Paternally inherited transgenes are down-regulated but retain low activity during early embryogenesis in *Arabidopsis*

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Abstract We investigated the timing of transgene activation after fertilisation in *Arabidopsis* following crosses and using two transgenic promoters (from the *AtCYCB1* and *AtLTP1* genes). Using both a transactivation system and direct transcriptional fusion to drive β-glucuronidase reporter expression, reciprocal crosses showed a lack of expression of the paternal components. This is consistent with a lack of paternal genome activity previously reported during early seed development in *Arabidopsis* [Viella-Calzada et al. (2000) Nature 404, 91–94]. However, transactivation experiments of the *BARNASE* gene gave evidence that at least some paternal loci retain transcriptional activity, though at a low level, during early embryogenesis.

Key words: Paternal gene expression; Embryo development; BARNASE; Transactivation; *Arabidopsis*

1. Introduction

Recent evidences suggested that the early stages of seed development are mainly under maternal control in *Arabidopsis*. A lack of expression of many, if not all, paternal alleles has been described, such that only maternal products are present following fertilisation and until the mid-globular embryo stage [1]. This phenomenon is referred to as parent-of-origin effects, and differential transcription of the parental alleles (genomic imprinting) may contribute to it. An interesting question remains as to whether the lack of paternal gene expression as detected so far corresponds to a global silencing phenomenon or whether some paternal loci retain transcriptional activity during the early stages of embryo development. We obtained evidence for the latter case by analysing the expression pattern of two transgenes in the embryo using a transactivation system. This led us to investigate further the activity of the transgenic promoters from the *AtCYCB1* and *AtLTP1* genes with regards to their parental origin. We could show that despite a process of repression of the paternally inherited transgenes, these could retain some transcriptional activity. Implications for models of paternal gene silencing are discussed.

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Abbreviations: DAP, days after pollination; GUS, β-glucuronidase; wt, wild-type

2. Materials and methods

2.1. Activator and reporter lines

The establishment of the activator lines (CYC::LhG4, LTP::LhG4) and reporter lines (pOp::GUS, pOp::BARNASE) based on the pOp/LhG4 transactivation system [2] are described elsewhere [3]. A summary is given in the text. For each activator and reporter construct, two independent lines were used. All the lines used for this work contain a single transgene insertion locus. The full names of the lines CYC:LhG4-H, -H* and -K, and pOp::BARNASE-F and -E are AL01-H1.3, AL01-H1.3.1, AL01-K3.3, RL03-F1.8.1 and RL03-E2.8.1, respectively.

2.2. GUS (β-glucuronidase) or BARNASE expression analysis

For GUS expression analysis, whole mount siliques were longitudinally cut, fixed for 1 h in −20°C acetone and washed three times with 100 mM phosphate buffer (NaH_2PO_4, Na_2HPO_4, pH 7.2) before incubation at 37°C into the reaction buffer for 24–72 h (1 mM 5-bromo,4-chloro,3-indolyl-D-glucuronide, 10 mM EDTA, 0.01% Triton X-100, 0.5 mM KFeCN, 100 mM phosphate buffer pH 7.2). Whole seeds were observed after clearing in Hoyers solution (2.5 g gum arabic, 100 g chloral hydrate, 16.7% glycerol in 30 ml H_2O) using Nomarski optics on a Zeiss Axioplan microscope. For BARNASE expression analysis, whole seeds were cleared and observed as described above. Images were acquired using a Nikon-Kodak DCS420 digital camera and processed with Adobe® Photoshop software.

3. Results

3.1. Parent-of-origin-dependent expression of transgenes in early embryo development

We aimed at analysing the timing of activation of two promoters during early embryogenesis that drive distinct expression patterns: the CYC promoter (from the cyclin B *AtCYCB1;1* gene [4]), and the LTP promoter (from the lipid transfer protein *AtLTP1* gene [5,6]). We used CYC::GUS and LTP::GUS lines containing a transcriptional fusion of the CYC and LTP promoters with the *uidA* reporter gene, encoding the GUS protein. In addition, we generated a set of transgenic lines referred to as activator and reporter lines, based on two components provided by the pOp/LhG4 transactivation system [2]. The CYC::LhG4 and LTP::LhG4 activator lines expressed the LhG4 chimeric transcription factor under the control of the CYC or the LTP promoter, respectively. The reporter pOp::GUS line carried the *uidA* gene under the control of the synthetic pOp promoter. Reporter expression is triggered upon binding of the LhG4 transcrip-

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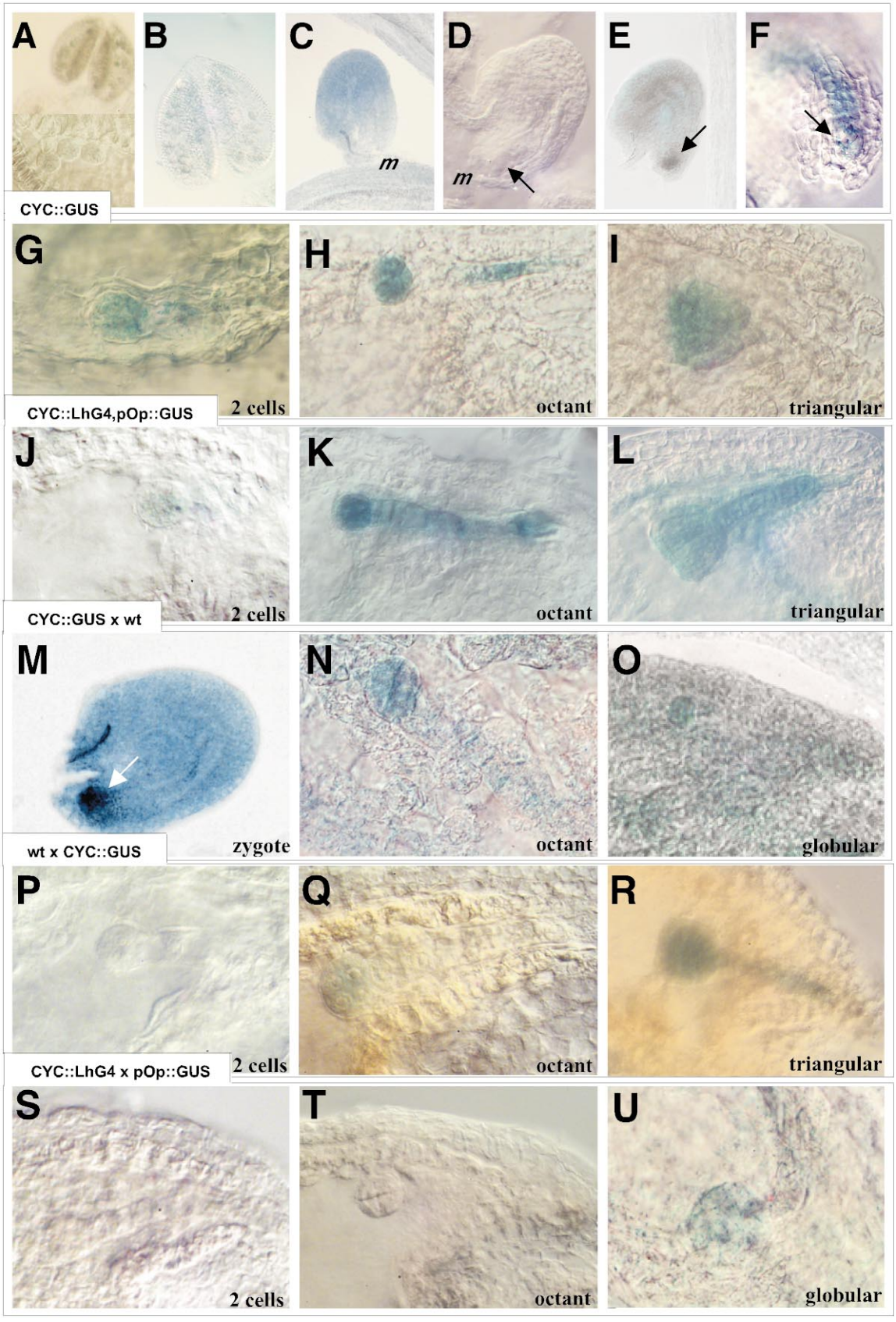
A
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CYC::LhG4,pOp::GUS
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Fig. 1. GUS expression under the CYC promoter is influenced by parent-of-origin effects during early embryogenesis. Flower organs and whole seeds were stained for GUS activity. A, C, E and G–I: CYC::GUS lines. B, D, F and J–L: CYC::LhG4; pOp:GUS double transgenic line. M–U: F1 seeds from crosses as indicated. A, B: Stamen; C, D: ovules; E, F: seeds at the zygote stage; G–U: embryos from the two-cell stage to the triangular stage as indicated. D: The arrow points out the egg cell. E, F, M: The arrows point out de novo GUS expression in the zygote. wt: wild-type plant. m: micropylar pole.
←

tion factor to the synthetic pOp promoter, following activator line × reporter line crossing [2].

Although the activity of the CYC promoter was described starting from the octant stage [4], we detected earlier activity through the analysis of both the CYC::GUS line and a CYC::LhG4; pOp::GUS double transgenic line ([3]; this study). Before fertilisation GUS activity is absent in the mature pollen (Fig. 1A, B), and is absent or weak in the ovule (Fig. 1C, D). After fertilisation, the zygote exhibits a strong staining (Fig. 1E, F), demonstrating de novo activity of the CYC promoter. In the embryo, the CYC promoter is active during cell division (Fig. 1G–L). When pollen of the CYC::GUS line was used to cross a wild-type (wt) plant, the first GUS signal was delayed up to the octant stage (wt × CYC:GUS, Fig. 1M–O), whereas the reciprocal cross showed a strong staining in the zygote (CYC::GUS × wt, Fig. 1P–R). In addition, embryos from CYC::LhG4 × pOp::GUS reciprocal crosses produced a first GUS signal at the late globular stage (Fig. 1S–U) instead of the zygote stage (Fig. 1F). The timing shown (Fig. 1S–U) was also observed in a wt × CYC::LhG4; pOp::GUS cross. Importantly, this phenomenon of delayed GUS detection following crosses was observed using another set of transgenic lines, the LTP::GUS (data not shown) and LTP::LhG4 lines (Fig. 2). Following reciprocal LTP::LhG4 × pOp::GUS crosses, GUS activity was absent at the globular stage and a weak activity was first noticed at the heart stage, becoming stronger at a later stage with its specific localisation at the apical pole (Fig. 2B) as previously described [6]. By contrast, embryos from self-fertilisation of LTP::LhG4; pOp::GUS double transgenic lines display reporter gene expression as early as the octant stage (Fig. 2C, D).

Altogether, we show here that the CYC::GUS transgene shows a parent-of-origin-dependent expression in the early embryo and a similar observation was made when analysing LTP::GUS lines. The absence of GUS activity in early stages of embryogenesis following activator × reporter crosses (Figs. 1 and 2) indicates that transactivation was impaired by the lack of expression of one or both transgenes after fertilisation. However, expression analysis in seeds from self-fertilisation clearly demonstrated that the components of the transactivation system are capable of inducing reporter gene expression after fertilisation when both constructs are transmitted by the female genome. Therefore, when transmitted separately to the zygote following a cross, the activator and reporter components were not expressed simultaneously after fertilisation. This was true irrespective of the nature of the paternal transgene, and for eight independent transgenic lines in total (one CYC::GUS line, one LTP::GUS line, two pOp::GUS lines, two CYC::LhG4 lines and two LTP::LhG4 lines). Therefore this phenomena is unlikely to be due to the nature of the promoter used, or to one particular locus. This lack of activity during early stages of embryogenesis was relieved at the mid-globular stage (CYC promoter) or heart stage (LTP promoter).

3.2. Evidence for early paternal transgene activity following fertilisation

The most straightforward explanation for our observations is that the paternal genetic components are not expressed during early seed development as suggested previously [1]. However, it cannot be ruled out that expression of the paternal components occurs early during embryo development but at a low and undetectable level (with the GUS reporter) compared to their maternal counterpart.

To address this question, we substituted the GUS gene contained within the pOp::GUS construct, with the *BARNASE* gene encoding a ribonuclease [7,8]. The rationale is that BARNASE expression is a better indicator of gene expression than the *uidA* gene, because fewer molecules of BAR-

Table 1
Reciprocal CYC::LhG4 × pOp::BARNASE crosses consistently produce 2–4-cell embryo arrest

Female	Male	Total F1 seeds	Abnormal embryos	2–4-cell embryos
CYC::LhG4	pOp::BARNASE			
H	F	133	87 (65.4%)	42 (31.5%)
H*	E	55	40 (72.7%)	17 (30.9%)
K	F	137	51 (37.2%)	32 (23.3%)
pOp::BARNASE	CYC::LhG4			
F	H	110	66 (60%)	21 (19%)
F	K	125	46 (36.8%)	24 (19.2%)
pOp::BARNASE	pOp::BARNASE			
F	F	200	2 (1%) (aborted)	0 (0%)

CYC::LhG4 × pOp::BARNASE reciprocal crosses were performed with different transgenic lines as indicated (CYC::LhG4 lines H, H* and K, pOp::BARNASE lines F and E). F1 seeds were collected at 7–8 DAP. The number of seeds containing abnormal embryos (indicating BARNASE toxicity) was scored (fourth column), and the number of seeds containing 2–4-cell arrested embryos among them is indicated (fifth column). The percentage of abnormal and early arrested seeds among the total F1 seeds scored is also indicated between brackets. The occurrence of the class of 2–4-cell arrested embryos indicates that transactivation of the *BARNASE* gene occurs as early as after the first division of the zygote. Self-fertilisation of CYC::LhG4 lines produce 100% normal seeds. Here, the frequency of abnormal embryos is lower than expected from the inheritance of both CYC::LhG4 and pOp::BARNASE transgenes. This is because in 7–8 DAP seeds not all *BARNASE*-expressing embryos are unambiguously distinguishable from their wt counterpart, except for the early arrested seeds. CYC::LhG4-H and -K are hemizygous. H* is a homozygous progeny from line H. pOp::BARNASE-F and E are homozygous.

NASE (compared to GUS protein) may be needed to be detected [9]. The question addressed here is the determination of the earliest stage at which cytotoxic effects are visible, corresponding to the earliest detectable stage of BARNASE transactivation. We have already published that, following crosses, the transactivation system used allowed BARNASE expression in all the seed progeny inheriting pOp::BARNASE and CYC::LhG4 transgenes. This resulted in seed abortion with a frequency that fitted a Mendelian ratio [3]. In this study, we conducted a time course analysis of seeds derived from reciprocal CYC::LhG4×pOp::BARNASE crosses. Because the analysis is carried out on early embryo stages, not all abnormal seeds are detected, explaining the numbers in Table 1 differing from expected Mendelian ratios (for instance 100% abnormal seeds are expected following a cross between homozygous lines). Importantly, a deviation of normal embryo development could be observed starting from the two-cell stage to the globular stage. Among the 7–8 DAP (days after pollination) seeds showing abnormal embryos, 20–30% contained 2–4-cell arrested embryos (Table 1; Fig. 3B, C). The percentages obtained depending on the direction of the cross are not significantly different. The remaining fraction displayed various lethal phenotypes at later embryo stages [3], probably reflecting individual variations in the level of BARNASE gene expression. Importantly, the same phenomena applied when LTP::LhG4×pOp::BARNASE reciprocal crosses were carried out (Fig. 3D). Although the frequency was much lower than with the CYC promoter (2–3%, $n = 268$), this phenotype indicates that the LTP promoter can also be activated soon after fertilisation. In control pOp::BARNASE×pOp::BARNASE crosses, up to 1% spontaneous seed abortion can occur in some siliques, but no abnormal zygote or 2–4-cell embryo was visible at the micropylar pole. Thus,

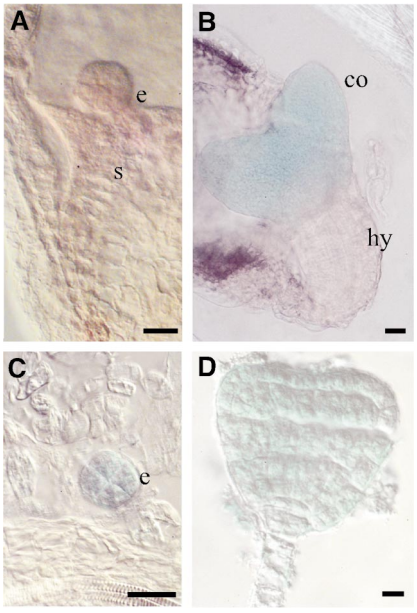

Fig. 2. GUS transactivation under the LTP promoter during early embryogenesis. Whole seeds were stained for GUS activity. A, B: Seeds from a LTP::LhG4×pOp::GUS cross. C, D: Seeds from self-fertilisation of a LTP::LhG4, pOp::GUS double transgenic line. A: Globular-stage embryo, B: torpedo-stage embryo, C: octant-stage embryo and D: heart-stage embryo. e: embryo proper; s: suspensor; co: cotyledons; hy: hypocotyle.

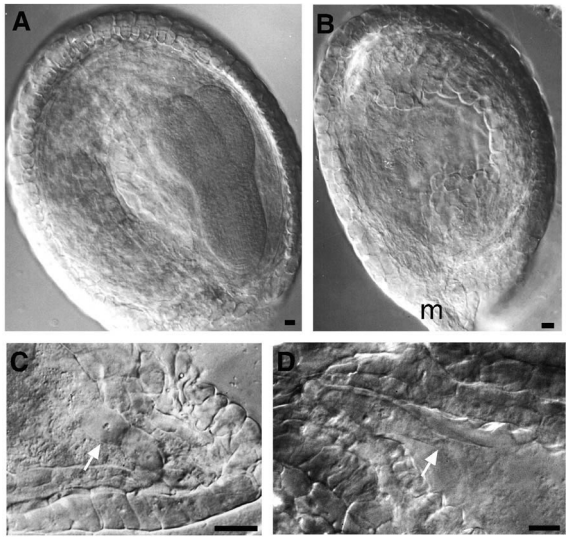

Fig. 3. Early embryo arrests following CYC::LhG4×pOp::BARNASE or LTP::LhG4×pOp::BARNASE crosses. A: wt seed. B: F1 seed from a CYC::LhG4×pOp::BARNASE or LTP::LhG4×pOp::BARNASE cross (7 DAP). C, D: Early arrested embryos, 7 DAP following a CYC::LhG4×pOp::BARNASE cross (C) or a LTP::LhG4×pOp::BARNASE cross (D). m: micropyle. Arrows: arrested embryos at the micropylar pole.

the embryo phenotypes observed following crosses with activator lines were specific to BARNASE expression under the CYC or the LTP promoter.

Because the cytotoxic effects of the BARNASE protein are first detected as early as the two-cell stage we deduced that both the maternal and paternal components were accessible and transcribed at this stage. This therefore differs from the data obtained with the GUS transgene. Again, this cannot be due to a particular promoter (the pOp, CYC and LTP promoter were used) or a particular integration locus of the components. Several loci were therefore able to show transcriptional activity, despite the proposed genome wide mechanism preventing expression of paternal genes ([1], this study).

4. Discussion

We present here evidence that several paternally inherited transgenes retain low activity during early embryogenesis in *Arabidopsis*. First, we demonstrated that transgene expression under the CYC and the LTP promoter is subjected to parent-of-origin effects during early embryogenesis. Several independent transgenic lines and several promoters (CYC, LTP and pOp) were used in this study demonstrating that these parent-of-origin effects are not due to a particular insertion locus or to the nature of the promoter. Therefore, although highlighted with a transgene expression system and not with a measure of the endogenous genes transcripts, this phenomenon is likely to correspond to the genome-wide parental effect on gene expression proposed in a previous report [1].

In our study, expression of the paternal transgenes was drastically repressed compared to their maternal counterparts that were de novo expressed in the zygote (e.g. the CYC promoter) or in the octant-stage embryo (e.g. the LTP promoter). This conclusion is based on the absence of GUS reporter detection during a definite developmental window, in F1 seeds resulting from several type of crosses (see Section 3.1

and Fig. 4). Since de novo GUS expression was detected in the corresponding developmental stages in F2 seeds, and because the GUS reaction product is not stored in the egg cell, we ruled out the possibility of misinterpretation of the GUS detection assays. Only differential expression of maternal and paternal transgenes can account for our observation, which thus provides an additional line of evidence for imprinting processes taking place during early seed development.

In the Vielle-Calzada et al. report [1], expression of paternal endogenous genes could not be detected in early embryo stages even using reverse transcription-polymerase chain reaction techniques on whole siliques, leading to the hypothesis of a genome-wide silencing of paternal genes. By contrast, in this study using a sensitive reporter system, we obtained evidence of paternal transgene expression soon after fertilisation, at the level of individual embryos. This was achieved by inducing

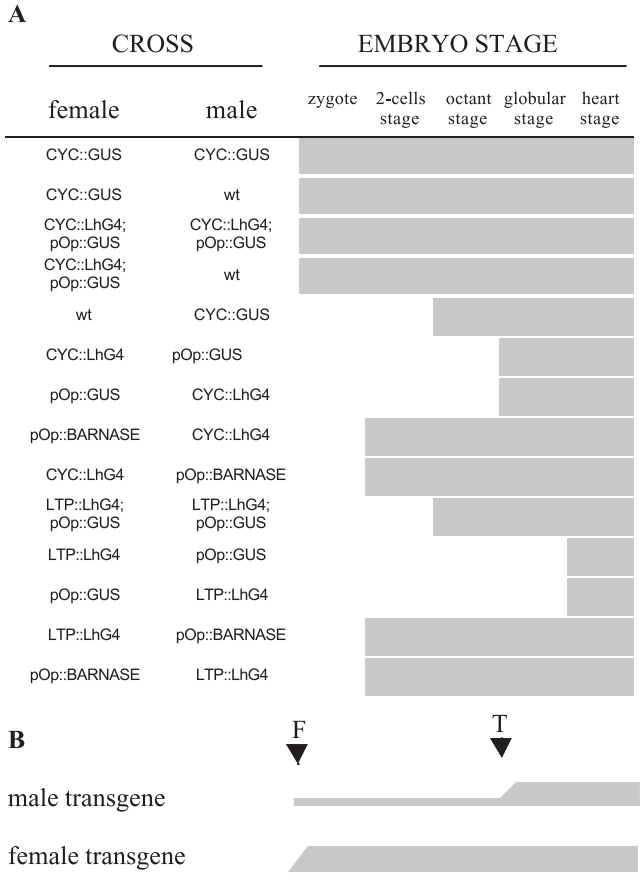

Fig. 4. Summary of the GUS and BARNASE expression study and model for differential expression of the paternal and maternal components during early embryogenesis. A: The timing of expression of the GUS reporter gene and the *BARNASE* gene during seed development, following self-fertilisation or reciprocal crosses of the transgenic lines, are indicated in grey (expression) or white (no detection of the expression). B: An interpretation of the above data is the occurrence of differential expression of the paternal and maternal transgene components (genomic imprinting). In a cross between two transgenic lines (such as those used in this study), while the maternal transgene is expressed soon after fertilisation (F), in the zygote (for instance according to the CYC promoter activity), the paternal transgene is expressed at a very low level. This level is not high enough to generate a detectable GUS activity, but is sufficient to generate detectable BARNASE activity. Between the octant to globular stage (transition point, T), the repression of paternal transgene expression is relieved and the GUS reporter gene is expressed at a detectable level.

expression of a *BARNASE* transgene in the zygote using a transactivation system. Again, this early paternal transgene activity is unlikely to be due to a particular transgene locus and we propose that it might reflect an endogenous situation where at least some paternal genes are expressed at the basal level following fertilisation. Since our GUS expression study did not allow detection of such an early expression, this indicates that paternal transgene activity as detected with BARNASE experiments must occur at a lower level than maternal transgene activity. On a different note, in this experiment not all *BARNASE*-expressing embryos were arrested at a 2–4-cell stage and a fraction displayed later lethal phenotypes, highlighting different *BARNASE* expression levels between embryos. This suggests that the activation of paternal transgene expression (and maybe endogenous gene expression) after fertilisation may be a process that varies between individuals. An interesting parallel could thus be made with stochastic gene activation events described during embryogenesis and specific cell-type differentiation in insects and animals [10–12].

The results from our GUS and BARNASE expression studies are summarised in Fig. 4A. A model is proposed (Fig. 4B), which reconciles both observations that crossing-based transactivation produces a delay in GUS detection but not in BARNASE detection, as explained above. This model proposes that at least some paternal loci can be expressed at a basal level after fertilisation despite a general mechanism leading to an under-representation of paternal gene expression compared to their maternal counterpart. In this model, paternal (trans)gene expression may gradually be activated to reach the level of maternal expression around the mid-globular stage.

Both the Vielle-Calzada et al. work [1] and this study raise the question of the nature of the underlying mechanisms leading to a lack of or down-regulation of paternal gene expression after fertilisation. DNA methylation changes and chromatin remodelling processes are known to affect the transcriptional state of the chromatin in yeast, insects and animals [13,14]. As proposed by Vielle-Calzada et al. [1], such epigenetic modifications could also occur in plants and alter the expression of the paternal genome. This is based on several reports describing changes in DNA methylation levels and in the composition of histones responsible for DNA-packaging in the sperm cells [15–18]. Alternatively, in the mouse embryo it is known that the absence of zygotic gene expression during the first 24 h after fertilisation is due to a lack of co-activators [19,20]. These proteins are known to enhance the activity of transcription factors [21]. Using an UAS/Gal4-VP16 system (essentially similar to the pOp/LhG4 system), the authors provided the egg cell with a transcription factor but despite this, no enhanced expression of the reporter was observed. It is tempting to speculate that plants use a similar co-activator-based system to control paternal genome expression during the early stages of seed development.

Both these mechanisms based either on epigenetic modifications altering chromatin structure and accessibility, or on the availability of co-activator proteins, are working hypothesis to explain a genome-wide lack of paternal gene expression or transgene expression as described by Vielle-Calzada et al. [1] and by this study, respectively. However, the occurrence of early paternal transgene activity (as observed with BARNASE transactivation experiments) necessarily implies that some or

most DNA domains in the paternal genome are down-regulated but still show basal activity. This suggests that the proposed paternal genome silencing does not correspond to an all-or-nothing mechanism. It remains to be shown whether for some specific genes, a low level paternal activity would produce enough protein to complement a maternal mutated allele or not. In conclusion, we believe that this study will open novel investigations on the underlying mechanisms of imprinted-based parent-of-origin effects during seed development.

On a different note, although this phenomenon was expected to be unfavourable towards the use of a two-component system in *Arabidopsis* embryo, we show that it is possible to activate transgenes of interest in the F1 progeny.

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