Cell-by-Cell Developmental Transition from Embryo to Post-Germination Phase Revealed by Heterochronic Gene Expression and ER-Body Formation in Arabidopsis leafy cotyledon Mutants

Akiko Yamamoto1, Masakatsu Yoshii1, Shoko Murase1, Masahiro Fujita2, Nori Kurata2, Tokunori Hobo1, Yasuaki Kagaya3, Shin Takeda1 and Tsukaho Hattori1,*

1Bioscience and Biotechnology Center, Nagoya University, Nagoya, 464-8601 Japan
2Plant Genetics Laboratory, National Institute of Genetics, Mishima, 411-8540 Japan
3Life Science Research Center, Mie University, Tsu, 514-8507 Japan

*Corresponding author: E-mail, hattori@agr.nagoya-u.ac.jp; Fax, +81-52-789-3124.
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LEC1, LEC2, FUS3 and ABI3 (collectively abbreviated LEC/ABI3 here) are required for embryo maturation and have apparent roles in repressing post-germinative development. lec mutant embryos exhibit some heterochronic characteristics, as exemplified by the development of true leaf-like cotyledons during embryogenesis. Although the roles of LEC/ABI3 as positive regulators of embryo maturation have been extensively studied, their roles in the negative regulation of post-germinative development have not been explored in detail. Based on microarray analyses, we chose PYK10, which encodes an endoplasmic reticulum (ER)-body-localized protein, as a molecular marker of post-germinative development. lec/abi3 embryos exhibited PYK10 misexpression and the formation of ‘constitutive’ ER-bodies, which develop specifically during the seedling stage, confirming the heterochronic nature of these mutants at both the gene expression and cellular levels. The PYK10 reporter expression in lec1 embryos started as early as the globular–heart transition stage. The onset of PYK10 promoter–enhanced green fluorescent protein (EGFP) reporter expression occurred in a stochastic, cell-by-cell manner in both developing lec/abi3 embryos and germinating wild-type seedlings. Additionally, clustered EGFP-positive cells were frequently found along cell files, probably representing the transmission of the expression state via cell division. These observations, together with the results of the experiments using PYK10-EGFP/PYK10-CFP double reporter transgenic lines and the analyses of H3K27me3 levels in the PYK10 chromatin, suggested the involvement of epigenetic mechanisms in repressing post-germinative genes during embryogenesis and derepressing these genes upon the transition to post-germinative development.

Introduction

During seed maturation, the embryo grows to fill the embryo sac, accumulates nutrition reserves including oil and storage proteins, and acquires dormancy and desiccation tolerance (Santos-Mendoza et al. 2008). Although these maturation-associated events start at different but overlapping times during seed development, they are considered to be controlled by a highly integrated genetic program because mutants have been identified in which these maturation events are globally but specifically disturbed at the same time (Santos-Mendoza et al. 2008). These mutants are leafy cotyledon1 and 2 (lec1, lec2), fusca3 (fus3) and ABA-insensitive3 (abi3) (Koornneef et al. 1984, Giraudat et al. 1992, Meinke 1992, Keith et al. 1994, Meinke et al. 1994, West et al. 1994). While LEC1 encodes a paralog of the NF-Y transcription factor subunit B, LEC2, FUS3 and ABI3 encode homologous transcription factors with highly conserved B3 DNA-binding domains that recognize the RY motif found in many seed-specific genes (Giraudat et al. 1992, Lotan et al. 1998, Luerssen et al. 1998, Stone et al. 2001). Because mutations in these genes have global effects on embryo maturation, they are considered master regulators of seed maturation (Vicente-Carbajosa and Carbonero 2005). Although apparently redundant functions are suggested by the high conservation of the B3 domain as well as by the experimental evidence, functional differentiation of these B3 master regulators is also recognized based on their mutant phenotypes (Kroj et al. 2003, To et al. 2006). However, the functional differences between these proteins are not clearly understood due to the complex regulatory networks they form (To et al. 2006).
In addition to the loss of seed maturation traits, lec1, lec2 and fus3 exhibit the leafy cotyledon phenotypes suggested by the names of the former two mutants. The cotyledons of these mutants exhibit some of the characteristics of true leaves (Meinke 1992, Keith et al. 1994, Meinke et al. 1994, West et al. 1994); they bear trichomes, which normally develop on true leaves but not on cotyledons, and the venation patterns and internal anatomies of the mutant cotyledons resemble those of true leaves. Because of these phenotypes, we will hereafter use the abbreviation LEC (lec) to represent these genes (mutants), including FUS3 (fus3), herein. Furthermore, lec embryos precociously develop stomata and xylem elements, and their shoot and root apical meristems (SAMs and RAMs, respectively) are activated during embryogenesis (Meinke 1992, Keith et al. 1994, Meinke et al. 1994, West et al. 1994). These features are all normally acquired during post-germinative seedling development. Because lec embryos express the traits of the subsequent developmental phase, they are considered to be heterochronic mutants (Keith et al. 1994). abi3 is also considered to be a heterochronic mutant due to precocious SAM activation and xylem differentiation (Nambara et al. 1995).

From a conceptual standpoint, the gene expression programs that define certain developmental phases involve two processes: the activation of genes that are required at a specific developmental phase and the repression of genes that specify other developmental phases. Therefore, the LEC/ABI3 factors do not simply direct embryo maturation; rather, they are also required to define the correct developmental phase. Although the mechanisms by which LEC/ABI3 positively regulate embryo-specific or maturation-associated genes have been extensively studied (Braybrook and Harada 2008, Santos-Mendoza et al. 2008), our knowledge concerning how they function to repress post-germinative genes remains limited. Aside from the morphological and anatomical traits mentioned above, the heterochronic traits of lec/abi3 mutants have not been studied in detail. Only a limited number of genes are known that are heterochronically expressed in developing lec/abi3 embryos (West et al. 1994, Nambara et al. 2000, Curaba et al. 2004). The gibberellin biosynthetic gene GA3ox2 has been reported to be misactivated in developing lec and fus3 embryos, and the elevated gibberellin level is thought to induce the formation of trichomes on cotyledons (Curaba et al. 2004). In addition, Gazzarrini et al. (2004) suggested that leafy cotyledon traits are caused by elevated and decreased levels of gibberellin and ABA, respectively, in fus3 embryos. However, it has still not been established whether the misregulation of these plant hormones is the sole cause of all the heterochronic traits of lec/abi3 embryos.

The importance of gene regulation by epigenetic mechanisms during developmental phase transitions has gained increasing attention recently (Holec and Berger 2012). The regulation of the flowering repressor FLC by Polycomb group (PcG) complexes, which generate and maintain the trimethylation of histone H3-Lys-27 (H3K27me3, a repressive chromatin modification), is a well-known example of epigenetic regulation of a developmental transition (Kim et al. 2012). PcG-based repression of the embryo maturation regulators LEC/ABI3 upon the transition to the post-germinative phase has also been revealed, and the underlying mechanisms have been studied (Makarevich et al. 2006, Berger et al. 2011, Bouyer et al. 2011, Kim et al. 2012, Müller et al. 2012, Zhang et al. 2012, Yang et al. 2013). Mutant seedlings defective in PcG repressive complex 2 (PRC2) components misexpress the embryo maturation program (Bouyer et al. 2011). Furthermore, the downstream genes of these master regulators were also shown to be targets of PRC2 (Kodama et al. 2007, Bouyer et al. 2011). An increasing number of related studies have recently been performed on the epigenetic mechanisms involved in this phase transition. For example, chromatin remodeling factors, including PKL and PPR2, and the novel B3 domain factors HS2/VAL1 and HSL1/VAL2 have been found to be involved in the repression of embryonic maturation genes (Ogas et al. 1999, Suzuki et al. 2007, Tsukagoshi et al. 2007, Suzuki and McCarty 2008, Aichinger et al. 2009). The mechanistic relationships between these factors and PcG regulation have also been studied further (Yang et al. 2013, Zhou et al. 2013).

In contrast to the growing body of knowledge concerning the mechanisms underlying the activation of embryo maturation-associated genes during embryogenesis and the repression of these genes during the subsequent developmental phases, little is known about how the post-germinative gene program is repressed during the previous developmental phase and how it is subsequently derepressed. Thus, more in-depth studies of the heterochronic nature of lec/abi3 embryos will enhance our understanding of the transition from embryo maturation to the post-germinative phase. In the present study, we identified PYK10, which encodes a $\beta$-glucosidase contained in the seedling-specific, endoplasmic reticulum-derived ER-body, as a molecular marker of the post-germinative gene program and used fluorescent protein-based reporter genes to perform a detailed study of PYK10 expression during embryo development in lec/abi3 mutants and during germination of wild-type seeds. We found that PYK10 reporter expression in lec1 embryos started as early as the embryo transition stage, when cotyledons are just beginning to form. The timing of the onset of PYK10 expression in the lec/abi3 mutants coincided with the order with which the corresponding wild-type genes reached peak expression. Remarkably, the onset of PYK10 reporter expression occurred in a stochastic and cell-by-cell manner in the embryos of all the lec/abi3 mutants and in germinating wild-type seedlings. Based on further analyses of PYK10 expression and H3K27me3 modification in developing seeds and seedlings, we discuss the roles of LEC/ABI3 and the possible involvement of epigenetic mechanisms in repressing the post-germinative gene program during embryogenesis.

**Results**

**Genes related to ER-bodies are highly up-regulated in lec seeds**

To characterize the heterochronic nature of lec mutants at the gene expression level, we compared the transcriptomes of developing seeds from lec mutants and wild-type plants by microarray analysis. Among the information that could be mined...
Table 1 List of top 15 lec-up-regulated genes at 8 DAF

<table>
<thead>
<tr>
<th>Rank</th>
<th>AGI locus</th>
<th>Signal ratio (mutant/wild type)*</th>
<th>Description</th>
<th>Relationship with ER-body</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AT3G09260</td>
<td>697</td>
<td>PYK10</td>
<td>Localized</td>
<td>Nagano et al. (2008)</td>
</tr>
<tr>
<td>2</td>
<td>AT3G16430</td>
<td>442</td>
<td>PYK10-BINDING PROTEIN 1 (PBP1)</td>
<td>Functional</td>
<td>Nagano et al. (2008)</td>
</tr>
<tr>
<td>3</td>
<td>AT3G51810</td>
<td>382</td>
<td>LATE EMBRYOGENESIS ABUNDANT 1 (EM1)</td>
<td>Localized</td>
<td>Ogasawara et al. (2009)</td>
</tr>
<tr>
<td>4</td>
<td>AT5G23820</td>
<td>292</td>
<td>MID-2-related lipid recognition domain-containing protein</td>
<td>Functional</td>
<td>Ogasawara et al. (2009)</td>
</tr>
<tr>
<td>5</td>
<td>AT3G04290</td>
<td>277</td>
<td>LI-TOLERANT LIPASE 1 (LTL1)</td>
<td>Functional</td>
<td>Nagano et al. (2008)</td>
</tr>
<tr>
<td>6</td>
<td>AT3G16460</td>
<td>244</td>
<td>JACALIN-RELATED LECTIN 34 (JAL34)</td>
<td>Localized</td>
<td>Ogasawara et al. (2009)</td>
</tr>
<tr>
<td>7</td>
<td>AT1G52400</td>
<td>190</td>
<td>BETA GLUCOSIDASE 18 (BGLU18)</td>
<td>Localized</td>
<td>Nagano et al. (2008)</td>
</tr>
<tr>
<td>8</td>
<td>AT1G52400</td>
<td>186</td>
<td>MLP-LIKE PROTEIN 423 (MLP423)</td>
<td>Localized</td>
<td>Nagano et al. (2008)</td>
</tr>
<tr>
<td>9</td>
<td>AT1G09260</td>
<td>697</td>
<td>PYK10</td>
<td>Localized</td>
<td>Nagano et al. (2008)</td>
</tr>
<tr>
<td>10</td>
<td>AT3G52310</td>
<td>125</td>
<td>Gibberellin-regulated family protein</td>
<td>Functional</td>
<td>Yamada et al. (2008)</td>
</tr>
<tr>
<td>11</td>
<td>AT1G66280</td>
<td>128</td>
<td>BGLU22</td>
<td>Functional</td>
<td>Nagano et al. (2008)</td>
</tr>
<tr>
<td>12</td>
<td>AT1G52400</td>
<td>190</td>
<td>JACALIN-RELATED LECTIN 34 (JAL34)</td>
<td>Localized</td>
<td>Ogasawara et al. (2009)</td>
</tr>
<tr>
<td>13</td>
<td>AT1G09260</td>
<td>697</td>
<td>PYK10</td>
<td>Localized</td>
<td>Nagano et al. (2008)</td>
</tr>
<tr>
<td>14</td>
<td>AT1G52400</td>
<td>186</td>
<td>MLP-LIKE PROTEIN 423 (MLP423)</td>
<td>Localized</td>
<td>Nagano et al. (2008)</td>
</tr>
<tr>
<td>15</td>
<td>ATG02380</td>
<td>101</td>
<td>SENESCENCE-ASSOCIATED GENE 21 (SAG21)</td>
<td>Functional</td>
<td>Nagano et al. (2008)</td>
</tr>
</tbody>
</table>

* The average of the normalized signals in the mutant is divided by that in the respective wild type (Col-0 for fusi; WS for lec1 and lec2). Values that do not represent significant up-regulation (P < 0.05; with false discovery rate correction) are indicated in italics.

...from these data, we focused on the genes that showed extremely high degrees of up-regulation in the mutant seeds. The genes (probe sets) that showed the highest and second highest expression ratios in lec1 seeds relative to wild-type W5 at 8 days after flowering (DAF) were PYK10 (AT3G09260) and PBP1 (AT3G16420/AT3G16430) (Table 1). Quantitative reverse transcription–PCR (qRT–PCR) analysis revealed that lec1 embryos accumulated PYK10 and PBP1 transcripts more abundantly than wild-type embryos by approximately three orders of magnitude or more (Fig. 1A; Supplementary Fig. S1A; the transcript levels of these two genes in wild-type embryos were close to the detection limit under the conditions used in these experiments). Although the expression levels of these genes were lower in lec2 embryos than in lec1 embryos, they were still higher than those in the wild-type by at least two orders of magnitude.

PYK10 encodes a β-glucosidase (BGLU23) that is a major component of the constitutive ER-body (Matsushima et al. 2003a, Matsushima et al. 2003b, Ogasawara et al. 2009). The ‘constitutive’ ER-body is a specialized, spindle-shaped form of ER that develops specifically in the epidermal cells of roots, hypocotyls and cotyledons in young Arabidopsis plants but not in older plants. PBP1 encodes a jacalin-like lectin family protein that forms a complex with PYK10 upon cell disruption by wounding (Nagano et al. 2005). Interestingly, based on the microarray data, the top 12 highly up-regulated genes (probe sets) in the 8 DAF lec1 seeds included another five genes encoding ER-body-localized proteins: two additional β-glucosidase genes; another jacalin-like lectin gene; and NAI2, which regulates ER-body shape and PYK10 accumulation (Nagano et al. 2008, Yamada et al. 2008, Ogasawara et al. 2009, Hakenjos et al. 2013) (Table 1).

Although detailed description and interpretation of the microarray data is beyond the scope of the present study, it is worth mentioning that the third highest up-regulated gene at 8 DAF is the Em1 gene, which is a typical late embryogenesis gene but not a post-germinative gene. The Em1 expression levels (microarray signals in lec1 and lec2 at 12 DAF were similar to that in the wild type. Therefore, the onset of the Em1 expression shifted earlier. This phenomenon corresponds to the observations reported earlier (Vicient et al. 2000) and it will be intriguing to pursue in the future.

Stochastic cell-by-cell onset of PYK10 expression in lec1 embryos

To characterize the heterochronic expression of PYK10 in lec1 mutants, the expression patterns of EGFP driven by the PYK10 promoter (PYK10-EGFP) were investigated during embryonic development. In the wild-type, EGFP signals above the background autofluorescence were not detected by laser scanning confocal microscopy (LSCM) in embryos at any stage during seed development (Fig. 1B, C) but were detected during and after seed germination (described later in Figs. 4, 5, 7). In lec1 embryos, however, PYK10-EGFP expression, which was restricted to epidermal cells, was observed with various timing and patterns during embryogenesis. The earliest onset of PYK10-EGFP expression occurred at the transition stage, when the future cotyledon regions were first apparent (Fig. 1D). This phenotype preceded all previously known lec1 phenotypes except for the suspensor cell division abnormality. Whereas not all lec1 embryos expressed EGFP at the transition stage, EGFP expression became detectable in every embryo at the heart stage (Fig. 1E).
Fig. 1 Heterochronic expression of PYK10 and PYK10-EGFP in developing lec1 and lec2 embryos. (A) Real-time RT–PCR quantification of PYK10 transcript levels in wild-type, lec1 and lec2 embryos dissected from 8 DAF Arabidopsis seeds. Error bars represent the SD of three biological replicates. (B–K) Representative EGFP fluorescence images of wild-type (Col-0; C), lec1 (D–G) and lec2 (H–K) embryos expressing PYK10-EGFP or
varied from only a few to >15 on the observable surface of embryos at apparently similar stages (Fig. 1E). There was a clear contrast between the EGFP-expressing cells and the neighboring non-expressing cells, indicating the bistable nature of PYK10 expression. While some single EGFP-expressing cells were isolated among equivalent non-expressing cells, some clusters of expressing cells formed a line along the cell file, possibly representing clonal sectors (Fig. 1D–F). The positions of the EGFP-expressing cells were variable, although they occurred more frequently in the region corresponding to the future hypocotyl than in the regions corresponding to the future cotyledons and root (Fig. 1D–F). Such variability indicated that the onset of PYK10 misexpression occurred stochastically and in a cell-by-cell manner. The features of PYK10-EGFP expression in lec1 embryos were consistently observed among several independent transgenic reporter lines. As the mutant embryos developed, the number of EGFP-expressing cells increased (Fig. 1F, G). At the late torpedo stage, most of the cells in the hypocotyl–root region were EGFP positive, although a few EGFP-negative cells or cell files still remained (Fig. 1G). However, in the cotyledons, EGFP-expressing cells remained dispersed (Fig. 1G). Throughout embryogenesis in the mutant, GFP expression was confined to the protodermal/epidermal cell layer.

PBP1 promoter–EGFP also exhibited expression patterns highly similar to those of PYK10-EGFP (Supplementary Fig. S1), suggesting that cell-by-cell expression was not specific to the PYK10 promoter but was in fact characteristic of a subset of genes that are tightly repressed during embryogenesis but highly activated during the post-germinative stages.

Different timing of the cell-by-cell onset of PYK10 expression among the lec/abi3 embryos

Stochastic PYK10-EGFP expression was also observed in other lec/abi3 embryos that have defects in the B3 transcription factors. The earliest stage at which EGFP expression was detected in lec2 embryos was the heart stage (Fig. 1H). Even at this stage, however, there were cases in which >10 EGFP-expressing cells were observed, including three consecutive cells in a cell file (Fig. 1H). Therefore, the actual onset could have occurred earlier. Nevertheless, the timing of EGFP expression was more variable in lec2 than in lec1 and the other lec/abi3 mutants (Fig. 1I–K). For example, a bent cotyledon embryo with only a few EGFP-expressing cells was found alongside a late heart embryo with substantial portions of EGFP-expressing cells (Fig. 1K, right; Fig. 1I, left). This variability may be related to the reported variability of the lec2 phenotype (To et al. 2006) and to the weaker effect of this mutation on the transcriptome.

Notably, an embryo was observed with five consecutive cells forming a single epidermal cell file out of the 10 EGFP-expressing cells found on the observation surface (Fig. 1, right).

In the fus3 background, the earliest onset of PYK10-EGFP expression was observed at the heart stage (Fig. 2A). At this stage, only one or two EGFP-positive cells, if any, were observed. As the embryo developed, the number of EGFP-expressing cells increased and varied, although not as much as in lec2 (Fig. 2B, C). Although some embryos showed dispersed EGFP expression in a single isolated cell or in two neighboring cells along the cell file at most (Fig. 2B, top), other embryos expressed EGFP in more consecutive cells in the hypocotyl epidermal cell files in torpedo embryos (Fig. 2B, bottom).

PYK10-EGFP expression was also observed in abi3 embryos; however, the onset of expression occurred at the green mature stage (Fig. 2D). The number of EGFP-positive cells increased as the embryo matured further (Fig. 2E, F). EGFP-expressing cells were more often found in N-cell files, which develop into non-hair cells in the root and non-stomatal cells in the hypocotyl. A similar tendency was also observed for PYK10-EGFP expression at earlier stages in other lec mutants.

Although the standard morphological criteria for embryo development do not strictly apply to lec mutants due to the tendency toward slow growth in lec2 and the distinct overall shapes of lec mutants, the onset of PYK10-EGFP expression in lec1 clearly preceded that in fus3, which was still earlier than in abi3. Notably, this apparent order of expression coincides with the peak expression of LEC1, FUS3 and ABI3 (Supplementary Fig. S2). Because the effects of lec2 on PYK10-EGFP expression are more variable than those of the other lec/abi3 mutants, it was actually difficult to determine the relative timing of PYK10-EGFP expression in lec2. However, the extremes of PYK10-EGFP onset in lec2 embryos appeared to precede those in fus3 and were closer to those in lec1, given that a heart stage lec2 embryo was found in which >10 cells expressed EGFP, whereas the maximal EGFP-expressing cell number was two in the observed fus3 heart embryos. Thus, the coincidence of the gene expression order may also extend to lec2.

The lec mutants develop ER-bodies during embryogenesis

An examination of PYK10-EGFP-expressing cells in lec1 embryos at higher magnifications revealed the presence of fluorescent, spindle-shaped structures from the heart stage onward (Fig. 3D). We doubted that the formation of ER-bodies in the epidermal cells of lec embryos developed because the EGFP
reporter contained the PYK10-derived ER-retention signal. To confirm this, the expression of 35S-GFP, which was previously used to label the ER-body (Hayashi et al. 2001), was examined in both wild-type and lec1 embryos (Fig. 3A–C). As expected, EGFP signals labeled ER-bodies in epidermal cells of young wild-type seedlings carrying 35S-GFP (Fig. 3A). In wild-type embryos, dot-like GFP signals were observed up to the torpedo or bent-cotyledon stage (6–7 DAF), and network-like structures were observed thereafter (Fig. 3B). In contrast, spindle-like GFP signals became detectable in lec1 epidermal cells at or beyond the torpedo stage (Fig. 3C). These results confirmed that lec embryos develop ER-bodies that are not present in wild-type embryos. Notably, cell to cell or cluster to cluster differences were observed in the stages of ER-body development in lec1 embryos (Fig. 3C, E). This result again confirmed the cell-by-cell switching of the developmental program.

**Possible involvement of epigenetic mechanisms in the regulation of PYK10**

Stochastic gene expression is often associated with epigenetic regulation (Hauser et al. 2011), and bistable gene expression is typically observed for epigenetically silenced loci (Takeda et al. 2004). The stochastic onset or termination of gene expression is also known to occur for epigenetically regulated loci during normal development. The transcriptional state of the flowering repressor FLOWERING LOCUS C (FLC) is switched by a Polycomb-based mechanism, and the

**PYK10 expression during wild-type seed germination**

To determine whether the cell-by-cell onset of PYK10-EGFP expression observed in the developing lec1 embryos is a feature of expression state conversion in the wild-type background, we examined the expression of PYK10-EGFP during wild-type seed germination (Fig. 4). PYK10-EGFP expression occurred in a cell-by-cell manner in the hypocotyl epidermis at approximately 10 h after the shift to the germination temperature (Fig. 4C). At approximately 14 h, the number of EGFP-expressing cells increased in the hypocotyl (Fig. 4D), and EGFP expression was observed in almost all cells in the N-cell files of the hypocotyl at 24 h (Fig. 4E). The cell-by-cell PYK10 expression was also observed in the cotyledons of germinating seedlings, although its onset was delayed (Supplementary Fig. S3). These results indicated that the cell-by-cell onset of PYK10 expression is not confined to its heterochronic expression pattern in the lec mutant but is actually intrinsic to normal development. Furthermore, in the 35-GFP-expressing seedlings at 18 h, cell-by-cell emergence of ER-body-containing cells was also observed (Fig. 4F). This result again confirmed the cell-by-cell switching of the developmental program.

**Fig. 2** Heterochronic expression of PYK10-EGFP in fus3 and abi3 developing embryos. Representative EGFP fluorescence images of fus3 (a–c) and abi3 (d–g) embryos carrying PYK10-EGFP. Images are presented as in Fig. 1. (A) Two examples of fus3 heart embryos (5 DAF; one early heart, one late heart). (B) Two examples of fus3 torpedo embryos (6 DAF). (C) An example of a fus3 embryo corresponding to the wild-type curled cotyledon stage (7 DAF). (D) Two examples of abi3 green mature embryos (12 DAF). (E) Two examples of abi3 mature embryos (14 DAF). (F) An example of an abi3 late mature embryo (16 DAF). Scale bar = 20 μm (A–C) or 50 μm (D–F).
cell-by-cell, bistable shift from repression during vernalization to reactivation at warmer temperatures was observed using an FLC-GUS (β-glucuronidase) reporter. Furthermore, probable clonal clusters of reactivated cells have also been described using this reporter (Angel et al. 2011). These features are shared by PYK10-EGFP (and PBP1-EGFP) expression in developing lec/abi3 embryos and in wild-type embryos upon germination.

Fig. 3 Formation of ER-bodies in lec1 embryos. (A) ER-bodies visualized by ER-localized GFP derived from 35S-GFP (left) or PYK10-EGFP in an epidermal cell from the hypocotyl of a wild-type seedling 2 d after germination. (B) GFP-labeled ER-related organelles in 35S-GFP-carrying wild-type developing embryos at the indicated stages. (C) GFP-labeled ER-bodies in 35S-GFP-carrying lec1 embryos at the indicated stages. (D) EGFP-labeled ER-related organelles in PYK10-EGFP-carrying lec1 embryos at the indicated stages. In the 4 DAF early heart embryos, cells that contained punctate ER-related organelles and cells that contained ER-body-like structures were observed. (E) A PYK10-EGFP-carrying lec1 heart embryo with different degrees of ER-body development; a fluorescent image is overlaid on the DIC image of the entire embryo, and magnified fluorescent images are shown that correspond to the regions indicated by rectangles. All the panels are single-cell-thick Z-stack images of the epidermal cells of the (future) hypocotyl. Typical consecutive EGFP-expressing cells in a cell file are marked with arrowheads in (h) and (i). Scale bar = 5 μm.
If the stochastic change in the state of PYK10 or PYK10-EGFP expression is in fact limited by the epigenetic state in cis, each of the multiple copies of the transgenic PYK10 reporter gene present in the same genome should behave independently. To test this, we introduced two fluorescent protein reporters [EGFP and cyan fluorescent protein (CFP)] under the control of the same PYK10 promoter sequence at different positions in the genome. If the epigenetic states of the PYK10 reporters are solely responsible for the cell-by-cell activation of PYK10 expression in lec embryos, EGFP and CFP expression should occur independently from cell to cell. However, if PYK10 expression in lec embryos depends on the cell-by-cell activation/derepression of a regulatory factor acting in trans on the PYK10 promoter, then the two fluorescent reporters would be synchronously activated in the same cell. The results showed that cells frequently expressed either EGFP or CFP alone, although a substantial number of epidermal cells simultaneously expressed EGFP and CFP in developing lec, fus3 or abi3 embryos (Fig. 5A–C) and germinating wild-type embryos (Fig. 5D). Two different combinations of PYK10-EGFP and PYK10-CFP loci derived from independent transgenic lines, as well as a combination of PBPI-EGFP and PBPI-CFP in lec, produced essentially the same results (Fig. 5E). Furthermore, the combination of PYK10-EGFP and PBPI-CFP also yielded similar results (Supplementary Fig. S4). Such independent behavior of the two reporters indicates that the cis state of each PYK10-EGFP/CFP locus in a cell governs the reporter expression state. The substantial overlap in the expression of the two reporters can be explained by assuming the presence of two cell states; one in which the state of each locus is more easily converted and one in which it is less easily converted. The cell states must be determined by some trans-acting regulators.

Changes in the H3K27Me3 levels in the PYK10 chromatin upon the developmental shift from the embryonic to the post-germiantive phase

One possible mechanism for epigenetic conversion of the transcriptional state upon developmental phase transition is histone modification, as in the case of the FLC locus (Angel et al. 2011). To test this possibility, the levels of histone H3 lysine-27 trimethylation (H3K27Me3), a Polycomb-associated repressive histone modification, in the PYK10 chromatin of developing seeds and germinated seedlings were compared (Fig. 6).
Although there was quite a large variation among the triplicate seed chromatin samples, significant differences were detected in the H3K27Me3 levels of the PYK10 chromatin. In developing seeds at 12 DAF, H3K27Me3 chromatin was detected on the PYK10 promoter at a level corresponding to 45–90% of the level detected on AGAMOUS (AG) chromatin, which has been reported as a Polycomb target (Turck et al. 2007), whereas the H3K27Me3 level on the chromatin of the constitutive ACT7 gene was much lower. The chromatin at the 5'-untranslated region and coding region of PYK10 also contained similar levels of H3K27Me3. Conversely, the levels of H3K27Me3 on the PYK10 chromatin, especially at the promoter, were significantly lower in the seedlings. Thus, the change in the H3K27Me3 status of PYK10 chromatin appeared to be associated with the change in the transcriptional state during the shift from the embryonic to the post-germinative phase. Notably, the H3K27Me3 states on the chromatin of a seed-specific gene (CRU3, a seed storage protein gene) showed an inverse pattern (i.e. high in seedlings and low in developing seeds). The results indicate that the state of PYK10 chromatin is actually converted upon the developmental transition from the embryonic to the post-germinative phase.

Gibberellin enhances the onset of PYK10 expression during normal development but is not required for heterochronic expression in lec mutants

It was reported that the misactivation of gibberellin signaling could underlie at least some of the heterochronic phenotypes of lec mutants. To determine the potential requirement for
gibberellin in the heterochronic expression of PYK10 in lec embryos, PYK10-EGFP expression was examined in seeds from the siliques of lec1 homozygous plants segregating for the gibberellin-deficient allele ga1-3. Notably, the EGFP expression patterns in lec1 ga1 double homozygous embryos were similar to those of lec1 single-mutant embryos (Fig. 7A, C). Similarly, fus3 ga1 mutant embryos and fus3 single mutant embryos exhibited a similar PYK10-EGFP expression pattern (Fig. 7B, D).
These results clearly indicate that gibberellin is not required for the heterochronic expression of PYK10 in lec embryos. Closer examination of the cotyledon epidermal cells from fus3 ga1 embryos revealed similar stomatal development to that in the fus3 single mutant (Fig. 7G). We also observed trichome development in lec1 ga1 embryos (Fig. 7C), and we observed the outgrowth of epidermal cells, which appeared to be under-developed trichomes, from the surface of fus3 ga1 embryos (Fig. 7H). Furthermore, the precocious activation of the SAM seen in lec mutant embryos was also observed in the lec1 ga1 and fus3 ga1 backgrounds (Fig. 7E, F). Therefore, the role of gibberellin in the expression of heterochronous phenotypes in lec mutants was considered to be limited.

Because publicly available microarray data suggested that gibberellin may be involved in the induction of PYK10 during germination (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), we examined PYK10–EGFP expression during wild-type germination in the presence or absence of paclobutrazol (PAC), a gibberellin biosynthesis inhibitor (Fig. 7I). PAC treatment considerably reduced the number of EGFP-expressing epidermal cells at an earlier time point (approximately 12 h). However, most of the epidermal cells switched to EGFP expression at later stages (18–24 h), and the EGFP signals did not appear to decrease substantially. Thus, PAC appeared to decrease the frequency or delay the cell-by-cell onset of EGFP expression. These results suggest that de novo synthesis of gibberellin is required to enhance the switching of PYK10 chromatin from the embryonic (repressed) to the post-germinative (derepressed) state rather than to increase the transcription rate.

Discussion

PYK10 expression and ER-body formation as markers for post-germinative development

We demonstrated that epidermal cells of lec and abi3 embryos developed ER-bodies, which normally form during germination in the epidermal cells of young seedlings. The formation of ER-bodies in the developing embryos of these mutants was associated with the expression of genes encoding major constituents of these organelles, including PYK10 and NA12, and genes for proteins such as PBP1, which localizes outside the ER-body but forms complexes with PYK10 or other ER-body-localized β-glucosidases upon cell disruption. ER-body formation and PYK10 expression were extensive in the mutants in the epidermal cells of the cotyledon, hypocotyl and root of the embryos as well as in the wild-type seedlings, although the timing differed between the different organs. These phenotypes, observed at the cellular and transcriptional levels, provide further evidence that these mutants are heterochronic. These results indicated that the developmental state of the mutant embryo epidermis was largely, if not entirely, converted to that of post-germinative seedlings. Because ER-body formation and PYK10 expression presented simple and robust phenotypes, they are considered to be suitable markers for the post-germinative developmental phase.

Early action and active roles of LEC1 in repressing the post-germinative program during embryogenesis

West et al. (1994) reported that the promoter activities of two representative post-germinative genes, isocitrate lyase and a lipid transfer protein, could first be detected at the linear cotyledon stage in lec1. Similarly, the earliest lec1 embryo abnormality reported by Meinke et al. (1994) was the vacuolation of hypocotyl cells at the torpedo stage, which also coincides with the timing of trichome initiation. In contrast, PYK10-EGFP expression in lec1 embryos could be observed as early as the transition stage. Thus, the timing of phenotypic expression in the embryo proper was far earlier than that observed in the above reports. The timing also preceded the expression of any of the seed maturation traits that are deficient in lec1 embryos. These findings suggest that the heterochronic expression of PYK10 is neither a reflection of the lack of the maturation program nor the mere result of the precocious progression of germinative processes; rather, these findings suggest more active roles for LEC1 in maintaining the repressed state of PYK10 and other post-germinative genes during embryogenesis.

Sequential action of LEC1 and B3 factors in the repression of post-germinative genes during embryogenesis

Previous studies indicated that LEC1 and the three B3 transcription factors LEC2, FUS3 and ABI3 regulate seed development via a complex regulatory network, which involves local cross- and self-regulation among the three B3 factors as well as hierarchical and combinatorial regulation between the B3 factors and LEC1 (Kagaya et al. 2005a, Kagaya et al. 2005b, To et al. 2006, Yamamoto et al. 2009). Due to the complexity of this network and the partially redundant functions of these transcription factors, which have the same target sequences, it is difficult to define their specific and overlapping roles clearly. However, the timing of the peak expression levels of the B3 and LEC1 genes are different, with the order being lec1/LEC1, FUS3 and ABI3, although the expression of all of these genes begins at very early stages (Supplementary Fig. S2). We showed that the timing of PYK10 expression onset in the lec1 and B3 factor mutant embryos roughly matched the order of peak expression of the respective transcription factors. This coincidence suggests that PYK10 repression relies on the redundant molecular functions of the B3 factors throughout embryonic development, further implying that the total concentration of the B3 factors may be important and that the B3 factors have a relatively direct role in maintaining the repressed state. However, the earlier action of LEC1 remains to be explained, as it is a different type of transcription factor. Because the transcript levels of FUS3 and ABI3 are reduced in lec1 embryos, the total concentration of B3 factors may not be sufficient at early stages. Another possibility is that LEC1 functions similarly to the B3 factors, although it does not contain a B3 domain. Alternatively, both LEC1 and the B3 factors, or their downstream regulators, may be simultaneously necessary to secure the repressed states of PYK10 and other post-germinative genes.
The sequential nature for LEC/ABI3 factors over the course of embryogenesis has also been demonstrated for their growth arrest function, which was measured by the ability of isolated embryos to undergo precocious growth (Raz et al. 2001). However, the temporal order of phenotypic expression of the respective mutants does not coincide with their gene expression sequence. Therefore, the growth arrest function of LEC/ABI3 may not be directly linked to their function as repressors of the post-germinative program.

Possible involvement of epigenetic mechanisms in defining the developmental states of the post-germinative genes

In this study, several lines of evidence indicated that epigenetic mechanisms are involved in the repression of post-germinative genes during embryogenesis and their subsequent derepression upon the transition to post-germinative development. The onset of PYK10-EGFP expression was stochastic during seed germination, and the transcriptional states often differed from cell to cell between PYK10-EGFP and PYK10-CFP present in the same genome at different loci. Notably, PYK10-EGFP expression in lec/abi3 embryos was often observed in cell clusters lined along a cell file, even when the total number of EGFP-positive cells was small. Because the expressed EGFP is sequestered in the ER or related organelles and thereby is cell autonomous, such clustered EGFP-positive cells most probably represent clonal sectors, which result from the inheritance of the active state of the reporter through cell division. These results suggest that the repressed state of PYK10 is maintained by epigenetic mechanisms during embryogenesis and is released upon the developmental transition from the embryonic to the post-germinative state. This was further supported by the observation that the H3K27me3 levels in PYK10 chromatin were significantly higher in developing embryos compared with those in young seedlings. We speculate that the heterochronic expression of PYK10, and probably that of other post-germinative genes, in lec/abi3 embryos occurs due to defects in the proper maintenance of this epigenetically repressed state. The heterochronic expression patterns of PYK10-EGFP (and other reporters) and the formation of ER-bodies in the lec/abi3 embryos described in this study were all in agreement with this hypothesis. Although the onset of PYK10-EGFP expression was stochastic, it appeared somewhat more synchronized in germinating seedlings compared with lec/abi3 developing embryos. This difference may be due to the fact that derepression during germination is an active event that is triggered by germination cues, whereas heterochronic expression in the mutant embryos occurs due to defects in the securing mechanism.

The double fluorescent reporter experiments suggested that trans-acting regulators are also involved in switching the PYK10 transcriptional state. The putative regulators could condition the cell states in a way that facilitates epigenetic changes in the target loci or that secures the repressed states, and are predicted to undergo cell-by-cell up-regulation or down-regulation in mutant embryos or germinating seedlings. Such regulators may be related to more synchronized derepression of the post-germinative genes after germination.

In precociously germinating Brassica napus seeds, extra cotyledons or chimeric organs with sectors of cotyledon and true leaf tissue are formed from the SAMs (Fernandez 1997). In situ hybridization analysis of such a chimeric organ using molecular markers of embryonic states revealed clear sectors of cells expressing these markers and those not expressing with clear boundaries. Such bistable or ‘all-or-nothing’ (Fernandez 1997) patterns of the embryonic identity in the precociously germinating B. napus seeds also appear to be a reflection of the cell-by-cell switching of developmental state as we observed in this study, and support the involvement of epigenetic mechanisms.

Role of gibberellin in switching of post-germinative genes

It has been reported that trichome development on the cotyledons of lec embryos depends on elevated gibberellin levels in these mutants (Curaba et al. 2004). The importance of the gibberellin–ABA ratio in the cotyledon–true leaf identity has also been reported (Gazzarini et al. 2004). However, PYK10-EGFP expression was observed in both lec1 ga1 and fus3 ga1 embryos. Thus, elevated gibberellin levels in these mutants were unlikely to be the cause of their heterochronic gene expression. Additionally, underdeveloped trichomes were found on the cotyledons of fus3 and lec1 embryos in the ga1 background. Nevertheless, experiments with the gibberellin biosynthesis inhibitor suggested that gibberellin increases the frequency of transcriptional state switching at PYK10 chromatin rather than simply increasing the rate of transcription during the germinative process. Gibberellin synthesized during imbibition may function to promote better synchronization of the cell-by-cell release of repression. The chromatin remodeling factor PKL is required to switch to or maintain the repressed state of the chromatin of seed-specific B3 factors, including FUS3 and LEC2 (Ogas et al. 1999, Zhang et al. 2008, Zhang and Ogas 2009, Zhang et al. 2012). Intriguingly, gibberellin is required to ensure this function of PKL (Ogas et al. 1999). Thus, one important role for gibberellin during developmental regulation may be to stimulate the alteration of chromatin states.

Materials and Methods

Genetic material and plant growth conditions

The alleles of the lec/abi3 mutants used in this study were lec1-1, lec2-1, fus3-3 and abi3-6. Additionally, a SALK T-DNA insertion allele of lec1 (Salk_000450) was used. For the microarray analysis and RNA expression studies, lec1-1 and lec2-1 in the WS background were used, as described in the original report (Meinke et al. 1994). In other experiments, lec1-1 and lec2-1 in the Col-0 background, which were produced by backcrossing five times to Col-0, were used. The plant growth conditions were described previously (Yamamoto et al. 2010). Lines homozygous for the respective mutant alleles were maintained by rescuing the seeds before desiccation. ga1-3 was described by Sun et al. (1992). To grow the plants that were homozygous for ga1-3, 2 μM GA4 solution was sprayed twice a week until flowering. PCR genotyping of GA4 was performed using the primers listed in Supplementary Table S1.
RNA isolation and qRT–PCR

The methods for total RNA isolation from developing seeds, embryos and seedlings and for qRT–PCR were described previously (Yamamoto et al. 2009). The primers used for qRT–PCR are listed in Supplementary Table S1.

ChIP and microarray analyses.

The procedures for chromatin isolation, chromatin immunoprecipitation (ChIP) analysis and microarray experiments are described in the Supplementary Information.

Production of transgenic plants

Agrobacterium-mediated Arabidopsis transformation was performed using the floral dip method (Clough and Bent 1998). Wild-type Arabidopsis (Col-0) was first transformed with pGWB501-PYK10-EGFP or pGWB501-PBP1spcEGFP. pGWB401 and pGWB-NB1 were used as backbone T-DNA vectors carrying kanamycin and BASTA® (Bayer Crop Science) resistance plant selection markers, respectively (Nakagawa et al. 2007, Nakamura et al. 2009). Construction of the plasmids used for transformation is described in the Supplementary Methods. The transgene carried by Col-0 was then crossed into the *lec*/abi3 mutants (all in the Col-0 background). The *lec1-sulk_000450* line was also directly transformed with pGWB501-PYK10-EGFP.

LSCM

Embryos were dissected from developing or germinating seeds and mounted between a slide and cover glass using Murashige and Skoog (MS) agar media. The embryos were then observed via LSCM (FV1000, OLYMPUS). EGFP and CFP fluorescence signals between 500 and 570 nm and between 460 and 480 nm, respectively, were collected for imaging. For most observations, differential interference contrast (DIC) images were acquired in parallel.

Accession number

The complete set of microarray data reported here was deposited in the Gene Expression Omnibus (GEO) repository at the NCBI under the accession number GSE61684.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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