) and SD (H-L) conditions. Pictures of WT and *hen3* plants at 18 d (A, B) and at flowering time (25 d (C) and 35 d (D)) under LD conditions, and at 20 d under SD (H, I). Silhouette of leaf #10 of representative WT and *hen3* plants (E, J). Length / width ratio (F, K) and number of serrations (G, L) on each of the first 15 leaves of WT and *hen3* plants.

Besides the delay in the acquisition of abaxial trichomes and flowering (Table 4, Figure 16 and Figure 17C&D), *hen3* mutants show other juvenile leaf traits. In LD conditions, *hen3* plants produce leaves with less serrations, since the 4th leaf, compared to wt plants (Figure 17 E&G), whereas the length/width ratio of the first leaves is similar between *hen3* and wt plants, but after the 10th leaf, *hen3* leaves are slightly more elongated than in wt (Figure 17 E&F). Similarly, in SD, the fewer serrations in *hen3* mutants are evident from the 6th leaf (Figure 17 J&L), whereas after the 8th leaf, *hen3* leaves are slightly more elongated than in wt (Figure 17 J&K) notwithstanding the production rate of leaves is lower in *hen3* under SD, having produced only 10 leaves while the wt bears at least 15 leaves. In addition, the leaves produced by *hen3* mutants clearly show a curled blade in both LD and SD conditions (Figure 17 B&I).

Table 4. Vegetative and flowering traits of CDK8 module mutants.

	1st leaf with abaxial trichomes	Flowering day	# of rosette leaves at flowering	# of cauline leaves at flowering	n
Long days (LD)					
wt Col	5.4 ± 0.5	26.3 ± 1.2	15.0 ± 0.7	3.2 ± 0.4	15
hen3	7.6 ± 0.5	35.8 ± 2.6	17.4 ± 1.2	5.8 ± 0.5	15
cct	12.2 ± 2.3	43.4 ± 0.7	30.4 ± 2.0	6.4 ± 0.9	11
hen3 cct	12.8 ± 1.9	53.0 ± 2.6	30.3 ± 3.0	6.5 ± 0.6	8
gct	13.3 ± 2.4	52.2 ± 3.7	35.6 ± 2.8	6.2 ± 0.8	12
Short days (SD)					
wt Col	12.3 ± 1.6	83.1 ± 6.6			
hen3	10.9 ± 1.1	76.7 ± 8.2			
gct	21.3 ± 2.9				

The juvenile phenotype of *hen3* plants is reminiscent of the phenotype of *cct* and *gct*, which are the corresponding mutants in *MED12* and *MED13*, the other CDK8 module components. However, the *hen3* phenotype is less severe than *cct* and *gct* (Figure 18 A-D). The acquisition of abaxial trichomes is delayed 7 and 8 leaves in *cct* and *gct* mutants respectively, whereas in *hen3* mutants it is only delayed by 2 leaves (Table 4). The same happens for flowering time and number of rosette leaves at flowering: *cct* and *gct* flower 17 and 26 days later than wt plants, whereas *hen3* mutants flower only 10 days later (Table 4, Figure 18E); *cct* and *gct* produce 15 and 20 more rosette leaves before flowering than wt plants, whereas *hen3* mutants produce only 2 more leaves (Table 4, Figure 18F). All *hen3*, *cct* and *gct* mutants produce approximately 6 cauline leaves before flowering, twice as

many cauline leaves produced by wt plants, which is a mark of delayed flowering (Table 4). Under SD conditions, *hen3* and *gct* mutants showed a drastic reduction in leaf growth rate and the rosette was consequently smaller than wt plants (Figure 18F), although later on they reach the same size (Figure 9C), suggesting that development in general is slower in the CDK8 module mutants. The abaxial trichomes on *gct* mutants under SD conditions appeared 9 leaves later than in wt plants (Table 4), which represents a delay of 75% compared to wt, this effect is smaller compared to the effect under LD conditions, where the delay was 145% compared to wt; on the other hand, *hen3* mutants under SD conditions acquired abaxial trichomes 1.4 leaves before than wt plants (Table 4), suggesting that the effects of mutations on the CDK8 module are partially counteracted in SD conditions.

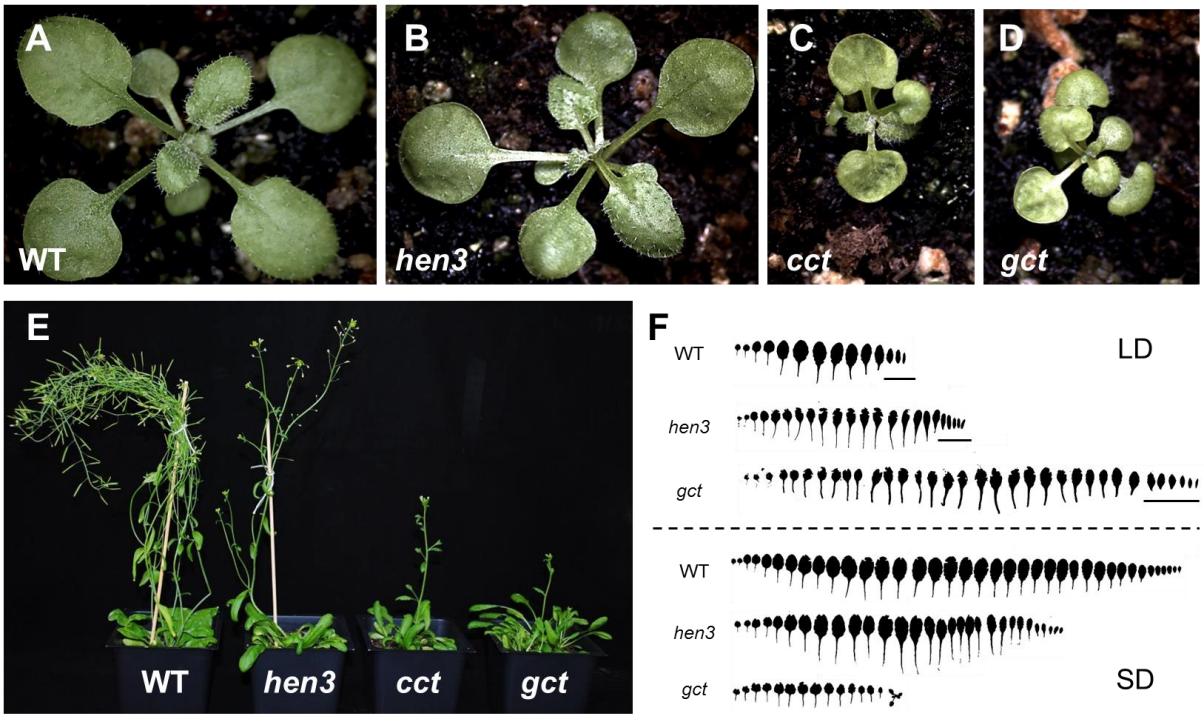

Figure 18. *hen3* mutants show a similar but less severe phenotype than *cct* and *gct* mutants. Pictures of wild type, *hen3*, *cct* and *gct* plants at 16 d (A-D) and 45 d (E). The complete rosette of a representative wild type, *hen3* and *gct* plants, grown under LD or SD conditions, dissected by consecutive leaves (F). Some leaves show incisions made to facilitate flattening them for scanning, because of their bent over nature; cauline leaves are underlined.

I generated *hen3 cct* double mutants by crossing *hen3*/+ to *cct*/+ and selecting double homozygous in the F3 generation (Figure 19). The *hen3 cct* double mutants show a similar phenotype to *cct* single mutants in abaxial trichomes, and number of rosette and cauline leaves at flowering, but the effect on flowering time seems to be additive, since *hen3* and *cct* single mutants are delayed 9.5 and 17 days respectively, whereas the double *hen3 cct* mutants are delayed 27 days compared to wt (Table 4). Similar to flowering, *hen3 cct* double mutants seems to produce leaves with much fewer serrations

compared to either *hen3* or *cct* single mutants (data not shown), suggesting that *HEN3* is particularly important for serrations and flowering.

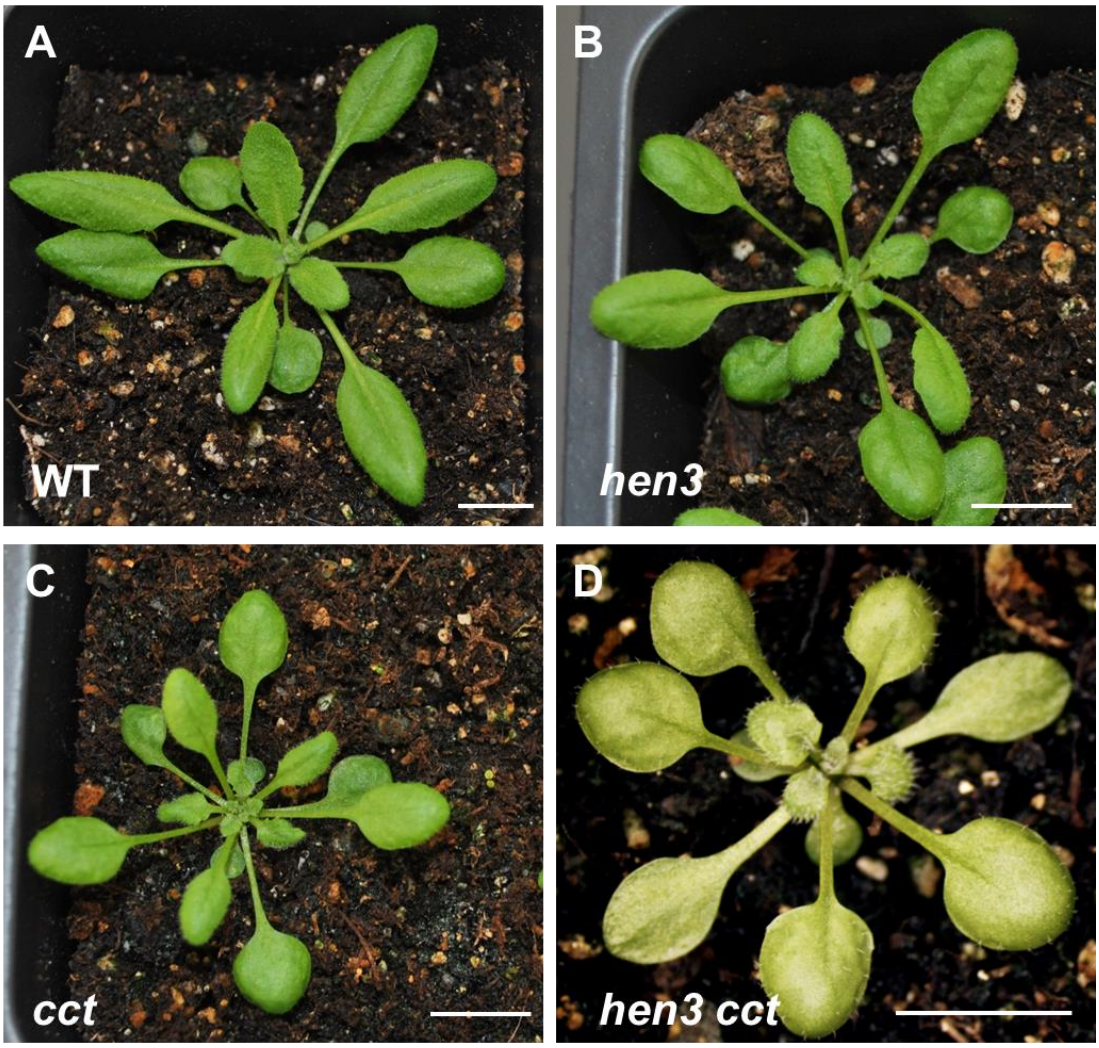

Figure 19. The vegetative phenotype of *hen3*, *cct* and *hen3 cct* mutants. Pictures of wild type (WT) plants (A), *hen3* (B), *cct* (C) and *hen3 cct* double mutants (D) at 20 days.

5.5.2. *HEN3* is dynamically expressed along vegetative development

For visualizing the spatio-temporal expression of *HEN3*, we generated a translational fusion of the *HEN3* gene, comprising the whole 5' region (starting at the end of the previous gene) and the coding region, to the coding region of *GUS* (see Methods), and transformed wild type plants with this construct (*gHEN3-GUS*). We obtained 17 different T2 basta-resistant lines, of which 5 lines showed exactly the same expression pattern, so we characterized the expression of those T2 lines. At early stages, *gHEN3* drives GUS expression at the shoot apical meristem and in the first leaf primordia, and faintly in the

vascular tissue of cotyledons (Figure 20A). As the first leaves elongate, *HEN3* is expressed higher at the leaf base and at low levels at the tips (Figure 20B). Later on, *HEN3* expression decreases in the first leaves and is higher in the shoot apical meristem and in the more recently-formed leaves (Figure 20C-E).

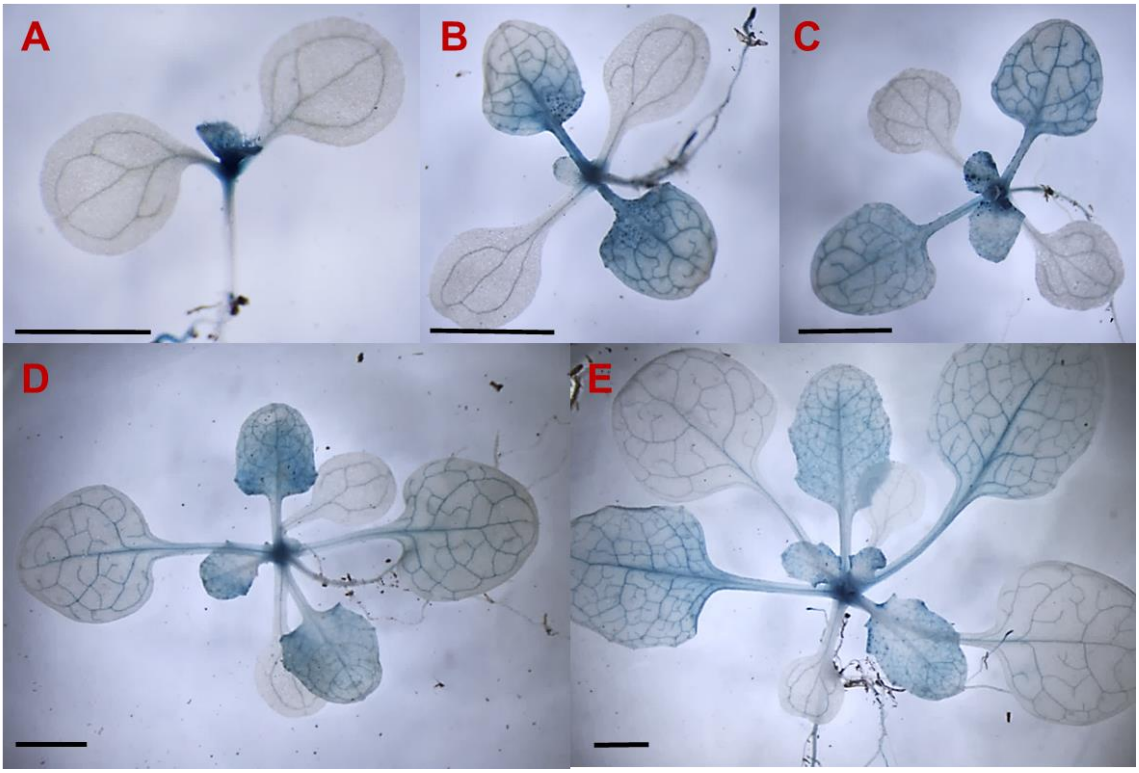

Figure 20. *HEN3* is dynamically expressed during vegetative development. The expression of gHEN3-GUS in 7 d (A), 10 d (B), 13 d (C), 16 d (D) and 20 d (E) plants. At each time point, one representative plant from four different T2 lines showing similar expression is shown.

5.5.3. The expression of specific *MIR156* and *SPL* genes is differentially affected in *hen3* mutants

The juvenile phenotype of *cct* and *gct* mutants is at least partially caused by an increase in the expression of miR156 and a decrease in the expression of *SPL3* and *SPL9* (Gillmor et al., 2014). Whether all the eight genes encoding miR156 (*MIR156A-H*) and all the miR156-targeted *SPL* genes (*SPL2, SPL3, SPL4, SPL5, SPL6, SPL9, SPL10, SPL11, SPL13* and *SPL15*) are regulated by the CDK8 module is currently unknown. The genes *MIR156A* and *MIR56C* are those that contribute the most to mature miR156, their expression is regulated by development and they are both the most important *MIR156* genes for the vegetative development (Yang et al., 2013; Yu et al., 2013). Among the *SPL* genes targeted by miR156, *SPL2*, *SPL9*, *SPL10*, *SPL11*, *SPL13* and *SPL15* contribute to both the juvenile-to-adult vegetative transition and the vegetative-to-reproductive transition, with *SPL9*,

SPL13 and *SPL15* the most important, whereas *SPL3*, *SPL4* and *SPL5* promote the floral meristem identity transition (Xu et al., 2016b).

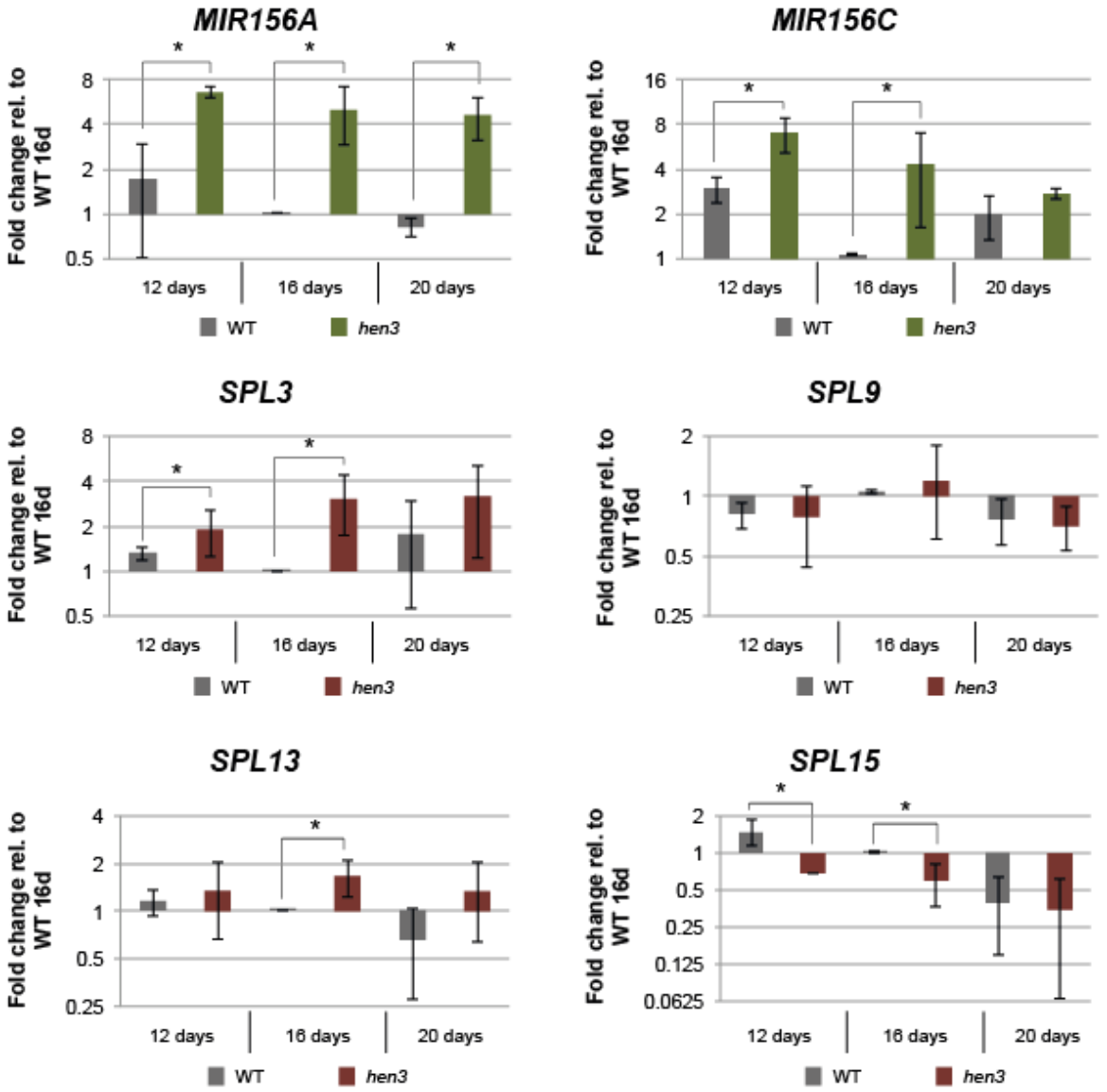

Figure 21. The expression of *MIR156A*, *MIR156C* and some *SPL* genes is affected in *hen3* mutants. Transcript levels of *pri-miR156A*, *pri-miR156C*, *SPL3*, *SPL9*, *SPL13* and *SPL15* in wt and *hen3* plants at 12, 16 and 20 long days. Fold change is shown relative to expression of wt at 16 days. Expression values were first normalized against *EIF4A* as a reference gene. Values shown are the mean of three technical replicates for three biological replicates. Standard deviation represented by bars. Asterisks indicate significant difference ($p<0.05$, Student's t test) between samples at the same time point.

In order to test if the juvenile phenotype of *hen3* mutants is due to changes in the expression of *MIR156* and/or *SPL* genes, we tested the expression of *MIR156A*, *MIR156C*, *SPL3*, *SPL9*, *SPL13* and *SPL15* in *hen3* mutants and wild type (wt) plants at

different time points (Figure 21). Both *MIR156A* and *MIR156C* showed higher transcript levels in *hen3* mutants from 12 to 20 days compared to wt plants (Figure 21), which is in accordance with the observed juvenile traits of *hen3* mutants, and is reminiscent of the regulation in *cct* and *gct* mutants. The expression of *SPL3* in wt plants show a slight increase at 20 days and is supposed to increase more at the flowering transition; whilst in *hen3* mutants, *SPL3* expression also increases along development but shows higher transcript levels than wt plants. The expression of both *SPL9* and *SPL13* did not show an evident increase or decrease from 12 to 20 days in both wt and *hen3* plants. The expression of *SPL15* was decreased in *hen3* mutants, particularly at 12 and 16 days; strikingly, *SPL15* expression showed a gradual decrease along development in both wt and *hen3* plants.

The fact that expression of most of the *SPL* genes tested did not decrease in *hen3* mutants could be the reason why the vegetative phenotype of *hen3* is not as severe as the phenotype of *cct* and *gct* mutants. In addition, the more elongated shape of *hen3* leaves compared to wt, which is a characteristic adult leaf trait, could be the result of up-regulating specific *SPL* genes, as is the case of *SPL3* in these samples. In agreement with these qPCR data, RNA-Seq data, obtained from 18 day-old seedlings in our lab (unpublished data from Abreu & Gillmor labs), shown that at least *SPL9*, *SPL11* and *SPL15* are significantly downregulated in both *cct* and *gct* mutants, but none of miR156-targeted *SPL* genes were significantly regulated in *hen3* mutants. However, these data were obtained using whole seedlings, so specific spatial increases or decreases in expression could be masked by the nature of the samples. Thus, for overcoming this sampling problem we took advantage of GUS-marker lines (described in Methods) expressing translational fusions of some *MIR156* and *SPL* genes. These marker lines also allow us to detect changes in the expression of *SPL* genes in the case that they were regulated at the translational level by miR156.

In wild type plants, the expression of both *MIR156A-GUS* and *MIR156C-GUS* is high in juvenile tissues like cotyledons and the first leaves, and then expression decreases in adult tissues, while still high in the meristem and in the petioles of new leaves (Figure 22 and 23). The expression of both *MIR156A-GUS* and *MIR156C-GUS* in the *hen3* mutant background increased in 10 d seedlings compared to their expression in wild type plants (Figure 22). Furthermore, the spatial expression pattern of both genes was extended in *hen3* mutants: at early stages (7 d to 13 d), *MIR156A* and *MIR156C* are highly expressed only in the cotyledons and mildly expressed in the tips of the first two leaves of wild type plants, whilst in *hen3* mutants, the expression of *MIR156A* and *MIR156C* is extended not only to the cotyledons and the two first leaf tips but to the whole first two leaves and the tips of the subsequent leaves. Although the development of *hen3* mutants was slower than wild type plants, it is evident at 16 d and 20 d that the expression of both *MIR156A* and *MIR156C* in the *hen3* background was high in more leaves than in the wt background. In addition, both genes are clearly expressed in the vascular tissue of both cotyledons and leaves in *hen3* mutants, tissues where they are never expressed in wt background. Similarly, the expression of both *MIR156A-GUS* and *MIR156C-GUS* is extended to more

than the first two leaves and both genes are clearly expressed in vascular tissue of cotyledons and leaves (Figure 23).

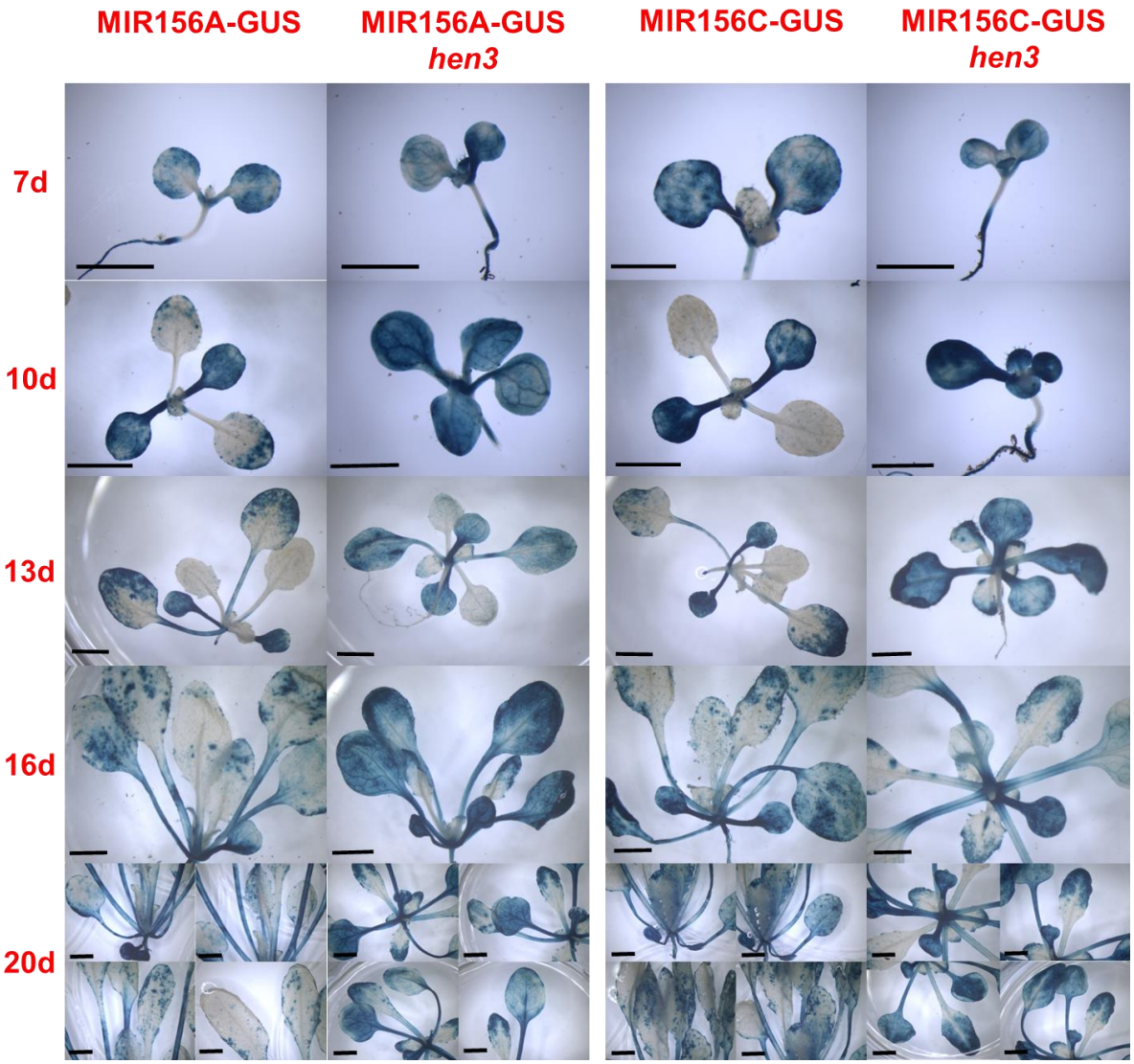

Figure 22. *MIR156* has an extended and increased expression pattern in *hen3* mutants. The expression of *MIR156A-GUS* and *MIR156C-GUS* in wild type and *hen3* backgrounds at 7, 10, 13, 16 and 20 days.

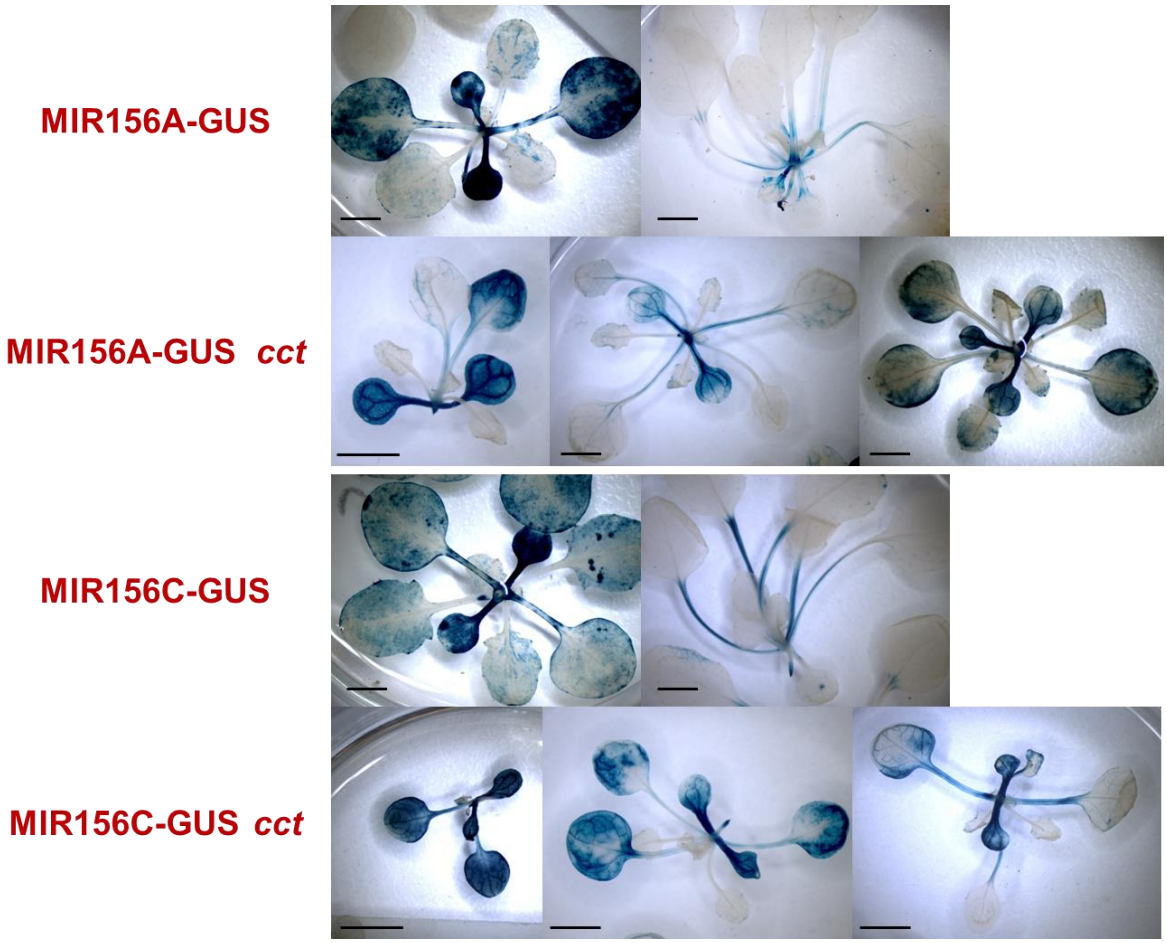

Figure 23. *MIR156* has an extended and increased expression pattern in *cct* mutants. The expression of *MIR156A-GUS* and *MIR156C-GUS* in wild type and *cct* backgrounds in plants at different developmental stages.

Since the expression pattern of *MIR156A* and *MIR156C* was increased and extended in the mutants of the CDK8 module, it is reasonable to think that the expression of the *SPL* genes targeted by miR156 would be decreased and restricted during development. However, in qPCR experiments using whole seedlings, the expression of several *SPL* genes did not decrease in *hen3* mutants (Figure 21). Besides regulation at the transcript level by miR156, translation regulation of SPL-GUS reporters is also possible. In addition, the expression of *SPL* genes could be transcriptionally regulated directly by the CDK8 module, as well as by other pathways.

We took advantage of GUS-marker lines, developed and provided by Scott Poethig's lab, that report the expression of *SPL3* and *SPL9* genes, in versions that are sensitive and resistant (r) to miR156 (see Methods). The resistant versions of *SPL* genes allow distinguishing whether the expression of such genes is regulated independently of miR156.

The expression of *SPL3* in wt plants at early stages is high at cotyledons and in first leaves, and then decreases, while maintaining high expression in the meristem and at the petioles and leaf margins (Figure 24). The expression of the resistant version, *rSPL3-*

GUS, is very similar to the sensitive version, suggesting that *SPL3* expression is not largely regulated by miR156. In *hen3* mutants, both *SPL3-GUS* and *rSPL3-GUS* show a slight increase in expression, most evident at adult stages (16 and 20 d) when *SPL3-GUS* and *rSPL3-GUS* are highly expressed in more leaves than in wild type plants (Figure 24).

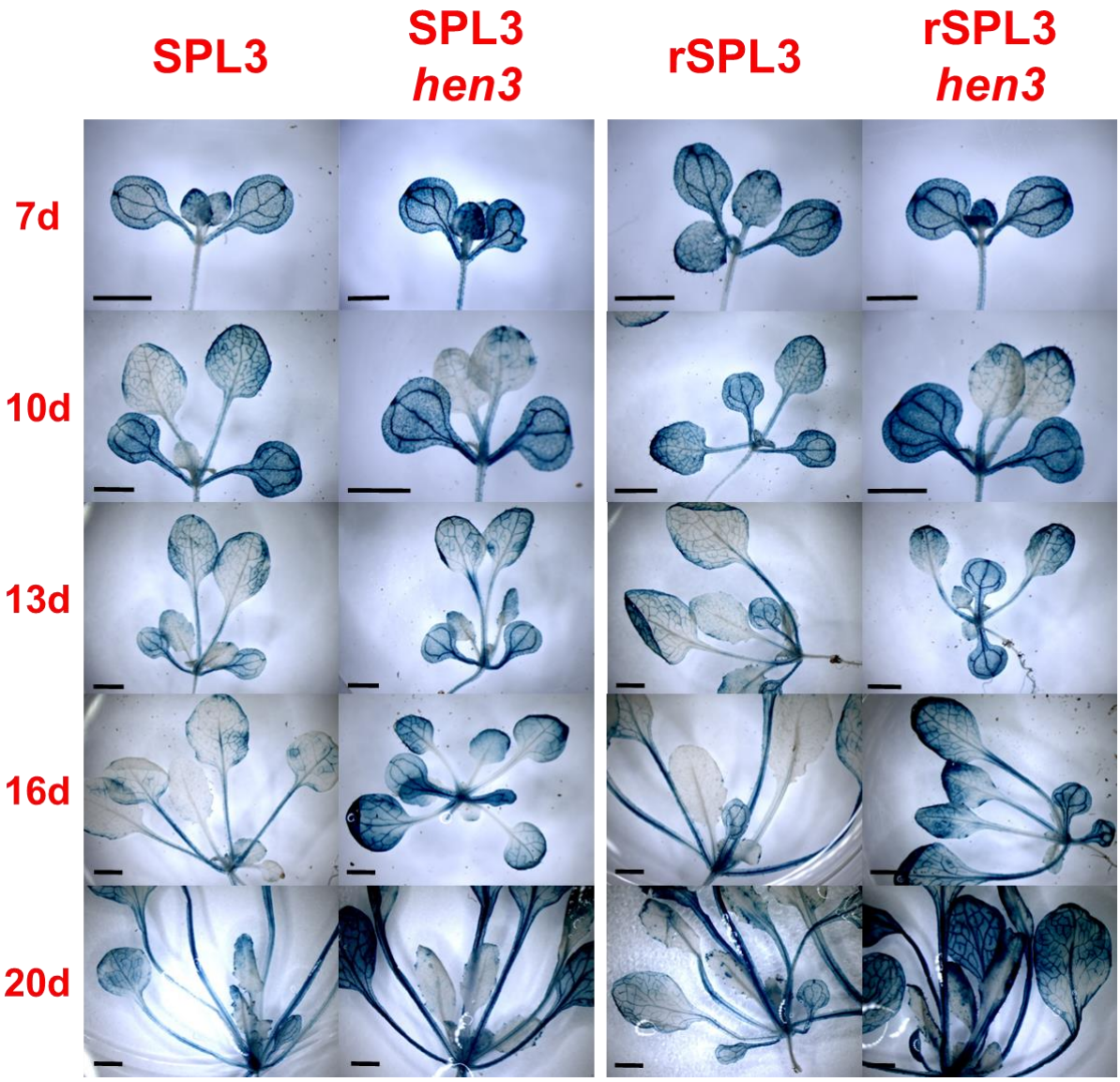

Figure 24. The expression pattern of *SPL3* and *rSPL3* is slightly extended and/or increased at specific time points in *hen3* mutants. The expression of *SPL3-GUS* and *rSPL3-GUS* in wild type and *hen3* backgrounds at 7, 10, 13, 16 and 20 days.

At juvenile stages, the expression of *SPL9* in wt plants is very low, and is restricted to only the shoot apical meristem, the vascular tissue of cotyledons and the leaf hydathodes; later on, its expression increases, being high at the shoot meristem, the petioles and in the leaf vascular tissue (Figure 25). The spatial expression pattern of *rSPL9-GUS* is similar to the sensitive version, but at much higher levels from early stages, indicating that *SPL9* is mainly regulated by miR156, especially early on development when

miR156 expression is high. In *hen3* mutants, the expression of both sensitive and resistant *SPL-GUS* versions is very low along vegetative development compared to wt plants, being barely detectable for the sensitive version at early stages, and being restricted mostly to the shoot meristem and principal vascular tissue for both sensitive and resistant versions (Figure 25). This drastic decrease in the expression of both *SPL9* and *rSPL9* in *hen3* mutants suggests that *HEN3* transcriptionally regulates the expression of *SPL9*, independent of miR156.

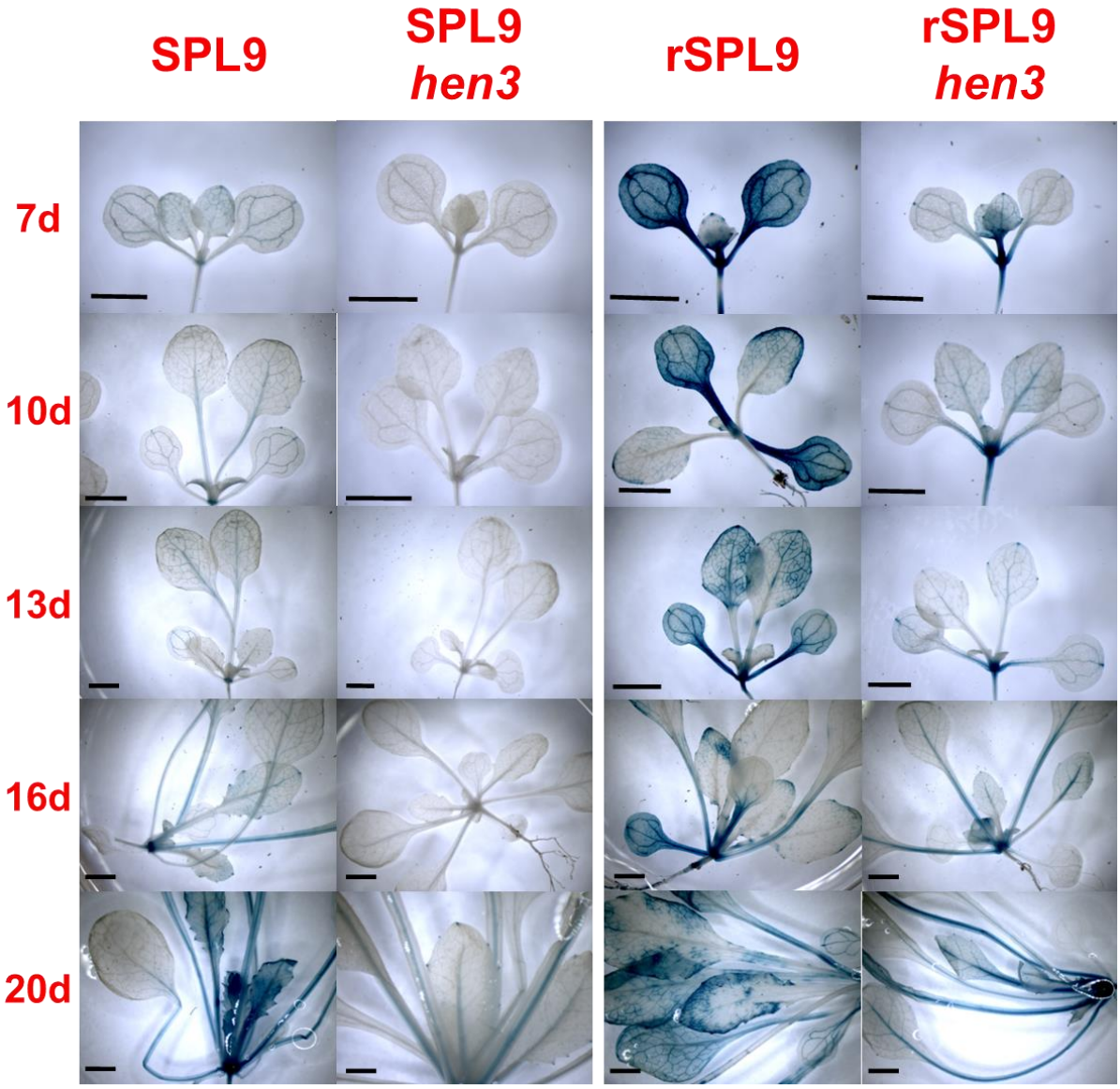

Figure 25. The expression pattern of *SPL9* and *rSPL9* is drastically decreased in *hen3* mutants. The expression of *SPL9-GUS* and *rSPL9-GUS* in wild type and *hen3* backgrounds at 7, 10, 13, 16 and 20 days.

5.5.4. Higher expression of miR156 is responsible for the juvenile phenotype of *hen3* mutants

The extended juvenile phenotype of *cct* and *gct* mutants was caused by higher expression of miR156, as demonstrated by *cct* MIM156 and *gct* MIM156 plants (Gillmor et al., 2014). MIM156 plants reduce miR156 function by overexpressing a transgene with an imperfect miR156 target site, sequestering this microRNA in inactive RISC complexes (Franco-Zorrilla et al., 2007). Such reduced miR156 function was completely epistatic to the *cct* and *gct* mutant phenotypes for abaxial trichomes, and partially epistatic for serrations and leaf shape (Gillmor et al., 2014).

To determine whether the higher expression of miR156 is responsible for the juvenile phenotype of *hen3* mutants, we crossed *hen3* plants to double *mir156a mir156c* and *mir157a mir157c* mutants. These double mutants of *MIR156* and *MIR157* genes have a specific reduction in the expression of the most important genes for the vegetative transition, and show a more subtle phenotype compared to MIM156 plants, allowing detecting phenotypic changes in *hen3* mutants caused by a more subtle reduction in *MIR156* / *MIR157* expression.

The *mir156a mir156c* plants show a shortened juvenile phase, acquiring adult leaf traits before than wild type plants; particularly, they produce abaxial trichomes starting with the 2nd leaf, while wt plants do not produce abaxial trichomes until the 6th leaf (Figure 26C). All the rosette leaves of *mir156a mir156c* plants are also evidently more elongated and serrated compared to those of wt plants (Figure 26A,G&H). They produce fewer rosette leaves (Figure 26A&E) and flower slightly before than wt plants (Figure 26B&D), whilst the number of cauline leaves is similar to wt plants (Figure 26F). On the other side, *hen3* mutants present an extended juvenile phenotype described earlier in this chapter, producing more leaves without abaxial trichomes and less serrated compared to wt plants (Figures 17 & 26). Triple *hen3 mir156a mir156c* mutants show an overall intermediate phenotype between *hen3* and *mir156a mir156c* mutants: the *mir156a mir156c* phenotype was epistatic to *hen3* for the leaf shape (Figure 26G) and partially epistatic for abaxial trichomes and number of rosette leaves (Figure 26C&E), whereas *hen3* was partially epistatic for flowering time and number of cauline leaves (Figure 26D&F), and the serrations phenotype was almost completely rescued to wt levels (Figure 26H).

The *mir157a mir157c* plants also have an early adult phenotype, but not as strong as the double *mir156a mir156c* mutants. They produce 1.5 less leaves without abaxial trichomes (Figure 26C), their leaves are more serrated (Figure 26H), produce fewer rosette leaves (Figure 26E) and flower slightly before than wt plants (Figure 26D), whilst the number of cauline leaves and the length/width ratio of their leaves are similar to wt plants (Figure 26F&G). Again, the triple *hen3 mir157a mir157c* mutants show an overall intermediate phenotype between *hen3* and *mir157a mir157c* mutants: the acquisition of abaxial trichomes and flowering time of triple mutants show intermediate numbers between those of *hen3* and *mir157a mir157c* mutants (Figure 26C&D), resulting in an abaxial trichomes phenotype similar to wt plants; the *hen3* phenotype seems to be largely

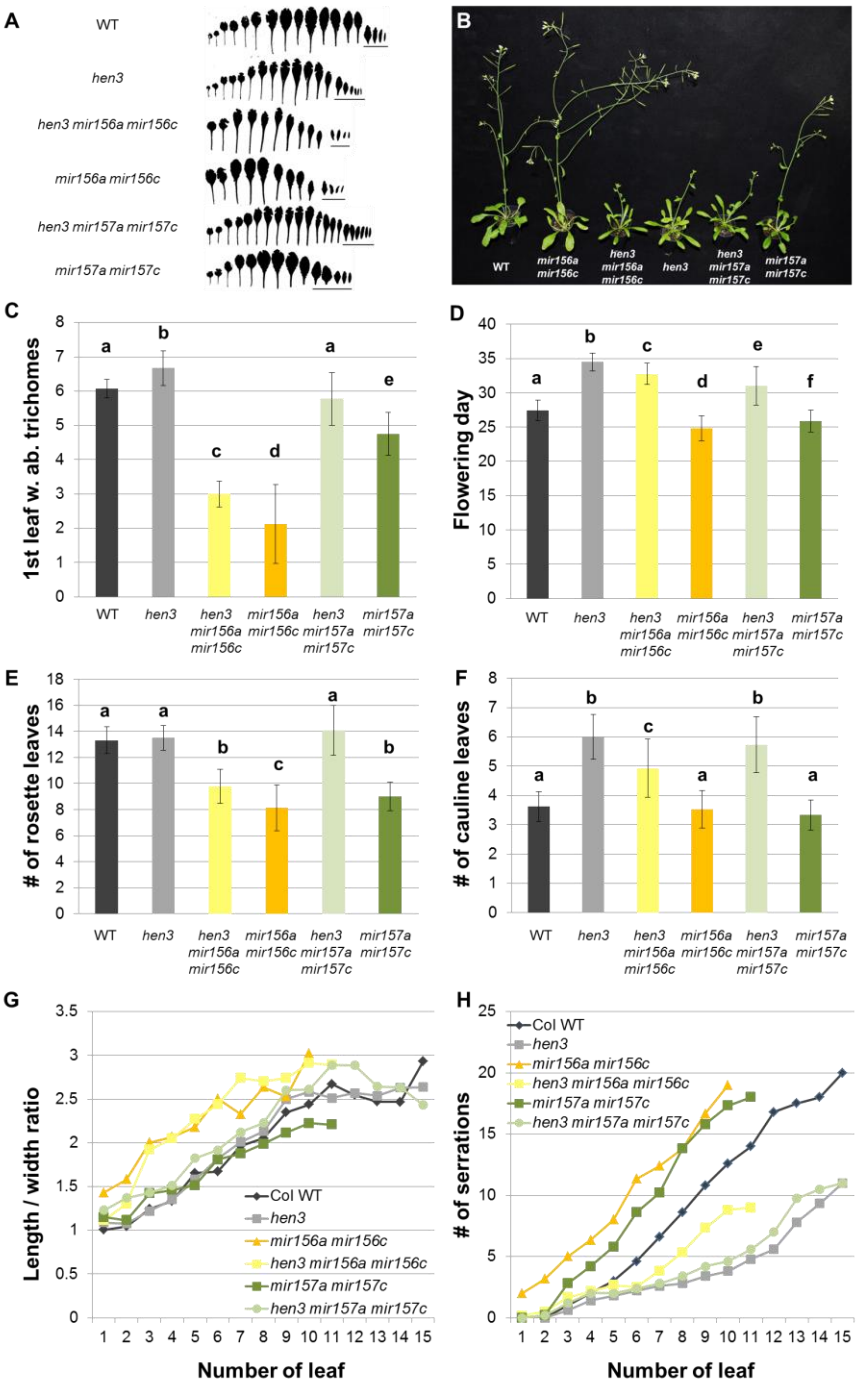

Figure 26. The *hen3* phenotype is partially due to overexpression of miR156. (A) The complete rosette of a representative plant from each wild type (WT), *hen3*, *mir156a mir156c*, *mir157a mir157c*, *hen3 mir156a mir156c* and *hen3 mir157a mir157c*, dissected by consecutive leaves. Some leaves show incisions made for facilitate to extend them because their bent over nature; cauline leaves are underlined. (B) Picture of 35 d of a representative plant from each genotype. (C-H) The number of the first leaf with abaxial trichomes (C), flowering day (D), the number of rosette leaves (E) and cauline leaves (F) at flowering time, and the length/width ratio (G) and number of serrations (H) of the first 15 leaves of each genotype shown in A and B. Genotypes that are not significantly different ($p>0.05$, Student's t test) for a particular trait share the same letter.

epistatic to *mir157a mir157c* for number of rosette and cauline leaves, and serrations (Figure 26E,F&H). Whether the expression of *MIR157* genes is increased in *hen3* mutants remains to be studied, though that is suggested by the intermediate phenotype of triple *hen3 mir157a mir157c* mutants.

The above results suggest that increased levels of miR156 are partially, but not totally responsible for the increased length of the juvenile phase in *hen3* mutants. However, my results also show that there is significant transcriptional regulation of *SPL3* and *SPL9* by *HEN3*, since the expression of rSPL3-GUS, and especially rSPL9-GUS, is greatly affected in *hen3* mutants. Thus, *HEN3* regulates vegetative development by both repressing miR156 levels, and by promoting transcription of certain *SPL* genes.

6. CONCLUSION

In this work I have expanded the knowledge of how the CDK8 module of Mediator controls the juvenile-to-adult transition in plants. Through molecular and genetic approaches, I have demonstrated that sugar and the CDK8 module regulate the vegetative transition in a convergent manner, as the photosynthetic gene *CH1*, and *CCT/MED12* and *HEN3/CDK8*, show additive effects on heteroblasty traits, and *MIR156* expression is synergistically increased in *ch1 cct* double mutants. The downregulation of *MIR156* expression by sugar in *cct* and *gct* mutants also supports the conclusion that sugar regulates miR156 separately from the CDK8 module. The inter-regulation between sugar signaling and the CDK8 module along vegetative development is not completely understood yet.

I have also shown that *HEN3/CDK8* has a similar function to *CCT/MED12* and *GCT/MED13* in promoting the vegetative and reproductive transitions, although the role of *HEN3/CDK8* seems to be more specific for certain heteroblasty traits (like serrations), and for flowering. Whether HEN3/CDK8 always works in complex with CCT/MED12 and GCT/MED13, or they could have independent functions, is currently unknown. The increased and/or extended expression pattern of *MIR156* genes in CDK8 module mutants is consistent with the extended juvenile phenotypes of such mutants, and with the consequent decreased and/or restricted expression pattern of some *SPL* genes. Interestingly, the expression of *SPL9* and most likely *SPL3* can be controlled by *HEN3/CDK8* independently of miR156, which implicates a transcriptional regulation of *SPL* genes by the CDK8 module. Furthermore, I demonstrated that miR156 upregulation was partly responsible for the juvenile phenotype of *hen3* mutants, since the vegetative phenotype of *hen3* plants was partially rescued by mutations in *MIR156/MIR157* genes, pointing out the functional importance of CDK8 module in the regulation of vegetative development.

7. FUTURE GOALS

This work has revealed some functions of the CDK8 module subunits in the regulation of vegetative development, but it also has raised some important questions, and the mechanism by which the CDK8 module plays such specific functions is far from being completely understood. For that reason, I generated transgenic plants expressing a version of *HEN3* tagged with GFP which will be used in ChIP experiments to determine the direct transcriptional targets of *HEN3*. The gHEN3-GFP construct was able to rescue the wild type phenotype in *hen3* mutants, demonstrating it to be functional (Figure 27). It is possible that *MIR156* and/or *SPL* gene expression could be directly regulated by the CDK8 module; alternatively, *HEN3* could regulate the epigenetic marks on *MIR156* genes settled by BRM and SWN. ChIp-Seq results will also reveal other gene pathways regulated by the CDK8 module.

Figure 27. The gHEN3-GFP construct complements *hen3* mutants. Pictures of 25 day-old wild type plants (A), *hen3* mutants (B) and T2 plants of *hen3* mutants transformed with the gHEN3-GFP construct (C).

Besides determining direct transcriptional targets of HEN3, it will be important to address the following questions:

To elucidate how the CDK8 module could promote miR156 downregulation by sugar. Photosynthesis, sugar metabolism, sugar sensing and/or sugar signaling could be regulated by the CDK8 module.

In order to know how *CCT* and *GCT* are regulated by sugar, the functionality of sugar response elements in the promoter regions of *CCT* and *GCT* genes needs to be evaluated.

To characterize in detail the phenotype of *hen3 cct* double mutants, and the expression of *MIR156* and *SPL* genes in such plants.

To test the expression of the other *MIR156/MIR157* and *SPL* genes in *hen3*, *cct* and *hen3 cct* mutants in order to know how specific is the regulation.

To evaluate the gene expression of other regulators of *SPL* genes, like DELLA proteins, in CDK8 module mutants, which could explain the miR156-independent regulation of *SPL* genes in *hen3* plants.

To evaluate the gene expression of auxin- and polarity-related genes in leaves of *hen3*, *cct* and *gct* mutants, which could explain their bent over shape.

To generate plants with HEN3 and CCT tagged with different fluorophores to determine if they are always expressed together, which would suggest they only act in complex. A similar strategy could be applied with plants expressing a *MIR156* reporter, to test if the CDK8 module and *MIR156* have complete or partially mutually excluding expression patterns, which provide evidence for strong spatial/temporal regulation.

To characterize the phenotype of *hen3* mutants only affected in their catalytic activity in order to determine if the role of HEN3 in vegetative development can be through phosphorylating a set of proteins, or just merely by forming the right structure of the CDK8 module.

To evaluate whether plants with a T-DNA insertion in between both Cyclin C genes (with reduced expression of both genes) show a vegetative phenotype and *MIR156* expression similar to *hen3* mutants.

Finally, given that Mediator, including its CDK8 module, is present in all eukaryotes, and that miR156-SPL pathway controls vegetative development in all plants, the understanding of miR156 regulation by the CDK8 module in other plants would be important to better know how to modulate the timing of the juvenile to adult transition for enhancing beneficial agricultural/forestal traits.

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9. APPENDICES

9.1. Research article #1 (Co-author)

Gillmor, C.S., Silva-Ortega, C.O., Willmann, M.R., **Buendía-Monreal, M.,** Poethig, R.S., 2014. The Arabidopsis Mediator CDK8 module genes CCT (MED12) and GCT (MED13) are global regulators of developmental phase transitions. Development 141, 4580–9. doi:10.1242/dev.111229

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RESEARCH ARTICLE

The *Arabidopsis* Mediator CDK8 module genes *CCT* (*MED12*) and *GCT* (*MED13*) are global regulators of developmental phase transitions

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ABSTRACT

Temporal coordination of developmental programs is necessary for normal ontogeny, but the mechanism by which this is accomplished is still poorly understood. We have previously shown that two components of the Mediator CDK8 module encoded by *CENTER CITY* (*CCT*; *Arabidopsis MED12*) and *GRAND CENTRAL* (*GCT*; *Arabidopsis MED13*) are required for timing of pattern formation during embryogenesis. A morphological, molecular and genomic analysis of the post-embryonic phenotype of *gct* and *cct* mutants demonstrated that these genes also promote at least three subsequent developmental transitions: germination, vegetative phase change, and flowering. Genetic and molecular analyses indicate that *GCT* and *CCT* operate in parallel to gibberellic acid, a phytohormone known to regulate these same three transitions. We demonstrate that the delay in vegetative phase change in *gct* and *cct* is largely due to overexpression of miR156, and that the delay in flowering is due in part to upregulation of *FLC*. Thus, *GCT* and *CCT* coordinate vegetative and floral transitions by repressing the repressors miR156 and *FLC*. Our results suggest that MED12 and MED13 act as global regulators of developmental timing by fine-tuning the expression of temporal regulatory genes.

KEY WORDS: miR156, *FLC*, MED12, MED13, Mediator, *Arabidopsis thaliana*

INTRODUCTION

Temporal coordination of developmental programs underlies many processes in plants and animals and plays a major role in evolution. For example, it is hypothesized that an extended phase of rapid brain growth (a characteristic of juvenile development) played a key role in the evolution of humans from their primate ancestors (Gould, 1977). In flowering plants, changes in the relative timing of juvenile and adult development can have major effects on traits such as leaf morphology and the onset of flowering (reviewed by Huijser and Schmid, 2011; Geuten and Coenen, 2013; Poethig, 2013). Resistance to herbivory, as well as changes in cell wall composition, also have an important temporal component (Abedon et al., 2006; reviewed by Boege and Marquis, 2005 and Chandler et al., 2011). Thus, the study of developmental timing is crucial to our understanding of plant and animal ontogeny, physiology and evolution.

Post-embryonic phases of plant development include germination, seedling growth, the juvenile and adult vegetative phases, and flowering. Transitions between some of these programs are abrupt, whereas other transitions are more gradual. For example, germination entails a drastic switch from dormancy and growth repression (seed) to active growth and metabolism (seedling) (Bassel et al., 2011). By contrast, gradual transitions occur during the transition from the juvenile to the adult vegetative phase: aspects such as shape, serrations and epidermal hairs change incrementally between successive leaves. Depending on the species, leaf morphological traits may become either more or less complex from the juvenile to adult vegetative phase, when plants become reproductively competent (Poethig, 2013). The reproductive transition is relatively rapid, as the shoot apical meristem changes from leaf initiation to producing branches and flowers (Amasino, 2010).

Studies of the model plants *Arabidopsis* and maize have been particularly important in elucidating some of the molecular mechanisms that regulate phase-specific developmental programs, and have implicated factors such as the phytohormone gibberellic acid (GA), microRNAs (miRNAs) and epigenetic marks in the regulation of genes involved in these processes. The role of GA in promoting developmental transitions in plants is well established. GA activates seed germination, when rapid cell elongation is required for the embryo to rupture the seed coat, and simultaneously represses the expression of seed-specific genes (Koornneef and van der Veen, 1980; Ogas et al., 1997). GA also promotes leaf expansion during vegetative growth, as well as the onset of adult epidermal characteristics such as trichomes, which are canonical markers of vegetative phase change in *Arabidopsis* and maize (Evans and Poethig, 1995; Telfer et al., 1997). In addition, GA is required for the transition from vegetative growth to flowering (Wilson et al., 1992; Yu et al., 2012). Similarly, epigenetic regulation of gene expression, in particular through Histone 3 lysine 27 (H3K27) methylation, is important for both the seed-to-seedling and vegetative-to-reproductive transitions in plants (reviewed by Crevillén and Dean, 2011). Loss of H3K27 methylation affects the embryo-to-seedling transition by increasing dormancy and causing expression of seed-specific genes in seedlings (Bouyer et al., 2011; Zhang et al., 2008), while decreased H3K27 methylation at the floral repressor *FLOWERING LOCUS C* (*FLC*) delays flowering (Bastow et al., 2004).

Another important temporal regulator in plants is miR156 (Poethig, 2013), which regulates leaf traits and flowering by repressing members of the SPL family of transcription factors. SPL proteins positively regulate the expression of miR172, which in turn represses AP2-like transcription factors that inhibit flowering

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(Aukerman and Sakai, 2003; Chuck et al., 2007; Wu and Poethig, 2006; Wu et al., 2009; Wang et al., 2009). It has recently been shown that miR156 exerts its effects on flowering in parallel with GA because GA induces degradation of DELLA repressor proteins that are bound to SPL transcription factors (Yu et al., 2012). miR156 is abundant in seedlings, and decreases during vegetative development, partly due to transcriptional repression by leaf-derived sugars (Wu and Poethig, 2006; Yang et al., 2011, 2013; Yu et al., 2013). However, miR156 levels decrease during vegetative development even in the absence of sugar signaling, indicating that there are additional, as yet unknown, factors that regulate miR156 abundance during vegetative development (Yang et al., 2013).

The transcriptional co-activator Mediator has recently emerged as a key integrator of signaling pathways during development in both animals and plants, capable of influencing RNA polymerase II transcription of mRNAs and miRNAs at all stages of the transcription process, including through epigenetic regulation (Kim et al., 2011; reviewed by Yin and Wang, 2014). Mediator is a large protein complex that is conserved in all eukaryotes and has two major components: Core Mediator, which acts as a transcriptional co-activator, and the Cyclin Dependent Kinase 8 (CDK8) module, which represses transcription by modulating the activity of Core Mediator (Bourbon, 2008; reviewed by Carlsten et al., 2013). The CDK8 module is usually thought of as a transcriptional repressor, since the active form of Core Mediator is never purified together with the CDK8 module (Carlsten et al., 2013); however, in some contexts, the CDK8 module can also act to stimulate transcription, usually through the kinase activity of the CDK8 protein on the C-terminal domain of RNA polymerase II (reviewed by Nemet et al., 2013).

Mediator is capable of integrating diverse signaling inputs to influence transcription of multiple genes. For example, the MED18 subunit of *Arabidopsis* Core Mediator regulates fungal resistance, responses to the phytohormone abscisic acid, and flowering time, by influencing transcription initiation, elongation and termination, as well as RNA polymerase II occupancy and deposition of Histone 3 lysine 36 (H3K36) methylation (Lai et al., 2014). Likewise, studies in yeast and humans have shown that the CDK8 module can inhibit transcription through different mechanisms, including preventing physical association of Core Mediator with RNA polymerase II, and recruiting Histone 3 lysine 9 (H3K9) and H3K27 methylation at target genes (Tsai et al., 2013; Ding et al., 2008; Chaturvedi et al., 2012). The CDK8 module is composed of CDK8, Cyclin C, MED12 and MED13 (Carlsten et al., 2013). In *Arabidopsis*, MED12 is encoded by *CENTER CITY* [*CCT*; also known as *CRYPTIC PRECOCIOUS* (*CRP*)], MED13 is encoded by *GRAND CENTRAL* [*GCT*; also known as *MACCHI-BOU 2* (*MAB2*)] and CDK8 is encoded by *HUA ENHANCER 3* (*HEN3*) (Gillmor et al., 2010; Imura et al., 2012; Ito et al., 2011; Wang and Chen, 2004). *CCT* and *GCT* have previously been implicated in temporal regulation of development, as *gct* and *cct* mutants affect the timing of radial pattern formation during early embryogenesis (Gillmor et al., 2010).

Here, we demonstrate that *GCT* and *CCT* are global regulators of developmental transitions, promoting the embryo-to-seedling, juvenile-to-adult and vegetative-to-reproductive transitions. We show that one of the most important post-embryonic roles of *GCT* and *CCT* is to repress seed-specific transcripts in seedlings, and to promote growth after germination. In addition, we demonstrate that *GCT* and *CCT* regulate the abundance of miR156 during vegetative development, and that the upregulation of miR156 in *gct* and *cct* plants plays a major role in extending the vegetative phase in these mutants. *GCT* and *CCT* also regulate levels of *FLC*, and overexpression of *FLC* is responsible for much of the delayed flowering phenotype in *gct* and *cct* mutants. Thus, *CCT* and *GCT* act as master regulators of developmental transitions during the plant life cycle by repressing the expression of key temporal control genes.

RESULTS

GCT and *CCT* are required for post-embryonic development

gct and *cct* mutants germinate well, but develop abnormally following germination (99.5% germination in progeny of *gct*/+, *n*=428; 99.1% germination in progeny of *cct*/+, *n*=441). Although loss-of-function mutations of both genes are fully penetrant, they display variable expressivity: some mutant seedlings have small leaves, short petioles and malformed cotyledons but continue to grow and develop (we refer to this as the 'grow' phenotype), whereas others germinate and produce a root, but do not form leaf primordia and arrest at this stage (we refer to this as the 'arrest' phenotype) (Fig. 1B,C). 54% of *gct* seedlings and 24% of *cct* seedlings display the arrest phenotype. This variable expressivity is not attributable to partial activity of the mutant gene products: all five alleles of *gct* and all three alleles of *cct* described by Gillmor et al. (2010) generate both grow and arrest phenotypes (data not shown). These *gct* and *cct* alleles include both RNA nulls and predicted functional nulls (supplementary material Fig. S1) (Ito et al., 2011; Imura et al., 2012). Taken together, these molecular and functional data demonstrate that null mutants for each gene produce both grow and arrest phenotypes.

Analysis of *GCT* and *CCT* expression in a public dataset revealed that both genes are broadly expressed at low levels throughout post-embryonic development (supplementary material Fig. S2) (Schmid et al., 2005; Winter et al., 2007). The functional relationship between *GCT* and *CCT* was investigated by constructing plants mutant for both genes. The genotypes of the phenotypically mutant progeny of a self-pollinated *gct*/+ *cct*/+ plant were determined using allele-specific molecular markers. No double mutants were identified among the 27 plants with the grow phenotype, but 3 out of 18 plants with the arrest phenotype were homozygous for

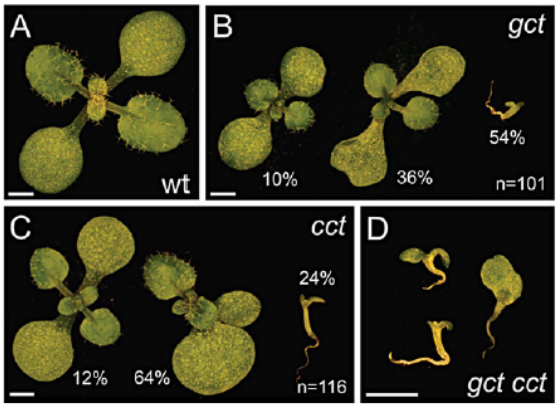

Fig. 1. *GCT* and *CCT* act together to promote post-embryonic growth. Wild-type (wt), *gct* and *cct Arabidopsis thaliana* seedlings after 10 d of growth on soil. (A) Wt seedling. (B) Phenotypic spectrum of *gct* seedlings: 10% showed regularly shaped cotyledons, 36% showed irregularly shaped cotyledons, and 54% arrested growth. (C) Phenotypic spectrum of *cct* seedlings: 12% showed regularly shaped cotyledons, 64% showed irregularly shaped cotyledons, and 24% arrested growth. (D) *gct cct* double-mutant seedlings show 100% expressivity of the arrested growth phenotype. Scale bars: 1 mm.

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both mutations; the phenotypes of these *gct cct* double mutants are shown in Fig. 1D. *GCT* and *CCT* are not linked, so the expected frequency of double mutants among progeny displaying a mutant phenotype is 1/7; the ratio of double mutants (0/27) among grow phenotype seedlings is significantly different from this ratio (χ^2 goodness of fit test, P=0.03), whereas the frequency of double mutants among arrest phenotype seedlings (3/18) is not significantly different from this ratio. We conclude that loss of both *GCT* and *CCT* consistently leads to growth arrest. These results, as well as the phenotypic similarity of *gct* and *cct* mutations, suggest that these genes operate together or in parallel to regulate a core set of processes required for post-embryonic development.

Genes misregulated in both *gct* and *cct* seedlings demonstrate a role for *GCT* and *CCT* in developmental transitions

To investigate the molecular basis for the post-embryonic phenotype of *gct* and *cct*, and the vegetative and reproductive changes described below, we used Affymetrix microarrays to compare steady-state mRNA levels in 7-day-old (7 d) wild-type (wt) seedlings and 9 d *gct* and *cct* seedlings, when the first two leaf primordia of these three genotypes were 1 mm in length (Fig. 2A). Compared with wt, transcripts for 1267 probe sets were more abundant in *gct* and 1373 were less abundant in *gct* (supplementary material Table S2), whereas transcripts for 925 probe sets were more abundant in *cct* and 1051 were less abundant in *cct* (supplementary material Table S3) (Limma, with a Benjamini-Hochberg adjustment, P<0.05). In total, 490 probe sets increased and 683 decreased in both *gct* and *cct* (supplementary material Table S4), an overlap that is greater than would be expected by chance (Fisher's exact test, P=0). Thus, *GCT* and *CCT* share a large, but not totally overlapping, set of direct and indirect gene targets.

An analysis of genes with transcripts that increase or decrease in both *gct* and *cct* is presented in supplementary material Table S5. These enriched terms (including GO terms) suggest a role for *GCT* and *CCT* in a number of growth-related processes, including the secretory pathway (such as GPI-anchored proteins and N-linked glycosylation), and cell wall remodeling. Among the most highly overrepresented GO terms were those related to seed-specific processes and flowering (supplementary material Table S5). An inspection of the genes that are most highly overexpressed in both *gct* and *cct* revealed many seed-specific transcripts, as well as the flowering repressor *FLC*; flowering genes that showed significant decreases in expression included *FLOWERING LOCUS T* (*FT*) and

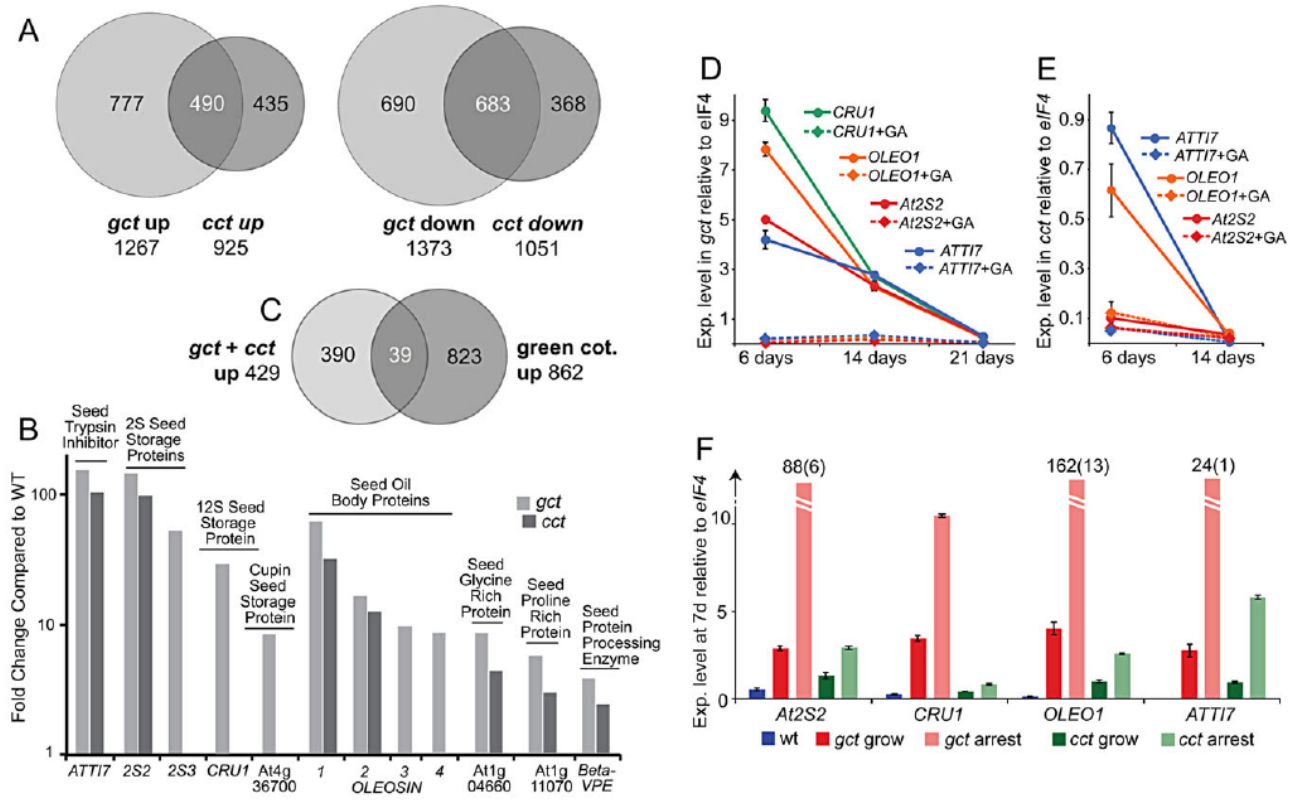

Fig. 2. *GCT* and *CCT* have many common targets and repress embryo-specific genes in seedlings. Microarray and qRT-PCR analyses of gene expression in wt, *gct* and *cct* seedlings. (A) Affymetrix microarray analysis of genes that change in 9 d *gct* and *cct* seedlings compared with 7 d wt (P<0.05). Shown is the number of genes that change in *gct* only (light gray circle), in *cct* only (medium gray circle) or in both (overlap), with the total number of genes that change in these mutants shown beneath. Three biological replicates were performed.16,826 non-control probe sets were present in at least three of the nine microarrays. Fisher's exact test, P=0 for significance of overlap of genes that increase or decrease in both *gct* and *cct*. (B) Affymetrix microarray data for seed-specific genes that are heterochronically misexpressed in *gct* and *cct* seedlings. Log_{10} scale. (C) Comparison of transcripts that are upregulated in both *gct* and *cct* (light gray) with transcripts upregulated from the torpedo to green cotyledon stage from Willmann et al. (2011) (medium gray); intersection shows the number of transcripts upregulated in both. 15,449 probe sets were present in both samples. Fisher's exact test, $P=1.6\times10^{-4}$ for significance of overlap of the two gene sets. (D,E) qRT-PCR validation of seed-specific genes expressed in *gct* seedlings at 6 d, 14 d and 21 d or in *cct* seedlings at 6 d and 14 d (solid lines); GA_3 treatment reduces the expression of these genes to negligible levels (dashed lines). (F) qRT-PCR expression analysis of seed-specific genes in 7 d wt seedlings, *gct* seedlings that grow or arrest, and *cct* seedlings that grow or arrest. For qRT-PCR experiments, the average and s.d. of three technical replicates for one representative experiment of two biological replicates is shown.

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SUPPRESSOR OF CONSTANS 1 (*SOC1*) (both of which are repressed by *FLC*), as well as *CONSTANS* (*CO*) (supplementary material Table S4). These results suggest that, in addition to controlling the timing of embryonic development (Gillmor et al., 2010), *GCT* and *CCT* regulate post-embryonic developmental transitions.

GCT and *CCT* promote the transition to post-embryonic development by repressing the expression of embryo-specific genes after germination

As mentioned above, a number of seed-specific transcripts were among the most highly overexpressed genes in both *gct* and *cct* seedlings (Fig. 2B; supplementary material Table S4). In support of a role for *GCT* and *CCT* in regulating the seed-to-seedling transition, we found a significant overlap between probe sets upregulated in both *gct* and *cct* and those increasing at the green cotyledon stage of embryogenesis (Fig. 2C; Fisher's exact test right-tail, $P \leq 1.61 \times 10^{-4}$) (Willmann et al., 2011). Thus, *gct* and *cct* seedlings show molecular signatures of late embryogenesis.

To confirm and extend these results, we used qRT-PCR to measure the abundance of four seed-specific transcripts (*At2S2*, *CRU1*, *OLEO1*, *ATTI7*) in 6 d, 14 d and 21 d wt and mutant plants; only mutant plants with the grow phenotype were used for this experiment. All four genes were expressed at high levels in 6 d *gct* seedlings, and their expression declined gradually in this mutant over the next 2 weeks (Fig. 2D). *At2S2*, *OLEO1* and *ATTI7* were also misexpressed in 6 d *cct* seedlings, but were much less elevated than in *gct* (Fig. 2E) and also disappeared faster in this genotype. Consistent with the results of the microarray analysis, *CRU1* was unaffected by *cct*. We also compared the expression of these genes in 7 d *gct* and *cct* seedlings with the grow and arrest phenotypes (Fig. 2F). In every case, these genes were expressed at significantly higher levels in seedlings with the arrest phenotype. For example, *At2S2* was elevated 3-fold in grow *gct* seedlings and 88-fold in arrest *gct* seedlings, while *OLEO1* was elevated 4-fold in grow *gct* seedlings and 162-fold in arrest *gct* seedlings. These results demonstrate that *GCT* and *CCT* repress the expression of embryo-specific genes during post-embryonic development.

The phytohormone GA has a similar function to GCT and CCT in that it promotes seed germination and represses embryonic identity in young seedlings (Koornneef and van der Veen, 1980; Ogas et al., 1997). To determine if GA operates via *GCT* and *CCT*, we examined the effect of this hormone on the expression of *At2S2*, *CRU1*, *OLEO1*, *ATTI7* in *gct* and *cct* mutants. These genes were strongly repressed by GA in both mutants (Fig. 2D,E), indicating that GA represses embryonic gene expression independently of *GCT* and *CCT*.

GCT and *CCT* promote vegetative phase change by repressing miR156

In *Arabidopsis*, the transition from the juvenile to the adult phase of vegetative development is marked by changes in leaf shape (round to elongated), leaf serration (smooth to serrated) and abaxial trichome production (absent to present) (Röbbelen, 1957; Chien and Sussex, 1996; Telfer et al., 1997). *gct* and *cct* delayed the appearance of the adult forms of all of these traits (Fig. 3). In long-day conditions, *gct* and *cct* plants had over twice as many leaves without abaxial trichomes as wt plants (Table 1). *gct* and *cct* also

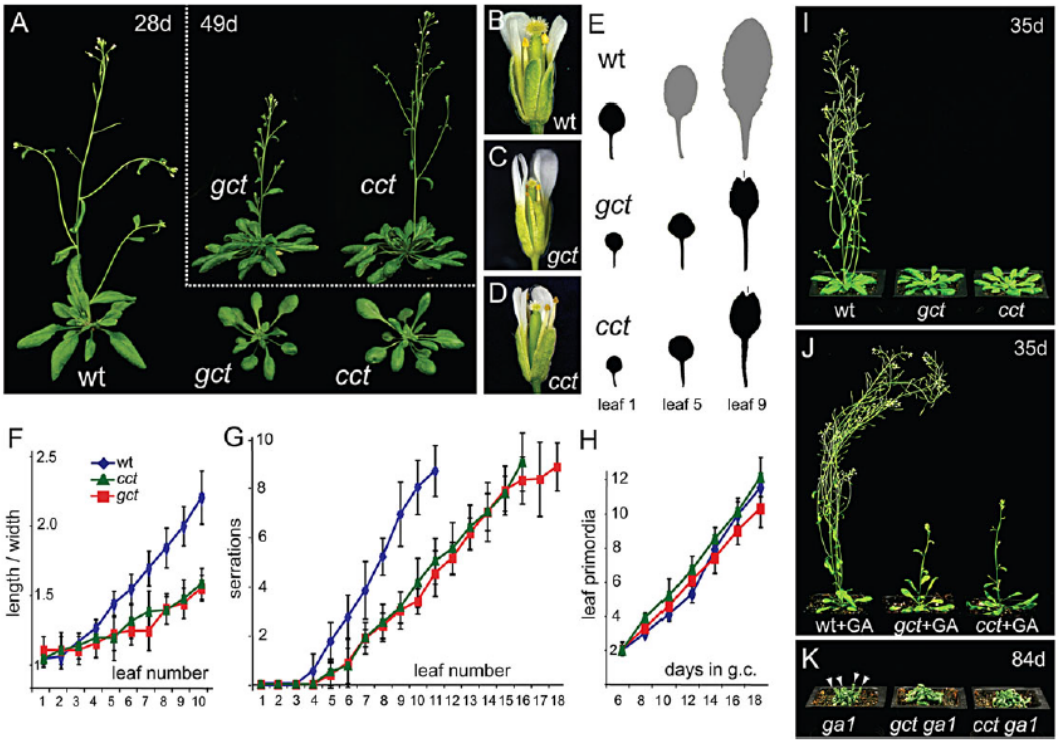

Fig. 3. *gct* and *cct* mutations delay the expression of adult leaf traits and the transition to flowering. (A) Wt, *gct* and *cct* plants after 28 d of growth and *gct* and *cct* after 49 d of growth (inset) in long-day conditions. (B) Wt flower at anthesis. (C) *gct* flower, no viable pollen is produced. (D) *cct* flower, almost no viable pollen is produced. (E) Silhouettes of fully expanded leaves of wt, *gct* and *cct*. Breaks at the distal end of *gct* and *cct* leaves (marked by short lines) are due to flattening of convex leaves for scanning. (F) Length-to-width ratios for the first ten leaves of wt, *gct* and *cct*. (G) Number of serrations per leaf for wt, *gct* and *cct*. (H) Rate of leaf initiation in wt, *gct* and *cct*. Error bars (F-H) represent s.d. ($n \geq 8$). (I,J) 35 d wt, *gct* and *cct* plants with (J) and without (I) daily GA_3 treatment. (K) 84 d *ga1-3*, *gct ga1-3* and *cct ga1-3* plants. Arrowheads point to inflorescences.

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Table 1. Leaf identity and flowering time data for wt, *gct*, *cct* in *flc-3* and *ga1-3* backgrounds or with GA_3 treatment

Genotype/treatment	Leaves without abaxial trichomes	Leaves with abaxial trichomes	Bracts	Days to first open flower	*n*
wt Col	4.9 (0.3)	4.8 (0.7)	3.1 (0.3)	24.7 (1.0)	10
gct	12.9 (0.9)	18.9 (3.9)	7.2 (1.2)	50.8 (5.0)	17
cct	11.6 (1.4)	13.2 (2.0)	7.8 (1.6)	41.3 (3.7)	15
wt Col+GA	2.0 (0.0)	4.2 (0.6)	3.5 (0.6)	18.5 (0.8)	12
gct+GA	5.5 (0.8)	6.2 (0.8)	12.1 (2)	32.3 (3.1)	10
cct+GA	4.8 (0.7)	5.1 (0.6)	12.5 (1.7)	31.4 (2.4)	10
ga1-3	>20	3 (2)	n.d.	70 (2)	11
gct ga1-3	>30	0	n.d.	>105	5
cct ga1-3	>30	0	n.d.	>91	13
flc-3	4.5 (0.6)	3.8 (0.8)	2.8 (0.5)	23.7 (0.9)	28
gct flc-3	12.6 (1.5)	9.1 (1.6)	5.4 (0.7)	43.2 (0.7)	15
cct flc-3	10.1 (1.0)	9.1 (2.1)	5.8 (1.4)	35.2 (2.4)	21

Plants were grown in long-day conditions. s.d. is shown in parentheses.
+GA, daily treatment with 100 μM GA_3.
n.d., not determined.

had rounder leaves than wt (Fig. 3E,F) and began to produce serrated leaves later and with a slower rate of increase in serration number than wt plants (Fig. 3E,G). Importantly, the rate of leaf initiation was almost identical in wt, *gct* and *cct*, indicating that the increased number of juvenile leaves in *gct* and *cct* is not due to an increase in the rate of leaf production (Fig. 3H).

The phytohormone GA promotes several of the processes regulated by *GCT* and *CCT*, including the onset of abaxial trichome production and the transition to flowering (Chien and Sussex, 1996; Telfer et al., 1997; Wilson et al., 1992). To determine whether *GCT* and *CCT* act through the GA pathway, we examined how variation in the amount of GA affects the phenotype of *gct* and *cct*. Exogenously applied GA_3 accelerated abaxial trichome production and flowering in *gct* and *cct*, whereas *ga1-3* – a mutation that blocks GA biosynthesis – further delayed abaxial trichome production and flowering time in these mutants (Table 1; Fig. 3I-K). The observation that *gct* and *cct* do not interfere with the sensitivity of plants to GA suggests that *GCT* and *CCT* regulate vegetative phase change and flowering in parallel to this hormone.

The vegetative phenotype of *gct* and *cct* is similar to that of plants overexpressing miR156, the miRNA that acts as a master regulator of the juvenile-to-adult transition in *Arabidopsis* (Wu and Poethig, 2006). To determine if *GCT* and *CCT* regulate vegetative phase change via miR156, we examined the effect of *gct* and *cct* on the expression of miR156, as well as the expression of direct (*SPL3* and *SPL9*) and indirect (miR172) targets of this miRNA (Wu et al., 2009) (Fig. 4). Northern blot analysis revealed that miR156 levels were approximately twice as high in *gct* and *cct* mutants as in wt plants at 7 d, 14 d and 21 d after germination. miR156 levels decreased steadily in all genotypes, so that at 21 d the amount of miR156 was about half that seen at 7 d. As reported previously, miR156 and miR172 show opposite patterns of accumulation (Wu et al., 2009). In agreement with this, we found that the increased expression of miR156 in *gct* and *cct* was associated with a decrease in the expression of miR172 at 7 d, 14 d and 21 d (Fig. 4A). Similarly, the mRNA levels of the transcription factors *SPL3* and *SPL9*, which are direct targets of miR156, were reduced in both *gct* and *cct* (Fig. 4C), although in the case of *SPL9* this effect was only observed at 21 d. *gct* and *cct* had no significant effect on the expression of miR159, miR161 and miR168 (Fig. 4C). These results suggest that *GCT* and *CCT* specifically regulate miR156, rather than being generally involved in miRNA transcription.

To determine if the increased expression of miR156 in *gct* and *cct* mutants is responsible for their effect on vegetative phase change, we reduced miR156 levels in these mutants using a 35S::MIM156 transgene. This transgene expresses an imperfect miR156 target site, which reduces free miR156 in the cell by sequestering this miRNA in inactive RISC complexes (Franco-Zorrilla et al., 2007). Consistent with previous reports (Wu et al., 2009; Wang et al., 2009), wt plants carrying a single 35S::MIM156 transgene failed to express juvenile leaf traits: they produced abaxial trichomes starting with leaf 1 (instead of leaf 5.5) (Fig. 5A), and all of their rosette leaves had the pattern of leaf serrations (Fig. 5B) and the elongated shape (Fig. 5C) characteristic of adult leaves. 35S::MIM156 had this same effect in the *gct* and *cct* mutant backgrounds: transgenic *gct* and *cct* plants produced abaxial trichomes on leaf 1 (instead of 16 and 15.5, respectively) (Fig. 5A), and had more serrated (Fig. 5B) and more highly elongated (Fig. 5C) leaves than wt plants. Although

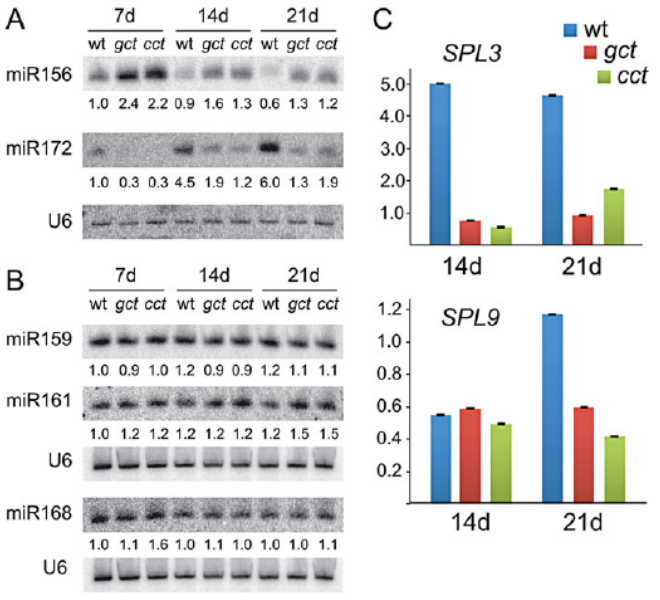

Fig. 4. *GCT* and *CCT* regulate miR156, miR172 and SPL genes. Northern blot and qRT-PCR analysis of 7 d, 14 d and 21 d wt, *gct* and *cct* plants grown in long-day conditions. (A) Northern blot detection of miR156 and miR172. One representative example of three biological replicates is shown. (B) Northern blot detection of miR159, miR161 and miR168. One experiment was performed. (A,B) Intensity values normalized to the *U6* loading control are given beneath each lane. (C) qRT-PCR analysis of *SPL3* and *SPL9* transcript levels, normalized to *EIF4A*. One of two biological replicates is shown. Error bars represent the s.d. of three technical replicates.

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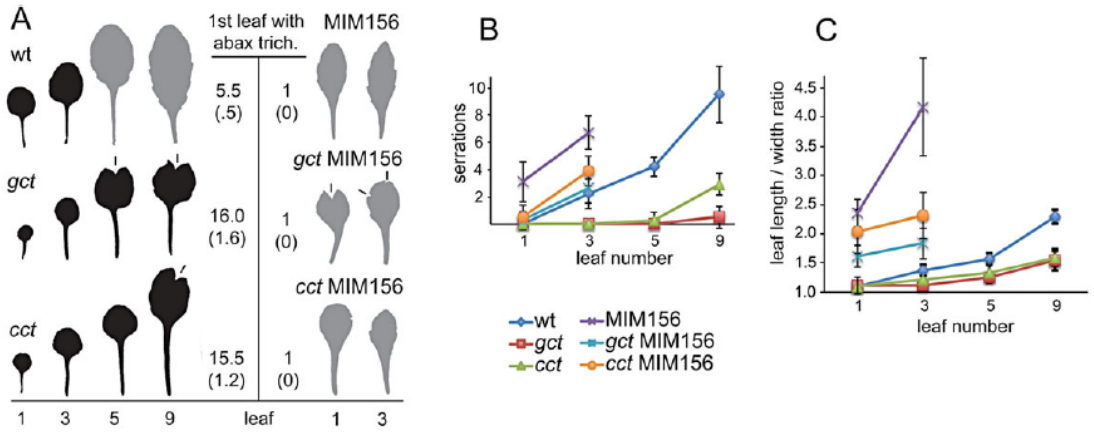

Fig. 5. Decrease in miR156 activity rescues the vegetative phase change phenotype of *gct* and *cct* plants. Phenotypic analysis of leaf shape, serrations and abaxial trichome onset in wt, *gct*, *cct*, MIM156, *gct* MIM156 and *cct* MIM156 plants grown in long-day conditions. (A) Morphology of leaves 1, 3, 5 and 9 (wt, *gct* and *cct*) and of leaves 1 and 3 (MIM156, *gct* MIM156, *cct* MIM156). Leaves with abaxial trichomes are shown in gray, leaves without abaxial trichomes in black. The average position of the first leaf with abaxial trichomes is shown, with s.d. in parentheses. Location of cuts made in leaves in order to flatten them for scanning are indicated with a short line. (B) Number of serrations in leaves 1, 3, 5 and 9 of each genotype. (C) Length-to-width ratios for leaves 1, 3, 5 and 9 of each genotype. Error bars indicate s.d.; $n \geq 12$.

a single copy of 35S::MIM156 was totally epistatic to *gct* and *cct* with regard to abaxial trichome production, it was unable to completely suppress their effect on leaf serrations and the leaf length-to-width ratio. This could mean that the pathways affecting these morphological traits are sensitive to very small amounts of miR156, or that *gct* and *cct* have both miR156-dependent and miR156-independent roles in leaf development.

In summary, these results demonstrate that miR156 is required for the juvenilized phenotype of *gct* and *cct*, and support the conclusion that this phenotype is largely attributable to the increased amount of miR156 in these mutants.

GCT and *CCT* promote the floral transition by repressing *FLC*

gct and *cct* also have a significant effect on the transition to flowering (Fig. 3A). During this transition, the shoot apex produces several flower-bearing branches (co-florescences) subtended by leaves (bracts), and then produces only flowers. In long days, wt plants made an average of 3.1 bracts and flowered 24.7 days after planting (DAP) (Table 1). *gct* plants made an average of 7.2 bracts and flowered 50.8 DAP, while *cct* plants produced an average of 7.8 bracts and flowered 41.3 DAP (Table 1). *gct* and *cct* produced smaller flowers than normal, with occasional fused stamen filaments and a variable number of petals and stamens (Fig. 3B-D; data not shown). *gct* flowers are completely male and female sterile, whereas the flowers of *cct* are initially completely sterile, but will produce a small amount of seed after several months of growth. Thus, as previously reported for *cct* (*crp*) by Imura et al. (2012), *GCT* and *CCT* promote floral induction and also play a role in floral morphogenesis.

Our microarray analysis revealed that the late flowering phenotype of *gct* and *cct* is associated with reduced expression of the floral inducers *FT*, *SOC1*, *SEPALLATA3* (*SEP3*), *FRUITFULL* (*FUL*) and *CO* and with elevated expression of the floral repressor *FLC* (Fig. 6A; supplementary material Tables S2-S4), which is consistent with the known regulatory interactions between these genes (Fig. 6B) (Amasino, 2010). To validate and extend these results, we quantified *FLC*, *FT* and *SOC1* levels in wt, *gct* and *cct* plants at 6 d and 21 d. In wt plants, *FLC* transcripts remained steady at 6 d and 21 d, whereas *SOC1* and *FT* increased dramatically (consistent with the vegetative-to-floral transition having occurred by 21 d) (Fig. 6C,D). In *gct* and *cct* plants, we found that levels of *FLC* transcripts were much higher than in wt at 6 d, and had decreased several fold by 21 d (Fig. 6C), whereas the levels of *FT* and *SOC1* increased slightly from 6 d to 21 d (Fig. 6D,E). These changes in *FT* and *SOC1* gene expression are not attributable to differences in leaf identity between wt, *gct* and *cct*, as *FT* and *SOC1* show essentially the same expression in juvenile and adult leaves (supplementary material Fig. S3). Although *FLC* is expressed more highly in juvenile than in adult leaves (supplementary material Fig. S3), a secondary effect of leaf identity is unlikely to explain the large differences in *FLC* levels between wt, *gct* and *cct* because the largest differences in *FLC* were observed at 6 d, when all three genotypes have only two juvenile leaf primordia (Fig. 3E-H). Thus, *gct* and *cct* mutants show large increases in expression of the floral repressor *FLC*, and, in turn, show decreased expression of the *FLC* targets *FT* and *SOC1*.

As shown in Fig. 4, *gct* and *cct* also have higher levels of miR156, a miRNA that has previously been shown to cause a small delay in flowering in long-day conditions when overexpressed (Wang et al., 2009). This raises the possibility that the increased level of *FLC* in *gct* and *cct* might be attributable to the increased level of miR156 in these mutants (Fig. 4). To test this, we examined *FLC* levels in 35S::miR156A plants and found a ~30% decrease in the level of *FLC* compared with wt (supplementary material Fig. S4). Because *gct* and *cct* show elevated levels of both miR156 and *FLC*, we conclude that the increase in *FLC* in *gct* and *cct* is not caused by the increase in miR156.

To determine whether the late flowering phenotype of *gct* and *cct* is indeed attributable to the increased expression of *FLC*, we crossed the *flc-3* null mutation into *gct* and *cct* mutant backgrounds. The flowering time of these double mutants was intermediate between the two parents: *gct flc-3* double mutants flowered ~7 days earlier than *gct*, while *cct flc-3* double mutants flowered 6 days earlier than *cct* (Table 1; Fig. 6F), but neither genotype flowered as early as *flc-3*. The levels of *FT* and *SOC1* in these double mutants were consistent with their intermediate phenotype. Whereas *flc-3* completely blocked the effect of *cct* on *SOC1* expression in 21 d plants (Fig. 6E), it had only a small effect on *FT* expression in *cct* mutants (Fig. 6D). *flc-3* had little or no effect on the expression of *FT* and *SOC1* in a *gct* mutant background (Fig. 6D,E). This result

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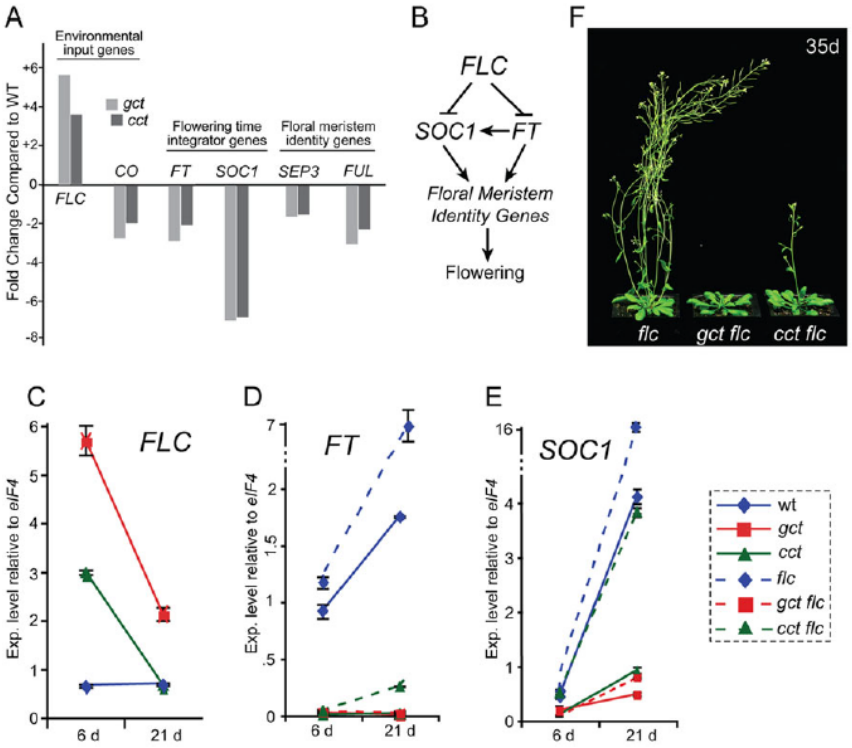

Fig. 6. *GCT* and *CCT* regulate *FLC* and its downstream targets. (A) Affymetrix microarray analysis of *CO*, *FLC* and the *FLC* targets *FT*, *SOC1*, *SEP3* and *FUL* in 9 d *gct* and *cct* seedlings compared with 7 d wt seedlings. (B) Regulatory interactions between *FLC*, *SOC1* and *FT*. (C-E) Expression of *FLC* (C), *FT* (D) and *SOC1* (E) in wt, *gct*, *cct*, *flc-3*, *gct flc-3* and *cct flc-3* plants at 6 d and 21 d. The average of three technical replicates for one representative experiment of two biological replicates is shown. Error bars indicate s.d. Results were normalized to expression of *EIF4A*. (F) *flc-3*, *gct flc-3* and *cct flc-3* 35 d plants. See Fig. 3I for 35 d wt, *gct* and *cct* plants.

indicates that the elevated expression of *FLC* contributes to the late flowering phenotype of *cct* and *gct*, but is not entirely responsible for this phenotype.

DISCUSSION

Multicellular organisms pass through several developmental phases during their life cycle. In *Arabidopsis*, these stages include embryogenesis and seed formation, germination, juvenile and adult phases of vegetative growth, and a reproductive phase that culminates in gamete production and fertilization. We have previously shown that the transcriptional repressors *GCT* (*MED13*) and *CCT* (*MED12*) regulate the timing of pattern formation during *Arabidopsis* embryogenesis (Gillmor et al., 2010). In the current study we show that *GCT* and *CCT* play a global role in the regulation of developmental timing, promoting the seed-to-seedling transition, vegetative phase change, and flowering.

Microarray analysis demonstrated that *GCT* and *CCT* share close to half of their direct or indirect gene targets, with 490 transcripts increasing in both *gct* and *cct* (out of a total 1267 genes increasing in *gct* and 925 in *cct*), and 683 transcripts decreasing in both mutants (out of 1373 genes decreasing in *gct* and 1051 in *cct*). The number of transcripts that change in level is comparable with the findings of RNA profiling in single cell types in *Drosophila* [S2 cells, where a total of 361 genes were found to change in *med12* (*kohtalo*) or *med13* (*skuld*) mutants] and yeast (where a total of 900 genes were found to increase or decrease), as is the highly significant overlap that we observed between the transcripts that change in common between *med12* and *med13* mutants (Kuuluvainen et al., 2014; Van de Peppel et al., 2005). It is commonly accepted that MED12 and MED13 primarily act as transcriptional repressors (Carlsten et al., 2013), and thus many of the transcripts whose steady-state levels decrease in *gct* and *cct* mutants are unlikely to be direct targets of *GCT* and *CCT* regulation. However, there are also reports of the CDK8 module acting as a transcriptional activator (Carrera et al., 2008; Donner et al., 2007). No genome-wide chromatin immunoprecipitation (ChIP) profiling experiment has been reported for either MED12 or MED13 in plants or animals. Despite the challenges inherent in ChIP analysis using such large proteins, determination of the direct targets of MED12 and MED13 is an important goal for future research in both *Arabidopsis* and animals.

In addition to the molecular data discussed above, genetic analysis provides functional evidence that *GCT* and *CCT* regulate many of the same processes. For example, *gct* and *cct* single mutants express either a weak 'grow' phenotype characterized by moderate defects in vegetative and reproductive timing, or a strong 'arrest' phenotype of arrested growth. By contrast, *gct cct* double mutants express only the arrest phenotype. This result suggests that, in the absence of either MED12 or MED13, the CDK8 module is partially functional and can still promote post-embryonic growth, albeit in a less effective manner, leading to the variable expressivity of the *gct* and *cct* grow and arrest phenotypes. This result, and the observation that *gct cct* double mutants only have the severe arrest phenotype, suggest that *GCT* and *CCT* have closely related, but not completely redundant functions. This is consistent with studies of MED12 and MED13 in yeast and animals, where they have been shown to affect transcription of many of the same genes and to have nearly identical mutant phenotypes (Samuelsen et al., 2003; Janody et al., 2003; van de Peppel et al., 2005; Yoda et al., 2005). Additionally, *in vitro* studies have demonstrated that, although MED12 and MED13 are capable of individually blocking transcription, they act most efficiently together (Knuesel et al., 2009).

The CDK8 module of Mediator represses transcription by at least two mechanisms. Structural studies in yeast and humans demonstrate that this module binds Core Mediator, blocking the Core Mediator-RNA polymerase II interaction (Elmlund et al., 2006; Tsai et al., 2013). The MED13, MED12 and CDK8 subunits all contribute to binding, with MED13 playing the most important role, followed by MED12 and CDK8 (Tsai et al., 2013). These results are consistent with the mutant phenotypes of these proteins in *Arabidopsis*, as the expressivity of the growth arrest phenotype is

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higher for *gct* (*med13*) than for *cct* (*med12*). Human MED12 also represses gene expression by recruiting H3K9 and H3K27 methylation to target loci through its interaction with the histone methyltransferase G9a (EHMT2) (Ding et al., 2008; Chaturvedi et al., 2009, 2012). Steric hindrance of RNA polymerase II-Core Mediator interactions represents a relatively flexible form of transcriptional repression, whereas H3K9 and H3K27 methylation is likely to have more stable effects on gene expression. The mutant phenotype of the *gct cct* double mutant suggests that both activities are necessary for full repression by the CDK8 module. H3K9 and H3K27 methylation have well-established roles in the seed-to-seedling and vegetative-to-reproductive transitions. The PRC2 complex represses the expression of embryonic genes after germination by recruiting deposition of H3K27 methylation (Bouyer et al., 2011). Likewise, the flowering repressor *FLC* is repressed by both H3K9 and H3K27 marks (Bastow et al., 2004). It will be important to determine whether the effect of *GCT* and *CCT* on these genes is mediated by these histone modifications.

Our results indicate that the delayed vegetative phase change phenotype of *gct* and *cct* is attributable to an increase in the abundance of miR156 and a corresponding decrease in levels of the downstream miRNA miR172. miR156 was elevated in *gct* and *cct* throughout shoot development, and declined at the same time in mutant and wt plants, suggesting that *GCT* and *CCT* might regulate the amplitude, rather than the temporal pattern, of miR156 expression. It will be important to determine if *GCT* and *CCT* directly regulate the transcription of specific MIR156 genes or whether they modulate the transcription of an upstream regulator of one or more MIR156 genes, in order to investigate the molecular mechanism of this effect. Whatever this mechanism might be, it is interesting that the relatively small change in the amount of miR156 in these mutants (~2-fold) has such a drastic effect on the expression of phase-specific traits. This suggests that fine-tuning of MIR156 expression by *GCT* and *CCT* is crucial for normal phase change, and raises the possibility that spatial or temporal variation in the activity of these proteins might contribute to natural variation in the timing of the vegetative phase change. Our results also suggest that *GCT* and *CCT* play an important role in controlling the timing of the vegetative-to-floral transition by direct or indirect regulation of *FLC*, as well as through additional, yet to be determined, pathways. Using physiological and genetic experiments, we demonstrated that *GCT* and *CCT* regulate the seed-to-seedling transition, vegetative phase change and flowering in parallel with the phytohormone GA.

Developmental transitions are characterized by a decrease in the expression of genes that specify the pre-existing developmental phase and by an increase in the expression of genes that promote the subsequent phase. In principle, the decline in gene expression during these transitions could be mediated by the loss of factors that promote gene expression, by the appearance of factors that repress gene expression, or by a combination of these events. The pleiotropic phenotype of *gct* and *cct* implies that active transcriptional repression by GCT (MED13) and CCT (MED12) plays a major role during many temporal transitions in *Arabidopsis*. This paradigm has been established for a few developmental transitions in *Arabidopsis* – the best example being the repression of *FLC* expression during vernalization – but the generality of transcriptional repression as a mechanism for developmental transition had not been fully appreciated until now. Our results demonstrate that *GCT* and *CCT* are not required for the downregulation of gene expression during phase transitions per se, but rather for the magnitude and perhaps the stability of the repressed state. It is reasonable to assume that they perform this function in association with temporally regulated transcription factors, and a major challenge for the future is to identify these transcription factors and the ways in which they interact with *GCT* and *CCT* to repress gene expression.

MATERIALS AND METHODS

Genetic stocks and growth conditions

All seed stocks were in the Columbia ecotype. Seeds were sown on soil (Farfard #2) and placed at 4°C for 3 days, before moving flats to growth chambers or laboratory growth racks under 16 h fluorescent illumination (140 µmol/m^2/s) at 22°C. Days to first open flower were measured beginning on the day flats were placed in the growth chamber. For GA treatments, plants were sprayed daily with 100 µM GA_3 (Sigma-Aldrich). For the microarray experiments, plants were grown under long-day conditions (16 h light:8 h dark; 100-125 µmol/m^2/s) in a Conviron growth chamber at a constant 23°C under a 1:1 ratio of T8 Sylvania Octron 4100K Ecologic and GroLite WS fluorescent lamps (Interlectric).

The reference alleles *gct-2* (ABRC stock CS65889; referred to as *gct* throughout the paper) and *cct-1* (ABRC stock CS65890; referred to as *cct* throughout the paper) described by Gillmor et al. (2010) were used for all analyses. The *gct-2* mutation was genotyped using the *gct-2* dCAPS primer pair, and the *cct-1* mutation was genotyped using the *cct-1* dCAPS primer pair. dCAPS primers were designed using dCAPS Finder 2.0 (Neff et al., 2002; http://helix.wustl.edu/dcaps/). The *ga1-3* allele, originally in the L*er* ecotype (Wilson et al., 1992), was introgressed six times into the Col ecotype, and was genotyped based on its morphological phenotype. The *flc-3* allele was used for analysis of the *FLC* gene (Michaels and Amasino, 1999). *flc-3* mutants were genotyped using the *flc-3* primer pair. The *35S::MIM156* target mimicry line for miR156 was genotyped based on its morphological phenotype (Franco-Zorrilla et al., 2007). Sequences of genotyping primers are listed in supplementary material Table S1.

RNA expression analyses

The microarray experiment was performed on three biological replicates of 7 d wt and 9 d *gct* and *cct* mutants with the 'grow' phenotype, each replicate being a pool of at least 20 seedlings. Different time points were used because they were when the first two leaf primordia of these three genotypes were 1 mm in length. Total RNA was extracted with TRI Reagent (Sigma-Aldrich), and further purified using RNeasy columns (Qiagen) with on-column DNase treatment (Qiagen). Biotin-labeled cDNA targets for hybridization to Affymetrix *Arabidopsis* ATH1 microarrays were prepared essentially as described by Willmann et al. (2011). The University of Pennsylvania Microarray Core Facility hybridized the arrays. Raw data have been deposited at Gene Expression Omnibus under accession number GSE56155.

The microarrays were gcRMA normalized in R (http://www.r-project.org/) and filtered using MAS5.0 presence/absence calls to remove any probe sets not expressed in at least three samples. The remaining 16,826 non-control probe sets were tested for differential expression in R using Limma (Bioconductor) with contrast and a Benjamini-Hochberg multiple test correction (MTC), $P \leq 0.05$. Enrichment of GO terms, SP_PIR keywords, PIR Superfamilies, and SMART, INTERPRO, COG_ONTOLOGY and UP_SEQ features within the lists of probe sets increasing and decreasing in both *gct* and *cct* was performed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp; Dennis et al., 2003; Huang et al., 2009) with the 16,826 expressed probe sets as a background. The probe sets differentially expressed in both *gct* and *cct* were also compared with the 862 probe sets shown to specifically increase in green cotyledon stage embryos by Willmann et al. (2011) (Cluster 9).

For quantitative RT-PCR (qRT-PCR), total RNA was isolated as described above for ten pooled seedlings per time point, per biological replicate. Reverse transcription was performed with the oligo(dT)$_{20}$ primer using the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems) on a StepOnePlus RT-PCR System (Applied Biosystems). Transcript levels were normalized against *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A* (*EIF4A*). Primers for qRT-PCR were designed using AtRTPrimer (http://pbil.kaist.ac.kr/AtRTPrimer; Han and Kim, 2006) and are listed in supplementary material Table S1.

For small RNA northern blots, 30 µg total RNA was separated on 8 M urea/15% denaturing polyacrylamide gels and electrically transferred to a Hybond-N+ nylon membrane (GE Healthcare). Blots were hybridized with [γ-^{32}P]ATP-labeled complementary oligonucleotide probes for 3 h at 40°C in Rapid-hyb hybridization buffer (GE Healthcare). A *U6* RNA-complementary oligonucleotide probe was used as a loading control. Blots were washed twice at 40°C in 5×SSC, 0.1% SDS for 20 min, and were scanned with a Storm 820 phosphorimager (Amersham Biosciences). Oligonucleotide probes used are listed in supplementary material Table S1. Signal intensities were quantified using ImageJ (NIH).

Morphological and histological analyses

Analysis of leaf traits, such as size, shape and the presence of abaxial trichomes, was performed at ~4 weeks in order to allow leaves to reach their final size. The presence of abaxial trichomes was scored using a dissecting microscope. Leaf length and width ratios, as well as the number of serrations, were quantified by taping leaves to paper, scanning and analyzing in Photoshop (Adobe).

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Competing interests

The authors declare no competing financial interests.

Author contributions

C.S.G., M.R.W., M.B.-M. and R.S.P. designed experiments. C.S.G., C.O.S.-O. and M.B.-M. performed all experiments, with the exception of the microarray experiments and analysis, which were performed by M.R.W. All authors analyzed data. C.S.G. and R.S.P. wrote the manuscript, with input from the other authors.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.111229/-/DC1

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Review article

Mediator: A key regulator of plant development

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ABSTRACT

Mediator is a multiprotein complex that regulates transcription at the level of RNA pol II assembly, as well as through regulation of chromatin architecture, RNA processing and recruitment of epigenetic marks. Though its modular structure is conserved in eukaryotes, its subunit composition has diverged during evolution and varies in response to environmental and tissue-specific inputs, suggesting different functions for each subunit and/or Mediator conformation. In animals, Mediator has been implicated in the control of differentiation and morphogenesis through modulation of numerous signaling pathways. In plants, studies have revealed roles for Mediator in regulation of cell division, cell fate and organogenesis, as well as developmental timing and hormone responses. We begin this review with an overview of biochemical mechanisms of yeast and animal Mediator that are likely to be conserved in all eukaryotes, as well as a brief discussion of the role of Mediator in animal development. We then present a comprehensive review of studies of the role of Mediator in plant development. Finally, we point to important questions for future research on the role of Mediator as a master coordinator of development.

1. Introduction

Mediator is a large protein complex that serves as a molecular bridge between gene-specific transcription factors bound at enhancers, and RNA polymerase II (RNA pol II). In yeast, Mediator consists of 25 subunits; in mammals approximately 31 subunits; and in plants, approximately 34 subunits (reviewed in Allen and Taatjes (2015), Samanta and Thakur (2015)). Mediator was first discovered in yeast as a large protein complex that was required for transcription (Kelleher et al., 1990; Flanagan et al., 1991), and was subsequently purified from human cells (Fondell et al., 1996), and from plant cells (Bäckström et al., 2007). Because of the low sequence conservation between Mediator subunits from different species (typically as low as 20% amino acid identity), many initial studies of Mediator in yeast and animals did not recognize that proteins that had been isolated based on their differing effects on transcription, were indeed Mediator components, and in some cases, the same Mediator subunit from different organisms (Sato et al., 2004; reviewed in Kornberg (2005)). This discovery led to a unified nomenclature for Mediator subunits in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and the animals *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens* (Bourbon et al., 2004), which was also used for the *Arabidopsis thaliana* Mediator (Bäckström et al., 2007). Shortly thereafter, Mediator components were identified from genomic sequences of many eukaryotes, indicating that Mediator has been widely conserved in evolution (Bourbon, 2008).

Structural studies of Mediator complexes have classified Mediator as having four different modules, referred to as the Head, Middle, Tail, and Cyclin Dependent Kinase 8 (CDK8) modules (reviewed in Chadick and Asturias (2005), Conaway et al. (2005)) (Fig. 1). The Head module is thought to have the most important initial interactions with RNA pol II, while the Middle module serves a structural function as well as interacting with RNA pol II once Mediator's conformation changes after its initial interaction with RNA pol II. The Tail module is thought to play an especially important role in interacting with gene-specific transcription factors (Tsai et al., 2014; Robinson et al., 2015). In yeast, animals, and plants, Mediator has been purified in two forms: as a complex of the Head, Middle and Tail modules (commonly referred to as Core Mediator), and as a larger complex containing Core Mediator and the CDK8 module. Core Mediator preparations support transcription in vitro, while Core Mediator preparations containing the CDK8 module do not (reviewed in Björklund and Gustafsson (2005)). The CDK8 module consists of 4 proteins: MED12, MED13, Cyclin C (CycC), and Cyclin Dependent Kinase 8 (CDK8). The MED12 and MED13 subunits are both about 2000 AA, much larger than most other Mediator subunits (Table 1) (Samuelsen et al., 2003). The large size of MED12 and MED13 may be related to their role as signal integrators, allowing large surface areas for protein interactions, as well as protein modifications that can affect their stability.

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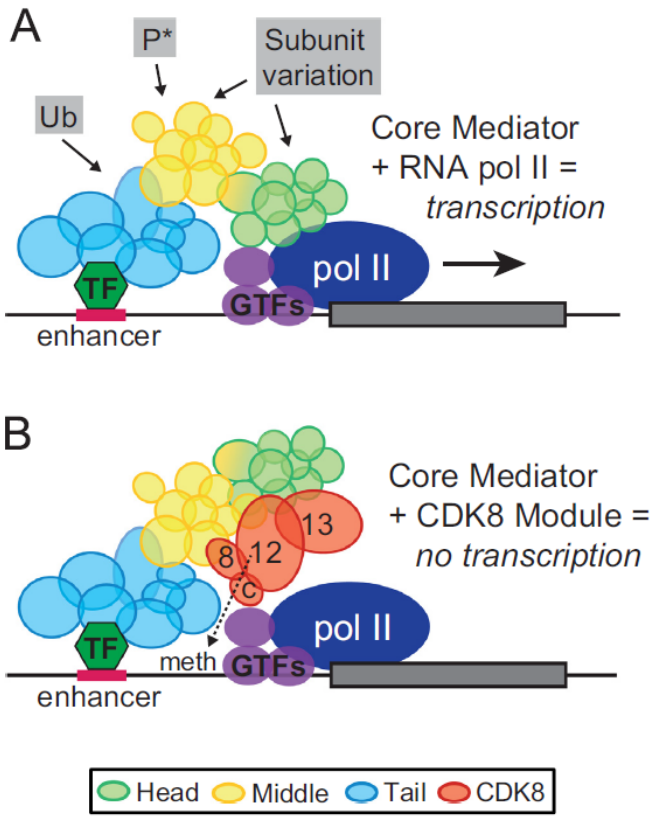

Fig. 1. Regulation of transcription by Core Mediator and the Cyclin Dependent Kinase 8 (CDK8) module of Mediator. A simplified representation of the role of Core Mediator and the CDK8 module of Mediator in regulation of transcription, based on literature cited in this review. (A) Core Mediator (composed of Head, Middle and Tail modules) serves as a molecular bridge between transcription factors (TF) bound at enhancers, and RNA polymerase II (pol II) and general transcription factors (GTFs) at the transcription start site. Individual subunits of each module are represented by colored circles. The composition of Core Mediator is dynamic, varying between different target genes (Subunit variation). Stability and activity of Mediator subunits can be regulated by ubiquitination (Ub), and by phosphorylation (P*). (B) The CDK8 module (composed of CDK8 (8), CyclinC (C), MED12 (12) and MED13 (13)) often acts to prevent transcription, either by steric inhibition of interactions between Core Mediator and RNA pol II, or through increasing epigenetic marks that inhibit transcription (such as H3K9me^2), or reducing epigenetic marks that promote transcription (such as H3K4me^3).

The size of MED12 and MED13 is also almost certainly related to their mechanism of action. Initial studies of the CDK8 module of Mediator reported that its effect was to prevent transcription by steric hindrance of interactions between Core Mediator and RNA pol II (Elmlund et al., 2006). A recent report expanded on earlier work by demonstrating that the yeast CDK8 module interacts with certain Head and Middle module Mediator subunits, in order to occupy the RNA pol II binding cleft of Core Mediator, preventing the initial association of RNA pol II and Core Mediator that leads to activation of transcription (Tsai et al., 2013) (Fig. 1). The MED13 protein plays the most important role in this interaction. The other CDK8 module components can repress gene expression through alternate methods, recruiting histone methylation marks that repress transcription, as well as decreasing histone marks that promote transcription (Gonzalez et al., 2007; Ding et al., 2008; Chaturvedi et al., 2012; Tsutsui et al., 2013; Law and Ciccaglione, 2015). In the absence of the CDK8 module, RNA pol II is able to interact with the Head and Middle domains in the RNA pol II binding pocket. Through mechanisms that are still poorly understood, the conformation of the Middle and Tail domains changes until RNA pol II occupies a site at the Middle domain, adjacent to the Tail domain (Tsai et al., 2013, 2014; Robinson et al., 2015). In addition to RNA pol II complex assembly, Core Mediator participates in multiple steps of transcription, such as RNA pol II initiation, pausing and elongation, and reinitiation. Core Mediator can also promote the formation of super enhancers, and alter genome architecture by looping DNA to put distant enhancers (with bound TFs) in close proximity to promoters, a mechanism that includes non-coding RNAs (Kagey et al., 2010; Whyte et al., 2013; Pelish et al., 2015; reviewed in Allen and Taatjes (2015)). Core Mediator has also been shown to be required for transcription of some siRNA precursors, as well as miRNA precursors (Kim et al., 2011).

Since the discovery of Mediator about 25 years ago, the vast majority of research has focused on biochemical and structural studies of Mediator preparations purified from yeast or human cells (comprehensively reviewed in Poss et al. (2013)). These studies have focused primarily on the activities of the whole Core Mediator complex as a transcriptional co-activator, or in the case of CDK8 module, as a repressor. Meanwhile, developmental biology studies, particularly genetic screens for mutants affecting a particular process of interest, have discovered discrete roles for animal Mediator subunits from all three modules of Core Mediator, and in particular for the Kinase (CDK8) module (reviewed in Yin and Wang (2014), Grants et al. (2015)). This research has demonstrated an essential role for Mediator as a signal integrator and specificity factor, with discrete Mediator subunits specific to certain developmental pathways. Mediator has been discovered to play an essential role in some of the most important signaling pathways in animals, including Wnt-β-catenin (Carrera et al., 2008; Rocha et al., 2010; Yoda et al., 2005), Hedgehog (Janody et al., 2003; Mao et al., 2014; Zhou et al., 2012), RAS-MAPK (Pandey et al., 2005; Balamotis et al., 2009; Grants et al., 2016), and TGFβ-SMAD signaling (Kato et al., 2002; Alarcón et al., 2009; Zhao et al., 2013; Huang et al., 2012). Mediator components have also been found to interact with several Sox transcription factors, which in turn bind to β-catenin and GLI, downstream components of the Wnt-β-Catenin and Hedgehog signaling pathways (reviewed in Kamachi and Kondoh (2013), Rau et al. (2006), Nakamura et al. (2011) and Hong et al. (2005)). Thus, Mediator serves as a transcriptional activator or repressor in a pathway-dependent manner, and can interact with components of signaling pathways like β-catenin (Kim et al., 2006), as well as cofactors of signaling pathway effectors such as Pygopus (Carrera et al., 2008), and Sox transcription factors (Zhou et al., 2002).

In plants, almost all research on Mediator has been performed with *Arabidopsis thaliana*; unless otherwise specified, all studies on plants mentioned in this review were conducted with that species. Mediator has been shown to regulate basic cellular processes such as cell proliferation, cell growth, and organ growth; as well as developmental timing, and hormone responses (Fig. 2 and Table 1). Similar to animals, transcription factors have been discovered which interact with specific plant Mediator components (Table 1), suggesting that many of the mechanisms of Mediator function are likely to be conserved between yeast, plants and animals, though the specific pathways in which they act differ between different kingdoms.

2. Mediator is involved in basic cellular processes in plants

2.1. Cell proliferation

In *hen3 [cdk8]* mutants, loss of CDK8 activity results in smaller leaves, which have approximately the same cell number per area as larger wild type leaves, indicating that CDK8 regulates cell proliferation at the level of the organ (Wang and Chen, 2004). *cct [med12]* and *gct [med13]* mutants also affect cell proliferation and organ growth, delaying the initiation of cotyledon primordia in embryos (Gillmor et al., 2010), and decreasing the rate of leaf

Table 1
Mediator functions in plant development and other processes.

Mediator submodule	Subunit	Arabidopsis gene names	Length (AA)		Functions in development	Other functions	Interacting Proteins
			At	Hs			
Head	MED6	At3g21350	298	246			
	MED8	At2g03070 SETH10 (Lalanne et al., 2004)	524	268	**Floral transition** (Kidd et al., 2009), **root development** (Sundaravelpandian et al., 2013), **cell expansion and organ size** (Xu and Li, 2012), **cell wall composition** (Seguela-Arnaud et al., 2015), **sugar signaling** (Seguela-Arnaud et al., 2015), **male gametophytic development** (Lalanne et al., 2004), **lateral root development** (Ito et al., 2016), **auxin responses** (Ito et al., 2016)		**ARF7, ARF19** (Ito et al., 2016)
	MED11	At3g01435	115	117			
	MED17	At5g20170	653	651	**Floral transition** (Kim et al., 2011), **lateral root development** (Ito et al., 2016)	**Non-coding RNA production** (Kim et al., 2011)	
	MED18	At2g22370	219	208	**Floral transition** (Lai et al., 2014; Zheng et al., 2013), **redox homeostasis** (Lai et al., 2014), **floral organ development** (Zheng et al., 2013), **ABA signaling in germination** (Lai et al., 2014)	**Non-coding RNA production** (Kim et al., 2011)	**SUF4, ABI4, YY1** (Lai et al., 2014); **SPL15** (Hyun et al., 2016)
	MED20a/ MED20b/ MED20c	At2g28230; At4g09070; At2g28020	219/ 219/ 70	212	**Floral transition (MED20a)** (Kim et al., 2011)	**Non-coding RNA production** (Kim et al., 2011)	
	MED22	At1g16430, MED22a; At1g07950, MED22b	154	200			
	MED28	At3g52860	156	178	**Redox homeostasis in root and senescence** (Shaikhali et al., 2015a, 2015b)		
	MED30	At5g63480	189	178			
Middle	CBP1 (MED1?)	At2g15890	203	1581	**Pollen tube guidance** (Li et al., 2015a)		**CCG, RNA Pol II, MED7a, MED7b, MED9, several AGL factors** (Li et al., 2015a)
	MED4	At5g02850	426	270	**Vegetative phase change (miR156 pathway)** (Li et al., 2015b)		**SAD1 (RPA34.5)** (Li et al., 2015b)
	MED7	At5g03220, MED7a; At5g03500, MED7b	168	233			
	MED9	At1g55080	244	146			
	MED10	At5g41910, MED10a; At1g26665, MED10b	189	135			
	MED19	At5g12230	221	244		**Pathogen resistance** (Caillaud et al., 2013)	**HaRxL44** (Caillaud et al., 2013)
	MED21	At4g04780	139	144		**Defense response to fungal pathogens** (Dhawan et al., 2009)	**HUB1** (Dhawan et al., 2009)
	MED26	At3g10820, MED26a; At5g05140, MED26b; At5g09850, MED26c	580 / 436 / 353	600			
	MED31	At5g19910	226	131			
Tail	MED14	At3g04740, SWP (Autran et al., 2002)	1703	1454	**Cell proliferation in leaf development** (Autran et al., 2002)	**Plant immunity** (Zhang et al., 2013), **cold response** (Hemsley et al., 2014)	**LUG** (Gonzalez et al., 2007)
	MED15	At1g15780, NRB4 (Canet et al., 2012)	1335	788	**Floral transition** (Canet et al., 2012)	**Salicylic acid response** (Canet et al., 2012), **lipid biosynthesis in seeds** (Kim et al., 2016)	**WRI1** (Kim et al., 2016)
	MED16	At4g04920, SFR6 (Knight et al., 2008), YID1(Yang et al., 2014), IEN1 (Zhang et al., 2012)	1278	877	**Floral transition** (Knight et al., 2008), **cell wall composition** (Sorek et al., 2015)	**Plant immunity** (Wang et al., 2015; Wathugala et al., 2012; Zhang et al., 2013, 2012), **iron homeostasis** (Yang et al., 2014), **cold response** (Hemsley et al., 2014; Knight et al., 2009)	

Table 1 (*continued*)

Mediator submodule	Subunit	Arabidopsis gene names	Length (AA)		Functions in development	Other functions	Interacting Proteins
			At	Hs			
	MED23	At1g23230	1615	1368			
	MED24/33/5	At3g23590, MED33a/MED5a/RFR1; At2g48110, MED33b/MED5b/REF4 (Bonawitz et al., 2012)	1309	989	**Cell wall composition** (Anderson et al., 2015; Bonawitz et al., 2014, 2012)		
	MED25	At1g25540; PFT1 (Cerdán and Chory, 2003)	836	747	**Floral transition** (Cerdán and Chory, 2003; Iñigo et al., 2012a, 2012b), **cell expansion and organ size** (Xu and Li, 2011), **auxin signaling** (Raya-González et al., 2014), **redox homeostasis in root** (Sundaravelpandian et al., 2013), **cell wall composition** (Seguela-Arnaud et al., 2015), **sugar signaling** (Seguela-Arnaud et al., 2015), **ABA signaling in germination** (Chen et al., 2012), **lateral root development** (Raya-González et al., 2014; Ito et al., 2016)	**Jasmonate-dependent defense** (Çevik et al., 2012; Chen et al., 2012; Kidd et al., 2009), **sulfate assimilation** (Koprivova et al., 2014), **iron homeostasis** (Yang et al., 2014)	**COP1&HY5** (Klose et al., 2012); **MBR1&MBR2** (Iñigo 2012b); **CDK8** (Zhu et al., 2014); **ERF1, MYC2, MYC3, MYC4, BZS1, POSF21, ORA59, WRKY10, MYB104, ERF15, DREB2A** (Çevik et al., 2012); **DREB2A, ZFHD1, PHL1** (Elfving et al., 2011); **MYC2 & ABI5** (Chen et al., 2012); **EIN3 & EIL1** (Yang et al., 2014); **ARF7, ARF19, IAA14** (Ito et al., 2016)
	MED27/3	At3g09180	402	311			
	MED29/32/2	At1g11760	151	200	**Redox homeostasis in root and senescence** (Shaikhali et al., 2015a, 2015b)	**Cold response** (Hemsley et al., 2014)	
CDK8	MED12	At4g00450, *CCT* (Gillmor et al., 2010), *CRP* (Imura et al., 2012)	2253	2177	**Embryo polarity and patterning** (Gillmor et al., 2010), **embryo to seedling transition** (Gillmor et al., 2014), **vegetative phase change (miR156 pathway)** (Gillmor et al., 2014), **floral transition** (Gillmor et al., 2014; Imura et al., 2012), **auxin signaling** (Gillmor et al., 2010; Imura et al., 2012; Ito et al., 2016), **lateral root development** (Ito et al., 2016)		
	MED13	At1g55325, *GCT* (Gillmor et al., 2010), *MAB2* (Ito et al., 2011)	2001	2174	**Embryo polarity and patterning** (Gillmor et al., 2010; Ito et al., 2011), **embryo to seedling transition** (Gillmor et al., 2014), **vegetative phase change (miR156 pathway)** (Gillmor et al., 2014), **floral transition** (Gillmor et al., 2014), **auxin signaling** (Gillmor et al., 2010; Ito et al., 2011; Imura et al., 2012; Ito et al., 2016), **lateral root development** (Ito et al., 2016)		**CYCC1;2** (Ito et al., 2011), **TPL** (Ito et al., 2016), **TPR2** (Ito et al., 2016), **IAA14** (via **TPL**) (Ito et al., 2016)
	CDK8 (CDKE)	At5g63610, *HEN3* (Wang and Chen, 2004)	470	464	**Floral organ development** (Wang and Chen, 2004), **auxin signaling** (Ito et al., 2016), **lateral root development** (Ito et al., 2016)	**Mitochondrial retrograde regulation** (Ng et al., 2013)	**RNA Pol II** (Wang and Chen, 2004); **CYC1;2** (Ito et al., 2011); **CYC1;1&CYCC1;2, WIN1** (Zhu et al., 2014); **LUG** (Gonzalez et al., 2007)
	CYCC1;1/CYCC1;2	At5g48640; At5g48630	253/256	283			**MED13+CYCC1;2, CDK8+CYCC1;2** (Ito et al., 2011); **CDK8+CYCC1;1&CYCC1;2** (Zhu et al., 2014)
Unassigned	MED34	At1g31360, RECQ2 (Kobbe et al., 2008)	705			**3′- > 5′ DNA helicase** (Kobbe et al., 2008)	
	MED35	At1g44910, PRP40a (Kang et al., 2009)	958			**RNA processing** (Kang et al., 2009)	**RNA Pol II** (Kang et al., 2009)
	MED36	At4g25630, FIB2 (Barneche et al., 2000)	320			**Processing of rRNA** (Barneche et al., 2000)	**PRMT1** (Barneche et al., 2000)
	MED37	At5g28540, BIP1 (Maruyama et al., 2010)	668		**Female gametophyte development** (Maruyama et al., 2010)		**BRI1–5** (Hong et al., 2008)

Length of Mediator subunits from *Homo sapiens* (Hs) was taken from Allen and Taatjes (2015). Length of Mediator subunits from *Arabidopsis thaliana* (At) was taken from TAIR database (www.arabidopsis.org).

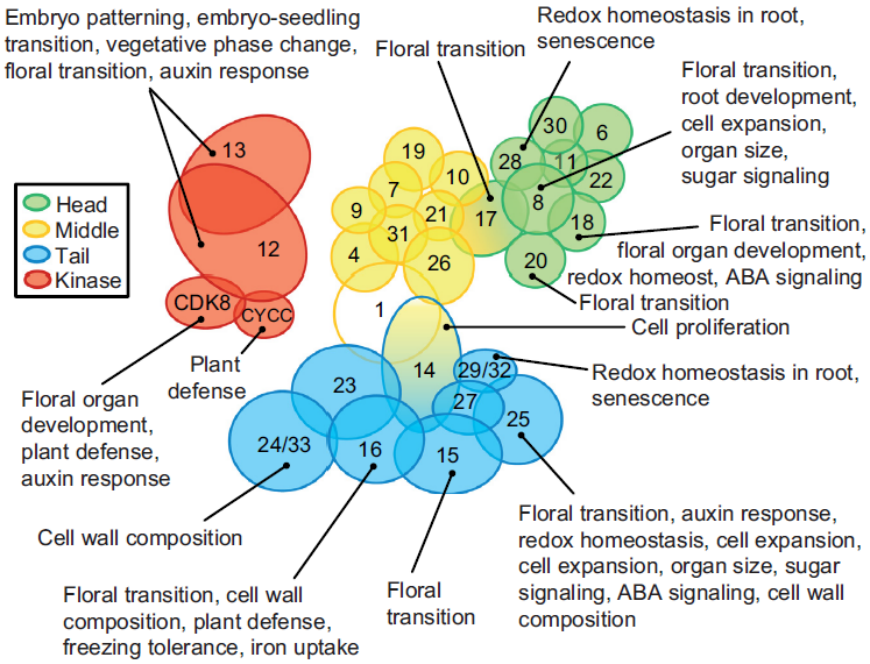

Fig. 2. Mediator functions in plant development. Submodular structure of the plant Mediator complex is depicted on the basis of tridimensional reported structures of yeast Mediator and human Mediator (Robinson et al., 2015; Tsai et al., 2014). Subunit sizes are according to predicted protein length (see Table 1). Note that Med14 and Med17 are represented in split color since the Med14 C terminal domain (CTD) belongs to the Tail module, and the Med14 N terminal domain (NTD) belongs to the Middle module. The Med17-NTD belongs to the Middle module and Med17-CTD to the Head. Med1 is absent in plants, although it has been suggested that CBP1 could act as a tetramer to play the role of Med1 in plants (Li et al., 2015a).

growth (Gillmor et al., 2014). *med8* mutants have smaller organs, due to reduced cell expansion (Xu and Li, 2012). By contrast, *med25* mutants have larger organs (with more and larger cells), as a result of an increased period of cell proliferation and cell expansion, but normal ploidy levels; plants overexpressing *MED25* show smaller organs (Xu and Li, 2011). *pft1 [med25]* mutants also have longer primary roots, and more and longer lateral roots as a result of increased cell division and cell elongation, indicating that MED25 restricts cell expansion and cell proliferation (Raya-González et al., 2014). *struwwelpeter (swp) [med14]* mutants reduce cell numbers in aerial organs by affecting the timing or window of cell proliferation, resulting in smaller aerial organs (Autran et al., 2002).

2.2. Cell wall and cell growth

MED25, along with *MED8*, *MED16*, *MED33A/MED5A*, and *MED33B/MED5B* regulate cell wall composition and growth in plants. Mutations in both *MED25* and *MED8* alter glucose-responsive gene expression, suppressing a cell elongation defect resulting from the arabinose deficiency of *mur4* seedlings (Seguela-Arnaud et al., 2015). Loss of *MED16* allows seedlings to be more resistant to perturbations in cellulose organization, partly through upregulation of pectin methyl esterification inhibitors (Sorek et al., 2015). *MED33A/MED5A* and *MED33B/MED5B* are important for synthesis of lignin, a class of phenylpropanoid polymer that plays an essential role in plant growth through its interaction with cellulose, but which also interferes with extraction of polysaccharides from plant biomass, which consists primarily of cellulose. *med33a* and *med33b* mutants result in increased expression of phenylpropanoid biosynthetic genes and hyperaccumulation of phenylpropanoids, the precursors of lignins (Bonawitz et al., 2012). The stunted growth and lignin biosynthesis mutant *reduced epidermal fluorescence 8 (ref8)*, is partially rescued by mutations in a *med5a;med5b* double mutant, because the loss of these Mediator subunits alters the lignin biosynthetic pathway, resulting in a novel lignin which interferes less with polysaccharide extraction from cellulose. Thus, *med5a;med5b;ref8* triple mutants allow production of biomass and facilitate polysaccharide extraction (Bonawitz et al., 2014).

MED25 also participates in regulation of reactive oxygen species (ROS). This was first discovered due to the role of *MED25* in root hair growth: *pft1* mutants have short root hairs, a defect that can be rescued by application of H_2O_2, which activates Ca^{2+} channels to focus tip growth (Foreman et al., 2003). MED25 was found to promote levels of H_2O_2 producing peroxidases, which in turn regulate cell wall modifying enzymes that promote cell elongation (Sundaravelpandian et al., 2013). A recent study provided a mechanistic link between ROS and Mediator. MED10A, MED28 and MED32, representatives of each core Mediator module (Fig. 2), form covalent oligomers linked by intermolecular disulfide bonds which can be reduced by thioredoxin (TRX)- and glutaredoxin (GRX)-dependent systems, implicating a redox regulation of Mediator function (Shaikhali et al., 2015b). *med28* and *med32* mutants show phenotypes in processes regulated by redox changes: senescence and root development, respectively. *med28* mutants showed an early senescent phenotype associated with earlier upregulation of the *SENESCENCE ASSOCIATED GENE 12 (SAG12)* and elevated levels of H_2O_2 in leaves. On the other hand, the reduction in root length in response to H_2O_2 treatment was significantly stronger in *med32* mutants compared to WT plants; this effect correlates with a defective redox behavior of MED32 protein, which is probably oligomerized in oxidizing conditions (Shaikhali et al., 2015a).

3. *MED12, MED13, MED18,* and *MED25* are major regulators of plant development

Since the biochemical identification of the Mediator complex in *Arabidopsis thaliana* (Bäckström et al., 2007), there have been an increasing number of reports on the role of Mediator in different

plant processes, mostly in Arabidopsis. Besides its function in plant immunity (reviewed in An and Mou (2013)) and sensing environmental nutrients like iron (Yang et al., 2014; Zhang et al., 2014), Mediator is important for regulation of developmental timing during the plant life cycle, as well as hormone responses.

3.1. Regulation of developmental phase transitions

Plants and animals go through multiple developmental phases during their life cycle, including embryogenesis, a post-embryonic (juvenile) phase, and an adult (or reproductive) phase. Seed plants have a more complicated transition from the embryo to juvenile phase, as seed desiccation and subsequent germination are superimposed upon the transition from embryogenesis to vegetative growth. After germination, the vegetative phase of plants consists of juvenile and adult stages, the length of which can vary greatly between species (reviewed in Poethig (2013)). In plants, much research on the role of Mediator has focused on regulating temporal aspects of development, with the majority of research on the timing of the vegetative to reproductive transition.

The Arabidopsis Mediator CDK8 module subunits *CENTER CITY (CCT) [MED12]* and *GRAND CENTRAL (GCT) [MED13]* were first identified based on their regulation of the timing of pattern formation in early embryogenesis: *cct* and *gct* mutants delay specification of the shoot apical meristem, vascular tissue, and ground tissue (Gillmor et al., 2010). Subsequently, *MED12* and *MED13* were shown to regulate the seed-to-seedling transition: *cct* and *gct* mutants show heterochronic misexpression of numerous late embryogenesis seed storage genes in seedlings, with *cct;gct* double mutants having a synergistic effect on seed gene misexpression (Gillmor et al., 2014). Surprisingly, *cct;gct* seedlings show a complete growth arrest. This result suggests that one of the principal roles of *MED12* and *MED13* is to promote the seedling (growth) program by repressing the seed (dormancy) program after germination (Gillmor et al., 2014). Phytohormones have long been known to play a key role in regulating this seed-to-seedling transition: abscisic acid (ABA) promotes late embryo identity and seed dormancy, while gibberellin (GA) promotes germination, and represses embryo identity (reviewed in Holdsworth et al. (2008)). *MED12* and *MED13* repress seed gene expression in seedlings in parallel with GA (Gillmor et al., 2014). *MED18* promotes ABA responses by increasing levels of *ABSCISIC ACID INSENSITIVE 4 (ABI4)* and *ABSCISIC ACID INSENSITIVE 5 (ABI5)*, two transcription factors required for ABA responses; loss of *MED18* makes seed insensitive to germination inhibition by ABA (Lai et al., 2014). *MED25* has the opposite role of *MED18*, as *MED25* represses transcriptional responses to ABA by decreasing ABI5 protein abundance. ABA responsive genes are greatly increased in the *med25* mutant (Chen et al., 2012), and thus *MED25* may serve to repress seed specific genes during seedling development, similar to *MED12* and *MED13*.

The transition from the juvenile to adult vegetative phase is controlled by the microRNA miR156 and its targets, the *SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL)* transcription factors. miR156 levels are high in the early vegetative stage, and decrease during shoot development, with a concomitant increase in *SPLs*. *SPLs* trigger adult leaf traits and flowering partly through increasing transcription of the microRNA miR172 (reviewed in Poethig (2013)). *MED12* and *MED13* regulate the juvenile to adult vegetative transition by fine tuning the levels of miR156 during vegetative development. *cct* and *gct* mutants have higher levels of miR156, decreased levels of *SPL* transcription factors, and decreased miR172, resulting in an extended juvenile vegetative phase (Gillmor et al., 2014) (Fig. 3). Similar to *cct* and *gct*, rice *super apical dormant (sad1)* mutants show higher levels of miR156 and a decrease of miR172, resulting in a delayed juvenile-to-adult transition. Interestingly, *SAD1* encodes an RNA Pol I subunit that interacts with Mediator through direct binding to the MED4 subunit, linking Mediator regulation of vegetative phase change to rRNA production (Li et al., 2015b).

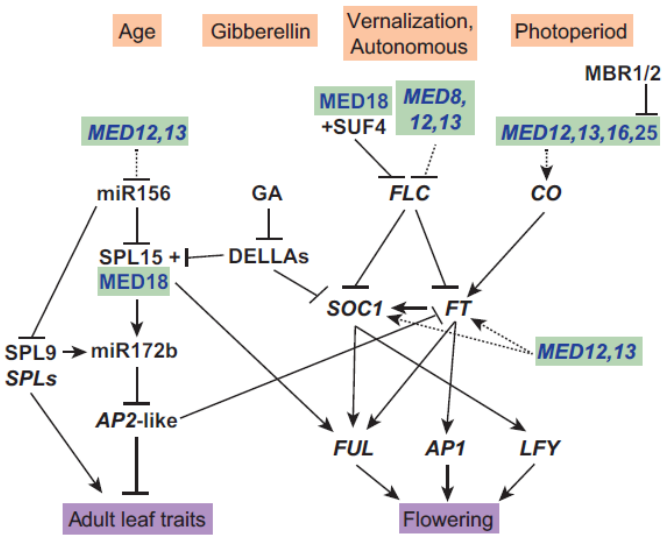

Fig. 3. Mediator regulation of vegetative and reproductive transitions. A simplified model of the genetic network regulating vegetative phase change and the transition to flowering, showing Mediator regulation of components of the network discussed in this review. Mediator regulation of transcription that has not been determined to be direct or indirect is shown with dotted lines. Direct regulation of transcription or protein stability is shown with solid lines. Protein-protein interactions are denoted with '+'. The different pathways controlling vegetative and reproductive transitions are shown with an orange background, Mediator components are shown with a green background, and phenotypic outputs are shown with a purple background. Figure modified from Kim et al., (2009).

The transition from the vegetative to the reproductive stage is controlled by at least five genetic pathways: photoperiod, vernalization, gibberellin, aging, and the autonomous pathway (Fig. 3). The photoperiod pathway responds to day length and light quality, through regulation of the flowering gene *CONSTANS* (*CO*) by photoreceptors and circadian clock-related genes. Vernalization refers to the induction of flowering by exposure to a long period of cold, which leads to epigenetic silencing of the flowering repressor *FLOWERING LOCUS C (FLC)*. Gibberellin signaling is essential for flowering, inducing the floral integrator genes *LEAFY (LFY)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*. The aging pathway is mediated by miR156, via *SPLs*, which promote transcription of the floral integrators *LFY* and *FRUITFULL (FUL)* and the microRNA miR172. The autonomous pathway represses *FLC* expression by regulating chromatin modification and RNA processing (reviewed in Srikanth and Schmid (2011)).

Various Mediator mutants have late flowering phenotypes, specifically *med8* (Kidd et al., 2009), *cryptic precocious (crp)/cct [med12]* (Imura et al., 2012; Gillmor et al., 2014), *macchi-bou2 (mab2)/gct [med13]* (Ito et al., 2011; Imura et al., 2012; Gillmor et al., 2014), *med15* (Canet et al., 2012), *med16* (Knight et al., 2008), *med17* (Kim et al., 2011), *med18* (Kim et al., 2011; Zheng et al., 2013; Lai et al., 2014), *med20a* (Kim et al., 2011) and *phytochrome and flowering time 1 (pft1) [med25]* (Cerdán and Chory, 2003). Due to the complex and interconnected nature of flowering control, some of these Mediator mutants affect multiple flowering pathways (Fig. 3).

MED25 participates in the photoperiod pathway. *pft1* mutants suppress the early flowering phenotype of *phyB* mutants, indicating that *MED25* is essential for the phyB regulation of flowering. *pft1* mutants have reduced transcript levels of *CO* and *FT*, whereas the protein levels of phyA and phyB remain unaffected, suggesting that *MED25* regulates *FT* downstream of *PHYB* (Cerdán and Chory, 2003). Given that the relative contributions of the three

phytochromes phyB, phyD and phyE remained unchanged in *pft1* mutants compared to WT plants, and that the quadruple *phyB phyD phyE pft1* mutant flowered significantly later than the triple *phyB phyD phyE* mutant, *MED25* can still promote flowering in the absence of these three phytochromes (Iñigo et al., 2012a). MED25 promotes flowering by enhancing light sensitivity (through its interaction with COP1 and HY5), and by modulating phyB function (Klose et al., 2012). The induction of flowering by MED25 is coupled to a mechanism called "activation by destruction", where two RING-H2 proteins, called MED25-BINDING RING-H2 PROTEIN1 (MBR1) and MBR2, target MED25 for degradation (Iñigo et al., 2012b).

MED16 is thought to act upstream of the circadian clock. The *sensitive to freezing6 (sfr6) [med16]* mutant is late flowering, and shows reduced expression of the circadian clock genes *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), *GIGANTEA (GI)*, *FLAVINBINDING, KELCH REPEAT, F-BOX1 (FKF1)*, *ZEITLUPE* (*ZTL)* and *TIMING OF CAB1 (TOC1)*, which in turn regulate the flowering genes *CO* and *FT* in the photoperiodic flowering pathway (Knight et al., 2008, 2009).

MED8, *MED18*, *MED12*, and *MED13* all act to promote flowering through downregulation of *FLC*, a floral repressor that participates in both the vernalization and autonomous pathways. *med8* and *med18* mutants are late flowering, and have increased levels of *FLC* and decreased levels of the *FLC* target *FT* (Kidd et al., 2009; Zheng et al., 2013; Lai et al., 2014). MED18 represses *FLC* expression by binding directly to the *FLC* promoter and interacting with SUPPRESSOR OF FRIGIDA 4, a transcription factor that promotes *FLC* expression (Lai et al., 2014). MED18 also acts downstream of miR156 in the age-dependent pathway, promoting flowering in short day (SD) conditions by interacting with SPL15 at the promoters of *FUL* and *MIR172B* (Hyun et al., 2016). MED18 and SPL15 directly increase expression of *FUL*, and indirectly increase expression of *FT* by promoting levels of mir172b, which represses APETALA 2-like (AP2-like) transcription factors which otherwise repress *FT* expression (Hyun et al., 2016). CDK8 module subunit mutants *crp/cct [med12]* and *mab2/gct [med13]* also result in delayed flowering. Analysis of dominant and loss of function mutants of *crp/cct* and *mab2/gct* show that *MED12* and *MED13* promote flowering in part by repressing the floral repressor *FLC*, allowing for transcription of the downstream floral activators *FT* and *SOC1* (Imura et al., 2012; Gillmor et al., 2014).

Indeed, *MED12* and *MED13* play an important role as integrators of multiple flowering pathways, and at multiple regulatory levels. In addition to allowing expression of *FT* and *SOC1* by repressing their repressor *FLC*, *MED12* and *MED13* also promote *FT* and *SOC1* expression independently of *FLC*, as *med12;flc* and *med13;flc* double mutants only partially restore *FT* and *SOC1* expression to WT levels (Imura et al., 2012; Gillmor et al., 2014). *MED12* and *MED13* also regulate the photoperiod pathway through increasing CO expression: flowering of *crp/cct* and *mab2/gct* mutants is greatly delayed under long day conditions, but is normal under SD conditions (Imura et al., 2012; Gillmor et al., 2014). *MED12* and *MED13* likely act in parallel with the gibberellin pathway, as GA treatment of *cct* and *gct* mutants can mitigate their late flowering phenotypes, while double mutants between *cct* or *gct*, and a deletion allele *(ga1–3)* of the enzyme encoding the first committed step to GA biosynthesis, have an additive effect on flowering (Gillmor et al., 2014). *MED12* is also required for normal transcript levels of *LFY*, one of the key targets of the gibberellin pathway (Imura et al., 2012). Finally, *MED12* and *MED13* regulate the aging pathway, through repression of miR156 levels, and promotion of *SPL* and miR172 expression. Part of the decrease in *LFY* and *AP1* levels in *crp/cct* and *gct* mutants is likely due to decreased levels of *SPL*s in these mutants (Imura et al., 2012; Gillmor et al., 2014).

3.2. Auxin responses

The phytohormone auxin controls many cellular and developmental processes in plants. Auxin is actively transported through plant tissues by PINFORMED (PIN) auxin efflux transporters and AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) influx transporters. Transcriptional responses to auxin are mediated by AUXIN RESPONSE FACTOR (ARF) transcription factors, and AUX/IAA proteins, which impede ARF function. Auxin responsive gene transcription occurs when auxin binds to TRANSPORT INHIBITOR RESPONSE (TIR1) receptors, which mark AUX/IAA proteins for degradation by the Ubiquitin pathway, allowing ARFs to function (reviewed in Enders and Strader (2015)). *MED12*, *MED13* and *MED25* have all been implicated in auxin transcriptional responses. In early embryogenesis, *cct [med12]* and *gct [med13]* mutants have phenotypes characteristic of *ARF* and *IAA* mutants such as *monopteros* and *bodenlos* (Gillmor et al., 2010). Consistent with this, *mab2 [med13]* embryos have decreased transcriptional auxin responses, *mab2* and *crp [med12]* plants have auxin deficient phenotypes, and *mab2* and *crp* enhance the phenotype of *pinoid*, a mutant that affects the polarity of auxin transport (Ito et al., 2011; Imura et al., 2012). Thus, directly or indirectly, *MED12* and *MED13* promote auxin transcriptional responses. *MED25* was recently shown to have the opposite role, repressing auxin dependent transcription. *pft1* mutants increase primary and lateral root growth (processes that are promoted by auxin), while overexpression of *PFT1* has the opposite effect. As expected from their phenotypes, *pft1* seedlings have increased auxin responsive transcription in their roots (including increased *PIN1* expression), while *35S::PFT1* seedlings have decreased auxin responsive transcription (Raya-González et al., 2014). These results raised the possibility that *MED12* and *MED13* act through *MED25* to regulate auxin-responsive gene transcription.

A very recent study examined the molecular basis of Mediator regulation of auxin-responsive gene expression in plants (Ito et al., 2016). Double mutants between *solitary root-1 (slr-1)* (a dominant mutant allele of *IAA14* which is immune to auxin-induced degradation), and *mab2 [med13]*, *crp [med12]* and *cdk8*, showed that these mutants are epistatic to *slr-1*. Using an auxin resistant IAA14 transgene, the Tail module component MED25, as well as the Head module subunit MED17, were also shown to be required to transmit the repressive signal from IAA14. These genetic data are consistent with a model in which IAA14 transmits its repressive function through the CDK8 module, to the Head and Tail module, to inhibit auxin-responsive gene expression via ARF7 and ARF19. Ito et al. (2016) demonstrated the molecular output of this system using *LATERAL ORGAN BOUNDARIES-DOMAIN16 (LBD16)*, a target of ARF7 and ARF19, whose expression is repressed by MED13, and promoted by the interaction of MED25 and ARF7. Both MED13, and MED25 (in cooperation with ARF7), were demonstrated to bind to the auxin-responsive element upstream of *LDB16*, in order to repress (MED13) or promote (MED25) auxin responsive gene expression. MED13 and IAA14 were shown to interact in vivo via the transcriptional co-repressor TOPLESS (TPL), which had previously been shown in regulate auxin responsive gene expression through interation with IAA proteins (Szemenyei et al., 2008). All together, these results suggest a model of auxin regulated gene expression via Mediator, where in low auxin conditions the repressive signal from an IAA protein is transduced through TPL to MED13 and the CDK8 module of Mediator, which sterically prevents interation of Core Mediator with RNA pol II, preventing transcription. Higher levels of auxin in the cell would induce degradation of the IAA protein, somehow causing disassociation of the CDK8 module and possibly conformational changes in Core Mediator, allowing ARF proteins, through their association with MED25, to promote transcription of auxin target genes such as LBD16 (Ito et al., 2016). These data are satisfying because they fit with the general idea of

the CDK8 module as a repressor of gene expression, and Core Mediator as a promoter of gene expression (Allen and Taatjes, 2015).

However, when we consider all functional data for the role of *MED12*, *MED13* and *MED25* in auxin-responsive gene expression, the picture becomes more complex. As mentioned above, *med12* and *med13* embryos have multiple phenotypes demonstrating that, during embryogenesis, *MED12* and *MED13* promote auxin responses (Gillmor et al., 2010; Ito et al., 2011; Imura et al., 2012). By contrast, *med25* mutants were shown to have an increase in the auxin responsive markers DR5::GFP and PIN1-GFP, while overexpression of *MED25* had the opposite effect, decreasing auxin responsive marker expression (Raya-González et al., 2014). Thus, the previously reported phenotypes of *med12*, *med13* and *med25* mutants are not what would be expected based on the molecular mechanism above. Taken together, the data suggest that Mediator subunits may promote or repress auxin-responsive gene expression in a tissue-specific manner, perhaps with outputs that depend on the specific IAA-ARF module active in a particular tissue.

4. Mechanistic studies of Mediator in plants

Several recent studies on the role of Mediator in regulating hormone and pathogen responses have contributed to a mechanistic understanding of how Mediator subunits function to regulate gene expression at the molecular level.

4.1. MED18, MED25 and CDK8

Lai et al. (2014) dissected the function of MED18 in repression of *FLC* and activation of *ABI5*. MED18 is found at the *SUPPRESSOR OF FRIGIDA 4 (SUF4)* binding site in the *FLC* promoter, the TATA box, the coding region, and the terminator region. *SUF4* promotes *FLC* expression, and MED18 can interact with SUF4, suggesting that MED18 interacts with SUF4 at the *FLC* promoter to prevent SUF4 activation of *FLC* transcription. Consistent with a repressive role of *MED18* on *FLC* transcription, Histone 3 Lysine 36 tri-methylation ($H3K36me^3$) in *FLC* was increased in *med18* mutants compared to WT, demonstrating that MED18 acts to decrease the presence of this positive mark of transcription. MED18 and SPL15 interact at the promoters of *FUL* and *MIR172B* to promote their expression; this interaction is inhibited in the presence of REPRESSOR OF GA (RGA) (Hyun et al., 2016). By contrast, MED18 promotes *ABI5* transcription, and is constitutively present at the ABI4 binding site in the *ABI5* promoter region. In the presence of ABA, MED18 was also recruited to the TATA box, coding region, and terminator of *ABI5*. MED18 interacts with ABI4, suggesting that perhaps ABA induces ABI4 to recruit MED18 to the *ABI5* gene. MED18 was also shown to be required for RNA Pol II occupancy at *ABI5*, and for recruitment of $H3K36me^3$, a positive mark for transcription. Thus, MED18 was shown to positively and negatively regulate transcription by interaction with transcription factors, occupancy at regulatory and coding regions of genes, RNA Pol II recruitment, and recruitment of epigenetic marks (Lai et al., 2014).

MED25 has also been shown to repress ABA-responsive transcription (Chen et al., 2012). *med25* seed are more sensitive than WT seeds to ABA inhibition of germination. This increase in ABA sensitivity is not due to an increase in *ABI5* mRNA transcripts in *med25* mutants, yet the ABA sensitivity of *med25* mutants is indeed due to *ABI5*, because analysis of *med25;abi5* double mutants showed that the ABA insensitive phenotype of *abi5* mutants is epistatic to the *med25* ABA sensitive phenotype. Further experiments showed that MED25 protein acts to decrease ABI5 protein levels in the absence of ABA, possibly through proteosome-mediated degradation. At low levels of ABA, MED25 and ABI5 interact strongly, and MED25 is present at higher levels at the promoter of the ABA responsive gene *EM6*. At high ABA concentration, there is less MED25-ABI5 interaction, and ABI5 predominates at the *EM6* promoter (Chen et al., 2012). These results suggest that, when ABA levels are low, MED25 exerts its effect on ABA responsive gene expression by targeting ABI5 for proteolysis, and also by preventing ABI5 access to the *EM6* promoter. High ABA levels would prevent this interaction. Whether ABA affects the stability of MED25 protein, or the ability of MED25 to cause degradation of ABI5, remains to be determined. MED25 has also been shown to interact with proteins involved in drought responses, through its ACID domain (Elfving et al., 2011). MED25 interacts with DROUGHT RESPONSIVE ELEMENT PROTEIN B 2A (DREB2A), ZINC FINGER HOMEODOMAIN 1 (ZFHD1), and PHOSPHATE STARVATION RESPONSE LIKE1 (PHL1); the genes encoding all of these proteins are induced in response to drought stress. *med25* mutants are more resistant to drought stress than WT plants, and show upregulation of drought responsive genes (Elfving et al., 2011). This is consistent with the role of *MED25* in repressing ABA-responsive transcription, as ABA is one of the principle hormones involved in promoting drought stress (Chen et al., 2012).

Another recent study of the role of Mediator in disease resistance has illuminated a number of ways in which *CDK8* regulates gene expression. *CDK8* (also known as *CDKE* in plants) was originally identified through mutant analysis as *HUA ENHANCER3 (HEN3). CDK8 [HEN3]* is required for AGAMOUS expression in flowers, and HEN3 was demonstrated to have CDK8 kinase activity (Wang and Chen, 2004). CDK8 was also demonstrated to repress gene expression by interaction with the co-repressor LEUNIG and its partner SEUSS (Gonzalez et al., 2007). Zhu et al. (2014) recently discovered that *CDK8*, as well as *CYCC*, *MED12*, and *MED13* regulate the response to both bacterial and fungal pathogens, though the four components did not always have the same function for all pathogens. CDK8 was shown to physically interact with MED25, and also with CYCCA (CYCC1;1) and CYCCB (CYCC1;2), the two CYCC proteins of Arabidopsis. Both CDK8 and MED25 were demonstrated to interact with WAX INDUCER1 (WIN1) in order to promote epidermal wax deposition; WIN1 is part of the ERF transcription factor family, many members of which have previously been shown to interact with MED25 (Çevik et al., 2012). In response to pathogen attack, CDK8 was shown to increase its occupancy at the upstream regulatory region, TATA box, and terminator sites of *PLANT DEFENSIN1.2 (PDF1.2)*. CDK8 was also shown to recruit RNA Pol II to these same sites, and this recruitment depended on the kinase activity of CDK8. However, kinase activity was not required for resistance to all pathogens, nor for all genes controlled by *CDK8*, demonstrating that CDK8 regulation of transcription can be both dependent or non-dependent on its kinase activity (Zhu et al., 2014).

4.2. Does Med1 exist in plants?

The middle module subunit Med1 plays multiple roles in animal development, due to its ability to interact with transcription factors such as PPAR-γ and GATA-1, through its LxxLL motif (Crawford et al., 2002; Zhu et al., 1997). Interestingly, this subunit is apparently absent in plants, with the exception of the red alga *Cyanidioschyzon merolae*, suggesting Med1 functions are lost in plants or they are carried out by other subunits (Mathur et al., 2011).

A recent study showed a role for Mediator in pollen tube guidance, and suggests that CCG BINDING PROTEIN 1 (CBP1) plays the role of Med1 in plants. CENTRAL CELL GUIDANCE (CCG) and CBP1 are essential proteins for pollen tube attraction; both genes positively co-regulate cysteine-rich peptides (CRPs) in the central cell and the synergid cells, contributing to pollen tube attraction. CBP1

interacts with CCG, Mediator subunits, RNA Pol II and central cell-specific AGAMOUS-like transcription factors. Thus, it has been proposed that the interaction of CBP1 with CCG recruits Mediator and the transcription initiation machinery to the promoters of *AGAMOUS*-like transcription factors to promote pollen tube guidance to the central cell. The interaction of CBP1 with AGL80 and AGL81, proteins involved in endosperm development, suggest that CBP1 also has a role in endosperm development (Li et al., 2015a). Since CBP1 interacts with MED7 and MED9, similar to Med1 in yeast and human, CBP1 may play the role of Med1 in plants. Although MED1 is five times larger than CBP1, the latter can form a tetramer in vitro. CBP1 shows almost no sequence similarity to MED1, but Mediator subunits between different species show low sequence identity due to the high proportion of disordered regions in Mediator proteins (Li et al., 2015a). The essential role of CBP1 in sexual reproduction resembles the essential role of Med1 in spermatogenesis, placenta and embryo development in animals (Huszar et al., 2015; Ito et al., 2000; Landles et al., 2003).

5. Mediator subunits with no known function in development

Plant-specific subunits such as MED34, MED35, MED36 and MED37 have functions distinct from transcription, such as DNA replication and RNA processing, and so far have no described function in plant development (Barneche et al., 2000; Kang et al., 2009; Kobbe et al., 2008). In addition, all subunits of the Middle module of Core Mediator, as well as several Head module subunits, have yet to be ascribed any function in development (Fig. 2 and Table 1). One possibility is that these subunits might serve a primarily structural (essential) function, where their loss would affect Core Mediator activity in many or all contexts. For example in animals, MED11 and MED22 serve a more ubiquitous function by stabilizing the transcription initiation complex (Seizl et al., 2011), MED26 has docking sites for both transcription elongation factors and for the general transcription initiation factor TFIID, and functions as a switch from initiation to elongation (Takahashi et al., 2011), and MED17 plays an essential role in switching between transcription and DNA repair by Nucleotide Excision Repair (NER) (Kikuchi et al., 2015). Mutations in subunits with a ubiquitous function might be expected to lead to gametophytic or embryo lethal phenotypes, and would not have been recovered in genetic screens targeting other phases of development. A simple way to test this hypothesis would be to systematically look for developmental phenotypes of mutations in all Mediator subunits in Arabidopsis.

6. Questions and challenges for identifying specific roles of Mediator in plant development

Research on Mediator in plants is currently confronting a universal issue in gene regulation and developmental biology: how are cellular and tissue-specific signals perceived and transduced into transcriptional outputs? In some cases, Core Mediator is recruited to target genes through interaction of one subunit of Mediator with a specific transcription factor already bound to a promoter or enhancer, for example in the case of MED18 recruitment by SPL15 (Hyun et al., 2016). Specific Mediator subunits may also continually present at their target genes, and act in cooperation with another transcription factor: MED18 is constitutively present at several sites in the *FLC* and *ABI5* genes, where it can interact with SUF4 or ABI4 to repress or promote transcription of *FLC* and *ABI5* (respectively) (Lai et al., 2014). In the future, it will be important to gain a mechanistic knowledge of interactions between the many TFs whose functions have been described in plants, and specific Mediator subunits.

Of all the Mediator subunits, the proteins of the CDK8 module may be the most mysterious, and also the most important for development. Biochemical and structural studies have demonstrated that the CDK8 module can act as a repressor by sterically blocking the initial association of Core Mediator with RNA pol II (Elmlund et al., 2006; Tsai et al., 2013). MED12 has also been demonstrated to recruit histone methylation in order to silence gene expression (Ding et al., 2008). Yet the CDK8 module, and in particular CDK8 itself, have also been shown to promote gene expression (reviewed in Nemet et al. (2014)). In yeast, the CDK8 module was shown to be present at most protein coding genes (Andrau et al., 2006). Thus, regulation by the CDK8 module may be more complex than just proximity to target genes. A couple of examples from yeast have shown that CDK8 module components regulate other Mediator components, or are themselves targeted by signaling pathways. CDK8 regulates the iron homeostasis pathway through phosphorylation of MED2 (van de Peppel et al., 2005), while MED13 itself is a target of the Ras pathway, which targets MED13 for degradation via Ubiquitination (Chang et al., 2004; Davis et al., 2013), demonstrating that targeted degradation is one mechanism for CDK8 module regulation.

Which components of Mediator play discrete roles in developmental biology, and which are factors required for general regulation of transcription? One difficulty in judging the extent of pleiotropy, at least based on morphological criteria, is masking of more subtle phenotypes by severe ones. In animals, especially Drosophila, it is common to study gene function using mosaics of wild type and mutant tissue (Xu and Rubin, 2012). In plants, there are fewer examples of sector analysis (e.g. Poethig and Sussex, 1985; Heidstra et al., 2004). Single tissue or inducible loss of function studies of Mediator subunits in plants would be one way to determine their role in discrete aspects of development, as have previously been useful in studying the role of Med1 in animal development (Landles et al., 2003; Chen et al., 2010; Huszar et al., 2015). Another important tool to determine interactions between individual Mediator subunits and their target DNA elements will be genome level molecular studies at single cell type resolution (Adrian et al., 2015; Efroni et al., 2015). Given the increasing interest in the role of Mediator in plants, the next few years should lead to important insights into the mechanism of Mediator function, as well as its role in development.

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9.3. Research article #2 (First author)

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Convergent repression of miR156 by sugar and the CDK8 module of Arabidopsis Mediator

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ABSTRACT

In Arabidopsis, leaves produced during the juvenile vegetative phase are simple, while adult leaves are morphologically complex. The juvenile to adult transition is regulated by miR156, a microRNA that promotes juvenility by impeding the function of *SPL* transcription factors, which specify adult leaf traits. Both leaf derived sugars, as well as the Mediator Cyclin Dependent Kinase 8 (CDK8) module genes *CENTER CITY (CCT)/MED12* and *GRAND CENTRAL (GCT)/MED13*, act upstream of miR156 to promote the juvenile to adult transition. However, it is not known whether sugar, *CCT* and *GCT* repress miR156 independently, as part of the same pathway, or in a cooperative manner. Here we show that sugar treatment can repress *MIR156* expression in the absence of *CCT* or *GCT*. Both *cct* and the photosynthetic mutant *chlorina1* (*ch1*) (which decreases sugar synthesis) exhibit extended juvenile development and increased *MIR156A* and *MIR156C* expression. Compared to *ch1* and *cct* single mutants, the *ch1 cct* double mutant has a stronger effect on juvenile leaf traits, higher *MIR156C* levels, and a dramatic increase in *MIR156A*. Our results show that sugar and the CDK8 module are capable of regulating *MIR156* independently, but suggest they normally act together in a synergistic manner.

1. Introduction

The timing of developmental transitions in plants is controlled by both endogenous and environmental signals (Bäurle and Dean, 2006; Huijser and Schmid, 2011; Poethig, 2013). The juvenile-to-adult vegetative transition, also called vegetative phase change, is primarily regulated by the microRNA miR156, which targets members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* transcription factor family (Rhoades et al., 2002; Wu and Poethig, 2006; Chuck et al., 2007; Wu et al., 2009). miR156 levels are high at early vegetative stages, and are necessary and sufficient for the juvenile phase (Wu and Poethig, 2006; Wu et al., 2009). miR156 expression decreases during development, allowing *SPL* transcription factors to promote adult vegetative development and flowering (Wang et al., 2009; Wu et al., 2009; Yamaguchi et al., 2009). In *Arabidopsis thaliana*, miR156 is encoded by 8 different genes (*MIR156A-MIR156H*), though *MIR156A* and *MIR156C* are the only miR156 genes that are developmentally regulated, and are primarily responsible for the timing of vegetative phase change (Reinhart et al., 2002; Kozomara and Griffiths-Jones, 2011; Yang et al., 2013).

In the early 20th century, Goebel hypothesized that vegetative phase change is driven by changes in the nutritional status of the shoot (Goebel, 1908). Subsequently, Allsopp demonstrated that sugar is necessary and sufficient to produce adult leaves (Allsopp, 1952, 1953), and Röbbelen reported that the products of photosynthesis promote the transition to the adult phase (Röbbelen, 1957). Recent studies have shown that leaves are the source of the signal that represses miR156 (Yang et al., 2011), and that this signal is sugar (Yang et al., 2013; Yu et al., 2013). How sugar represses miR156 remains to be completely understood, though *HEXOKINASE1 (HXK1)* and Trehalose-6-phosphate (T6P) likely play important roles in this developmental transition, since they have an effect on miR156 expression (Wahl et al., 2013; Yang et al., 2013). The Arabidopsis *CENTER CITY (CCT)* and *GRAND CENTRAL* (*GCT)* genes also repress miR156 during vegetative development. Due to an increase in miR156 levels, *cct* and *gct* mutants show a delay in the juvenile to adult vegetative transition (Gillmor et al., 2014). *CCT* and *GCT* encode the Arabidopsis homologs of MED12 and MED13, components of the CDK8 module of Mediator that regulate transcription by modulating the association of Core Mediator with RNA polymerase II (Ding et al., 2008; Gillmor et al., 2010; Tsai et al., 2013; reviewed in Allen and Taatjes, 2015). Thus, both sugar and the CDK8 module of Mediator control the timing of vegetative development by modulating miR156 levels. Whether sugar and the CDK8 module interact to regulate miR156 is unknown.

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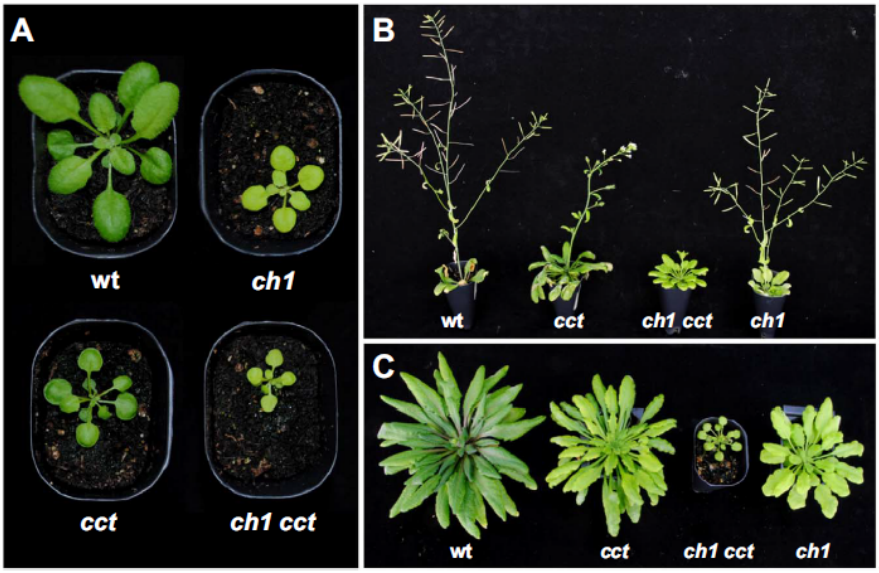

Fig. 1. *ch1* and *cct* show an additive delay on vegetative and reproductive transitions. Phenotype of wt Col, *ch1*, *cct* and *ch1 cct* plants grown in long days (LD) (A and B) or short days (SD) (C) for 20 days (A), 60 days (B) and 80 days (C).

We used functional genetic and gene expression analyses to test whether sugar and the CDK8 module of Mediator interact in their regulation of miR156. We found that *ch1* and *cct* mutants delay vegetative phase change and increase *MIR156* expression, and that the phenotypes for leaf identity, flowering, and *MIR156* levels are stronger in *ch1 cct* double mutants than in single *ch1* and *cct* mutants. Furthermore, we demonstrate that sugar is able to repress *MIR156* expression in the absence of *CCT/MED12* or *GCT/MED13* function. Our results suggest that *CCT* and sugar regulate miR156 cooperatively during vegetative development.

2. Results and discussion

2.1. ch1 cct double mutants show an increased delay in vegetative and reproductive transitions compared with ch1 and cct single mutants

In *Arabidopsis thaliana*, juvenile vegetative leaves are round with smooth margins and lack abaxial trichomes (leaf hairs), while adult leaves are elongated with abaxial trichomes and serrated margins (Fig. 1 and Table 1) (Telfer et al., 1997; Tsukaya et al., 2000). Perturbations in photosynthesis affect sugar production and consequently delay the juvenile to adult transition (Yang et al., 2013). *chlorina1* (*ch1*) plants have a mutation in the *CHLOROPHYLL A OXYGENASE* gene (*AtCAO*, At1g44446), which encodes the key enzyme for chlorophyll b biosynthesis (Espineda et al., 1999). Under long day (LD) growth conditions (16 h light), *ch1-4* mutants are yellow, grow more slowly, produce abaxial trichomes 1.5 leaves later, and flower 7 days (d) later compared to wild type (wt) plants (Fig. 1A and B and Table 1). *cct/med12* plants are also delayed in the juvenile-to-adult and flowering transitions: *cct* mutants produce 7 more leaves without abaxial trichomes and flower 22 d later than wt plants (Fig. 1A and B and Table 1). The number of both rosette and cauline leaves in *cct* mutants is significantly higher compared to wt plants, consistent with the delayed flowering transition (Table 1). By contrast, *ch1* mutants produce less rosette leaves and slightly fewer cauline leaves, yet flower later than wt (Table 1). Decreased photosynthesis in *ch1* mutants may constitute a physiological stress, inducing the switch to a reproductive meristerm earlier than in wt (leading to fewer rosette leaves), while the slow growth rate of the *ch1* inflorescence may delay flowering (as measured by the first open flower).

In order to test the genetic interaction between sugar and *cct/med12*, we constructed *ch1 cct* double mutants, and assayed their effect on vegetative and reproductive transitions in LD and short day (SD, 10 h light) conditions. The interaction between sugar and *gct/med13* was not analyzed, because we were unable to recover *ch1 gct* double mutant seedlings, perhaps due to a strong effect of the double mutant on germination or growth. Compared to *ch1* and *cct* single mutants, the effect of the *ch1 cct* double mutant on vegetative and reproductive transitions was increased (Fig. 1 and Table 1). When compared to wt in LD conditions, abaxial trichomes were delayed 1.5 leaves in *ch1*, 7.0 leaves in *cct*, and 9.7 leaves in *ch1 cct*. Flowering was delayed 6.9 days in *ch1*, 21.5 days in *cct*, and 28.9 days in *ch1 cct*. The number of both rosette and cauline leaves in *ch1 cct* double mutants was lower than in

Table 1
Vegetative and flowering traits of single and double mutants. Standard deviation is shown in brackets. Every genotype showed significantly different traits (Student's *t*-test $p < 0.05$) with the exception of those with asterisks.

	1st leaf with abaxial trichomes	n	Flowering day	n	# of rosette leaves at flowering	n	# of cauline leaves at flowering	n
Long Days (LD)								
wt Col	7.1 (0.7)	22	30.5 (1.3)	11	15 (0.9)	12	3.7 (0.5) *	12
ch1	8.6 (0.8)	21	37.4 (1.7)	11	12.6 (1.2)	10	3.0 (0.5) *	10
cct	14.1 (1.2)	16	52.0 (3.2)	13	33.0 (0.6)	7	7.6 (1.2)	12
ch1 cct	16.8 (1.9)	11	59.4 (3.1)	10	26.5 (1.7)	8	5.8 (0.7)	8
Short Days (SD)								
wt Col	11 (0.9)	6	73.2 (3.2)	6				
ch1	19.9 (1.6)	9	>90	9				
cct	24 (1.1)	6	>90	6				
ch1 cct	30 (3.6)	6	>90	6				

cct plants, an additive interaction, since *ch1* mutants produce less rosette and cauline leaves than wt plants (Table 1). SD conditions allow evaluation of effects on vegetative phase change in the absence of flowering. Similar to LD conditions, in SD *ch1 cct* plants had an additive effect on the number of leaves without abaxial trichomes, compared to *ch1* and *cct* single mutants (Fig. 1C and Table 1). The additive interactions observed between *ch1* and *cct* mutants suggest that sugar and *CCT/MED12* act separately to promote the timing of vegetative and reproductive morphological traits.

2.2. ch1 cct *double mutants have increased* MIR156A *and* MIR156C *transcript levels compared to* ch1 *or* cct *single mutants*

The transition from the juvenile to the adult vegetative phase is controlled by miR156 (reviewed in Huijser and Schmid, 2011). *MIR156A* and *MIR156C* play dominant roles within the miR156 gene family: they are the only miR156 genes that are developmentally regulated, and *mir156a mir156c* double mutants shorten the juvenile phase of development (Yang et al., 2013; Yu et al., 2013). To test if the delayed vegetative transitions seen above correlate with higher miR156 levels, we measured pri-*MIR156A* and pri-*MIR156C* transcript levels in wt, *ch1*, *cct* and *ch1 cct* mutants grown in LD (Fig. S1). In these conditions, *MIR156A* and *MIR156C* show a steady decrease from 12 to 20 d in both wt and *ch1* plants, whereas in *cct*, the decrease is much slower, and in *ch1 cct*, *MIR156* levels remain steady from 12 to 20 d (Fig. S1 A and B). At 12 days, *MIR156A* and *MIR156C* show similar expression levels among all genotypes tested; at 16 days, the expression is significantly higher in *cct* and *ch1 cct* compared to wt and *ch1* plants. At 20 days, *MIR156* levels are increased in *ch1* and *cct* single mutants compared to wt, and are even higher in *ch1 cct* double mutants (Fig. S1 C and D).

To better quantify the interaction between *ch1* and *cct* on *MIR156* expression, we measured *MIR156A* and *MIR156C* levels in SD (to avoid the effect of flowering), and over a longer time period (10–40 d), so that the relationship between *ch1* and *cct* would be more clear (Fig. 2). Wt and *ch1* showed a gradual decrease in *MIR156A* and *MIR156C* levels over the time period examined, with slightly elevated *MIR156* expression in *ch1* compared to wt (Fig. 2A and B). *MIR156A* levels at 10 days and 20 days were 8 and 16 times higher in *cct* compared to *ch1* and wt (Fig. 2C), while *MIR156C* was more than twice as high (Fig. 2D). Thus, the effect of loss of *CCT* on *MIR156* is much greater in SD than in LD, in agreement with the greater effect of *cct* on abaxial trichomes in SD compared to LD (Table 1). The larger effect of *cct* on *MIR156A* than *MIR156C* suggests that *MIR156A* contributes more than *MIR156C* to the *cct* vegetative phenotype.

The *ch1 cct* double mutant showed a dramatic effect on *MIR156A* expression. At 10 d, *MIR156A* levels were twice as high in *ch1 cct* as in *cct*; at 30 d, *MIR156A* levels are about 3 times higher; and at 40 d, *MIR156A* levels were more than 6 times higher (Fig. 2C). The *ch1 cct* double mutant showed a twofold increase in *MIR156C* compared to *cct* at 20 d, and a slight increase at 40 d (Fig. 2D). The increase in *MIR156* expression in *ch1*, *cct* and *ch1 cct* is consistent with the effects of *ch1*, *cct*, and *ch1 cct* on morphological traits of vegetative phase change (Fig. 1 and Table 1). The increase in *MIR156A* expression in *ch1 cct* double mutants is much greater than the additive interaction that would be expected if *CH1* and *CCT* regulated *MIR156A* strictly independently, suggesting that *CH1* and *CCT* interact synergistically in their regulation of *MIR156A*.

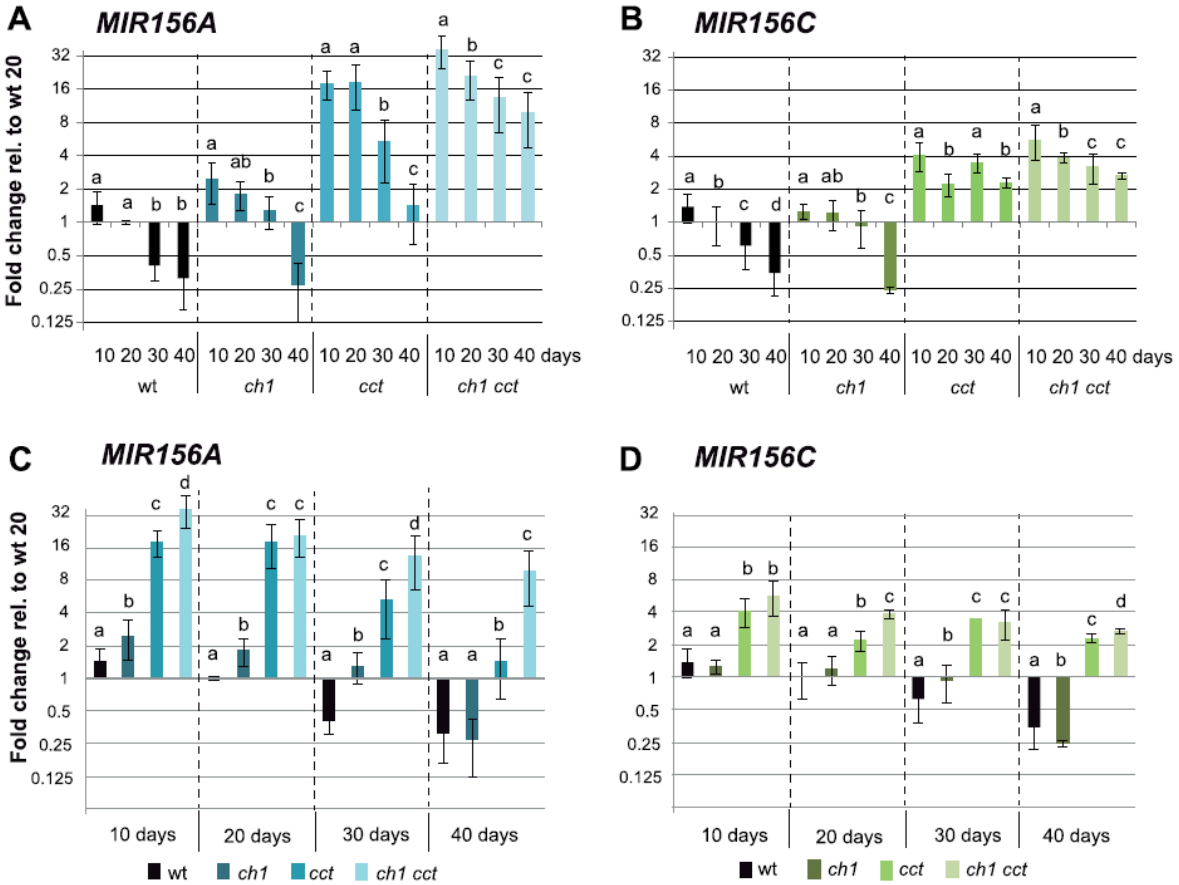

Fig. 2. *ch1* and *cct* interact in their regulation of *MIR156*. Transcript levels of *pri-miR156A* (A & C) and *pri-miR156C* (B & D) in wt, *ch1*, *cct* and *ch1 cct* plants at 10, 20, 30 and 40 days, in SD conditions. Fold change is shown relative to expression of wt at 20 days. Expression values were first normalized against *EIF4A* as a reference gene. Values shown are the mean of three technical replicates for three biological replicates. Standard deviation represented by bars. Data are grouped by genotype (A & B) and by time point (C & D). Samples that are not significantly different ($p > 0.05$, Student's *t*-test) share the same letter.

3. Sugar can repress *MIR156* in the absence of *CCT* and *GCT*

Both glucose and fructose have previously been demonstrated to reduce miR156 levels in 12d seedlings of Arabidopsis (Yang et al., 2013). To test whether glucose can repress miR156 levels in the absence of *CCT/MED12* or *GCT/MED13* function, we measured *MIR156A* and *MIR156C* transcripts by qPCR in 12d wt, *cct,* and *gct* seedlings grown on MS medium with no sugar (MS NS) and on MS medium with 10 mM glucose (MS GLU), in LD conditions (Fig. 3). In the absence of glucose, *MIR156C* levels were elevated 3–4 fold in both *cct* and *gct* seedlings compared to wt, while *MIR156A* increased ~1.5 fold in *cct* compared to wt. Interestingly, *MIR156A* levels were not significantly different in *gct* compared to wt, suggesting that *GCT* does not play an important role in regulating *MIR156A* (Fig. 3). Growth of seedlings on glucose caused a significant decrease of *MIR156A* in wt, although no significant decrease was observed for *MIR156C*. The lack of effect of glucose on *MIR156C* in wt may be attributable to rapid processing of pri-*MIR156C* transcripts: a previous study of the effect of glucose on *MIR156C* was conducted in *serrate-1* mutants, in order to slow the processing of pri-*MIR156C* transcripts, so they are better substrates for qPCR (Yang et al., 2013). Importantly, glucose did cause a significant decrease in steady-state levels of *MIR156C* in *cct* and *gct* mutants, and of *MIR156A* in *cct* mutants, demonstrating that glucose repression of these genes does not require *CCT* (*MIR156A* and *MIR156C*) or *GCT* (*MIR156C*) (Fig. 3).

3.1. Significance

Both sugar and the Mediator CDK8 module subunits *CCT/MED12* and *GCT/MED13* have previously been shown to regulate the juvenile to adult transition, through their effect on miR156, the master regulator of the timing of vegetative development (Yang et al., 2013; Yu et al., 2013; Gillmor et al., 2014). Because of the importance of miR156 for control of vegetative development in both herbacious and woody species (reviewed in Poethig, 2013), knowledge of miR156 regulation is crucial for a mechanistic understanding of developmental timing in plants. We have shown that sugar can repress *MIR156C* in the absence of *CCT* and *GCT*, and *MIR156A* in the absence of *CCT*. Loss of the *CH1* or *CCT* genes causes an increase in *MIR156* expression, as well as a delay in adult vegetative traits. Simultaneous loss of *CH1* and *CCT* has a synergistic effect on *MIR156A* levels, and an increased delay in vegetative development, compared to *ch1* and *cct* single mutants. These results suggest that sugar and the CDK8 module are capable of regulating *MIR156* independently, but have a stronger effect when they act together. This convergent regulation of miR156 by sugar and the CDK8 module of Mediator may better allow fine-tuning of miR156 levels during vegetative development, perhaps through feedback regulation between sugar signaling and Mediator subunit transcription or protein stability. A twofold increase in miR156 levels was previously shown to triple the length of the juvenile vegetative phase (Gillmor et al., 2014), demonstrating the functional importance of relatively small adjustments in miR156 levels. Multiple inputs to miR156 regulation may be useful to the plant in coordinating the vegetative phase of development with the biotic and abiotic environment.

4. Materials and methods

4.1. Genetic stocks and growth conditions

All seed stocks were in the Columbia ecotype. The CDK8 module mutant lines used in this article were *gct-2* (ABRC stock #CS65889) and *cct-1* (ABRC stock #CS65890), which were described in Gillmor et al. (2010). *ch1-4* was provided by Scott Poethig. *cct/+* and *gct/+* plants were crossed to *ch1/-* plants to obtain the double homozygous mutants in the F3 generation; *cct* and *gct* mutations were genotyped using dCAPS markers (Gillmor et al., 2014). Seeds were sown on a mixture of vermiculite (GRACE MAN-FIN), perlite (AGROL125) and sunshine mix (PREMEZ FWSS3) (1:1:3 v/v/v); or ½ MS plates; and placed at 4 °C for 3 days, before moving flats or plates to Percival growth chambers. Plants were grown either under long days (LD) (16 h light) or short days (SD) conditions (10 h light) at a constant 22 °C under a 3:1 ratio of standard Philips F17T8/TL741 lamps and Osram Lumilux Deluxe Daylight 18W/954 fluorescent lamps (170–180 mol/m^2/s). For measuring *MIR156* expression in sugar, seedlings were grown in LD on plates with MS medium with no sugar and plates with MS medium containing 10 mM Glucose.

4.2. Morphological analyses

Heteroblasty traits such as number of leaves and the presence of abaxial trichomes were measured at flowering time, to allow leaves to reach their final size and shape. Flowering time was counted from the day seeds were placed in the growth chamber, to the day of the first open flower. The presence of abaxial trichomes was scored using a dissecting microscope.

4.3. Expression analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and reverse transcribed into cDNAs using Super Script II Reverse Transcriptase (Invitrogen). *MIR156A* and *MIR156C* expression was tested by real-time PCR using SYBR Green I in a Light Cycler 480 instrument II from Roche following the instrument instructions. Transcript levels were normalized against *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A* (*EIF4A*). Relative quantifi-

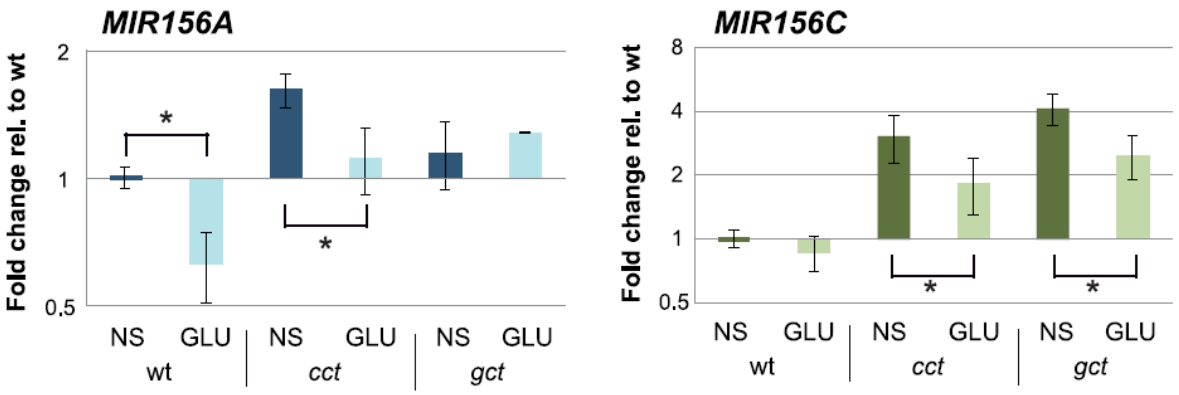

Fig. 3. Sugar can repress *MIR156* in the absence of *CCT* and *GCT*. Transcript levels of *pri-miR156A* and *pri-miR156C* in WT Col, *cct* and *gct* plants at 12 days grown in long days in the absence of sugar (No Sugar, NS) or in the presence of 10 mM Glucose (GLU). Expression values were first normalized against the reference gene *EIF4A*. Values shown are the mean of three technical replicates for three biological replicates. Standard deviation represented by bars. Asterisks indicate significant difference ($p < 0.05$, Student's *t*-test) between samples.

cation was analyzed by Pfaffl method (Pfaffl, 2001). Sequences of the primers are listed in the Supplementary Table 1.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2017.01.007.

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