Published in final edited form as:
Plant J. 2003 July ; 35(1): 33–43.

Coordinate Repression of Regulators of Embryonic Identity by *PICKLE* During Germination in Arabidopsis

Stanley Dean Rider Jr.[1], **James T. Henderson**[1], **Ronald E. Jerome**[2], **Howard J. Edenberg**[2], **Jeanne Romero-Severson**[3], and **Joe Ogas**[1,*]

1*Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-2063*

2*Department of Biochemistry and Molecular Biology and Center for Medical Genomics, Indiana University School of Medicine, Indianapolis, Indiana 46202*

3*Department of Forestry and Natural Resources and Computational Genomics Center, Purdue University, West Lafayette, Indiana 47907-1159*

Abstract

In angiosperms, germination represents an important developmental transition during which embryonic identity is repressed and vegetative identity emerges. *PICKLE* (*PKL*) encodes a CHD3-chromatin remodeling factor necessary for the repression of expression of *LEAFY COTYLEDON1* (*LEC1*), a central regulator of embryogenesis. A candidate gene approach and microarray analysis identified nine additional genes that exhibit *PKL*-dependent repression of expression during germination. Transcripts for all three *LEAFY COTYLEDON* genes, *LEC1*, *LEC2*, and *FUS3*, exhibit *PKL*-dependent repression, and all three transcripts are elevated more than 100-fold in *pkl* primary roots that inappropriately express embryonic traits (“pickle roots”). Three other genes that exhibit *PKL*-dependent regulation have expression patterns correlated with zygotic or somatic embryogenesis, and one gene encodes a putative LIM domain transcriptional regulator that is preferentially expressed in siliques. Genes that exhibit *PKL*-dependent repression during germination are not necessarily regulated by *PKL* at other points in development. Our data suggest that *PKL* selectively regulates a suite of genes during germination to repress embryonic identity. In particular, we propose that *PKL* acts as a master regulator of the *LEAFY COTYLEDON* genes, and that joint derepression of these genes is likely to contribute substantially to expression of embryonic identity in *pkl* seedlings.

Keywords

CHD3; chromatin remodeling factors; developmental transition; embryo; seed; germination

INTRODUCTION

The germination of a seed is a dramatic developmental event that is dependent on the plant growth regulators gibberellin (GA) and abscisic acid (ABA) and on multiple other factors (Koornneef *et al.*, 2002). In the *pkl* mutant of Arabidopsis, this developmental transition is perturbed; *pkl* seedlings continue to express embryonic identity after germination (Ogas et al., 1997). *PKL* is predicted to encode a member of the CHD3 family of chromatin remodeling proteins (Eshed *et al.*, 1999; Ogas *et al.*, 1999) suggesting that the inability of *pkl* seedlings to repress embryonic identity is a result of a defect in transcriptional regulation. The name ‘CHD’

*To whom correspondence should be addressed (phone 765-496-3969, fax 765-494-7897, e-mail ogas@purdue.edu)

is derived from the predicted domain structure of these proteins, which includes two copies of a chromatin organization modifier domain (chromodomain), a SWI/SNF - ATPase domain, and a motif with sequence similarity to a DNA-binding domain (Woodage *et al.*, 1997). In addition to these domains, CHD3 proteins also contain one or more plant homeodomain (PHD) zinc fingers.

Ongoing work with CHD3 proteins in animal systems indicates that CHD3 proteins mediate transcriptional repression and that they play specific roles in development (Ahringer, 2000). A CHD3 protein is a component of the multisubunit Mi-2/NURD complex, a major histone deacetylation complex in Xenopus and mammalian cell lines that possesses nucleosome remodeling activity (Tong *et al.*, 1998; Wade *et al.*, 1998; Xue *et al.*, 1998; Zhang *et al.*, 1998). This complex is hypothesized to play a role in establishment and/or maintenance of hypoacetylated domains of chromatin that result in transcriptional repression of target genes. *Drosophila* that are homozygous for a mutation in the CHD3-encoding gene *dMi-2* die at the first or second larval instar (Kehle *et al.*, 1998). *dMi-2* and the corresponding CHD3 protein have been shown to interact genetically and physically with key transcriptional regulators of embryo development and neuronal cell fate determination (Kehle *et al.*, 1998; Murawsky *et al.*, 2001). In particular, dMi-2 binds to the gap protein Hunchback and participates in the repression of the homeotic *HOX* genes (Kehle *et al.*, 1998). Thus, dMi-2 appears to function as a transcriptional repressor that is recruited to different promoters through interaction with a number of proteins and plays a role in several developmental pathways. In a similar vein, *C. elegans* Mi-2 genes are necessary for the proper specification of developmental identity in vulval cell precursors (Solari *et al.*, 2000; von Zelewsky *et al.*, 2000).

Phenotypic characterization of the *pkl* mutant reveals that CHD3-chromatin remodeling factors also play a role in plant development. *PKL* is necessary for the repression of embryonic identity in Arabidopsis seedlings (Ogas *et al.*, 1997; Ogas *et al.*, 1999). The most striking phenotype of *pkl* seedlings is the presence of large, swollen primary roots (referred to as pickle roots) that express many embryonic characteristics, including the ability to undergo somatic embryogenesis. GA represses the expression of the pickle root phenotype. Furthermore, the ability of GA to repress penetrance of the pickle root phenotype is restricted to the developmental period of 24 to 36 h following imbibition (seed wetting) (Ogas *et al.*, 1997). Thus *PKL* and GA function early during germination to prevent the misexpression of embryonic identity that is evident later in seedling development.

Characterization of gene expression in *pkl* mutants suggests that PKL acts as a negative regulator of transcription, analogous to animal CHD3 proteins. Expression of *LEC1*, a key promoter of embryonic identity that is normally expressed only in seeds (Lotan *et al.*, 1998; Meinke, 1992), is derepressed in the *pkl* mutant during germination (Ogas *et al.*, 1999). Furthermore, the *LEC1* transcript is elevated during the time at which derepression of embryonic identity in *pkl* seedlings has been shown to be dependent on GA, suggesting that *LEC1* may be a direct target of *PKL*.

The objective of this study was the identification of other genes that might contribute to derepression of embryonic identity in *pkl* seedlings. Examination of candidate gene expression and analysis of microarrays identified nine additional genes that exhibited robust *PKL*-dependent repression of expression during germination, five of which have been previously associated with embryogenesis. The data suggest that *PKL* selectively represses key promoters of embryonic identity during germination so that vegetative identity can become fully established in the developing *Arabidopsis* seedling.

RESULTS

PKL regulates expression of *LEC2* in germinating seeds

We examined whether other genes associated with zygotic or somatic embryogenesis exhibited *PKL*-dependent repression similar to *LEC1*. We examined the expression of all three *LEC* genes, *LEC1* (Lotan *et al.*, 1998), *LEC2* (Stone *et al.*, 2001), and *FUS3* (Luerssen *et al.*, 1998), as well as another master regulator involved in embryo maturation *ABI3* (Giraudat *et al.*, 1992). We looked at the expression of another transcriptional regulator, *AGL15* (Heck *et al.*, 1995), that is preferentially expressed in embryos but for which a corresponding loss-of-function allele has yet to be phenotypically characterized. We also examined the expression of two genes that promote somatic embryogenesis when overexpressed, *AtSERK1* (Hecht *et al.*, 2001) and *WUSCHEL* (Zuo *et al.*, 2002). These genes were not meant to be an exhaustive list of genes associated with embryogenesis but rather a representative selection with which we could address the generality of *PKL*-dependent repression.

We examined the relative transcript level of these genes in germinating seeds that were developmentally staged such that 50% of the seeds had cracked seed coats. This developmental stage was selected based on our previous characterization of the behavior of *LEC1* transcript in germinating *pkl* seed (Ogas *et al.*, 1999). Quantitative RT-PCR (qRT-PCR) was performed using total RNA from wild-type and *pkl* seed imbibed in either the absence or presence of uniconazole-P, an inhibitor of GA biosynthesis (Izumi *et al.*, 1985). We examined whether or not transcript levels were dependent on uniconazole-P to address the possibility that altered expression of one or more of these genes might contribute to the increased penetrance of the pickle root phenotype that is observed when *pkl* seeds are germinated in the presence of uniconazole-P (Ogas *et al.*, 1997).

We observed that transcript levels of both *LEC1* and *LEC2* were greatly increased in germinating *pkl* seeds (Figure 1). The *LEC2* transcript was elevated more than 8-fold in germinating *pkl* seeds relative to germinating wild-type seeds, whereas the *LEC1* transcript was elevated more than 5-fold. The mRNA level of both genes was largely insensitive to uniconazole-P. Although other studies have shown that the *LEC2* transcript was present during germination (Stone *et al.*, 2001) and that the *LEC1* transcript was not (Lotan *et al.*, 1998;Ogas *et al.*, 1999), qRT-PCR analysis revealed that transcripts for both genes are present during germination. The *FUS3* transcript was only elevated in *pkl* seeds imbibed in the presence of uniconazole-P. Subsequent analyses, however, revealed that the level of the *FUS3* transcript is also *PKL*-dependent in the absence of uniconazole-P when assayed at a later stage of development (100% germination versus 50% cracked seed coats; see below). Thus the transcript level of all three *LEC* genes is *PKL*-dependent.

Expression of the other candidate genes was largely *PKL*- and uniconazole-P-insensitive, with the notable exception of *AGL15* and *AtSERK1*. Surprisingly, the mRNA level of both of these genes is substantially reduced (> 10-fold) in germinating *pkl* seeds if they are imbibed in the presence of uniconazole-P (Figure 1). Thus expression of both of these putative positive regulators of embryonic identity is decreased under conditions that lead to increased expression of the pickle root phenotype. Although this result suggests that *AGL15* and *AtSERK1* do not contribute to expression of the pickle root phenotype, a loss-of-function mutation in these genes would be necessary to test this hypothesis. Such a loss-of-function mutation has not been described for either of these loci. The level of *ABI3* and *WUSCHEL* transcripts (Figure 1) are not *PKL*-dependent at this point in development. Consequently, altered expression of these genes is unlikely to contribute to determination of the potential to express embryonic identity in *pkl* seedlings.

Microarray analysis reveals additional genes that exhibit *PKL*-dependent expression

The finding that two known master regulators of embryonic identity (*LEC1* and *LEC2*) exhibit *PKL*-dependent expression relatively early in germination whereas another well-characterized regulator (*ABI3*) does not was consistent with our hypothesis that *PKL* acts to repress certain genes that promote embryonic identity. To identify additional putative targets of PKL, we utilized oligonucleotide-based microarrays from Affymetrix that represent 8256 genes covering approximately 30% of the *Arabidopsis thaliana* transcriptome. We examined transcript levels in *pkl* and wild-type seeds imbibed in the presence or absence of uniconazole-P until 50% of the seeds had cracked seed coats as above. A portion of seeds from each treatment was retained to determine germination percentages, and to determine the effect that uniconazole-P had on pickle root penetrance. Greater than 99% of the seeds germinated and became seedlings for all treatments. Pickle root penetrance increased from 7.1% on MS media to 59.4% on MS media supplemented with uniconazole-P. The microarray experimental design then consisted of four treatments: untreated wild type (Wt), untreated *pkl* mutant (Pkl), uniconazole-P treated wild type (Uwt) and uniconazole-P treated *pkl* mutant (Upkl). There were four replications per treatment, for a total of 16 microarrays.

Our analyses of *LEC1* and *LEC2* revealed that expression of these genes is strongly derepressed in *pkl* seeds and unaffected by uniconazole-P treatment (Figure 1). Based on these results, we queried our data set for genes that exhibited altered expression in germinating *pkl* seeds regardless of uniconazole-P treatment. We compared expression values using the *t*-test.

The *t*-test criterion ($p < 0.05$) identified 759 genes that were expressed at different levels in wild-type and *pkl* seeds on MS media. For *pkl* and wild-type seeds imbibed on MS media supplemented with uniconazole-P the same criterion identified 1188 genes. We took the intersection of the two sets of genes described (293 genes). A Venn diagram depicting this intersection is shown in Figure 2. Of the genes represented by the intersection, 185 had higher transcript levels in *pkl* seeds in the presence or absence of uniconazole-P whereas 101 genes had decreased transcript levels (see http://www.biochem.purdue.edu/research/ogas_lab/arrays/). The subset of genes identified by this analysis as exhibiting *PKL*-dependent expression represented a little more than 3.5% of the total number of elements present on the array. Among the genes that code for a protein with a known or putative function (158), there was little change in the relative abundance of the basic types of protein function represented on the arrays overall, with the exception of heat shock proteins and proteins involved in translation (Figure 2). The percentage of both of these functional classes was found to be increased approximately four-fold among genes that exhibit *PKL*-dependent expression when compared to their representation among all of genes found on the microarrays.

The *LEC1* and *LEC2* transcripts are elevated 4-fold or more in *pkl* seeds in both the absence and presence of uniconazole-P when assayed by qRT-PCR (Figure 1). Of the 185 genes with increased mRNA levels in *pkl* seed identified by microarray analysis, transcripts of 56 were elevated two-fold or more in *pkl* seeds in both the absence and presence of uniconazole-P. Based on this similarity of expression pattern, these genes were considered to be strong candidates for genes whose expression might show a dependence on *PKL* analogous to that of *LEC1* and *LEC2*.

We wished to verify that a subset of these genes exhibited *PKL*-dependent expression when assayed by an alternative method and then follow expression of these genes under several other conditions to explore the potential role of these genes in embryo development and in generation of *pkl*-associated phenotypes. Although it was tempting to pick genes based on putative function - transcriptional regulators for example - we were concerned that this approach might lead to an inaccurate assessment of the validity of the methods we used to identify the 56 genes

from the microarray dataset. We therefore randomly selected 12 of the 56 genes (Figure 3a) for further analysis for an unbiased test of the value of our selection criteria.

We re-examined the transcript levels for these 12 genes by qRT-PCR using the same total RNA pools from germinating seeds that were used for microarray analysis (Figure 3c). We found that transcripts of eight of the genes were significantly ($p < 0.05$) elevated in *pkl* seeds by qRT-PCR. Thus independent experimental verification of our candidates suggests that approximately 2/3 of the genes that we identified by microarray analysis will exhibit *PKL*-dependent expression when assayed by another means.

Transcripts of seven of the genes that we assayed were elevated by at least 4-fold in *pkl* seeds relative to wild-type seeds (Figure 3c). Thus the expression of these genes when assayed by qRT-PCR is dependent on *PKL* to an extent that is comparable to that of *LEC1* and *LEC2*. We decided to follow expression of these 7 genes and *LEC1* and *LEC2* to determine if the 9 genes exhibited similar transcriptional regulation under other conditions. We also included *FUS3* in this analysis based on the observation that the *FUS3* transcript was elevated in *pkl* seeds treated with uniconazole-P (Figure 1) and the fact that *FUS3* is also a *LEC* gene (Keith et al., 1994; Meinke et al., 1994).

The LIM domain transcription factor is preferentially expressed in developing siliques

We wished to test the possibility that one or more of the seven genes that exhibit at least a 4-fold elevation of transcript level in *pkl* seeds might be preferentially expressed in developing seeds in a manner analogous to *LEC1, LEC2*, or *FUS3* (Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001). Such a result would be consistent with the hypothesis that one or more of these genes might function as a positive regulator of embryonic identity analogous to the *LEC* genes. We therefore examined the relative abundance of transcripts in wild-type roots, leaves, and developing siliques using qRT-PCR (Figure 4). We included *FUS3* in this analysis as a positive control. We found that one gene (*At2g39900*) was preferentially expressed in siliques in a manner similar to *FUS3*, suggesting that it might also be involved in embryo development. This gene, previously identified as *AtWLIM2*, is predicted to encode a LIM domain transcription factor (Eliasson *et al.*, 2000). *At2g05520*, which encodes the glycine rich protein atGRP3, was preferentially expressed in leaves as observed previously (de Oliveira *et al.*, 1990). The remaining genes did not show significant changes in transcript levels in the tissues examined.

Some genes that exhibit *PKL*-dependent regulation during germination are highly expressed in pickle roots

To determine if the gene expression that is derepressed during germination in *pkl* mutant seeds is also elevated in pickle roots, we compared transcript levels in wild-type roots (non embryonic) to pickle roots (embryonic). If the transcript level of a gene was found to be elevated in a pickle root, this result would suggest that altered expression of the gene might play a role in maintenance of pickle root identity.

Among the eleven genes examined, transcripts for *LEC1, LEC2, FUS3, HSP 17.6A* and *atGRP3* are significantly elevated in pickle roots. qRT-PCR was performed using total RNA isolated from wild-type roots and pickle roots with gene-specific primers for the gene of interest (Figure 5). The *LEC1, LEC2*, and *FUS3* transcripts were each elevated more than 100-fold in pickle roots in comparison to wild-type roots. *LEC1, LEC2*, and *FUS3* encode transcriptional regulators that promote embryogenesis during both morphogenesis and maturation (Harada, 2001). Thus their enhanced expression in pickle root tissue is consistent with their known roles in promotion of embryonic identity. The transcripts for *HSP 17.6A* and *atGRP3* were elevated approximately 12-fold and 8-fold respectively in pickle root tissue, an expression pattern

consistent with previous observations. The *HSP 17.6A* transcript is elevated in maturing seeds (Sun *et al.*, 2001), and increased transcript levels for GRPs are correlated with somatic embryogenesis (Magioli et al., 2001;Sato et al., 1995). The other six genes did not have significantly higher transcript levels in pickle roots. This observation indicates that *PKL* is not constitutively required to maintain proper transcript levels of these six genes. Furthermore, although altered expression of these genes may play a role in establishment of pickle root identity, altered expression is apparently not required for maintenance of pickle root identity.

PKL is required for proper expression in leaves

Adult *pkl* plants are dark green late-flowering semi-dwarfs that exhibit many other shoot phenotypes that are consistent with a defect in GA signal transduction (Ogas *et al.*, 1997). Although previous analyses had indicated that organs generated post-embryonically in *pkl* plants do not express embryonic traits, we wished to determine whether or not the genes that exhibited *PKL*-dependent expression during germination might also require *PKL* for proper regulation of expression in leaf tissue. If the transcript level of a gene was found to be elevated in *pkl* leaves, the gene might play a role in generation of the adult shoot phenotype.

Three of the genes that exhibit *PKL*-dependent repression during germination are also overexpressed in *pkl* leaves. qRT-PCR was performed using total RNA isolated from wild-type leaves and *pkl* leaves with gene-specific primers for the gene of interest (Figure 6). Transcripts for *FUS3, GASA1*, and *Unknown1* were significantly ($p < 0.05$) higher in *pkl* leaves whereas transcript levels for the remaining genes were not *PKL*-dependent in leaves. The *LEC1* transcript was not detected in either *pkl* or wild-type leaves. The implication of this analysis is that inappropriate expression of *FUS3, GASA1*, and *Unknown1* contributes to the adult shoot phenotypes observed in *pkl* plants (see Discussion). In addition, analysis of gene expression in *pkl* leaves, as in pickle roots, reveals that *PKL* is only required at specific points in development for proper expression of many target genes.

Derepression of transcript levels in *pkl* occurs during germination

We have proposed that *LEC1* is directly repressed by PKL because of a correlation in the timing of two distinct events related to expression of embryonic identity in *pkl* seedlings. The transcript level of *LEC1* is elevated in *pkl* seeds during that time at which GA is necessary to repress expression of embryonic identity in *pkl* seeds, between 24 and 36 hours after imbibition (Ogas et al., 1997; Ogas et al., 1999). If the transcripts of the nine other genes that exhibit *PKL*-dependent repression were elevated in germinating *pkl* seeds with the same timing as the *LEC1* transcript, this result would suggest that these genes are also direct targets of PKL. For the subsequent analysis, it is important to note that we used seeds that were 100% germinated by 36 hours and that represent the same seed batch for which the timing of GA action was determined (Ogas et al., 1997) and in which the *LEC1* transcript was derepressed between 24 and 36 hours after imbibition (Ogas et al., 1999). The seeds used to derive the data in Figures 1-3 germinate at a slower rate (100% germination at 48 hours) and so various time points should not be compared in these experiments, only the relative state of germination.

We found that transcripts of all nine genes were elevated in germinating *pkl* seeds in a manner similar to that of *LEC1*. qRT-PCR was performed using the total RNA isolated from wild-type and *pkl* seeds pre-imbibition and 12, 24, and 36 hours after imbibition (Figure 7). All of the genes tested showed an increase in the relative expression in *pkl* seeds between 24 and 36 hours after imbibition. Thus the transcript level of these genes is coordinately derepressed during the time in which the fate of the primary root in *pkl* seedlings is determined. This observation is consistent with the hypotheses that inappropriate expression of these genes contributes to the observed inability of *pkl* seedlings to repress embryonic identity and that some or all of these genes are directly regulated by PKL.

DISCUSSION

PKL codes for a putative CHD3 chromatin-remodeling factor (Eshed *et al.*, 1999; Ogas *et al.*, 1999) that is required for repression of embryonic identity in seedlings and for repression of *LEC1* expression during germination (Ogas *et al.*, 1997; Ogas *et al*., 1999). Ongoing work in animal systems indicates that CHD3 proteins function as negative regulators of transcription that are recruited to specific promoters. It was recently found that a CHD3 protein plays a role in development of *C. elegans* that is strikingly similar to that of PKL in Arabidopsis; worm embryos in which expression of *LET-418* (a CHD3 gene) has been decreased by RNAi are defective in repression of germline fate in somatic cells (Unhavaithaya et al., 2002). Characterization of the role of *PKL* enables us to explore the role of a CHD3 protein in regulation of gene expression in plants in the context of an analogous developmental function (repression of embryonic identity).

Identification of additional genes that exhibit *PKL*-dependent expression

Wild-type levels of GA are necessary between 24 and 36 hours after seed imbibition to repress expression of embryonic identity in *pkl* primary roots (the “pickle root” phenotype). Similarly, the *LEC1* transcript is first elevated in germinating *pkl* seedlings between 24 and 36 hours after imbibition. The implication of these two observations is that *PKL* and GA are acting during this time to determine the ability of *pkl* seedlings to express embryonic identity later in development. Thus we chose to screen for genes that exhibited *PKL*-dependent transcript levels during this stage of development to give us the greatest chance to identify i) genes whose inappropriate expression contribute to derepression of embryonic identity and ii) genes that are directly regulated by *PKL*.

A candidate gene approach was undertaken to examine the role of *PKL* in regulation of expression of other genes previously implicated in embryogenesis (Figure 1). Of the six genes examined, only the *LEC2* transcript behaved similarly to the *LEC1* transcript and was strongly elevated in germinating *pkl* seed. In contrast, expression of the seed-specific maturation regulatory factor *ABI3* (Giraudat *et al.*, 1992) was not significantly altered under any condition examined. Thus these data reveal that *PKL* is not universally required for repression of all regulatory genes involved in seed formation at this point in development.

The microarray data provided evidence that 293 genes (3.5% of the genes queried) may exhibit *PKL*-dependent expression before germination is complete. These genes do not tend to occur in tandem arrays on a chromosome, an observation that supports the hypothesis that *PKL* acts in a locus-specific manner. To our knowledge, these data represent the first attempt to characterize the effect of a mutant CHD3-encoding gene on global transcript levels in the context of a developmental transition. In this light, it is intriguing to note that the representation of genes that code for predicted heat shock proteins is increased among the genes that exhibit *PKL*-dependent expression in comparison to their representation among all of the genes on the arrays. The expression of heat shock proteins is induced during seed maturation (Wehmeyer *et al.*, 2000). Similarly, we found that **12** of the 56 genes identified by microarray analysis as having transcript levels increased by two-fold or more in *pkl* seeds may be associated with some aspect of embryo or seed development, an observation consistent with the established role of *PKL* in repression of embryonic differentiation traits (See http://www.biochem.purdue.edu/research/ogas_lab/arrays/ Supplementary Table 1a). These data are also consistent with observations made by others regarding the role of CHD3 proteins in repressing alternative differentiation states in animal systems (Kehle et al., 1998; Solari et al., 2000; Unhavaithaya et al., 2002; von Zelewsky et al., 2000)

We have also identified 69 genes that exhibit uniconazole-P dependent expression that is not dependent on the state of the *PKL* locus as well as 88 genes that are expressed at different levels

in *pkl* seeds germinated in the presence of uniconazole-P. (See http://www.biochem.purdue.edu/research/ogas_lab/arrays/ for a complete list of these genes.) Our observations regarding analysis of these genes will be described elsewhere.

We chose to focus our subsequent analysis on genes that exhibit robust *PKL*-dependent expression analogous to that of *LEC1* and *LEC2*. By analogy to the known roles of *LEC1* and *LEC2* in embryogenesis (Lotan *et al.*, 1998; Stone *et al.*, 2001), we felt that genes exhibiting similar *PKL*-dependent regulation were likely to contribute substantively to the derepression of embryonic identity in *pkl* seedlings. Of the 293 genes that we identified as exhibiting *PKL*-dependent expression by microarray analysis, transcript levels for 56 were elevated by at least two-fold in *pkl* seeds. We selected 12 genes for verification of *PKL*-dependent expression by qRT-PCR and observed that expression of 8 of the 12 genes was elevated in *pkl* seedlings. These results indicated that our approach worked well. It should be noted, however, that the final determination of whether or not our 66% success rate is a reliable predictor of future success would be dependent on repeated trials and/or computer simulation studies. Transcript levels of 7 of the genes were elevated four-fold or more in germinating *pkl* seed. These genes thus exhibited *PKL*-dependent expression that was comparable to that of *LEC1* and *LEC2* when assayed by qRT-PCR (Figure 1) and were therefore selected for follow-up analysis.

Identification of a potential transcriptional regulator of embryogenesis

We examined whether or not the seven genes that had transcript levels that were elevated four-fold or more in *pkl* seed were preferentially expressed in developing siliques. We carried out this analysis in part to address the hypothesis that one or more of the genes might have a seed-specific pattern of expression analogous to the *LEC* genes. We observed that *AtWLIM2* was preferentially expressed in developing siliques in a manner analogous to the *LEC* gene *FUS3* (Figure 4).

It should be noted that our expression analysis of whole siliques was designed only to identify genes whose transcripts were strongly elevated in developing seeds similarly to the *LEC* genes. Such an analysis would not identify genes for which the corresponding transcript level was more modestly elevated in embryos, or those genes for which the corresponding transcript was elevated at a different point in development. In fact, the *HSP 17.6A* transcript has been shown to be elevated in maturing seeds (Sun *et al.*, 2001). In addition, it is intriguing to note that two of the seven genes are related to genes whose expression is elevated during somatic embryogenesis; elevated expression of glycine-rich proteins such as GRP3 is associated with somatic embryogenesis (Magioli et al., 2001; Sato et al., 1995) and the apospory-related gene has sequence similarity to a gene whose expression is elevated during apomixis in buffelgrass (Gustine et al., 1993).

In an attempt to explore the possibility that expression of genes that exhibit *PKL*-dependent expression during germination is correlated with embryogenesis, we examined the transcript levels for all 56 genes that we identified as exhibiting 2× or greater dependence on *PKL* in a previously generated microarray dataset of developing seeds (Girke et al., 2000). This analysis was not very informative; only 3 of 56 genes were represented in the developing seed microarray dataset. None of the 3 genes were represented in the 12 genes that we examined by qRT-PCR. Transcript levels for these three genes were not elevated in developing embryos and did not change during embryogenesis (Girke et al., 2000; Ruuska et al., 2002) (See http://www.biochem.purdue.edu/research/ogas_lab/arrays/ Supplementary Table 1a).

If expression of one or more genes that exhibit *PKL*-dependent expression during germination were not elevated at some point during seed development, this observation would not rule out the possibility that these genes may play a significant role during embryogenesis. In this regard, it is interesting to note that comparison of microarray data with phenotypic characterization of

deletion mutants of *Saccharomyces cerevisiae* in various growth conditions has revealed that relative expression of a gene in a particular context is not sufficient to serve as a robust predictor of the functional significance of the gene in that context (Giaever *et al.*, 2002).

The observation that *AtWLIM2* is expressed preferentially in developing siliques is intriguing. Transcription factors that contain LIM domains are important regulators of development in animal systems (Dawid *et al.*, 1998). The AtWLIM2 protein has greater than 50% sequence identity to Ntlim2, which has been demonstrated to bind to DNA in a sequence-specific manner and to regulate transcription of genes involved in phenylpropanoid biosynthesis in tobacco (Kawaoka *et al.*, 2000). This level of sequence conservation in combination with the observed expression profile suggests that AtWLIM2 may be functioning as a transcriptional regulator in Arabidopsis embryo development in a manner analogous to LEC transcriptional regulators. In support of a potential role for *AtWLIM2* in embryogenesis, we have observed that plants that are heterozygous for a T-DNA tagged allele of *AtWLIM2* exhibit a segregating embryo-lethal phenotype but have yet to determine whether or not this phenotypic trait is linked to the mutant *Atwlim2* allele (unpublished data).

PKL is a negative regulator of the *LEAFY COTYLEDON* genes

Analysis of transcript levels in pickle roots suggests that derepression of the *LEC* genes is likely to play a significant role in generation of the pickle root phenotype. Transcript levels for *LEC1*, *LEC2*, and *FUS3* are elevated more than 100-fold in pickle roots in comparison to wild-type roots. Transgenic seedlings in which *LEC1* or *LEC2* are ectopically expressed continue to exhibit embryonic differentiation traits after germination (Lotan *et al.*, 1998; Stone *et al.*, 2001). Based on the combined observations that all three *LEC* genes are coordinately regulated by *PKL* during germination (Figure 7) and that expression of all three genes is greatly increased in pickle roots (Figure 5), we propose that *PKL* is acting as a master regulator that is required for repression of all three genes during germination. We furthermore propose that inappropriate expression of these three genes is likely to contribute substantively to the ability of *pkl* primary roots to continue to express embryonic differentiation traits after germination. Analysis of the expression pattern of the *LEC* genes in germinating *pkl* seeds through a GUS reporter or in situ RNA analysis might shed light on why embryonic identity is preferentially expressed in the primary roots of *pkl* seedlings. We are unaware of any other transcriptional regulator that has been shown to be necessary for proper expression of all three *LEC* genes.

Identification of a putative *PKL*-dependent regulon specifically associated with germination

Genes that exhibit *PKL*-dependent expression during germination do not necessarily exhibit a similar dependence on *PKL* at other times during plant development. Expression of five of 10 of the genes is not elevated in pickle root tissue (Figure 5). Thus although inappropriate expression of these genes may play a role in determining the potential to express the pickle root phenotype, inappropriate expression of these genes is not a necessary differentiation characteristic of the pickle root itself. In contrast, we observe that three genes exhibit *PKL*-dependent expression in leaves (Figure 6), suggesting that inappropriate expression of these genes may play some role in generation of the shoot phenotype. Derepression of embryonic identity has not been observed in *pkl* leaves. Instead, *pkl* shoots express phenotypes that are consistent with a defect in GA signal transduction (Ogas *et al.*, 1997;Ogas *et al.*, 1999). In this regard, further exploration of the role of *PKL* in repression of *GASA1*, a gene that is known to exhibit GA-dependent expression (Herzog *et al.*, 1995), may shed light on the role of *PKL* in GA-dependent responses.

Despite the fact that these genes do not exhibit uniform *PKL*-dependent expression at other points in development, expression of these genes is remarkably similar during germination of *pkl* seeds. The transcripts of all eleven genes, not only the *LEC* genes, are elevated in *pkl* seeds

between 24 and 36 hours after imbibition. It remains to be determined if the *pkl*-dependent derepression of all of these genes will continue to appear as uniform if the transcript levels are measured at more frequent intervals. Nonetheless, the implication of this observation is that these genes identify a *PKL*-dependent regulon that requires *PKL* for repression of transcription during germination but not necessarily at other points in development.

This result is particularly striking given that the genes were initially identified based solely on their relative transcript level in *pkl* seeds at a single point in germination. It seemed quite reasonable to anticipate that some transcripts might exhibit continuous elevation in the absence of *PKL*, rather than elevation at a particular stage of the germination process. Our hypothesis is that the transcript level of these genes is elevated specifically at this point in germination because PKL is necessary at this time to directly repress transcription of one or more of these genes. Consistent with such a proposed time of action, we have observed that the *PKL* transcript is expressed during imbibition (unpublished data) in addition to other stages of the life cycle (Ogas et al., 1997). Analysis of the sequence of the upstream regions of these *PKL*-dependent genes does lead to the identification of common sequence motifs (data not shown), but it is not yet known if any of these motifs are involved in conferring *PKL*-dependent regulation.

The data presented has not illuminated the role of GA in penetrance of the pickle root phenotype. We have shown that the transcript levels of a number of genes, some of which are known positive regulators of embryonic identity, are coordinately derepressed during germination in *pkl* seedlings. Our working model is that inappropriate expression of these genes contributes to the inability of *pkl* seedlings to repress embryonic identity and that one or more of these genes are directly repressed by PKL, a CHD3-chromatin remodeling factor. Strikingly, although inhibition of GA biosynthesis through the application of uniconazole-P greatly increases penetrance of the pickle root phenotype (Ogas *et al.*, 1997), the presence of uniconazole-P either has no effect on or slightly decreases the transcript level of the *PKL*-dependent genes examined (Figure 3). Our interpretation of these observations is that the absence of *PKL* leads to the transcriptional derepression of a set of genes that have the potential to generate embryonic differentiation traits. In some seedlings, the inappropriate expression of this suite of genes leads to the establishment of the pickle root phenotype, in which the primary root terminally differentiates into an organ that expresses both root-like and embryo-like differentiation traits. Inhibition of GA biosynthesis during germination increases the probability that the transcriptional derepression of the *PKL*-dependent genes will lead to establishment of the pickle root phenotype by a mechanism that has yet to be identified. Further characterization of genes that are expressed at significantly different levels when *pkl* seeds are germinated in the presence of uniconazole-P may be informative in this regard (Figure 1, see http://www.biochem.purdue.edu/research/ogas_lab/arrays/).

In summary, we have identified a number of genes that exhibit *PKL*-dependent repression during germination. Limited characterization of these genes suggests that *PKL* is necessary for repression for some but not all regulators of embryogenesis. In particular, *PKL* is necessary for repression of the *LEC* genes. We anticipate that further characterization of the genes that we have identified will lead to the discovery of novel regulators of embryogenesis as well as to uncovering genes that are directly regulated by *PKL*.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions

Seeds or plants incubated on synthetic media (Ogas *et al.*, 1997) were grown in a CU36L5 incubator (Percival Scientific) under 24h illumination. Plants in pots were grown in AR75L growth chambers (Percival Scientific) under 24h illumination. For expression analysis during germination by both qRT-PCR and microarray (Figures 1, 2, and 3), seeds were sown at a

density of 100 mg of seeds (~3000 seeds) per 150 mm diameter petri dish. The media was supplemented with 10^{-8} M uniconazole-P or solvent alone (0.01% methanol final concentration). Samples were collected when 50% of the seeds had cracked seed coats (approximately 32 h post imbibition). Alternatively, samples were collected at 0, 12, 24, and 36 h post imbibition (Figure 7). Root, leaf, and silique tissue was collected from 32-day old plants. Siliques in the early stages of development at which both heart stage and torpedo stage embryos could be observed (which consisted of the first 5 silique pairs after the siliques were greater than 1 cM in length) were collected for RNA extraction. For expression analysis of pickle root tissue and wild-type root tissue plants were grown on media containing 10^{-8} M uniconazole-P and for 4 d and then transferred to new plates containing 10^{-6} M cytokinin. Root tissue was collected after 3 wk on these plates.

RNA Isolation and RNA Hybridization

Total RNA was isolated as described previously (Verwoerd *et al.*, 1989). All subsequent experimental manipulations were carried out as per manufacturers instructions. Total RNA was further purified with Qiagen RNeasy columns (catalog 74904). cDNA was generated from 10 μg of total RNA using the Superscript Choice System (Gibco BRL, Life Technologies). A T7-$(dT)_{24}$ oligonucleotide was used for first strand synthesis. Double stranded cDNA was cleaned using phenol:chloroform extraction and Phase Lock Gel (Eppendorf-5 Prime). Biotin labeled cRNA was generated using the BioArray High Yield RNA Transcript Labeling Kit (ENZO), cleaned and fragmented. Affymetrix Gene Chips (Arabidopsis Genome Array, catalog #900292) were hybridized with labeled cRNA at 45°C for 16 hours in an Affymetrix GeneChip Hybridization Oven 640. Washing and staining of the chips was performed using the Affymetrix Gene Chip Fluidics Station 400. Arrays were scanned in an HP GeneArray Scanner and data was analyzed with the Affymetrix Microarray Suite v. 4.0 software.

Quantitative RT-PCR

Total RNA (6 μg) was treated with RQ1 DNase (Promega) in a 10 μl reaction using the manufacturer's protocol. DNase treated RNA (4 μg) was reverse transcribed using SuperScript II and 100 ng random hexamers. The cDNA (~2μg) was diluted to ~2.6 ng/μl prior to quantitative PCR. Primers were designed using Primer Express 2.0 software (Applied Biosystems). Quantitative PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) as recommended by the manufacturer. Reaction volumes were scaled to 20 μl final volume and were comprised of 10 μl SYBR Green PCR master mix (Applied Biosystems), 5 μl primer mix, and 5 μl (~13 ng) template cDNA. All reactions were repeated in triplicate. 18S ribosomal RNA was used as a normalization control for the relative quantification of transcript levels. Oligonucleotide primer sequences and primer concentrations used as well as critical threshold values for the figures presented can be found at http://www.biochem.purdue.edu/research/ogas_lab/arrays/.

Statistical Analysis and Archiving of Array Data

See Note 1, supplemental material at http://www.biochem.purdue.edu/research/ogas_lab/arrays/ for a complete description of analyses performed. The complete array data set has been deposited at the Gene Expression Omnibus (GEO) of NCBI, accession number GPL244.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We would like to thank Rick Westerman, Jeff Gustin, Monica Alvarez, and Linda Quach for their assistance in sequence analysis. We thank Heather Hostetler and Clint Chapple for thoughtful discussions. This work was supported in part by grants from the National Institutes of Health (R01GM059770-01A1 and 5R01GM59770-02). Microarray analyses were partly supported by a grant to HJE from the Indiana 21st Century Research and Development Fund and by the Indiana Genomics Initiative, partly supported by the Lilly Endowment, Inc. Additional support was obtained by a grant to JRS from the Indiana 21st Century Research and Development Fund. SDR. was supported by funds from BASF. This is journal paper number 16933 of the Purdue University Agricultural Experiment Station.

REFERENCES

Ahringer J. NuRD and SIN3 histone deacetylase complexes in development. Trends Genet 2000;16:351–356. [PubMed: 10904264]

Dawid IB, Breen JJ, Toyama R. LIM domains: multiple roles as adapters and functional modifiers in protein interactions. Trends Genet 1998;14:156–162. [PubMed: 9594664]

de Oliveira DE, Seurinck J, Inze D, Van Montagu M, Botterman J. Differential expression of five Arabidopsis genes encoding glycine-rich proteins. Plant Cell 1990;2:427–436. [PubMed: 2152168]

Eliasson A, Gass N, Mundel C, Baltz R, Krauter R, Evrard JL, Steinmetz A. Molecular and expression analysis of a LIM protein gene family from flowering plants. Mol. Gen. Genet 2000;264:257–267. [PubMed: 11085265]

Eshed Y, Baum SF, Bowman JL. Distinct mechanisms promote polarity establishment in carpels of Arabidopsis. Cell 1999;99:199–209. [PubMed: 10535738]

Giaever G, Chu AM, Ni L, Connelly C, Riles L, Veronneau S, Dow S, Lucau-Danila A, Anderson K, Andre B, et al. Functional profiling of the Saccharomyces cerevisiae genome. Nature 2002;418:387–391. [PubMed: 12140549]

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM. Isolation of the Arabidopsis ABI3 gene by positional cloning. Plant Cell 1992;4:1251–1261. [PubMed: 1359917]

Girke T, Todd J, Ruuska S, White J, Benning C, Ohlrogge J. Microarray analysis of developing Arabidopsis seeds. Plant Physiol 2000;124:1570–1581. [PubMed: 11115875]

Gustine, DL.; Sherwood, RT.; Hulce, DA. A strategy for cloning apomixis-associated cDNA markers from buffelgrass. In: Baker, MJ.; Crush, JR.; Humphreys, LR., editors. Proceedings of the XVII International Grassland Congress. 2. Dunmore Press; Palmerston North: 1993. p. 1033-1034.

Harada JJ. Role of Arabidopsis *LEAFY COTYLEDON* genes in seed development. J. Plant. Physiol 2001;158:405–409.

Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt ED, Boutilier K, Grossniklaus U, de Vries SC. The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol 2001;127:803–816. [PubMed: 11706164]

Heck GR, Perry SE, Nichols KW, Fernandez DE. AGL15, a MADS domain protein expressed in developing embryos. Plant Cell 1995;7:1271–1282. [PubMed: 7549483]

Herzog M, A.-M. D, Grellet F. *GASA*, a gibberellin-regulated gene family from *Arabidopsis thaliana* related to the tomato *GAST1* gene. Plant Mol. Bio 1995;27:743–752. [PubMed: 7727751]

Izumi K, Kamiya Y, Sakurai A, Oshio H, Takahashi N. Studies of sites of action of a new plant growth retardant (E)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol (S-3307) and comparative effects of its stereoisomers in a cell-free system from *Cucurbita maxima*. Plant Cell Physiol 1985;26:821–827.

Kawaoka A, Kaothien P, Yoshida K, Endo S, Yamada K, Ebinuma H. Functional analysis of tobacco LIM protein Ntlim1 involved in lignin biosynthesis. Plant J 2000;22:289–301. [PubMed: 10849346]

Kehle J, Beuchle D, Treuheit S, Christen B, Kennison JA, Bienz M, Muller J. dMi-2, a hunchback-interacting protein that functions in polycomb repression. Science 1998;282:1897–1900. [PubMed: 9836641]

Keith K, Kraml M, Dengler NG, McCourt P. fusca3: A Heterochronic Mutation Affecting Late Embryo Development in Arabidopsis. Plant Cell 1994;6:589–600. [PubMed: 12244252]

Koornneef M, Bentsink L, Hilhorst H. Seed dormancy and germination. Curr. Opin. Plant Biol 2002;5:33–36. [PubMed: 11788305]

Lotan T, Ohto M, Yee KM, West MA, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ. Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. Cell 1998;93:1195–1205. [PubMed: 9657152]

Luerssen H, Kirik V, Herrmann P, Misera S. FUSCA3 encodes a protein with a conserved VP1/AB13-like B3 domain which is of functional importance for the regulation of seed maturation in Arabidopsis thaliana. Plant J 1998;15:755–764. [PubMed: 9807814]

Magioli C, Barroco RM, Rocha CAB, de Santiago-Fernandes LD, Mansur E, Engler G, Margis-Pinheiro M, Sachetto-Martins G. Somatic embryo formation in Arabidopsis and eggplant is associated with expression of a glycine-rich protein gene (Atgrp-5). Plant Science 2001;161:559–567.

Meinke DW. A homeotic mutant of Arabidopsis thaliana with leafy cotyledons. Science 1992;258:1647–1650. [PubMed: 17742538]

Meinke DW, Franzmann LH, Nickle TC, Yeung EC. *Leafy Cotyledon* mutants of Arabidopsis. Plant Cell 1994;6:1049–1064. [PubMed: 12244265]

Murawsky CM, Brehm A, Badenhorst P, Lowe N, Becker PB, Travers AA. Tramtrack69 interacts with the dMi-2 subunit of the Drosophila NuRD chromatin remodelling complex. EMBO Rep 2001;2:1089–1094. [PubMed: 11743021]

Ogas J, Cheng J-C, Sung ZR, Somerville C. Cellular differentiation regulated by gibberellin in the *Arabidopsis thaliana pickle* mutant. Science 1997;277:91–94. [PubMed: 9204906]

Ogas J, Kaufmann S, Henderson J, Somerville C. PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in Arabidopsis. Proc. Natl Acad. Sci. USA 1999;96:13839–13844. [PubMed: 10570159]

Ruuska SA, Girke T, Benning C, Ohlrogge JB. Contrapuntal networks of gene expression during Arabidopsis seed filling. Plant Cell 2002;14:1191–1206. [PubMed: 12084821]

Sato S, Toya T, Kawahara R, Whittier RF, Fukuda H, Komamine A. Isolation of a carrot gene expressed specifically during early-stage somatic embryogenesis. Plant Mol. Biol 1995;28:39–46. [PubMed: 7787186]

Solari F, Ahringer J. NURD-complex genes antagonise Ras-induced vulval development in Caenorhabditis elegans. Curr. Biol 2000;10:223–226. [PubMed: 10704416]

Stone SL, Kwong LW, Yee KM, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB, Harada JJ. LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. Proc. Natl Acad. Sci. USA 2001;98:11806–11811. [PubMed: 11573014]

Sun W, Bernard C, van de Cotte B, Van Montagu M, Verbruggen N. At-HSP17.6A, encoding a small heat-shock protein in Arabidopsis, can enhance osmotolerance upon overexpression. Plant J 2001;27:407–415. [PubMed: 11576425]

Tong JK, Hassig CA, Schnitzler GR, Kingston RE, Schreiber SL. Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. Nature 1998;395:917–921. [PubMed: 9804427]

Unhavaithaya Y, Shin TH, Miliaras N, Lee J, Oyama T, Mello CC. MEP-1 and a Homolog of the NURD Complex Component Mi-2 Act Together to Maintain Germline-Soma Distinctions in C. elegans. Cell 2002;111:991–1002. [PubMed: 12507426]

Verwoerd TC, Dekker BM, Hoekema A. A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res 1989;17:2362. [PubMed: 2468132]

von Zelewsky T, Palladino F, Brunschwig K, Tobler H, Hajnal A, Muller F. The C. elegans Mi-2 chromatin-remodelling proteins function in vulval cell fate determination. Development 2000;127:5277–5284. [PubMed: 11076750]

Wade PA, Jones PL, Vermaak D, Wolffe AP. A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase. Curr. Biol 1998;8:843–846. [PubMed: 9663395]

Wehmeyer N, Vierling E. The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. Plant Physiol 2000;122:1099–1108. [PubMed: 10759505]

Woodage T, Basrai MA, Baxevanis AD, Hieter P, Collins FS. Characterization of the CHD family of proteins. Proc. Natl Acad. Sci. USA 1997;94:11472–11477. [PubMed: 9326634]

Xue Y, Wong J, Moreno GT, Young MK, Cote J, Wang W. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol. Cell 1998;2:851–861. [PubMed: 9885572]

Zhang Y, LeRoy G, Seelig HP, Lane WS, Reinberg D. The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell 1998;95:279–289. [PubMed: 9790534]

Zuo J, Niu QW, Frugis G, Chua NH. The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 2002;30:349–359. [PubMed: 12000682]

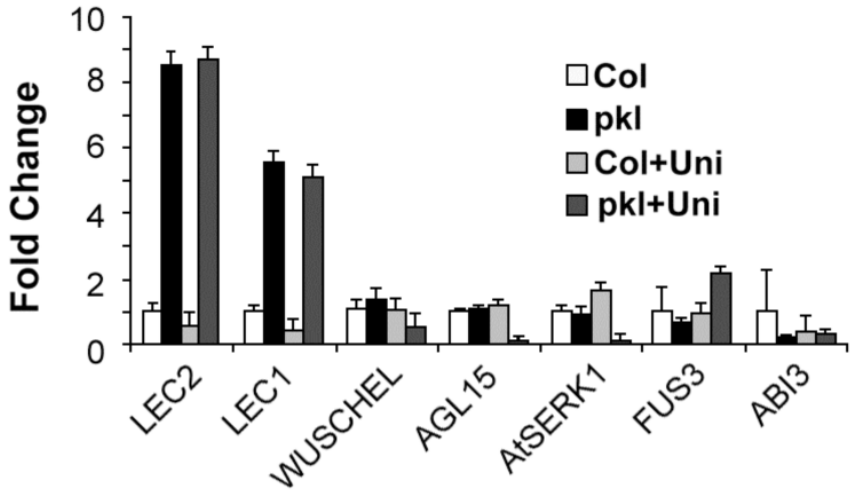

Figure 1.

LEC1 and *LEC2* transcripts are elevated in germinating *pkl* seeds. Quantitative RT-PCR was used to determine the relative transcript levels of the genes of interest in germinating wild-type and *pkl* seeds in the absence or presence of uniconazole-P. The transcripts examined represent genes known or suspected to be involved in embryogenesis (see text). 18s rRNA was used as a standardization control, and expression levels are normalized to wild-type seeds imbibed in the absence of uniconazole-P. Error bars represent the standard deviation of the mean.

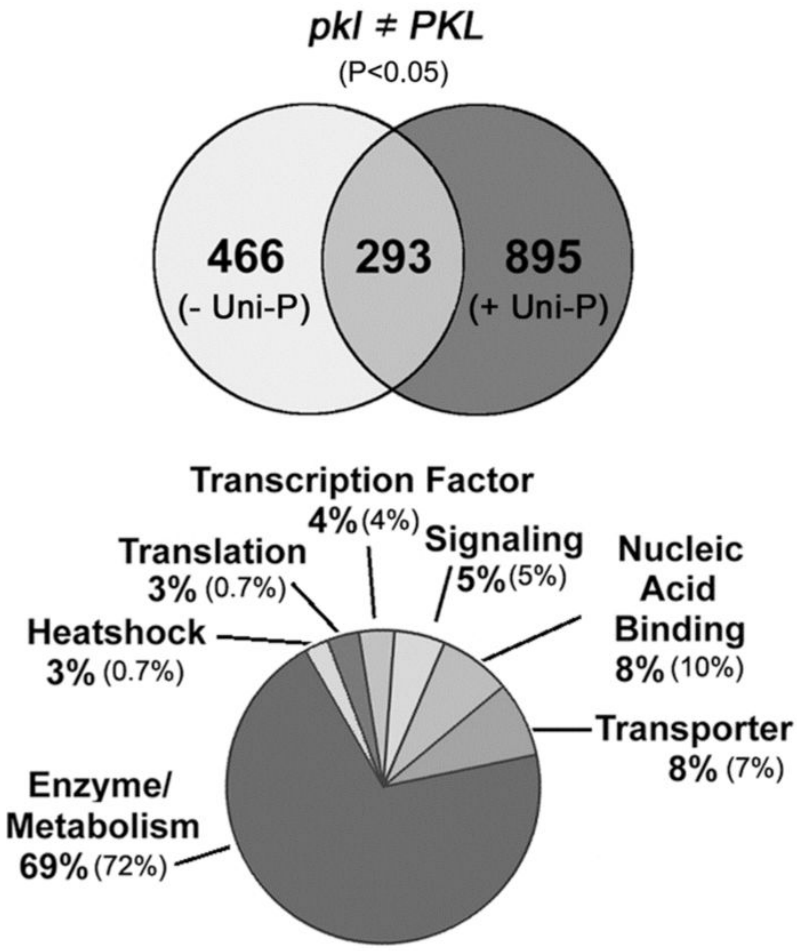

Figure 2.

Identification of genes that exhibit *PKL*-dependent transcript levels. The Venn diagram (top) indicates the number of loci for which the corresponding transcript is expressed at significantly different ($p < 0.05$) levels in wild-type (*PKL*) versus *pkl* seeds in the absence (- Uni-P) or presence (+ Uni-P) of uniconazole-P. The intersection of both data sets represents the 293 genes that showed significantly different expression ($p < 0.05$, uncorrected for multiple comparisons) in the *pkl* mutant regardless of the presence of uniconazole-P. The 293 genes constituted about 3.5% of the total number of loci interrogated with the array, and 54% of the genes identified have a known or putative function. Functional categories assigned to the 54% with known or putative functions are represented in the chart at the bottom. The smaller number in parentheses indicates the percentage of all of the genes represented on the microarray that were assigned to that functional category.

(a)

AGI Code	Affy Code	Wt Mean ± SD	pkl Mean ± SD	Uwt Mean ± SD	Upkl Mean ± SD	Annotation in Text	Putative Function
At1g75750	16014_at	377 ± 873	1730 ± 236	311 ± 609	2050 ± 188	GASA1	gibberellin response
At2g05520	12763_s_at	89 ± 298	512 ± 90	22 ± 137	402 ± 163	atGRP3	cell wall / signaling
At1g62480	17909_at	625 ± 583	2029 ± 522	676 ± 186	1935 ± 170	Unknown1	unknown
At4g25900	13880_s_at	313 ± 272	810 ± 197	256 ± 190	791 ± 88	Apospory	carbohydrate metabolism
At5g12030	13278_f_at	137 ± 140	630 ± 223	20 ± 26	491 ± 56	HSP 17.6A	heat shock protein
At2g35810	20024_s_at	1181 ± 877	2727 ± 362	1033 ± 335	2653 ± 201	Unknown2	unknown
At2g39900	17003_at	351 ± 289	875 ± 179	369 ± 108	918 ± 118	AtWLIM2	transcription factor
At2g16060	17832_at	2032 ± 1213	4064 ± 559	1512 ± 328	3212 ± 389	HB1	hemoglobin
At1g09460	14383_at	82 ± 63	322 ± 158	85 ± 48	446 ± 188	Glucanase	glucanase
At2g04780	16513_at	1330 ± 793	3059 ± 557	1105 ± 212	2765 ± 278	FLA7	cell wall / signaling
At4g31550	16893_at	284 ± 193	656 ± 178	315 ± 190	713 ± 98	WRKY11	transcription factor
At1g03420	20054_at	597 ± 365	1206 ± 188	559 ± 105	1234 ± 230	Unknown3	unknown

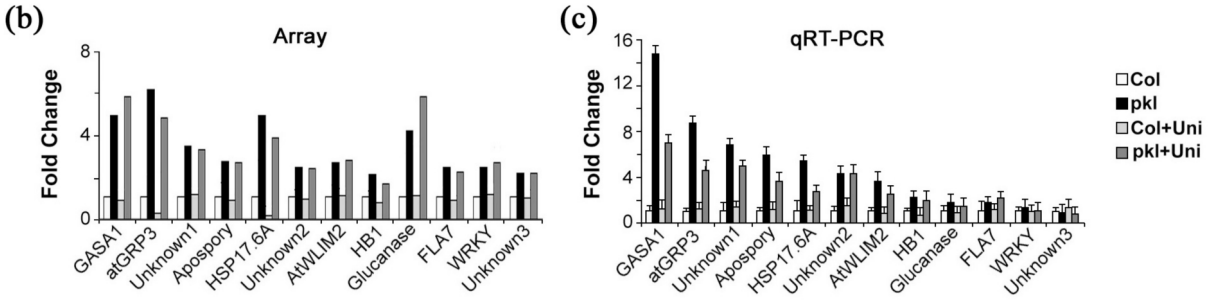

Figure 3.

Transcript level of several genes is strongly *PKL*-dependent during germination. Quantitative RT-PCR was used to determine the relative transcript levels of the genes of interest in germinating wild-type and *pkl* seeds in the absence or presence of uniconazole-P. The transcripts examined represent twelve genes that exhibit robust *PKL*-dependent expression based on microarray analysis (see text).

(a) Microarray data for the twelve candidate genes that were analyzed. Both the AGI code and the corresponding Affymetrix identification code are given. The mean values derived from the microarray analysis for these genes under the four treatments are shown. The last two columns indicate the name of the gene as referred to in the text of this paper along with the predicted function of the gene based on published data and/or sequence similarity.

(b) Relative transcript levels of the twelve genes as indicated by microarray analysis. Expression levels are normalized to wild-type seeds imbibed in the absence of uniconazole-P.

(c) Relative transcript levels of the twelve genes as indicated by quantitative RT-PCR. 18s rRNA was used as a standardization control, and expression levels are normalized to wild-type seeds imbibed in the absence of uniconazole-P. Genes are ordered by relative expression in *pkl* seeds from highest to lowest from left to right. Error bars represent the standard deviation of the mean.

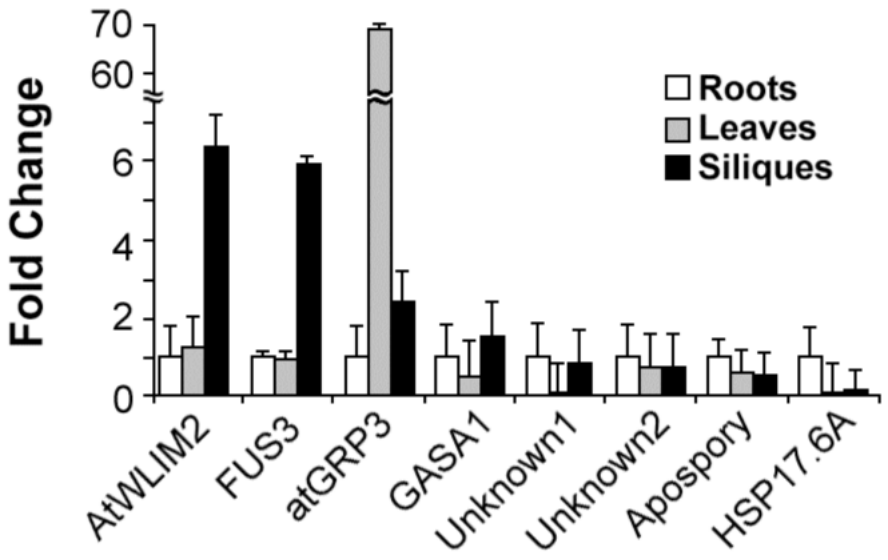

Figure 4.

The LIM-encoding gene is preferentially expressed in siliques. Quantitative RT-PCR was used to determine the relative transcript levels of the genes of interest in wild-type roots, rosette leaves, and siliques. The transcripts examined represent genes that exhibit *PKL*-dependent expression during germination. Genes are ordered by relative expression in siliques from highest to lowest from left to right. 18s rRNA was used as a standardization control, and expression levels are normalized to roots. Error bars represent the standard deviation of the mean.

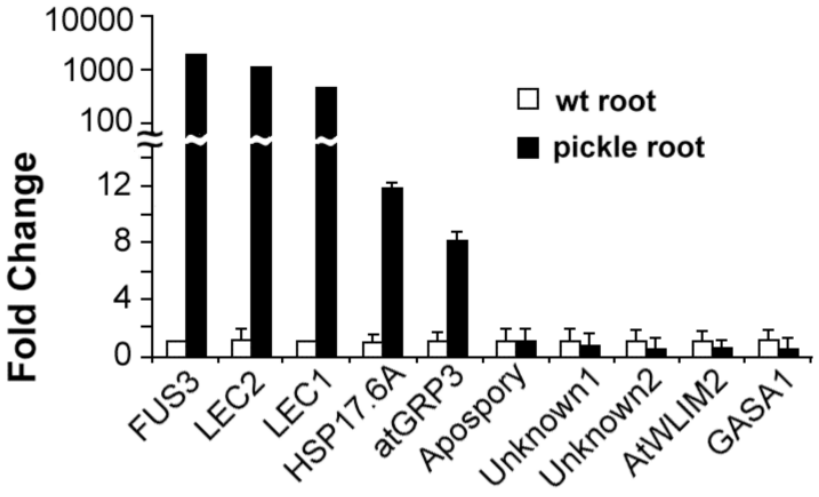

Figure 5.
The *LEC* genes are highly expressed in pickle roots. Quantitative RT-PCR was used to determine the relative transcript levels of the genes of interest in wild-type roots and pickle roots. The transcripts examined represent genes that exhibit *PKL*-dependent expression during germination. Genes are ordered by relative expression in pickle roots from highest to lowest from left to right. 18s rRNA was used as a standardization control, and expression levels are normalized to wild-type roots. Error bars represent the standard deviation of the mean.

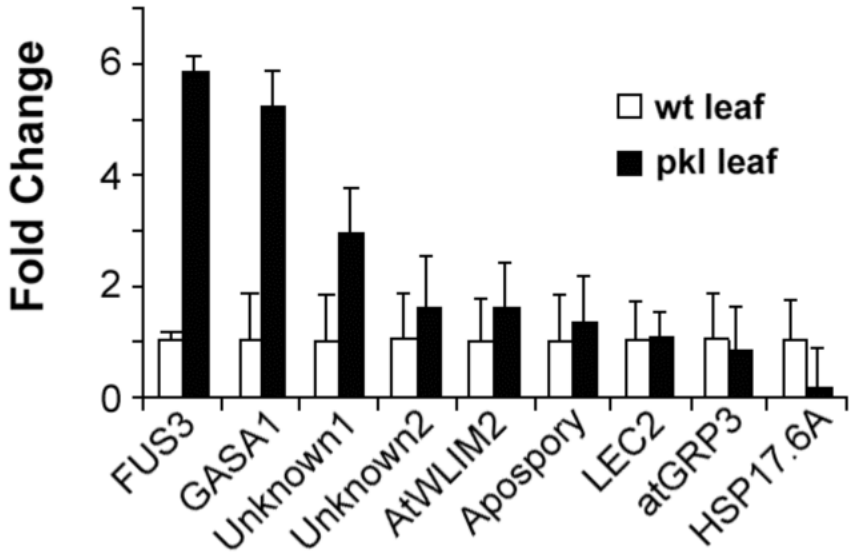

Figure 6.

The *FUS3* transcript is elevated in *pkl* leaves. Quantitative RT-PCR was used to determine the relative transcript level of the genes of interest in wild-type leaves and *pkl* leaves. The transcripts examined represent genes that exhibit *PKL*-dependent expression during germination. Genes are ordered by relative expression in *pkl* leaf tissue from highest to lowest from left to right. The *LEC1* transcript was not detected in either tissue and is therefore not included on the graph. 18s rRNA was used as a standardization control, and expression levels are normalized to wild-type leaves. Error bars represent the standard deviation of the mean.

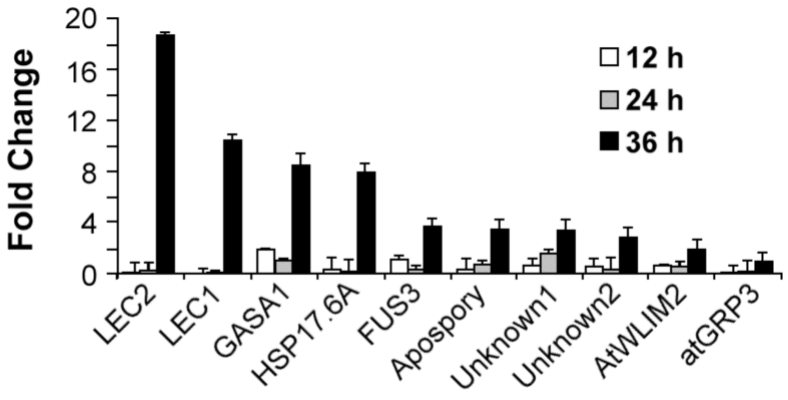

Figure 7.

Genes exhibit coordinate regulation by *PKL* during germination. Quantitative RT-PCR was used to determine the relative transcript level of the genes of interest in wild-type seeds and *pkl* seeds that were desiccated or imbibed for 12, 24, and 36 hours. The transcripts examined represent genes that exhibit *PKL*-dependent expression during germination. Genes are ordered by relative expression in *pkl* seeds at 36 hours after imbibition from highest to lowest from left to right. 18s rRNA was used as a standardization control, and expression levels are normalized to wild-type seeds at each time point. Error bars represent the standard deviation of the mean.