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The Arabidopsis transcription factor NAI1 is required for enhancing the active histone mark but not for removing the repressive mark on *PYK10*, a seedling–specific gene upon embryonic-to-postgerminative developmental phase transition

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Abbreviations: DAF, days after flowering; DAG, days after germination; H3K27me3, histone H3 lysine 27 trimethylation; H3K4me3, histone H3 lysine 4 trimethylation; LAFL, transcription factors LEC1, ABI3, FUS3 and LEC2.

We have recently shown that the expression onset of a seedling-specific gene, *PYK10*, occurs in a cell-by-cell manner upon the transition from the embryonic to the postgerminative phase and during embryogenesis in seed maturation regulator mutants such as *lec1*, and implicated epigenetic mechanisms in the process. Here, the role of the NAI1 transcription factor required for *PYK10* expression in the developmental switching of *PYK10* was investigated. The cell-by-cell onset of *PYK10-EGFP* in *lec1* embryo was still observed in the *nai1* background, but at greatly reduced levels. Decreases in the level of the repressive histone mark, H3K27 trimethylation observed upon the transition to the postgeminative phase normally occurred in *nai1*. However, concomitant increases in the level of the active mark, H3K4 trimethylation observed in wild type was significantly compromised in *nai1*. These results indicate that the switching of *PYK10* upon developmental phase transition involves 2 separable steps of chromatin state change.

Upon the developmental transition from the embryonic to the postgermitive seedling phase, gene expression programs are dramatically reorganized. Postgerminative gene expression programs that only operate after seed imbibition/germination are tightly repressed during embryo formation and maturation, and programs specific for embryonic development become tightly repressed after the transition to the postgerminative phase. The mechanisms for the activation and repression of embryonic phase-specific genes, particularly those involving the LAFL (transcription factors LEC1, LEC2, FUS3 and ABI3) seed maturation master regulators have been extensively studied.[1-11] However, our knowledge remains limited concerning how postgerminative genes are repressed during the embryonic phase and derepressed upon the phase transition. Our recent study[12] together with previous studies by others[13-17] revealed that LAFL factors also play active roles in the maintenance of repressed states of the postgerminative genes. From comparative transcriptome analyses between the wild type and *lafl* mutant embryos, we identified a set of seedling-specific genes related to ER-body function that are tightly repressed in the wild type, but expressed at high levels in these mutants during embryogenesis.[12] Among these genes, *PYK10* encoding ER-body-localized β-glucosidase showed a remarkably high level of upregulation in the mutants relative to the wild type and was therefore chosen as a marker for the postgerminative gene expression program that is tightly repressed during embryogenesis. Analyses using promoter-EGFP/CFP reporter genes revealed that the expression of *PYK10* and other ER-body-related genes begins in a stochastic and cell-by-cell manner during germination in the wild type and during embryogenesis in *lafl* mutants.[12] Clusters of cells expressing the reporter were also observed along cell files. In agreement with these observations, the degree of ER-body development differed from cell to cell during embryo development in the mutants and during seed germination in the wild type, indicating that the developmental transition take places on a cell-by-cell basis. Furthermore, the level of a repressive epigenetic mark, histone H3-Lys27 trimethylation (H3K27me3), at the *PYK10* locus was high during embryogenesis, but was significantly reduced in early seedlings.[12]

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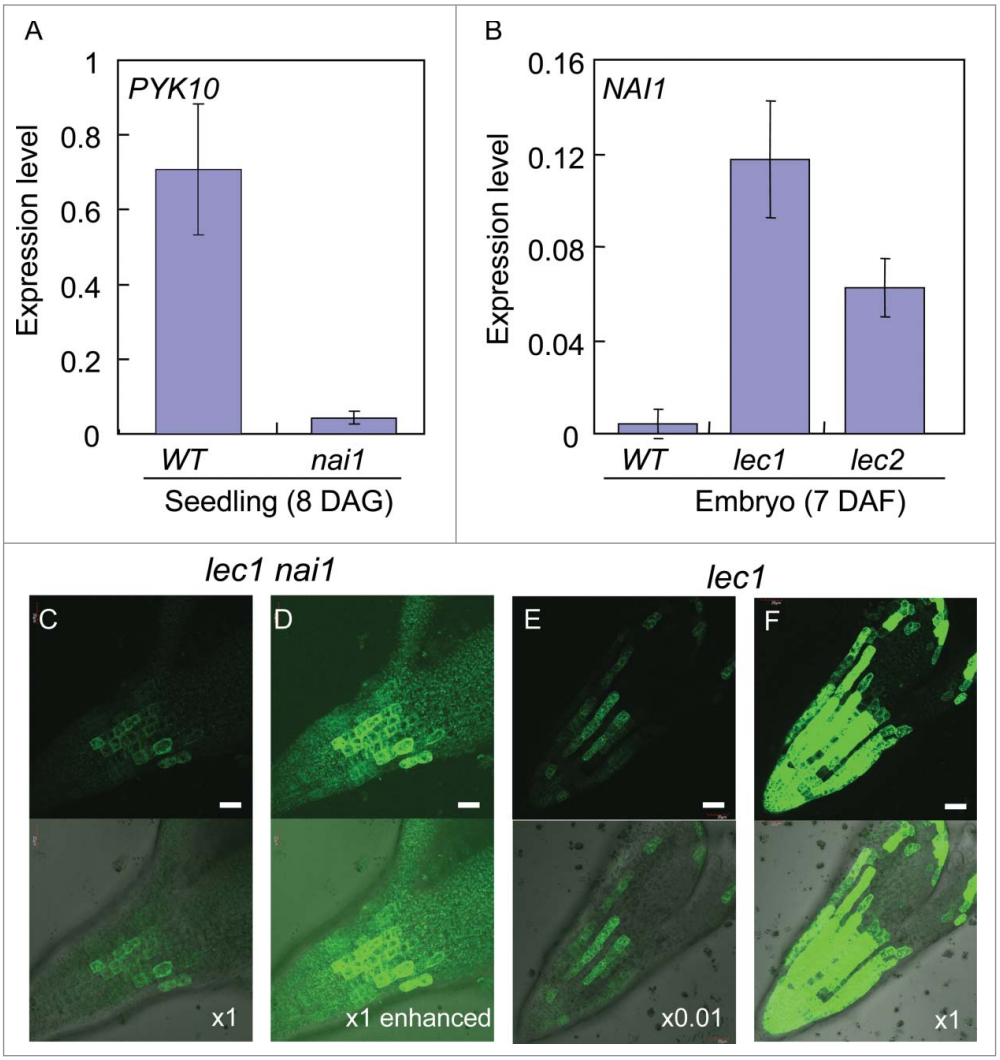

Figure 1. Effects of the *nai1* mutation on *PYK10* expression and of *lec1/lec2* on *NAI1* expression. (**A**, **B**) Real-time RT-PCR quantification of *PYK10* and *NAI1* transcript levels in wild-type (ecotype Col-0; WT) and *nai1* seedlings at 8 days after germination (8 DAG; A), and in wild-type (ecotype WS; WT) and *lec1/lec2* embryos at 7 days after flowering (7 DAF; B), respectively. Error bars represent the SD of biological replicates (n = 3). (**C**–**F**) Expression of *PYK10-EFGP* in *lec1 nai1* double (**C**, **D**) or *lec1* single (**E**, **F**) mutant embryos at 7 DAF. The top and bottom of each paired panel show a Z-stack image consisting of several EGFP optical sections from the surface to the approximate median of the embryo and the corresponding DIC image overlaid with the EGFP image, respectively. The image in (**D**) is a graphically enhanced image of (**C**). Images in (**E**) and (**F**) are of the same embryo. However, the image in (E) was acquired with an excitation laser intensity reduced by 2 orders of magnitude relative to that applied to the image acquisition in (**C**) and (**F**). All other conditions for image acquisition were identical. Bar = 20 μm. The *lec1 nai1 PYK10-EGFP* embryos were obtained by crossing *lec1 PYK10-EGFP* into *nai1*. Thus the embryo of each genotype carried the same allele of the transgenic reporter. RNA preparation, real-time RT-PCR, and laser confocal microscopic observation were performed as described previously.[12] The mutant alleles used in the experiments were *nai1-1*, *lec1-1* and *lec2-1*.[12,19] For the *PYK10-EGFP* reporter gene experiments, the *lec1-1* allele in the Col-0 background was used.[12]

These observations led us to postulate that tight repression and its release upon the developmental transition of the postgeminative genes, as represented by *PYK10*, is controlled by epigenetic mechanisms.[12] In addition, in transgenic lines carrying 2 independent reporters, *PYK10-EGFP* and *PYK10-CFP*, cells expressing only EGFP or CFP were observed together with a substantial number of cells expressing both reporters in developing *lafl* embryos as well as in germinating wild-type embryos. These results led us to postulate that the expression state of *PYK10* is limited not only by the local chromatin state but also factors acting in trans, which may facilitate the change in the chromatin states.[12]

NAI1, a basic-helix-loop-helix (bHLH) transcription factor, has been identified as being required for the expression of *PYK10* and the formation of ER-bodies.[18] No visible phenotypes beside the ER-body defects have been detected in *nai1* mutants under normal growth conditions.[19] As reported, *PYK10* transcript levels were greatly reduced in the *nai1* seedlings compared to those in the wild type seedlings (**Fig. 1A**). Thus, a question is raised about how NAI1 is involved in the switching of *PYK10* upon the developmental transition from the embryonic to the postgeminative phase. Examination of the transcriptome data comparing 8 days-after-flowering (DAF) wild type and *lec1* or *lec2* embryos[12] and quantitative RT-PCR revealed high levels of upregulation of *NAI1* in the mutant embryo (**Fig. 1B**).

To see whether NAI1 could be a trans-acting factor responsible for the onset of *PYK10* expression in *lec* embryos, we examined the effect of the *nai1* mutation on *PYK10-EGFP* expression in *lec1* embryos. Although the fluorescent signals in *lec1* embryos were substantially reduced, the cell-by-cell onset of expression of the same *PYK10-EGFP* transgene was still recognized in a *nai1* background (**Figs. 1C–F**). Thus, NAI1 was unlikely to be a factor that conditions or triggers the conversion of *PYK10* chromatin from a repressed to a derepressed state. Instead, it appeared to be required for quantitative regulation. In accordance with this, the H3H27me3 level on the *PYK10* promoter region chromatin in the *nai1* seedlings was similarly lowered as in the wild type seedlings (**Fig. 2A**). Thus, the repressed state of *PYK10* appeared to be converted to a derepressed state in the absence of *NAI1* without a high level of transcriptional activation. We also examined levels of H3K4me3, an active histone mark, in the *PYK10*

chromatin. As expected from the expression state, an increased level of H3K4me3 on the *PYK10* chromatin of wild type seedlings was detected compared to that in developing seeds (**Fig. 2B**). However, in *nai1* seedlings, such an increase in H3K4me3 level was significantly reduced (**Fig. 2B**). Thus, 3 *PYK10* chromatin states were distinguished, high H3K27me3/low H3K4me3, low H3K27me3/lowH3K4me3 and low H3K27me3/high H3K4me3. These results together indicate that NAI1 is required for converting the *PYK10* chromatin to an active state, but not to a derepressed state, and/or maintaining the active state.

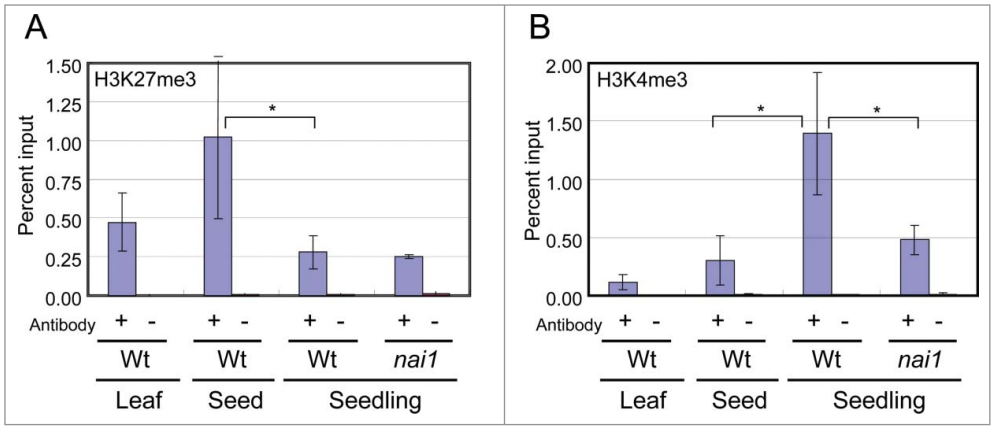

Figure 2. Effect of the *nai1* mutation and developmental phases on the histone methylation states of *PYK10* chromatin. Chromatin immunoprecipitation analyses of H3K27me3 (**A**) and H3K4me3 (**B**) on *PYK10* chromatin (promoter region) from developing seeds at 10 DAF, seedlings at 3 DAG and rosette leaves obtained immediately after bolting were performed as described previously.[12] Results from the immunoprecipitation with the specific antibody and the mock precipitation are indicated by (+) and (−), respectively. Data are expressed as a percentage of input DNA, with error bars representing the SD of biological replicates (n = 2–5). Asterisks indicate values that are significantly different between the treatments ($p < 0.05$, Student's t test).

Considering the tight repression of *NAI1* expression during the embryonic phase under the control of LAFL, *NAI1* may also be repressed at the chromatin level in a similar way to that of its target genes such as *PYK10*, and derepressed in a cell-by-cell manner. This assumption can explain the discrete fluorescent intensity levels of expression among the *PYK10-EGFP*-expressing cells or cell clusters. Other quantitative regulators that are regulated in a similar manner to that of NAI1 may be involved in such multistable expression of *PYK10*.

In conclusion, we have shown that the switching of *PYK10* expression upon the embryonic-to-postgerminative developmental phase transition is controlled by at least 2 steps of histone modification: removal of H3K27me3 and stimulation of H3K4me3. We have also shown that the bHLH transcription factor NAI1 is required for the latter but not for the former event. Although the 2 processes are experimentally uncoupled, they may not take place independently during the actual development because repression of *NAI1* during the embryonic phase may be controlled in a similar manner to that of *PYK10*. Detailed analyses of *NAI1* regulation as done for *PYK10* will apparently be important for clarifying this point. Currently the molecular links between LAFL and the maintenance of the repressive state of postgerminative genes are totally missing. Possible mechanisms may range from direct interaction of LAFL with chromatins of postgerminative genes to transcriptional regulation of genes for chromatin metabolism. Biochemical and genetic examination of such possibilities regarding *PYK10* and *NAI1* regulation will eventually shed light on more general understanding of the molecular mechanisms underlying the robust switching of gene expression program upon developmental phase transitions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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