Published in final edited form as:
Plant J. 2005 December ; 44(6): 1010–1022.

PICKLE acts during germination to repress expression of embryonic traits

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SUMMARY

PICKLE (*PKL*) codes for a CHD3 chromatin remodeling factor that plays multiple roles in Arabidopsis growth and development. Previous analysis of the expression of genes that exhibit *PKL*-dependent regulation suggested that *PKL* acts during germination to repress expression of embryonic traits. In this study, we examined the expression of PKL protein to investigate when and where PKL acts to regulate development. A PKL:eGFP translational fusion is preferentially localized in the nucleus of cells, consistent with the proposed role for PKL as a chromatin remodeling factor. A steroid-inducible version of PKL - a fusion of PKL to the glucocorticoid receptor (PKL:GR) - was used to examine when PKL acts to repress expression of embryonic traits. We found that activation of PKL:GR during germination was sufficient to repress expression of embryonic traits in the primary roots of *pkl* seedlings whereas activation of PKL:GR after germination had little effect. In contrast, we observed that PKL is required continuously after germination to repress expression of *PHERES1*, a type I MADS box gene that is normally expressed during early embryogenesis in wild-type plants. Thus PKL acts at multiple points during development to regulate patterns of gene expression in Arabidopsis.

Keywords

CHD3; chromatin remodeling factor; embryo; seedling; germination; developmental transition

INTRODUCTION

Germination of seeds marks a dramatic developmental transition in the life cycle of the plant. Significantly different developmental programs are expressed by the young plant prior to and following germination (Baud *et al.*, 2002; Bewley and Black, 1994; Goldberg *et al.*, 1994; Holdsworth *et al.*, 1999). The latter phase of seed development is referred to as the maturation phase and includes processes such as the accumulation of storage reserves and the acquisition of desiccation tolerance. A young growing seedling, in contrast, undergoes cell expansion and division, mobilizes seed storage reserves, and initiates photosynthesis. Consistent with the distinct developmental characteristics exhibited by maturing seeds and developing seedlings, microarray analysis and proteomics have revealed that vast repertoires of genes are differentially expressed at each of these two stages of development (Gallardo *et al.*, 2001; Gallardo *et al.*, 2002; Girke *et al.*, 2000).

PICKLE (*PKL*) is necessary to ensure that traits expressed during embryogenesis and seed formation are not expressed after germination (Ogas *et al.*, 1997). *pkl* seedlings are capable of

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expressing embryo-associated traits throughout the plant. In particular, the primary roots of *pkl* seedlings have been demonstrated to express many embryo specific traits after germination, including the accumulation of seed storage reserves (oil, protein, and phytate) and the ability to undergo somatic embryogenesis (Henderson *et al.*, 2004; Ogas *et al.*, 1997; Rider *et al.*, 2004). *pkl* primary roots that express these traits are referred to as "pickle roots" based on their swollen green appearance.

PKL encodes a SWI/SNF class chromatin remodeling factor that belongs to the CHD3 group (Eshed *et al.*, 1999; Ogas *et al.*, 1999). Consistent with the presence of an ATPase domain that is the hallmark of SWI/SNF proteins, animal CHD3 proteins have been demonstrated to exhibit ATP-dependent chromatin remodeling activity in vitro (Brehm *et al.*, 2000; Guschin *et al.*, 2000; Wang and Zhang, 2001). In addition, CHD3 proteins from animal systems have been shown to associate in multisubunit complexes (Mi-2 or NURD) that contain a histone deacetylase, a result that suggests that CHD3 proteins function as negative regulators of transcription (Tong *et al.*, 1998; Wade *et al.*, 1998; Xue *et al.*, 1998; Zhang *et al.*, 1998). Characterization of mutants from a variety of model systems in which CHD3 activity has been compromised also indicates that CHD3 proteins generally act as repressors of transcription, often of genes that are developmentally regulated (Ahringer, 2000; Kehle *et al.*, 1998; Unhavaithaya *et al.*, 2002; von Zelewsky *et al.*, 2000).

Previous characterization of *pkl* plants suggested that *PKL* acts to repress genes that promote embryonic identity (Ogas *et al.*, 1997; Ogas *et al.*, 1999; Rider *et al.*, 2003). The *LEAFY COTYLEDON* class of genes (*LEC1*, *LEC2*, and *FUS3*) encode transcription factors that are preferentially expressed in developing seeds and act as positive regulators that play a critical role in promoting seed development (Harada, 2001). In particular, *LEC1* and *LEC2* have the ability to promote somatic embryogenesis when ectopically expressed during germination (Lotan *et al.*, 1998; Stone *et al.*, 2001). In *pkl* plants, expression of *LEC1* and *LEC2* is substantially derepressed during germination (Ogas *et al.*, 1999; Rider *et al.*, 2003). Furthermore, expression of all three *LEC* genes is elevated more than 100-fold in pickle roots, suggesting that the elevated expression of the *LEC* genes contributes substantially to the manifestation of this unique developmental state (Rider *et al.*, 2003).

Previous data have suggested that *PKL*and gibberellin (GA) act in concert during germination to establish repression of genes that promote embryonic identity. Inhibition of GA biosynthesis in *pkl* seedlings results in increased penetrance of the pickle root phenotype, but only if the inhibition occurs during germination (Ogas *et al.*, 1997). Expression analysis of 10 genes that exhibit *PKL*-dependent expression revealed that the transcript level of all 10 genes were coordinately upregulated during germination of *pkl* seeds (Rider *et al.*, 2003). Furthermore, transcript levels of *PKL* increase during germination (Henderson *et al.*, 2004). These observations provide tantalizing yet indirect evidence that *PKL* acts specifically during germination to repress expression of embryonic traits.

Prior characterization of CHD3 proteins in animal systems makes a strong biochemical prediction that PKL interacts with the promoter of target genes as a component of a multisubunit complex. Yet these data also reveal that CHD3 proteins are directed to a multitude of targets and can function in multiple complexes. In Arabidopsis, for example, characterization of the *gymnos* mutant (which is allelic to *PKL*) revealed that *PKL* also acts to repress ectopic formation of placental meristems during carpel development (Eshed et al., 1999). Determination of when PKL acts to repress embryonic traits would greatly facilitate biochemical characterization of how PKL acts by enabling us to focus on that specific developmental window to evaluate potential targets and co-factors of PKL. We therefore undertook an analysis of *PKL* expression in an effort to investigate the timing of PKL action. In particular, we fused *PKL* to the glucocorticoid receptor to generate a conditional version of

PKL (*PKL:GR*) that is dependent on the presence of dexamethasone (Dex) for activity. We then used this fusion to examine when PKL acts to repress embryonic traits as well as to investigate when PKL acts to repress traits that arise during post-germinative development.

RESULTS

PKL protein preferentially accumulates in differentiating tissue

Polyclonal antibodies to PKL recognize a protein of an apparent molecular weight of about 200 kD in crude extracts from 3-day-old seedlings of wild-type plants (Figure 1a, lane 1). The observed molecular weight of PKL is substantially different from the predicted molecular weight of 158 kD; however, western analysis of PKL protein produced in *S. cerevisiae* and in *E. coli* indicates that it migrates with the same apparent molecular weight as observed in extracts from Arabidopsis (data not shown), suggesting that the increased apparent molecular weight of PKL is unlikely to be due to some modification of PKL protein that occurs in plant cells. PKL protein is absent in plants carrying a fast neutron-derived allele of *PKL, pkl-7* (Ogas *et al.*, 1999) (Figure 1a, lane 3). PKL is substantially reduced in abundance in extracts of carrying an EMS-derived allele of *PKL, pkl-1* (Ogas *et al.*, 1997) (Figure 1a, lane 2). Sequence analysis of the *pkl-1* allele revealed a G to A transition that alters the consensus sequence for the 3′ splice site upstream of exon #15 leading to the use of an alternate splice site that results in a 9 nucleotide in frame deletion of the *PKL* transcript. This deletion corresponds to amino acids 633-635 from the PKL protein, which lie in the conserved ATPase domain necessary for the remodeling activity of SWI/SNF proteins.

We next used the α-PKL antibodies to examine developmental regulation of PKL protein (Figure 1b). The greatest levels of PKL protein were observed in tissue undergoing significant differentiation such as young seedlings, inflorescent tissue, and young siliques. This pattern of expression is consistent with previous transcript analysis (Eshed *et al.*, 1999) and with the hypothesis that one of the primary roles of PKL is to act as a determination factor during differentiation. Decreasing amounts of PKL were observed as organs aged. Markedly less PKL protein is found in older siliques than in younger siliques (lane 8 versus lanes 6 and 7) and in 15-day-old rosette tissue versus 3-day-old seedlings (lane 4 versus lane 2). Previous data had suggested that *PKL* acted during germination to repress expression of genes that promote embryonic identity (Rider *et al.*, 2003), so we used western analysis to examine the level of PKL protein in greater detail after seed imbibition (Figure 1c). As in our previous analyses (Ogas et al., 1997; Rider et al., 2003), we consider germination to begin with seed imbibition (defined as when the seeds are sterilized) and to end with emergence of the radicle from the seed coat. We observed that PKL protein was expressed during germination (0.5d versus 1.5d), consistent with the hypothesis that PKL acts during germination to repress transcription of target genes. The peak of PKL protein accumulation, however, occurred after seedlings had completed germination (2.5d - 4d after seed imbibition). This observation indicated that PKL is also likely to play a significant role in gene expression in young seedlings after germination.

PKL protein accumulates in the nucleus

PKL codes for a CHD3 chromatin remodeling factor that is predicted to function in the nucleus. To examine the localization of the PKL protein, we fused *eGFP* to the 3′ end of the *PKL* ORF and expressed the resulting *PKL:eGFP* fusion under the control of the endogenous *PKL* promoter and terminator. This fusion construct is capable of rescuing all of the associated mutant phenotypes when transformed into a *pkl-1* plant (Figure 2a-b). Thus localization of the PKL:eGFP translational fusion is likely to reflect that of the endogenous PKL protein. We observed that GFP fluorescence was nuclear localized in transgenic plants carrying the *PKL:eGFP* translational fusion (Figure 2c, e). Identification of the nuclear compartment was confirmed by staining the roots with DAPI (Figure 2f). In contrast, a *PKL:eGFP* transcriptional

fusion in which expression was directed by the same genomic sequences is detected in both the cytoplasm and nucleus of cells (Figure 2g), indicating that the nuclear localization exhibited by the translational fusion is conferred by the PKL amino acid sequence. The transcriptional and translational fusions otherwise exhibit a similar pattern of expression, with the exception that transgenic lines carrying the transcriptional fusion exhibit much greater fluorescence than lines carrying the translation fusion (data not shown). The presence of the PKL:eGFP translational fusion in trichomes (Figure 2c) is consistent with the observation that the trichomes of *pkl* plants exhibit a reduced branching phenotype. This observation also demonstrates that *PKL* is expressed even in highly differentiated cells. Although eGFP reporter constructs are ubiquitously expressed (Figure 2c, d, h), the greatest expression was observed in tissue undergoing differentiation (Figure 2h-i). These data are consistent with the western analysis (Figure 1b).

Recent work has highlighted the contribution of the endosperm of the mature seed to storage reserve mobilization and post-germinative growth of dark-grown seedlings (Penfield *et al.*, 2004). The implication of this work is that gene expression occurs in two distinct developmental compartments during germination: the embryo and the endosperm. To address the possibility that PKL functions in the endosperm as well as the seedling during germination, we examined expression of the *PKL:eGFP* translational fusion in germinating seeds. We were unable to detect GFP fluorescence in the endosperm/seed coat of germinating seeds despite robust detection of GFP fluorescence in the seedling (data not shown). These data suggest that *PKL* is unlikely to play a role in regulation of gene expression in the endosperm during germination and that *PKL*-dependent expression that occurs during this time is most likely due to the action of *PKL* in the developing embryo.

Activation of PKL during germination is sufficient to suppress expression of embryonic traits

The discovery that PKL protein accumulation is constitutively nuclear localized suggested that we might be able to use the glucocorticoid receptor (Lloyd *et al.*, 1994; Picard *et al.*, 1988) to generate an inducible version of PKL protein. We fused the ligand-binding domain of the glucocorticoid receptor to full length *PKL* ORF to generate a *PKL:GR* translational fusion. This construct was then placed under the control of the endogenous *PKL* promoter and terminator as described above for the *PKL:eGFP* fusions.

We isolated transgenic *pkl-1* lines carrying the *PKL:GR* fusion, selected lines in which segregation of the resistance marker indicated that a single insertion event had taken place, and scored homozygous T3 progeny of these lines for penetrance of the pickle root phenotype in the presence and absence of dexamethasone (Dex). We observed that penetrance of the pickle root phenotype varied in individual T3 lines, which is consistent with prior characterization of this trait (Ogas et al., 1997 and unpublished observations). Penetrance of the pickle root phenotype depends on the genotype and ecotype of the plants, how the parent plants are grown, the age of the seeds, and the conditions under which the seeds are plated. As a result, penetrance of the pickle root phenotype does vary from experiment to experiment as it is technically impossible to carry out all of the experiments with the same batch of seed at the same time. In any given experiment, all seeds are obtained from plants grown in the same chamber at the same time so as to provide a suitable control, analogous to what must be done for studies of seed germination. In the case of characterization of penetrance of the pickle root phenotype in T3 lines, the position of the T-DNA integration event as well as any extraneous mutations introduced by transformation are also likely to have contributed to the observed variation in pickle root penetrance.

We examined 6 T3 lines in all, of which 5 exhibited at least a 30% Dex-dependent decrease in pickle root penetrance (Figure 3a). The ability of Dex to rescue the pickle root phenotype of transgenic *pkl-1* plants carrying the *PKL:GR* fusion was consistent with the hypothesis that

PKL functions in the nucleus. This result furthermore demonstrated that activation of PKL:GR after imbibition is sufficient to rescue the pickle root phenotype, an observation that is consistent with the hypothesis that PKL acts during germination to repress genes that promote embryonic identity.

To determine if there was a particular phase of development during which PKL:GR was capable of rescuing the pickle root phenotype, *PKL:GR* transformants of *pkl-1* plants (henceforth referred to as *pkl-1*/*PKL:GR* plants) were transferred to or from Dex-containing media at different times during the first 96 hours after seed imbibition. Specifically, seed from the GR-3 T3 line (Figure 3a) was used in this and all subsequent analyses involving *pkl-1*/*PKL:GR* plants. Previous work has demonstrated that transgenic plants expressing a GR fusion can respond to application of Dex prior to germination (Sanchez and Chua, 2001), indicating that the initial presence of an intact seed coat would not prevent Dex from reaching the seedling. In fact, we observed that as time after imbibition increased, the *pkl-1*/*PKL:GR* seedlings became less responsive to induction/repression of PKL:GR (Figure 3b). We found that after 60 hours in either the absence or the presence of Dex, the fate of the primary root of *pkl-1*/*PKL:GR* seedlings was largely determined even though 100% germination was not obtained until 96 hours. Thus PKL:GR can only suppress expression of the pickle root phenotype if induced prior to the completion of germination. Conversely, transient activation of PKL:GR during germination is sufficient to establish repression of embryonic traits; continuous exposure to Dex is not required for repression of the pickle root fate in *pkl-1*/*PKL:GR* seedlings.

Although these data indicate that PKL can act during germination to repress expression of embryonic traits, they do not exclude the possibility that PKL can also act during seed formation to enable subsequent repression of embryonic traits during germination. To test this hypothesis, we examined pickle root penetrance in the progeny of *pkl-1*/*PKL:GR* plants that had been grown in the absence or presence of Dex (Figure 4). A GR fusion expressed in the seed has previously been demonstrated to exhibit Dex-dependent activity (Baudry et al., 2004), and PKL is abundantly expressed in young embryos (Figure 2d), suggesting that the PKL:GR fusion was likely to be Dex-responsive during this period of development. Consistent with this supposition, we found that siliques of *pkl-1*/*PKL:GR* plants grown in the presence of Dex were restored to nearly wild-type length, indicating that application of Dex can rescue *pkl*-associated traits in developing *pkl-1*/*PKL:GR* siliques (Figure 4a). Penetrance of the pickle root trait in the subsequent generation, however, was insensitive to application of Dex to the parent (Figure 4b). Application of Dex during *pkl-1*/*PKL:GR* seed formation did not decrease pickle root penetrance in the resulting progeny (black bars denote absence of Dex during seed formation whereas white bars denote presence of Dex during seed formation). In fact, application of Dex during seed formation resulted in an increase in pickle root penetrance in a manner that was not dependent on the presence of the GR gene as it occurred in both *pkl-1* and *pkl-1*/*PKL:GR* lines. This effect, albeit surprising at first glance, is consistent with prior characterization of the pickle root penetrance as being responsive to the conditions under which the parent plant is grown. In contrast, application of Dex during germination of *pkl-1*/*PKL:GR* seeds suppressed pickle root penetrance regardless of the treatment during seed formation but had no measurable effect on pickle root penetrance of *pkl-1* seed. These data reveal that action of PKL during seed formation is not sufficient to repress expression of embryonic traits in the subsequent generation.

Taken together, our data strongly suggest that PKL acts specifically during germination to repress the potential of the young seedling to express embryonic traits and thus enable the developmental switch to post-germinative growth.

PKL can also act after germination to regulate development

In addition to the pickle root trait, *pkl* plants exhibit pleiotropic defects in shoot development including dark green leaves, reduced stature, and delayed flowering (Henderson *et al*., 2004; Ogas *et al.*, 1997). We examined the shoot phenotype of *pkl-1*/*PKL:GR* plants that were grown in the presence or absence of Dex. We found that Dex-treated *pkl-1*/*PKL:GR* plants exhibited partial to complete suppression of various *pkl*-associated traits assayed, including rescue of the rosette size, height, and flowering time (Figure 5a, b, data not shown). These data suggest that the PKL:GR fusion protein can also act in the shoot to regulate gene expression.

Our analysis of PKL protein expression (Figures 1 and 2) in combination with previous analyses of *PKL* transcript accumulation (Eshed *et al.*, 1999;Ogas *et al*., 1999) indicated that *PKL* was likely to act at other points in the plant life cycle in addition to germination to regulate gene expression. Our characterization of the time of PKL action (Figure 3b), however, was consistent with an alternative hypothesis that the *pkl* shoot phenotypes that arise after germination are primarily due to the establishment of improper patterns of gene regulation during germination of *pkl* seedlings. In support of such a hypothesis, it has previously been observed that *pkl* leaves accumulate elevated levels of the *FUS3* transcript (Rider *et al.*, 2003) and that transgenic plants exhibiting ectopic expression of *FUS3* in the L1 layer of the shoot exhibit GA-deficient phenotypes (Gazzarrini *et al.*, 2004) reminiscent of those exhibited by *pkl* plants. To examine the contribution of PKL action during germination to post-germinative development, we examined the shoot phenotype of *pkl-1*/*PKL:GR* plants that were only treated with Dex after seed germination. We found that these Dex-treated plants exhibited a substantially wild-type shoot phenotype whereas plants that were treated with Dex only during germination but not afterwards did not rescue the pickle shoot phenotype as effectively (Figure 5c). Thus these data suggest that the shoot phenotype of *pkl* plants is primarily due to the absence of PKL activity after germination.

PKL acts continuously after germination to repress *PHE1* expression in the shoot

In order to examine when PKL acted after germination to regulate development, we needed a distinct marker for PKL action in the shoot similar to that of the pickle root phenotype. Ongoing analysis of candidate genes for *PKL*-dependent expression in young seedlings (selected on the basis of their predicted/demonstrated role in embryo development) uncovered 2 genes that exhibited robust *PKL*-dependent expression after germination (Table 1, data not shown). *PHERES1* (*PHE1*) and *PHERES2* (*PHE2*) code for type I MADS box proteins that are transiently expressed in young embryos (Kohler *et al*., 2003). We extracted RNA from plants that were staged at 50% germination (i.e. 50% of the seedlings had completed germination based on emergence of the radicle from the seed coat), 4-day-old seedlings, and from 21-day-old rosette tissue from wild-type and *pkl* plants and analyzed relative transcript levels by qRT-PCR (Table 1). We observed that *PHE1* and *PHE2* exhibit strong *PKL*-dependent expression, but only after germination. This observation is in striking contrast to previously characterized *PKL*-dependent genes such as *LEC1* and *LEC2* that exhibit strong *PKL*-dependent expression during germination but do not exhibit *PKL*-dependent expression in the mature shoot (Rider *et al.*, 2003).

The discovery that two type I MADS box genes exhibited *PKL*-dependent expression after germination prompted us to examine whether other type I MADS box genes exhibited similar regulation. The expression of 45 type I MADS box genes (Alvarez-Buylla *et al.*, 2000)(De Bodt *et al.*, 2003) was examined by qRT-PCR at the same developmental stages assayed for *PHE1* and *PHE2*. We identified 4 other predicted type I MADS box genes for which the level of the corresponding transcript was altered at least 5-fold in the absence of *PKL*. Unlike *PHE1* and *PHE2*, however, none of these genes exhibited *PKL*-dependent expression at more than one of the developmental stages that were assayed. *At5gt26630* and *At3g66656* transcripts

were elevated 5- and 7-fold respectively in germinating seedlings whereas *At2g28700* transcripts were up 17-fold in 4-day-old seedlings. In contrast, *At1g18750* was 12-fold decreased at 50% germination, suggesting that *PKL* is a positive regulator of this locus. Our analysis failed to reveal a large effect of the *pkl* mutation on expression of the other type I MADS box genes (Supplementary Data, Table S1), indicating that *PKL* does not play a general regulatory role for this class of transcription factor.

The identification of *PHE1* and *PHE2* as genes that exhibit robust *PKL*-dependent expression in the shoot provided us with the opportunity to determine if *PKL* could also act after germination to regulate gene expression. We examined *PHE1* transcript levels in *pkl-1*/*PKL:GR* plants grown continuously in the presence or absence of Dex. These experiments demonstrated that application of Dex to *pkl-1*/*PKL:GR* plants results in 3-fold repression of *PHE1* transcript levels relative to growth in the absence of Dex (Figure 6a). Thus the PKL:GR fusion is capable of partial rescue of *PHE1* expression in *pkl* plants, an observation that is consistent with the observation that application of Dex to *pkl-1*/*PKL:GR* plants can completely rescue some *pkl*-associated traits (Figures 4a and 5b) but only partially rescue others (Figure 5a). Similarly, application of Dex to *pkl-1*/*PKL:GR* plants results in less than 2-fold repression of *PHE2* transcript levels (data not shown).

We were able to use the more robust Dex-dependent *PHE1* expression to examine when PKL is capable of acting to regulate gene expression in the shoot. *pkl-1*/*PKL:GR* seedlings were grown in the absence or presence of Dex for 10 days and then transferred to or from Dex-containing media. Transcript levels of *PHE1* were followed by qRT-PCR at 0.5, 1, and 3 days after transferring the seedlings as well as in plants that were subjected to a mock transfer (Dex to Dex or no Dex to no Dex). We found that after 10 days of growth in the absence of Dex, treatment of *pkl-1*/*PKL:GR* seedlings with Dex resulted in repression of *PHE1* transcript levels within 1 day (Figure 6b). Conversely, we found that transfer of 10-day-old *pkl-1*/*PKL:GR* seedlings from media containing Dex to Dex-free media resulted in elevation of transcript levels within 1 day (Figure 6c). Thus in contrast to what is observed during germination, PKL appears to be continuously needed to repress expression of at least one gene in the shoot after germination. In both cases, 3 days of the new treatment was largely sufficient to switch *PHE1* transcript levels to reflect the new regime. Similar results were observed if *pkl-1*/*PKL:GR* plants are transferred after 21 days of growth (data not shown). Thus these data reveal that *PKL* continuously acts after germination to regulate expression of some genes in the shoot.

PKL acts during germination to repress expression of *LEC1*

Our success at examining the effect of activation of PKL:GR on *PHE1* expression in the shoot prompted us to examine the effect of activation of PKL:GR on gene expression during germination. Although the previous analysis indicated that PKL acted during germination to repress expression of embryonic traits in the primary root (Figure 3b), it did not address when PKL was capable of acting to repress gene expression. We have previously observed that transcript levels of *LEC1* are elevated during germination of *pkl* seeds (Ogas *et al.*, 1999; Rider *et al.*, 2003). To test the hypothesis that PKL acted early during germination to repress expression of *LEC1*, we used qRT-PCR to examine *LEC1* transcript levels in germinating *pkl* and *pkl-1*/*PKL:GR* seedlings that were treated with Dex either during early germination or late germination. We also examined *PHE1* transcript levels as a control for developmental specificity.

We found that imbibition of *pkl-1*/*PKL:GR* seedlings in the presence of Dex until they reached 50% germination resulted in a >5-fold decrease in transcript levels of *LEC1* relative to *pkl-1*/*PKL:GR* seedlings imbibed in the absence of Dex (Figure 7a). In contrast, if *pkl-1*/*PKL:GR* seeds were imbibed in the absence of Dex until they had reached 75% germination and then treated with Dex for 48 hours, we observed a < 2-fold decrease in transcript levels of *LEC1*

relative to *pkl-1*/*PKL:GR* seedlings that were not exposed to Dex (Figure 7b). Thus these data reveal that the ability of PKL:GR to repress expression of *LEC1* (Figure 7) and to repress expression of the pickle root phenotype (Figure 3b) is largely restricted to early germination, consistent with the hypothesis that expression of the pickle root trait is a reflection of the ability of PKL to act on *LEC1* and related genes during early germination.

PHE1 transcript levels exhibited a pattern of expression that was reciprocal to that of *LEC1*. *PHE1* transcript levels were relatively unchanged when *pkl-1*/*PKL:GR* seedlings were imbibed in Dex until 50% germination (Figure 7a) whereas they were repressed nearly 3-fold when *pkl-1*/*PKL:GR* seedlings were treated with Dex after they had reached 75% germination (Figure 7b). These data are consistent with our observation that *PKL* is necessary after germination for repression of *PHE1* expression and further demonstrate that PKL:GR is capable of acting to regulate gene expression in response to Dex after 75% germination.

As expected, *LEC1* and *PHE1* transcript levels were not responsive to either regimen of Dex application in *pkl* seedlings.

DISCUSSION

Activation of PKL during germination is sufficient to repress expression of embryonic traits

We have shown that PKL is a nuclear localized protein that is preferentially expressed in differentiating tissue and specifically acts during germination to repress genes that promote embryonic identity, in strong agreement with our previous observation that genes that exhibit *PKL*-dependent expression exhibit elevated transcript levels during germination of *pkl* seedlings (Ogas *et al.*, 1999; Rider *et al.*, 2003). We have previously demonstrated that GA also acts during germination to repress expression of the pickle root trait (Ogas *et al.*, 1997). Thus our data reveal that *PKL* and GA act during the same period of development to repress expression of embryonic traits. The implication of our data is that a GA-dependent *PKL*-independent repression pathway exists that operates concurrently with the *PKL*-dependent pathway and that can in some way ameliorate the effect of loss of PKL on repression of embryonic traits. Given that PKL is a chromatin remodeling factor, it seems reasonable to anticipate that the mechanism by which GA suppresses the consequences of loss of *PKL* is likely to be chromatin-based. Determination of whether or not this GA-dependent *PKL*-dependent repression pathway plays a similar role in the presence of *PKL* has yet to be determined. Suppression of GA biosynthesis in the presence of a wild-type copy of *PKL* is not sufficient to generate a pickle root phenotype or to elevate expression of any of the *LEC* genes during germination (JO and JH unpublished observations). Nonetheless, these observations do not preclude the possibility that reduced levels of GA lead to an alteration of chromatin that is not sufficient to alter expression of affected genes except in the absence of *PKL*.

Our data are consistent with a model in which the seed is developmentally reprogrammed during germination in a *PKL*-and GA-dependent manner to repress expression of embryonic traits and thereby ensure the switch to the developmental status of a seedling. Thus the ability to express embryonic potential is not silenced during seed maturation but rather during seed germination. This type of regulatory control would provide an explanation of why treatments that promote somatic embryogenesis during germination are not effective during subsequent shoot development. For example, ectopic expression of *LEC1* during germination is sufficient to promote somatic embryogenesis (Lotan *et al.*, 1998; Zuo *et al.*, 2002), whereas it is not sufficient after germination (Zuo *et al.*, 2002). By the time germination is complete, PKL and GA have acted to render the differentiation state of an embryo less accessible, most likely through alteration of the chromatin structure of genes that promote embryonic identity.

PKL acts continuously during shoot development to repress expression of *PHE1*

Our analysis of *pkl-1*/*PKL:GR* plants clearly demonstrates that PKL also acts after germination. Although activation of PKL:GR during germination is sufficient to suppress pickle root penetrance (Figure 3b), it is not sufficient to robustly rescue *pkl*-associated shoot traits (Figure 5c). In contrast, we observe that activation of PKL:GR after germination is sufficient to substantially rescue the shoot phenotype of *pkl* plants, suggesting that the shoot phenotype of *pkl* plants is largely due to the loss of *PKL*-dependent regulation after germination. An interesting implication of this observation is that subsequent shoot development appears rather insensitive to a substantial prior perturbation of the expression of developmentally regulated genes. Based on our expression analysis and phenotypic characterization of *pkl-1*/*PKL:GR* plants, it is likely that many of the genes that exhibit elevated transcript levels in *pkl* plants exhibit similarly elevated transcript levels in *pkl-1*/*PKL:GR* plants imbibed in the absence of Dex. Yet despite this extensive alteration in the pattern of gene expression during germination, *pkl-1*/*PKL:GR* plants are substantially restored to wild-type appearance when treated with Dex after germination.

Our analysis of *PKL*-dependent regulation of *PHE1* and *PHE2* clearly illustrates that PKL can act after germination to regulate gene expression. *PHE1* and *PHE2* code for closely related type I MADS box proteins that are transiently expressed during early embryogenesis (Kohler *et al*., 2003). Previous analysis of embryo-related genes for *PKL*-dependent expression, such as *LEC1* and *LEC2*, identified genes that exhibited *PKL*-dependent expression during germination (Rider *et al*., 2003). *PHE1* and *PHE2*, in contrast, do not exhibit *PKL*-dependent expression during germination but instead exhibit strong *PKL*-dependent expression after germination (Table 1). Furthermore, we observed that PKL acts continuously during shoot development to regulate expression of *PHE1*. Transcript levels of *PHE1* in 10-day-old *pkl-1*/*PKL:GR* plants responded within one day of addition or removal of Dex (Figure 6).

One interesting implication of our data is the existence of multiple regulatory pathways for expression of embryonic identity. Both GA and *PKL* act during germination to repress expression of the pickle root trait, whereas *PHE1* and *PHE2* transcript levels are elevated after germination. *PHE1* and *PHE2* were initially identified in an effort to identify targets of MEDEA (MEA), a PcG protein that plays an essential role in developing embryos (Kohler *et al.*, 2003). The characterization of *PHE1* and *PHE2* expression in wild-type and *mea* embryos suggested that transient elevated expression of *PHE1* and *PHE2* was likely to play a significant role in early embryogenesis (Kohler *et al*., 2003). The observation that expression of *PHE1* and *PHE2* is not elevated during germination of *pkl* seedlings reveals that altered expression of these genes does not contribute to the establishment of embryonic identity in this context.

Our data also suggest that the response of the plant to altered *PHE1* expression is likely to be dependent on the stage of development. Suppression of elevated *PHE1* levels in *mea* seeds is sufficient to rescue *mea*-associated seed abortion, suggesting that elevated *PHE1* expression is lethal in this context (Kohler *et al.*, 2003). In contrast, we observe that elevated *PHE1* levels (Table 1) are not sufficient to lead to termination of development in the *pkl* shoot. Furthermore, we also find that Dex can substantially rescue the shoot phenotype of *pkl-1*/*PKL:GR* plants despite the fact that transcript levels of *PHE1* are elevated approximately 50-fold in Dex-treated *pkl-1*/*PKL:GR* plants relative to wild-type plants (Table1, Figure 6b, and data not shown). This observation reveals that elevated *PHE1* levels are not sufficient to generate the *pkl* shoot phenotype. Determination of whether or not elevated *PHE1* expression is necessary for development of the *pkl* shoot phenotype will require reducing *PHE1* transcript levels in *pkl* plants by mutational analysis or RNAi.

Although these data do not reveal if PKL directly regulates expression of *LEC1* and *PHE1*, it is intriguing to note that our data are consistent with the possibility that PKL acts in a similar

fashion to regulate expression of both genes. The level of *PHE1* transcript was determined in whole plants. Possible expression patterns range from *PKL*-dependent expression occurring in every cell throughout development to *PKL*-dependent expression occurring in a specific subset of cells during a particular stage of leaf development. The latter type of regulation would be very similar to the manner in which *PKL* acts during germination to repress expression of embryonic traits in the developing root. This model would also explain why PKL is only required after germination to repress expression of *PHE1*; PKL acts after germination during organogenesis to shut down transient expression of *PHE1*. More detailed characterization of developmental regulation of *PHE1* expression in *pkl-1*/*PKL:GR* plants with the assistance of reporter genes and/or in situ hybridization will be necessary to distinguish between these possible patterns of expression.

CHD3 chromatin remodeling factors are known to play a role in promoting various developmental transitions in eukaryotes (Ahringer, 2000). In the absence of a conditional version of a CHD3 protein, however, it has not been possible to address whether or not CHD3 action during a specific developmental window is sufficient to prevent inappropriate expression of prior differentiation traits during subsequent developmental stages. Our studies demonstrate that the CHD3 protein PKL can act during a specific developmental window to determine fate in plants: PKL is required during germination to repress embryonic identity. Determination of the timing of PKL action greatly facilitates elaboration of other aspects of the mechanism of PKL action. It is now known when to undertake biochemical characterization of the PKL protein (e.g. identification of interacting proteins and direct targets) to understand how PKL promotes the developmental transition that occurs during germination.

The identity of differentiated plant tissue is commonly perceived as being significantly more malleable than that of differentiated animal tissue. It is less often recognized that limitations to this developmental plasticity occur quite frequently. Many agronomically important plants are resistant to in vitro manipulation (e.g. many species of forest trees) (Merkle and Dean, 2000). Although it is relatively easy in Arabidopsis to transform roots into shoots and vice versa by manipulating hormone concentrations, reacquisition of embryonic identity after germination is much more difficult to achieve (Zuo *et al.*, 2002). *PKL* plays a critical role in repression of embryo-associated differentiation traits. It seems reasonable to anticipate that *PKL* may play a general role in restricting access to alternate differentiation states in plants. Characterization of the role of CHD3 proteins in development and differentiation in animal systems is consistent with such a hypothesis (Ahringer, 2000; Fujita *et al.*, 2004; Unhavaithaya *et al.*, 2002). Further characterization of the mechanism by which *PKL* regulates gene expression during germination may thus provide general insights into how plants - like animals - restrict developmental potential.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

Wild type, *pkl-1* (Ogas *et al.*, 1997), and *pkl-7* (Ogas *et al.*, 1999) are in the Col background. Plants were grown on synthetic media or in pots as previously described, with exceptions noted below (Ogas *et al.*, 1997). Plants were transformed by using an *in planta* transformation protocol with the *Agrobacterium tumefaciens* strain GV3101 (Clough and Bent, 1998).

For the analysis of PKL protein expression during development (Figure 1b), the seedling tissue and the 15-day-old shoot and root tissue was collected from plants grown on synthetic media. The inflorescences and siliques were collected from plants that were grown for 3 weeks on plates and then transferred to pots and grown for another 5 weeks. Siliques were numbered sequentially where the first open flower corresponded to silique number one, the second open

flower to silique number two, and etc. Siliques #10 and #20 contained embryos at the globular stage and the mature embryo stage respectively (Bowman, 1993).

For the analysis of Dex-dependent shoot traits, plants were imbibed 2 days with an aqueous solution containing 10^{-5}M Dex or with a mock solution containing 0.2% methanol. Seedlings were rinsed with water for 5 times, sown on soil, grown in a Percival growth chamber with continuous illumination (150-180 μE m^{-2} s^{-1}) at 22°C. Plants were sprayed every other day with an aqueous solution containing 10^{-5} M Dex and 0.05% (w/v) Tween 20 or with a mock solution containing 0.02% methanol and 0.05% (w/v) Tween 20. The rosette size of *pkl-1*, *PKL:GR*, and WT were compared after 3 weeks. The length of siliques was measured when seed maturation was complete. Eight to ten siliques were measured for each treatment. Plants for Dex-dependent flowering time analysis were grown in a Percival growth chamber with 16 hours of illumination. Flowering was scored as the number of days until the first flower opened. For analysis of the effect of application of Dex during seed formation on subsequent penetrance of the pickle root phenotype, seeds were plated on MS media supplemented with 10^{-8}M uniconazole or 10^{-8}M uniconazole and 10^{-5}M Dex, and the pickle root trait was determined for 144 seedlings 10 days after imbibition.

For analysis of *PHE1* expression in *pkl-1*/*PKL:GR* transgenic lines (Figure 6), seeds were plated on MS media for 10 days, then transferred to MS media supplemented with 10^{-5}M Dex, and harvested 0.5, 1, or 3 days after transfer.

Plasmid construction

A complete description of the construction of all recombinant DNA molecules generated for this study can be found in Supplementary Material.

Antibody preparation

Polyclonal anti-PKL antiserum was obtained from rabbits immunized with the N-terminal (1-291aa) or C-terminal (740-1385aa) region of PKL. *E. coli* Tuner DE3 cells (Novagen, Madison, WI) were used for the production of recombinant antigen. Growth of the expression host strain and induction of protein expression were performed according to the manufacturer's recommendations. Whole cell lysates from *E. coli* expressing recombinant PKL peptide were separated on 10% SDS-PAGE (Laemmli, 1970). Gels were stained with copper chloride (Lee *et al*., 1987) and the band corresponding to the PKL N- or C-terminal fragment was excised and equilibrated in 40 volumes of 0.5 M EDTA (pH 8.0) for 30 minutes, followed by 3 washes (30 minutes each) in 40 volumes of Tris-Glycine gel running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) to remove the copper stain. The recombinant PKL peptides were electroeluted from the gel slices using an S&S Elutrap apparatus (Schleicher and Schuell, Inc., Keene, NH) according to the manufacturers instructions. Eluted peptides were concentrated using Microcon concentrators (Millipore Corp., Billerica, MA), quantified, and adjusted to a final concentration of approximately 1 mg/ml. The partially purified PKL fragments (~5 mg) were used for the production of polyclonal antibodies (Alpha Diagnostic, San Antonio, TX).

Protein extraction

For analysis of PKL protein in *E. coli*, crude whole cell extracts were prepared from cells that were resuspended in 0.5 ml PBS with 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 1 mM PMSF. Equal volumes of hot (80°C) 1x PBS and hot 2x sample (2% SDS, 80mM Tris pH6.8, 0.6% bromophenol blue, 15% glycerol and 0.1M DTT) buffer were added, and samples were heated at 80°C for 15 min.

For analysis of PKL protein in *S. cerevisiae*, crude whole cell extracts were prepared from cells grown to saturation in minimal media that were lysed with an equal volume of 2x sample buffer

(0.2 M Tris pH6.8, 20% β-mercaptoethanol, 4% SDS, 10% glycerol, 0.002% bromophenol blue) supplemented with 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 1 mM PMSF. Cells were beat twice for 30 sec with 0.5 mm zirconia beads (Biospec Products, Inc., Bartlesville, OK), boiled for 3 min, and supernatants were transferred to a new tube.

Crude whole cell protein extracts of *A. thaliana* were prepared as previously described (Rider *et al*., 2004).

Protein quantification and western analysis

Total proteins were quantified with Bio-Rad RCDC protein assay (Bio-Rad Lab., Hercules, CA) as per manufacturer's recommendation. Equal amounts of total proteins were separated on 6% SDS-polyacrylamide gels and transferred to immobilon-P membrane (Millipore Corp., Billerica, MA). Membranes were blocked with 1x BSA (0.01 M Tris pH8, 0.1 M NaCl, 0.1% Tween-20, 1% BSA), and probed with anti-PKLN or anti-PKLC terminal antiserum in 1:5000x dilution. Anti-PEP carboxylase antibody at a dilution of 1:5000 (Rockland, Gilbertsville, PA) was used as a control. Secondary antibody (goat anti-rabbit IgG-HRP conjugate; Pierce Biotech. Inc., Rockford, IL) was used at a dilution of 1:10000.

PKL:eGFP characterization

Arabidopsis seed, seedlings and leaves were mounted in water and observed with a fluorescence microscope (model DMR HC, Leica Inc., Deerfield, IL). eGFP was visualized with EN GFP filter from Chroma Technology (part # 41017). Nuclei were visualized with DAPI as described by Copenhaver (http://www.biology.wustl.edu/pikaard/protocols/chromcount.html).

pkl-1/*PKL:GR* inducible lines

Seedlings were grown on synthetic media supplemented with 10^{-7}M uniconazole-P and with or without 10^{-5} M Dex (Sigma) dissolved in methanol. Seedlings were shifted from plates containing Dex to plates that did not contain Dex or vice versa using fine point tweezers in a sterile hood. At each time point 144 seeds of *pkl-1*/*PKL:GR* (transgenic line GR-3), 72 seeds of *pkl-1*, and 72 wild-type seeds were transplanted from Dex to no Dex plates or vice versa. Seedlings were scored for the pickle root trait at 15 days post seed imbibition. The experiment was carried out at 22°C in continuous light (60 μE m^{-2} s^{-1}). Three biological replicates of this analysis were performed.

RNA isolation and quantitative RT-PCR

Total RNA was isolated as described previously by Verwoerd *et al.* (Verwoerd *et al*., 1989). Quantitative PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), as previously described (Rider *et al.*, 2003). 18S ribosomal RNA was used as a normalization control for the relative quantification of transcript levels. Data presented are derived from three unpaired technical replicates as recommended by ABI. All qRT-PCR experiments were repeated with a biological replicate with similar results (data not shown). All oligonucleotide primer sequences and primer concentrations used, the critical threshold values for the figures presented, and the complete analysis of type I MADS box genes can be found in the Supplementary Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Sean Cutler for sharing the pEGAD vector and Alan Lloyd for sharing the pBI-ΔGR vector. We thank Clint Chapple for thoughtful discussion. We thank Vasundhara Kandachar for assistance with some of the GR-related work. We thank Jody Banks for use of her microscope. We thank the undergraduates in the laboratory for numerous contributions. This work was supported by the National Institutes of Health (grant no. R01GM059770 to J.O.) and by the National Science Foundation (IGERT Fellowships to K.C. and to J.T.H.). This is journal paper no. 17751 of the Purdue University Agricultural Experiment Station.

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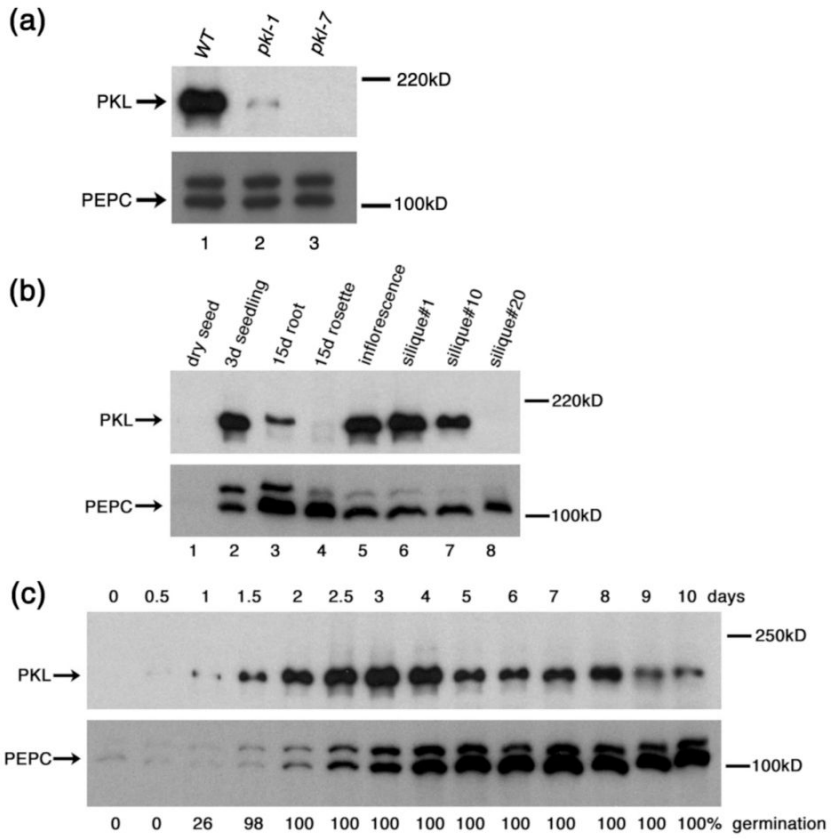

Figure 1.

PKL protein preferentially accumulates in differentiating tissue. Western blot analysis of total protein extracts with a polyclonal antibody to the N-terminus of PKL was used to examine the amount of PKL protein levels in a variety of tissues. Levels of PEP carboxylase were similarly determined as a loading control. The size (in kD) of protein molecular weight standards is indicated to the right of each gel.

(a) Levels of PKL protein found in 3-day-old WT (lane 1), *pkl-1* (lane 2), and *pkl-7* (lane 3) seedlings.

(b) Levels of PKL protein found in dry seed (lane1), 3-day-old seedlings (lane2), 15-day-old roots (lane3), 15-day-old rosettes (lane4), the inflorescence (lane5), silique #1 (lane6), silique #10 (lane7), and silique #20 (lane8). Please see Experimental Procedures for a description of the numbering scheme used for siliques.

(c) Levels of PKL protein found in seedlings that had been imbibed for up to 10 days. The percentage of seedlings that had germinated at each time point is indicated below each lane. 25μg of protein were added per lane in all panels. During this analysis, we observed that PEP carboxylase was only effective as a loading control at 4 days after imbibition and thereafter. Prior to this time, expression of PEP carboxylase is significantly upregulated relative to a desiccated seed as the young seedling develops.

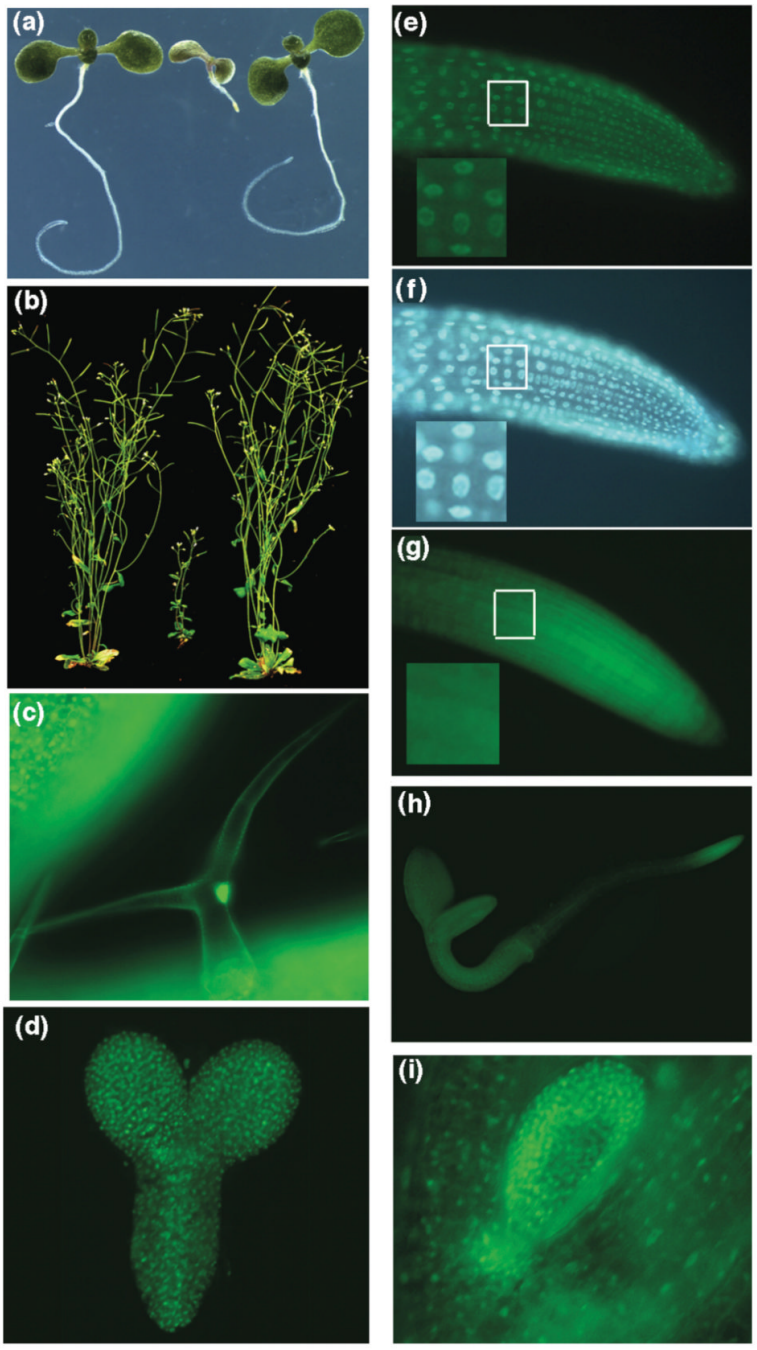

Figure 2.

The PKL:eGFP translational fusion rescues *pkl-1* and is nuclear localized. Complementation of *pkl-1* seedlings (a) and mature *pkl-1* plant (b). For both panels, the plant on the left is wild-type, the plant in the center is *pkl-1*, and the plant on the right is *pkl-1* transformed with the *PKL:eGFP* translational fusion. The plants in panel (a) were grown in the presence of 10^{-8} M uniconazole-P in continuous light for 14 days, whereas the plants in panel (b) were grown in the presence of 10^{-8} M uniconazole-P in continuous light for 23 days then moved to soil and grown under continuous light for an additional 21 days.

(c-i) Transgenic plants expressing *PKL:eGFPM* transcriptional (g,h) or translational (c-f,i) fusions were observed using fluorescence microscopy. (c) GFP fluorescence in a trichome from

the first leaf of 9-day-old plant carrying a translational fusion. (d) GFP fluorescence in torpedo stage embryo in a plant expressing a translational fusion. (e) GFP fluorescence in the root tip of a 3-day-old plant expressing a translational fusion. (f) DAPI fluorescence in the same root tip depicted in panel (e). (g) GFP fluorescence in the root tip of a 3-day-old plant expressing a transcriptional fusion. The white box in panels e-g highlights the area magnified 2.5X in the lower left corner of each panel. (h) GFP fluorescence of an entire 3-day-old plant expressing a transcriptional fusion. (i) GFP fluorescence in the first leaf of a 5-day-old plant expressing a translational fusion.

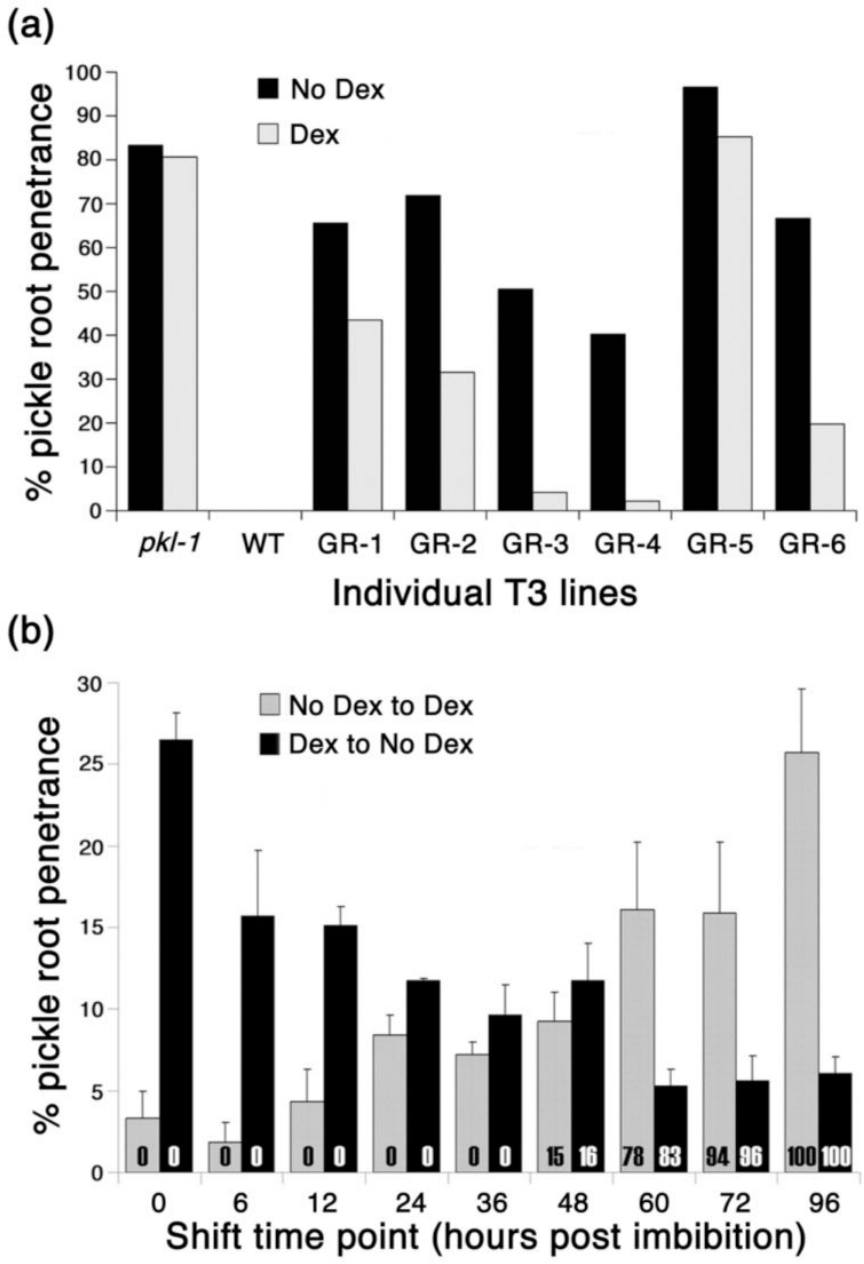

Figure 3.

Action of PKL during germination is sufficient to suppress embryonic traits.

(a) 6 independent T3 *pkl-1* lines that were homozygous for *PKL:GR* were scored for pickle root penetrance when grown in the presence or absence of Dex.

(b) Seeds from the GR-3 *pkl-1/PKL:GR* T3 line were imbibed in water and immediately plated on media not containing Dex and then shifted at the indicated times to media containing Dex (gray columns), or on media containing Dex and then shifted at different times to media without Dex (black columns). The *x* axis indicates the times at which seeds were shifted. Error bars represent the standard error of the mean. The numbers within the columns represent the percent of germinated seeds at the time the seeds were shifted.

For both panels, pickle root penetrance was scored fifteen days after imbibition. All media used contained 10^{-7} M uniconazole-P to increase penetrance of the pickle root phenotype.

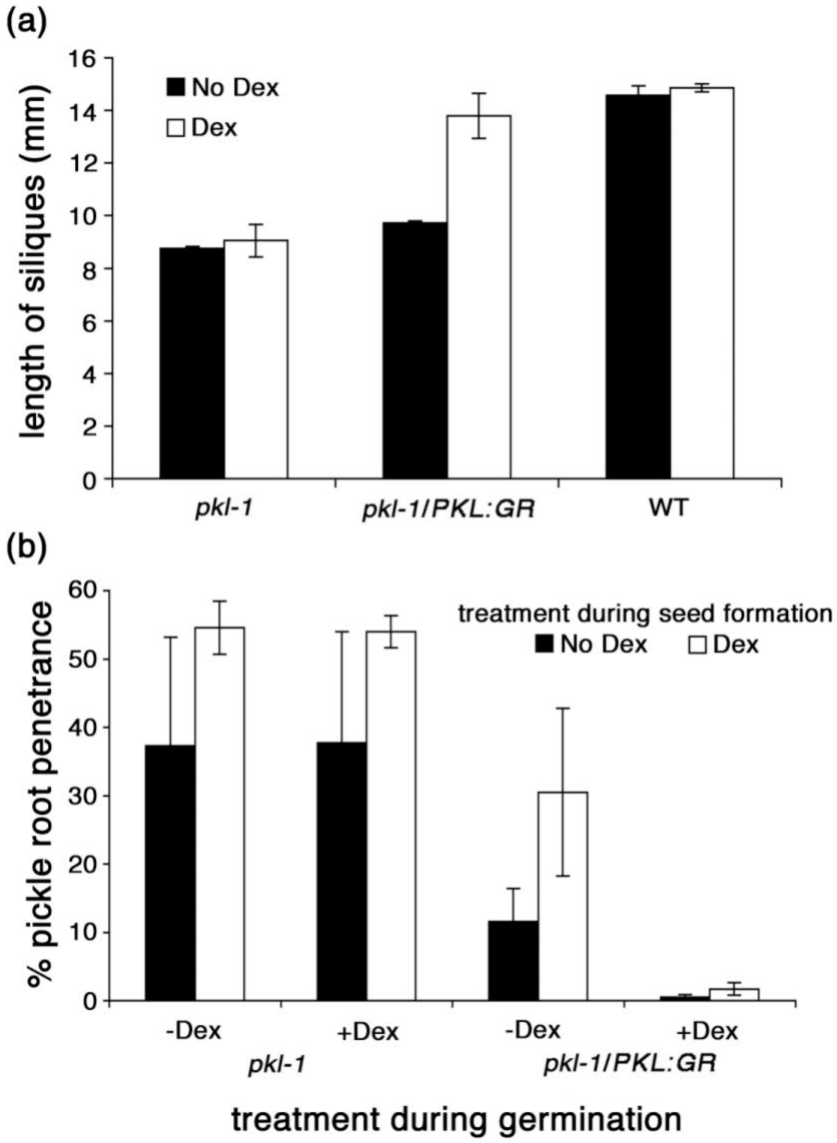

Figure 4.

Action of PKL during seed formation does not suppress embryonic traits.

(a) The length of siliques was determined of *pkl-1*, *pkl-1/PKL:GR* T3, and WT plants that were grown in the absence or presence of Dex. The values plotted are the mean ± SD of three biological replicates, in which 10 siliques were measured for each replicate.

(b) Seeds from *pkl-1* and *pkl-1/PKL:GR* T3 plants that were grown in the absence or presence of Dex were scored for pickle root penetrance when imbibed in the presence or absence of Dex. All seeds were imbibed on media that contained 10^{-8} M uniconazole-P to increase penetrance of the pickle root phenotype. Expression of the pickle root trait was scored fifteen days after imbibition. The values plotted are the mean ± SD of three biological replicates, in which 144 seedlings were scored for each replicate.

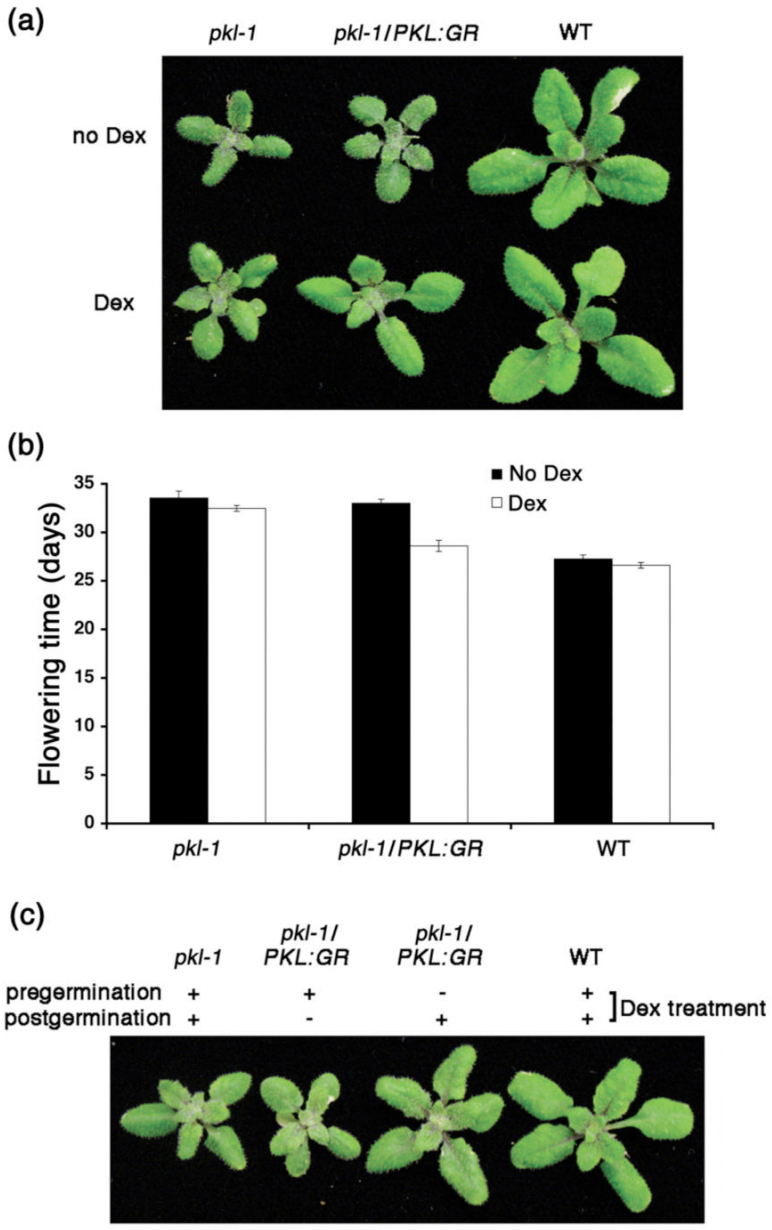

Figure 5.

PKL:GR can act after germination to rescue the shoot phenotype of *pkl* plants.

(a) *pkl*, *pkl-1/PKL:GR*, and wild-type plants were grown for 19 days in the absence or presence of Dex.

(b) Flowering time was determined for *pkl*, *pkl-1/PKL:GR*, and wild-type plants grown in the presence or absence of Dex. The values plotted are the mean +/- SE of 15 plants measured.

(c) Comparison of shoots of 19-day-old *pkl-1/PKL:GR* plants that were imbibed in the presence of Dex until completion of germination (2 days) and then not treated with Dex subsequently (-/+) or that were imbibed in the absence of Dex until completion of germination (2 days) and then sprayed with Dex subsequently (+/-). 19-day-old *pkl-1* and wild-type plants that were treated continuously with Dex (+/+) are shown for comparison.

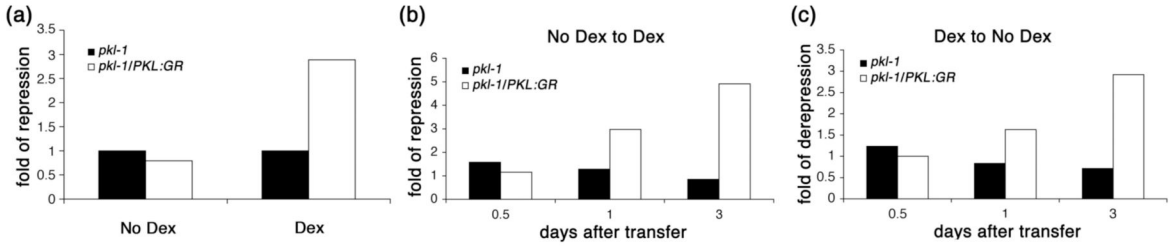

Figure 6.

PKL can act after germination to determine expression of *PHE1*. Quantitative RTPCR was used to determine the relative transcript levels of *PHE1* in plants in response to a variety of Dex treatments. 18S rRNA was used as a standardization control.

(a) Relative *PHE1* transcript levels were determined in 21-day-old *pkl-1/PKL:GR* plants and *pkl* plants that were grown in the presence and absence of 10^{-5} M Dex. Expression levels are normalized relative to *pkl* plants.

(b) Relative *PHE1* transcript levels were determined in *pkl-1* and *pkl-1/PKL:GR* seedlings that were grown on media that did not contain Dex for 10 days and then transferred to media containing 10^{-5} M Dex and allowed to grow for the number of days indicated on the *x* axis. Expression levels (fold repression) are normalized relative to plants of the same genotype that underwent a mock treatment regime (transfer to no Dex media).

(c) Relative *PHE1* transcript levels were determined in *pkl-1* and *pkl-1/PKL:GR* seedlings that were grown on media that contained 10^{-5} M Dex for 10 days and then transferred to media that did not contain Dex and allowed to grow for the number of days indicated on the *x* axis. Expression levels (fold derepression) are normalized relative to plants of the same genotype that underwent a mock treatment regime (transfer to media that contained 10^{-5} M Dex).

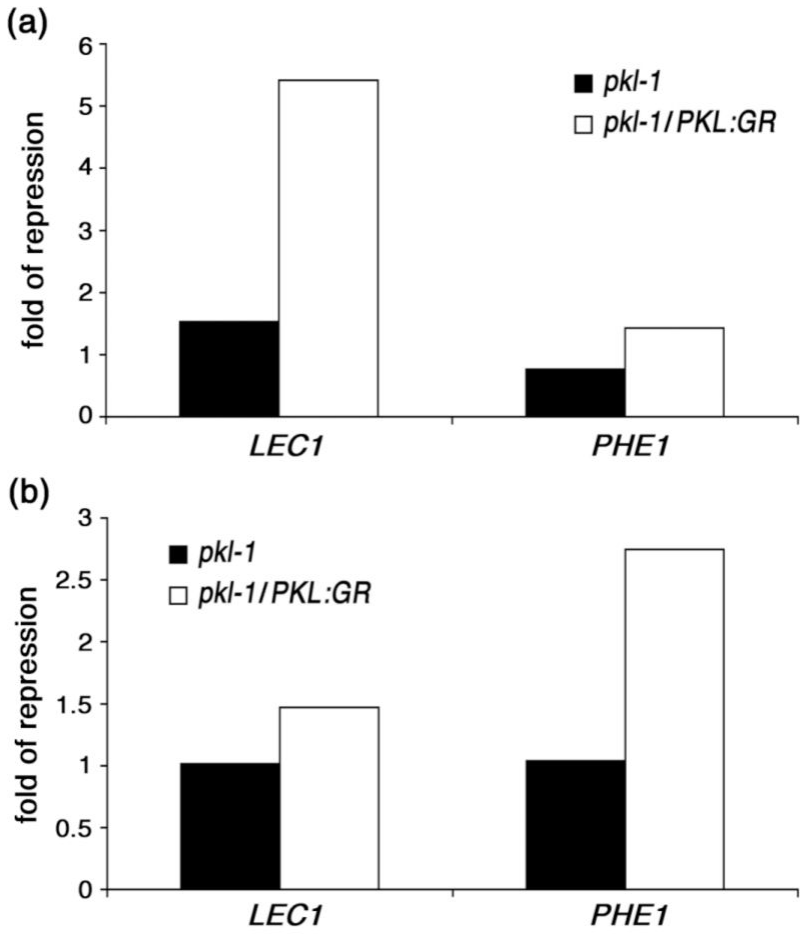

Figure 7.

PKL acts early during germination to determine expression of *LEC1*. Quantitative RT-PCR was used to determine the relative transcript levels of *LEC1* and *PHE1* in *pkl* and *pkl-1/PKL:GR* seedlings in response to a variety of Dex treatments. 18S rRNA was used as a standardization control.

(a) Relative *LEC1* and *PHE1* transcript levels were determined in *pkl* and *pkl-1/PKL:GR* seedlings that had been imbibed in the absence or presence of 10^{-5} M Dex until they reached 50% germination. Expression levels in Dex-treated plants are normalized relative to plants grown in the absence of Dex.

(b) Relative *LEC1* and *PHE1* transcript levels were determined in *pkl* and *pkl-1/PKL:GR* seedlings that had been imbibed in the absence of Dex until they reached 75% germination and then exposed to 10^{-5} M Dex for 48 hours or grown for 48 hours in the continued absence of Dex. Expression levels in Dex-treated plants are normalized relative to plants grown in the absence of Dex.

Table 1

Relative transcript levels for *PHERES1* and *PHERES2* in *pkl* mutants. Transcript levels were determined for *pkl-1* and wild-type plants at 50% radicle emergence (Germination), in young seedings (4 day) and in 21-day-old leaves (21 day). 18s rRNA was used as a standardization control. levels are normalized relative to wild-type plants.

AGI Code	Gene	Fold Change (*pkl/wt*)		
		Germination	4 day	21 day
At1g65330	PHERES1	0.9	161	151
At1g65300	PHERES2	3	40	27

Table 2

Relative transcript levels for Type I MADS-box transcription factors in *pkl* mutants. Transcript levels were determined for *pkl-1* and wild-type plants at 50% radicle emergence (Germination), in young seedlings (4 day) and in 21-day-old leaves (21 day). 18s rRNA was used as a standardization Expression levels are normalized relative to wild-type plants. Only those loci that showed greater a 5-fold change are presented. ND indicates transcript not detected.

		Fold Change (*pkl*/*wt*)		
AGI Code	Gene	Germination	4 day	21 day
At2g28700		ND	17	3.1
At3g66656		7.1	1.5	0.83
At5g26630	AGL 35	5.1	0.57	0.74
At1g18750	AGL 65	0.08	0.96	0.71