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**Acknowledgements**

We thank R. G. Foster, J. S. Takahashi, M. Menaker, S. Reppert, N. Cermakian, D. De Cesare, U. Strähle and P. Blader for discussions, advice and gifts of materials, and E. Heitz, D. Biellman, O. Nkundwa and N. Fisher for technical assistance. D.W. was supported by an EEC TMR fellowship. Our studies are funded by grants from CNRS, INSERM, CHUR, Rhône-Poulenc Rorer (Bioavenir), Fondation pour la Recherche Médicale and Association pour la Recherche sur le Cancer (P. S.-C.).

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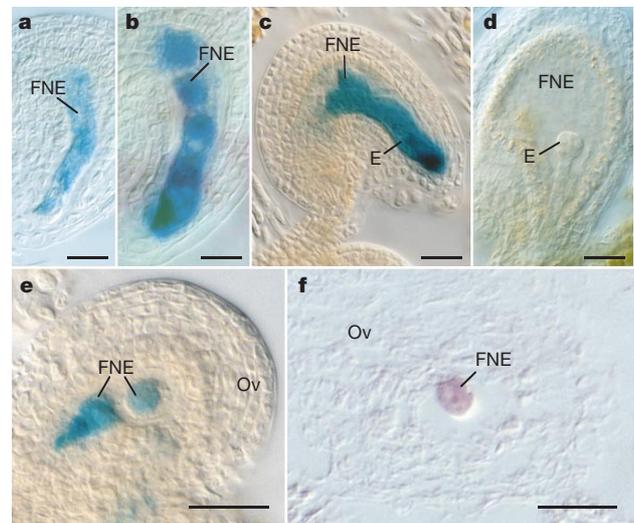
**Delayed activation of the paternal genome during seed development**

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Little is known about the timing of the maternal-to-zygotic transition during seed development in flowering plants. Because plant embryos can develop from somatic cells or microspores<sup>1</sup>, maternal contributions are not considered to be crucial in early embryogenesis<sup>2</sup>. Early-acting embryo-lethal mutants in *Arabidopsis*, including *emb30/gnom* which affects the first zygotic division<sup>3,4</sup>, have fuelled the perception that both maternal and paternal genomes are active immediately after fertilization. Here we show that none of the paternally inherited alleles of 20 loci that we tested is expressed during early seed development in *Arabidopsis*. For genes that are expressed at later stages, the paternally inherited allele becomes active three to four days after fertilization. The genes that we tested are involved in various processes and distributed throughout the genome, indicating that most, if not all, of the paternal genome may be initially silenced. Our findings are corroborated by genetic studies showing that *emb30/gnom* has a maternal-effect phenotype that is paternally rescuable in addition to its zygotic lethality. Thus, contrary to previous interpretations, early embryo and endosperm development are mainly under maternal control.

In flowering plants, double fertilization involves two sperm cells: one fuses with the egg cell to form a diploid zygote; the second fuses

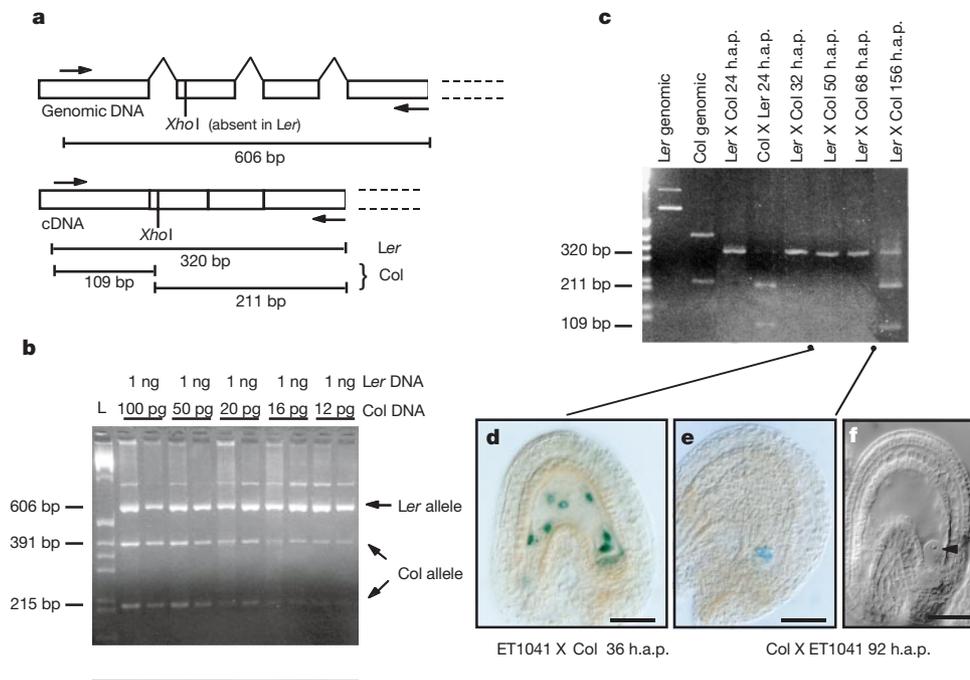


**Figure 1** Silencing of paternally inherited genes during seed development in *Arabidopsis*. **a**, If an ET2612 female is crossed to a wild-type male, *GUS* expression is detected in the free nuclear endosperm 12 h.a.p. **b,c**, F<sub>1</sub> seeds of the same cross show more intense *GUS* expression in embryo and endosperm 48 h.a.p. **d**, If ET2612 is crossed as the male to wild-type plants, *GUS* expression is not detectable in embryo and free nuclear endosperm 48 h.a.p. **e**, Transverse optical section through a seed of a self-pollinated ET2612 plant 48 h.a.p. **f**, *In situ* hybridization to mRNA of the putative basal transcription factor tagged in ET2612; the pattern of mRNA and *GUS* expression are identical 48 h.a.p. E, embryo; FNE, free nuclear endosperm; Ov, ovule. Scale bars, 17 μm (**a,b**); 23 μm (**c**); 45 μm (**d**); 40 μm (**f**).

with the binucleated central cell to give rise to the triploid primary endosperm nucleus<sup>5</sup>. Double fertilization triggers rapid proliferation of the endosperm and slow cell divisions of the zygote, which usually undergoes an asymmetrical division<sup>6</sup>. In *Arabidopsis*, as in most plant species, the primary endosperm nucleus undergoes divisions without cytokinesis, giving rise to a syncytium that eventually cellularizes<sup>7</sup>. In contrast to animals<sup>8–10</sup>, the timing of transcriptional activation of the genome in the plant embryo and endosperm has not been intensively studied<sup>11</sup>. The identification of a large group of early-acting embryo-lethal *Arabidopsis* mutants that segregate as sporophytic recessive traits<sup>12</sup> suggested that the activation of the zygotic genome occurs before the first division of the zygote; however, a spatial and temporal pattern for zygotic genome activation has not yet been determined.

We generated a library of enhancer detector and gene trap lines (transposants) that harbour *Ds* elements with a *uidA* reporter gene encoding β-glucuronidase (*GUS*) by using the system of Sundaresan *et al.*<sup>13</sup>. Screening for genes that act during ovule and early seed development in *Arabidopsis* (U.G. *et al.* unpublished data), we identified 19 transposants that show *GUS* expression in the developing embryo and/or endosperm after fertilization. *GUS* is expressed in the egg and/or central cell and persists for several rounds of cell division in either one or both fertilization products. To determine whether *GUS* expression was the result of transcription from one or both parental alleles in each of these lines, we performed reciprocal crosses between wild-type plants and the 19 transposants. When wild-type plants were used as male parents, the resulting F<sub>1</sub> seeds showed *GUS* expression in a pattern identical to the one found in developing seeds resulting from self-pollination in all 19 lines (Fig. 1a–c). In contrast, if the transposants were used as male parents, *GUS* expression was absent from all F<sub>1</sub> seeds and remained undetectable up to 80 hours after pollination (h.a.p.) (Fig. 1d). This indicates that the paternally inherited allele may not be expressed during early stages of embryo and endosperm development. To verify whether the pattern of *GUS* expression truly reflects the expression of genes neighbouring the insertion, we performed

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**Figure 2** Allele-specific expression profile of *PRL* during early seed development. **a**, Schematic of *PRL* showing the position of exons (squared boxes), introns (lines) and the SNP in an *XhoI* site which is present in Col but absent in *Ler*. **b**, Allele-specific PCR analysis using titrated mixtures of genomic DNA each of which is represented twice. Both *PRL* alleles are consistently amplified throughout the dilution series. **c**, Allele-specific RT-PCR analysis of *PRL* in reciprocal crosses between Col and *Ler*. Only transcripts derived

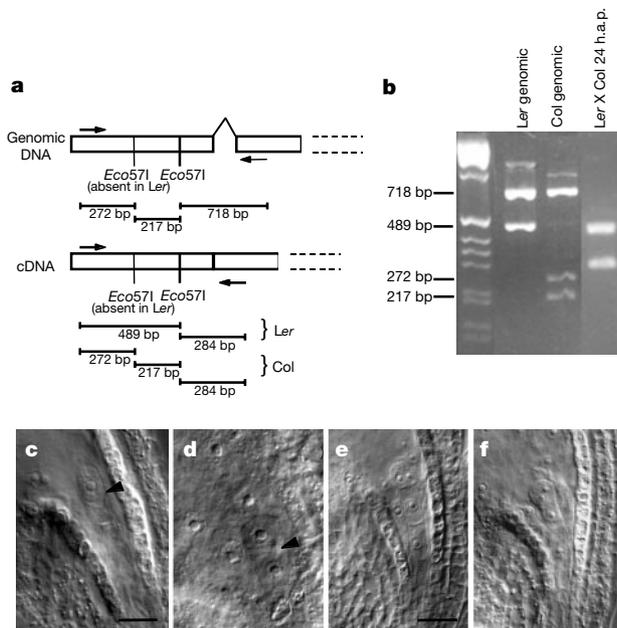
*in-situ* hybridization with digoxigenin-labelled probes of a gene encoding a putative basal transcription factor that was tagged by ET2612 (see Table 1). Messenger RNA was detected in the developing endosperm (Fig. 1f) and the early globular embryo (data not shown) in a pattern identical to the one observed in *GUS* assays (Fig. 1e). Uniparental expression could be the consequence of transgene silencing specifically affecting the paternally inherited allele. Alternatively, it could indicate that our enhancer detection screen identified a subclass of genes that are transcribed maternally before fertilization and are subject to paternal silencing. A third possibility is that the paternally inherited genome remains silent during the first days after double fertilization, and that the activation of the paternal genome occurs after several rounds of cell division in both the embryo and endosperm.

To determine whether the absence of paternally derived *GUS* expression was due to sex-specific inactivation of the enhancer detector transgene or whether it reflected sex-specific silencing of endogenous genes, we designed a reverse transcription polymerase chain reaction (RT-PCR) assay that depends on single-nucleotide polymorphisms (SNPs) between Columbia (Col) and Landsberg *erecta* (*Ler*) ecotypes. Single-nucleotide polymorphisms in endonuclease restriction sites allow the distinction of transcripts derived from Col and *Ler* alleles. *PROLIFERA* (*PRL*) is an MCM2-3-5-like replication licensing factor that is required for the initiation of DNA replication<sup>14</sup>. We have identified a new enhancer detector insertion (ET1041) in the coding region of *PRL* (see Table 1), and reporter gene expression confirmed that *PRL* is expressed in the developing embryo and free nuclear endosperm. An SNP in the second exon of *PRL* creates a *XhoI* site in Col but not in *Ler* (Fig. 2a). To test the sensitivity of our assay, we amplified a region spanning exon 2 in titrated mixtures of genomic DNA. After digesting the PCR products with *XhoI*, we could consistently amplify both *PRL* alleles even when the amount of Col DNA used as a template was 80 times less than that of *Ler* DNA (Fig. 2b). We examined *PRL* expression by RT-PCR on RNA isolated 24, 32, 50, 68 and 156 h.a.p. from developing

from the maternal *PRL* allele are detected up to 68 h.a.p. **d**, If *PRL* enhancer detector ET1041 is used as the female in crosses to Col, *GUS* is detected in the endosperm 36 h.a.p. **e**, If *PRL* enhancer detector ET1041 is used as the male parent, *GUS* is detected in a small portion of the chalazal endosperm 92 h.a.p. **f**, Cleared seed showing the nodular cyst (arrowhead) in the chalazal endosperm. Scale bars, 53  $\mu$ m (**d**); 50  $\mu$ m (**e**); 55  $\mu$ m (**f**).

siliques derived from reciprocal crosses between *Ler* and Col plants. During the first three days after pollination (68 h.a.p.) only transcripts derived from the maternally inherited allele could be detected (Fig. 2c). These results confirm that the paternally inherited *PRL* allele is not transcribed in either embryo or endosperm during early seed development. Transcripts from both *PRL* alleles can be detected at 156 h.a.p. (Fig. 2c), indicating that the activation of the paternally inherited *PRL* allele occurs only later during seed development. Additional histochemical analysis of ET1041 showed that paternally derived *GUS* expression can be observed earlier, in seeds containing embryos at the mid-globular stage 92 h.a.p. (Fig. 2e). Notably, *GUS* expression is initially restricted to a small region of the chalazal chamber in the nodular cyst of the developing endosperm (Fig. 2d–f), and progressively expands to include cellularizing tissue (data not shown). These results indicate that the paternal silencing that we observed using transposants affects endogenous loci and is independent of the presence of a transgene.

To test whether a particular subset of genes was affected by this epigenetic regulation, we determined the molecular nature of the genes identified by enhancer detection. We isolated genomic regions flanking the *Ds* insertion using thermal asymmetric interlaced PCR (TAIL-PCR)<sup>15</sup>. We subcloned and sequenced a total of 16 flanking fragments representing 12 loci (Table 1). For all of them, the insertion site could be confirmed by the presence of a 3' - or 5' -*Ds* end bordering the genomic sequence. We identified eight insertions within the regulatory region or coding sequence of genes that are represented in GenBank, and two insertions in new genomic sequence. Whereas sequences from four insertions show no homology to sequences in public databases or have similarity to genes of unknown function, the remaining eight genes encode proteins with a wide variety of functions. Some of them are similar to genes that are involved in basic cellular functions such as cell-cycle regulation, the basal transcription machinery or the assembly of protein secondary structure. Others encode putative signal transduction proteins or transcription factors that may have regulatory roles



**Figure 3** Silencing of the paternally inherited *EMB30* allele during early embryogenesis. **a**, Schematic of *EMB30* showing the position of exons (squared boxes), introns (lines) and the SNP in an *Eco57I* restriction site which is present in *Col* but absent from *Ler*. **b**, Allele-specific RT-PCR analysis of *EMB30*. In reciprocal crosses between *Col* and *Ler* plants, only the transcript derived from the maternal *EMB30* allele is detected 24 h.a.p. **c**, Two-cell wild-type embryo; the first division of the zygote (arrowhead) gives rise to a small apical and an elongated basal cell. **d**, Two-cell *emb30<sup>mut</sup>/EMB30<sup>P</sup>* embryo showing a nearly symmetrical plane of division (arrowhead). **e**, Wild-type embryo with the division plane of the apical cell parallel to the apical-basal axis. **f**, *emb30<sup>mut</sup>/EMB30<sup>P</sup>* embryo with an oblique division plane of the apical cell. Scale bars, 20 μm (**c,d**); 18 μm (**e,f**).

during seed development. The *Ds* insertions are located on at least four out of the five chromosomes of *Arabidopsis* (Table 1). These results indicate that the genes identified in our screen do not encode members of a specific family of proteins and are distributed throughout the *Arabidopsis* genome.

Our observations suggest that most, if not all, of the paternal genome is silenced during early seed development. We expect that other genes known to be expressed at early stages are also affected by this regulation; therefore, we examined *EMB30/GNOM*<sup>16,17</sup> expression using allele-specific RT-PCR. Embryos homozygous for *emb30* are defective in the establishment of the apical-basal axis. In some *emb30* embryos, the zygote divides almost symmetrically, giving rise to an enlarged apical cell that subsequently forms an abnormal globular embryo<sup>4</sup>. Self-pollinated heterozygous *emb30/EMB30* individuals produce 25% of aborted embryo-lethal seeds, suggesting a

zygotic requirement. The phenotype of *emb30* has been interpreted as being caused by a recessive mutation which affects a gene that is active during the earliest diploid (sporophytic) phase of embryogenesis<sup>18</sup>. The *emb30* embryo phenotype has been the strongest argument in favour of early genome activation in *Arabidopsis*. *EMB30* encodes a Sec-7-like protein that has been shown to be essential for auxin transport<sup>19</sup>. Although *EMB30* is expressed throughout the plant, its allele-specific pattern of expression has not been investigated during seed development. An SNP in the first exon of *EMB30* creates an *Eco57I* site present in *Col* but not in *Ler* (Fig. 3a). We examined the allelic pattern of *EMB30* expression using RT-PCR followed by endonuclease digestion with *Eco57I*. At 24 h.a.p., *EMB30* transcripts from the paternal allele could not be detected (Fig. 3b), suggesting that the initial post-fertilization expression of *EMB30* is exclusively dependent on transcription from the maternally inherited allele. Because *EMB30* may be expressed in sporophytic tissues such as the silique or seed coat, however, we could not discard the possibility that transcripts from the *EMB30* maternal allele are present in vast excess over the paternally derived transcripts, obscuring their detection. Therefore, we tested for genetic activity of the paternal *EMB30* allele by crossing heterozygous *emb30/EMB30* plants with wild-type pollen. If the paternally inherited *EMB30* allele is active after fertilization, heterozygous *emb30<sup>mut</sup>/EMB30<sup>P</sup>* embryos should develop normally. Owing to low expressivity of the *emb30* phenotype at young stages, only 12.2% (*n* = 131) of the embryos derived from self-fertilization of *emb30/EMB30* plants show morphological defects 24 to 48 h.a.p. If the same plants were crossed to wild-type pollen, 12.9% (*n* = 116) of the F<sub>1</sub> seeds showed the same morphological defects, which strongly suggests that the paternally inherited wild-type allele does not provide *EMB30* activity. No defective F<sub>1</sub> embryos were observed when female wild-type plants were crossed with the same heterozygous *emb30/EMB30* individuals (*n* = 53). The defects in *emb30<sup>mut</sup>/EMB30<sup>P</sup>* embryos include a nearly symmetrical plane of division of the zygote (Fig. 3c, d), an oblique plane of division of the apical cell (Fig. 3e, f) and delayed differentiation of the epidermal precursor cells (data not shown). All these abnormalities have previously been described in *emb30* embryos<sup>4</sup>. At maturity, all F<sub>1</sub> seeds resulting from *emb30/EMB30* plants crossed to the wild type are morphologically normal and viable (*n* = 157). Thus, late expression of a paternally derived *EMB30* allele is sufficient to rescue embryos inheriting a maternal *emb30* allele, or, in genetic terms, *emb30* is a paternally rescuable maternal-effect mutant and not a purely zygotic embryo lethal.

Our results indicate that the activity of many genes acting during early embryo and endosperm formation may depend solely on transcription from the maternally inherited allele. These maternal transcripts probably represent a combination of mRNAs transcribed before and after fertilization. The identification of enhancer detector lines in which *GUS* expression is detected in the central cell

**Table 1** Identification of genes expressed during early seed formation in *Arabidopsis*

Line	Post-fertilization <i>GUS</i> expression*	Localization of the insertion	Chromosome position†	Accession number‡
ET1041	Embryo and endosperm	PROLIFERA <sup>14</sup>	IV, 12 cM	L39954
ET1051	Embryo and endosperm	Homology to rape mRNA	IV, 69 cM	AL031326 (CAA20464)
ET1275	Embryo	Cop1-interacting protein CIP8 <sup>29</sup>	V, 125 cM	AF162150
ET1278	Embryo	Isoflavone reductase homologue		New sequence
ET1811	Embryo and endosperm	14-3-3 GF14 mu	II, 83 cM	AC007087 (AAD 23005)
ET2209	Endosperm	Transmembrane protein homologue	II, 79 cM	AC004261 (AAD12006)
ET2612	Embryo and endosperm	Basal pol III transcription factor homologue	II, 1 cM	AC006200 (AAD14528)
ET2567	Endosperm	Hypothetical protein	IV, 77 cM	AL021749 (CAA16882)
ET3536	Endosperm	AT-hook DNA-binding protein	IV, 69 cM	AL021635 (CAA16562)
ET3988	Embryo and endosperm	Disulphide isomerase homologue	II, 87 cM	AC002535 (AAC62863)
ET3992	Embryo and endosperm	No homology	I, 70 cM	New sequence
ET4336	Embryo and endosperm	Germinating seed mRNA		AF162845

\* All lines show post-fertilization *GUS* expression only when self-pollinated or used as females in reciprocal crosses to the wild type.

† The map position is based on the nearest genetic marker referenced in the recombinant inbred list of Lister and Dean (see <http://genome-www.stanford.edu/Arabidopsis/>).

‡ Accession numbers correspond to nucleotide sequences in GenBank; protein accession numbers are indicated in brackets.

only after fertilization or in specific cells of the embryo (data not shown) indicates that at least some of the detected genes are transcribed zygotically from the maternal allele only, and thus are regulated by genomic imprinting as has been shown for the *meadea* locus, which displays a true maternal effect<sup>20,21</sup>. Together, these results strongly suggest that early seed formation in *Arabidopsis* is characterized by a delayed transcriptional activation of the paternally inherited genome. Reporter gene expression and RT-PCR analyses indicate that transcription of paternally inherited alleles initiates late after fertilization, when the embryo consists of 32–64 cells.

Although a case of global paternal silencing has not been reported in the plant kingdom, it is reminiscent of genome-wide heterochromatinization in scale insects<sup>22,23</sup> and parent-specific X-chromosome inactivation in the extra-embryonic tissues of mammals<sup>24</sup>. The silencing of the paternal genome probably occurs during sperm cell differentiation and may be related to the tight packaging of sperm chromatin involving specific histones<sup>25,26</sup>, or changes in methylation levels of sperm DNA as compared with DNA in the vegetative nucleus<sup>27</sup>. Whatever the mechanism, paternal silencing prolongs the functionally haploid phase, which serves to eliminate deleterious mutations<sup>2</sup>, leading to a more stringent selection against such mutations inherited from the mother. This mechanism is imperfect, however, because only a subset of genes are expressed in the female gametophyte and early seed, and some maternal defects can be paternally rescued as illustrated by *emb30*. The genetic analysis of *emb30* indicates that some of the early embryo-lethal mutants, which have been interpreted as affecting zygotically transcribed genes, represent loci that are only transcribed from the maternal allele. The delayed transcription of a paternally inherited wild-type allele is sufficient to rescue heterozygous embryos carrying a maternally inherited mutant allele, resulting in the 25% aborted seeds observed for many mutants that act during the pre-globular stage of embryogenesis. Thus, paternal silencing is not necessarily reflected in the segregation ratio of aborted seeds in mature siliques. Our results strongly suggest that the first few days of embryogenesis and endosperm development are mostly, if not exclusively, under maternal control. This finding should lead to a reinterpretation of the genetic basis and molecular mechanisms that regulate early seed development in plants. □

## Methods

### Plant material and growth conditions

Plant growth conditions and insertional mutagenesis have been described<sup>13,28</sup>. The wild-type strains used were *Arabidopsis thaliana* (L.) Heynh. var. Landsberg (*erecta* mutant: Ler) and *A. thaliana* (L.) Heynh. var. Columbia (Col.). Seeds of *emb30-3/EMB30-3* plants were obtained from the Arabidopsis Biological Resource Center (CS6322).

### GUS Assays

Developing carpels and siliques were dissected to expose the ovules and incubated in GUS staining buffer (10 mM EDTA, 0.1% Triton X-100, 2 mM Fe<sup>2+</sup>CN, 2 mM Fe<sup>3+</sup>CN, 100 µg ml<sup>-1</sup> chloramphenicol, 1 mg ml<sup>-1</sup> X-Gluc (Biosynth) in 50 mM sodium phosphate buffer pH 7.0) for 3 days at 37 °C. The tissue was cleared in 20% lactic acid/20% glycerol and observed on a Leica DMBR microscope under Nomarski optics.

### TAIL-PCR

Genomic fragments flanking *Ds* insertions were isolated by TAIL-PCR as described<sup>15,20</sup> and sequenced at the IOWA State University DNA Sequencing and Synthesis Facility after subcloning into pCRII-TOPO (Invitrogen).

### In-situ hybridization

Subcloned TAIL-PCR fragments were linearized with restriction enzymes cutting in the polylinker (*Xho*I, *Bam*HI). Probe synthesis using 1 µg as template and *in situ* hybridization were done as described<sup>2</sup>.

### RT-PCR

For RNA preparation, young siliques were harvested in liquid nitrogen at specific time points after pollination. RNA preparation, complementary DNA synthesis and PCR amplification of one-third of the cDNA were done as described<sup>20,21</sup>. For *PRL*, the primers used were PRLS1 (5'-CAGTCACTGGTTCATTCCT-3') and PRLAS1 (5'-GTAA-

CAACTCGTCAACAGC-3'). For *EMB30*, the primers used were EMB30S2 (5'-CGCCTAAAGTTGCGATTCTG-3') and EMB30AS3 (5'-AATCACTGTCTACTCCAGC-3'). PCR products were digested with *Xho*I (*PRL*) and *Eco*57I (*EMB30*) overnight at 37°C.

## Histological analysis

Siliques were dissected with hypodermic needles (Becton-Dickinson, 1 ml insulin syringes) and fixed in FAA (4% formaldehyde, 5% acetic acid, 50% ethanol). Dissected siliques or individual seeds were cleared in Herr's solution (2.2:2.2:1 lactic acid:chloral hydrate:phenol:clove oil:xylene, by volume) and observed on a Leica DMBR microscope under brightfield or Nomarski optics.

Received 17 November 1999; accepted 4 January 2000.

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## Acknowledgements

We thank J. Moore and W. Gagliano for help in generating transposants, J. Thomas, A. Coluccio and D. Page for technical assistance, E. Vollbrecht and M. Affolter for reviewing the manuscript, and R. Pruitt, V. Sundaresan and E. Vollbrecht for continuous interest in this project. This work was supported in part by the Cold Spring Harbor Laboratory President's Council, a grant from the NRICG Program of the US Department of Agriculture to U.G., a fellowship of the Fonds National Suisse de la Recherche Scientifique to J.-P.V.-C., and scholarships of the Janggen Poehn-Foundation and the Searle Family Trust to U.G.

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