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SERRATE: a new player on the plant microRNA scene

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MicroRNAs (miRNAs) function as sequence-specific guides that control gene expression by post-transcriptional gene silencing. Many miRNAs influence plant development by regulating the accumulation of transcripts that encode transcription factors. Mutants defective in miRNA accumulation, such as *dcl1*, *hen1*, *hyl1* and *ago1*, have pleiotropic developmental phenotypes. The *serrate-1* (*se-1*) mutant of *Arabidopsis* also shows a highly pleiotropic phenotype, which overlaps with the phenotypes of mutants defective in miRNA accumulation. Although it has been proposed that SERRATE (SE) functions specifically in miRNA-mediated repression of the leaf polarity genes *PHABULOSA* and *PHAVOLUTA*, microarray analysis shows upregulation of many genes known to be the targets of miRNAs in *se-1*. We show that SE is a general regulator of miRNA levels affecting the processing of primary miRNA to miRNA.

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INTRODUCTION

MicroRNAs (miRNAs) and short interfering RNAs (siRNAs) function as sequence-specific guides to silence genes, transposons and viruses, and to modify the chromatin structure. MiRNAs are involved in the control of various plant developmental processes, including leaf morphogenesis (Palatnik *et al*, 2003), floral development (Chen, 2004), root development (Guo *et al*, 2005), vascular development (Kim *et al*, 2005) and the transition from vegetative to reproductive phases (Lauter *et al*, 2005). Many miRNAs regulate plant development by delimiting the regions of accumulation of transcripts encoding transcription factors that function in development (Kidner & Martienssen, 2005). This explains the pleiotropic developmental phenotypes of plant mutants defective in miRNA accumulation, such as *dicer-like1* (*dcl1*), *hua enhancer 1* (*hen1*), *hyponastic leaves 1* (*hyl1*) and *argonaute 1* (*ago1*) (Park *et al*, 2002; Boutet *et al*, 2003; Vaucheret *et al*, 2004; Vazquez *et al*, 2004). The maturation of both types of small RNA is catalysed by double-stranded RNA (dsRNA)-specific RNaseIII-like enzymes called DICER-LIKE (Park *et al*, 2002). DCL1 processes miRNA precursors (Park *et al*, 2002; Kurihara & Watanabe, 2004) with two other proteins HEN1 (Boutet *et al*, 2003; Yu *et al*, 2005) and HYL1 (Han *et al*, 2004). The short dsRNAs produced by DCL activity are assembled into effector complexes and they guide sequence-specific degradation of complementary target messenger RNAs, translational repression of target mRNAs or condensation of heterochromatin (Meister & Tuschl, 2004). ARGONAUTE (AGO) proteins are components of these silencing effector complexes.

The *serrate-1* (*se-1*) mutant shows a highly pleiotropic phenotype that overlaps those of miRNA-defective mutants. *se-1* has abnormal embryogenesis like *short integument 1-2* (*sin1-2*; Ray *et al*, 1996); is hypersensitive to germination inhibition by abscisic acid (ABA) like *hyl1* (Lu & Fedoroff, 2000; Bezerra *et al*, 2004); and has delayed leaf initiation, accelerated phase change and aberrant phyllotaxy in the inflorescence like *hasty* (*hst*) alleles (Clarke *et al*, 1999; Prigge & Wagner, 2001; Bollman *et al*, 2003). Mutations in *SERRATE* (*SE*) also lead to defects in leaf number, leaf shape and flower development. Despite its pleiotropic effects on development, *se-1* is thought to cause only a partial loss of *SE* function. Two other *se* alleles (*se-2* and *se-3*) show more severe defects in leaf development, with adaxial leaf curling, loss of asymmetric differentiation of abaxial and adaxial cell types, and development of trumpet-shaped or radial leaves (Grigg *et al*, 2005). These effects are correlated with increased expression of the adaxial determinant *PHABULOSA* (*PHB*), an HD-ZIP III gene, and with reduced levels of two miRNAs, miR165 and miR166, which mediate cleavage of HD-ZIP III transcripts.

We investigated those genes whose expression is altered in response to changes in SE activity. Many upregulated genes are targets of miRNA regulation. Increased levels of transcripts of these target genes are correlated with decreased levels of at least eight mature miRNAs and a consequent decrease of target gene miRNA cleavage products. The levels of most primary miRNAs (pri-miRNAs) are increased in *se-1*. A new allele of *se* is embryonic lethal, which serves to underline the importance of miRNA-based regulation in all stages of plant development.

RESULTS

MicroRNA target genes are misexpressed in *serrate-1*

We used ATH1 arrays (Affymetrix, Santa Clara, CA, USA) to compare the transcript profiles of *se-1* with those of wild type. A total of 303 genes showed significantly changed expression between wild type and *se-1* (at least a twofold change and a *P*-value cutoff of 0.01), with 135 being upregulated and

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Table 1 | Changes in the expression levels of genes targeted by microRNA and *trans*-acting short interfering RNA assayed on ATH1 microarrays

Accession number	Fold increase	Gene description	Targeted by
miRNA and ta-siRNA target genes			
At1g51670	6.35	Expressed protein	ta-siRNAs 255 and 752
At3g05690	4.66	AtHAP2B	miRNA 169
At1g62930	4.65	PPR repeat protein	miRNA 161
At2g02850	3.85	Plastocyanin-like protein	miRNA 408
At1g63080	3.18	PPR repeat protein	miRNA 161
At5g18040	3.00	Expressed protein	ta-siRNAs 752, 255, 289 and 850
At2g22840	2.70	GRF1	miRNA 396
At2g33860	2.70	ETTIN/ARF3	ta-siRNAs 2141 and 2142
At1g52150	2.68	Athb-15	miRNA 165/166
At2g34710	2.62	Athb-14, PHB	miRNA 165/166
At5g60450	2.60	ARF4	ta-siRNAs 2141 and 2142
At5g37020	2.43	ARF8	miRNA 167
At1g63130	2.36	PPR repeat protein	miRNA 161; ta-siRNA 2140
At2g28190	2.33	Superoxide dismutase	miRNA 398
At1g30330	2.31	ARF6	miRNA 167
At1g27340	2.18	F-box protein	miRNA 394
At5g50570	2.16	SPL13	miRNA 156/157
At1g30490	2.12	Athb-9, PHV	miRNA 165/166
At2g36400	2.07	GRF3	miRNA 396
ARGONAUTE-like proteins			
At1g31290	7.39	AGO3	Not known
At1g31280	4.15	AGO2	miRNA 403

AGO, ARGONAUTE; ARF, AUXIN RESPONSE FACTOR; Athb, *Arabidopsis thaliana* homeobox; GRF, GROWTH-REGULATING FACTOR; HAP, HEME ACTIVATOR PROTEIN; miRNA, microRNA; PHB, PHABULOSA; PHV, PHAVOLUTA; PPR, PENTATRICOPEPTIDE REPEAT; SPL, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; ta-siRNA, *trans*-acting short interfering RNA.
The data show the relative increase in the expression of each gene in the *serrate-1* mutant compared with wild type.

168 downregulated (supplementary Fig 1 and supplementary Table 1 online).

Analysis of upregulated genes showed a total of 20 genes (16% of the upregulated genes; Table 1) to be known targets of miRNAs and/or *trans*-acting siRNAs (ta-siRNAs), as well as two *AGO* genes (*AGO2* and *AGO3*). None of the downregulated genes was a known target of miRNAs. The differentially expressed miRNA target genes included those encoding transcription factors involved in leaf growth and leaf morphology. As has been reported for *se-2* and *se-3* (Grigg *et al*, 2005), we observed an upregulation of *PHB* and *PHAVOLUTA* (*PHV*) in *se-1*. Another HD-Zip III gene, *ATHB15*, was also upregulated in *se-1*. Other miRNA and ta-siRNA targets upregulated in *se-1* included the transcriptional activators GRF1 (AtGRF1) and GRF3 (AtGRF3) (Kim *et al*, 2003), which are targets of miR396, AUXIN RESPONSE FACTOR (ARF)3/ETTIN and ARF4 (Pekker *et al*, 2005; Fahlgren *et al*, 2006).

The microarray data were verified for selected genes using real-time reverse transcription–PCR (RT–PCR) (supplementary Table 2 online). The misregulation of multiple miRNA targets in *se-1* suggested that SE is a general regulator of miRNA-based silencing.

SERRATE regulates microRNA accumulation

We used northern blotting to compare the abundance of miRNAs in wild type and *se-1*. Three miRNAs (miR156, miR165 and miR167), the target genes of which showed increased expression in the microarray experiments, showed reduced levels of accumulation in *se-1* compared with wild type (Fig 1A). To assess whether *se-1* has any effect on specific miRNAs or more generally on all miRNAs, we examined the accumulation of other miRNAs—miR163, miR164, miR168, miR171 and miR-JAW—because most of these were shown to be expressed in leaves. The levels of all miRNAs studied were reduced in *se-1* (Fig 1A).

In addition to miRNAs, *Arabidopsis* produces endogenous siRNAs (Llave *et al*, 2002a; Xie *et al*, 2004). The *se-1* mutation had no effect on the abundance of *AtSN1* siRNA, derived from the short interspersed repeated sequence retroelement *AtSN1* (Fig 1B). The accumulation of *siRNA 1003* was slightly reduced in *se-1*, but

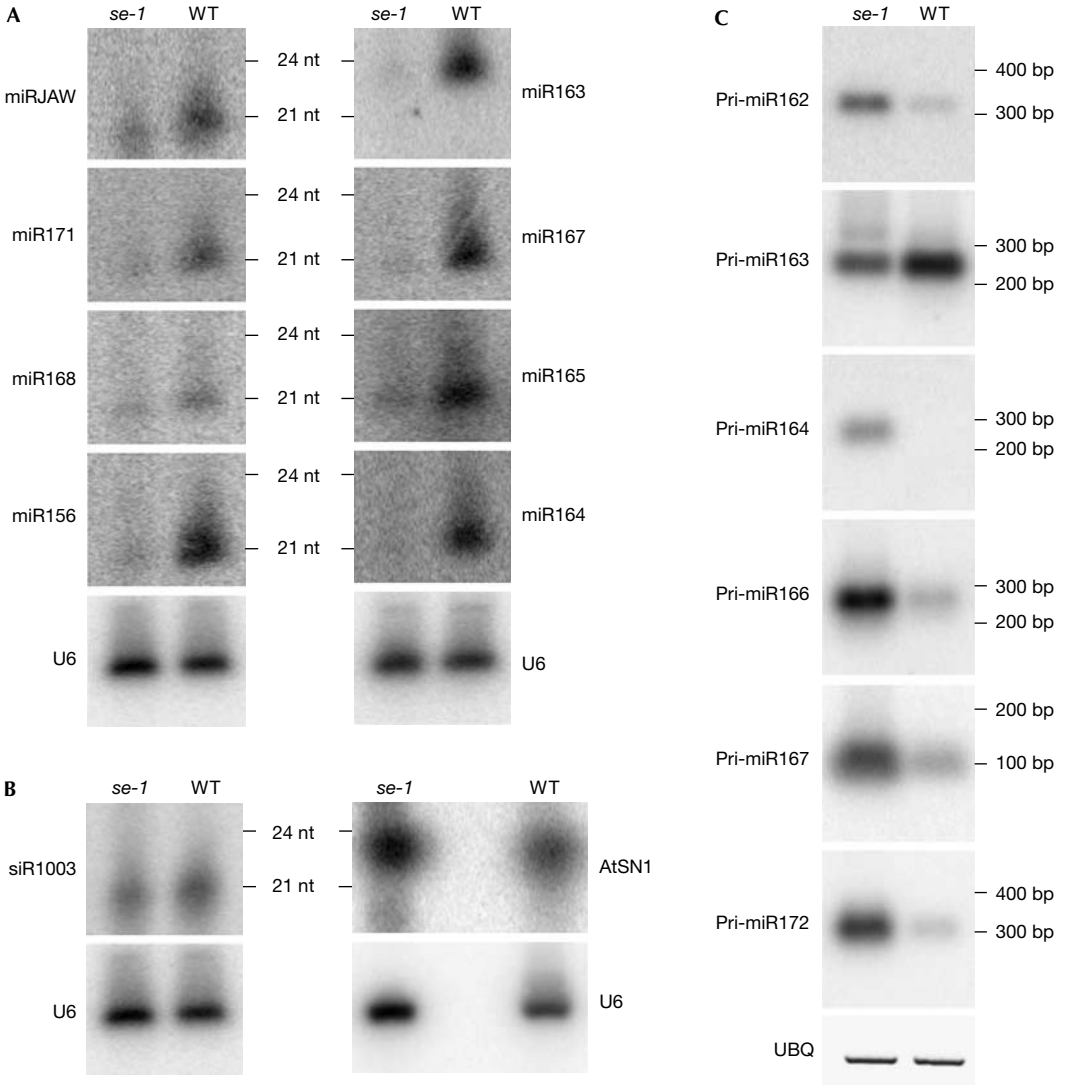

Fig 1 | *serrate-1* decreases the accumulation of mature microRNAs and increases the accumulation of primary microRNA transcripts. (**A**) Northern blotting of total RNA from wild-type and *serrate-1* (*se-1*) seedlings. For all microRNAs (miRNAs) tested (miRJAW, miR171, miR168, miR156, miR163, miR167, miR165, miR164), the *se-1* mutant showed a reduced level of miRNA accumulation compared with wild type. (**B**) Accumulation of short interfering RNA (siRNA) in *se-1* compared with wild type. The *se-1* mutation had little effect on the accumulation of these siRNAs. The corresponding *U6* loading control is shown below each blot. (**C**) Reverse transcription–PCR assay comparing levels of primary miRNAs (pri-miRNAs) in wild type and *se-1*. PCR products were blotted and hybridized with random-primed probes. The *UBIQUITIN* (UBQ) loading control is shown below the blots.

the reduction was negligible compared with the effects on miRNA accumulation. Thus, SE seems to be required for the accumulation of miRNAs in general, but has little effect on the accumulation of the siRNAs tested.

MicroRNA cleavage products are reduced in *serrate-1*

The effects of *se-1* on the accumulation of miRNA target gene cleavage products for three miRNA target genes, *PHB*, *NAC1* and *ARF8*, were analysed by detection of exposed 5′ RNA ends (Llave *et al*, 2002b). For all three genes, cleavage products were less abundant in *se-1* than in wild type (Fig 2). These results confirmed that the reduced accumulation of miRNAs in *se-1* results in less cleavage directed by miRNAs in *se-1* compared with wild type.

Primary microRNAs accumulate in *serrate-1*

To investigate whether SE might be involved in miRNA biogenesis, we examined the levels of pri-miRNA transcripts in *se-1*. Grigg *et al* (2005) showed that miR165 and miR166 primary transcripts were elevated in *se-3* compared with wild type, which was confirmed for *se-1* (Fig 1C). We also examined the levels of pri-miR162, pri-miR163, pri-miR164, pri-miR167 and pri-miR172 (Fig 1C); all, except pri-miR163, accumulated in *se-1*. These results indicate that SE is involved in the processing of most pri-miRNA transcripts in miRNA biogenesis.

SERRATE interacts with HYL1

It has been suggested that the HYL1 protein is part of a macromolecular complex (Han *et al*, 2004) and interacts with

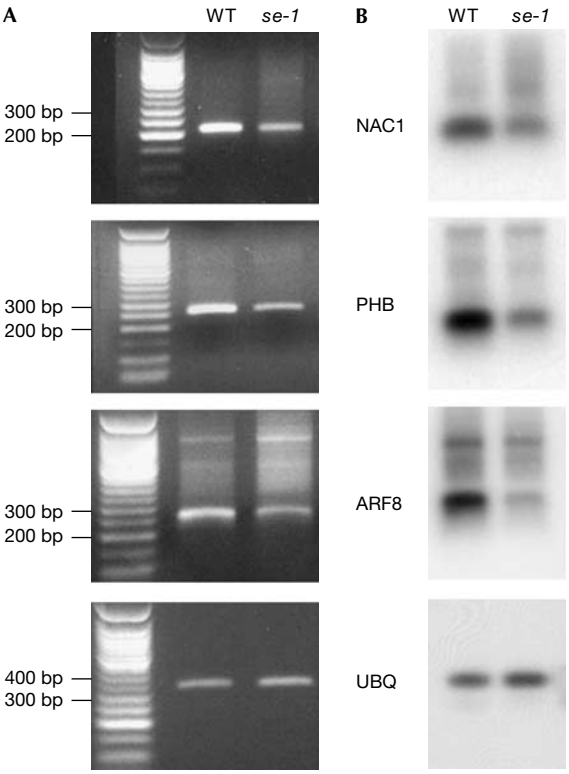

Fig 2 | *serrate-1* decreases the accumulation of microRNA cleavage products. (**A**) Agarose gels showing the nested PCR products of *PHB*, *NAC1* and *ARF8* microRNA (miRNA) cleavage products. (**B**) Southern blot analysis of the *PHB*, *NAC1* and *ARF8* miRNA cleavage products hybridized with a probe corresponding to the 5′ rapid amplification of cloned ends nested PCR products. The *UBIQUITIN* (UBQ) loading control is shown below. ARF, AUXIN RESPONSE FACTOR; NAC, for NAM, ATAF1-2, CUC2; PHB, PHABULOSA; *se-1*, *serrate-1*; WT, wild type.

DCL1 (Hiraguri *et al*, 2005; Kurihara *et al*, 2006) to process pri-miRNA in the nucleus. SE is localized in the nucleus (supplementary Fig 2 online), as are DCL1 and HYL1 (Hiraguri *et al*, 2005). We used a yeast two-hybrid system to test for interaction of SE with HYL1. SE interacted with HYL1 in yeast (supplementary Fig 3 online). To determine which domains are important for protein–protein interactions of HYL1 and SE, truncated HYL1 and SE fragments were used. Both dsRNA-binding motifs of HYL1 interacted with SE in yeast, whereas the full-length SE protein was required for interaction with HYL1. These studies indicate that SE and HYL1 might interact *in vivo* as well and that SE is another basic component of the miRNA processing complex in plants.

ago2 and *ago3* do not rescue the *serrate-1* phenotypes

To determine whether the phenotypic effects of *se-1* work through direct effects of SE on *AGO2* and *AGO3* genes, we created double *se-1 ago2* and *se-1 ago3* mutants. If the phenotypes of the *se-1* mutation were caused by the increase in *AGO2* and *AGO3* transcripts, we predicted suppression of the *se-1* phenotype in the double mutants.

We used lines with a T-DNA insertion in the *AGO2* gene (SALK_003380) and a dSpm insertion in *AGO3*. In both mutants, we detected shorter transcripts of the respective genes compared with wild type. Neither *ago2* nor *ago3* showed any obvious phenotypic abnormalities. Double mutants between *se-1* and *ago2* or *ago3* did not suppress the *se-1* phenotype. These data indicate that the upregulation of *AGO2* or *AGO3* in *se-1* does not cause the *se-1* phenotype.

A strong *serrate* allele is embryonic lethal

As the *se-1* allele is predicted to confer only a partial loss of function, we characterized a new *se* allele to assess the role of SE in plant development. The SALK_059424 line carries a T-DNA insertion in the fifth intron of SE (*se-4*). We were unable to identify plants homozygous for this insertion. When *se-4* heterozygotes were crossed with *se-1*, 50% of the progeny had *se* mutant phenotypes, including aerial rosettes, serrated leaves and adaxial leaf curling. To determine whether *se-4* affected embryo viability, siliques of plants heterozygous for *se-4* were examined. At later stages of development, 25% of seeds were paler in colour than wild-type seeds. Wild-type and mutant embryos at the heart, torpedo and cotyledon stages of development were examined using Nomarski optics (Fig 3). Developmental stages of mutant embryos were established by comparison with wild-type embryos in the same silique. In wild type, the globular-stage embryo consists of an embryo proper and the suspensor (West & Harada, 1993). Abnormal development and cell divisions of *se-4* mutant embryos were first visible at the heart stage, compared with wild-type embryos (compare Fig 3A and D). From the heart stage onwards, cotyledon primordia could not be recognized by cell shape or arrangement in *se-4* embryos (Fig 3D–F). Irregular cell divisions continued throughout the abnormal embryo during the torpedo stage (Fig 3E), to produce irregularly shaped mature embryos (Fig 3F). Twenty-five per cent of the embryos in siliques of heterozygotes were 'mutant'. These data show that the strong *se-4* allele confers lethality, when homozygous, under the growth conditions we used. Characterization of the *se-3* allele (Grigg *et al*, 2005) showed that it also confers embryonic lethality under some environmental conditions. This underlines the importance of the *SE* gene product to plant development, and in turn emphasizes the significance of miRNA regulation to all stages of plant development.

DISCUSSION

se-1 shares phenotypes with miRNA pathway mutants

The phenotype of *se-1* shares many similarities with developmental defects in general miRNA pathway mutants (*dcl1*, *hen1* and *hyl1*). Similar to *se-1*, all three mutants reduce miRNA accumulation and increase uncleaved target mRNA levels (Park *et al*, 2002; Boutet *et al*, 2003; Vazquez *et al*, 2004). The weak *sin1-2* allele shows that *DCL1* activity is essential very early on in diploid maternal cells for normal embryogenesis. A homozygous *sin1* maternal plant gives rise to a few viable seed irrespective of the genotype of the embryo, and seedlings derived from crosses with *sin1-2/sin1-2* show various morphological abnormalities, including seedlings with zero, one or three cotyledons, or a funnel-shaped cotyledon (Ray *et al*, 1996). The only other locus with a similar mutant phenotype in *Arabidopsis* is *SE*; progeny of selfed *se-1* plants often have either a single fused cotyledon or extra cotyledons (Prigge & Wagner, 2001). The strong *se-4* allele is embryonic lethal as are *dcl1* null alleles (Schwartz *et al*, 1994).

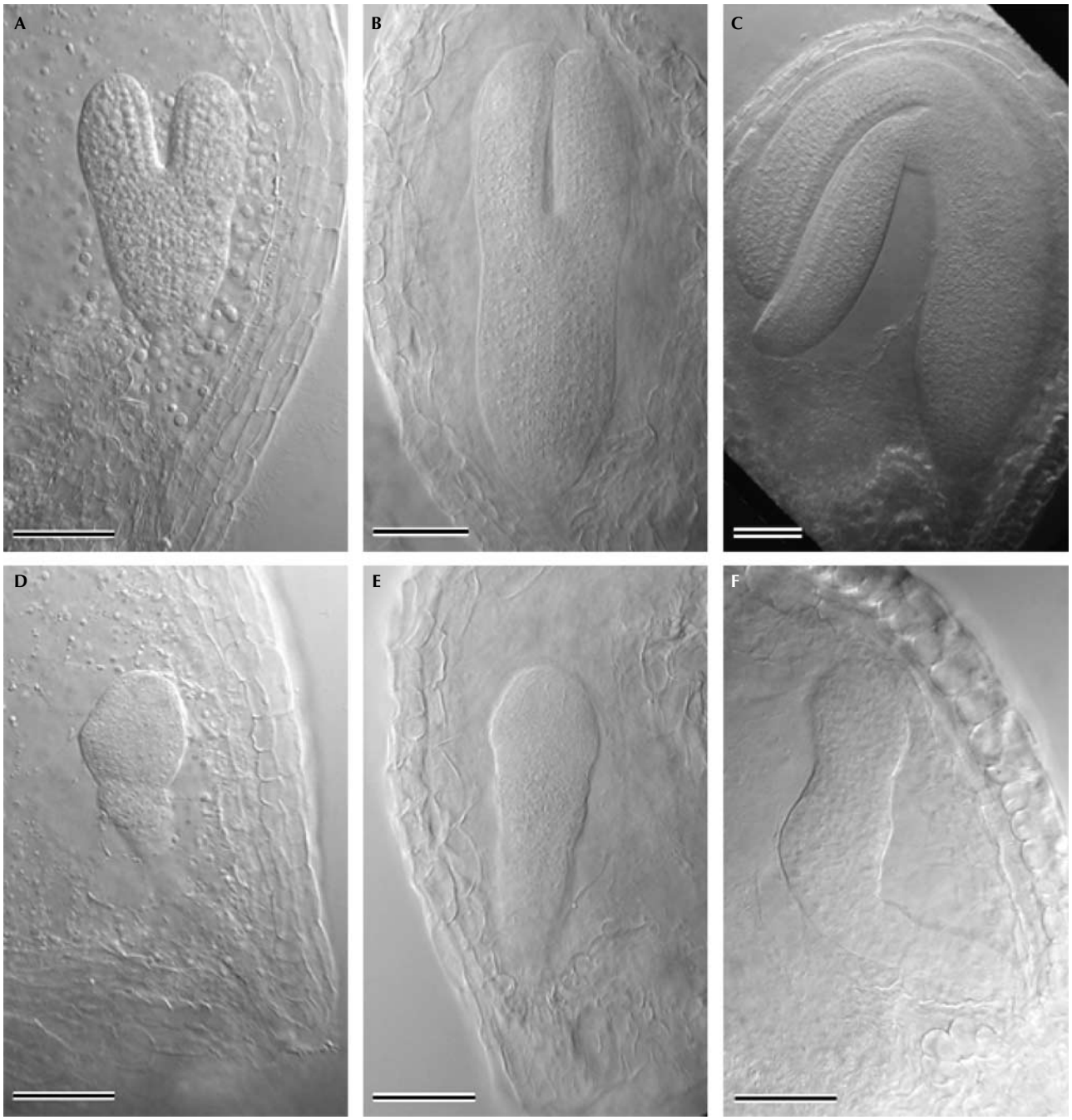

Fig 3 | The *serrate-4* mutant allele confers embryonic lethality. The development of wild-type embryos (**A–C**) and *serrate-4* (*se-4*) mutant embryos (**D–F**) is shown. Each column depicts embryos from seeds of the same age. Scale bars, 50 μm. (**A**) Wild-type, heart-stage embryo with cotyledon primordia. (**B**) Wild-type, torpedo-stage embryos showing elongating cotyledons. (**C**) Wild-type, curled cotyledon-stage embryo. (**D**) *se-4*, heart-stage embryo. (**E**) *se-4*, torpedo-stage embryo. (**F**) *se-4*, curled cotyledon-stage embryo.

The *Arabidopsis HYL1* gene encodes a nuclear dsRNA-binding protein and the *hyl1* mutation is pleiotropic. *hyl1* mutant plants show reduced sensitivity to exogenous cytokinin and auxin and are hypersensitive to germination inhibition by ABA (Lu & Fedoroff, 2000). Hypersensitivity to ABA has also been shown for *se-1* (Bezerra *et al*, 2004).

Mutations in *HASTY* (*HST*) share phenotypic similarities with *se-1*, with delayed leaf initiation, accelerated vegetative phase change and aberrant phyllotaxy in the inflorescence (Clarke *et al*, 1999; Prigge & Wagner, 2001; Bollman *et al*, 2003). HST probably exports miRNAs from the nucleus (Park *et al*, 2005).

SERRATE does not work through AGO2 or AGO3

Similar to *AGO1*, *AGO2* has been identified as a target of miRNA gene silencing (miR403; Allen *et al*, 2005). This suggests that the upregulation of *AGO2* in *se-1* is caused by the reduced accumulation of its miRNA. Double mutant analysis showed that the *se-1* phenotype is not due to upregulation of *AGO2* or *AGO3* expression alone, although it is possible that AGO2 and AGO3 act redundantly, as they are closely related structurally. In this model, SE might regulate the transcription of *AGO2* and *AGO3* and their upregulation might then disturb the miRNA biogenesis pathway (as shown for *AGO1*; Vaucheret *et al*, 2004). However, triple mutants of *se-1*, *ago2* and *ago3* would be necessary to test the effect of both on the *se-1* phenotype, and as *AGO2* and *AGO3* are very closely linked (only 3.2 kb apart), it would be very difficult to test this effectively.

SERRATE is a regulator of miRNA-based gene regulation

Microarray analysis showed misexpression of 20 mRNAs that are targets of miRNAs or ta-siRNAs in the *se-1* mutant; eight mature miRNAs have been tested and all were decreased in *se-1*, whereas the levels of six pri-miRNAs were increased compared with wild type. In total, we tested pri-miRNA, mature miRNA or miRNA target mRNA levels of 19 different miRNAs and showed that all were affected by *se-1*.

Our results differ from those of Grigg *et al* (2005), who proposed that SE functions specifically in miRNA-mediated repression of *PHB/PHV* gene expression. Our data show that genes targeted by miRNA-based regulation in 10-day-old seedlings are upregulated in *se-1* in association with reduced miRNA levels. Consequently, we propose that SE is a general regulator of miRNA-based gene silencing. In humans, it has been shown that single C_2H_2 zinc-fingers can bind specifically to RNA (Friesen & Darby, 2001). One possibility is that SE binds to miRNA and acts together with DCL1 and HYL1 to process miRNAs. Supporting this, pri-miRNA transcripts are elevated in *se-1*, as also observed in *hyl1-2* and *dcl1-7* mutants (Kurihara *et al*, 2006). In addition, SE interacts with HYL1 in yeast, indicating that both proteins might interact *in vivo* as well and that SE is a basic component of the miRNA processing complex in plants.

Speculation

Analysis of *SE* gene expression has shown that *SE* is not constitutively expressed, but restricted to specific tissue types and developmental stages (Prigge & Wagner, 2001). This leads us to propose that the presence or absence of SE activity might therefore promote or limit miRNA production. In support of this idea, overexpression of *SE* causes variable phenotypes both within and between overexpression lines, showing that the level of expression of *SE* greatly influences development. A significant portion of overexpression lines showed seedling lethality (Prigge & Wagner, 2001). Other phenotypes involved sterility, an increased rate of leaf production, inflorescence phyllotaxy defects, adaxial leaf curling, and disorganized flowers with variable numbers of floral organs and radially symmetric filaments. Consequently, SE activity defines a new level of potential developmental regulation: general, post-transcriptional regulation of miRNA levels.

METHODS

Plant material. *Arabidopsis thaliana* (Col-4 and *se-1*) was grown under 16 h light and 8 h dark conditions at 20 °C for 10 days.

Microarray analysis. Affymetrix GeneChip (ATH1) array expression profiling was carried out according to the Affymetrix Expression Analysis Technical Manual II. Microarray data are available on the ArrayExpress database under accession number E-MEXP-838.

Real-time reverse transcription–PCR. Total RNA was isolated from the aerial parts of wild-type and *se-1* seedlings using TRI reagent (Sigma, St Louis, MO, USA), and poly(A) + RNA was purified using the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). Real-time RT–PCR using first-strand complementary DNA was performed with a DNA Engine Opticon® 2 Continuous Fluorescence Detector (MJ Research, Waltham, MA, USA).

Northern blotting. For analysis of miRNAs and siRNA 1003, total RNA was separated on 8% denaturing polyacrylamide gels. The *AtSN1* blot was generated by using low-molecular-weight RNA.

5′ rapid amplification of cloned ends. Target cleavage products were detected using a modified 5′ rapid amplification of cloned ends protocol (Llave *et al*, 2002b).

Detection of pri-miRNAs. cDNA synthesis from wild-type and *se-1* seedlings was performed as described for real-time RT–PCR. UBIQUITIN5 was amplified as a control for cDNA synthesis and amplification. Pri-miR166 PCR primers have been described by Juarez *et al* (2004), and primer sequences for UBIQUITIN5, pri-miR162, pri-miR163, pri-miR164, pri-miR167 and pri-miR172 can be found as supplementary information online.

Yeast two-hybrid assay. A yeast two-hybrid assay was performed using the Matchmaker GAL4 two-hybrid system (Clontech, Mountain View, CA, USA) for detecting protein–protein interactions in yeast.

Differential interference contrast microscopy of cleared seeds. Seeds were removed from siliques, cleared for 16 h in Hoyer's solution (7.5 g gum Arabic, 100 g chloral hydrate, 5 ml glycerol in 30 ml water) and examined using a Nikon Microphot-SA microscope equipped with Nomarski optics.

Further information about the mutant lines, sample preparation, primer sequences, reactions and data analysis can be found in supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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