Distinct Roles of LAFL Network Genes in Promoting the Embryonic Seedling Fate in the Absence of VAL Repression

Haiyan Jia, Donald R. McCarty*, and Masaharu Suzuki*

Horticultural Sciences Department, Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, Florida 32611

The transition between seed and seedling phases of development is coordinated by an interaction between the closely related ABSCISIC ACID-INSSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON2 (LEC2; AFL) and VIVIPAROUS1/ABI3-LIKE (VAL) clades of the B3 transcription factor family that respectively activate and repress the seed maturation program. In the val1 val2 double mutant, derepression of the LEC1, LEC1-LIKE (L1L), and AFL (LAFL) network is associated with misexpression of embryonic characteristics resulting in arrested seedling development. We show that while the frequency of the embryonic fate in val1 val2 seedlings depends on the developmental timing of seed rescue, VAL proteins repress LAFL genes during germination, but not during seed development. Quantitative analysis of LAFL mutants that suppress the val1 val2 seedling phenotype revealed distinct roles of LAFL genes in promoting activation of the LAFL network. LEC2 and FUS3 are both essential for coordinate activation of the network, whereas effects of LEC1, L1L, and ABI3 are additive. Suppression of the val1 val2 seedling phenotype by the B3 domain-deficient abi3-12 mutation indicates that ABI3 activation of the LAFL network requires the B3 DNA-binding domain. In the VAL-deficient background, coordinate regulation of the LAFL network is observed over a wide range of genetic and developmental conditions. Our findings highlight distinct functional roles and interactions of LAFL network genes that are uncovered in the absence of VAL repressors.

In Arabidopsis (*Arabidopsis thaliana*), the seed maturation program is controlled by a network of transcription factors that includes the ABSCISIC ACID (ABA)-INSSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON2 (LEC2; AFL) clade of B3 domain transcription factors (Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001) and two LEC1-type HAP3 family CCAAT-binding factors, LEC1 and LEC1-LIKE (L1L; Lotan et al., 1998; Kwong et al., 2003), which we here designate the LEC1/AFL (LAFL) network.

Mutations in LAFL network genes partially or completely block the seed maturation program, which includes accumulation of storage reserves, developmental arrest of the embryo, and acquisition of desiccation tolerance (Keith et al., 1994; Meinke et al., 1994; Parcy et al., 1994, 1997; West et al., 1994; Nambara et al., 1995; Raz et al., 2001; Kroj et al., 2003; To et al., 2006). While LAFL mutant seeds are typically desiccation intolerant, they can produce viable homozygous mutant plants if seed are rescued prior to desiccation. Mutants in maize (*Zea mays*) VIVIPAROUS1 (VPI) and its ortholog in Arabidopsis, ABI3, display reduced sensitivity to ABA during seed development (McCarty et al., 1991; Giraudat et al., 1992). To varying degrees, overexpression of individual LAFL genes during postembryonic development causes ectopic activation of embryonic pathways. Ectopic expression of LEC1 or LEC2 is sufficient to induce sporadic somatic embryo development in the vegetative tissues (Lotan et al., 1998; Stone et al., 2001). Epidermal overexpression of FUS3 induces formation of cotyledonlike leaves (Gazzarrini et al., 2004). For ABI3 and L1L overexpressors, activation of embryonic pathways is less pronounced, but induction of seed storage protein (SSP) genes is detected in the leaf tissues (Parcy et al., 1994; Parcy and Giraudat, 1997; Kwong et al., 2003).

The AFL B3 transcription factors regulate seed maturation by activating genes encoding SSPs, the lipid biosynthetic pathway, and late embryogenesis abundant proteins. Target gene activation by VP1 and AFL proteins is mediated by B3 domain-specific binding to the Sph/Ry motif (Suzuki et al., 1997; Reidt et al., 2000; Kroj et al., 2003; Mönke et al., 2004; Braybrook et al., 2006). The N-terminal Co-Activator/Co-Repressor (COAR)
domain of VP1 and ABI3 is required for activation of ABA-responsive genes as well as for repression of genes induced during germination (Hoekker et al., 1995, 1999; Carson et al., 1997; Nakamura et al., 2001; Nambara et al., 2002). The COAR domain is capable of mediating ABA-dependent activation of late embryogenesis abundant genes independent of the B3 domain (Carson et al., 1997).

LAFL genes have distinct temporal patterns of expression during seed development. LEC1 and LEC2 expression reaches a maximum at the heart stage of embryogenesis, and FUS3 expression peaks during early seed maturation, while ABI3 is expressed throughout the maturation phase (Lotan et al., 1998; Stone et al., 2001; Kroj et al., 2003; To et al., 2006; Suzuki et al., 2007). Genetic analyses reveal that temporal and spatial regulation of the network is refined by mutual interactions among the LAFL genes. As a consequence, the organization of the LAFL network is neither strictly hierarchical nor linear. LEC1 and LEC2 can activate ABI3 and FUS3 expression (Kroj et al., 2003; Kagaya et al., 2005; To et al., 2006; Stone et al., 2008). LEC1 expression is up-regulated by LEC2 (Stone et al., 2008). LEC1 and FUS3 positively regulate LIL expression (Yamamoto et al., 2009, 2010). ABI3 and FUS3 exhibit autoregulation and interact through mutual activation (To et al., 2006).

Analyses of mutants that cause ectopic expression of seed maturation genes in seedling tissues suggest that repression of the LAFL network during germination is necessary for the transition from seed to seedling development. The VP1/ABI3-LIKE (VAL) B3 proteins, which form a sister clade to AFL B3 factors, are required for repression of LAFL network during germination. In val1 and val2 double mutant seedlings, derepression of the LAFL network is associated with a characteristic embryonic seedling phenotype that includes callus proliferation in shoot and root regions as well as arrested shoot apical meristem development that prevents formation of leaves and progression to vegetative development (Suzuki et al., 2007). The VAL genes were independently identified as mutants in sugar signaling (HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENES [HS12/VAL1], HS12-LIKE1 [HSL1/VAL2], and HS12-LIKE2 [HSL2/VAL3; Tsukagoshi et al., 2005, 2007]) and as a mutant causing constitutively elevated expression of the Glutathione S-transferase F8 gene (Veerapann et al., 2012). LEC1, LEC2, and FUS3 were shown to be up-regulated by Suc in the hsi2 hs1 mutant (Tsukagoshi et al., 2007). In addition to the VAL factors, a plant-specific trihelix factor (Gao et al., 2009) as well as multiple chromatin-remodeling proteins are implicated in maintaining repression of the LAFL network in vegetative tissues. The latter include polycomb group complexes (Makarevich et al., 2006; Aichinger et al., 2009; Chen et al., 2010; Kim et al., 2010, 2012), histone deacetylase complexes (Tanaka et al., 2008), CHD3 chromatin-remodeling factors PICKLE and PICKLE-RELATED2 (Ogas et al., 1997, 1999; Dean Rider et al., 2003; Aichinger et al., 2009), RETINOBLASTOMA-RELATED PROTEIN (Gutzat et al., 2011), and SNF2 chromatin-remodeling ATPase BRAHMA (Tang et al., 2008).

Although derepression of the LAFL network is implicated in the expression of embryonic seedling phenotype in the val1 val2 double mutant, the roles and interactions of individual LAFL genes in blocking the transition to normal seedling development have not been delineated. If derepression of the LAFL network in val1 val2 seedlings is responsible for ectopic expression of embryonic characteristics, we would expect that mutations in one or more LAFL genes will suppress the embryonic seedling phenotype.

Here, we show that mutants in the LAFL network suppress the embryonic seedling phenotype of the val1 val2 double mutant to varying degrees. The lec1, l1l, and abi3 mutants are partial suppressors, whereas fus3 and lec2 mutants are complete suppressors that allow seedlings to transition to normal development. Both FUS3 and LEC2 are absolutely required for maintenance of the activated state of LAFL network in the absence of VAL repressors, whereas LEC1, L1L, and ABI3 have additive effects on the activity of the network. Quantitative changes in expression of the LAFL network overall correlate with the penetrance of embryonic seedling phenotypes under the variety of genetic and developmental conditions examined.

RESULTS

The Frequency of Embryonic Phenotypes in val1 val2 Seedlings Depends on the Time of Rescue in Seed Development

By genotyping progeny from val1 val2/+ plants, we confirmed that around 20% of mature val1 val2 double mutant seeds could escape the embryonic seedling fate and produce viable seedlings (Supplemental Fig. S1A). The frequency of escapes was sufficient to allow maintenance of a homozygous val1 val2 double mutant line and hence in depth study of the seedling phenotype. We noted that the expression of embryonic seedling phenotypes varied depending on the developmental stage at which seeds were rescued.

To quantify the influence of the timing of seed rescue on the phenotype of val single and double mutant seedlings, developing seeds were harvested at 1-d intervals between 7 and 15 d after flowering (DAF) and placed on Murashige and Skoog media. Under our growth conditions, seeds of the ecotype Columbia (Col-0) wild type as well as the val single and double mutants reached physiological maturity by 15 DAF. Seeds harvested at 15 DAF were completely desiccation tolerant and fully capable of germinating. Seeding phenotypes were recorded following 12 d of growth on Murashige and Skoog media at 23°C to 25°C with continuous light. Based on the observed range of expression, seeding phenotypes were classified into three types (Fig. 1A): embryonic, partial embryonic, and normal. The embryonic type seedlings had callus formation in both shoot and root regions together with arrested shoot apical meristem development that prevented production of true leaves. The partial embryonic class included seedlings that formed callus in either the cotyledon and/or root but produced true leaves from the apex. Seedlings with no
visible callus formation were classified as normal. Most wild-type and val single mutant seeds rescued at mid to late stages (11–15 DAF) produced normal seedlings (Fig. 1B). Nongreen and abnormal growth phenotypes were observed in wild-type and val single mutant seedlings rescued early in seed development (7–10 DAF). Occasional embryonic callus formation (up to 30% of rescued seedlings) was observed in val1 mutant seedlings rescued between 7 and 10 DAF, though rarely at later stages (Fig. 1B). By contrast, the majority of val1 val2 double mutant seedlings (70%–100%) rescued at all seed developmental stages (7–15 DAF) showed strong embryonic callus seedling phenotypes, with a shift toward higher frequencies of partial embryonic (12%–21%) and normal (up to about 20%) phenotypes occurring in seedlings rescued at later stages of seed development (Fig. 1B and C). Plants grown from val1 val2 double mutant seedlings that escaped the embryonic seedling fate exhibited pleiotropic phenotypes, including delayed germination, variegated leaves, late flowering, and reduced fertility (Supplemental Fig. S1, B and C; Suzuki et al., 2007).

In addition, to address the influence of Suc on the val1 val2 seedling phenotype (Tsukagoshi et al., 2007), mature seeds of val1 val2 mutant plants were placed on Murashige and Skoog media supplemented with different concentrations of Suc (0.1%, 1%, 2%, 3%, 4%, and 5% [w/v]). At all Suc concentrations tested, the majority of val1 val2 seedlings (74%–98%) exhibited embryonic callus formation (Supplemental Fig. S2), indicating that seedling fate was only moderately affected by Suc.

VAL B3 Factors Repress the LAFL Genes during Germination But Not during Seed Development

We previously showed that the embryonic callus phenotype in val1 val2 seedlings grown from mature seeds is associated with dramatic up-regulation of LAFL genes (Suzuki et al., 2007). To determine whether expression of LAFL genes in val mutant seedlings was affected by the stage of seed development at which seedlings were rescued, we used a quantitative PCR assay to measure expression of LAFL genes and two downstream SSP genes (At2S1 and CRUCIFERIN C [CRC]) in wild-type and val mutant seedlings grown from seed rescued at 9, 11, 13, and 15 DAF. The transcript levels of all marker genes were strongly up-regulated in the val1 val2 mutant seedlings across all stages of rescue relative to wild-type and val single mutants (Fig. 2A). Consistent with the occasional occurrence of the embryonic callus phenotype, the marker genes were also elevated in val1 single mutant seedlings rescued at 9 DAF compared with the wild type and val2, while expression of the LAFL genes was at or below the threshold for detection in wild-type and single mutant seedlings rescued at later stages (11–15 DAF; Fig. 2A). These results confirm that VAL1 and VAL2 are also required for repression of the LAFL network in seedlings rescued prior to seed maturity.

To determine if VAL B3 factors also regulate the expression of LAFL genes during seed development, we examined transcript levels of the marker genes in wild-type and val mutant developing seeds harvested...
at 9 and 11 DAF. The transcript levels of all genes assayed were comparable or slightly lower in val1 val2 mutant seeds compared with the wild type and val single mutants (Fig. 2B). This result indicated that in contrast to their role in seedlings, VAL1 and VAL2 do not repress \textit{LAFL} gene expression during seed development.

**Suppression of the Embryonic Seedling Phenotype by Mutants in \textit{LAFL} Network Genes**

We hypothesized that derepression of the \textit{LAFL} genes during germination is responsible for the embryonic seedling phenotype of the val1 val2 mutant. To test this hypothesis, we constructed a series of triple mutants by introducing \textit{lec1}, \textit{l1l}, \textit{lec2}, \textit{fus3}, and \textit{abi3} mutants, respectively, into the val1 val2 mutant background (Supplemental Fig. S3). The \textit{lec1}, \textit{l1l}, and \textit{lec2} alleles used in this analysis are transfer DNA insertion mutants. \textit{abi3}-6 allele is a deletion mutation that produces a truncated, non-functional transcript (Nambara et al., 1994). The \textit{fus3}-3 allele is a point mutation that blocks intron splicing to produce nonfunctional aberrant transcripts (Luerssen et al., 1998).

As shown in Figure 3, the triple mutants containing \textit{fus3} or \textit{lec2} had uniformly normal seedling phenotypes across all times of rescue during seed development, revealing that the \textit{fus3} and \textit{lec2} mutants are complete suppressors of the val1 val2 embryonic seedling phenotype. This result demonstrates that both \textit{fus3} and \textit{lec2} are essential for the embryonic seedling fate. By contrast, the embryonic seedling phenotype of val1 val2 was only partially suppressed in the triple mutants containing \textit{l1l}, \textit{lec1}, and \textit{abi3}, respectively. In the \textit{l1l} and \textit{abi3}-6 triple mutants, the seedling phenotype was dependent on the timing of seed rescue, where the predominant seedling phenotype gradually shifted from embryonic to partial to normal in seeds rescued.
during early (7–9 DAF), mid (10–12 DAF), and late (13–15 DAF) stages, respectively (Fig. 3, A and B). The maximum proportions of normal seedlings reached approximately 50% and 85% in \( l_{1l} \) and \( abi3-6 \) triple mutants, respectively (Fig. 3B). In the \( l_{1l} \) and \( abi3 \) triples, most of seedlings rescued after 13 DAF transitioned to a normal developmental fate; whereas for the \( lec1 \) triple mutant, nearly one-half of the seedlings rescued as early as 7 DAF had a normal seedling fate, reaching a maximum of about 85% normal in seedlings rescued at 15 DAF (Fig. 3, A and B).

**Promotion of Embryonic Seedling Fate by ABI3 Requires the B3 Domain**

In contrast to the LEC2 and FUS3 B3 proteins, ABI3 also contains a COAR domain that mediates interactions with ABA-regulated transcription factors (Nakamura et al., 2001). To examine the effect of COAR on the \( val1 \) \( val2 \) embryonic seedling phenotype, we constructed a triple mutant using the \( abi3-12 \) mutation (Supplemental Fig. S3). \( abi3-12 \) is a nonsense mutation that encodes a mutant protein that lacks the B3 domain but retains an intact COAR domain (Nambara et al., 2002). This mutation confers a desiccation-tolerant seed phenotype analogous to dormant, colorless alleles of maize \( vp1 \) (Carson et al., 1997).

Interestingly, we found that \( abi3-12 \) triple mutant seedlings exhibited a seed rescue time-dependent phenotype similar to the \( abi3-6 \) triple mutant, indicating that the B3 domain-deficient \( abi3-12 \) allele is a partial suppressor of \( val1 \) \( val2 \) embryonic seedling phenotype (Fig. 3, A and B). This result implies that the promotion of the embryonic seedling fate in the \( val1 \) \( val2 \) double mutant specifically requires the B3 DNA-binding domain of ABI3. Moreover, for seed rescued at later stages (11–15 DAF), the percentage of normal seedlings (11 DAF, 22% versus 6%; 13 DAF, 82% versus 54%; and 15 DAF, 92% versus 85%) in \( abi3-12 \) triple was higher than in \( abi3-6 \) triple mutants (Fig. 3B), indicating that the presence of a functional COAR domain in the \( abi3-12 \) mutation enhanced suppression of the \( val1 \) \( val2 \) embryonic phenotype compared with the \( abi3-6 \) null mutation. This result suggests that COAR domain of ABI3 may have a role in negative regulation of the LAFL network.

**Figure 3.** Seedling phenotypes of LAFL-VAL triple mutants. Triple mutant seeds were rescued between 7 and 15 DAF. The culture conditions are described in Figure 1. A, Representative phenotypes of triple mutant (\( val1 \) \( val2 \) \( l_{1l} \), \( val1 \) \( val2 \) \( lec1 \), \( val1 \) \( val2 \) \( abi3-6 \), \( val1 \) \( val2 \) \( abi3-12 \), \( val1 \) \( val2 \) fus3-3, and \( val1 \) \( val2 \) lec2) seedlings from seeds rescued at 7 to 15 DAF. Bar = 1 cm. B, Histogram of the three phenotypic classes of triple mutant seedlings grown from seeds rescued at different developmental stages (7–15 DAF). The phenotypic classes are described in Figure 1. Values are means ± se of the mean (\( n = 6–10 \)).
Distinct Functions of LAFL Factors in Activation of the Network

To determine the effects of genetic suppressors on regulation of the LAFL network, we examined transcript levels of LAFL and downstream SSP genes in seedlings of the triple mutants. In the fully suppressed fus3 and lec2 triple mutant seedlings, transcript levels of all LAFL genes were at or below the limit of detection across all stages of seedling rescue (Fig. 4). These results indicated that both FUS3 and LEC2 are necessary for mutual activation as well as activation of LEC1, L1L, and ABI3. Thus, FUS3 and LEC2 are absolutely required for activating the LAFL network in val1 val2 mutant seedlings. By contrast, lec1, abi3-6, and l1l mutants caused less dramatic quantitative reductions in LAFL and SSP gene expression in the val1 val2 mutant background. The lec1 triple mutant seedlings had reduced LAFL expression at all stages of seed rescue (Fig. 4), whereas effects of abi3-6 and l1l were most evident in triple mutant seedlings rescued late in seed development (13 and 15 DAF; Fig. 4). Altogether, our results show that the effects of fus3, lec2, lec1, l1l, and abi3 mutations in suppression of val1 val2 embryonic seedling phenotype were correlated with quantitative changes in expression of other LAFL genes.

Moreover, consistent with the seedling phenotype, transcript levels of LAFL genes were also substantially reduced in abi3-12 triple mutant seedlings relative to the val1 val2 mutant, indicating that ABI3 activation of LAFL genes specifically requires the B3 domain (Fig. 4). The slight reduction of LAFL transcripts in the abi3-12 triple mutant seedlings relative to the abi3-6 triple lends further support to the notion that the COAR domain may have a role in negative regulation of the LAFL network.

Interestingly, in the partially suppressed triple mutant genotypes, the sensitivity of the LAFL network to repression increased with the stage of seed rescue. It remains to be determined whether this temporal pattern reflected residual VAL activity, perhaps due to VAL3 or the effects of other mechanisms that control repression of LAFL network.

Partial Suppression of val1 val2 val3 Embryonic Callus Phenotype by abi3-6

To address the role of VAL3 in regulating the seed to seedling transition, we constructed and analyzed the val1 val2 val3 triple mutant. Nearly all val1 val2 val3 triple mutant seedlings recovered had severe embryonic seedling phenotypes (Fig. 5A). In rare cases, triple mutant seedlings with normal phenotypes were identified but none produced fertile plants. This result indicated that the low frequency of normal seedling escapes observed in the val1 val2 double mutant is at least partly attributable to residual activity of VAL3.

Next, we asked whether VAL3 is also responsible for the increased frequency of normal seedlings observed in the partially suppressed val1 val2 abi3-6 triple mutant. To address this question, we compared the phenotypes and LAFL gene expression in the val1 val2 abi3-6 triple and val1 val2 val3 abi3-6 quadruple mutants. val1 val2 val3 abi3-6 quadruple mutant seedlings had a seed rescue time-dependent phenotype similar to the val1 val2 abi3-6 triple mutant (Fig. 5B). The frequency of normal seedlings in the two genotypes was comparable for seed rescued between 12 to 15 DAF (Figs. 5C and 3B). This result indicated that the abi3-6 null mutation also partially

Figure 4. Expression of LAFL network genes in LAFL-VAL triple mutant seedlings. Q-PCR analysis was performed to quantify transcript levels of LAFL network genes (LEC1, L1L, LEC2, FUS3, and ABI3) and downstream targets (At2S1 and CRC) in 6-d-old triple mutant (val1 val2 l1l, val1 val2 lec1, val1 val2 abi3-6, val1 val2 abi3-12, val1 val2 fus3-3, and val1 val2 lec2) seedlings grown from seeds rescued at 9, 11, 13, and 15 DAF. Bars show copies of mRNA per nanogram total RNA. Values are means ± se of the mean (n = 3). n.d., Not detected.
suppressed the embryonic seedling phenotype of the val1 val2 val3 triple mutant. Moreover, for same-stage-rescued seeds, transcript levels of LAFL genes and downstream target genes were slightly lower in val1 val2 val3 abi3-6 seedlings than in the val1 val2 abi3-6 triple mutant (Fig. 5D). These results indicated that in contrast to the evident role of VAL3 in allowing some val1 val2 mutant seedlings to escape the embryonic fate, VAL3 is not involved in genetic suppression of the val1 val2 embryonic seedling phenotype by abi3-6.

The abi3, l1l, and lec1 Mutations Have Additive Roles in Suppression of the Embryonic Seedling Phenotype

To dissect the genetic interactions of abi3, l1l, and lec1 as partial suppressors of the embryonic seedling
phenotype, we made a series of quadruple mutants that included the pairwise combinations of abi3-6 with l1l and lec1 in the val1 val2 background. We found that the embryonic seedling phenotype of val1 val2 mutant is not fully suppressed in val1 val2 abi3-6 l1l quadruple mutant (Fig. 6A). The maximum proportion of normal seedlings in the abi3-6 l1l quadruple mutant (about 80%) was comparable to the abi3-6 triple mutant (about 85%; Figs. 6B and 3B), suggesting the addition of l1l had little phenotypic effect in the abi3-6 triple mutant background. However, expression of LAFL factors and downstream markers was reduced slightly in the abi3-6 l1l quadruple mutant relative to abi3-6 triple mutant (Fig. 7). This result suggests that l1l and abi3-6 mutants act additively to partially suppress the activity of LAFL network. In contrast to the abi3-6 l1l combination, the val1 val2 abi3-6 lec1 quadruple mutant produced normal seedlings across all stages of seed rescue, demonstrating that lec1 and abi3-6 combined fully suppressed the embryonic seedling phenotype of val1 val2 (Fig. 6, A and B). In line with the fully suppressed seedling phenotype, the expression of LAFL factors and downstream markers were almost completely suppressed in the val1 val2 abi3-6 lec1 quadruple mutant (Fig. 7). These results showed that the additive effects of abi3, l1l, and lec1 mutations in suppression of the val1 val2 embryonic seedling phenotype are correlated with quantitative changes in expression of LAFL genes.

These findings suggest that ABI3, L1L, and LEC1 act redundantly to activate FUS3 and LEC2 in val1 val2 mutant seedlings. If this is correct, then we would expect fus3 and lec2 mutations to be epistatic to the other genes in the network that confer partial suppression. This was confirmed by construction and analysis of the val1 val2 abi3-6 fus3-3, val1 val2 abi3-6 lec2, val1 val2 l1l fus3-3, val1 val2 l1l lec2, val1 val2 lec1 fus3-3, and val1 val2 fus3-3 lec2 quadruple mutants and the val1 val2 abi3-6 fus3-3 lec2 quintuple mutant (Fig. 6; Supplemental Figs. S4–S6). All combinations had fully suppressed seedling phenotypes as well as expression profiles of LAFL genes that were similar to the lec2 and fus3 triple mutants analyzed above (Figs. 3 and 4). Hence, both fus3 and lec2 are epistatic to abi3, l1l, and lec1 in suppression of the val1 val2 mutant embryonic seedling phenotype.

Coordinate Regulation of the LAFL Network

To quantify coordinate regulation of LAFL genes under diverse genetic and developmental circumstances, we calculated pairwise correlations of LAFL gene expression levels in seedling of all genotypes rescued at various stages of seed development, excluding data points where

Figure 6. Seedling phenotypes of LAFL-VAL quadruple mutants. Quadruple mutant seeds were rescued from 7 to 14 DAF using culture conditions described in Figure 1. All quadruple mutants except val1 val2 abi3-6 l1l that were rescued after 14 DAF failed to germinate. A, Representative phenotypes of quadruple mutant (val1 val2 abi3-6 l1l, val1 val2 abi3-6 lec1, val1 val2 abi3-6 fus3-3, and val1 val2 abi3-6 lec2) seedlings from seeds rescued at 7 to 14 DAF. Bar = 1 cm. B, Histogram of the three phenotypic classes of val1 val2 abi3-6 l1l and val1 val2 abi3-6 lec1 quadruple mutant seedlings grown from seeds rescued at different developmental stages (7–15 DAF) using phenotypic classes described in Figure 1. Values are means ± se of the mean (n = 6–10).
one of the two genes is mutant (Fig. 8A). Overall, we found there were relatively strong correlations of expression among all pairs of LAFL genes ($R^2 = 0.72-0.97$; Fig. 8A), confirming that the LAFL genes are coordinately regulated as a network over a range of genetic and developmental conditions. Notably, expression of LEC2 was more closely correlated with LEC1 and L1L ($R^2 = 0.93$ and 0.91, respectively) than with FUS3 ($R^2 = 0.72$) and ABI3 ($R^2 = 0.76$, Fig. 8A and B; whereas expression of FUS3 and ABI3 were tightly correlated [$R^2 = 0.97$], Fig. 8A). This difference is consistent with the genetic evidence that FUS3 and LEC2 have distinct nonredundant activities that are each essential for maintenance of the LAFL network expression in the absence of VAL repression.

**DISCUSSION**

Genetic analyses indicate that derepression of the LAFL network during germination prevents the transition between embryonic and vegetative phases of plant development. Our results reveal that the LAFL genes have distinct functions in promoting expression of the LAFL network in VAL-deficient seedlings. The contribution of ABI3 to activation of LAFL network in this context requires the B3 DNA-binding domain. Moreover, we have shown that expression of the LAFL network overall is coordinately regulated and quantitatively well correlated with the frequency of embryonic seedling phenotypes in VAL-deficient seedlings.

**LAFL Factors Have Distinct Roles in Promoting Activation of the LAFL Network**

Gene expression analyses suggest that derepression of the LAFL network during seed germination is responsible for the embryonic seedling phenotype of the val1 val2 mutant (Figs. 1 and 2; Suzuki et al., 2007; Tsukagoshi et al., 2007). This hypothesis is confirmed by our demonstration that the embryonic seedling phenotype is suppressed in a series of triple and quadruple mutants that include various combinations of LAFL network mutants (lec1, lec2, fus3, and abi3) in the val1 val2 background (Figs. 3 and 6; Supplemental Figs. S4A–S6A). Suppression of val1 val2 embryonic seedling phenotypes in various triple and quadruple mutants was correlated with reduced or complete absence of LAFL gene expression (Figs. 4 and 7; Supplemental Figs. S4B–S6B). Hence, ectopic expression of the LAFL network in the VAL-deficient background is maintained by regulatory interactions among genes in the network. Our results further show that the LAFL genes have distinct functional roles in promoting activity of the LAFL network in val1 val2 seedlings. 1) Both LEC2 and FUS3 are shown to have essential nonredundant functions in coordinately activating the LAFL genes. They evidently mutually activate each other as well as LEC1, L1L, and ABI3. 2) LEC1, L1L, and ABI3 have additive or partially redundant roles in promoting activity of the network, most likely by contributing to activation of LEC2 and FUS3.

Nevertheless, the developmental timing of seed rescue strongly influenced seedling fate in the partially...
excluded genotypes where one of the two genes is mutant. Red, LAFL of each of the other cated for data from each comparison.

Interestingly, this contrast coincides with the differential temporal expression patterns of LEC1 and L1L, and ABI3 factors causes the activity of LAFL network to decrease gradually in rescued seedlings as the developing seeds approach maturity, whereas loss of either LEC2 or FUS3 causes the network to collapse completely in seedlings rescued across all stages of transition from seed to seedling development rather than at a particular stage of seed development. Because VAL genes are transcribed at a low level throughout plant development (Suzuki et al., 2007; Tsukagoshi et al., 2007), initiation of VAL-mediated repression is probably regulated by posttranscription and/or post-translational mechanisms in coordination with chromatin-remodeling factors (Jia et al., 2013).

Some of the interactions among LAFL genes observed in val1 val2 seedlings have also been described in developing embryos. These include LEC1 and LEC2 activation of FUS3 and ABI3 (Kroj et al., 2003; To et al., 2006) and LEC1 and FUS3 activation of L1L (Yamamoto et al., 2009, 2010), as well as mutual positive interactions between ABI3 and FUS3 (To et al., 2006). While LEC2 was shown to act upstream of ABI3 and FUS3 in developing embryos (To et al., 2006), no clear hierarchical relationship between LEC2 and FUS3 was detected in val1 val2 seedlings. Our results indicate that both LEC2 and FUS3 are required for mutual activation of each other as well as ABI3, LEC1, and L1L in val1 val2 seedlings. Additional interactions among LAFL genes implied by our results, including LEC1 activation of LEC2, FUS3 activation of LEC1, and the additive interactions between LEC1, L1L, and ABI3, have thus far not been described in developing embryos. One reason may be that in developing embryos, mutual genetic interactions within the network are strongly shaped by the differential temporal and regional expression patterns of LAFL genes (Parcy et al., 1994; Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001; Kroj et al., 2003; Kwong et al., 2003; To et al., 2006), whereas in the absence of VAL repression, the LAFL network is coordinately up-regulated in seedling tissues. Hence, the lack of normal temporal ordering in val1 val2 mutant seedlings may enable interactions among LAFL factors that ordinarily do not arise in seed development. The molecular basis for differential temporal regulation of genes in the network during seed development is unclear. However, our results indicate that the VAL genes do not play a major role in regulation of the network during seed development.

Our correlation analysis is consistent with a model in which FUS3 and LEC2 function in distinct subnetworks, both of which must be maintained to produce the embryonic seedling fate in the VAL-deficient background. The expression levels of the partial suppressors LEC1, L1L, and ABI3 showed very high correlations ($R^2 \approx 0.90$) with either LEC2 or FUS3 but not both (Fig. 8A). Hence, LEC1, L1L, and ABI3 may serve to coordinate activities of the LEC2 and FUS3 subnetworks. In that case, LEC2 and FUS3 may activate LEC1, L1L, and ABI3 via positive feedback interactions (Fig. 4). Consistent with this idea, in the val1 val2 mutant background, eliminating any one of the LEC1, L1L, and ABI3 factors causes the activity of LAFL network to decrease gradually in rescued seedlings as the developing seeds approach maturity, whereas loss of either LEC2 or FUS3 causes the network to collapse completely in seedlings rescued across all stages of

Figure 8. Correlation of LAFL gene expression under various genetic and developmental treatments. A, Matrix of pairwise correlations ($R^2$) of LAFL gene expression data across all genotypes and treatments, excluding genotypes where one of the two genes is mutant. Red, $R^2 \geq 0.90$; yellow, $R^2 < 0.90$. B, Scatterplot of LEC2 expression versus each of the other LAFL genes. Regression equations and $R^2$ are indicated for data from each comparison.
seed development. Overall, our results are consistent with emerging evidence for direct molecular interactions among LAFL genes. All LAFL network genes contain Sph/RY motifs in their promoter and intron sequences consistent with direct regulation by the B3 transcription factors (Suzuki et al., 2007). LEC1, FUS3, and ABI3 were identified as potential direct targets of FUS3 (Wang and Perry, 2013), while FUS3 is a probable target of ABI3 (Mönke et al., 2012). In addition, LEC1 associates with the LIL promoter (Junker et al., 2012). However, other direct interactions among the LAFL factors implied by our results as well as previous genetic analyses (Kagaya et al., 2005; To et al., 2006; Stone et al., 2008) are not yet confirmed. These include LEC1 regulation of LEC2 and FUS3, LEC2 regulation of LEC1 and ABI3, and mutual interactions between LEC2 and FUS3. It is possible that these interactions are mediated by indirect mechanisms. Suppression of the val1 val2 embryonic seedling phenotype by mutations in LAFL genes suggests that LAFL genes are targets of the VAL repressors. Consistent with this hypothesis, LEC1 and LEC2 were identified as HISTONE DEACETYLASE19 (HDA19) targets in a chromatin immunoprecipitation analysis, and VAL2 was further shown to interact with HDA19 in a yeast (Saccharomyces cerevisiae) two-hybrid assay (Zhou et al., 2013).

The B3 Domain- and COAR Domain-Dependent Functions of ABI3

Functional analyses have identified three distinct modes of VP1/ABI3 regulation of gene expression. First, direct binding to regulatory regions of target genes mediated by the B3 domain is implicated in VP1 activation of C1 in maize (Hattori et al., 1992; Kao et al., 1996; Suzuki et al., 1997) and ABI3 activation of CRC (Mönke et al., 2012). Second, the COAR domain is capable of mediating B3 domain-independent activation of ABA-regulated genes (Carson et al., 1997). This activation function is mediated by physical interaction of the COAR domain with ABA-regulated G-box-binding bZIP factors (Hobo et al., 1999; Nakamura et al., 2001). Finally, the COAR domain is necessary and sufficient for VP1-mediated repression of seed germination-specific genes in maize aleurone (Hoecker et al., 1995, 1999). Our results show that ABI3 promotion of LAFL network expression in VAL-deficient seedlings requires the B3 domain. Thus, the B3-deficient abi3-12 allele is capable of suppressing the val1 val2 phenotype. Our finding is consistent with ABI3 binding directly to the Sph/RY motif (Mönke et al., 2004) that is enriched in the promoter and intron regions of LAFL genes (Suzuki et al., 2007). In addition, there is evidence that FUS3 is positively regulated (To et al., 2006) as a potential direct target of ABI3 (Mönke et al., 2012).

In this context, it is interesting that the abi3-12 mutation, which retains a functional N-terminal COAR domain, is a stronger suppressor of the val1 val2 embryonic seedling phenotype than the abi3-6 null mutation. This result suggests that the COAR domain may contribute to down-regulation of LEC1, LIL, LEC2, and FUS3 observed during late embryo development (Suzuki et al., 2007; Supplemental Fig. S4).

Ablation of the AFL and VAL B3 Networks Eliminates the Desiccation-Tolerant Phase of the Plant Life Cycle

Our results indicate that the primary function of the VAL B3 factors is to mediate repression of the LAFL network during germination, thereby enabling the transition from seed to seedling development. Our analysis of triple and quadruple mutants indicates that val double and triple mutant plants, which typically do not develop beyond the seedling stage, are viable when the LAFL network is also disabled (Figs. 3 and 6). These results suggested that complete elimination of LAFL and VAL B3 networks would produce viable plants that are capable of completing the life cycle. The quadruple and quintuple mutants constructed in this study provide a direct test of this hypothesis. The val1 val2 abi3-6 fus3-3, val1 val2 fus3-3 lec2, and val1 val2 abi3-6 lec2 quadruple mutants as well as val1 val2 abi3-6 fus3-3 lec2 quintuple mutants were rescued successfully as homozygous plants prior to seed desiccation. All homozygous val afl mutant seed rescued between 7 to 14 DAF produced normal seedlings that lacked expression of downstream seed maturation markers (Supplemental Fig. S6, A and B). In addition to desiccation intolerance, quadruple and quintuple val afl mutant plants exhibited a suite of pleiotropic phenotypes, including leaf variegation and reduced fertility, consistent with VAL genes having other nonessential functions outside of the seed. Nevertheless, these results confirm that if the AFL B3 network is absent, the VAL B3 network is no longer essential for plant survival. This conclusion is consistent with the hypothesis that the VAL B3 genes coevolved with the LAFL network primarily to provide a mechanism to enable the germinating seed to transition from the maturation phase to reserve remobilization and seedling development.

In conclusion, our results indicate that LAFL factors have distinctive functional roles in promoting activity of the LAFL network in val1 val2 mutant seedlings. In rescued seedlings, the activity of the LAFL network correlates with the occurrence of the embryonic seedling fate. Ablation of the LAFL and VAL B3 networks eliminates the desiccation-tolerant phase of the plant life cycle, suggesting that the AFL and VAL B3 factors have coevolved to regulate initiation of and exit from the seed maturation program, respectively.

MATERIALS AND METHODS

Plant Material and Growth Conditions

For characterization of postgermination seedling phenotypes at various stages of seed development, siliques were rescued at 7 to 15 DAF and sterilized immediately after harvest. And then, seeds were plated on Murashige and Skoog media containing 1× Murashige and Skoog salt, 0.05% MES, 1% Suc sterilized by filtration, and 0.15% of phytagel (Sigma). Plated seeds were...
stratified at 4°C in dark for 3 d. To record seedling phenotypes and to sample plant materials for RNA isolation, stratified seeds were incubated at 23°C to 25°C for 12 and 6 d under continuous light, respectively. Prior to genetic and phenotype analysis, all the higher order mutants were genotyped among progeny of the quadruple mutants. To establish the genotype all the alleles were listed in Supplemental Table S1.

Genetic Analysis and Construction of Mutant Lines

The transfer DNA insertion alleles (Supplemental Fig. S3), val1-2 (SALK_088606), val2-1 (SALK_015582), lec2 (SALK_015228), lec1 (SALK_000450), and l1l (GABI_670E09) in col-0 were obtained from the Arabidopsis Biological Resource Center. The val2-1 allele was described in Suzuki et al. (2007). ab3-6, ab3-12, and fus3-3 stocks were provided by Eiji Nambara and Peter McCourt (University of Toronto). val1, val2-2/+, and abi3-6 fus3-3 mutant lines were established previously in our lab. val1 val2 homogenous double mutant seed were created by self-pollination of val1 val2/+ plants. val1 val2 ab3-6 and val1 val2 fus3-3 genotypes were created by crossing val1 val2/+ with ab3-6 and fus3-3, respectively. val1 val2 ab3-12 was created by crossing val1 val2 with ab3-12. val1 val2 l1l, val1 val2 lec1, and val1 val2 lec2 were created by crossing val1 val2 ab3-6 with lec1, lec2, and l1l, respectively. val1 val2 val3 and val1 val2 ab3-6 were created by crossing val1 val2 ab3-6 with val3. Quadruple mutants (val1 val2 ab3-6 l1l, val1 val2 ab3-6 lec1, val1 val2 ab3-6 fus3, and val1 val2 ab3-6 lec2) were created by crossing val1 val2 ab3-6 with l1l, lec1, ab3-6 fus3, and lec2, respectively. Prior to genetic and phenotype analysis, all the higher order mutants were first confirmed by PCR genotyping, including testing of F1 progeny resulting from outcrosses of each genotype to the Col-0 wild type. The primers used for genotyping all the alleles were listed in Supplemental Table S1. ab3-6 and fus3-3 alleles were genotyped following the description in To et al. (2006) with different set of primers. To establish the val1 val2 ab3-12 double mutant, ab3-6 homozygous gai mutants were identified among progeny of val1 val2/+ ab3-12/+ plants by PCR and sequencing of ABI3 alleles (Supplemental Fig. S3).

Q-PCR

Plant total RNA was prepared from 6-d-old seedlings grown from seeds rescued at 9, 11, 13, and 15 DAF using the Plant RNeasy Kit (Qiagen). For developing seeds (9 or 11 DAF), total RNA was extracted from whole siliques as described by Sangha et al. (2010). RNA concentration was measured by NanoVue Plus (GE Healthcare). Total RNA from each sample was treated with RQ1 DNase-Free DNase (Promega). The abundance of target gene transcript was determined by Q-PCR using a Power SYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems) with a StepOnePlus system (Applied Biosystems). For analysis of gene expression, an absolute quantification method was used. Standard curves were constructed using plasmids containing the target gene sequences. The primers used for quantitative PCR are listed in Supplemental Table S2.

Supplemental Data

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

Supplemental Figure S1. val1 val2 mutant phenotypes at seedling, rosette, and mature plant stages.

Supplemental Figure S2. Effects of Suc on val1 val2 seedling phenotype.

Supplemental Figure S3. Structures of the LAFL and VAL genes and the mutant alleles used in this study.

Supplemental Figure S4. Seedling phenotypes and LAFL network gene expression in val1 val2 l1l fus3-3 and val1 val2 l1l lec2 quadruple mutants.

Supplemental Figure S5. Seedling phenotypes and LAFL network gene expression in the val1 val2 lec1 fus3-3 quadruple mutant.

Supplemental Figure S6. Seedling phenotypes and LAFL network gene expression of higher order null mutant combinations in the val1 val2 background.

Supplemental Table S1. The genotyping primers used in this study.

Supplemental Table S2. Primers for quantitative real-time PCR.
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