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Identification of the maize Mediator CDK8 module and transposon-mediated mutagenesis of *ZmMed12a*

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ABSTRACT Mediator is a conserved transcriptional co-activator that links transcription factors bound at enhancer elements to RNA Polymerase II. Mediator-RNA Polymerase II interactions can be sterically hindered by the Cyclin Dependent Kinase 8 (CDK8) module, a submodule of Mediator that acts to repress transcription in response to discrete cellular and environmental cues. The CDK8 module is conserved in all eukaryotes and consists of 4 proteins: CDK8, CYCLIN C (CYCC), MED12, and MED13. In this study, we have characterized the CDK8 module of Mediator in maize using genomic, molecular and functional resources. The maize genome contains single copy genes for *Cdk8, CycC,* and *Med13,* and two genes for *Med12*. Analysis of expression data for the CDK8 module demonstrated that all five genes are broadly expressed in maize tissues, and change their expression in response to phosphate and nitrogen limitation. We performed *Dissociation (Ds)* insertional mutagenesis, recovering two independent insertions in the *ZmMed12a* gene, one of which produces a truncated transcript. Our molecular identification of the maize CDK8 module, assays of CDK8 module expression under nutrient limitation, and characterization of transposon insertions in *ZmMed12a* establish the basis for molecular and functional studies of the role of these important transcriptional regulators in development and nutrient homeostasis in *Zea mays*.

KEY WORDS: *Zea mays, Mediator, Cdk8, Med12, Med13, CycC*

Introduction

Transcriptional regulation plays an essential role in almost all aspects of development and physiology, including responses to biotic and abiotic environments. One key regulator of transcription is Mediator, a multiprotein complex conserved in yeast, plants and animals, which was initially identified based on its requirement for transcription of virtually all protein-coding genes (Kelleher *et al.*, 1990; Flanagan *et al.*, 1991; Bourbon, 2008). The Core Mediator consists of Head, Middle and Tail domains, and typically functions as a transcriptional co-activator, linking transcription factors bound at upstream enhancer elements to RNA polymerase II (RNA pol II) (reviewed in Yin and Wang, 2014; Allen and Taatjes, 2015). The Head and Middle domains interact with RNA pol II, while the Tail domain is thought to interact with specific transcription factors (Tsai *et al.*, 2014; Robinson *et al.*, 2015; Plaschka *et al.*, 2015; reviewed in Larivière *et al.*, 2012). A fourth Mediator module shows transient association with Core Mediator and often acts to repress transcription. This Cyclin Dependent Kinase 8 (CDK8) module is composed

Abbreviations used in this paper: BLAST, basic local alignment search tool; CDK8, cyclin-dependent kinase 8; UTR, untranslated region.

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of the proteins MED12, MED13, CYCLIN C (CYCC), and CDK8 (reviewed in Björklund and Gustafsson, 2005). In agreement with the variable association of the CDK8 module with Core Mediator, purification of Mediator from *Arabidopsis thaliana* yielded both conserved Core Mediator subunits, as well as subunits unique to *Arabidopsis*, but did not include components of the CDK8 module (Bäckström *et al*., 2007).

In yeast and animals, components of the CDK8 module can regulate transcription in several ways, with different subunits playing different roles. One mechanism for transcriptional repression involves steric inhibition, where the CDK8 module occupies the Core Mediator pocket that binds RNA pol II, thereby preventing interaction of Core Mediator and RNA pol II (Elmlund *et al*., 2006; Tsai *et al*., 2013). Transcriptional repression by this steric mechanism has the potential to be dynamic, as the occupancy of the RNA pol II binding pocket can be modulated during subsequent rounds of assembly of the Mediator-RNA pol II holoenzyme (reviewed in Allen and Taatjes, 2015). This steric mechanism involves all four units of the CDK8 module, with the MED13 subunit playing the most important role, interacting directly with the Middle domain of Core Mediator (Knuesel *et al*., 2009; Tsai *et al*., 2013). The MED13 subunit also serves an important function in regulation of CDK8 module stability: phosphorylation of a conserved phosphodegron site in MED13 can lead to recognition by a ubiquitin ligase complex, and subsequent degradation (Davis *et al*., 2013).

In *Arabidopsis*, components of the CDK8 module were initially identified by their requirement for development, and affect the response to fungal pathogens and cellular stress. Mutations in *CDK8* were identified as enhancers of the phenotype of the floral homeotic mutant *hua1hua2,* and thus were named *hua enhancer 3 (hen3)*. *hen3* mutants affect floral organ identity, as well as leaf size and cell shape, and the HEN3 protein was demonstrated to have CDK8 kinase activity (Wang and Chen, 2004). CDK8 also regulates retrograde signaling from the mitochondria to the nucleus in response to H_2O_2 and cold stress (Ng *et al*., 2013), and *CDK8*, *MED12* and *MED13* are required for the response to both fungal and bacterial pathogens (Zhu *et al*., 2014).

Mutations in *MED12* and *MED13* were initially reported from a genetic screen for regulators of pattern formation in *Arabidopsis* embryogenesis, and were named *center city (cct)* and *grand central (gct),* to reflect the increased size of the shoot apical meristem (SAM) in these mutants. *cct* and *gct* mutants delay the timing of pattern formation during embryogenesis, rather than affecting pattern formation *per se*- the increased size of the SAM in *cct* and *gct* mutants can be attributed to its formation later in embryogenesis compared to the wild type (wt) (Gillmor *et al*., 2010). The delayed formation of the SAM may be related to auxin signaling, as both the *med13* allele *macchi-bou2 (mab2)*, and the *med12* allele *cryptic precocious* (c*rp*) act as enhancers of a mutation in the auxin dependent kinase *PINOID* (Furutani *et al*., 2004; Ito *et al*., 2011; Imura *et al*., 2012), and *med12* and *med13* mutants display reduced auxin responses in roots and leaf primordia (Raya-González *et al*., 2017). Importantly for mechanistic studies of CDK8 module function in *Arabidopsis*, Ito *et al.,* (2011) demonstrated that the MED13 and CDK8 proteins are both able to interact with Cyclin C, as has previously been demonstrated in *Drosophila* (Loncle *et al*., 2007). Consistent with studies showing auxin-related phenotypes for CDK8 module mutants, *MED12, MED13*, and *CDK8* are involved in auxin transcriptional responses, and MED13 relays signals from IAA14 to repress the auxin responsive transcription factors ARF7 and ARF19 (Ito *et al*., 2016).

In addition to affecting the timing of pattern formation in embryogenesis, *MED12* and *MED13* also regulate the timing of post-embryonic phase transitions in *Arabidopsis*. A dominant allele of *med12* (c*rp-1D*) was isolated in a genetic screen for enhancers of the early flowering phenotype conditioned by overexpression of the florigen *FT* (Imura *et al*., 2012). Loss of function mutants in *crp/cct* and *gct* show late flowering due to overexpression of the floral repressor *FLOWERING LOCUS C (FLC)*, as well as decreased expression of the floral promoters *FLOWERING LOCUS (FT)*, *TWIN SISTER OF FT (TSF)*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANTS 1 (SOC1)*, *APETALA 1 (AP1)* and *FRUITFULL (FUL)* (Imura *et al*., 2012; Gillmor *et al*., 2014). *cct* and *gct* mutants also misexpress seed specific genes during seedling development, and have an elongated vegetative phase due to overexpression of the microRNA miR156 (Gillmor *et al*., 2014), a master regulator of the vegetative phase in plants (Wu *et al*., 2009). Taken together, these results demonstrate that *MED12* and *MED13* act as master regulators of developmental timing in plants, regulating the timing of pattern formation in embryogenesis, the seed-to-seedling transition, vegetative phase change, and the transition to flowering (Gillmor *et al*., 2010; Ito *et al*., 2011; Imura *et al*., 2012; Gillmor *et al*., 2014).

Due to its importance in plant development and physiology, we have extended studies of the CDK8 module to the crop plant maize (*Zea mays*). Establishment of molecular and genetic resources for the study of the maize CDK8 module will allow evaluation of its role in the regulation of agricultural traits such as timing of flowering and seed development, as well as responses to biotic and abiotic stresses. One of the primary goals of this work was isolation of loss of function mutant alleles of maize CDK8 module-encoding genes. In maize, resources based on endogenous DNA transposons constitute the most accessible and widely used technology for reverse genetics (McCarty and Meeley, 2009). The two major transposon systems used for gene tagging in maize are *Activator*/D*issociation* (A*c/Ds*) and *Mutator* (M*u)* (Candela and Hake, 2008). These systems consist of an autonomous or master element that encodes a transposase (TPase) and a second non-autonomous or receptor element. The receptor elements are frequently derived from a master element by mutations within the TPase gene. Lacking TPase, non-autonomous elements are stable, unless mobilized by TPase supplied *in trans* by an autonomous element (Kunze *et al*., 1997). *Activator (Ac)* is a member of the hAT transposon superfamily (named after the founding members *hobo*, *Ac* and *Tam3*; Calvi *et al*., 1991) and moves via a cut-and-paste mechanism (Bai *et al.,* 2007), with a preference for transposition to linked sites, making the system ideal for local mutagenesis (Greenblatt, 1984; Dooner and Belachew, 1989; Brutnell and Conrad, 2003). *Dissociation (Ds)* is a non-autonomous element whose transposition relies on the TPase encoded by *Ac*. To exploit the *Ac*/D*s* system for reverse genetics, *Ds* elements have been distributed throughout the genome to provide potential "launch pads" for mutagenesis of nearby genes (Vollbrecht *et al.,* 2010).

In this study, we identify five genes encoding components of the CDK8 module in maize, present experimentally determined gene structures, report expression of corresponding transcripts, and show that all components of CDK8 module respond to phosphate and nitrogen availability, consistent with a role for the CDK8 module

TABLE 1

COMPONENTS OF HUMAN, *ARABIDOPSIS*, AND MAIZE CDK8 MODULES

Human		Arabidopsis			Maize			
CDK8 module component	***Hs* GenBank mRNA**	**CDK8 module component**	***At* Model[1]**	***At* GenBank mRNA**	**CDK8 module component**	***Zm* Model[2]**	***Zm* GenBank Locus**	***Zm* GenBank mRNAs[3]**
CDK8	P49336	*HEN3*	AT5G63610.1	AAT36644	*ZmCDK8*	Zm00001d014438 (GRMZM2G166771)	LOC100284562	EU968864, NM_001157457, BT018448; BT039744, XR_552425
CYCC	P24863	*CYCC1;1*	AT5G48640.1	BX833973	*ZmCYCC*	Zm00001d021031 (GRMZM2G408242)	LOC100193909	BT040922, BT033427, XM008652706; AY105730, EU972675; BT036293
		CYCC1;2	AT5G48630.1 AT5G48630.2	AY085977 BT024473				
MED12	NP_005111	CCT/CRP	AT4G00450.1	AB690341	*ZmMed12a*	Zm00001d027299 (GRMZM2G114459)	LOC103630556	KP455660
					ZmMed12b	Zm00001d048541 (GRMZM5G828278/ GRMZM5G844080)[4]	LOC100384108	KP455661
MED13	NM_005121	*GCT/MAB2*	AT1G55325.2	N/A	*ZmMed13*	Zm00001d045603 (GRMZM2G053588/ GRMZM2G153792)[4]	LOC100279985	KP455662

[1]TAIR gene models [www.*Arabidopsis*.org] [2]Maize gene models B73 Reference Genome v4 [maizegdb.org] [3]Independent mRNAs containing full length coding sequences are listed for each splice product. Different spice products are separated by a semi-colon. [4]Previous split gene annotation from Reference Genome v3.

in abiotic signal integration in maize. We performed *Ds* mutagenesis of the *ZmMed12a* gene, identifying two novel insertional alleles, one of which results in a truncation of the *ZmMed12a* transcript. These insertional mutant alleles will facilitate determination of the biological roles of the CDK8 module in maize development and stress responses.

Results

The maize genome encodes all four components of the CDK8 module of Mediator

A previous effort to identify Mediator genes from many plant species identified a single maize homolog for all four CDK8 module genes (*CDK8*, *CYCC*, *MED12* and *MED13*) (Mathur *et al*., 2011). In order to conclusively define the number and identity of CDK8 module homologs in maize, we performed BLAST searches to identify all maize gene-models (B73 reference genome v4; www.maizesequence.org) whose putative protein products exhibit a high degree of similarity to the entire predicted *Arabidopsis* proteins of the CDK8 module of Mediator: CDK8 (encoded by *HEN3*) (Wang and Chen, 2004); CYCC1;1 or CYCC1;2 (Wang *et al*., 2004); MED12 (encoded by *CCT/CRP*) (Gillmor *et al*., 2010; Imura *et al*., 2012); and MED13 (encoded by *GCT/MAB2*) (Gillmor *et al*., 2010; Ito *et al*., 2011) (Table 1). Using the translated experimentally verified coding sequences for all maize CDK8 module genes (see below), all potential orthologous relationships were further validated by reciprocal searching of the *Arabidopsis* genome using maize sequences, and by inspection of the next-best-hit in both *Arabidopsis*-to-maize and maize-to-*Arabidopsis* searches (data not shown).

A single maize gene (Zm00001d014438) was identified as a potential ortholog of *HEN3/CDK8*, and designated *ZmCDK8*. Two different full-length splice products were identified for this gene (EU968864 and BT039744), predicted to encode a full-length and a truncated maize CDK8 protein (Fig. 1A; Fig. S1). The full-length ZmCDK8 protein is 471 amino acids (AA), and shows 73% identity with the 470 AA *Arabidopsis* CDK8 protein, and 43% identity with the 464 AA human CDK8 protein (Fig. S1). The smaller ZmCDK8

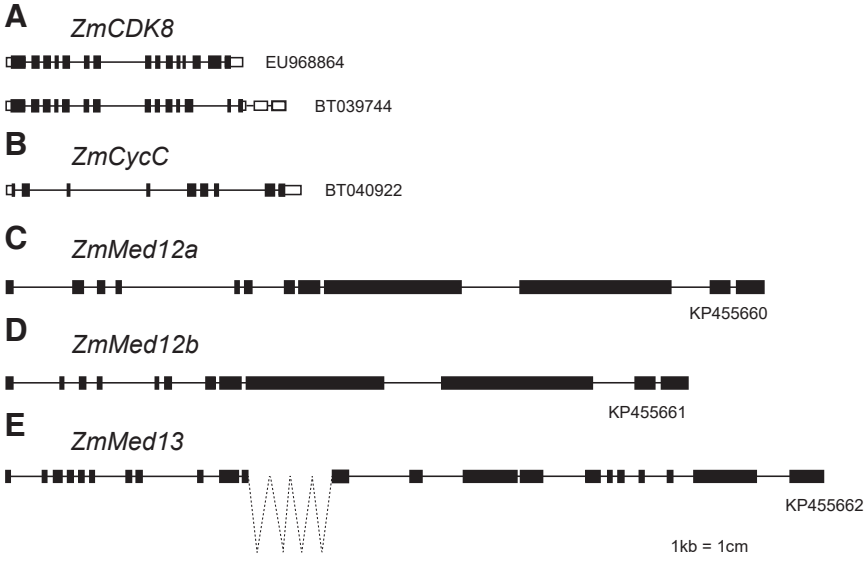


Fig. 1. The CDK8 module of maize consists of *CDK8, CyclinC, Med12a, Med12b,* and *Med13*. (A) *Exon-intron structure for two different splice products (EU968864 and BT039744) of the* ZmCDK8 *gene (Genbank LOC100284562). EU968864 encodes a 471AA protein, while BT039744 encodes a 385AA protein, truncated after the CDK8 kinase catalytic domain (cd07842)* **(B)** *Exon-intron structure of mRNA sequence BT040922 for the* ZmCycC *gene (Genbank LOC100193909), encoding a predicted protein of 257AA.* **(C)** *Exon-intron structure of mRNA sequence KP455660 for* ZmMed12a *(Genbank LOC103630556), which encodes a 2193 AA protein.* **(D)** *Exon-intron structure of mRNA sequence KP455661 for* ZmMed12b *(Genbank LOC100384108), which encodes a 2202 AA predicted protein.* **(E)** *Exon-intron structure of mRNA sequence KP455662 for* ZmMed13 *(Genbank LOC100279985), which encodes an 1892 AA protein. Intron 11 of* ZmMed13 *is approximately 10kb (dotted lines). Intron sizes for all genes were determined using corresponding maize genomic sequence. Exons are represented by black boxes, untranslated regions by open boxes, and introns by solid black lines.*

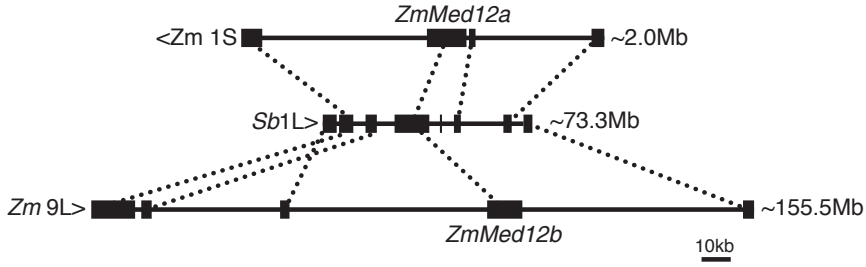


Fig. 2. Synteny between maize and sorghum genomic regions surrounding *Med12*. *The* Med12 *gene is conserved across sorghum and maize syntenic regions. Upper and lower rows: annotated genes in syntenic regions on maize chromosome 1S (*Zm*1S) at ~2Mb (upper row), and maize chromosome 9L (*Zm*9L) at ~155Mb (lower row). Middle row: annotated genes in the region of* SbMed12 *at ~73Mb on sorghum chromosome 1L (*Sb*1L). Orthologous genes are connected by dashed lines.* Sb*1L and* Zm*9L run left to right,* Zm*1S runs right to left. Genes are shown as black boxes, and the chromosomes are represented by horizontal lines. Regions are shown to scale.*

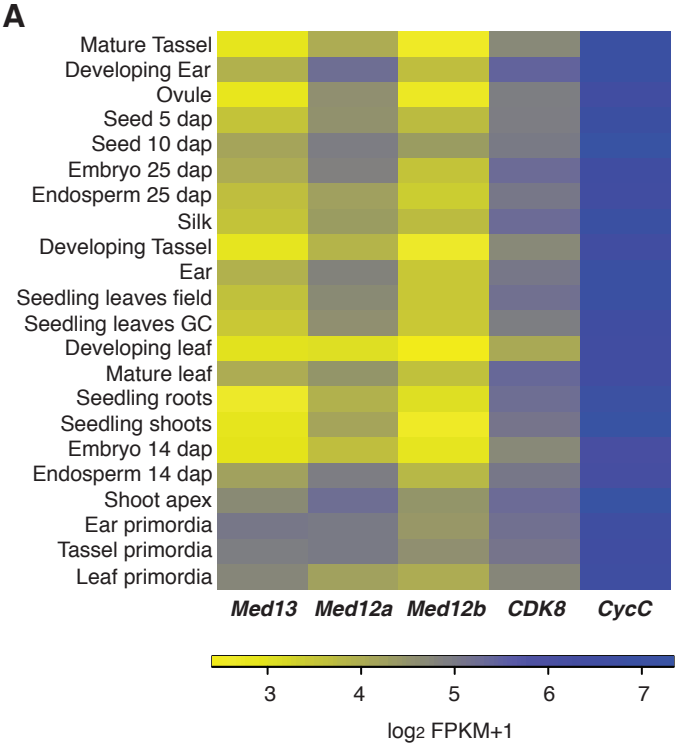
protein is 385 AA, primarily because of a truncation of the C terminal domain, and shows 75% identity with *Arabidopsis* CDK8, and 43% identity with human CDK8. This truncation occurs after the CDK8 kinase catalytic domain (cd07842), and is thus unlikely to interfere with the kinase function of the protein (Fig. S1).

Although *Arabidopsis* CYCC is encoded by a tandem-duplicated gene pair (Wang *et al.*, 2004), a single potential maize ortholog of *CYCC* (Zm00001d021031) was identified and designated *ZmCycC* (Fig. 1B), corresponding to the splice product represented by the full-length cDNA clone BT040922. The 257AA BT040922 protein is 42% identical to human CycC and 67% identical to *Arabidopsis* CycC1;1 (Fig. S2), and contains the Cyclin domain (cd00043) that is present in human and *Arabidopsis* CycC (Fig. S2).

BLAST searches using the *Arabidopsis* CCT/MED12 protein identified two putative full-length maize genes (Zm00001d027299 on chromosome 1, and Zm00001d048541 on chromosome 9), which were designated *ZmMed12a* and *ZmMed12b* (Fig. 1 C,D). Partial cDNA sequences were publicly available for *ZmMED12a* and *ZmMed12b*. These sequences, as well as coding sequences predicted by the maize database, were used to experimentally determine mRNA coding sequences for both genes by RT-PCR. The exon-intron structure of both genes is very similar, with the only differences occurring in the length and position of exons 2, 3 and 4 (Fig. 1 C,D). These splicing differences lead to several small insertions or deletions in the N-terminal portions of the ZmMed12 proteins, with *ZmMed12a* encoding a protein of 2193AA, and *ZmMed12b* encoding a protein of 2202AA. The two ZmMed12 proteins are 91% identical (Fig. S3). ZmMed12a is 19% identical to human Med12, and 46% identical to *Arabidopsis* MED12. ZmMed12b is 20% identical to human Med12, and 46% identical to *Arabidopsis* MED12 (Fig. S3). The region of highest identity is that comprising the Med12 domain (pfam09497), located in the N-terminus of the Med12 proteins (Fig. S3).

A single maize gene corresponding to *GCT/MED13* was identified (Zm00001d045603), and designated *ZmMed13*. Partial cDNA sequences were publicly available for *ZmMed13*. These sequences were used as the basis for RT-PCR experiments to identify full-length mRNA and coding sequences. *ZmMed13* encodes a protein of 1892 AA, with 20% identity to human Med13, and 49% identity to *Arabidopsis* MED13 (Fig. 1E and Fig. S4).

Maize Med12 is encoded by the duplicated gene pair *ZmMed12a* and *ZmMed12b* The high degree of similarity between *ZmMed12a* and *ZmMed12b* suggests that they are the result of a recent duplication event (Fig. S5). *ZmMed12a* and *ZmMed12b*



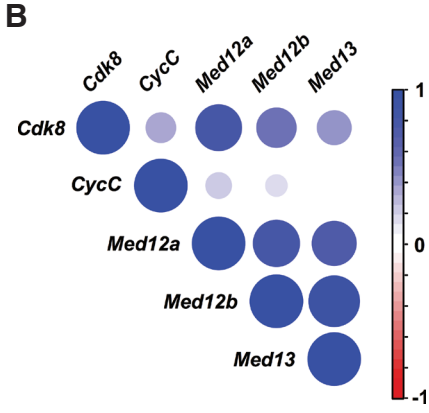


Fig. 3. CDK8 module genes are broadly expressed in development. (A) *Expression of* Med13, Med12a, Med12b, CDK8 *and* CycC *are shown as* $\log_2$*(FPKM+1) (Fragments Per Kilobase of exon per Million reads mapped). Data are from the following sources: Mature tassel, Developing ear, Ovule, Seed 5 dap, Seed 10 dap, Embryo 25 dap, Endosperm 25 dap, Silk, Developing tassel, Ear, Seedling leaves field, Seedling leaves gc (growth chamber) from Davidson* et al., *(2011). Developing leaf and Mature leaf from Li* et al., *(2010). Seedling roots and Seedling shoots from Wang* et al., *(2009). Embryo 14 dap and Endosperm 14 dap from Waters* et al., *(2011). Shoot apex, Ear primordia, Tassel primordia and Leaf primordia from Bolduc* et al., *(2012).* **(B)** *Correlation of expression patterns for pairwise combinations of members of CDK8 module. Positive correlations are shown as blue circles, with larger circles and darker blue signifying greater correlations between the two genes. Gene-by-gene comparisons for all tissue samples are shown in Figure S6, from which r values to make this plot were taken.*

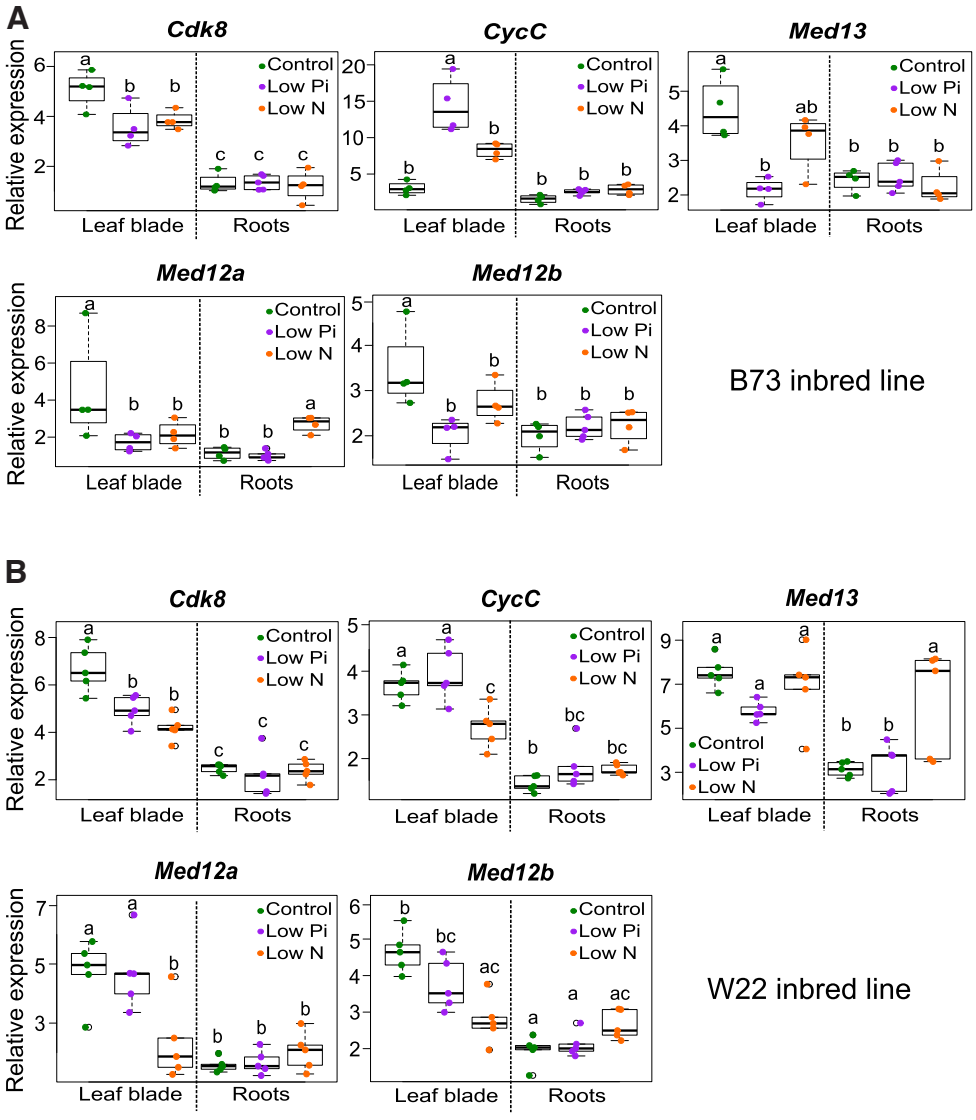


Fig. 4. CDK8 module genes in the B73 and W22 inbred backgrounds show differential responses to low phosphorus (P) and low nitrogen (N). *RT-qPCR was used to quantify all 5 five CDK8 module genes in leaf blades and roots of 15 day old* **(A)** *B73 and* **(B)** *W22 maize seedlings. All plants had 3 true leaves at harvesting time. Each point in the boxplots represents an independent biological replicate. Different letters indicate significant differences between samples using Tukey's HSD test (P < 0.05).*

Maize CDK8 module genes are expressed throughout development

In organisms where the CDK8 module has been studied, the gene pairs *CDK8* and *CyclinC,* and *Med12* and *Med13*, have similar expression patterns and mutant phenotypes (Yoda *et al.*, 2005; Loncle *et al.*, 2007; Gillmor *et al.*, 2010; Gillmor *et al.*, 2014). To determine whether the *CDK8*, *CycC*, *Med12a, Med12b* and *Med13* genes have similar expression patterns in maize, we used publicly available RNA sequence data to quantify CDK8 module gene expression in different tissues and at different developmental stages (see Materials and Methods). As seen in the heatmap in Fig. 3A, *CycC* was expressed at much higher levels in all tissues than the other CDK8 module genes, with *CDK8* and *Med12a* the next highest expressed genes, and *Med13* and *Med12b* with the lowest expression levels.

To more precisely compare tissue-specific expression between the different CDK8 module genes, we made pairwise comparisons for all five genes (Fig. 3B and Fig. S6). Expression was most highly correlated for *Med13* and *Med12b* (Pearson's r = 0.93), where the expression ratio between the two genes was close to 1 (compare dotted red line for r, with solid black line representing a 1:1 expression ratio) (Fig. 3B and Fig. S6). *Med12a* and *Med12b* (r = 0.77); *Med12a* and *Med13* (r = 0.7); and *CDK8* and *Med12a* (r = 0.76) also had high Pearson's coefficients for pairwise comparisons (Fig. 2B and Fig. S5). By contrast, *CycC* showed almost no correlation with any of the other CDK8 module genes (Fig. 3B and Fig. S6). The fact that *CycC* shows little expression correlation with the other CDK8 module genes, and is expressed at higher levels than *CDK8*, and many times higher than *Med13*, *Med12a* and *Med12b*, suggests that *CycC* may play more varied roles in development and physiology than the other CDK8 module genes

are in syntenic regions of the genome (1S and 9L, respectively), which derive from a polyploidy event that occurred 5-12 million years ago, after the divergence of maize and sorghum lineages (Fig. 2). Although gene loss has reduced the number of genes in present-day maize close to pre-duplication levels, in certain cases both syntenic paralogs have been retained (Schnable *et al.*, 2011). Further inspection revealed a sorghum *Med12* gene (Sb01g050260; *SbMed12*) to be present in a region on Chromosome 1L syntenic to the two maize *ZmMed12* containing regions. Moving up- and downstream from *SbMed12*, micro-synteny was conserved, although for any given sorghum gene usually only one candidate ortholog was identified in maize, in either the 1S or 9L region, presumably as the result of gene-loss among paralogous pairs following whole genome duplication (Fig. 2).

CDK8 module genes respond to nutrient deficient conditions

To determine if the expression of the maize CDK8 module genes are affected by nitrogen (N) and phosphorus (P) stress, we analyzed the expression pattern of all the CDK8 module genes in B73 and W22 plants grown under normal, N deficient or P deficient conditions. The expression analysis was performed in roots and leaf blades of 15 day old seedlings. All five CDK8 module genes are more highly expressed in leaf blades than in roots, and change their expression patterns in response to nutrient deficiency, which is more evident in leaf blades than in roots (Fig. 4). Subtle differences

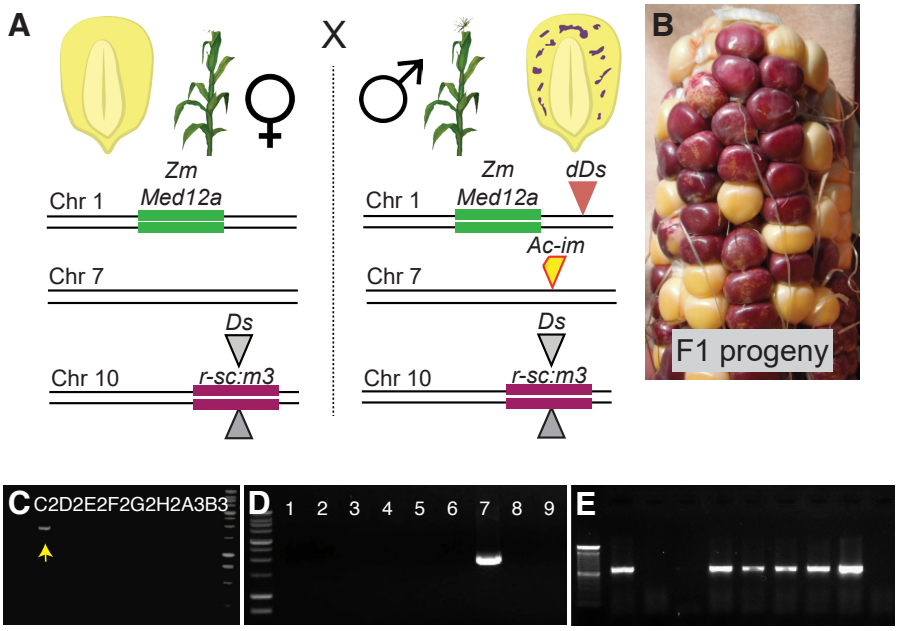


Fig. 5. Generation and identification of *Ds* insertional alleles of *ZmMed12a*. (A) *Crossing scheme for generating plants homozygous for the* r-sc:m3 *reporter allele, and heterozygous for* Ac-immobilized *(Ac-im) and the* Ds *insertion linked to* ZmMed12a. **(B)** *The presence of* r-sc:m3 *allows for selection of spotted F1 kernels, indicating the presence of* Ac-im, *required for remobilizing the* Ds *insertion linked to* ZmMed12a. **(C)** *Initial pools of 10-18 seedlings were screened by PCR for the presence of a* Ds *element in* ZmMed12a, *using gene specific primer E10.2 and* Ds *specific primer JGp3. A 1.8 kb fragment (yellow arrow) was amplified from pool C2.* **(D)** *Individual plants from pool C2 were tested for the presence of the same fragment, which was amplified from plant 7, labeled C2.7 (*Zmmed12a-2::Ds*)* **(E)** *This 1.8kb band segregated in nine progeny of the selfed plant C2.7, demonstrating that it was a heritable germinal insertion.*

were found between the two inbred genotypes tested, but in general more changes are observed in response to low nitrogen conditions. In the B73 inbred line, expression of *CDK8*, *Med12a* and *Med12b* decreased in leaf blades under low P and low N conditions, while their expression only changed in leaf blades of W22 in low N. *CycC* and *Med13* expression levels changed in response to low P in leaf blades of B73, while in W22 *CycC* decreased under low N conditions, and *Med13* showed no change (Fig. 4). *Med12a* and *Med12b* showed a similar expression pattern in leaf blades but not in roots. In B73 leaf blades, expression of both genes decreased under low N and low P, while in W22 leaf blades they changed only in low N. In response to low N in B73 roots, *Med12a* expression changed, but *Med12b* did not. While in W22 roots, *Med12a* expression did not change in response to low N, but expression of *Med12b* did (Fig. 4). Thus, CDK8 module genes respond differently to P and N stress, and these responses vary between the B73 and W22 inbred backgrounds.

Reverse genetics strategies to target maize CDK8 components

To initiate functional analysis of the maize CDK8 module, we identified publicly available seed stocks carrying *Ac/Ds* or *Mu* family transposons inserted into, or close to, genes encoding components of the maize CDK8 module (Table 2). Based on this search, we selected *ZmMed12a* as our first target for reverse genetics: at ~56kb, the closest potential *Ds* donor was nearer to *ZmMed12a* than to any of the other genes. In addition, the availability of a well-characterized *med12* mutant in *Arabidopsis* provides for the possibility of comparative studies (Gillmor *et al.*, 2010; Imura *et al.*, 2012; Gillmor *et al.*, 2014). Finally, the retention of two *Med12* syntenic paralogs in maize suggests that the roles of *ZmMed12a* and *ZmMed12b* might be functionally different, a question which can be addressed by characterization of maize *med12* mutant alleles.

Identification of novel Ds insertions into ZmMed12a

To use the *Ac/Ds* transposon system to generate mutant alleles of *ZmMed12a*, we first obtained donor *Ds* (d*Ds*) stocks carrying the *Ds* element *dDs-B.S07.0835*, located 56.2 kb from *ZmMed12a* (acdstagging.org). The position of the linked *Ds* element was confirmed by a PCR assay (see Materials and Methods) (Conrad and Brutnell, 2005). Presence of *Ac-im* in testcross progenitor seed stocks was monitored by somatic excision of a second *Ds* from the *r1-sc:m3* marker locus, resulting in variegated spotting of the kernel aleurone and scutellar tissues (Fig. 5 A,B). Spotted kernels were planted and seedlings genotyped for the presence of *dDs* using a PCR assay (Materials and Methods). To generate novel germinal insertions into *ZmMed12a*, individuals carrying the *dD* and the *Ac-im* transposase source were used as males to pollinate T43 (*r-sc:m3*/r-*sc:m3*) females. A test cross population of 59 ears was obtained for the *ZmMed12a* screen (Fig. 5 A,B).

The test-cross population was screened for *Ds* insertions in *ZmMed12a* using combinations of gene specific and *Ds* specific PCR primers (see Materials and Methods). Pools of 10-18 seedlings were assayed for amplification of putative *Ds*-flanking junction products (see Fig. 5C for an example for the *Zmmed12a-2::Ds* insertion).

TABLE 2

REVERSE GENETICS RESOURCES FOR MAIZE CDK8 MODULE GENES

Maize	Maize Accession	Position (kb)	Closest *Ac/Ds*[1]	Uniform *Mu*[2]
ZmCDK8	Zm00001d014438 cdk8	Chr5: 45,538,294-45,544,117	8311.3 kb	3' UTR: mu1053539 Flanking: mu1077819
ZmCYCC	Zm00001d021031 cyc12	Chr7: 137,095,918-137,100,956	420 kb	Upstream: mu1060662 mu1092706 mu1092707
ZmMED12a	Zm00001d027299 polm1	Chr1: 2,088,572-2,102,312	56.2 kb	UTR: mu1013385 Flanking: many
ZmMED12b	Zm00001d048541 polm2	Chr9: 155,361,528-155,373,747	717.1 kb	Exon: mu1058248 Intron/UTR/Flanking: many
ZmMED13	Zm00001d045603 polm3	Chr9: 28,392,287-28,413,513	226.7 kb	Flanking: mu1054840

[1]acdstagging.org; [2]*Mutator* resources available at maizeGDB.org

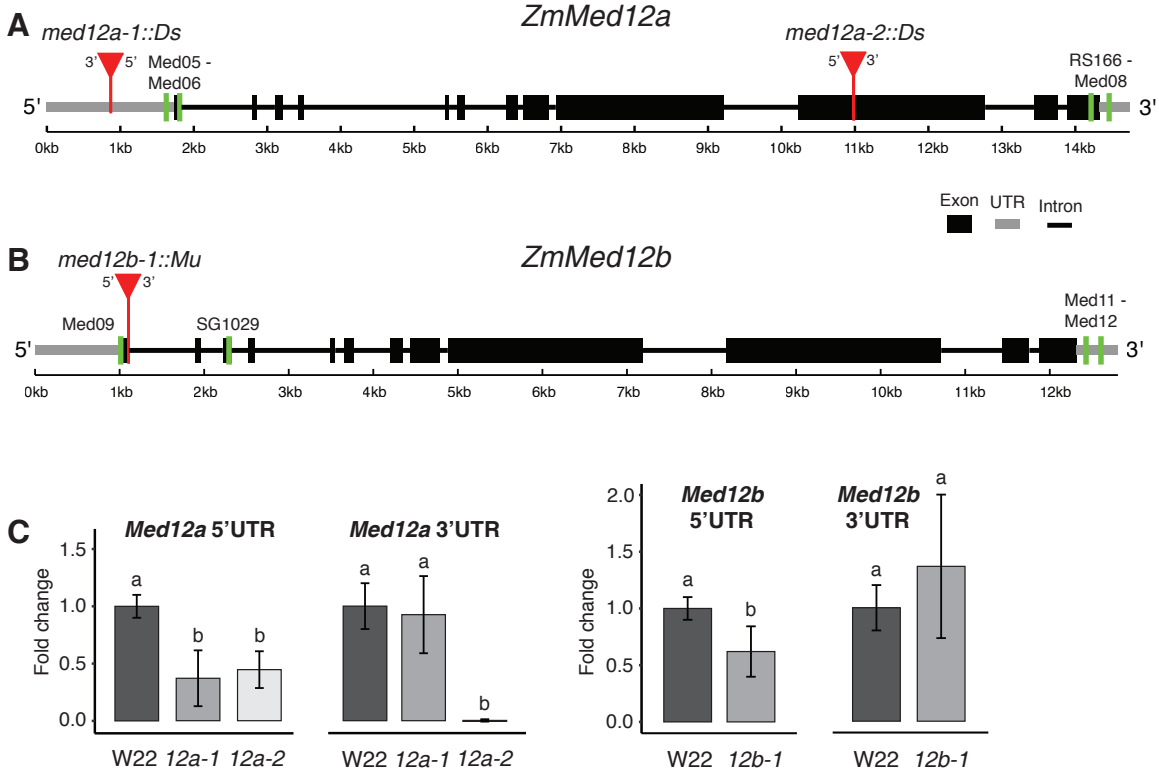


Fig. 6. Transposon insertions in *ZmMed12a* affect its transcript levels. (A) ZmMed12a *gene structure, including 5′ and 3′ UTRs, based on Genbank mRNA XM_008651608.2. The position of the* 1::Ds *insertion in the 5′UTR at 918bp upstream of the ATG, and the* 2::Ds *insertion in exon 10 at 4,236 bp of the coding sequence, are shown in red. The insertion of the* 2::Ds *element results in the introduction of a stop at codon 1459.* **(B)** *RT-qPCR analysis of the effect of the* 1::Ds *and* 2::Ds *insertions on the* ZmMed12a *transcript. The location of the primer pairs used to amplify the transcript are indicated in the gene diagram in green.* ZmCDK *(GRMZM2G149286) was used as a control gene.* **(C)** *For all RT-qPCR experiments, the average and standard deviation of three biological replicates, each with three technical replicates, are shown.*

Seedlings constituting the pools from which products were amplified were re-screened separately to identify positive individuals (Fig. 5D). This second PCR was performed using DNA extracted from a leaf different from that used for the pool PCR to reduce the rate at which we recovered somatic transposition events. We screened a total of 3,049 seedlings and identified two novel insertions into *ZmMed12a*: *zmmed12a-1::Ds*, located 918 bp upstream of the translational start, and *zmmed12a-2::Ds* located in exon 10. We performed additional PCR reactions to recover both flanks of the *zmmed12a-1::Ds* and *zmmed12a-2::Ds* insertions. Flanking DNA products were sequenced, confirming the location of the insertions and identifying characteristic 8bp target site duplications. The seedlings carrying the two novel *zmmed12a* insertional alleles were grown to maturity and propagated by both self-pollination and out-crossing. Progeny were germinated and genotyped, confirming the heritability of each novel *Ds* insertion (Fig. 5E).

The med12a-2::Ds insertion causes a truncation in the ZmMed12a transcript

The positions of the *Ds* insertions in the *ZmMed12a* gene are shown Fig. 6A. We performed RT-qPCR analysis of plants homozygous for the wild type and *Ds* alleles, using primer pairs that amplify 5′ and 3′UTRs (Fig. 6B). Using primers in the 5′UTR, both *med12a-1::Ds* and *med12a-2::Ds* alleles showed a significant expression decrease of *ZmMed12a*, suggesting the *Ds* insertion affects abundance of the *Med12a* transcript. To determine if the *Med12a* mutant transcripts were full length, we quantified the abundance of transcripts at the 3′UTR for both mutant alleles. The *med12a-1::Ds* insertion had no effect on transcript length or accumulation, while the normal 3′UTR of *Med12a* was undetectable from the *med12a-2::Ds* insertion allele. This is consistent with the position of the *med12a-2::Ds* insertion in exon 10, which is predicted to introduce a stop at codon at position 1,459, likely resulting in a truncated transcript and protein (Fig. 6).

Discussion

We have identified the five genes encoding the CDK8 module of Mediator in maize, determined their coding sequences, characterized their expression in maize tissues during development, evaluated their response to phosphate and nitrogen deficiency, and examined their synteny in maize and sorghum. Additionally, we have mutagenized the *ZmMed12a* gene using the *Ac/Ds* transposon system created by Vollbrecht *et al.,* (2010), and determined the effect of *Ds* insertions on *ZmMed12a* transcripts.

In our analysis of CDK8 module genes, we identified two alternative transcripts for *CDK8* (Fig. 1). One predicted CDK8 protein is significantly shorter than the other, lacking the C-terminal 86 AA.

This truncation seems unlikely to affect enzyme activity *per se*, as the kinase domain is intact (Fig. S1). However, the lack of this domain may alter regulation of the kinase activity. Alternatively, the truncation may modify the interaction of CDK8 with CycC, or affect the formation of the four protein CDK8 complex. This complex sterically inhibits the interaction of Core Mediator with RNA pol II, by making direct contact with Core Mediator (Tsai *et al*., 2013). For CycC, only one isoform was represented by multiple independent cDNAs. In our cloning and sequencing of cDNAs for *Med12a*, *Med12b* and *Med13,* only single splice products were identified (Fig. 1). One explanation for this is that there is indeed only one splice product for each gene in maize. It is also possible that the very large size of the mRNAs for these three genes (6-7 kb) makes cloning of multiple splice products difficult, due to technical limitations in cloning large cDNAs.

MED12 is a single copy gene in *Arabidopsis*, with mutant phenotypes in both development and pathogen responses (Gillmor *et al*., 2010; Imura *et al*., 2012; Gillmor *et al*., 2014; Zhu *et al*., 2014). Two *Med12* genes were identified in maize, consistent with whole genome duplication of the maize lineage, sometime after its divergence with sorghum (Schnable *et al*., 2011). While most duplicate gene copies have been lost in maize, syntenic paralog pairs have been retained for ~10% of the original gene set (Hughes *et al*., 2015). The genomic location of *ZmMed12a* and *ZmMed12b* is consistent with them representing such a paralogous pair (Fig. 2). In the region of synteny between maize and sorghum, other genes surrounding *Med12* have been reduced to a single copy, suggesting that the retention of both paralogs of *Med12* in maize may have functional significance. In the future, this possibility can be explored by phenotypic analysis of the *med12a-2::Ds* allele (Fig. 6).

Analysis of the effects of the two new *Ds* insertions described in this study showed that both insertions cause changes in the expression of *ZmMed12a* (Fig. 6). As measured by primers in the 5´UTR, the *med12a-1::Ds* insertion significantly decreases the expression of *ZmMed12a*, suggesting that *Ds* insertion affects *Med12a* transcription. Curiously, using primers in the 3´UTR, the expression of *Med12a* transcript in *med12a-1::Ds* is the same as in wild type plants. This observation suggests that transcripts detected in *med12a-1::Ds* using primers in the 5´UTR are fully transcribed, and may have the same length as wild type transcripts. In addition, it is possible that alternative transcripts have been detected using primers in the 3´UTR. Several studies have shown that transposable elements able to generate new transcription initiation sites, alternative splicing sites, introduce regulatory cis-elements and recruit epigenetic marks (Weil *et al*., 1990; Bai and Brutnell, 2011; Wei and Cao, 2016). Generation of different transcription initiation sites or alternative splicing sites in *med12a-1::Ds* mutant could be analyzed in order to determine if transcripts with different sequences near the 5´UTR but same at the 3´UTR are produced.

The truncation of the *ZmMed12a* transcript in the *2::Ds* allele makes it very likely that this allele causes a loss of function. A T-DNA insertion in a similar location of the *CCT* (M*ED12*) gene of *Arabidopsis* results in a truncated transcript and a strong loss of function phenotype (Gillmor *et al*., 2010; Gillmor *et al*., 2014). An advantage of using *Ds* as a mutagen is that novel transpositions occur at linked sites. This will allow for the remobilization of the *Ds* insertions in *ZmMed12a* to create additional allelic variation in *ZmMed12a*. In addition to mutant alleles that cause a complete loss of function, subsequent *Ds* mutagenesis of *ZmMed12a* may result in hypomorphic alleles that reduce (but do not eliminate) the function of *ZmMed12a*, or that inactivate specific functional domains of Med12. Alleles that eliminate only certain parts of the Med12 protein could be especially useful in understanding the function of different domains of Med12, currently one of the most interesting, and least explored, aspects of Mediator biology.

In our analysis of the relative expression of CDK8 module genes, we found *CDK8* and *CycC* to be more highly expressed in all tissues than *Med12a*, *Med12b* or *Med13*. *CycC* showed the highest expression in all tissues, consistently 3-4 times higher than *CDK8* (Fig. 3). This high relative expression of *CycC* is consistent with roles for CycC beyond regulating transcription as part of the CDK8 module (Allen and Taatjes, 2015). For example, CycC has been shown to promote the G0 to G1 cell cycle transition through phosphorylation of Retinoblastoma, allowing quiescent cells to enter the cell cycle. CycC achieves this through interaction with CDK3, a kinase that is not associated with transcriptional activation, but instead promotes cell cycle entry (Ren and Rollins, 2004). CycC has also been demonstrated to be a haploinsuficient tumor suppressor in mammals, whose loss of function in mice is lethal during embryogenesis (Li *et al*., 2014). The haploinsufficiency of CycC may require its mRNA or protein levels to be stably maintained, suggesting an explanation for its high expression in all the tissues that we examined (Fig. 3 and Fig. S6). *Med12a*, *Med12b*, and *Med13* show much lower expression levels, which also vary considerably between different tissues (Fig. 3 and Fig. S6). The similar expression profiles for *Med12* and *Med13* in maize are consistent with *Arabidopsis*, where similar expression profiles for these two genes were reported (Gillmor *et al*., 2010; Ito *et al*., 2011; Imura *et al*., 2012; Gillmor *et al*., 2014). The widely varying expression levels for *Med12* and *Med13* in different tissues suggest various roles for these genes in development, both in primordia (where they show the highest expression), as well as in differentiating and mature tissue.

The CDK8 module genes respond differently to nitrogen and phosphorus stress (Fig. 4). In B73 leaf tissue, four out five CDK8 module genes decrease their expression under both nitrogen and phosphorus limitation, while *CycC* increases its expression in phosphorus deficiency. In W22 leaf blades, the CDK8 module maintains similar expression in normal and phosphorus deficient conditions, while in nitrogen limitation the expression of *CDK8*, *CycC, Med12a* and *Med12b* decreases. In *Arabidopsis*, many Mediator subunits have been reported to be important for environmental adaptation. For example, MED25 regulates defense responses, and has an important role in hormone signaling (Kidd *et al*., 2009; Raya-González *et al*., 2014); MED16 plays an important role in iron deficiency responses and is essential for the expression of iron uptake genes (Zhang *et al*., 2014); and MED12 and MED13 are required for correct auxin responses (Ito *et al*., 2011; Ito *et al*., 2016; Raya-González *et al*., 2017).

The fine-tuning of gene expression under unfavorable environmental conditions is essential for plant survival. Plants need to prioritize responding to phosphorus and nitrogen limitation rather than continue growing, so many metabolic pathways are downregulated under nutrient stress. Since the active form of Core Mediator has been purified without the CDK8 module, and *in vitro* experiments containing the Core Mediator plus CDK8 module

cannot maintain transcription activity, the CDK8 module is usually considered as a transcriptional repressor (Elmlund *et al*., 2006). This is consistent with a scenario where CDK8, Med13, Med12a and Med12b repress low nutrient responses under normal growth conditions, and are downregulated in low nutrient conditions so that low nutrient response genes can be de-repressed. CycC may play distinct roles in response to low nutrient conditions, perhaps by regulating cell cycle progression under nutrient limiting conditions. A role for the CDK8 module in response to P or N limitation has not thus far been reported (Buendía-Monreal and Gillmor, 2016). In the future, it will be interesting to test the functional role of the CDK8 module in nitrogen and phosphate homeostasis in maize.

Materials and Methods

Identification of maize CDK8 module genes

Maize CDK8 module genes were identified by BLAST searches using the predicted *Arabidopsis Thaliana* protein sequences for HEN3/CDK8 (AT5G63610), CYCC1;1 (At5g48640), CCT/MED12 (At4g00450), and GCT/MED13 (At1g55325) available at The *Arabidopsis* Information Resource (TAIR) (www.*Arabidopsis*.org). Reciprocal BLAST searches were conducted between all maize and *Arabidopsis* sequences, establishing that the five maize genes *ZmCDK8*, *ZmCycC*, *ZmMed12a*, *ZmMed12b*, and *ZmMed13* were the only full length CDK8 homologs present in maize.

TABLE 3

PCR PRIMERS USED IN THIS STUDY

Name	Sequence	Purpose
B.S07.0835	5'-GACGCACACACGTCAGTATAG-3'	Donor Ds site
JGp3	5'-ACCCGACCGGATCGTATCGG-3'	Ds specific
JSR01	5'-GTTCGAAATCGATCGGGATA-3'	Ds specific
JSR05	5'-CGTCCCGCAAGTTAAATATGA-3'	Ds specific
5'UTRF	5'-TGCACTGCTGCTGTCCTATT-3'	*ZmMed12a* specific- Ds tagging
E03R	5'-TGGTCCATAACTCAGACATACTTGT-3'	*ZmMed12a* specific- Ds tagging
E03F	5'-CTCCCTAATACCCCTGTATTTCA-3'	*ZmMed12a* specific- Ds tagging
E07R	5'-GCATTTGGTAGTAAACAAGAGATGG-3'	*ZmMed12a* specific- Ds tagging
E06F	5'-CCTTGTTAGAATGCGGTCAA-3'	*ZmMed12a* specific- Ds tagging
E09.2R	5'-TCAGGACGAACATACCTAAGCA-3'	*ZmMed12a* specific- Ds tagging
INT02F	5'-ACCAAGTTTGTCAGGTCAACG-3'	*ZmMed12a* specific- Ds tagging
E10.2R	5'-CTACCGAAAACCCATGTTGG-3'	*ZmMed12a* specific- Ds tagging
E10.2F	5'-GCAGCTTTTGAGAGGTTTGAA-3'	*ZmMed12a* specific- Ds tagging
E12R	5'-GCAACTTCCGTCAGCCTTAG-3'	*ZmMed12a* specific- Ds tagging
C2.7 F	ACCCAGGAATCCACTCACTTTT	Genotyping F2 for *2::Ds*
C2.7 R	TGCAATCAATAATAGCGTCCAG	Genotyping F2 for *2::Ds*
A5.12 F	AACGTGTAGACCTTGGGTTGAAT	Genotyping F2 for *1::Ds*
A5.12 R	AGGCGTATAGCGGCTAAGGA	Genotyping F2 for *1::Ds*
TIR6	CGCCTCCATTTCGTCGAATCCCCTS	Mutator TIR specific
Med_01	AGCCTTCAACCAACTCAACC	qPCR CDK8 Fwd (3´UTR)
Med_02	ATCCGCCTTCCTTCTCAACT	qPCR CDK8 Rev (3´UTR)
Med_03	GATTTGGCGAGCTTAGGAGTTG	qPCR CycC Fwd (3´UTR)
Med_04	AACTCCAGCAGCATACACAAAC	qPCR CycC Rev (3´UTR)
Med_13	CAACAGGTCGTGAGTGTGAGAT	qPCR Med13 Fwd (3´UTR)
Med_14	CTACAAAGCTCGTCAAATGTGC	qPCR Med13 Rev (3´UTR)
RS166	AAGCAATGGCTCGAGTGGAGTGAC	qPCR Med12a Fwd (3´UTR)
Med_08	CATATGGTTTCCATTCCACCTT	qPCR Med12a Rev (3´UTR)
Med_05	CGGCTGAGTGAGGACAAATAA	qPCR Med12a Fwd (5´UTR)
Med_06	GTAACCAGAGGAGGAGGAGGAC	qPCR Med12a Rev (5´UTR)
Med_11	GGATGGTTGTTGTTGGTGTAGA	qPCR Med12b Fwd (3´UTR)
Med_12	ACTGCATGGCTTCCTACTTCTC	qPCR Med12b Rev (3´UTR)
Med_09	GCATAGGTACTCCAGCAACAGC	qPCR Med12b Fwd (5´UTR)
SG1029	CCTTGTACCCAGCTTGTGCATACT	qPCR Med12b Rev (5´UTR)
MS174	CCGTCATCGCCTCACGAAGAG	qPCR CDK Fwd - housekeeping gene used for normalization
MS175	AGAGCCTGCCTTACGGAATTGG	qPCR CDK Rev - housekeeping gene used for normalization
MS182	GCGTGCTCTTTCGTCAGATGTG	qPCR Hypothetical protein Fwd - housekeeping gene used for normalization
MS183	CCTACTGTTGGCTGGAGACTGG	qPCR Hypothetical protein Rev - housekeeping gene used for normalization

Determination of coding sequences for ZmCDK8, ZmCycC, ZmMed12a, ZmMed12b, and ZmMed13

Multiple mRNA sequences with full-length coding sequences (as well as upstream and downstream untranslated regions) were identified from the NCBI database for both *ZmCDK8* and *ZmCycC*. For *CDK8*, cDNAs for two alternative splice products were identified: EU968864, NM_001157457 and BT018448 correspond to one splice variant, and BT039744 and XR_552425 correspond to the other splice variant. For *CycC*, three independent cDNAs (BT040922, BT033427, and XM008652706) were identified for the one splice variant. Two independent cDNAs (AY105730 and EU972675) represented another *CycC* splice variant with an identical coding sequence but with slight differences in the 3'UTR. A third splice variant was represented by a single cDNA (BT036293); this mRNA has two upstream open reading frames, and encodes a truncated CycC protein. For *ZmMed12a*, *ZmMed12b* and *ZmMed13*, partial sequences were obtained from the maize database (maizegdb.org), which were then confirmed and extended by RT-PCR using RNA extracted from seedlings of the B73 inbred line. To confirm the *ZmMed12a,* Zm*Med12b,* and *ZmMed13* gene models, we amplified cDNA products covering the entire predicted coding regions. Given their large expected size, *ZmMed12a, ZmMed12b,* and *ZmMed13* cDNAs were amplified in multiple overlapping fragments. Sequencing of cDNA products was generally consistent with gene models based on genomic sequence analysis. Coding sequences were deposited in the NCBI database with the following accession numbers: *ZmMed12a* (KP455660), *ZmMed12b* (KP455661), and *ZmMed13* (KP455662).

In addition, numerous short genes that are predicted to encode highly truncated ZmMed12 proteins of 199 to 431 residues were identified. These short *ZmMed12* genes are predicted to encode the Med12 domain (pfam09497) and many have corresponding expressed sequence tags (EST), which do not cover the entire body of these short genes. Analysis of genomic sequences around these predicted coding sequences did not identify additional *Med12* exons (data not shown), suggesting that these are indeed truncated versions of *ZmMed12*, and not mis-annotated genes with nearby exons that would constitute the middle and C-terminal portions of Med12 proteins.

In silico expression profiles of maize CDK8 module genes

RNA-seq expression data from 22 maize tissues were obtained from the https://qteller.maizegdb.org database, in the form of Fragments Per Kilobase of transcript per Million (FPKM). To look for correlations between pairs of genes across the tissues, the data was $\log_2$ transformed (first adding 1, to avoid the logarithm of 0) and normalized using the normalizeQuantiles function from the limma package (Bolstad *et al*., 2003). The expression values were selected for the 5 CDK8 module genes: *CDK8* (GRMZM2G166771), *CycC* (GRMZM2G408242), *Med12a* (GRMZM2G114459), *Med12b.1* (GRMZM5G828278), *Med12b.2* (GRMZM5G844080), *Med13.1* (GRMZM2G053588), and *Med13.2* (GRMZM2G153792). Since *Med12b.1* and *Med12b.2* as well as *Med13.1* and *Med13.2* are spliced versions of the same gene, the geometric mean was calculated to obtain an average estimate of their expression. These data were employed to produce Fig. 3A, using the heatmap.2 function from the gplots package (Warnes *et al*., 2015). All pairwise combinations of the 5 genes across all tissues were plotted using the generic plot function in R (R Core Team, 2015) (Fig. S6). The Pearson correlations for all possible pairs of genes were calculated with the cor function, and these data were used as the empirical null to calculate p-values. Correlations for CDK8 module genes were calculated separately. The blob plot in Fig. 3B was generated with the corrplot for R.

Description of maize stocks

All stocks were maintained in the common genetic background of a color-converted W22 inbred line (Dooner & Kermicle, 1971). A stable source of *Ac* transposase was provided by *Ac-immobilized* (A*c-im*), an *Ac* derivative which has lost 10 bp at the 5' end of the element, preventing excision (Conrad and Brutnell, 2005). Activity of *Ac* transposase was monitored using the mutable *Ds* reporter *r1-sc:m3* that carries a *Ds6*-like insertion in the *r1* locus that controls anthocyanin production in the aleurone and scutellum tissues (Alleman and Kermicle, 1993): when *Ac* transposase is present, excision of *Ds* from *r1* restores gene function, producing colored sectors (Brutnell and Dellaporta, 1994). The donor *Ds* (d*Ds*) stock *dDs-B. S07.0835* was generated by isolation of novel transpositions from *r1-sc:m3* (Vollbrecht *et al*., 2010). Presence of *dDs-B.S07.0835* was assayed by PCR (Vollbrecht *et al*., 2010) using a combination of the *Ds* end primer JSR05 and a primer specific to the genomic site of B.S07.0835 (5'-GACGCACACACGTCAGTATAG-3'). To generate the test-cross population, plants verified as carrying the donor *dDs-B.S07.0835* with *Ac-im* in the genetic background were used as males to pollinate *r1-sc:m3*/r*1-sc:m3* female plants.

Seedling screen for transposon insertions in ZmMed12a

Testcross progeny were germinated and screened for novel *Ds* insertions in *ZmMed12a* using a PCR-based strategy. Tissue was collected between 7 and 10 days after planting from pools of 10-18 seedlings using a ~3mm hole punch, and DNA was isolated following a CTAB-based extraction protocol (Weigel and Glazebrook, 2009). A total of 10 *ZmMed12a* gene-specific primers were designed, covering a region extending from 1.8kb upstream of the translational start to the stop codon. These were used in conjunction with the 5' and 3' *Ds*-end primers JSR01 and JGp3, respectively, to amplify DNA adjacent to novel *Ds* insertions in *ZmMed12a* (Table 1). Pools amplifying a product were de-convoluted by screening individuals separately; this second round of PCR used DNA extracted from a different seedling leaf than that sampled for the pool to reduce the chances of recovering somatic transposition events. The PCR products of the second PCR were cleaned (Sambrook and Russell, 2006) and the DNA concentration was adjusted for sequencing by the GENEWIZ Company (South Plainfield, New Jersey, USA). Seedlings carrying putative *med12a* insertional alleles were grown to maturity and propagated by both self-pollination and out-crossing to W22 and B73 inbred lines.

Expression analysis of CDK8 module genes in leaf blades and roots under different nutrient conditions

Maize B73 and W22 inbred lines were planted in sand and watered with Hoagland solution in greenhouse conditions (Hoagland and Broyer, 1936). The control condition contained complete Hoagland solution with a final PO_4 (Pi) concentration of 333 μM and 175 mM of NO_3 (N); the low Pi condition contained 10 μM of Pi and 175 mM of N; and the low N condition contained 333 μM Pi and 1.6 μM N. Leaf blades and roots were harvested at 15 days after germination (dag) for control and low Pi conditions, and at 20 dag for low N condition. All plants had 3 true leaves at harvesting time. Tissue was frozen with liquid nitrogen and stored at -80°C until processing.

Total RNA was extracted from ~200 mg of frozen tissue with the Trizol® reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA samples (1 μg) were then reverse-transcribed to generate first-strand cDNA using an oligo dT20 primer and 200 units of SuperScript II reverse transcriptase (Invitrogen). qRT-PCR was carried out on a Bio-Rad CFX96 using the SYBR GREEN FAST kit (Kapa Biosystems), using the following program: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s; 60 °C for 20 s; 72°C for 20 s. As an internal control, the average expression levels of two housekeeping genes were used: *ZmCDK* (GRMZM2G149286) and a gene of unknown function (GRMZM2G047204). Both have been reported to be constitutively expressed under different nutrient conditions and in different genotypes (Lin *et al.,* 2014). The Threshold Cycle (Ct) method was employed to quantify the relative gene expression of CDK8 module genes in different tissues, at different developmental stages and in response to nutrient deficient conditions. It was calculated according to the following equation: $2^{\Delta C}t$, where ΔCt = (Average Ct of reference genes – Ct of target gene). Four and five biological replicates were used for B73 and W22, respectively. The list of primer pairs that were used to quantify each gene are shown in Table 1.

Expression analysis of *Zmmed12a* mutant alleles DNA was extracted from 10 day old greenhouse grown seedlings of F2 populations segregating the *med12a-1::Ds* and *med12a-2::Ds* insertions. Seedlings were genotyped using primers to identify homozygous wild type and homozygous insertion alleles for *1::Ds* (primer pair A5.12F and A5.12R for wild type and A5.12F and JGp3 for *Ds* insertion) and *2::Ds* (primer pair C2.7F and C2.7R for wild type allele and C2.7R and JGp3 for *Ds* insertion). Total RNA was then extracted for wild type and homozygous insertion alleles. RNA isolation and RT-qPCR were performed as described above. Two sets of primers were used to analyze the effect of the transposon insertions on *ZmMed12a* transcripts. One pair of primers was in the 5´UTR and the other pair was in the 3´UTR (Table 3). Relative gene expression was normalized to levels of control genes in WT plants (normalized to 1). Values reported in qPCR analysis are the mean of three biological replicates and error bars are standard deviations. Each biological replicate consisted of one plant.

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