Repression of the *LEAFY COTYLEDON 1/B3* Regulatory Network in Plant Embryo Development by *VP1/ABSCISIC ACID INSENSITIVE 3-LIKE* B3 Genes[1[C][W]]

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Plant embryo development is regulated by a network of transcription factors that include *LEAFY COTYLEDON 1* (*LEC1*), *LEC1-LIKE* (*L1L*), and B3 domain factors, *LEAFY COTYLEDON 2* (*LEC2*), *FUSCA3* (*FUS3*), and *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) of Arabidopsis (*Arabidopsis thaliana*). Interactions of these genes result in temporal progression of overlapping B3 gene expression culminating in maturation and desiccation of the seed. Three *VP1/ABI3-LIKE* (*VAL*) genes encode B3 proteins that include plant homeodomain-like and CW domains associated with chromatin factors. Whereas *val* monogenic mutants have phenotypes similar to wild type, *val1 val2* double-mutant seedlings form no leaves and develop embryo-like proliferations in root and apical meristem regions. In a *val1* background, *val2* and *val3* condition a dominant variegated leaf phenotype revealing a *VAL* function in vegetative development. Reminiscent of the *pickle* (*pkl*) mutant, inhibition of gibberellin biosynthesis during germination induces embryonic phenotypes in *val1* seedlings. Consistent with the embryonic seedling phenotype, *LEC1*, *L1L*, *ABI3*, and *FUS3* are up-regulated in *val1 val2* seedlings in association with a global shift in gene expression to a profile resembling late-torpedo-stage embryogenesis. Hence, VAL factors function as global repressors of the *LEC1/B3* gene system. The consensus binding site of the ABI3/FUS3/LEC2 B3 DNA-binding domain (Sph/RY) is strongly enriched in the promoters and first introns of *VAL*-repressed genes, including the early acting *LEC1* and *L1L* genes. We suggest that *VAL* targets Sph/RY-containing genes in the network for chromatin-mediated repression in conjunction with the PKL-related CHD3 chromatin-remodeling factors.

Embryo development in plants is regulated by a network of transcription factors that include the *LEAFY COTYLEDON 1* (*LEC1*) and *LEC1-LIKE* (*L1L*) genes belonging to the HAP3 family CCAAT-binding factors and a subgroup of the plant-specific B3 domain protein family composed of the *LEC2*, *FUSCA3* (*FUS3*), and *ABSCISIC ACID* (*ABA*)*-INSENSITIVE 3* (*ABI3*) genes (Giraudat et al., 1992; Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001; Kwong et al., 2003).

A series of studies have detailed the complex sequential and regional expression patterns and mutual interactions among these regulators, which underlie the process of embryo formation (Parcy et al., 1997; Nambara et al., 2000; Raz et al., 2001; Brocard-Gifford et al., 2003; Baumbusch et al., 2004; Kagaya et al., 2005; Santos Mendoza et al., 2005; To et al., 2006). *LEC1* and *L1L* act early in embryogenesis and initiate a complex progression of B3 transcription factor expression associated with the transition from embryo morphogenesis to embryo maturation and acquisition of desiccation tolerance. RNA interference of *L1L* function has been shown to cause embryo arrest during morphogenesis (Kwong et al., 2003), whereas *lec1* embryos complete morphogenesis and are capable of further development into plants if rescued prior to desiccation of the seed (Meinke, 1992, 1994; West et al., 1994). Ectopic expression of *LEC1* or *L1L* is sufficient to induce embryo formation in vegetative organs (Lotan et al., 1998; Kwong et al., 2003). The *lec1*, *fus3*, and *lec2* mutations cause partial transformation of cotyledons to leaf-like organs (Meinke, 1992, 1994; Keith et al., 1994; West et al., 1994). The *fus3* mutant embryos, if rescued, are also viable (Keith et al., 1994; Meinke et al., 1994). Overexpression of *LEC2* or *FUS3* causes ectopic expression of embryonic traits in vegetative tissues, albeit not as strongly as induced by *LEC1* or *L1L* misexpression (Stone et al., 2001; Tsuchiya et al., 2004). Finally, *ABI3* is required for maturation-related and ABA-regulated gene expression and establishment of embryo dormancy during mid-to-late embryo development (Koornneef et al., 1984). Null *abi3* mutant embryos are morphologically almost normal and viable if rescued prior to desiccation (Giraudat et al., 1992; Nambara et al., 1992, 1994). Plants that ectopically express *ABI3* or the maize (*Zea mays*) ortholog *VP1* develop normally,

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but have dramatically altered ABA-dependent gene expression in vegetative tissues (Parcy et al., 1994; Suzuki et al., 2001).

A major function of the *LEC2*, *FUS3*, and *ABI3* B3 transcription factors is activation of genes involved in accumulation of storage protein and lipid reserves in the embryo during seed maturation. Activation of downstream genes is mediated by specific binding of the B3 domain (Suzuki et al., 1997; Kroj et al., 2003; Carranco et al., 2004; Monke et al., 2004; Braybrook et al., 2006) to the Sph/RY cis-element (Hattori et al., 1992; Kao et al., 1996; Ezcurra et al., 1999; Reidt et al., 2000; Chandrasekharan et al., 2003; Nag et al., 2005). In addition, *ABI3* mediates ABA-regulated gene expression in the seed through interaction with specific basic Leu-zipper transcription factors that bind ABA response elements (Finkelstein et al., 2002). *FUS3* and *LEC2* are implicated in repression of GA biosynthesis during seed development (Curaba et al., 2004; Gazzarrini et al., 2004). The B3 factors themselves are regulated in part through mutual interactions, as well as unidentified region-specific signals within the embryo (Kagaya et al., 2005; Santos Mendoza et al., 2005; To et al., 2006).

The *LEC1/B3* network is repressed prior to germination and resumption of vegetative development. Chromatin-based repression of the pathway is implicated by *pickle* (*pkl*), a conditional recessive mutant that causes expression of embryonic characteristics in roots of seedlings treated with GA biosynthesis inhibitors (Ogas et al., 1997). The *PKL* gene encodes a CHD3 chromatin-remodeling factor (Ogas et al., 1999). Several of the embryonic regulatory genes, including *LEC1*, *LEC2*, and *FUS3*, are derepressed in roots of *pkl* seedlings (Dean Rider et al., 2003).

Here we show that the *VP1/ABI3-LIKE* (*VAL*) family of B3 domain transcription factors that form a sister clade to the *ABI3/FUS3/LEC2* family are required for repression of the *LEC1/B3* transcription factor network in germinating seedlings. The *val1 val2* double mutant exhibits strong expression of embryonic characteristics in shoot as well as root tissues. Moreover, germination of *val1* monogenic mutant seeds on low doses of paclobutrazol, a GA biosynthesis inhibitor, induces embryonic phenotypes reminiscent of *pkl* (Ogas et al., 1997). Derepression of the *LEC1/B3* network in seedlings lacking *VAL* function is associated with a shift in global gene expression to a profile characteristic of late-torpedo-stage embryos. The Sph/RY consensus motif is highly enriched in promoters and introns of *VAL*-repressed genes, including *LEC1* and *L1L*.

RESULTS

VAL Genes and Proteins

The three Arabidopsis (*Arabidopsis thaliana*) *VAL* genes encode proteins that contain B3 domains that are closely related to the ABI3/FUS3/LEC2 family of B3 transcription factors. The *VAL1* gene is identical to *HSI2*, which was recently identified as a transcriptional repressor for a sugar-inducible gene (Tsukagoshi et al., 2005). In addition, PSI-BLAST analysis (Altschul et al., 1997; Schaffer et al., 2001) of the proteins identified two other conserved domains, a CW domain of unknown function described previously (Perry and Zhao, 2003) and a putative plant homeodomain (PHD)-like zinc (Zn)-finger domain (Fig. 1A). CW and PHD domains are frequently found in chromatin factors (Aasland et al., 1995; Perry and Zhao, 2003). An alignment of the PHD-like region with a canonical PHD domain showed that, in addition to the conserved His and Cys residues that comprise the interleaved Zn-finger structure, the VAL domain potentially includes a third, interleaved Zn finger (Fig. 1B). While there is precedent for similar expansion of the first loop in known PHD domains, to our knowledge the hypothetical third finger is novel though consistent with structural constraints of the canonical PHD fold. Except for the *VAL3* sequence inferred from the Arabidopsis genome annotation that lacks an intact PHD-like domain, the domain architecture is conserved in VAL orthologs identified in rice (*Oryza sativa*), maize, and poplar (*Populus* spp.; data not shown). These structural features imply that VAL proteins are DNA-binding proteins that potentially have chromatin-related functions. The Zn-finger-like domain in the C-terminal region is highly novel and the function of this domain is unknown. Interestingly, the only closely related sequence detected by PSI-BLAST occurs in an isoform of CAAT binding factor subunit C found only in primates (data not shown).

VAL Genes Regulate Plant Development in a Functionally Redundant Manner

To determine the functions of *VAL* genes in plant development, we identified multiple T-DNA insertion alleles for each of the three *VAL* genes (Fig. 1C). The single mutants lack discernible morphological phenotypes, albeit *val1* homozygous plants tend to grow more slowly and flower slightly later than wild type.

Because genetic redundancy could mask essential functions of the *VAL* genes, we generated double mutants between *VAL1*, *VAL2*, and *VAL3* as well as triple mutants. The *val2-1 val3-3* double mutant and all pairwise combinations of *val1* alleles with *val2* and *val3* alleles, except *val1-2 val3-2*, were tested. Whereas the *val2 val3* double mutant did not have a discernible phenotype, the double mutants involving *val1* showed altered development. In the *val1* homozygous mutant background, plants with *val2/+*, *val3/+*, or *val3/val3* genotypes exhibited pronounced variegation of rosette leaves (Fig. 2, A–C; Supplemental Fig. S1). Under a long-day growth regime (16-h light/8-h dark), variegation was evident in rosette leaves produced immediately prior to flowering and in cauline leaves, but was absent during early vegetative growth. The variegation phenotypes produced by *val3/+* heterozygous,

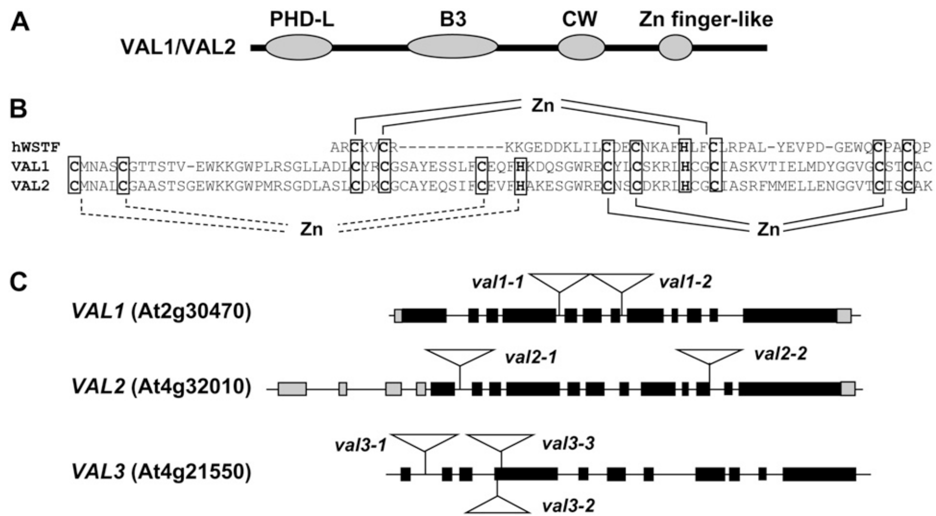

Figure 1. Structures of *VAL1*, *VAL2*, and *VAL3*. A, The four conserved domains of VAL1 and VAL2 include PHD-like (PHD-L), B3, CW (Perry and Zhao, 2003), and a putative Zn-finger-like motif. B, PHD-L sequences of VAL1 and VAL2 are aligned with the canonical PHD domain of human Williams syndrome transcription factor (hWSTF; AAC97879). The two interleaved Zn fingers, which are conserved in canonical PHD domains, are indicated with solid brackets. A potential third finger structure of VAL factors is shown by a dashed bracket. C, Gene structures of the *VAL* genes as well as the T-DNA insertion mutations in each gene are shown. Filled and gray boxes represent coding exons and untranslated transcribed regions, respectively. The *VAL2* gene has four noncoding exons in the 5′ region. The *VAL1* gene is identical to *HSI2* (Tsukagoshi et al., 2005).

val2/+ heterozygous, and *val3* homozygous plants exhibited a similar range of variation and were visually indistinguishable. Hence, *val2* and *val3* mutations had similar dominant effects in the *val1* homozygous background.

The *val1*/+ *val2*/*val2*, and *val1*/*val1 val2*/+ plants segregated seedlings that arrested in development shortly after germination (Fig. 2D). Progeny tests of normal segregants and molecular genotyping confirmed that the arrested seedling phenotype was due to the *val1 val2* double homozygous mutant. Germination of double-mutant seeds was delayed by at least 2 d compared to wild type. Double-mutant seedlings survived for at least 30 d following germination on

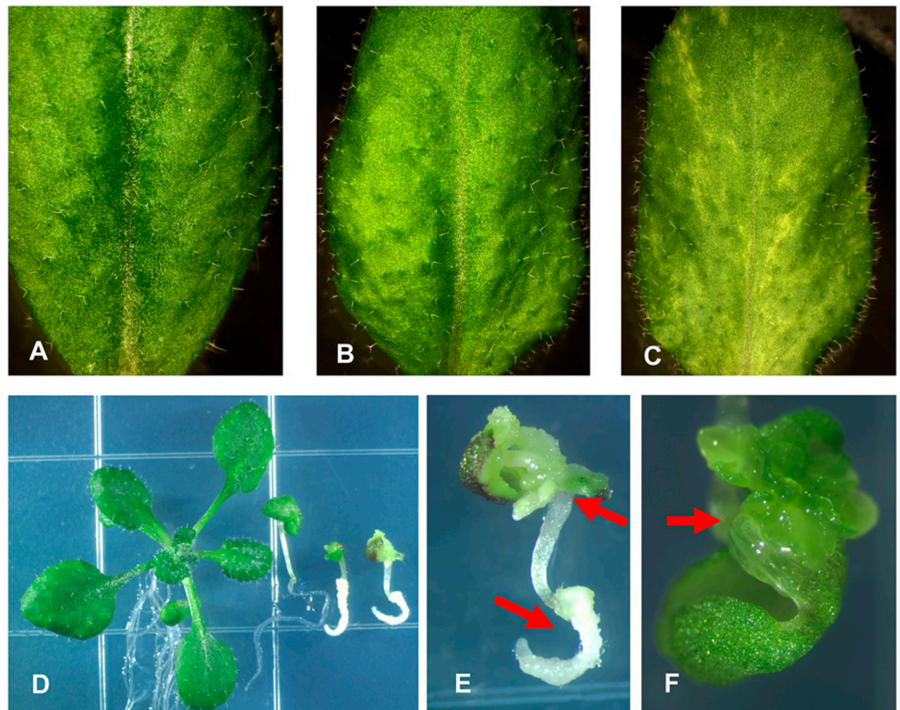

Figure 2. Phenotypes of *val1 val3* and *val1 val2* double mutants. A to C, Leaf variegation of the *val1 val3* double mutant. The first cauline leaves from Col wild type (A), *val1-2*/*val1-2* (B), *val1-2*/*val1-2*; *val3-1*/+ (C) plants are shown. D, Wild-type seedling and three *val1-2 val2-1* double-mutant seedlings at 12 d are arranged left to right. E, Close image of the rightmost *val1-2 val2-1* mutant seedling shown in D. Embryonic callous formation at shoot and root regions is highlighted by red arrows. F, Shoot apical region of the leftmost *val1-2 val2-1* double-mutant seedlings shown in D. In this seedling representing the mildest expression of the double-mutant phenotype, embryo-like outgrowths are present in the shoot apical meristem region.

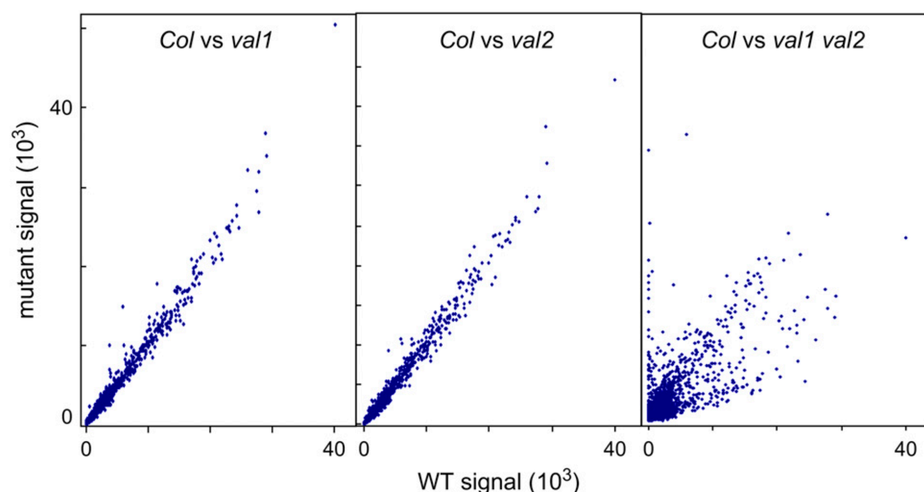

Figure 3. Altered gene expression in seedlings of wild type and *val* mutants. Global alteration of gene expression in *val1 val2* double-mutant seedlings. Means for all elements, excluding controls from two biological replicate hybridizations, are plotted for Col wild type versus *val1-2*, *val2-1*, and the *val1-2 val2-1* double mutant, respectively. [See online article for color version of this figure.]

Murashige and Skoog medium, but failed to produce leaf primordia, indicating absence of a functional apical meristem. Root growth was stunted, resulting in a thick club-like root (Fig. 2E). Double-mutant seedlings developed cell proliferations that have embryonic characteristics, including callous formation from both shoot and root regions, formation of embryo-like structures at the position of the apical meristem and on cotyledon margins (Fig. 2F). Penetrance of the phenotype varied slightly among allele and ecotype combinations. The embryonic seedling phenotype was stronger in the *val1-1 val2-1* and *val1-1 val2-2* combinations constructed in the Wassilewskija (Ws) background (Supplemental Fig. S2) compared to the *val1-2 val2-1* double mutant in Columbia (Col; Fig. 2D). In the Ws background, monogenic *val1-1* homozygous seedlings formed embryonic callous at the cotyledon margins at low frequency (1% to 8%) when germinated on Murashige and Skoog medium, whereas this rare phenotype was not observed in *val1-2* Col seedlings (Supplemental Fig. S2). Moreover, leaf variegation was more pronounced in *val1-1 val3-3* Ws plants compared to *val1-2 val3-3* Col plants (Supplemental Fig. S2).

Triple-mutant seedlings produced by self pollination of *val1/+ val2/val2 val3/val3* plants had an embryonic seedling phenotype similar to the *val1 val2* double mutant, although the triple-mutant seedlings germinated more slowly and typically arrested growth before emergence of the cotyledon from the seed coat (based on comparison of *val1-1 val2-1 val3-3* and *val1-1 val2-1* in Ws; data not shown). Hence, *val1* behaved as a recessive in the *val2* and *val2 val3* homozygous backgrounds. Consistent with genetic evidence that *VAL3* has a minor role in plant development, expression of *VAL3* mRNA was low compared to *VAL1* and *VAL2* in seedlings (Supplemental Fig. S3) and other tissues (Arabidopsis Gene Atlas database; The Arabidopsis Information Resource [TAIR]).

Our analysis of double and triple mutants indicated that *VAL* genes are functionally redundant and required for development and/or maintenance of a functional apical meristem and for repression of embryonic development prior to or during seedling development.

VAL Is Required for Global Repression of Embryonic Gene Expression

To obtain a global picture of gene expression changes conditioned by *val* mutants and to detect subtle gene expression differences in the *val1* and *val2* single mutants, we performed microarray analysis of mutant and wild-type seedlings using the Affymetrix 22K chip (Supplemental Table S1). Pairwise comparisons detected a limited number of gene expression differences between the wild type and either of the single mutants, whereas global gene expression patterns were profoundly altered in the *val1 val2* double mutant relative to wild-type and single mutants (Fig. 3). Sets of 837 genes and 656 genes were more than 4-fold up-regulated and down-regulated, respectively, in the double mutant compared to wild type (Supplemental Tables S2 and S3, respectively). Genes implicated in regulation of seed development, including *LEC1*, *L1L*, and the *ABI3* and *FUS3* B3 transcription factors, were strongly up-regulated in the double mutant (Fig. 4; Supplemental Table S4). *LEC2* expression was below the threshold for detection in all microarray treatments and in quantitative reverse transcription (RT)-PCR assays (Fig. 4). Consistent with the deregulation of *FUS3* and *ABI3*, downstream targets of the B3 factors, such as the 2S albumin and cruciferin genes (Baümlein et al., 1994; Parcy et al., 1994, 1997; Nambara et al., 1995, 2000; Vicient et al., 2000; Suzuki et al., 2003; Tsuchiya et al., 2004; Braybrook et al., 2006), were also expressed at very high levels in the double mutant compared to levels below detection in wild type (Fig. 4; Supplemental Table S2). These two seed storage protein genes are among 61 of the 837 up-regulated genes that were also at least 2-fold up-regulated in the *val1* single mutant. In contrast, only 11 of the 837

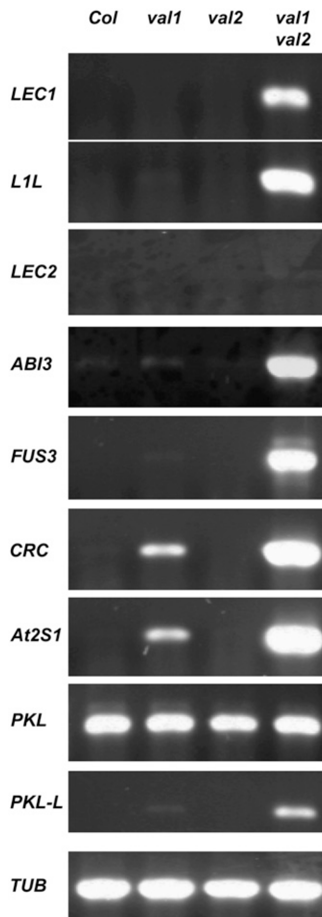

Figure 4. Confirmation of gene expression differences in wild type and *val* mutants. Expression of seed-expressed genes in the seedlings of wild type and *val* mutants. RT-PCR analysis was performed to verify expression of seed-specific genes as well as *PKL* and *PKL-like* (*PKL-L*) genes. *Tubulin* (*TUB*) was used as a control.

up-regulated genes showed >2-fold response in the *val2* single mutant. Recently, the *HSI2* gene, which is identical to *VAL1*, was identified as a transcriptional repressor for sugar-inducible genes (Tsukagoshi et al., 2005). In the *hsi2* mutant, activation of a sugar-inducible seed storage protein gene was observed under noninducing conditions. A similar weak derepression of several seed storage protein genes was observed in the *val1* single mutant.

Overall, our results suggested that failure to achieve or maintain repression of the *LEC1*-related and ABI3/FUS3 B3 transcription factors during seed germination is likely responsible for the embryonic seedling phenotype of the *val1 val2* double mutant. A correlation analysis of our microarray data in comparison to the Arabidopsis Gene Atlas dataset (described in "Materials and Methods") indicated that the global gene expression profile of the double mutant was strongly correlated ($r^2 = 0.9$) with late-torpedo-stage embryo development (Fig. 5). Based on the Gene Atlas dataset, the late torpedo stage corresponds to the peak in expression of *VAL1* during embryo development and marks a temporal transition from peak expression of early embryonic regulators, *LEC1*, *L1L*, and *LEC2*, to the induction of the maturation phase B3 transcription factors, *FUS3* and *ABI3* (Supplemental Fig. S4).

LEC2, *FUS3*, and *ABI3* activation of downstream maturation genes is mediated in part by specific binding of the B3 domain to Sph/RY cis-elements (Suzuki et al., 1997; Kroj et al., 2003; Carranco et al., 2004; Monke et al., 2004; Braybrook et al., 2006). Because the VAL B3 domain is closely related, there is potential for direct interaction with downstream genes as well as indirect regulation via derepression of the B3 activators. We analyzed the frequency of consensus Sph/RY motifs (CATGCA; Suzuki et al., 2003) in the promoter regions (500 and 1,000 bp upstream of the annotated coding sequence) and introns of *VAL*-regulated genes. Genes derepressed 4-fold or greater in the *val1 val2* double mutant showed significant enrichment of the Sph/RY motif in promoters as well as first introns (Table I). The presence of the Sph/RY consensus in the first intron was the strongest single correlate ($P < 3.6 \times 10^{-56}$) of *VAL*-repressed genes. This set of Sph/RY-containing genes included the non-B3 *LEC1* and *L1L* genes, as well as *FUS3*, *ABI3*, *VAL1*, and *VAL2* (Table II). *LEC2*, on the other hand, which did not respond to loss of *VAL* function, lacks a CATGCA consensus motif. Overall, about 54% (353/655 = 0.539) of genes >4-fold up-regulated in the double mutant contained at least one consensus motif in the 500-bp promoter region or first intron.

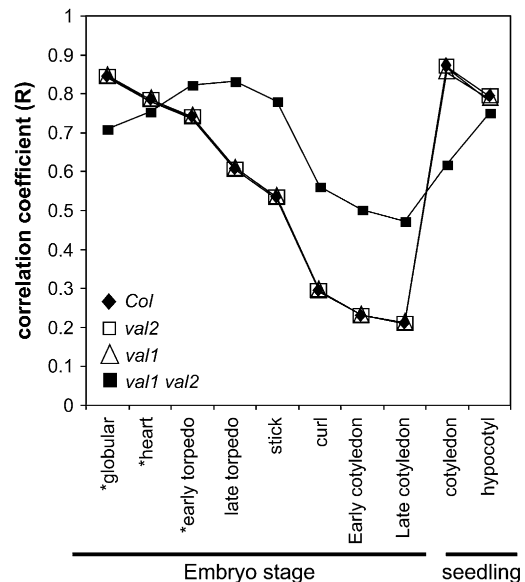

Figure 5. Correlation analysis of gene expression profiles of wild-type and *val* mutant seedlings with wild-type embryo development. Correlations of microarray data with Affymetrix 22K microarray data for staged embryos contained in the Arabidopsis Gene Atlas dataset (TAIR). Globular, heart, and early torpedo stages (shown by asterisks) of the Gene Atlas include silique tissue.

Table I. *Occurrence of Sph (CATGCA) motif in the noncoding regions of VAL-regulated genes*

Gene Region	Total[a]	>4-Fold Up	>4-Fold Down	% Up[b]	*P* Value
Sph/RY promoter[c] (500 bp)	1,563	228	61	14.7	1.7×10^{-30}
Sph/RY first intron	888	184	22	20.7	3.6×10^{-56}
Sph/RY promoter and first intron	167	59	5	35.3	1.1×10^{-43}
Sph/RY promoter[c] (1,000 bp)	3,424	350	149	10.2	2.5×10^{-13}
All genes[d]	9,333	655	473	7.0	–

[a]Total number of genes that have at least one consensus motif in either orientation in the indicated regions. [b]Percent of Sph-containing genes that were up-regulated. [c]500 and 1,000 bp upstream of annotated coding sequence extracted from the TAIR promoter database. Intron sequences were extracted from the TAIR intron database. [d]Elements on the chip selected for analysis had unambiguous gene assignments and absolute signal values of >200 in either the wild-type or *val1 val2* double-mutant treatment.

Inhibition of GA Synthesis Induces an Embryonic Seedling Phenotype in the *val1* Single Mutant

The embryonic seedling phenotype of the *val1 val2* double mutant is reminiscent of the *pkl* mutant (Ogas et al., 1997). To test whether *VAL* function, like *PKL*, is also conditioned by GA biosynthesis, we tested germination of *val1* single-mutant seeds on medium containing paclobutrazol, an inhibitor for GA biosynthesis (Fig. 6). On 5 nM paclobutrazol, a dosage below the threshold for inhibition of germination (Ogas et al., 1997), 23% of the seedlings exhibited strong embryonic transformations, including embryonic callous on cotyledon and leaf margins, ectopic embryo formation on leaves, and partial or complete transformation of leaves to cotyledon-like organs (Fig. 6, B and C). Whereas *VAL* repression of embryonic development was also affected by GA signaling, *PKL* expression was not affected in the double mutant, indicating that *VAL* genes do not act upstream of *PKL* (Fig. 4). However, a related CHD3 remodeling gene, *PKL-like*, was up-regulated in the *val1 val2* double mutant.

DISCUSSION

Our results show that *VAL* genes are essential for repression of embryonic pathways, as well as maintenance and/or differentiation of functional apical meristem function during seedling development. The three *VAL* genes are expressed throughout the plant life cycle (Arabidopsis Gene Atlas) and the seedling and variegated leaf phenotypes of double-mutant genotypes indicate that *VAL* genes have redundant functions in vegetative as well as seed development.

Among the three genes, *VAL1* alone is sufficient for normal development. A nonredundant function of *VAL1* is revealed by the variegated leaf phenotype that is manifest in *val1 val3*/+ and *val1 val2*/+ plants, but not in the *val2 val3* double mutant. Dominant leaf variegation could arise from a variety of mechanisms, including haploinsufficiency, interference by defective VAL2 or VAL3 proteins, or transmission of an epigenetic state established in the haploid gametophyte phase. The similarity of *val3* and *val2* heterozygotes to the *val3* homozygote in the *val1* background does not suggest dosage sensitivity consistent with haploinsufficiency. The lack of allele-specific differences among the *val3* and *val2* insertion mutations argues against expression of a dominant inhibitory product, although this possibility is not ruled out. The possibility of epigenetic origin is intriguing in light of circumstantial evidence linking *VAL* to chromatin regulation and the classical relationship between variegation and epigenetic phenomena (Wakimoto, 1998; Schotta et al., 2003).

In addition to the functional relationship with *PKL*, the domain architecture of the VAL factors is consistent with a possible role in chromatin regulation. Whereas the VAL PHD-like domain bears the most resemblance to PHD domains of chromatin factors, the structure is quite divergent from canonical PHD domains known to bind chromatin. Four aromatic residues that are conserved in VAL orthologs, for example, evidently do not align with conserved Tyr and Trp residues implicated in the binding of specific methylated histones by the ING2 PHD domain (Peña et al., 2006). The CW domain is also frequently associated with chromatin factors (Perry and Zhao, 2003). For example, a histone methyltransferase equipped with a CW domain has recently been shown to regulate flowering in Arabidopsis (Zhao et al., 2006). Intriguingly, chromatin remodeling has been implicated in gene regulation mediated by other B3 domain factors, including ABI3 (Li et al., 1999; Levy et al., 2002; Fukaki et al., 2006; Ng et al., 2006), with association of PKL function (Fukaki et al., 2006). Hence, VAL B3 factors may associate with general chromatin factors, perhaps

Table II. *Occurrence of the Sph (CATGCA) motif in the noncoding regions of embryonic regulators*

Promoter (1,000 bp) and first intron regions are obtained from the TAIR database.

	LEC1	*L1L*	*LEC2*	*FUS3*	*ABI3*	*VAL1*	*VAL2*	*VAL3*	*PKL*	*PKL-L*
Promoter	2	0	0	1	0	5	0	0	0	3
First intron	2	2	0	0	1	0	1	0	0	0

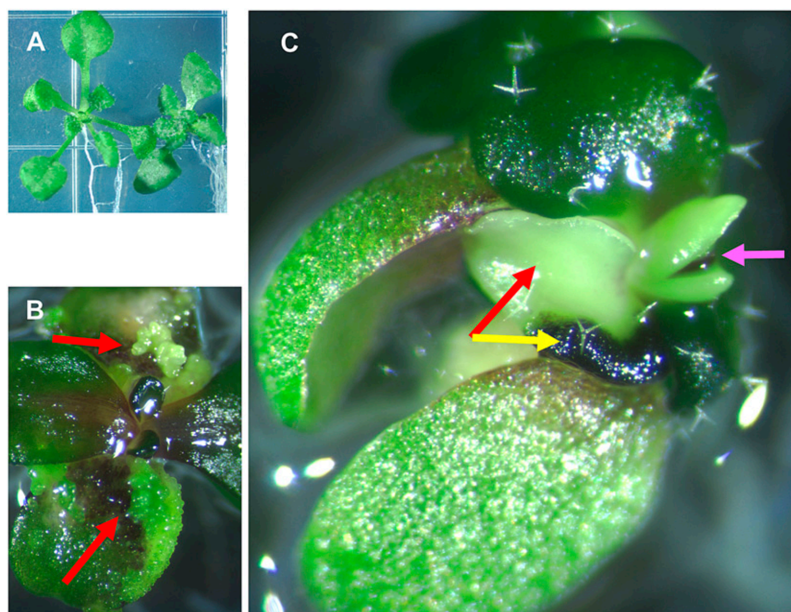

Figure 6. Phenotype of the *val1* mutant seedling treated with an inhibitor of GA biosynthesis. A, 14-d-old seedlings of control (left) and 5 nM paclobutrazol-treated *val1-2* mutant (right). The mild dwarf phenotype of the GA inhibitor-treated seedling was typical of the majority of seedlings (77%). B, Embryonic characteristics observed in 23% of the paclobutrazol-treated *val1-2* seedlings are highlighted by a red arrow. C, Paclobutrazol-treated *val1* seedling showing an emerging leaf with normal (yellow arrow) and embryonic (red arrow) sectors as well as a pair of transformed cotyledon-like leaves (pink arrow) is shown.

as part of the protein complex, to repress expression of a specific set of genes.

At a minimum, *VAL* function is required for maintenance of repression of embryonic gene expression during germination. The conditional phenotype of *val1* mutant seedlings treated with the GA synthesis inhibitor, paclobutrazol, strongly implies that *VAL* function during germination is necessary. Whereas we suggest that *VAL* function is also required for establishing repression of the embryo pathway during seed development, the evidence for *VAL* action during embryo development is indirect. The global profile of gene expression in *val1 val2* double-mutant seedlings resembles late-torpedo-stage embryogenesis, albeit combined with aspects of seed maturation and normal seedling development. This correlation is consistent with down-regulation of the early regulators of embryogenesis, *LEC1* and *L1L*, and the relative peak in *VAL1* expression at the late torpedo stage, suggesting that *LEC1* and *L1L* may be direct targets of VAL.

The *pkl* mutant is the only other recessive mutant known to cause derepression of the embryonic program in vegetative organs. PKL is a CHD3 homolog implicated in establishing chromatin-based silencing of the embryo pathway during germination. Like *pkl* (Ogas et al., 1997), the *val1* embryonic seedling phenotype is conditioned by inhibition of GA synthesis, suggesting these genes function in a common pathway. However, the *val1 val2* double-mutant phenotype differs from *pkl* in at least three respects: (1) the embryonic seedling phenotype of the *val* double mutant is not dependent on GA synthesis inhibitor treatment; (2) whereas in *pkl*, embryonic characteristics are predominantly expressed in the distal region of the root (Ogas et al., 1997; Dean Rider et al., 2003), the *val1 val2* phenotype is strongly expressed in shoot as well as root tissues; and (3) *pkl* and the *val* loss-of-function phenotypes affect different sets of embryo pathway genes. For instance, in contrast to the *val* double mutant, *ABI3* is not derepressed in *pkl* mutant seedlings, whereas *LEC2* is activated in the *pkl*, but not in the *val* double mutant (Dean Rider et al., 2003). These differences may be partly due to differential expression of *PKL* and *VAL* genes in root and shoot regions of the embryo. Although we have not determined the spatial distribution of *VAL* gene expression during development, *PKL* is predominantly expressed in the distal region of the root where the embryonic phenotype is manifest, as well as at the shoot apical meristem (Li et al., 2005). In contrast, the *LEC2* gene is strongly expressed in the root axis, but not in the shoot apical meristem during seed development (To et al., 2006). Consistent with these expression patterns, the paclobutrazol-induced phenotype of *val1* is most prominent in the shoot apical region. Hence, the *LEC2* gene, which also lacks of a consensus Sph/RY, may be regulated independently of *VAL1* and *VAL2*.

The conditional embryonic seedling phenotypes of the *val1* and *pkl* mutants implicate GA signaling in repression of embryonic pathways. One interpretation is that, on normal medium, GA signaling enhances the function of *VAL2* in the *val1* single mutant sufficiently to allow normal development, whereas *VAL2* activity is insufficient under GA-deficient conditions. The *VAL* genes may, in turn, at least indirectly regulate GA synthesis during seed development. *AtGA3ox1*, one of the key 3-oxidase genes expressed during seed germination (Yamaguchi et al., 1998; Mitchum et al., 2006), is down-regulated more than 10-fold in the *val* double mutant compared to wild type (Supplemental Table S4). Simultaneous strong up-regulation in the double mutant (approximately 1,700-fold for *AtGA20ox3*) of genes encoding earlier steps in GA biosynthesis is consistent with loss of negative feedback regulation of

the pathway by biologically active GA (Mitchum et al., 2006). *VAL* regulation of the GA pathway may occur indirectly through regulation of other B3 factors. Synthesis of active GA in the developing seed is enhanced by the *lec2* and *fus3* mutants (Curaba et al., 2004). *FUS3* is proposed to repress GA biosynthesis (Curaba et al., 2004; Gazzarrini et al., 2004) through direct binding to Sph/RY elements in the promoter of a biosynthesis gene, *AtGA3ox2* (Curaba et al., 2004). Hence, the delayed germination of *val* double- and triple-mutant seeds compared to wild type may be due to prolonged expression of *FUS3* in the mutant seed.

Because many of the *VAL*-repressed genes are transcription factors, including representatives of most major families in the Arabidopsis genome, the prospects for distinguishing primary and secondary targets among the large number of affected genes detected by microarray analysis are seemingly limited. In spite of this complexity, we find that a majority of *VAL*-regulated genes contain a consensus Sph/RY element (CATGCA) either upstream of transcription initiation or within the first intron, and this enrichment is highly significant. This result suggests two limiting cases: (1) VAL and ABI3/FUS3/LEC2 families of B3 proteins may recognize the same or overlapping sets of downstream targets, the former functioning as repressors and the latter primarily as activators; and (2) *VAL* genes may primarily effect global repression of the pathway indirectly through repression of the B3 factors, or one step further removed, through repression of *LEC1* and *L1L*. An intermediate view that we find attractive is that there is not necessarily a clear distinction between upstream and downstream targets within the B3 network. Initially proposed by Parcy et al. (1997), there is mounting evidence of mutual interactions among *LEC1*, *FUS3*, *LEC2*, and *ABI3* during embryo development (Brocard-Gifford et al., 2003; Kagaya et al., 2005; Santos Mendoza et al., 2005; To et al., 2006). Moreover, our findings imply that the set of Sph/RY-regulated genes includes the early acting *LEC1* and *L1L* genes in the embryo pathway. Recently, deletion of the 5′ region of *LEC1* in the *tnp* mutant is shown to induce ectopic expression of this gene (Casson and Lindsey, 2006). The deleted region of the *LEC1* promoter in this mutant contains a consensus Sph (ACATGCAT) at 666 bp upstream of the translation initiation, suggesting that this motif may mediate *VAL* repression function.

For these reasons, we suggest that B3-regulated genes comprise a gene system that includes two classes of B3 factors that recognize the Sph/RY element leading to activation and repression, respectively. Many, but not all, of these genes are involved in seed development. We further suggest that, by enabling recognition of Sph/RY in an active chromatin context, VAL proteins target genes in the system for repression by recruiting a chromatin-remodeling complex that includes PKL and related CHD3 proteins. Identification of cis-elements recognized by the VAL B3 domains is critical to understanding the mechanism of VAL-mediated repression. Our preliminary experiments aimed at testing specific binding of VAL1 and VAL2 B3 domains to the Sph motif in vitro are so far inconclusive (M. Suzuki and D.R. McCarty, unpublished data). Conceivably, the DNA-binding activity of the VAL B3 domain may depend on a chromatin context.

MATERIALS AND METHODS

Plant Growth Conditions

For phenotypic characterization of seedlings of *val* mutants, seeds were sterilized and sown on plates containing 1× Murashige and Skoog salt, 0.05% MES, 1% Suc, and 0.15% of Phytagel (Sigma). Seedlings were grown for 5 to 18 d at 22°C under continuous light. For phenotypic characterization of mature plants, seeds were germinated on sterile plates and transferred to soil or sown directly on soil after sterilization and cold stratification treatment and grown at 23°C to 25°C in a 16-h light/8-h dark regime.

Genetic Analysis and Construction of *val* Mutant Lines

We obtained T-DNA insertion alleles, *val1-1*, *val2-1*, *val2-2*, and *val3-3* in Ws ecotype from the University of Wisconsin Arabidopsis Knockout facility (Sussman et al., 2000). The *val3-3* allele, which has a T-DNA insertion within a coding exon of the *VAL3* gene, was derived from an activation tag population. Alleles derived from the SALK Institute population (Alonso et al., 2003), *val1-2* (SALK_088606), *val3-1* (SALK_015582), and *val3-2* (SALK_013113) in Col-0 ecotype, were obtained from the Arabidopsis Biological Resource Center. The mutant lines were backcrossed at least twice prior to genetic and phenotype analysis. The double and triple mutants were constructed by PCR-based genotyping. Because *val2* and *val3* loci are weakly linked, we screened recombinants to establish a *val2 val3* double homozygous mutant. The primers used for genotyping the *val* alleles were listed in Supplemental Table S5.

Microarray Analysis

For Affymetrix ATH1 GeneChip microarray analysis, total RNA was prepared from two independent biological replicates using the RNeasy Plant mini kit (Qiagen). cDNA synthesis, in vitro transcription reactions, and hybridization were performed at the University of Florida Interdisiplinary Center for Biotechnology Research Core facility. Five-day-old whole seedlings were sampled from Col-0, *val1-2*, and *val2-1*, and 7.5-d-old whole seedlings were sampled for the *val1-2 val2-1* double mutants. Due to their delayed germination relative to wild type, the double-mutant seedlings were grown 2.5 d longer to obtain comparable postgermination development. Because *val2-1* was originally generated in Ws ecotype background, we backcrossed this allele with *Col-0* five times. The backcrossed *val2-1* mutant was used to generate *val2* and *val1 val2* mutants for microarray analysis. The *val1 val2* double mutants were sampled based on the phenotypic differences among the seedlings segregating *val2*/+ in a *val1* homozygous background. The genotypes of RNA samples were confirmed by RT-PCR before microarray hybridization (Supplemental Fig. S5).

RT-PCR Analysis

A 100-ng sample of total RNA was used for RT-PCR reactions (28 cycles) in a total volume of 12 μL with the One-Step RT-PCR kit (Qiagen). The primers used for RT-PCR are listed in Supplemental Table S5.

Statistical Analysis of cis-Elements and Microarray Data

Statistical analysis of Affymetrix microarray data derived experimentally or obtained from the Gene Atlas dataset of TAIR was performed using Excel spreadsheet functions. Motif frequencies in promoters and introns of coregulated genes were determined using a simple word search implemented in a custom java program and analyzed using chi-square (Suzuki et al., 2005). Gene sequence subregions were extracted from TAIR promoter (500 bp and 1,000 bp) and intron sequence compilations, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Leaf variegation.

Supplemental Figure S2. *val* double mutants.

Supplemental Figure S3. *VAL* gene expression.

Supplemental Figure S4. *LEC1/B3* gene expression.

Supplemental Figure S5. Sample profiles for ATH1 microarray analysis.

Supplemental Table S1. Affymetrix ATH1 all genes.

Supplemental Table S2. *VAL*-repressed genes.

Supplemental Table S3. *VAL*-up-regulated genes.

Supplemental Table S4. Selected set of genes.

Supplemental Table S5. Primers in this study.

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