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The role of Mediator in small and long noncoding RNA production in *Arabidopsis thaliana*

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Mediator is a conserved multi-subunit complex known to promote the transcription of protein-coding genes by RNA polymerase II (Pol II) in eukaryotes. It has been increasingly realized that Pol II transcribes a large number of intergenic loci to generate noncoding RNAs, but the role of Mediator in Pol II-mediated noncoding RNA production has been largely unexplored. The role of Mediator in noncoding RNA production in plants is particularly intriguing given that plants have evolved from Pol II two additional polymerases, Pol IV and Pol V, to specialize in noncoding RNA production and transcriptional gene silencing at heterochromatic loci. Here, we show that Mediator is required for microRNA (miRNA) biogenesis by recruiting Pol II to promoters of miRNA genes. We also show that several well-characterized heterochromatic loci are de-repressed in Mediator mutants and that Mediator promotes Pol II-mediated production of long noncoding scaffold RNAs, which serve to recruit Pol V to these loci. This study expands the function of Mediator to include Pol II-mediated intergenic transcription and implicates a role of Mediator in genome stability.

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Introduction

Mediator is a multi-subunit complex first identified in yeast as required for activator-dependent stimulation of RNA polymerase II (Pol II) transcription (Kelleher *et al*, 1990; Flanagan *et al*, 1991). It promotes transcription initiation by bridging transcription activators that bind to upstream promoter or enhancer elements and the Pol II general transcriptional machinery located at the basal promoter. Structural studies show that the 21-subunit yeast Mediator forms three submodules, the head, middle, and tail, with the head submodule serving as the Pol II-interacting interface and the tail submodule interacting with sequence-specific transcription factors (reviewed in Chadick and Asturias, 2005). While some studies support a strict activator-dependent role of Mediator in transcription (Fan *et al*, 2006; Fan and Struhl, 2009), others favour an additional role of Mediator in basal transcription as a general transcription factor (Thompson and Young, 1995; Holstege *et al*, 1998; Ansari *et al*, 2009). Mediator is highly conserved in a wide range of eukaryotes including mammals and plants, although the mammalian and *Arabidopsis* Mediator contains more subunits than the yeast Mediator (Malik and Roeder, 2005; Backstrom *et al*, 2007). Almost all studies on yeast and mammalian Mediator have been focused on the role of Mediator in the expression of protein-coding genes. A recent study shows that the mouse Mediator promotes the expression of small nuclear RNAs (snRNAs) (Krebs *et al*, 2010). But the role of Mediator in noncoding RNA transcription remains largely unexplored.

The *Arabidopsis* Mediator was biochemically characterized only recently and found to contain 21 subunits with yeast or metazoan counterparts and six plant-specific subunits (Backstrom *et al*, 2007). A few *Arabidopsis* genes encoding Mediator subunits have been genetically characterized. Loss-of-function mutants in *MED21* are embryo lethal (Dhawan *et al*, 2009), indicating that Mediator is essential. On the other hand, mutants in *STRUWWELPETER*/*MED14* and *PHYTOCHROME AND FLOWERING TIME1*/*MED25* are viable but defective in specific developmental processes or responses to environmental stimuli (Autran *et al*, 2002; Cerdan and Chory, 2003; Kidd *et al*, 2009). This led to the hypothesis that Mediator integrates various signalling pathways at the molecular level (Backstrom *et al*, 2007). Although these studies suggest that the plant Mediator is important in gene expression, the scope of Mediator's function in transcription remains unknown.

Transcriptome studies in fungi, plants, and animals have revealed pervasive transcription of the genome to generate a multitude of noncoding RNAs. microRNAs (miRNAs) constitute a class of well-studied noncoding RNAs that regulates gene expression at posttranscriptional levels (reviewed in Chen, 2009; Kim *et al*, 2009). Small interfering RNAs (siRNAs) constitute another class of endogenous small RNAs that maintains genome stability by triggering heterochromatin formation at repeats and transposons in plants and *Schizosaccharomyces pombe* (Reinhart and Bartel, 2002; Volpe *et al*, 2002; Xie *et al*, 2004). In these organisms, long noncoding RNAs are also produced at repeats and transposons and serve to recruit siRNAs to these loci to result in heterochromatin formation (Volpe *et al*, 2002; Wierzbicki *et al*, 2008; Zheng *et al*, 2009). The role of Mediator in the production of these small and long noncoding RNAs has not been evaluated. The fact that plants have evolved from Pol II two new polymerases, Pol IV and Pol V, to produce siRNAs and long noncoding RNAs, respectively, to silence

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repeats and transposons (reviewed in Chen, 2009), raises the question of whether Mediator acts in transcriptional gene silencing (TGS) and whether Mediator promotes the activities of Pol IV or Pol V.

Here, we evaluate the effects of three Mediator mutations in the expression of protein coding as well as noncoding RNA genes in *Arabidopsis*. We show that Mediator is likely a general transcription factor that promotes Pol II transcription of a large number of protein-coding genes. We also show that Mediator promotes the transcription of miRNA genes (*MIR*) by recruiting Pol II to their promoters. In addition, we reveal a previously unsuspected role of Mediator in noncoding RNA production at, and TGS of, loci regulated by endogenous siRNAs. These findings broaden our knowledge of the role of Mediator in gene expression and genome defense.

Results

Arabidopsis *mutants in three Mediator subunit genes display pleiotropic developmental defects*

A mutant with pleiotropic developmental phenotypes was isolated in a forward genetic screen in the Columbia (wild type) background. The mutant was smaller than wild type in stature and late flowering (Figure 1A). The leaves had shorter petioles and curled downward (Supplementary Figure S1A), the phyllotaxy of flowers was abnormal (Supplementary Figure S1B), and fertility was reduced (Figure 1B; Supplementary Figure S1D). At a frequency of ∼20%, the mutant developed three cotyledons and three first true leaves instead of a pair of each in wild type (Supplementary Figure S1A).

The mutation was mapped to a 120-kb region covered by the BACs T3B23 and T1B3 on chromosome 2. Sequencing candidate genes in this region revealed a C-to-T mutation in At2g28230, which encodes a subunit of the head submodule in Mediator (Figure 1C). The mutation is at the 58th nucleotide of the coding region in the second exon and results in a premature stop codon (Figure 1C). A genomic construct covering 2.5 kb of the promoter and the entire coding region of At2g28230 was introduced into the mutant—the morphological phenotypes of the mutant were rescued in all 39 independent transgenic lines (Supplementary Figure S1C and data not shown). Therefore, the *med20a* mutation was responsible for the morphological defects. A homology-based search identified two more paralogs in *Arabidopsis*: At2g28020 and At4g09070. Therefore, At2g28230, At4g09070, and At2g28020 were named *MED20a*, *MED20b*, and *MED20c*, respectively (Figure 1C). *MED20a* and *MED20b* have 85% identity at the nucleotide level. *MED20*c is shorter than *MED20a* and *MED20b*; there is 93% identity at the nucleotide level in exon 2 among the three genes. Transcriptome studies using Affymetrix ATH1 microarrays found *MED20a* and *MED20c* to be expressed throughout the

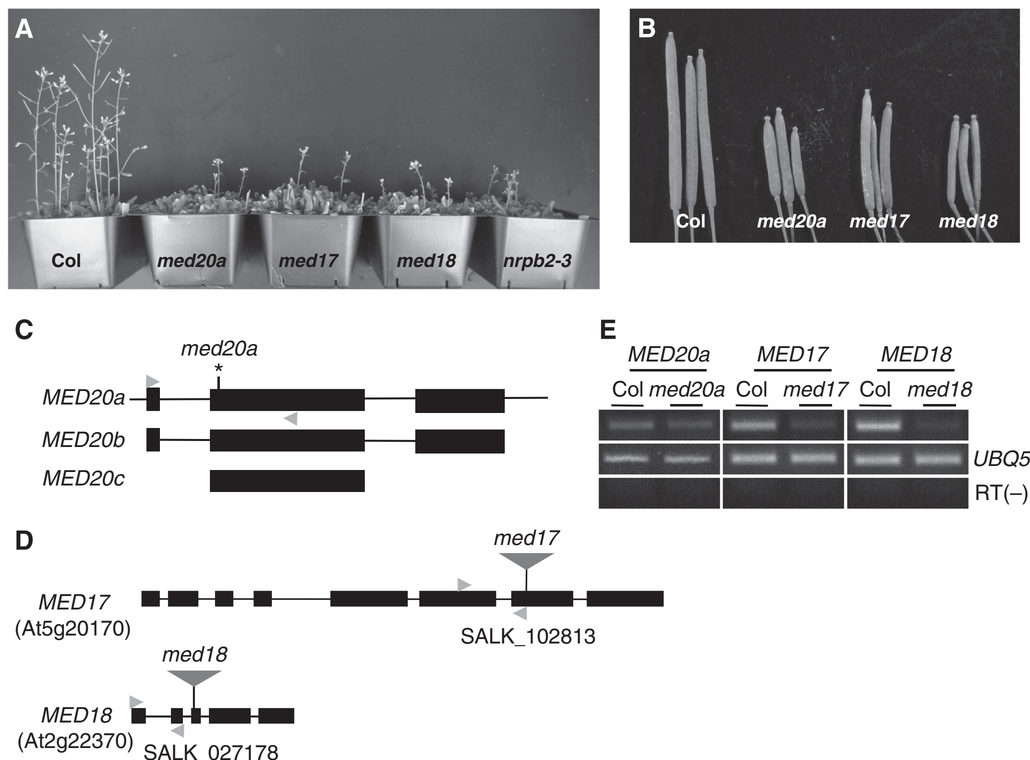

Figure 1 Isolation and characterization of mutants in Mediator genes. (**A**) Four-week-old plants of wild type (Col), *med20a*, *med17*, *med18*, and *nrpb2-3*. (**B**) Siliques from Col, *med20a*, *med17*, and *med18* plants. (**C**) Schematic diagrams of *MED20* paralogs *MED20a*, *MED20b*, and *MED20c*. The asterisk indicates the mutation causing a premature stop codon in the *med20a* mutant. Black rectangles represent exons and lines represent introns. *MED20a* and *MED20b* are composed of three exons showing 85% identity at the nucleotide level. *MED20c* only encodes the conserved second exon compared with *MED20a* and *MED20b*. (**D**) Schematic diagrams of T-DNA insertion mutants of *MED17* and *MED18*. Large triangles represent T-DNA insertions. Black rectangles represent exons. (**E**) RT–PCR analysis of *MED20a*, *MED17*, and *MED18* expression in *med20a*, *med17*, and *med18* mutants, respectively. The images in the top row represent the indicated Mediator genes. *UBIQUITIN5* (*UBQ5*) was used as an internal loading control. The '−RT' reactions were performed with the *UBQ5* primers. The PCR primers used are indicated by small arrowheads in (**C**, **D**).

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plant but probes for *MED20b* were not present in the microarray (Winter *et al*, 2007). Given the high degree of sequence similarity among the genes, it is unlikely that the microarray studies were able to differentiate the paralogs.

To determine whether the developmental phenotypes of the *med20a* mutant reflect the function of the head submodule of Mediator, we obtained two T-DNA mutant lines of genes encoding two other head submodule subunits, *MED17* and *MED18*, from the SALK collection and analysed their phenotypes (Figure 1D). Transcript levels of *MED17* and *MED18* were significantly reduced in *med17* and *med18*, respectively, indicating that the T-DNA insertions caused at least a partial loss of function of *MED17* and *MED18* genes (Figure 1E). The *med17* and *med18* mutants exhibited similar developmental defects as *med20a*, although the phenotypes of *med17* were weaker overall as compared with those of the other two mutants (Figure 1A and B; Supplementary Figure S1A, B, and D). These results indicate that Mediator is broadly involved in developmental processes in *Arabidopsis*.

The plant Mediator is likely a general transcription factor

Mediator is a conserved multi-protein complex in animals, fungi, and plants. Even for the yeast Mediator, which has been studied much more extensively than the plant Mediator, there is still controversy as to whether Mediator promotes basal transcription as a general transcriptional factor for Pol II (Thompson and Young, 1995; Holstege *et al*, 1998; Fan *et al*, 2006; Ansari *et al*, 2009; Fan and Struhl, 2009). A few studies on mutants in several *Arabidopsis* Mediator genes implicate a role of Mediator in integrating various environmental signals (Cerdan and Chory, 2003; Backstrom *et al*, 2007; Kidd *et al*, 2009), but it is unknown whether the plant Mediator serves as a general transcription factor or as a bridge between sequence-specific transcription factors and Pol II.

To investigate the molecular function of the plant Mediator, we compared the phenotypic as well as transcriptomic effects of the *med20a* mutation with those of *nrpb2-3*, a weak allele in the second largest subunit of Pol II that we had previously isolated (Zheng *et al*, 2009). *med20a* and *nrpb2-3* exhibited similar morphological phenotypes—both mutants were small in stature, had reduced fertility, and exhibited altered phyllotaxy and delayed leaf emergence indicative of abnormal shoot apical meristem activity (Figure 1A, Supplementary Figure S1A, and data not shown). Affymetrix ATH1 microarray-based transcript profiling using inflorescence tissues from wild type and *nrpb2-3* revealed that 448 genes were downregulated and 95 genes were upregulated by two-fold in *nrpb2-3* (Zheng *et al*, 2009). To examine the overlap between Pol II- and Mediator-dependent genes, we conducted similar transcript profiling between wild-type and *med20a* inflorescences. We found that a total of 754 genes were downregulated and 140 genes were upregulated in *med20a* by two-fold in three biological replicates (Supplementary Tables 1 and 2). Genes upregulated in the two mutants were unlikely direct targets of Pol II or Mediator and thus were not studied further. The number of genes downregulated in *med20a* was almost twice that in *nrpb2-3*, which was not surprising given that *nrpb2-3* is a weak allele. Intriguingly, 84% (377 genes) of the downregulated genes (448 genes) in *nrpb2-3* were also downregulated in *med20a* (Supplementary Figure S2), suggesting a large degree of overlap between Pol II and Mediator in gene expression and supporting the role of Mediator as a general transcription factor. The small number of genes (754) downregulated in *med20a* is likely due to the presence of two other *MED20* paralogs in the genome.

Pol II and Mediator are required for miRNA accumulation in Arabidopsis

Mediator is well known for its role in Pol II-mediated transcription of protein-coding genes and has been recently shown to promote the expression of snRNAs (Krebs *et al*, 2010), but its role in noncoding RNA transcription remains largely unexplored. Although the structures of *MIR* genes, such as the promoters and introns, and the presence of polyA tails in primary precursors of miRNAs (pri-miRNAs) suggest that *MIR* genes are transcribed by Pol II in plants (Xie *et al*, 2005), direct evidence for Pol II transcription of *MIR* genes is still lacking. We explored the role of Pol II and Mediator in miRNA biogenesis using the *nrpb2-3* and *med* mutants.

We first examined the accumulation of various mature miRNAs in *nrpb2-3* and *med20a* mutants by northern blotting. siR1511, a *trans*-acting siRNA (ta-siRNA), whose biogenesis requires a miRNA (Peragine *et al*, 2004; Vazquez *et al*, 2004; Allen *et al*, 2005; Yoshikawa *et al*, 2005), was also examined. Nine out of 12 tested miRNAs and siR1511 were present at reduced levels in *nrpb2-3*, with the exceptions being miR160, miR163, and miR166 (Figure 2A; Supplementary Figure S3A). In the *med20a* mutant, 8 out of 11 examined miRNAs (with miR158, miR160, and perhaps miR390 being the exceptions) and siR1511 were at lower levels (Figure 2B). The reduced accumulation of miRNAs in *med20a* was rescued by the introduction into *med20a* a *MED20a* transgene (Supplementary Figure S3B). We also examined the levels of four miRNAs in *med17* and *med18*. Consistently, all four miRNAs were at reduced levels in *med17* and *med18* (Figure 2C). Therefore, both Pol II and Mediator are required for miRNA biogenesis in *Arabidopsis*.

Mediator regulates MIR gene expression at the transcriptional level

The reduced miRNA accumulation in Mediator mutants may be due to reduced transcription of *MIR* genes, compromised posttranscriptional processing of the precursors, or decreased stability of the mature miRNAs. Given the known role of Mediator in promoting transcription, it is likely that the reduced miRNA accumulation in Mediator mutants is attributable to reduced transcription of *MIR* genes. To test this hypothesis, we first examined the levels of six pri-miRNAs by real-time RT–PCR. Indeed, the levels of all examined pri-miRNAs were decreased in the *med20a* mutant to 20–70% of the wild-type levels (Figure 3A; Supplementary Figure S4). We further examined the levels of individual pri-miRNAs from each member of the miR166 family (Supplementary Figure S5). Four of the six *MIR166* loci were expressed at lower levels in *med20a* as compared with wild type, suggesting that different *MIR* family members have different degrees of dependence on *MED20a* for their expression.

To directly demonstrate that the *med20a* mutation affected the activity of an *MIR* promoter, we crossed a *GUS* transgene under the control of the *MIR167a* promoter (*pMIR167a*::*GUS*) (Wu *et al*, 2006) into *med20a*. In the F2 population, 3 to 1 segregation of the transgene was observed, which indicated

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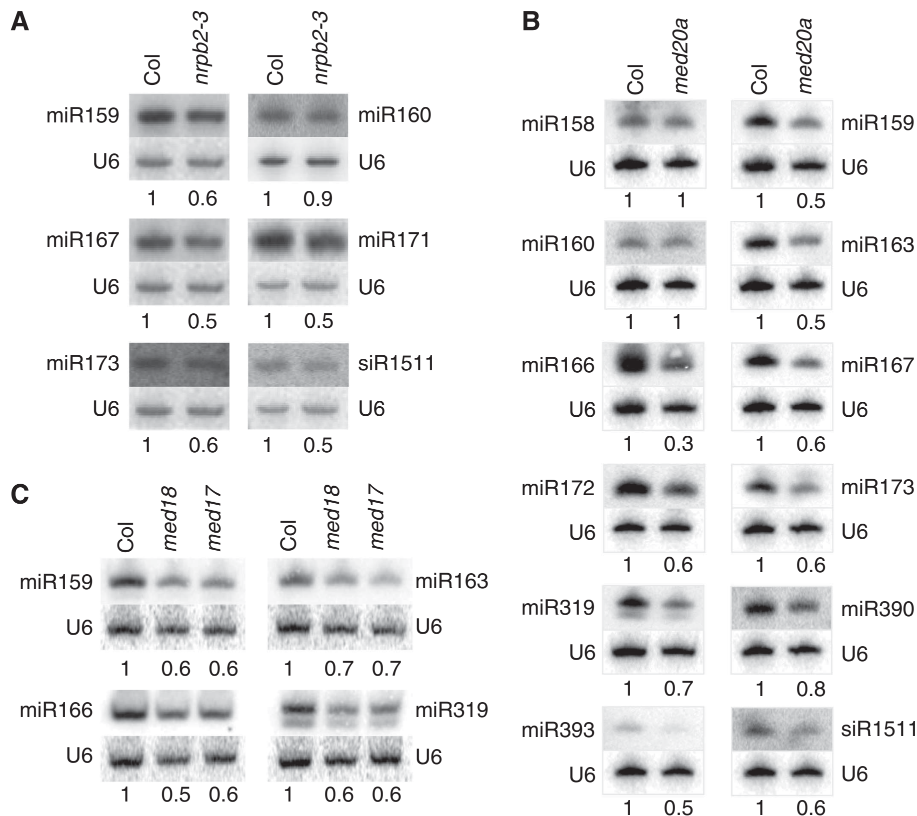

Figure 2 The accumulation of miRNAs and a ta-siRNA in *nrpb2-3* and Mediator mutants as determined by northern blotting. (**A**) The accumulation of five miRNAs and a ta-siRNA (siR1511) in wild type (Col) and *nrpb2-3*. (**B**) The accumulation of 11 miRNAs and one ta-siRNA (siR1511) in Col and *med20a*. (**C**) The accumulation of four miRNAs in Col, *med17*, and *med18*. Total RNAs were extracted from inflorescences. The levels of each small RNA were normalized to those of U6 and compared with Col. The numbers below the gel images indicate the relative abundance of the small RNAs. Two to three biological replicates were performed for all small RNAs except for miR390 and miR159 and yielded similar results.

that the transgene was at a single locus. Wild-type and *med20a* plants containing the transgene were obtained from the F2 population of the cross and their progeny were screened to identify ones that were homozygous for the transgene. GUS staining performed on these single insertion, isogenic *med20a* and wild-type plants revealed decreased *GUS* expression in *med20a* compared with wild type, especially in old flowers (Figure 3B). Since GUS staining was not a quantitative measure of reporter gene expression, we performed real-time RT–PCR to determine the levels of *GUS* mRNA in inflorescences. Indeed, *GUS* mRNA levels were lower in *med20a* than in wild type (Figure 3C), indicating that Mediator acts through the promoter of *MIR167a*.

The plant Mediator has been implicated as an integrator in response to environmental cues in *Arabidopsis* (Cerdan and Chory, 2003; Backstrom *et al*, 2007; Kidd *et al*, 2009). Meanwhile, the expression of some *MIR* genes is known to be regulated by abiotic stresses. For example, the expression of miR398b is transcriptionally downregulated by oxidative stress (Sunkar *et al*, 2006), whereas the expression of *MIR167a* and *MIR171a* is transcriptionally upregulated by cold stress and that of *MIR159a* is upregulated by salt stress (Liu *et al*, 2008). To determine whether Mediator is required for the stress-induced changes in *MIR* gene expression, we monitored levels of pri-miRNAs of these stress-inducible *MIR* genes in response to stress treatments in wild type, *med20a*, and *med18*. Our semi-quantitative RT–PCR reproduced the induced expression of *MIR159a*, *MIR167a*, and *MIR171a* and the repressed expression of *MIR398b* by various stresses (Supplementary Figure S6). The stress-induced changes in the expression of these *MIR* genes occurred also in *med20a* and *med18* mutants (Supplementary Figure S6). Given that *med20a* is likely a null allele and *med18* is a strong allele in that *MED18* RNA levels were strongly reduced in this mutant (Figure 1E), these results suggest that *MED20a* and *MED18* are dispensable for stress-induced changes in *MIR* gene expression.

Mediator promotes Pol II recruitment to MIR genes

The reduced pri-miRNA and mature-miRNA levels in the *med20a* mutant are unlikely attributable to reduced expression of miRNA biogenesis genes because none of the known genes in miRNA biogenesis were affected in *med20a* as determined by microarray analyses (see Supplementary Table 3 for a list of the genes). This prompted us to examine whether Mediator has a direct role in the transcription of *MIR* genes. We examined Pol II occupancy at promoters of several *MIR* genes by chromatin immunoprecipitation (ChIP) using an antibody against the second largest subunit of Pol II (RPB2). We monitored three *MIR* genes, *MIR167a*, *MIR171a*, and *MIR166a*, of which pri-miRNAs were at reduced levels in the *med20a* mutant (Figure 3A). Pol II was enriched at the regions encompassing the transcription start sites at these three loci, as compared with the 'no antibody' ChIP that

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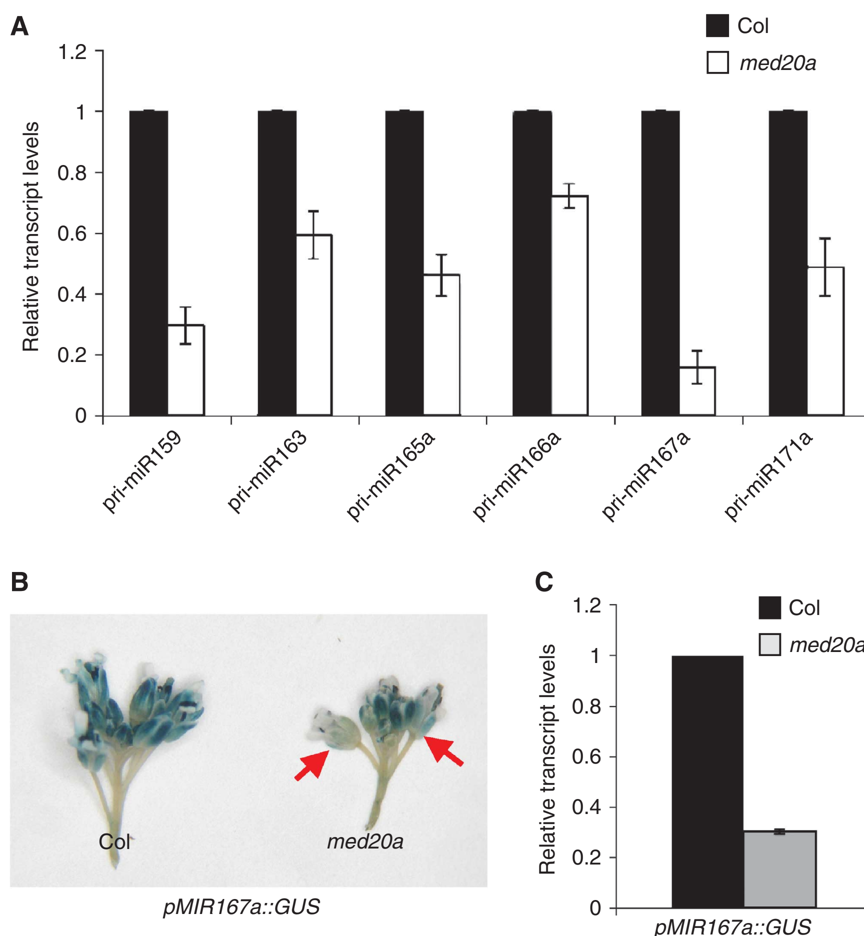

Figure 3 *med20a* affects the transcription of *MIR* genes. (**A**) The accumulation of six pri-miRNAs was determined by real-time RT–PCR in Col and the *med20a* mutant. Total RNAs were extracted from inflorescences. The pri-miRNA levels were normalized to those of *UBQ5* and compared with Col. Standard deviations were calculated from three technical replicates. Three biological replicates yielded similar results. (**B**) *GUS* expression driven by the *MIR167a* promoter was monitored in isogenic Col and *med20a* transgenic lines through GUS staining. *GUS* expression in old flowers was reduced in *med20a* compared with Col (arrows). (**C**) The accumulation of *GUS* mRNA from the lines in (**B**) was determined by real-time RT–PCR. The *GUS* mRNA levels were normalized to those of *UBQ5* and compared with Col. Standard deviations were calculated from three technical replicates. Two biological replicates yielded nearly identical results.

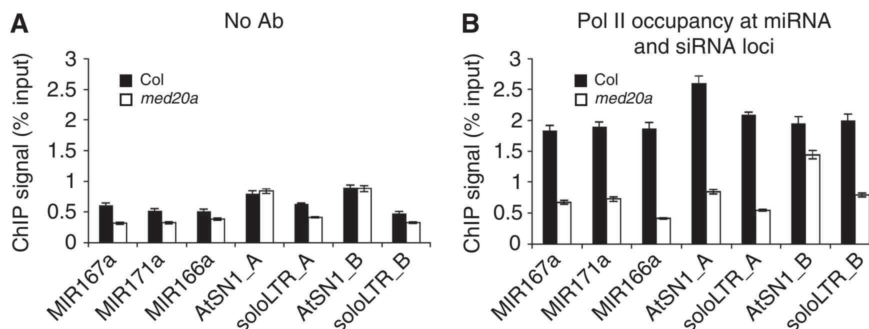

Figure 4 Pol II occupancy at miRNA and siRNA loci. Pol II occupancy at several miRNA and siRNA loci was determined by ChIP using anti-RPB2 antibodies in Col and *med20a*. DNA present in the immunoprecipitates was quantified by real-time PCR relative to total input DNA. (**A**) ChIP performed with no antibodies as negative controls. (**B**) ChIP with anti-RPB2 antibodies. The results were reproduced in two biological replicates. Standard deviations were calculated from three technical repeats. AtSN1_A and soloLTR_A, regions A of these two loci as depicted in Figure 5B. AtSN1_B and soloLTR_B, regions B of these two loci as in Figure 5B.

served as a negative control (Figure 4A and B). Pol II occupancy at these loci was significantly reduced in the *med20a* mutant (Figure 4B). These results indicate that Mediator promotes Pol II recruitment to *MIR* genes.

Mediator is required for TGS and Pol II-mediated long noncoding RNA production at repeats and transposons

Plants have evolved from Pol II two polymerases, Pol IV and Pol V, to specialize in endogenous small RNA-based genome

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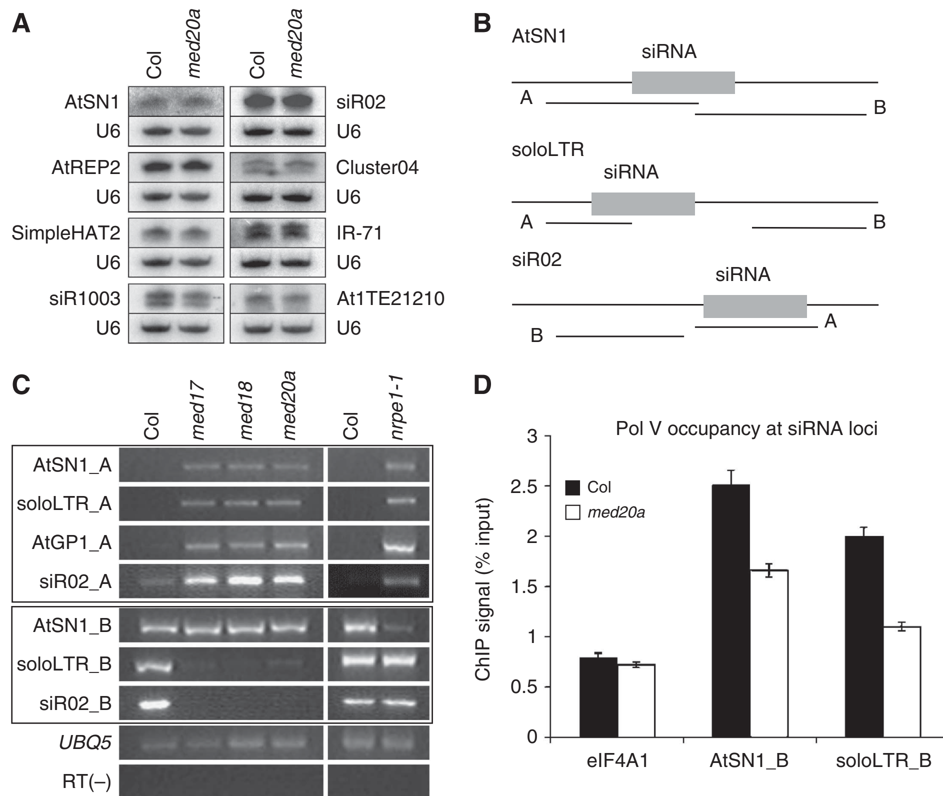

Figure 5 Mediator in TGS of repeats and transposons. (**A**) The accumulation of endogenous siRNAs from heterochromatic loci. Small RNA northern blotting was performed with total RNAs extracted from inflorescences from Col and *med20a*. Small RNA accumulation was not affected in the *med20a* mutant. The U6 blots served as loading controls for the overlying small RNA blots. (**B**) Diagrams of AtSN1, soloLTR, and siR02 genomic regions. These regions are based on analysis of transcription units by Wierzbicki *et al* (2008) and Zheng *et al* (2009). The 'A' regions are where the siRNAs are derived, while the 'B' regions are where scaffold transcripts are produced. (**C**) RT–PCR analysis of noncoding transcripts from regions A and B at siRNA loci in Col and Mediator mutants. *UBQ5* was used as an internal control. The RT (−) control PCR was performed with *UBQ5* primers. *nrpe1-11* is a loss-of-function allele in the largest subunit of Pol V. The results shown were reproduced in three biological replicates. (**D**) Pol V occupancy at regions B of AtSN1 and soloLTR was determined by ChIP using anti-FLAG antibodies in *pNRPE*::*NRPE1-FLAG* and *pNRPE*::*NRPE1-FLAG med20a*. DNA that co-purified with Pol V was measured by real-time PCR against total input DNA. *eIF4A1*, a gene not bound by Pol V, served as a negative control. Standard deviations were calculated from three technical repeats. The results were reproduced in two other biological replicates shown in Supplementary Figure S8.

defense against repeats and transposons (Herr *et al*, 2005; Kanno *et al*, 2005; Onodera *et al*, 2005; Pontier *et al*, 2005; Huang *et al*, 2009; Ream *et al*, 2009). These repeated elements generate 24 nt endogenous siRNAs in a Pol IV-dependent manner (Herr *et al*, 2005; Onodera *et al*, 2005; Pontier *et al*, 2005) and the siRNAs are thought to be recruited back to these elements by nascent transcripts, also known as scaffold transcripts, generated by Pol V from these elements (Herr *et al*, 2005; Kanno *et al*, 2005; Wierzbicki *et al*, 2008, 2009). At some loci, such as solo LTR and siR02, Pol II is responsible for the production of noncoding scaffold transcripts and TGS of the loci (Zheng *et al*, 2009). The presence of two additional polymerases derived from Pol II and the recent finding that Pol II generates long noncoding RNAs at heterochromatic loci raised the question of whether Mediator acts with any of the three polymerases in TGS.

We first determined whether Mediator is required for the production of 24 nt siRNAs, a process that requires Pol IV. We examined the levels of 24 nt siRNAs from eight loci known to undergo siRNA production and siRNA-mediated TGS (Herr *et al*, 2005; Onodera *et al*, 2005; Pontier *et al*, 2005; Huettel *et al*, 2006) in wild type and *med20a* by northern blotting. The levels of the siRNAs were not affected in *med20a* (Figure 5A). These results do not support a role of Mediator in promoting Pol IV-mediated production of endogenous siRNAs, although such a role cannot be excluded given that the *med20a* mutation is unlikely to completely eliminate Mediator activity.

Next, we examined the expression of these loci as a measure of their silencing status by real-time RT–PCR. It is known that these loci are de-repressed in Pol IV and Pol V mutants (Herr *et al*, 2005; Onodera *et al*, 2005; Pontier *et al*, 2005; Huettel *et al*, 2006). At all tested loci, transcript levels from regions A, to which siRNAs map (Figure 5B), were higher in *med17*, *med18*, and *med20a* mutants as compared with wild type, although the degree of de-repression was not as large as in *nrpe1-11*, a mutant in the largest subunit of Pol V (Pontier *et al*, 2005) (Figure 5C). Note that among these loci, AtSN1 and AtGP1 are known as type I loci that are silenced in a Pol V-dependent manner whereas solo LTR and siR02 are known as type II loci that are silenced in a Pol II- and Pol V-dependent manner (Zheng *et al*, 2009). Pol V generates scaffold transcripts at type I loci, whereas Pol II generates these RNAs at type II loci (Wierzbicki *et al*, 2008; Zheng *et al*, 2009). Since siRNA levels were not affected in *med20a* (Figure 5A), we suspected that the de-repression of

these loci in the Mediator mutants was due to reduced levels of Pol II- or Pol V-dependent scaffold RNAs from these loci. We examined the levels of scaffold RNAs in wild type, *med17*, *med18*, *med20a*, and *nrpe1-11* (a control for type I loci) corresponding to regions B (Figure 5B) at these loci. At type II loci (soloLTR and siR02), the levels of Pol II-dependent scaffold transcripts were obviously reduced in all *med* mutants (Figure 5C). However, at the type I locus AtSN1, the scaffold transcripts were reduced in abundance in *nrpe1-11* but not affected in any of the Mediator mutants (Figure 5C). The levels of Pol V-dependent scaffold transcripts at two other loci, IGN6 (Wierzbicki *et al*, 2008) and MEA-ISR (Law *et al*, 2010), were also not affected in the Mediator mutants (Supplementary Figure S7).

To determine whether Mediator has a direct role in promoting Pol II-based noncoding transcription at the type II locus soloLTR, we performed ChIP to examine Pol II occupancy at this locus in wild type and *med20a*. At both region A and region B, from which siRNAs and scaffold transcripts are produced, respectively (Figure 5B), Pol II was enriched at soloLTR in comparison to the 'no antibody' control (Figure 4A and B), consistent with previous observations (Zheng *et al*, 2009). Pol II occupancy at both regions A and B at this locus was reduced in *med20a*, supporting a role of Mediator in recruiting Pol II to this locus. This, together with the reduced levels of Pol II-dependent scaffold transcripts at soloLTR and siR02 (Figure 5C), indicates that Mediator promotes Pol II-mediated noncoding RNA production at loci undergoing siRNA-mediated TGS.

Intriguingly, although analysis of the weak *nrpb2-3* mutant did not reveal a role of Pol II in TGS at type I loci (Zheng *et al*, 2009), Pol II was enriched at the type I locus AtSN1 (Figure 4A and B), which was also observed previously (Zheng *et al*, 2009). Moreover, Pol II occupancy at both regions A and B at AtSN1 was reduced in *med20a* (Figure 4B). In addition, although Pol V-dependent scaffold transcripts at AtSN1 were not affected in the *med20a* mutant (Figure 5C), ChIP detected reduced occupancy of Pol V at AtSN1 region B (Figure 5D; Supplementary Figure S8), where Pol V-dependent scaffold transcripts are derived (Wierzbicki *et al*, 2008). At soloLTR, which requires Pol V for TGS and to which Pol V recruitment to region B relies on Pol II-dependent scaffold transcripts (Zheng *et al*, 2009), Pol V occupancy was reduced in *med20a* (Figure 5D; Supplementary Figure S8), consistent with the reduced scaffold RNAs in this mutant (Figure 5C). There are two possibilities regarding the relationship between Mediator and Pol V. In one, Mediator promotes Pol V-mediated noncoding transcription at type I loci, but the weak nature of the *med*20a, *med17*, and *med18* mutants (due to the presence of paralogs or partial loss of function) precluded the assignment of such a role to Mediator. The second possibility is that Pol II also acts in the TGS of type I loci, and the de-repression of these loci in *med* mutants reflects the role of Mediator acting with Pol II.

Discussion

In this study, we provided evidence supporting that MED20a, a subunit of the head submodule of the plant Mediator, is broadly required for Pol II transcription of protein-coding genes. Transcript profiling with *med20a* and *nrpb2-3* mutants revealed a high degree of overlap in genes that are decreased in expression in the two mutants. Although only 750 genes were affected in the *med20a* mutant, it is likely that these genes represent only a portion of those requiring *MED20* since two other *MED20a* paralogs may compensate for the absence of *MED20a*. It is likely that the plant Mediator, or at least the head submodule, functions as a general transcription factor to promote basal Pol II transcription.

Mediator also promotes Pol II transcription of *MIR* genes. Plant *MIR* genes contain canonical basal promoter elements and produce polyadenylated pri-miRNAs, suggesting that *MIR* genes are transcribed by Pol II. In this study, we provided direct evidence for a role of Pol II in *MIR* gene expression by showing that miRNAs are reduced in abundance in the *nrpb2-3* mutant and that Pol II is present at the transcription start sites of *MIR* genes. The reduced accumulation of mature miRNAs in mutants in three head submodule subunits of Mediator suggests a general role of Mediator in *MIR* gene expression. The role of Mediator in promoting Pol II transcription of *MIR* genes is supported by the reduced levels of pri-miRNAs, compromised *MIR167a* promoter activity, and decreased Pol II occupancy at *MIR* transcription start sites in *med20a*.

This work also revealed a previously unknown role of Mediator in silencing repeats and transposons. Well-characterized elements that undergo siRNA-mediated TGS were found to be de-repressed in all three *med* mutants. The decreased Pol II occupancy and reduced levels of noncoding scaffold RNAs transcribed by Pol II support a role of Mediator in Pol II-mediated noncoding RNA production in genome defense. While a previous study with the weak *nrpb2-3* mutant established a role of Pol II in TGS only at type II loci, the analysis of three Mediator mutants in this study shows that Mediator is involved in TGS at both type I and type II loci. How Mediator acts in TGS of type I loci remains to be determined. One possibility is that Mediator acts with Pol II, which also acts at type I loci but such a function of Pol II has not been established since it is not compromised by the weak *nrpb2-3* mutation. Another possibility is that Mediator acts with Pol V. This is consistent with the observation that Pol V occupancy is reduced in *med20a* (Figure 5D). Intriguingly, the plant-specific Mediator subunit MED36 (Backstrom *et al*, 2007) was found in a proteomic study to co-purify with the largest subunit of Pol V (Huang *et al*, 2009).

Materials and methods

Plant materials

All mutants are in the Col background. *Arabidopsis* plants were grown at 23°C under continuous light. T-DNA insertion lines of *MED17* and *MED18* were obtained from the Salk T-DNA insertion collection. *nrpb2-3* was isolated from our laboratory (Zheng *et al*, 2009). *nrpe1-11* (formerly known as *nrpd1b-1*) (Pontier *et al*, 2005) and the *pMIR167a::GUS* transgenic line (Wu *et al*, 2006) were gifts from Dr Thierry Lagrange and Dr Jason W Reed, respectively. For *med20a* genotyping, genomic DNA was amplified with the primers At2g28230-F (CATACCTCAATTTCGATTGGG) and At2g28230-R4 (GAAGAATCAGCTTCCAAGAC) and PCR products were digested with *Alu*I. The PCR products from wild type could be digested by *Alu*I, whereas those from *med20a* could not.

For NaCl and cold stress treatments, seeds were surface sterilized and sown on MS-agar plates. Seeds were stratified at 4°C for 2 days and transferred to 22°C for 10 days. For salt stress treatments, seedlings were transferred to filter paper saturated with either water (control) or 300 mM NaCl and incubated for 6 h. For cold stress

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treatments, seedlings on plates were incubated either at 22°C (control) or at 4°C for 6 h. For copper treatment, surface-sterilized seeds were sown on MS-agar plates with or without 10 μM Cu^{2+} and 10-day-old seedlings were harvested.

Map-based cloning of MED20a

med20a (Col) was crossed to L*er*. In the F2 population, plants showing *med20a* phenotypes were identified and used as the mapping population. Rough mapping using 27 *med20a* plants showed that *MED20a* is linked to the marker nga168 on chromosome 2. For fine mapping, we designed new SSLP or CAPS markers in this region according to polymorphisms between L*er* and Col according to Monsanto *Arabidopsis* polymorphism database (http://www.arabidopsis.org/Cereon). Using these markers and ~500 plants of *med20a* phenotypes, we mapped *MED20a* to a 120-kb region covered by the BACs T3B23 and T1B3. Among the 23 genes in this region, sequencing analysis revealed a point mutation in *MED20a*.

Plasmid construction

MED20a genomic region was amplified with primers At2g28230-pro2 (CACCCTTGGATTGTACTGCTGGT) and At2g28230-R2 (GCCTC TCACAGC-TTGAACG) and cloned into the pEG301 gateway vector (Earley *et al*, 2006) to generate *pMED20a*::*MED20a-HA*. The plasmid was used to transform *med20a* plants by agroinfiltration.

RT–PCR

Total RNA from seedlings or inflorescences was extracted with TRI reagent (Molecular Research Center) and reverse transcribed using SuperScriptII reverse transcriptase (Invitrogen) and an oligo-d(T) primer according to the manufacturer's instructions. For pri-miRNA detection, quantitative PCR was carried out in triplicate on a Bio-Rad IQcycler apparatus with the Quantitech SYBR green kit (Bio-Rad). To detect Pol II- or Pol V-dependent noncoding transcripts, RT–PCR was done according to Zheng *et al* (2009). Primers used are listed in Supplementary Table 4.

Small RNA northern blot analysis

RNA isolation and hybridization for miRNA and endogenous siRNA detection were performed as described (Park *et al*, 2002; Pall *et al*, 2007). In all, 10 μg total RNA and 5 μg small RNA-enriched RNA from inflorescences were used in northern blotting to detect miRNAs and endogenous siRNAs, respectively. 5′-End-labelled ^{32}P antisense DNA or LNA oligonucleotides were used to detect miRNAs and endogenous siRNAs. Oligonucleotide probes used are listed in Supplementary Table 5.

Histochemical staining

GUS staining was performed as described (Jefferson *et al*, 1987; Rodrigues-Pousada *et al*, 1993). Inflorescences were fixed in 90% cold acetone for 15–20 min and rinsed with the rinse solution (50 mM $NaPO_4$ pH 7.2, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$). Then, they were vacuum infiltrated in the staining solution (50 mM $NaPO_4$ pH 7.2, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 2 mM X-Gluc)) and incubated at 37°C.

ATH1 Affymetrix microarray analysis

GeneChip arrays were hybridized according to the manufacturer's instructions (Affymetrix). Data analysis was done according to Horan *et al* (2008). Normalization of raw intensities across all probe sets was performed in R using RMA algorithms. To calculate *P*-values for increases or decreases in expression, the Wilcoxon signed-rank test was applied to each pair of chips after normalization using R & Bio Conductor (developed by Dr Thomas Girke). A *P*-value of $\leqslant 0.05$ in combination with a two-fold difference was used to define changes in gene expression.

Chromatin immunoprecipitation

ChIP was performed according to Zheng *et al* (2009). The results shown were reproduced in three biological replicates. Commercial anti-RPB2 (catalog no. ab10338, Abcam) and anti-FLAG (F7425, Sigma) antibodies were used. Quantitative real-time PCR was performed on bound and input DNAs. The primer sets used for the PCR are listed in Supplementary Table 4.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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