

Overexpression of *PGA37/MYB118* and *MYB115* promotes vegetative-to-embryonic transition in *Arabidopsis*

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Formation of somatic embryos from non-germline cells is unique to higher plants and can be manipulated in a variety of species. Previous studies revealed that overexpression of several *Arabidopsis* genes, including *WUSCHEL* (*WUS*)/*PLANT GROWTH ACTIVATOR6* (*PGA6*), *BABY BOOM*, *LEAFY COTYLEDON1* (*LEC1*), and *LEC2*, is able to cause vegetative-to-embryonic transition or the formation of somatic embryos. Here, we report that a gain-of-function mutation in the *Arabidopsis* *PGA37* gene, encoding the MYB118 transcription factor, induced vegetative-to-embryonic transition, the formation of somatic embryos from root explants, and an elevated *LEC1* expression level. Double mutant analysis showed that *WUS* was not required for induction of somatic embryos by *PGA37/MYB118*. In addition, overexpression of *MYB115*, a homolog of *PGA37/MYB118*, caused a *pga37*-like phenotype. A *myb118 myb115* double mutant did not show apparent developmental abnormalities. Collectively, these results suggest that *PGA37/MYB118* and *MYB115* promote vegetative-to-embryonic transition, through a signaling pathway independent of *WUS*.

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Introduction

In higher plants, life cycle starts from the fertilization of an egg cell by a sperm to produce a zygotic embryo. After fertilization, a zygotic embryo develops into a mature embryo through a series of developmental processes referred to as embryogenesis. In addition to the zygotic embryogenesis pathway, embryos can also arise from somatic tissues without undergoing meiosis and fertilization. Asexual embryos can be produced by manipulating somatic or gametophytic cells from a variety of species [1, 2]. Moreover, the process of somatic embryo development is similar to that of zygotic embryos which are usually embedded inside maternal tissues, making them inaccessible to analysis. Thus, somatic embryos provide

an attractive model for studying embryogenesis.

In vitro induction of somatic embryo formation is initiated by inducing de-differentiation of somatic cells, usually requiring high concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). Subsequently, the de-differentiated cells or calli re-differentiate and develop as somatic embryos by an unknown mechanism. Whereas phytohormones are generally believed to activate a signaling pathway for the induction of embryogenic competence, a number of genetic loci have also been shown to play critical roles during somatic embryo development [2-4]. Mutations in the *Arabidopsis* *LEAFY COTYLEDON1* (*LEC1*) and *LEC2* genes cause similar defects in embryo maturation and partially transform cotyledons into leaves [5, 6]. *LEC1* and *LEC2* encode a CCAAT-binding factor-type transcription factor and a plant-specific B3 transcription factor, respectively [7, 8]. Ectopic expression of these two embryo-specific genes causes the formation of somatic embryos on vegetative tissues [4, 7, 8]. A recent study shows that the auxin biosynthetic genes *YUC2* and *YUC4* are rapidly induced by *LEC2*, and *YUC4* is likely a direct target of *LEC2* [4]. These observations establish

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a molecular link between the regulation of embryogenic competence and the auxin activity. On the other hand, whereas *LEC1* and *LEC2* are key regulators controlling embryonic identity, the *Arabidopsis* *PICKLE* (*PKL*) gene, encoding a CHD3 chromatin-remodeling factor, appears to play an opposite role. The *pkl* mutant displays some embryonic traits after germination, including a higher capacity of embryogenic competence, the production of somatic embryos from root explants, and an elevated *LEC1* expression [9–11]. Because CHD3 proteins are involved in transcriptional repression, *PKL* has been proposed to prevent re-expression of the embryonic developmental state during germination, likely through the regulation of gibberellin (GA) signaling [10, 11]. A more recent study identified three functionally redundant *VPI/ABI3-LIKE* (*VAL*) genes encoding B3 domain-containing proteins [12]. Whereas *val1* shows a *pkl*-like phenotype, the *val1 val2* double mutant produces embryo-like structures around root and apical meristem regions, which is associated with increased expression of *LEC1*, *LIL*, *ABI3*, and *FUSCA3* (*FUS3*). It has been proposed that *VAL* factors may function as transcriptional repressors of the *LEC1/B3* genes in conjunction with *PKL* or related factors [12].

Using reverse genetic approaches, several genes have been shown to have the capacity to induce the formation of embryonic calli or somatic embryos from vegetative tissues. The carrot *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*) gene was shown to mark vegetative-to-embryonic transition in carrot suspension cell cultures [13], and its *Arabidopsis* orthologous gene was found to enhance embryogenic competence in cell cultures as well [14]. The *BABY BOOM* (*BBM*) gene, encoding an AP2/ERF-type transcription factor, was identified from *Brassica napus* and *Arabidopsis thaliana*. Ectopic expression of *BBM* results in the formation of somatic embryos and cotyledon-like structures on seedlings [15]. Moreover, overexpression of the *Arabidopsis* *AGAMOUS-Like 15* (*AGL15*) gene and its homologous gene in soybean, which encode MADS domain-containing proteins, enhances the formation of secondary embryos and somatic embryos [16, 17]. The promotion of somatic embryo formation by *AGL15* is attributed, at least in part, to a reduced GA level [18]. This notion is consistent with the observation made in the *pkl* mutant that displays a GA-deficiency phenotype [9, 10], and the observation that *FUS3*, encoding a B3 transcription factor critical for embryogenesis, negatively regulates GA biosynthesis [19].

We have used a functional screening strategy to identify *Arabidopsis* gain-of-function mutants, *plant growth activator* (*pga*), by culturing T-DNA-transformed root

explants on a medium containing auxin but without cytokinin. In this screen, a chemical-inducible activation tagging vector was used, and therefore the expression of the mutant phenotype was strictly dependent on the chemical inducer estradiol [20, 21]. Mutants identified from this screen were presumed to conditionally overexpress key components that regulate somatic embryo formation or the cytokinin response [21, 22]. The *pga6* mutant was found to efficiently produce somatic embryos on seedlings and explants in the presence of the inducer. Molecular analysis demonstrated that *PGA6* was identical to the homeobox gene *WUSCHEL* (*WUS*) [21], which regulates stem cell fate in shoot and floral meristems [23–25]. Moreover, ectopic expression of *WUS* also induces shoot and floral stem cell activities in roots and stems, respectively [26, 27]. These observations suggest that *WUS/PGA6* plays a critical role in the maintenance of the identity of embryonic stem cells. Here, we report the characterization of the *pga37* mutant, which displays a similar but distinctive phenotype compared to *pga6*. The *PGA37* gene, encoding a MYB transcription factor, induces embryogenic competence in a *WUS*-independent manner.

Results

The pga37 mutant phenotype

In previous genetic screens for *pga* mutants, we have identified two groups of mutants that displayed the ability to form either somatic embryos or shoots [21, 22]. For example, overexpression of the *PGA6/WUS* gene causes vegetative-to-embryonic transition [21]. The *PGA22* gene, encoding isopentenyl transferase 8 (IPT8), catalyzes the first rate-limiting step of cytokinin biosynthesis, and the *pga22* gain-of-function mutant shows a cytokinin overproduction phenotype, including the regeneration of shoots in the absence of externally supplied cytokinin [22].

Here, we report the characterization of *pga37*, which showed a phenotype similar to that of *pga6*. When cultured on the screening medium (SCM; containing IAA, kanamycin, and the chemical inducer 17- β -estradiol but without cytokinins; see Materials and Methods) for 7–10 days, *pga37* root explants produced green-yellowish embryonic calli (Figure 1A), which were not found on control medium without the chemical inducer estradiol (Figure 1B). Upon culturing for a longer period of 3–5 weeks, somatic embryos were generated from embryonic calli (Figure 1C and 1D). Scanning electron microscopic analysis revealed the presence of somatic embryos at a variety of developmental stages, including the globular, heart, and cotyledon stage (Figure 1E–1G). Similar to

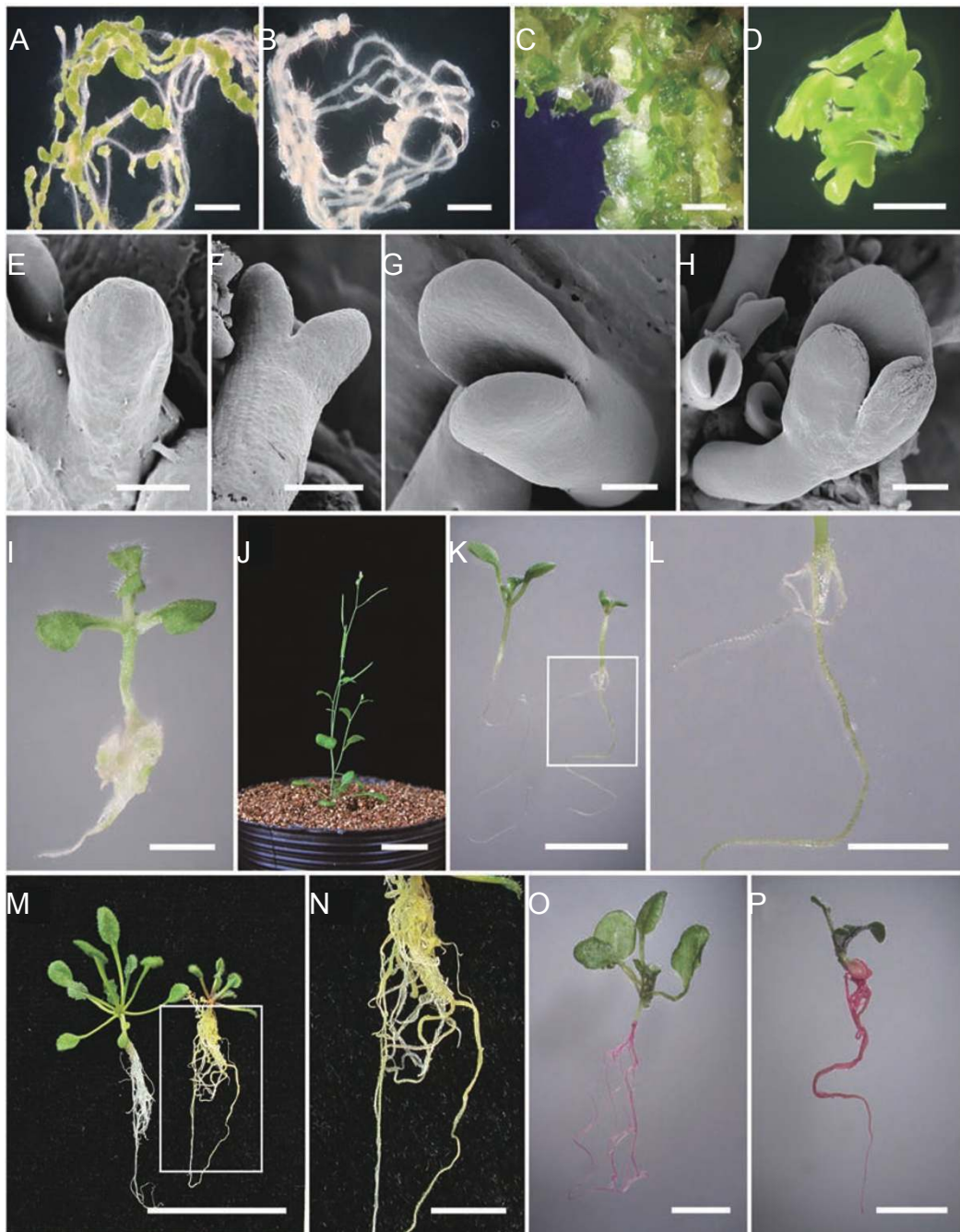


Figure 1 The *pga37* gain-of-function mutant phenotype. **(A)** *pga37* root explants cultured on SCM containing 10 μ M estradiol for 7 days. Note: yellowish green embryonic calli are visible. **(B)** *pga37* root explants cultured on SCM without estradiol for 7 days. **(C)** *pga37* root explants cultured on SCM containing 10 μ M estradiol for 5 weeks. Somatic embryos are formed on the explants. **(D)** An enlarged view of panel (C). **(E-H)** Scanning electron microscopy of somatic embryos at various developmental stages. Images were taken from *pga37* root explants cultured on SCM for 3-5 weeks. **(E)** A somatic embryo at the globular stage. **(F)** A somatic embryo at the heart stage. **(G)** A somatic embryo at the cotyledon stage. **(H)** An abnormal somatic embryo with three cotyledons. **(I)** A *pga37* seedling germinated from a somatic embryo. **(J)** A soil-grown *pga37* seedling derived from a somatic embryo. **(K)** Seven-day-old *pga37* seedlings germinated and grown on a MS medium in the absence (left) or presence (right) of 10 μ M estradiol. **(L)** An enlarged view of the boxed region in panel (K). **(M)** Three-week-old *pga37* seedlings germinated and grown on a MS medium in the absence (left) or presence (right) of 10 μ M estradiol. **(N)** An enlarged view of the boxed region in panel (M). **(O and P)** Fat Red 7B staining of 2-week-old *pga37* seedlings germinated and grown on MS medium in the absence (O) or presence (P) of 10 μ M estradiol. Bar, 0.5 mm (A-D), 50 μ m (E), 100 μ m (F-H), 2 mm (I and L), 5 mm (K, N-P), and 2 cm (J and M).

that observed in the *pga6* mutant, somatic embryos with three cotyledons were occasionally observed (Figure 1H).

After transfer from SCM to MS medium [28] without the inducer estradiol, these somatic embryos were able to continue development into healthy seedlings, and plantlets were fertile and set seeds (Figure 1I and 1J). When seeds harvested from *pga37* plantlets were germinated and grown in the absence of the inducer, *pga37* plants were indistinguishable from wild-type (WT) plants. However, when germinated and grown in the presence of inducer, *pga37* seedlings were smaller than WT seedlings, and produced dark green cotyledons and true leaves (Figure 1K and 1M). Moreover, *pga37* roots and the junction region between the root and the hypocotyl turned light green (Figure 1K-1N) similar to that previously observed in *pickle* (*pkl*) mutant roots [9, 10]. Notably, the *pga37* mutation also caused the accumulation of storage compounds such as lipids, which were normally found in embryos and seeds, in roots and other vegetative tissues (Figure 1O and 1P).

Taken together, these observations suggest that the *pga37* mutation is capable of promoting vegetative-to-embryonic transition, thus likely representing a key genetic locus regulating *Arabidopsis* embryogenesis.

Molecular characterization of the *PGA37* gene

To determine the genetic basis of *pga37* (in the Wasilewskija (WS) background), we crossed the mutant with WT (WS). All tested F1 progeny from the cross showed the mutant phenotype in an estradiol-dependent manner. In an F2 population obtained from self-pollinated F1 plants, the phenotype of forming somatic embryos on explants segregated in a 3:1 ratio (mutant:WT = 85:26). These results indicated that *pga37* is a dominant mutation in a single nuclear gene, and its mutant phenotype was strictly dependent on the inducer. Because of the dependency on the inducer, the mutated gene should be tightly linked to a T-DNA insert carrying the O^{LexA} -46 promoter/enhancer. We identified the *Arabidopsis* genomic sequences flanking the O^{LexA} -46 promoter/enhancer by inverse PCR [29]. A T-DNA was found to be inserted approximately 7.8 kilo base pairs (kb) upstream from the putative translation start codon of At3g27785, which encodes the transcription factor MYB118. The left border (LB) of the T-DNA insertion was adjacent to the 5'-end of *MYB118*, thus allowing the inducible O^{LexA} -46 promoter/enhancer to activate the expression of the transcription factor gene (Figure 2A). Using an F2 population obtained from a cross between *pga37* (WS) and WT (Columbia-0 or Col-0), the mutation was roughly mapped onto chromosome III, approximately 4.4 cM

from the simple sequence length polymorphism marker *ciw11* (45 F2 plants were tested). *MYB118* is approximately 4.0 cM south of *ciw11* (Figure 2A). These results suggest that the *pga37* mutation was likely caused by the identified T-DNA insertion.

In this 7.8-kb region between the insertion site and *MYB118*, neither protein- nor putative small RNA-encoding genes could be annotated using various prediction programs or by a manual search. Northern blot analysis showed that *MYB118* expression was inducible in the *pga37* mutant by estradiol in a dose-dependent manner (Figure 2B); moreover, the severity of the mutant phenotype was correlated with the *MYB118* expression level (Figure 2B and 2C). A *MYB118* cDNA fragment spanning the entire coding sequences was cloned into the estradiol-inducible expression vector pER10 [20] and the resulting construct pER10-*MYB118* was transformed into WT plants (WS). Expression of the *MYB118* transgene was highly inducible by estradiol (Figure 2D). Upon induction, the *pga37* mutant phenotype was recapitulated in pER10-*MYB118* transgenic plants, including the somatic embryo formation phenotype (Figure 2E) and retarded growth and development of the transgenic seedlings (Figure 2F). Notably, when germinated under higher concentrations of inducer, growth and development of pER10-*MYB118* transgenic plants were completely arrested, and the treated plants produced callus-like tissues without any recognizable structures of roots and hypocotyls (Figure 2F and 2G). Note that multiple independent pER10-*MYB118* transgenic lines were analyzed and similar results were obtained.

A stronger phenotype of the pER10-*MYB118* transgenic plants compared to *pga37* was most likely caused by higher transgene expression levels. In most transgenic lines, *MYB118* expression was substantially higher than that in *pga37*, as shown in Figure 2D for a representative transgenic line. Moreover, because of the relative distal position of the T-DNA insertion from *MYB118* in the *pga37* genome (approximately 7.8 kb), the O^{LexA} -46 sequence was unlikely to directly activate the *MYB118* transcription as a promoter, but rather it might act as a transcriptional enhancer. Consistent with this notion, only a single transcript was detected in inducer-treated *pga37* plants, which was different from that observed in *pga6* and *pga22*. In *pga6* and *pga22*, two species of transcripts were detected, of which the shorter species presumably originated from the native promoters whereas the longer one likely initiated from the O^{LexA} -46 promoter [21, 22]. Nevertheless, the data presented above confirmed that overexpression of *MYB118* was sufficient to recapitulate the *pga37* mutant phenotype.

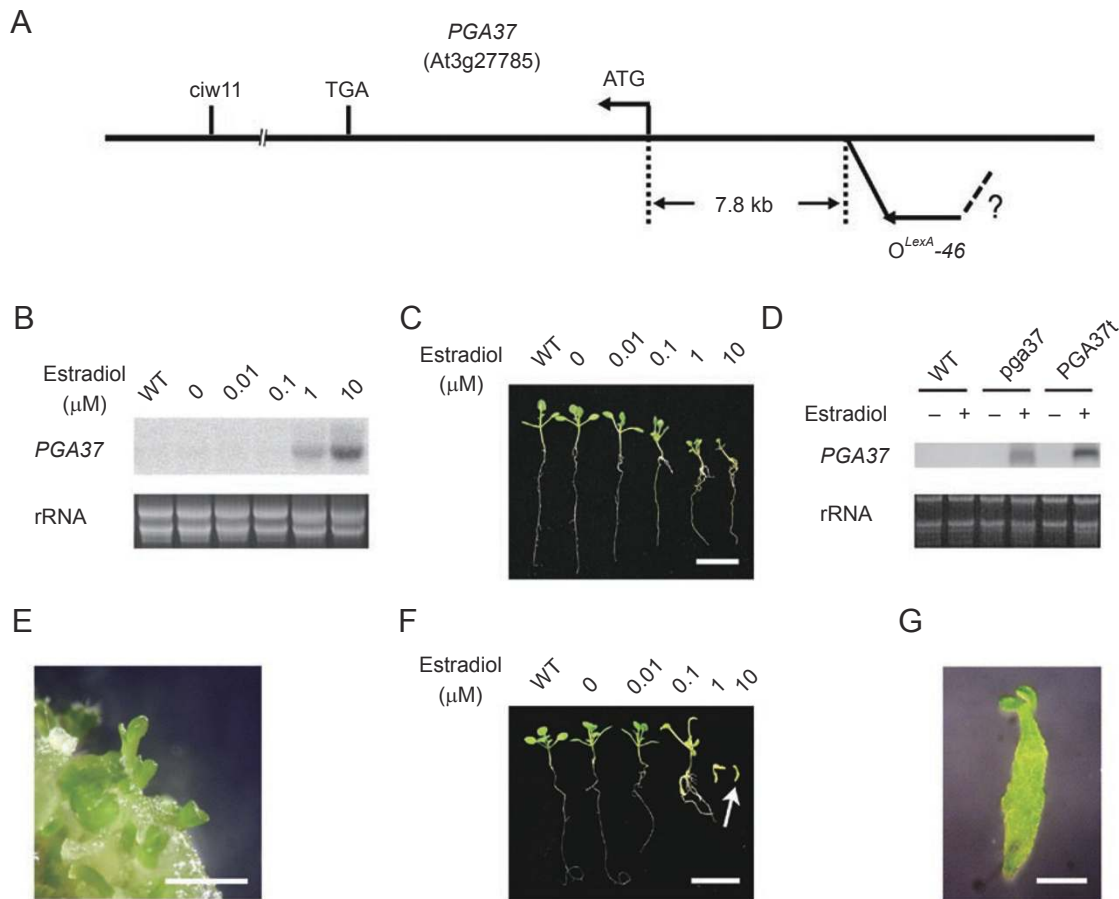


Figure 2 Molecular characterization of *PGA37*. **(A)** A schematic map of the *pga37* mutant genome (not in scale). The insertion position is indicated by an arrow that also shows the orientation of left border (LB) of the T-DNA. The insertion position of the right border (RB) is unclear, as shown by a question mark. The *pga37* mutation was mapped to approximately 4.4 cm from the *ciw11* marker. **(B)** Expression of *PGA37* in the *pga37* mutant. Two-week-old *pga37* seedlings were treated with different concentrations (μM) of estradiol for 16 h as indicated on the top of the panel. Total RNA was analyzed by northern blot hybridization using a *PGA37* cDNA fragment as a probe. WT: wild type (Wassilewskija or WS) seedlings treated with dimethyl sulfoxide (DMSO; a solvent for estradiol). **(C)** Dose-dependency of the *pga37* mutant phenotype. Two-week-old *pga37* seedlings germinated and grown on MS medium containing various concentrations of estradiol (μM) as indicated on the top of the panel. WT: DMSO-treated WS seedlings. **(D)** Expression of *PGA37* in pER10-*PGA37* transgenic plants. Two-week-old seedlings of WT, *pga37* and pER10-*PGA37* transgenic plants (*PGA37t*) were treated with (+) or without (-) 10 μM estradiol for 16 h, and total RNA was analyzed by northern blot hybridization as described in panel (B). **(E)** Formation of somatic embryos on root explants of a pER10-*PGA37* transgenic line. The explants were cultured on SCM for 5 weeks. **(F)** Phenotype of pER10-*PGA37* transgenic plants. Two-week-old pER10-*PGA37* seedlings germinated and grown on MS medium containing various concentrations of estradiol (μM) as indicated on the top of the panel. WT: DMSO-treated WS seedlings. Note that the transgenic plants showed a stronger phenotype than *pga37* (see panel (C) and text for details). **(G)** An enlarged view of a pER10-*PGA37* transgenic seedling in panel (F) marked by an arrow (grown in the presence of 10 μM estradiol). No distinctive root and hypocotyl structures were found, and embryonic calli were formed on the plantlet. Bar, 1 cm (C and F) and 1 mm (E and G).

A PGA37::GUS reporter gene is preferentially expressed in embryos and endosperm

A reverse transcription-PCR (RT-PCR) analysis detected *PGA37* expression in siliques but not in any other tissues/organs (Figure 3A). To more precisely follow the *PGA37* expression pattern, a *PGA37* promoter:: β -glucuronidase (*PGA37::GUS*) reporter gene was trans-

formed into WT plants. A 2.5-kb *Arabidopsis* genomic DNA fragment, including the putative promoter sequence, 5'-untranslated region (UTR), and sequences encoding the first 12 amino acid residues of MYB118, was fused in frame to the *GUS* coding sequence. The *PGA37::GUS* expression was specifically detected in developing embryos, starting approximately at the early

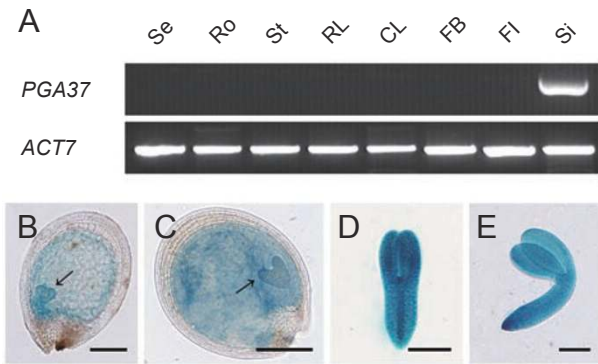


Figure 3 Expression pattern of *PGA37*. **(A)** Total RNA from various tissues/organs of WT plants was subjected to reverse transcription (RT)-PCR analysis. Two micrograms of RNA was used for RT using oligo-dT as a primer, and the PCR was cycled for 30 times. Primers used in PCR were located in exons 2 and 4 of *PGA37*, respectively (see Figure 6I for the positions and orientation of the primers). *Actin7* (*ACT7*; At5g09810) was used as an internal control. Se: seedling; Ro: root; St: stem; RL: rosette leaf; CL: cauline leaf; FB: flower bud; Fl: flower; Si: silique. **(B-E)** Analysis of β -glucuronidase (GUS) activity in *PGA37::GUS* transgenic plants. At least five independent transgenic lines were analyzed and similar results were obtained. **(B)** Early heart stage; **(C)** late heart stage; **(D)** torpedo stage; **(E)** cotyledon stage. Bar = 100 μ m.

heart stage and throughout embryogenesis (Figure 3B-3E). GUS activity was restricted to endosperm and embryos, but not detectable in the seed coat (Figure 3B and 3C). Upon entering the cotyledon stage, GUS expression was also detected in embryos (Figure 3D and 3E), but became undetectable after germination. Consistent with the results of RT-PCR analysis (Figure 3A), the *PGA37::GUS* expression was not detected in any other tissues/organs at various developmental stages. The embryo- and endosperm-specific expression pattern of *PGA37* suggests that it plays a role during embryogenesis.

Increased expression of embryonic genes in *pga37*

Data presented above show that *PGA37/MYB118* is capable of inducing vegetative-to-embryonic transition, leading to the formation of somatic embryos. To better understand the molecular mechanism underpinning this transition, we investigated the expression of several embryo- or seed-specific genes in *pga37*. Root explants derived from WT and *pga37* plants were cultured on SCM for different times. In *pga37* explants, green-yellowish embryonic calli were usually formed around 7-10 days, and somatic embryos were produced around 20-30 days. Expression of *LEC1* was detectable around day 1, whereas expression of *LEC2*, *FUS3* and *WUS* became detect-

able in later stages (Figure 4A). A similar expression pattern was also observed in explants derived from pER10-*PGA37* transgenic plants. To confirm this result, pER10-*PGA37* transgenic plants germinated and grown on MS medium were treated with inducer for varying times, and *LEC1* expression was analyzed by RT-PCR. *LEC1* expression could be detected after 6 h of induction, and a higher expression level was found at later time points (Figure 4B). Under these assay conditions, expression of *WUS*, *FUS3*, and *LEC2* was not detected (data not shown). These results suggest that *PGA37* is able to enhance *LEC1* expression in non-embryonic tissues.

Because *pga37* roots showed a phenotype similar to

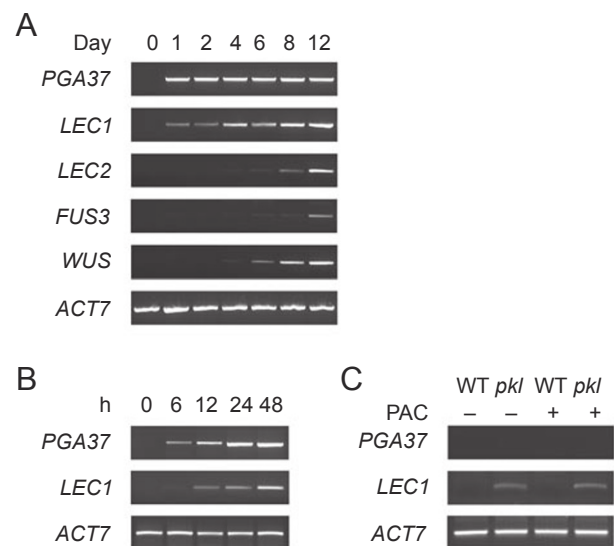


Figure 4 *PGA37*-regulated expression of *LEC1*. **(A)** RT-PCR analysis of *PGA37*, *LEC1*, *LEC2*, *FUS3*, and *WUS* expression in *pga37* root explants. Root explants cultured on CIM medium (containing 2,4-D; see Materials and Methods) for 3 days were transferred onto SCM. Root explants collected at the time points indicated on the top of the panel were used for the preparation of total RNA. RT-PCR was carried out as described in Figure 3A. PCR was cycled 25 times for *PGA37* and *ACT7*, and 30 times for other genes. **(B)** RT-PCR analysis of the *LEC1* expression in *pga37* seedlings. Two-week-old seedlings germinated and grown on MS medium were treated with 10 μ M estradiol for different times (hours; as indicated on the top of the panel). Expression of *LEC2* and *WUS* was not detected under the assay conditions. **(C)** Expression of *PGA37* and *LEC1* in WT and *pkl* mutant plants. Two-week-old seedlings were germinated and grown on MS medium with (+) or without (-) 20 nM paclobutrazol (PAC). PAC is an inhibitor of gibberellin biosynthesis, which increases the phenotype penetrance of *pkl* and *val1* mutants [9, 12]. Total RNA was used for RT-PCR analysis as described in Figure 3. RNA prepared from *pga37* seedlings treated with estradiol was used as a positive control for the analysis of the *PGA37* expression (not shown).

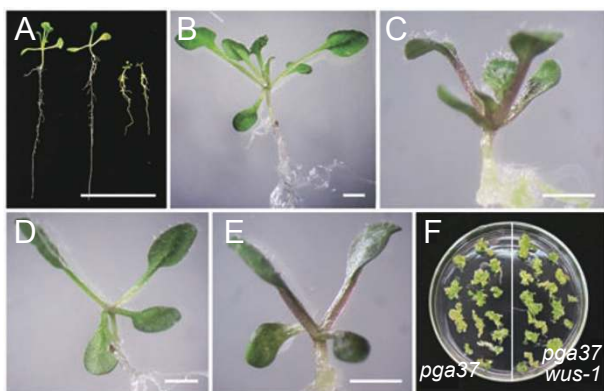


Figure 5 *PGA37* functions independently from *WUS*. **(A)** Two-week-old seedlings germinated and grown on MS medium in the presence or the absence of 10 μ M estradiol. Note that the *pga37 wus-1* double mutant shows a phenotype similar to that of *pga37* in the presence of estradiol. Double mutant plants were smaller than WT plants and produced green roots. From left to right: *pga37* on MS medium, *pga37 wus-1* on MS medium, *pga37* germinated on estradiol-containing medium, and *pga37 wus-1* germinated on estradiol-containing medium. **(B-E)** Enlarged views of 2-week-old seedlings under different conditions. **(B)** A *pga37* seedling germinated and grown on MS medium. **(C)** A *pga37* seedling germinated and grown on MS medium containing 10 μ M estradiol. **(D)** A *pga37 wus-1* seedling germinated and grown on MS medium. **(E)** A *pga37 wus-1* seedling germinated and grown on MS medium containing 10 μ M estradiol. **(F)** Root explants derived from *pga37* (left) and *pga37 wus-1* double mutant (right) plants were cultured as described in panel (A) (pretreated with 2,4-D for 3 days). Bar, 2 cm (A) and 2 mm (others).

that of *pkl*, which produced green roots and had an elevated *LEC1* expression level [9, 10], it is possible that *MYB118* expression might be elevated in *pkl* as well. However, *MYB118* expression was not detectable under various conditions in *pkl*, whereas *LEC1* expression was readily detected as reported previously (Figure 4C) [10], suggesting that *MYB118* is not directly regulated by *PKL*.

PGA37 acts independently from *WUS*

The *pga37* mutant shares some similarities with the *pga6* mutant with respect to their capacity to promote vegetative-to-embryonic transition. To investigate possible interactions between *WUS/PGA6* and *PGA37*, we constructed a *pga37 wus-1* double mutant by genetic crosses of these two mutants. *wus-1* has been previously shown as a null mutation [24]. In the double mutant, *PGA37* expression was inducible by estradiol in the *wus-1* loss-of-function mutant background. When germinated and grown in the presence of estradiol, *pga37 wus-1* showed a *pga37*-like phenotype, with characteristics of

dark-green leaves, light green roots and growth inhibition (Figure 5A). Notably, developmental defects in the shoot apical meristem in *wus-1* could not be rescued by *PGA37* overexpression (Figure 5B-5E). To test if somatic embryo formation promoted by *PGA37* is dependent on *WUS*, *pga37 wus-1* root explants were cultured on a callus induction medium (CIM) and then transferred onto SCM (see Materials and Methods). Root explants derived from both *pga37 wus-1* and *pga37* plants were able to efficiently form somatic embryos (Figure 5F), suggesting that *WUS* is not required for *PGA37* function in promoting somatic embryo formation.

Functional characterization of *MYB115*, a homologous gene of *PGA37*

PGA37 encodes a R2R3-type MYB transcription factor (MYB118) with unknown function [30, 31]. *PGA37/MYB118* belongs to a small subgroup of six members, classified on the basis of the relatively conserved carboxyl terminal sequences outside the MYB domain [30, 32]. In this family, *MYB115* (At5g40360) is most closely related to *MYB118* [30]. These two proteins share approximately 32% identity (45% similarity) in the 150-amino acid segment located at the carboxyl terminal half. To functionally characterize *MYB115*, an estradiol-inducible expression construct pER10-*MYB115* was generated, which was stably transformed into WT plants. Expression of the *MYB115* transgene in transgenic plants was highly inducible by estradiol (Figure 6A).

When germinated in the presence of inducer, growth and development of pER10-*MYB115* transgenic plants were significantly retarded, and the phenotypic strength was dependent on the transgene expression level (Figure 6A-6C). Overexpression of *MYB115* caused a complete arrest of plant growth and development and the transformation of the plantlet into callus-like structures (Figure 6C), a phenotype similar to that of pER10-*MYB118* (see Figure 2G). In root explants derived from pER10-*MYB115* transgenic plants, somatic embryos could be efficiently formed in an inducer-dependent manner (Figure 6D-6G). Similar to that observed in *pga37* and pER10-*MYB118* transgenic plants, expression of *LEC1*, but not of *LEC2* and *WUS*, was increased in pER10-*MYB115* transgenic plants treated with the inducer for 1-2 days (data not shown). The results suggest that the structurally similar *MYB118/PGA37* and *MYB115* genes have similar functions.

A RT-PCR analysis showed that *MYB115* was expressed in flowers and siliques, but its expression was not detectable in other tissues (Figure 6H). The expression pattern is similar to that of *PGA37/MYB118* but in a more extended domain (see Figure 3), suggesting that

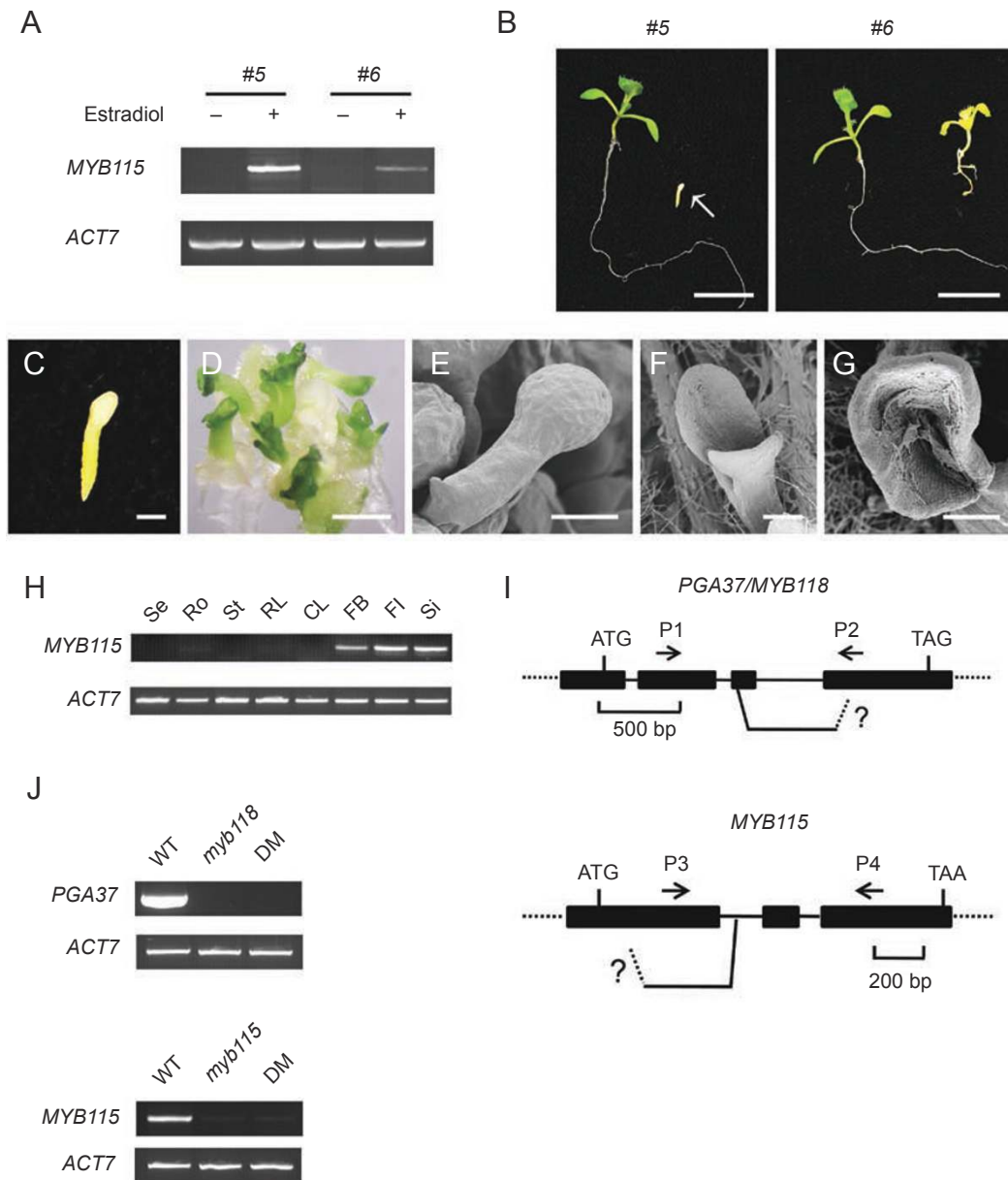


Figure 6 *MYB115* functions similarly as *PGA37/MYB118*. **(A)** Expression of *MYB115* in pER10-*MYB115* transgenic plants. Two-week-old pER10-*MYB115* transgenic seedlings germinated and grown on MS medium were treated with (+) or without (–) 10 μ M estradiol for 16 h. Total RNA used for RT-PCR analysis as described in Figure 3A. The PCR was cycled for 25 times. Two transgenic lines (indicated on the top of the panel) with different *MYB115* expression levels are shown. **(B)** Two-week-old pER10-*MYB115* transgenic seedlings germinated and grown on MS medium supplemented with (right in each panel) or without (left in each panel) 10 μ M estradiol. Two transgenic lines (#5 and #6) are shown. **(C)** An enlarged view of a plantlet shown in panel (B) marked by an arrow. **(D)** Germinating somatic embryos from pER10-*MYB115* root explants cultured on SCM for 4 weeks. **(E–G)** Scanning electron microscopy of somatic embryos derived from pER10-*MYB115* root explants cultured on SCM for 4 weeks. **(E)** A somatic embryo at the globular stage. **(F)** A somatic embryo at the cotyledon stage. **(G)** A somatic embryo with fused cotyledons. **(H)** Expression pattern of *MYB115* analyzed by RT-PCR. Se: seedling; Ro: root; St: stem; RL: rosette leaf; CL: cauline leaf; FB: flower bud; Fl: flower; Si: silique. **(I)** Schematic maps of the *myb118* (top) and *myb115* (bottom) mutant genomes. A T-DNA was inserted in exon 3 of *PGA37/MYB118*, and a T-DNA was inserted in intron 1 of *MYB115*. Filled boxed and lines denote exons and introns, respectively. Dashed lines indicate non-transcribed sequences. Arrows above exons show the positions and orientation of primers used for RT-PCR analyses shown in panel (J). **(J)** Expression of *PGA37/MYB118* (top) and *MYB115* (bottom) in the *myb118 myb115* double mutant. Total RNA prepared from immature siliques of WT, single, and double mutant plants was used for RT-PCR analysis. DM: the *myb118 myb115* double mutant. Bar, 0.5 cm (B), 0.5 mm (C and D), 50 μ m (E), and 200 μ m (F and G).

these two genes have similar but distinctive functions during plant growth and development. To further investigate the function of *PGA37/MYB118* and *MYB115* in plant growth and development, we identified two T-DNA insertion lines SALK_111812 and SALK_044168 [33]. These two lines contained T-DNA insertions in exon 3 of *PGA37/MYB118* and intron 1 of *MYB115*, respectively (Figure 6I). Expression of *PGA37/MYB118* was not detected in siliques of *myb118* and *myb118 myb115* plants (Figure 6J), suggesting that the T-DNA insertion likely caused a null mutation. However, reduced expression of *MYB115* was detected in *myb115* and *myb118 myb115* siliques (Figure 6J), which presumably resulted from cryptic splicing of *myb115* pre-mRNAs. Homozygous *myb118* and *myb115* plants were phenotypically indistinguishable from WT plants. In addition, no abnormalities were found in the *myb118 myb115* double mutant at different developmental stages. When germinated and grown in the presence of auxin, cytokinin, abscisic acid or sucrose, the *myb118 myb115* double mutant showed a normal response to these compounds as WT.

Discussion

In this study, we show that overexpression of *PGA37/MYB118* and *MYB115*, two homologous *Arabidopsis* genes, efficiently induces the formation of somatic embryos from root explants, resulting in elevated expression levels of the embryo-specific gene *LEC1*. Moreover, overexpression of *PGA37/MYB118* and *MYB115* also causes the accumulation of lipids in vegetative tissues, the formation of green roots, and the disruption of post-germination morphogenesis. These observations suggest that *PGA37/MYB118* and *MYB115* are able to promote reprogramming of cell fate, acting as positive regulators of vegetative-to-embryonic transition.

The *pga37* mutant shows phenotypic similarities with *pga6* and transgenic plants overexpressing *BBM* [15, 21]. The most striking phenotype of these mutants and transgenic plants is their capacity to form somatic embryos from vegetative tissues. Several lines of evidence suggest that *PGA37* may act independently from *WUS/PGA6*. First, *WUS* expression is restricted to the organizing center during embryogenesis [24]. However, *PGA37* is expressed in a more expanded domain, including both the embryo and the endosperm, similar to the expression pattern of *BBM* [15]. Although a non-cell-autonomous regulatory mechanism could not be excluded, it is unlikely that *WUS* directly targets *PGA37*. Second, overexpression of *PGA37* is unable to rescue the shoot apical meristem defects in *wus-1*, thus disfavoring a model that *PGA37* acts downstream of *WUS*. Third, *WUS* represses

[21], whereas *PGA37* promotes, *LEC1* expression in root explants or embryogenic cells. Moreover, in *pga6* root explants, *LEC1* expression was found to be induced around 14 days after cultured on SCM [21]. By contrast, *LEC1* expression is detectable in pER10-*PGA37* seedlings treated with estradiol for 12 h. These observations lead to the speculation that *LEC1* expression observed in *pga6* may represent a characteristic of embryogenic calli or somatic embryos. A similar observation was recently made by finding that *LEC2*-induced expression of genes encoding the seed proteins oleosin and storage proteins is prior to that of *LEC1* and *FUS3* [4]. In contrast to that of *WUS/PGA6* and *LEC2*, *PGA37* may act upstream of *LEC1* to regulate vegetative-to-embryonic transition. Therefore, these different regulatory modes do not support the hypothesis that *WUS* and *PGA37* act in a linear pathway. Instead, different mechanisms may be deployed by *WUS/PGA6* and *PGA37* to regulate vegetative-to-embryonic transition.

Compared to the previously characterized genetic loci involved in the regulation of vegetative-to-embryonic transition such as *WUS/PGA6* and *BBM*, *PGA37* employs a unique regulatory mechanism. The *PGA37* activity is independent of *WUS* in the regulation of vegetative-to-embryonic transition. Moreover, *PGA37/MYB118* is capable of enhancing *LEC1* expression, but does not appear to be regulated by *PKL*. Whereas *PKL* has been shown to repress *LEC1* expression [10], the expression of *PKL* itself is, in turn, positively regulated by *LEC1*, suggesting the involvement of a feedback regulatory mechanism (J Mu and J Zuo, unpublished data). Similarly, expression of *LEC1*, but not of *PGA37/MYB118* and *MYB115*, is remarkably elevated in the *val1 val2* double mutant [12]. Notably, overexpression of *PGA37/MYB118* and *MYB115* induces the expression of *LEC1* but not *LEC2*, consistent with the observation made in previous studies that these two loci may act independently in the control of embryo development and seed maturation [34]. Collectively, these results suggest that *PGA37/MYB118* and *MYB115* may regulate *LEC1* expression in a *PKL*-independent manner. *LEC1* is a key regulator of embryogenesis and seed maturation [5-7, 35]. Overexpression of *LEC1* is sufficient to induce vegetative-to-embryonic transition [7], indicating a critical role of *LEC1* in the initiation and/or maintenance of embryonic cell identity. Therefore, the promotion of vegetative-to-embryonic transition by *PGA37/MYB118* and *MYB115* should be attributed, at least partly, to an elevated *LEC1* expression level.

PGA37/MYB118 is preferentially expressed in embryos and endosperm, implying a possible role of the MYB transcription factor in embryogenesis and seed development. This notion is supported by the phenotype

of the gain-of-function mutants and of transgenic plants overexpressing *PGA37*. However, neither *myb118* nor the *myb118 myb115* double mutant displays any developmental defects under our assay conditions. The lack of an apparent phenotype may be caused by functional redundancy of this small class of MYB transcription factors and, in particular, owing to the residual expression of *MYB115* in the double mutant. Alternatively, genetic redundancy may be present between the *MYB118/MYB115* family and other transcription factor genes. Despite the lack of an apparent phenotype in *myb118* and *myb118 myb115* mutants, overexpression of these two genes causes strong inhibitory effects on plant growth and development and promotes the formation of embryonic calli on seedlings, suggesting that these two functionally redundant genes likely play a role in embryo or seed development.

In summary, we have identified and characterized *PGA37/MYB118* and *MYB115* that are capable of inducing vegetative-to-embryonic transition and the formation of somatic embryos. Because *PGA37/MYB118* acts independently from *WUS*, the finding presented in this study thus reveals a new regulatory mechanism of somatic embryogenesis in *Arabidopsis*, which should have substantial impacts on both plant biotechnology and our understanding of this fundamental question in plant biology.

Materials and Methods

Plant material, growth conditions, and plant transformation

The *Arabidopsis thaliana* ecotypes Col-0, WS, and Landsberg *erecta* (*Ler*) were used in this study. Screening of *pga* mutants has been described in detail previously [21, 22]. The *myb118* (SALK_111812; Col-0), *myb115* (SALK_044168; Col-0), and *wus-1* (CS15; *Ler*) mutants were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, Columbus, OH). The *pkl-1* mutant (Col-0) [9] was kindly provided by J Ogas.

Plants were grown at 22 °C under a 16-h-light/8-h-dark cycle in soil or on agar plates containing 1/2 MS medium (Sigma Hong Kong and China), 2% sucrose, and 0.8% agar supplemented with or without the inducer 17- β -estradiol. Treatment of plants with 17- β -estradiol was carried out as described previously [20]. Transformation of root explants was carried out using *Agrobacterium tumefaciens* (the ABI strain) as described previously [21]. Unless indicated otherwise, root explants were cultured on a CIM (1 \times B5 salts, 2% glucose, 0.5 g/L MES, 0.5 mg/L 2,4-D, 0.05 mg/L kinetin and 0.25% phytigel, pH 5.7) for 3 days, and subsequently transferred onto a SCM (1 \times MS salts, 1% sucrose, 0.5 g/L MES, 0.15 mg/L IAA, 10 μ M 17- β -estradiol, and 0.25% phytigel, pH 5.7) for 3-5 weeks. *In planta* transformation was done by the flower-dip method [36] using the *Agrobacterium* GV3101 strain.

Staining of seedlings with Fat Red 7B was carried out according to [9]. Scanning electron microscopy was performed as de-

scribed previously [37].

Molecular manipulations

Molecular cloning was performed according to standard methods [38]. Genomic sequences flanking the *O^{LexA}-46* promoter in the *pga37* genome were identified by inverse PCR [29]. A *PGA37/MYB118* cDNA clone and a *MYB115* (AT5g40360) genomic clone were obtained by PCR using PWO DNA polymerase (Roche Diagnostics Hong Kong and China) and appropriate primer pairs (all primers used in this study are listed in Supplementary information, Table S1). The PCR fragments were cloned into the *AscI* (*PGA37/MYB118*) or *XhoI* (*MYB115*) and *SpeI* sites of the estradiol-inducible vector pER10 [20].

To generate the *PGA37::GUS* reporter construct, a 2.5-kb genomic fragment, which spanned nucleotides -2 535 to +36 with respect to the putative translation start codon, was amplified from Col-0 genomic DNA using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) and primers *PGA37PF* and *PGA37PB*. The PCR fragment, containing the coding sequence for the first 12 amino acid residues of *PGA37*, was in-frame fused to the *GUS* coding sequence in pBI101.1 using the *XbaI* and *BamHI* sites. All constructs were verified by extensive restriction digests and DNA sequencing.

Analysis of gene expression by RT-PCR was performed essentially as described previously [22].

Analysis of GUS activity

GUS staining was performed as described [39]. Histochemical staining of siliques was carried out as described [40] with minor modifications. Briefly, siliques were slit twice longitudinally and incubated in 90% acetone for 20 min at room temperature. After extensive wash, the sample was incubated in a GUS assay buffer (50 mM phosphate buffer [pH 7.2], 0.1% Triton X-100, 2 mM X-Gluc, 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide) for about 6 h at 37 °C. After staining, siliques were cleared in ethanol-acetic acid and then mounted in Hoyer's medium. Dissected ovules were observed and photographed under an Olympus BX51 microscope equipped with Nomarski optics and a cooled KX85 CCD camera.

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