Running Head: Promotion of the embryonic seedling fate by LAFL genes

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

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One-sentence summary: The LEC1, ABI3, FUS3 and LEC2 genes that regulate seed maturation comprise a self-activated network.
Footnotes:

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ABSTRACT
The transition between seed and seedling phases of development is coordinated by an interaction between the closely related AFL [ABSCISIC ACID (ABA)-INSENSITIVE 3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON 2 (LEC2)] and VAL (VP1/ABI3-LIKE) clades of the B3 transcription factor family that respectively activate and repress the seed maturation program. In the val1 val2 double mutant, de-repression of the LAFL [LEC1, LEC1-LIKE (L1L), and AFL] network is associated with mis-expression 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.
INTRODUCTION

In Arabidopsis, the seed maturation program is controlled by a network of transcription factors that includes the AFL clade of B3 domain transcription factors [ABSCISIC ACID (ABA)-INSENSITIVE 3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON 2 (LEC2)] (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 LAFL (LEC1/AFL) 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 VIVIPAROUS 1 (VP1) 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, over-expression of individual LAFL genes during post-embryonic 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 over-expression of FUS3 induces formation of cotyledon-like leaves (Gazzarrini et al., 2004). For ABI3 and L1L over-expressors, 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 et al., 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 (LEA) 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 (Hoecker 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 (LEA) 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, 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 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; Mu et al., 2008; Stone et al., 2008). LEC1 expression is up-regulated by LEC2 (Stone et al., 2008). LEC1 and FUS3 positively regulate L1L 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 val2 double mutant seedlings, de-repression 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 (SAM) 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 GENE 2 (HSI2/VAL1), HSI2-LIKE1 (HSL1/VAL2), and HSI2-LIKE2 (HSL2/VAL3) (Tsukagoshi et al., 2005, 2007). LEC1, LEC2, and FUS3 were shown to be up-regulated by sucrose in the hsi2 hsl1 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 (PKL) and PICKLE-RELATED 2 (PKR2) (Ogas et al. 1997,1999; Rider et al., 2003; Aichinger et al., 2009), RETINOBLASTOMA-RELATED PROTEIN (RBR) (Gutzat et al., 2011) and SNF2 chromatin-remodeling ATPase BRAHMA (BRM) (Tang et al., 2008).
Although de-repression of the LAFL network is implicated in the expression of embryonic seedling phenotype in the \textit{val1 val2} double mutant; the roles and interactions of individual \textit{LAFL} genes in blocking the transition to normal seedling development have not been delineated. If de-repression of the LAFL network in \textit{val1 val2} seedlings is responsible for ectopic expression of embryonic characteristics, we would expect that mutations in one or more \textit{LAFL} genes will suppress the embryonic seedling phenotype.

Here we show that mutants in the LAFL network suppress the embryonic seedling phenotype of the \textit{val1 val2} double mutant to varying degrees. The \textit{lec1, l1l, and abi3} mutants are partial suppressors; whereas, \textit{fus3 and lec2} mutants are complete suppressors that allow seedlings to transition to normal development. Both \textit{FUS3} and \textit{LEC2} are absolutely required for maintenance of the activated state of LAFL network in the absence of VAL repressors; whereas, \textit{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.

\textbf{RESULTS}

\textbf{The Frequency of Embryonic Phenotypes in \textit{val1 val2} Seedlings Depends on the Time of Rescue in Seed Development}

By genotyping progeny from \textit{val1 val2/+} plants, we confirmed that around 20\% of mature \textit{val1 val2} double mutant seeds could escape the embryonic seedling fate and produce viable seedlings (Fig. S1A). The frequency of escapes was sufficient to allow maintenance of a homozygous \textit{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 \textit{val} single and double mutant seedlings, developing seeds were harvested at one day intervals between 7 and 15 days after flowering (DAF) and placed on MS media. Under our growth conditions, seeds of \textit{Col-0} wild type (WT) as well as the \textit{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. Seedling phenotypes were recorded following 12d of growth on MS media at 23-25°C with continuous light. Based on the observed range of expression, seedling phenotypes were classified into 3 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 (SAM) 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 WT and val single mutant seeds rescued at mid to late stages (11-15 DAF) produced normal seedlings (Fig. 1B). Non-green and abnormal growth phenotypes were observed in WT 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 a 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 (Fig. S1B and C; see also Suzuki et al., 2007).

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

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 qPCR assay to measure expression of LAFL genes and two downstream SSP genes (At2S1 and CRC) in WT 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 WT 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 to WT and val2, while expression of the LAFL genes was at or below the threshold for detection in WT and single mutant seedlings rescued at later stages (11 to 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 WT 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 to the WT and val single mutants (Fig. 2B). This result indicated that in contrast to their role in seedlings, VAL1 and VAL2 do not repress LAFL gene expression during seed development.

**Suppression of the Embryonic Seedling Phenotype by Mutants in LAFL Network Genes**

We hypothesized that de-repression of the 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 lec1, l1l, lec2, fus3 and abi3 mutants, respectively, into the val1 val2 mutant background (Fig. S3). The lec1, l1l, and lec2 alleles used in this analysis are T-DNA insertion mutants. abi3-6 allele is a deletion mutation that produces a truncated, non-functional transcript (Nambara et al., 1994). fus3-3 allele is a point mutation that blocks intron splicing to produce non-functional aberrant transcripts (Luerssen et al., 1998).

As shown in Fig. 3, the triple mutants containing fus3 or lec2 had uniformly normal seedling phenotypes across all times of rescue during seed development revealing that the fus3 and lec2 mutants are complete suppressors of the val1 val2 embryonic seedling phenotype. This result demonstrates that both FUS3 and 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 l1l, lec1, and abi3, respectively. In the l1l and 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. 3A and B). The maximum proportions of normal seedlings reached approximately 50% and 85% in l1l and abi3-6
triple mutants, respectively (Fig. 3B). In the l1l and abi3 triples, most of seedlings rescued after 13 DAF transitioned to a normal developmental fate; whereas, for the lec1 triple mutant, nearly 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. 3A 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 (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 also a partial suppressor of val1 val2 embryonic seedling phenotype (Fig. 3A 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% vs 6%; 13 DAF, 82% vs 54%; and 15 DAF, 92% vs 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 in comparison to the abi3-6 null mutation. This result suggests that COAR domain of ABI3 may have a role in negative regulation of the LAFL network.

**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). All together, 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 (Fig. 5C and 3B). This result indicated that the *abi3-6* null mutation also partially suppressed the
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 pair-wise 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%) (Fig. 6B and Fig. 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. 6A 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, S4, S5 and 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 (Fig. 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 pair-wise correlations of LAFL gene expression levels in seedling of all genotypes rescued at various stages of seed development excluding data points where 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, non-redundant activities that are each essential for maintenance of the LAFL network expression in the absence of VAL repression.

**DISCUSSION**

Genetic analyses indicate that de-repression 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 de-repression of the LAFL network during seed germination is responsible for the embryonic seedling phenotype of the val1 val2 mutant (Fig. 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, l1l, lec2, fus3 and abi3) in the val1 val2 background (Fig. 3, 6, S4A, S5A and 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 (Fig. 4, 7, S4B, S5B, and 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, non-redundant 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 suppressed val1 val2 abi3 and val1 val2 lec1 triple mutants. The residual formation of embryonic tissues in these triple mutants is likely attributable to continued expression of LEC2 and FUS3. While for both genotypes the frequency of normal seedlings increased in seedlings rescued at later stages of seed development, the difference between seedlings rescued at early (7 DAF) and late (15 DAF) stages of seed development is more pronounced for the val1 val2 abi3 triple. Thus, the abi3-6 mutant is only effective in suppressing the embryonic phenotype in seeds rescued at later stages of seed development (after 13 DAF, Fig. 3B); whereas, partial suppression by lec1 was evident in seedlings rescued at 7 DAF, the earliest time point sampled in our analysis. Interestingly, this contrast coincides with the differential temporal expression patterns of LEC1 and ABI3 in seed development. LEC1 expression peaks during early seed development and declines during the maturation phase where ABI3 expression is high. This indicates that the additive effect of LEC1 and ABI3 in promoting the embryonic seedling fate is partly due to their complementary temporal patterns of expression. Hence, seedling fate is determined by which of the two genes is more active at the time of rescue. This pattern is consistent with evidence that VAL repression occurs at the 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 post-transcription 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), 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; To et al., 2006; Kwong et al., 2003); 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 sub-networks, 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 ($R^2 \geq 0.90$) 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 sub-networks. 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 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 L1L 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 HDA19 targets in a chromatin immunoprecipitation (ChIP) analysis, and VAL2 was further shown to interact with HDA19 in a yeast two hybrid (Y2H) 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 co-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*, *L1L*, *LEC2*, and *FUS3* observed during late embryo development (Suzuki et al., 2007, 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 (Fig. 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 7-14 DAF produced normal seedlings that lacked expression of downstream seed maturation markers (Fig. S6A 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 non-essential 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 co-evolved with the LAFL network primarily to provide a mechanism to enable the germinating seed to transition from the maturation phase to reserve re-mobilization 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 co-evolved to regulate initiation of and exit from the seed maturation program, respectively.
MATERIALS AND METHODS

Plant Material and Growth Conditions

For characterization of post-germination-seedling phenotypes at various stages of seed development, siliques were rescued at 7 to 15 days after flowering (DAF) and sterilized immediately after harvest. And then seeds were plated on MS media containing 1X Murashige and Skoog salt, 0.05% MES, 1% sucrose sterilized by filtration, and 0.15% of phytagel (Sigma). Plated seeds were stratified at 4°C in dark for 3 days. To record seedling phenotypes and to sample plant materials for RNA isolation, stratified seeds were incubated at 23-25°C for 12 and 6 days under continuous light, respectively. When harvested at 15 DAF, seeds of WT, val1, val2, val1 val2, val1 val2 l1l, and val1 val2 abi3-12 genotypes are brown in color, not fully dry and desiccation tolerant. Seeds of the other triple mutants and two quadruple mutants (val1 val2 abi3-6 l1l and val1 val2 val3 abi3-6) harvested at 15 DAF were green or dark in color and not completely dry, while all other quadruple and quintuple mutants failed to germinate after 14 DAF. To test the effect of sugars on expression of the seedling phenotype, mature seeds of WT and val mutants were germinated on MS media supplemented with different concentrations of filter sterilized sucrose (0.1%, 1%, 2%, 3%, 4%, and 5%). Arabidopsis thaliana Col-0 WT and all mutant plants used in this study were grown in soil by transplanting from MS plates to plastic pots at 25°C with a continuous light.

Genetic Analysis and Construction of Mutant Lines

The T-DNA insertion alleles (Fig. S3), val1-2 (SALK_088606), val3-1 (SALK_015582), lec2 (SALK_015228), lec1 (SALK_000450), and l1l (GABI_670E09) in Col-0 ecotype, were obtained from the Arabidopsis Biological Resource Center. The val2-1 allele was described in Suzuki et al. (2007). abi3-6, abi3-12 and fus3-3 stocks were provided by Eiji Nambara and Peter McCourt (University of Toronto). val1 val2/+ and abi3-6 fus3-3 mutant lines were established previously in our lab. val1 val2 homozygous double mutant seed were created by self-pollination of val1 val2/+ plants. val1 val2 abi3-6 and val1 val2 fus3-3 genotypes were created by crossing val1 val2/+ with abi3-6 and fus3-3, respectively. val1 val2 abi3-12 was created by crossing val1 val2 with abi3-12. val1 val2 l1l, val1 val2 lec1, and val1 val2 lec2, were created by crossing val1 val2 abi3-6 with lec2, lec1, and l1l, respectively. val1 val2 val3 and val1 val2 val3 abi3-6 were created by crossing val1 val2 abi3-6 with val3. Quadruple mutants (val1 val2 abi3-6 l1l, val1 val2 abi3-6 lec1, val1 val2 abi3-6 fus3, and val1 val2 abi3-6 lec2) were created by crossing val1 val2 abi3 with l1l, lec1, abi3-6 fus3-3, and lec2,
respectively. Quadruple mutants (val1 val2 l1l fus3-3 and val1 val2 l1l lec2) were created by crossing val1 val2 fus3-3 with l1l, and val1 val2 lec2 with val1 val2 l1l, respectively. val1 val2 lec1 fus3-3 was created by crossing val1 val2 fus3-3 with lec1. val1 val2 fus3-3 lec2 and val1 val2 abi3-6 fus3-3 lec2 were created by crossing val1 val2 fus3-3 with val1 val2 lec2, and val1 val2 abi3-6 fus3-3 with val1 val2 abi3-6 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 WT. The primers used for genotyping all the alleles were listed in Supplemental Table S1. abi3-6 and fus3-3 alleles were genotyped following the description in To et al. (2006) with different set of primers. To establish the val1 val2 abi3-12 triple mutant, abi3-12 homozygotes were identified among progeny of val1 val2+/abi3-12/+ plants by PCR and sequencing of ABI3 alleles (Fig. S3).

Quantitative Real-Time RT-PCR (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 RNase-free DNase (Promega). The abundance of target gene transcript was determined by quantitative real-time RT-PCR using a Power SYBR Green RNA-to-Ct 1-step kit (Applied Biosystems) with a step one plus system (Applied Biosystems). For analysis of gene expression, an absolute quantification method was used. Standard curves were constructed using plasmids that containing the target gene sequences. The primers used for quantitative PCR are listed in Supplemental Table S2.

Supplemental materials

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

Supplemental Figure S2. Effects of sucrose 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 l1 l fus3-3* and *val1 val2 l1 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 *afl* 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

**ACKNOWLEDGEMENTS**

We are indebted to Eiji Nambara and Peter McCourt (University of Toronto) for providing the *abi3-6, abi3-12* and *fus3-3* stocks. We thank Shan Wu for assistance with genotyping, and Dr. Karen Koch and her lab for advice and access to real-time PCR equipment.
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FIGURE LEGENDS

Figure 1. The embryonic seedling phenotype of the val1 val2 double mutant. Col-0, val1, val2, and val1 val2 seeds were rescued at one day increments between 7 and 15 days after flowering (DAF). Sterilized seeds were sown on MS phytagel media and stratified by incubation at 4°C in the dark for 3 days. After treatment, they were grown for 12 days at 23-25°C under continuous light, and the seedling phenotypes were recorded. A, Based on the observed variation, the phenotypes of val1 val2 mutant seedling are classified into 3 types: embryonic, partial, and normal. The scale bar represents 1 cm. B, Representative phenotypes of Col-0, val1, val2, and val1 val2 seedlings rescued at different stages of seed development (7-15 DAF). The scale bar represents 1 cm. C, Histogram of the three phenotypic classes of val1 val2 seedlings grown from seed rescued at different seed developmental stages (7 to 15 DAF). Values are means ± SEM (n=6-10).

Figure 2. Expression of LAFL network genes in wild type and rescued val mutant seedlings (A) and developing seeds (B). Q-PCR analysis was performed to quantify the transcript level of LAFL network genes (LEC1, L1L, LEC2, FUS3 and ABI3) and downstream targets (At2S1 and CRC). Bars show copies of mRNA per nanogram total RNA. Values are means ± SEM (n=3), and n.d. indicates not detected. Asterisks indicate significant differences (Student’s t test; P< 0.05) in the transcript levels of WT, val1, val2 compared to the corresponding val1 val2 double mutant mean. A, Marker gene expression in 6-day-old wild-type and val (val1, val2, and val1 val2) mutant seedlings grown from seeds rescued at different developmental stages (9, 11, 13, and 15 DAF). B, Marker gene expression in wild-type and val (val1, val2, and val1 val2) mutant developing seeds (9 and 11 DAF).

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 l1l, val1 val2 lec1, val1 val2 abi3-6, val1 val2 abi3-12, val1 val2 fus3-3, and val1 val2 lec2) seedlings from seeds rescued at 7-15 DAF. The scale bar represents 1cm. 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 ± SEM (n=6-10).
**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-day-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 ± SEM (n=3), and n.d. indicates not detected.

**Figure 5.** Seedling phenotypes and LAFL network expression in val1 val2 val3 triple and val1 val2 val3 abi3-6 quadruple mutants. The val1 val2 val3 triple mutants were confirmed by genotyping seedlings grown from val1 val2 val3/+ triple mutant plants harvested at maturity. val1 val2 val3 abi3-6 quadruple mutant seeds were rescued at intervals between 7 and 15 DAF. The culture conditions are as described in Figure 1. A, Representative phenotypes of val1 val2 val3 triple mutant seedlings grown from mature seeds. The scale bar represents 1cm. B, Representative phenotypes of quadruple mutant val1 val2 val3 abi3-6 seedlings grown from seeds rescued at 7-15 DAF. The scale bar represents 1cm. C, Histogram of the three phenotypic classes of val1 val2 val3 abi3-6 mutant seedlings grown from seeds rescued at 7-15 DAF. The phenotypic classes are as described in Figure 1. Values are means ± SEM (n=6-10). D, Expression of seed specific marker genes in val1 val2 abi3-6 triple and val1 val2 val3 abi3-6 quadruple mutant seedlings. Q-PCR analysis was performed to quantify the transcript levels of LAFL network genes (LEC1, L1L, LEC2, FUS3 and ABI3) and downstream targets (At2S1 and CRC) in 6-day-old mutant (val1 val2 abi3-6 and val1 val2 val3 abi3-6) seedlings grown from seeds rescued at 9, 11, 13, and 15 DAF. Bars show copies of mRNA per nanogram total RNA. Values are means ± SEM (n=3).

**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 7-14 DAF. The scale bar represents 1cm. 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 ± SEM (n=6-10).
Figure 7. Expression of LAFL network genes in LAFL-VAL quadruple mutant seedlings. Q-PCR analysis was performed to quantify the transcript levels of LAFL network genes (LEC1, L1L, LEC2, FUS3 and ABI3) and downstream targets (At2S1 and CRC) in 6-day-old quadruple mutant (val1 val2 abi3-6 l1l, val1 val2 abi3-6 lec1, val1 val2 abi3-6 fus3-3, val1 val2 abi3-6 lec2) seedlings grown from seeds rescued at 9, 11, 13, and 15 DAF. Note that with the exception of abi3-6 l1l, all quadruple mutants failed to germinate after 14 DAF. Bars show copies of mRNA per nanogram total RNA. Values are means ± SEM (n=3), and n.d. indicates not detected.

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 >= 0.90; yellow R^2 < 0.90. B, Scatter plot of LEC2 expression versus each of the other LAFL genes. Regression equations and R^2 are indicated for data from each comparison.
Figure 2

(A) Rescued seedlings

- LEC1
- L1L
- LEC2
- FUS3
- ABI3
- At2S1
- CRC

(B) Developing seeds

- LEC1
- L1L
- LEC2
- FUS3
- ABI3
- At2S1
- CRC

Copies/ng total RNA

9 DAF 11 DAF 13 DAF 15 DAF


Col-0 val1 val2 val3

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Figure 3
Figure 4
Figure 7

The figure shows the expression levels of various genes in different developmental stages. The x-axis represents different days after fertilization (DAF), and the y-axis represents the number of copies per total RNA. The bars for each gene indicate the expression levels across different genotypes and developmental stages, with error bars indicating variability. The genes shown include LEC1, L1L, LEC2, FUS3, ABI3, At2S1, and CRC. The bars are color-coded for different genotypes (val1, val2, abr3-6) and stages (9 DAF, 11 DAF, 13 DAF, 15 DAF).
Figure 8

A

Correlation matrix of LAFL factor co-expression

<table>
<thead>
<tr>
<th></th>
<th>R²</th>
<th>FUS3</th>
<th>ABI3</th>
<th>L1L</th>
<th>LEC1</th>
<th>LEC2</th>
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<td>0.93</td>
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B

Graph showing correlation between LEC2 (Copies/ng total RNA) and other factors, with regression lines and R² values.