bZIP67 Regulates the Omega-3 Fatty Acid Content of Arabidopsis Seed Oil by Activating FATTY ACID DESATURASE3

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Arabidopsis thaliana seed maturation is accompanied by the deposition of storage oil, rich in the essential ω-3 polyunsaturated fatty acid α-linolenic acid (ALA). The synthesis of ALA is highly responsive to the level of FATTY ACID DESATURASE3 (FAD3) expression, which is strongly upregulated during embryogenesis. By screening mutants in LEAFY COTYLEDON1 (LEC1)–inducible transcription factors using fatty acid profiling, we identified two mutants (lec1-like and bizp67) with a seed lipid phenotype. Both mutants share a substantial reduction in seed ALA content. Using a combination of in vivo and in vitro assays, we show that bizp67 binds G-boxes in the FAD3 promoter and enhances FAD3 expression but that activation is conditional on bZIP67 association with LEC1-LIKE (L1L) and NUCLEAR FACTOR-YC2 (NF-YC2). Although FUSCA3 and ABSCISIC ACID INSENSITIVE3 are required for L1L and bizp67 expression, neither protein is necessary for [bizp67:L1L:NF-YC2] to activate FAD3. We conclude that a transcriptional complex containing L1L, NF-YC2, and bZIP67 is induced by LEC1 during embryogenesis and specifies high levels of ALA production for storage oil by activating FAD3 expression.

INTRODUCTION

Seed maturation in higher plants is associated with the deposition of storage reserves, such as oil (triacylglycerol), carbohydrates, and proteins (Baud and Lepiniec, 2010). The physiological role of these reserves is to serve as a source of carbon (and nitrogen) to fuel postgerminative growth, thereby enabling seedling establishment and completion of the plant’s life cycle (Graham, 2008). However, seed storage reserves also provide a major source of nutrition for humans and livestock, and serve as feedstock for a broad variety of industrial applications (Lu et al., 2011). As such, seeds have a significant social and economic importance. Understanding how seed storage reserve content and composition are controlled is of considerable basic and strategic interest.

Genetic studies have revealed that a complex network of transcriptional master regulators orchestrates the seed maturation program, of which storage reserve deposition is an integral part (Vicente-Carbajosa and Carbonero, 2005; Santos-Mendoza et al., 2008). In Arabidopsis thaliana, forward genetic screens have identified four loci, in particular, that act as positive regulators: namely LEAFY COTYLEDON1 (LEC1), LEC2, FUSCA3 (FUS3), and ABSCISIC ACID INSENSITIVE3 (ABI3) (Giraudat et al., 1992; Meinke, 1992; Keith et al., 1994; West et al., 1994; Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001). LEC1 encodes a protein that is homologous to the Saccharomyces cerevisiae HEME ACTIVATOR PROTEIN3 or mammalian NUCLEAR FACTOR YB subunit of the heterotrimeric CCAAT box binding factor (Lotan et al., 1998; Lee et al., 2003). LEC2, FUS3, and ABI3 encode plant-specific transcription factors (TFs) that are closely related and contain a conserved B3 DNA binding domain (Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001).

Ectopic embryogenesis can be induced in vegetative tissues of Arabidopsis by expression of LEC1 or LEC2 (Lotan et al., 1998; Santos Mendoza et al., 2005; Mu et al., 2008). This developmental shift is accompanied by the differential expression of several hundred genes, including many that encode the metabolic apparatus for reserve synthesis and storage (Santos Mendoza et al., 2005; Mu et al., 2008). The regulation of seed storage protein (SSP) synthesis has been studied in some detail, and there is evidence to support a role for B3 domain proteins (such as ABI3) in the transactivation of SSP gene expression, either directly by binding RY/Sph cis-elements (Kroj et al., 2003; Stone et al., 2008) or through association with TFs from other families, such as basic Leu zippers (bZIP) domain proteins (Alonso et al., 2009). LEC1 has also been implicated in transcriptional activation of SSP genes through an association with bZIPs (Yamamoto et al., 2009).

In contrast with SSPs, the transcriptional regulation of many key genes involved in storage lipid biosynthesis is less well understood. These genes may be direct targets of LEC1, LEC2, ABI3, and FUS3, or they may be controlled by other TFs that interact directly or lie downstream in the regulatory network (Baud and Lepiniec, 2010). An important example is WRINKLED1 (WRI1) (Focks and Benning, 1998; Cernac and Benning, 2000).
and following ectopic expression of LEC1 were selected using 
lie downstream of or function together with LEC1 and are re-
(Li-Beisson et al., 2013). (ELONGASE1 (include
for the expression of several key enzymes in the pathways of fatty
acid modification and triacylglycerol (TAG) assembly in the
endoplasmic reticulum (Baud et al., 2007; To et al., 2012). These
include FATTY ACID DESATURASE2 (FAD2), FAD3, FATTY ACID
ELONGASE1 (FAE1), and DIACYLGlycerol ACYLTRANSFER-
ASE1 (DGAT1) (To et al., 2012), which are critical for determining the
determination and/or quantity of seed storage oil in Arabidopsis
(Li-Beisson et al., 2013).

The aim of this study was to identify and characterize TFs that
lie downstream of or function together with LEC1 and are re-
sponsible for regulating key enzymes of TAG synthesis during
Arabidopsis seed maturation. To do this, TF genes that are sub-
stantially upregulated both during wild-type embryo maturation and
following ectopic expression of LEC1 were selected using public
microarray data (Winter et al., 2007; Mu et al., 2008). T-DNA insertion
mutants were then obtained (Alonso et al., 2003) and mature seeds were
screened for informative changes in fatty acid composition. Arabidopsis seed oil contains six major
fatty acid species, namely, palmitic acid (16:0), stearic acid (18:0),
oleic acid (18:1\(^n9\)), \(\alpha\)-linolenic acid (18:2\(^n6\)), \(\alpha\)-linolenic acid (18:3\(^n3\)),
and eicosanoic acid (20:1\(^n11\)), and a number of minor species
(<3 mol % each). The composition is highly heritable and genetic
variation (both induced and natural) has been used extensively as a
tool to elucidate gene function in seed oil metabolism
(Lemieux et al., 1990; O’Neill et al., 2003). Two TF mutants were
identified that showed a seed lipid phenotype. Further experimen-
tation revealed the molecular mechanism by which both TFs
cooperate to regulate the expression of FAD3 and, therefore, the level of the \(\omega-3\) polyunsaturated fatty acid 18:3\(^n3\), which is an essential fatty acid for human and livestock nutrition.

RESULTS

L1L and bZIP67 Are Regulators of Seed Storage Oil Composition

Several studies have established that ectopic expression of LEC1
leads to the induction of genes associated with the embryo
maturation program, and results in accumulation of storage oil
(Lotan et al., 1998; Mu et al., 2008). To identify TFs that might lie
downstream of LEC1 in the regulatory network that controls seed
oil content and composition, published Affymetrix ATH1 chip microarray data were used to select those genes listed on the
Arabidopsis Transcription Factor Database (http://Arabidopsis.
med.osu-state.edu/AITFDB/) that are more than fourfold upreg-
ulated in both LEC1-overexpressing seedlings (Mu et al., 2008) and
wild-type developing seeds (stages 7 to 9; Winter et al., 2007). In total, 30 genes were shortlisted (see Supplemental Table
1 online), including AB13, FUS3, and WRI1, which have been well
characterized and are known to play a role in storage oil synthesis
(Baud and Lepiniec, 2010). LEC2 is absent from this list because it is
not upregulated in LEC1-overexpressing seedlings (Mu et al., 2008).

To screen the shortlisted TFs for a role in storage oil synthesis,
publically available T-DNA mutants (see Supplemental Table 1
online) were identified on the SIGnAL T-DNA Express website
(Alonso et al., 2003), four homozygous plants of each genotype
were grown in a controlled environment, and their seeds were
profiled for alterations in fatty acid composition (Lemieux et al.,
1990). Five mutants (fus3-3, ab13-7, wr1-3, l1l-1, and bzip67-1)
were found to exhibit significant differences in their seed fatty acid
composition (P < 0.05) in comparison to their wild-type genetic
background Columbia-0 (Col-0; Figure 1A). The alterations in fatty
acid profiles are relatively complex; therefore, canonical variate
analysis (CVA) was used to assist in the assessment of overall
differences (and similarities) between the genotypes (Figure 1B).
CVA showed that the first two CVs were sufficient to account for
the majority of the variance (93.21%) and possible discrimination.
The loadings indicated that 20:1\(^n11\), followed by 18:3\(^n3\) and 18:3\(^n6\),
were most important in the discrimination observed in the CV1
direction. 18:3\(^n6\) and 18:3\(^n3\) also had influence in the CV2 direction,
along with 18:0. It is clear that ab13-7 is very different from all other
genotypes in the CV1 direction, with a large negative CV1 score
indicating a strong influence of 18:3\(^n3\) and 18:3\(^n6\). The four other
genotypes appear to be different in the CV2 direction. The most
different of these is wr1-3, with a negative CV2 score. Interestingly,
the other three genotypes (bzip67-1, fus3-3, and l1l-1) have similar
positive CV2 scores, and fus3-3 and l1l-1 are not significantly
different given their 95% confidence circles, while bzip67-1 is separ-
ated from these two on CV1 with a positive score (Figure 1B),
suggesting a stronger influence of 20:1\(^n11\) for this genotype.

L1L (LEC1-LIKE), AB13, FUS3, and WRI1 have all been im-
licated in storage oil synthesis previously (Baud and Lepiniec,
2010), and ab13, fus3, and wr1 seeds have already been re-
ported to exhibit substantial changes in fatty acid content/ composition (Finkelstein and Somerville, 1990; Keith et al., 1994;
Focks and Benning, 1998). However, a lipid phenotype pre-
viously has not been reported for l1l (Yamamoto et al., 2009),
and bzip67 has not been reported to have any anatomical or
biochemical phenotype (Bensmihen et al., 2005; Le et al., 2010).
L1L is closely related to LEC1 and is capable of functionally
complementing the lec1 mutant, when expressed under the
LEC1 promoter (Kwong et al., 2003). However, the spatial and
temporal pattern of L1L expression during seed development is
substantially different from that of LEC1 (Winter et al., 2007), and
L1L induction is also dependent on both LEC1 and FUS3 (Mu
et al., 2008). The dependence of L1L expression on FUS3 may
explain why the fatty acid profiles of l1l-1 and fus3-3 cannot be
discriminated by CVA (Figure 1B).

bzip67 is a seed-specific basic Leu zipper protein from group
A that is closely related to AB15 (Jakoby et al., 2002). Despite the
lack of a reported phenotype of bzip67 (Bensmihen et al., 2005;
Le et al., 2010), modeling studies have placed bzip67 as a central
hub in the gene regulatory networks that govern seed maturation
(Belmonte et al., 2013). CVA suggests that the fatty acid profile of
bzip67-1 is similar (but not identical) to that of l1l-1 and fus3-3
(Figure 1B). Because little is known about the physiological role of
bzip67, we decided to characterize mutants in this gene in
greater detail and investigate its relationship with L1L and FUS3, within the context of seed lipid metabolism.

Molecular Characterization of Two T-DNA Mutants in \textit{bZIP67}

To confirm that disruption of \textit{bZIP67} does alter seed metabolism, two independent T-DNA mutant alleles were characterized for this locus (Figure 2A). PCR was performed on genomic DNA using gene-specific primers straddling the insertion site alone and in combination with T-DNA border primers (Figure 2B) and the flanking sequences were sequenced. In \textit{bzip67-1} (SALK\_085497), the insertion was found to consist of an inverted T-DNA repeat situated in the second intron at +1033 bp 3' of the start codon and results in a further 10-bp deletion. In \textit{bzip67-2} (GABI\_314D04), the T-DNA insertion site is in the third intron at +1356 bp 3' of the start codon and results in a further 22-bp deletion.

To investigate what impact the two T-DNA insertions have on \textit{bZIP67} transcript, RT-PCR was performed on RNA extracted from stage 8 (Winter et al., 2007) developing siliques of the wild type (Col-0), \textit{bzip67-1}, and \textit{bzip67-2} (Figure 2C). No PCR product was amplified from \textit{bzip67-1} and \textit{bzip67-2} using primers that straddle the T-DNA insertion sites, showing that either no transcript is present or that the RNA is incorrectly spliced. Even if mis-spliced transcripts are produced by these alleles, they are likely to be null because the conserved basic Leu zipper domain of \textit{bZIP67} is partially coded for by the last exon (Bensmihen et al., 2002) that lies downstream of the insertion sites. RT-PCR performed on separated seed and silique tissue of wild-type plants showed that \textit{bZIP67} transcripts are only detected in the seed (Figure 2D).

Public microarray data also suggests that \textit{bZIP67} expression is restricted to seed tissues (Winter et al., 2007).

To investigate whether \textit{bzip67-1} and \textit{bzip67-2} exhibit the same seed fatty acid composition phenotype, they were grown together with the wild type in a controlled environment and the seed was analyzed (Li et al., 2006). As observed previously (Figure 1A), the fatty acid composition of seed from \textit{bzip67-1} was clearly different from the wild type, and there was also no significant difference (P > 0.05) between the two \textit{bzip67} alleles (see Supplemental Figure 1A online). On a percentage basis, the levels of 18:1\textit{n}9 and 18:3\textit{n}3 are decreased in both \textit{bzip67} mutants, and the level of 18:2\textit{n}6 is increased (Figure 1A; see Supplemental Figure 1A online). Finally, a \textit{ProbZIP67}:GFP-\textit{bZIP67} construct from a transgenic line (Bensmihen et al., 2005) was introduced into the \textit{bzip67-1} background by crossing in order to perform a complementation test. Expression of \textit{bZIP67} recovered the seed fatty acid composition of \textit{bzip67-1} to a profile that is very similar to the wild type (see Supplemental Figure 1B online).

Disruption of \textit{bZIP67} Alters TAG and Phosphatidylcholine Composition, but Total Fatty Acid, Protein Content, and Seed Weight Are Unchanged

To investigate the effect of \textit{bZIP67} disruption on seed lipid composition in more detail, electrospray ionization–tandem mass spectrometry (Devaiah et al., 2006; Krank et al., 2007) was used to profile neutral and polar lipid species in mature and developing (stage 8) seeds, respectively, of \textit{bzip67-1} and the wild type (Figure 3).
Analysis of TAG showed that molecular species containing 18:3n3 (e.g., 52:6, 54:7, and 56:7) are significantly (P < 0.05) less abundant in bzip67 seeds and those containing 18:2n6 (e.g., 56:4, 56:5, and 58:4) are more abundant (Figure 3A). Similarly, analysis of phosphatidylcholine (PC) molecular species also showed that 36:4 is significantly more abundant in bzip67-1 (P < 0.05), while 36:5 and 36:6 are less abundant (Figure 3B). Smaller increases in 36:4 were also observed in phosphatidylethanolamine and phosphatidic acid, but the compositions of phosphatidylglycerol and mono- and digalactosyldiacylglycerol did not appear to be changed (see Supplemental Table 2 online). In contrast with the changes in lipid composition detected in bzip67-1 seed, the total quantity of fatty acids was not significantly different (P > 0.05) from the wild type (see Supplemental Table 3 online). Measurements of total protein content and fresh weight also suggested that there are no significant differences (P > 0.05) from the wild type (see Supplemental Table 3 online).

Disruption of bZIP67 Suppresses FAD3 and Enhances ROD1 Expression

To identify genes that may be targets of bZIP67 regulation, microarray experiments were performed on RNA from whole siliques of wild-type and bzip67-1 plants using the Affymetrix Ath1 chip. Siliques were selected that contained developing seeds at stage 8 when bZIP67 (Winter et al., 2007) and many genes involved in storage lipid synthesis are strongly expressed (Baud and Lepiniec, 2010). In whole siliques, bZIP67 expression is only present in the seed (Figure 2D); therefore, any changes in transcript abundance that are detected in bzip67-1 siliques are likely to have arisen from the seed. Analysis of data from three biological replicates suggested that disruption of bZIP67 leads to a relatively small number of changes in gene expression at this stage in development (see Supplemental Data Set 1 online). Using Extration of Differential Gene Expression software (Leek et al., 2006) only 37 genes were found with significant changes (P < 0.01) that were >1.5-fold up or downregulated. The signal for bZIP67 was reduced 12-fold in bzip67-1, which is consistent with the characterized gene defect in this mutant (Figure 2).

Cross-referencing those transcripts that are significantly (P < 0.01) up- or downregulated with a recent census of Arabidopsis genes involved in storage oil metabolism (Li-Beisson et al., 2013) flagged a single gene: FAD3. FAD3 transcript abundance was approximately twofold lower in whole bzip67-1 siliques (see Supplemental Data Set 1 online). FAD3 encodes a microsomal 18:2n6 desaturase that uses 18:2n6 esterified to PC as its substrate and is required for the majority of 18:3n3 produced in Arabidopsis seeds (Lemieux et al., 1990; Browse et al., 1993). The abundance of 18:3n3 is known to be highly responsive to the level of FAD3 expression (Shah et al., 1997; Puttick et al., 2009; O'Neil et al., 2011) and FAD3 transcript abundance increases approximately fivefold during the maturation stage of embryo development (Winter et al., 2007). Reduced expression of FAD3 could therefore explain the higher level of 18:2n6 and lower level of 18:3n3-containing species in both PC and TAG, in bzip67
seeds (Figure 3). FAD3 is also expressed in vegetative tissues; therefore, the change in gene expression detected in whole siliques might underrepresent the size of the effect in seeds. To measure FAD3 expression in stage 8 developing seeds, real-time RT-PCR experiments were performed (Figure 4). These experiments suggest that FAD3 expression is reduced fourfold in bzip67-1 seeds.

A small but significant (P < 0.05) reduction in 18:1n9 level is also observed in bzip67 seeds (Figure 1A), and this cannot easily be explained by misregulation of FAD3 (Browse et al., 1993; O’Neill et al., 2011). Therefore, real-time RT-PCR was also used to measure the transcript abundance of several other important genes associated with 18:1n9 metabolism in stage 8 developing seeds (i.e., ROD1, FAD2, FAE1, and DGAT1). None of these genes appeared to be differentially expressed in whole siliques, based on our microarray data (see Supplemental Data Set 1 online). However, the transcript abundance of ROD1 was significantly higher (P < 0.05) in real-time RT-PCR experiments performed on seeds (Figure 4). ROD1 encodes a PC:diacylglycerol cholinephosphotransferase that provides FAD2 with 18:1n9-PC substrate for desaturation to 18:2n6-PC (Lu et al., 2009). Mutations in both ROD1 and FAD2 lead to an increase in the 18:1n9 content of seed oil (Okuley et al., 1994; Lu et al., 2009). It is therefore possible that increased expression of ROD1 might contribute to the lower level of 18:1n9 in bzip67 seeds.

Disruption of bZIP67 Also Reduces Seed Storage Protein Gene Expression

Microarray analysis on bzip67-1 siliques also revealed that, unlike storage lipid synthesis, many SSP genes and several late embryogenesis abundant (LEA) genes were significantly repressed (P < 0.01) at stage 8 (see Supplemental Data Set 1 online). In Arabidopsis seeds, four genes encode the major 12S globulins/ crucifers (CRU1/ CRA1, CRB, CRU2, and CRU3/CRC) and five genes encode the 2S albumins (2S1 to 2S5) (Baud et al., 2008). The microarray data suggested that transcript abundance of all of them was reduced in bzip67-1, and in particular CRU2, 2S1, and 2S5 were downregulated more than 10-fold (see Supplemental Data Set 1 online). Ectopic expression of bZIP67, in combination with other seed-specific TFs, has been reported to activate CRU3 expression (Yamamoto et al., 2009). Real-time PCR analysis of the 12S globulins (including CRU3) and the 2S albumin genes in bzip67-1 stage 8 developing seeds confirmed that the transcript abundance of all nine genes is significantly (P < 0.05) reduced (Figure 4). Analysis of the bzip67-1 mutant therefore provides genetic evidence that bZIP67 plays a nonredundant physiological role in regulating SSP expression (Yamamoto et al., 2009).

![Figure 3. Effect of bZIP67 Disruption on Seed Lipid Composition.](image)

(A) Mature seed TAG composition. WT, the wild type. (B) Developing seed PC composition. Classes are defined by the total number of carbons and double bonds within the acyl groups. Values are the mean ± se of measurements on seed batches from four plants of each genotype. Asterisk denotes a significant difference between the wild type and bzip67-1 (P < 0.05).

![Figure 4. Analysis of Gene Expression in Developing bzip67-1 Seeds.](image)
bZIP67 Can Transactivate FAD3 in the Presence of L1L + NF-YC2

Yamamoto et al. (2009) have previously shown that several bZIP TFs from group A, including bZIP67, can transactivate the CRU3 SSP promoter in Arabidopsis protoplasts, but only when coexpressed with LEC1 (or L1L) and a NF-YC subunit, such as NF-YC2, which is seed specific. Our own analysis of the bzip67-1 mutant shows that bZIP67 is necessary for full CRU3 expression in stage 8 developing seeds (Figure 4). Furthermore, it is apparent from seed fatty acid profiling that both bzip67-1 and l1l-1 share common features; in particular, a substantial reduction in the level of 18:3n3 verses 18:2n6 in their seed oil (Figure 1A). We therefore decided to investigate whether bZIP67 might transactivate FAD3, together with L1L and NF-YC2. A 600-bp region of the FAD3 promoter was cloned upstream of β-glucuronidase (GUS), and the construct was transfected into Arabidopsis mesophyll protoplasts, alone and in combination with bZIP67, L1L, and NF-YC2 constructs driven by the 35S promoter (Yamamoto et al., 2009). Transfection with ProFAD3::GUS alone resulted in significant (P < 0.05) GUS activity, which is consistent with the fact that FAD3 is expressed and functions in vegetative tissues as well as in seeds (Browse et al., 1993). Cotransfection with bZIP67 individually did not enhance ProFAD3::GUS expression significantly (P > 0.05), while cotransfection with L1L plus NF-YC2 led to a twofold increase (Figure 5A). When bZIP67, L1L, and NF-YC2 were cotransfected, ProFAD3::GUS activity was enhanced more than sixfold (Figure 5A).

Other Group A bZIPS Can Also Transactivate FAD3 in the Presence of L1L + NF-YC2

ENHANCED EM LEVEL (EEL), AREB3 (bZIP66), and ABI5 are group A bZIPS that are closely related to bZIP67 and are also expressed in maturing seeds (Bensmihen et al., 2005). To determine whether these TFs could also function as activators of ProFAD3::GUS, each was cotransfected together with L1L and NF-YC2. As with bZIP67, EEL, AREB3, and ABI5 could enhance FAD3 expression in the presence of L1L and NF-YC2 (Figure 5B). These data suggest that strong transactivation of FAD3 can potentially be achieved by the combined action of one of several group A bZIPS, L1L, and NF-YC2. Of the three additional group A bZIPS that are known to be expressed in developing seeds, EEL is the only one that is also more than fourfold upregulated in both LEC1-overexpressing seedlings and wild-type developing seeds (see Supplemental Table 1 online). However, analysis of a bzip67-1 eel-3 double mutant showed that disruption of EEL has no additional effect on seed fatty acid composition, suggesting that this gene is unlikely to contribute to the regulation of FAD3 in vivo (see Supplemental Figure 1C online).

FUS3 and ABI3 Are Not Required for Activation of FAD3 by bZIP67-L1L-NF-YC2

Mu et al. (2008) have shown that FUS3 (and to a lesser extent ABI3) are required for LEC1 induction of FAD3. However, FUS3 and ABI3 are also required for bZIP67 and L1L expression (Kagaya et al., 2005; Mu et al., 2008). Therefore, it is not clear whether FUS3 and ABI3 participate directly in transactivation of the FAD3 promoter or act through their control of bZIP67 and L1L expression. To investigate this question, cotransfection experiments were performed using mesophyll protoplasts from wild-type, fus3-3, and abi3-7 leaves. When bZIP67, L1L, and NF-YC2 were cotransfected together, ProFAD3::GUS activity was enhanced in all three genetic backgrounds (Figure 5C). Therefore, FUS3 and ABI3 are not directly required for induction of FAD3 by bZIP67-L1L-NF-YC2.

Transactivation of FAD3 by bZIP67-L1L-NF-YC2 Is Dependent on G-Box Elements

Transactivation of CRU3 by bZIP67, LEC1 (or L1L), and NF-YC2 has been shown to rely on G-box elements in the promoter (Yamamoto et al., 2009). The FAD3 promoter contains two such
bZIP67 Can Bind FAD3 G-box1 in Vitro

Although it has been established that several members of bZIP group A can bind G-boxes in vitro, this has not been demonstrated previously for bZIP67 (Bensmihen et al., 2002; Kim et al., 2002). To confirm that bZIP67 is able to bind G-box1 from the FAD3 promoter, in vitro experiments were performed using an ELISA-based DNA binding assay (Alonso et al., 2009). Epitope-tagged recombinant bZIP67 was incubated with immobilized double-stranded DNA oligonucleotides containing the putative cis-element (G-box1), and binding was determined by immunodetection. The strength of the ELISA signal increased with the concentration of T7-tagged bZIP67 applied to the G-box1 oligonucleotide, indicating that the protein can bind to it (Figure 7A). Furthermore, in competition experiments, the addition of free G-box1 oligonucleotides could significantly suppress the ELISA signal (P < 0.05), while addition of G-box1 oligonucleotides with a mutated ACGT core could not (Figure 7B).

In Vivo Association of L1L-NF-YC2 with the FAD3 Promoter Requires bZIP67 Binding

Yamamoto et al. (2009) have shown via coimmunoprecipitation experiments that L1L and bZIP67 associate with one another in vivo. However, binding with the CRU3 promoter was not demonstrated directly. To investigate whether L1L, NF-YC2, and bZIP67 can bind the FAD3 promoter, in vivo chromatin immunoprecipitation (ChIP) experiments were performed on transgenic Arabidopsis mesophyll protoplasts using the method of Lee et al. (2007). Constructs containing either myc-tagged or untagged bZIP67, L1L, and NF-YC2 were transfected into protoplasts, and after 24 h, chromatin complexes were cross-linked using formaldehyde. After shearing by sonication, the fragmented chromatin was incubated with monoclonal anti-myc antibodies and immunoprecipitated complexes were captured using magnetic protein G beads. DNA eluted from the beads was analyzed by real-time PCR using primers corresponding to regions of the FAD3 promoter and a negative control gene (Figure 8A; see Supplemental Table 4 online).
homozygous seeds from three separate lines with the strongest effect are shown in Figure 9. The fatty acid composition of bZIP67 overexpressors was significantly different (P < 0.05) from that of the wild type. In the case of 18:1n9, levels were increased to values significantly greater than the wild type (P < 0.05), consistent with a predicted gain-of-function effect. However, 18:3n3 levels were not increased and were actually significantly decreased, relative to the wild type (P < 0.05). The total fatty acid, protein, and fresh weight of seeds from overexpressor lines was not altered (see Supplemental Table 3 online), and the plants have no obvious morphological phenotypes. These data confirm that changes in the level of bZIP67 expression do affect storage oil composition. However, the alterations caused by reduction and increase in bZIP67 expression do not simply mirror each other. The data can best be explained by a model in which bZIP67 abundance is colimiting for 18:3n3 production and where changes influence multiple protein and DNA interactions, which give rise to both positive and negative effects on gene expression.

DISCUSSION

Seed storage reserves not only serve as a major source of calories in the human diet but also provide essential metabolic precursors that we lack the capacity to synthesize for ourselves. The ω-3 polyunsaturated fatty acid α-linolenic acid (18:3n3) is an important example. The enzyme primarily responsible for the production of 18:3n3 in seeds is the microsomal ω-3 fatty acid desaturase FAD3 (Browse et al., 1993). In Arabidopsis seeds, the expression level of FAD3 determines the abundance of the fatty acid in storage oil (Puttick et al., 2009; O’Neill et al., 2011), but the precise molecular mechanism for the transcriptional regulation of FAD3 is not known. In this study, we addressed this question by identifying three components of a transcriptional complex that govern FAD3 expression in developing seeds.

We used a targeted reverse genetic screen to identify five TFs that are induced during embryo maturation, act downstream of LEC1, and lead to significant changes in seed fatty acid profile.
bZIP67, all share a characteristic reduction in the ratio of 18:3n3 to FAD3 association with L1L and NF-YC2. Furthermore, although FUS3 binding and enhanced transcription are conditional on bZIP67 mutants exhibited a unifying feature. Namely, fus3 when they are knocked out. The fatty acid profiles of three mutants exhibited a unifying feature. Namely, fus3, I1L, and bzip67 all share a characteristic reduction in the ratio of 18:3n3 to 18:2n6, potentially implicating them in the regulation of FAD3. Disruption of FUS3 is known to affect seed lipid metabolism and block FAD3 expression (Mu et al., 2008). However, I1L and bzip67 have not been shown to exhibit a seed lipid phenotype previously, and in the case of bzip67, no phenotype of any kind has so far been ascribed to the mutant (Bensmihen et al., 2005; Le et al., 2010). This is despite models that place bzip67 near the center of gene regulatory networks that govern seed maturation (Belmonte et al., 2013). Given this fact, we focused primarily on characterizing bzip67.

Microarray and real-time PCR experiments performed on developing siliques and seeds of bzip67-1 confirmed that FAD3 is downregulated, while the expression of many other genes involved in lipid metabolism is unaffected. Disruption of bZIP67 had a pronounced negative impact on the expression of all the major SSP genes. Interestingly, Yamamoto et al. (2009) have shown that bZIP TFs from group A, including bZIP67, can transactivate the CRU3 SSP promoter in Arabidopsis protoplasts, but only when coexpressed with LEC1 (or L1L) and a NF-YC subunit such as NF-YC2. Given that I1L and bzip67 share a reduction in the ratio of 18:3n3 to 18:2n6, we hypothesized that FAD3 might also be regulated by the cooperative actions of these three proteins. Using a combination of in vivo and in vitro assays, we show that bZIP67 binds G-box cis-elements in the FAD3 promoter and enhances FAD3 expression but that DNA binding and enhanced transcription are conditional on bZIP67 association with L1L and NF-YC2. Furthermore, although FUS3 (and to a lesser extent ABI3) are known to be required for L1L, bZIP67, and FAD3 expression during seed maturation (Mu et al., 2008), using fus3 and abi3 null backgrounds, we show that these proteins need not participate directly in transactivation of FAD3 by [L1L:NF-YC2:bZIP67].

Based on our data, we propose a model (Figure 10) whereby LEC1 specifies high levels of 18:3n3 production during Arabidopsis seed development by inducing FUS3 which then, either independently or cooperatively with LEC1, triggers the induction of L1L, NF-YC2, and bzip67. The latter three gene products then combine to form part of a transcriptional complex that binds the FAD3 promoter via bZIP67 interaction with G-boxes and drives FAD3 expression. It is likely that some redundancy exists among the isoforms that make up the complex, since I1L and bzip67 seeds only show a partial reduction in 18:3n3 content and nf-yc2 does not exhibit a significant change in fatty acid composition (Figure 1).

LEC1 and L1L are considered to be functionally equivalent (Kwong et al., 2003), and the complex may potentially contain either protein. However, the temporal and spatial patterns of expression of the two genes during seed development differs (Kwong et al., 2003), and while LEC1 expression peaks sharply in heart stage embryos (stage 4) and also endosperm, L1L is most strongly expressed in torpedo and walking stick stage embryos (stage 6-7), when both bzip67 and FAD3 expression are also approaching their peak (see Supplemental Figure 3 online; Winter et al., 2007). Other group A bZIPs, in addition to bzip67, may also form part of the complex that regulates FAD3 expression. We show that EEL, AREB3, and ABI5 can also transactivate FAD3 expression in combination with L1L and NF-YC2. EEL is the only one of these three genes that has a similar temporal pattern of expression during seed development to bzip67 (Winter et al., 2007) and is also more than fourfold enhanced in comparison to wild type (Figure 1).

Figure 9. Effect of bZIP67 Overexpression on Seed Fatty Acid Composition.

Values are the mean of measurements of seeds from six plants of each genotype. All se are <1% of the mean. For each fatty acid, the columns from left to right correspond to the numbered genotypes (1 to 5). Wild-type (Col-0) plants were transformed with a construct containing bZIP67 under the control of the glycmin promoter, and three independent homozygous T3 lines with the strongest effect were selected and analyzed (OE1-3). Asterisk denotes a significant difference from the wild type (WT) (P < 0.05).

Figure 10. A Model Illustrating the Proposed Role of LEC1, L1L, FUS3, ABI3, NF-YC2, and bZIP67 in the Transcriptional Regulation of FAD3 during Arabidopsis Embryo Maturation.

Loss- and gain-of-function genetic studies have established that L1L, FUS3, ABI3, NF-YC2, and bZIP67 are essential for FAD3 expression and are induced by LEC1 (Mu et al., 2008). FAD3 expression is also dependent on FUS3 and ABI3 (Mu et al., 2008). Here, we show that bZIP67 binds the FAD3 promoter via G-box elements and that trans-activation depends on complex formation between bZIP67, L1L, and NF-YC2 (and not ABI3 and FUS3). Analysis of I1L seeds suggests that the complex contains L1L rather than LEC1. This would be consistent with expression data that show that L1L is expressed more strongly in embryonic tissues than LEC1 during the stages when FAD3 is also expressed (Winter et al., 2007).
induced by ectopic expression of LEC1 (Mu et al., 2008). However, analysis of the bzip67-1 eel-3 double mutant seed suggested that EEL does not participate in the regulation of fatty acid composition. Bensmihen et al. (2005) reported that simultaneous RNA interference–targeted suppression of bZIP67, EEL, and AREB3 leads to no macroscopic changes in seed morphology, but no analysis of storage reserves was described.

Although we show that bZIP67 plays a specific role in regulating the level of 18:3n3 in seeds through its regulation of FAD3, it is apparent from our analysis of bzip67 mutants and bZIP67 overexpression lines that manipulating the abundance of this TF has broader and more complex effects on lipid metabolism. Seeds of bzip67 contain slightly less 18:1n9 as well as less 18:3n3, and this cannot be explained simply by misregulation of FAD3 (Puttick et al., 2009; O’Neill et al., 2011). Real-time PCR experiments performed on developing seeds suggest that ROD1 is upregulated in bzip67, and this might explain the low 18:1n9 content, since the rod1 mutant has elevated 18:1n9 levels (Lu et al., 2009). Interestingly, ROD1 expression peaks earlier in seed development than bZIP67 and the transcript abundance of the two genes is inversely correlated (Winter et al., 2007). To et al. (2012) recently showed that ROD1 expression is reduced in the wr1 wr3 wr4 mutant, suggesting that WRI family TFs regulate this gene. How bZIP67 represses ROD1 expression is currently unclear. However, it is possible that bZIP67 might block the interaction of other TFs (such as WR1) with the ROD1 promoter via either DNA or protein binding. Group A bZIPs are thought to be capable of forming dimers with one another (Dempmann et al., 2004), and both negative and positive regulatory roles have been shown previously. For example, the abi5 mutant has reduced expression of the LEA genes EM1 and EM6, while expression of these genes is enhanced in the eel mutant (Finkelstein and Lynch, 2000; Bensmihen et al., 2002). Overexpression of bZIP67 in wild-type plants leads to an increase in 18:1n9, but, surprisingly, 18:3n3 is not increased and is actually slightly decreased. This result suggests that bZIP67 is not the rate-limiting component for trans-activation of FAD3 expression. Indeed, an overabundance of bZIP67 might even inhibit FAD3 expression by sequestering other components of the complex. Interestingly, the fatty acid profile of l1l seeds mimics that of bZIP67 overexpression with increased 18:1n9 and decreased 18:3n3, so L1L function might be negatively affected (Figures 1A and 9).

The observation that all major SSP genes are substantially downregulated in stage 8 developing bzip67 seeds supports a physiological role of this TF in regulating SSP expression (Yamamoto et al., 2009) as well as FAD3. Although ab15 and eel are affected in the expression of certain LEA genes, such as EM1 and EM6 (Finkelstein and Lynch, 2000; Bensmihen et al., 2002), these genes do not appear to be misregulated in bzip67, while some other LEA genes are affected (see Supplemental Data Set 1 online). These data suggest some specificity of function for bZIP67, ABI5, and EEL. The impact of bZIP67 on SSP expression does not translate into a reduced level of total protein in mature seeds. However, SSPs are still expressed in bzip67-1. It is probable that other TFs partially compensate for a defect in bZIP67. Other group A bZIPs might contribute in this instance (Yamamoto et al., 2009). However, bZIP53 (from group S1) has also been reported to activate CRU3 via G-box binding as a heterodimer with group B bZIPs, and a bzip53 mutant was also shown to have reduced CRU3 and 2S2 expression during seed development (Alonso et al., 2009). It appears that bZIP53 is not induced by ectopic expression of LEC1 based on microarray analysis (Mu et al., 2008) and was therefore not investigated in this study. Interestingly, severe disruption of SSPs in soybean (Glycine max) seeds does not alter the protein content of the seed because other proteins are produced to compensate (Schmidt et al., 2011). It is therefore possible that detailed proteomic analysis of seeds from mutants such as bzip53 and bzip67 might reveal quantitative differences in specific SSPs. In addition to FAD3 and SSPs, our microarray data suggest that several other genes with well-defined developmental and metabolic functions in seeds might be positively regulated by bZIP67, and these await further study (see Supplemental Data Set 1 online). Among the genes is SUCCROSE SYNTHASE2, which Yamamoto et al. (2009) previously showed could be activated by [L1L-NF-YC2:bZIP67]. We investigated two previously characterized T-DNA mutants in this gene (Bieniawska et al., 2007) but did not observe any effect on seed fatty acid composition.

In conclusion, we show that the LEC1-inducible transcriptional regulators L1L and bZIP67 both play a significant and nonredundant role in storage reserve accumulation during Arabidopsis seed maturation. Focusing on the regulation of storage oil composition, we demonstrate that bZIP67 is required for FAD3 expression and, thus, the production of the essential fatty acid 18:3n3, which is a major component of many seed oils. We show that bZIP67 binds the FAD3 promoter directly via interaction with a G-box but that bZIP67 also requires both L1L and a NF-YC subunit, such as the seed-specific NF-YC2, to drive gene expression. LEC1 induction of bZIP67, L1L, and NF-YC2 is known to require FUS3 and ABI3, but we show that neither protein is directly necessary for the protein complex to trans-activate FAD3. Hence, we provide a model to explain how the production of 18:3n3 for incorporation into seed oil is ultimately coupled to embryogenesis in Arabidopsis. Further work will be required to address how the expression of other important structural genes involved in fatty acid modification and TAG assembly are integrated with the embryogenesis developmental program.

METHODS

Plant Material and Growth Conditions

All T-DNA insertion mutants were identified on the SIGnAL T-DNA Express website (http://signal.salk.edu/cgi-bin/tdnaexpress), and seeds were obtained from the European Arabidopsis Stock Centre (NASC; University of Nottingham, UK). All mutants were from the SALK, GABI-Kat, or SAIL collections, and the appropriate wild-type genetic backgrounds were used for comparison. The bZIP67;GFP-bZIP67 reporter line used in this study is described by Bensmihen et al. (2005). For plant growth experiments, the seeds were sown on moist Levingtons F2 compost in 7-cm pots, and the pots were stored in the dark for 4 d before being transferred to a growth chamber set to 21°C, 70% relative humidity (16 h light [22°C]/8 h dark [18°C]; PPFD = 150 μmol m−2 s−1). The genotype of the T-DNA lines was confirmed by genomic PCR using gene-specific primers in combination with T-DNA left and right border primers (Alonso et al., 2003). Primers are listed in Supplemental Table 4 online.
Metabolite Measurements

The fatty acid content and composition of seeds was measured by gas chromatographic analysis after combined digestion and fatty acid methyl ester formation using the method of Browse et al. (1996). Pentadecanoin (Sigma-Aldrich) was added to the samples as an internal control. Seed total protein and storage protein content were determined using the methods of Baud et al. (2002). TAG and phospholipid analyses were performed on developing seeds (between stages 7 and 9; Winter et al., 2007) and mature dry seeds using a 4000 OTRAP LC-MS/MS (ABSciex). Seeds were ground in 1 mL of ethanol/chloroform (9:1 v/v), shaken for 1 h, and centrifuged for 2 min at 3500 rpm, and the supernatant was transferred to a new vial and dried down under N2. The lipids were dissolved in methanol and centrifuged for 2 min at 3500 rpm, and the supernatant was transferred to a new vial and dried down under N2. The lipids were dissolved in chloroform/methanol/0.3 M ammonium acetate (300:665:35 v/v) for phospholipid analysis following the methods described by Devaiah et al. (2006). For TAG, the lipids were dissolved in IMAD buffer (isopropl alcohol/methanol/50 mM ammonium acetate/dichloromethane [4:3:2:1 v/v]), and analysis was performed following the method of Krank et al. (2007), except that 12 periods of 2 min were used for neutral loss scans. The data were subsequently analyzed using Lipidview (V1.1) software.

Gene Expression Analysis

DNase-treated total RNA was isolated from developing siliques and seeds using the RNeasy kit from Qiagen with modifications described by Wu et al. (2002). The synthesis of single-stranded cDNA was performed using SuperScript II RNase H+ reverse transcriptase from Invitrogen. Real-time PCR was performed in a MyQ Single-Color real-time PCR detection system (Bio-Rad) using qPCR Mastermix Plus from Eurogentec. Data were analyzed using Bio-Rad iQ5, Optical System Software, version 2.0. The primer pairs used for real-time PCR are listed in Supplemental Table 4 online. Microarray analysis was performed by NASC using the Affymetrix ATH1 chip, according to the manufacturer’s protocols. Raw data were normalized by MAS5 (www.affymetrix.com) to a target signal of 500. P and Q values for the comparison of genotypes were calculated using Extraction of Differential Gene Expression software (http://www.genomine.org/edge/), and a P = 0.1 cutoff was used to select the genes that are differentially regulated (Liu and Howell, 2010). All key findings were replicated in independent experiments using real-time PCR. The microarray data (NASCARRAYS-606) are publicly available at the NASC website (http://Arabidopsis.info/).

In Vitro Protein-DNA Binding Assays

Protein-DNA binding assays were performed using the method of Alonso et al. (2009), with the following modifications. A cDNA encoding bzIP67 was cloned into pET23a (Novagen), and the T7 epitope-tagged protein was expressed in Escherichia coli. Biotinylated oligonucleotides derived from the FAD3 promoter G-box1 sequence (5′-GTTTACCTTATTGTTAAGGATTTAAC-3′ and 5′-GTTTATACCTTATTGTTAAGGATTTAAC-3′) or a mutated version (5′-GTTTACCTTATTGTTAAGGATTTAAC-3′ and 5′-GTTTATACCTTATTGTTAAGGATTTAAC-3′) were annealed and used for binding assays.

Transient Expression and Chromatin Immunoprecipitation

Arabidopsis thaliana mesophyll protoplasts were prepared, transfected, and cultured according to the methods of Yoo et al. (2007). After polyethylene glycol–calcium transfection with plasmid DNA carrier, promoter constructs (ProFAD3::GUS and Pro35S::LUC) and combinations of effector constructs (Pro35S::bZIP67, Pro35S::EEL, Pro3SS::ABI5, Pro3SS::AREB3, Pro3SS::L1L, and Pro3SS::NF-YC2), cells were cultured for 16 h before luciferase (LUC) and GUS activities were determined using methods described by Yamamoto et al. (2009).

For ChIP assays, Arabidopsis mesophyll protoplasts were transfected with various combinations of Pro35S::bZIP67-myc, Pro35S::L1L-myc, and Pro35S::NF-YC2. After 24 h, protoplasts were harvested and ChIP assays were conducted following the procedure of Lee et al. (2007), with minor modifications. After formaldehyde fixation, the chromatin of the protoplasts was isolated and extensively sheared by sonication to obtain fragment sizes between 300 and 400 bp. Rat anti-myc monoclonal antibodies (Roche) and Dynabeads Protein G magnetic beads (Invitrogen) were used to immunoprecipitate the genomic fragments. Real-time PCR was performed on the immunoprecipitated DNA as described above using primer sets corresponding to four regions of FAD3 and a control gene (ACTIN7) and were corrected for their individual PCR amplification efficiencies. The primer pairs are listed in Supplemental Table 4 online.

Creation of DNA Constructs and Arabidopsis Transformation

bzIP67 was amplified by PCR from cDNA using primer pairs 5′-CGTCTGAAATGCTGTTTTGGAAATCGAGAC-3′ and 5′-GCCCCGGGTTTACACCCGGTGCACCTGCGACATCCCT-3′. The product was cloned into the TA vector pCR2.1 (Invitrogen) following the manufacturer’s instructions. The gene was then excised using XbaI and Smal (restriction sites underlined) and inserted into the destination vector pBiNHygRed3 using T4 DNA ligase. The construct was transformed into Agrobacterium tumefaciens strain GV3101 by heat shock and subsequently into bzip67-1 using the floral dip method (Clough and Bent, 1998). Transformed seeds were identified by detection of the DsRed marker using fluorescence microscopy.

Statistical Analysis

One-way analysis of variance was used to assess the differences between genotypes or treatments. Following significant (P < 0.05) F-test results, means of interest were compared using the appropriate LSD value at the 5% (P = 0.05) level of significance, on the corresponding df. CVA was also used to assess overall differences in seed fatty acid composition between genotypes. As the data were compositional, account of this dependence was taken using a centering transformation. The GenStat (2011, 14th edition; VSN International) statistical system was used for these analyses.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: LEC1, At1g21970; L1L, At5g47670; ABI3, At3g24650; FUS3, At3g26790; WRI1, At3g44460; ROD1, At3g29980; CRU1, At5g54320; FAD3, At3g29980; CRU3, At5g15820; CRU2, At1g03880; CRU1, At1g03890; CRU3, At4g28520; 2S1, At4g27140; 2S2, At4g27150; 2S3, At4g27160; 2S4, At4g27170; 2S5, At5g54740; NF-YC2, At1g56170; ABI5, At2g36270; EEL, At4g11070; and AREB3, At3g56850. The microarray data (NASCARRAYS-606) are publicly available at the NASC website (http://Arabidopsis.info/).

Supplemental Data

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

Supplemental Figure 1. Seed Fatty Acid Composition of Various Genotypes.

Supplemental Figure 2. The FAD3 Promoter Region Used for Promoter-Reporter Studies.

Supplemental Figure 3. Temporal and Spatial Expression Patterns of LEC1, L1L, bzip67, and FAD3 in Seeds.

Supplemental Table 1. A List of Putative Transcription Factors That Are >4-Fold Upregulated in LEC1-Overexpressing Seedlings and Maturing Wild-Type Embryos.

Supplemental Table 2. Composition of Polar Lipid Classes in Developing bzip67-1 Seeds.
Supplemental Table 3. Effect bZIP67 Disruption and Overexpression on Seed Weight and Storage Reserve Content.

Supplemental Table 4. List of Primers Used in This Study.

Supplemental Data Set 1. ATH1 Microarray Data from Developing Siliques of bzip67-1.

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AUTHOR CONTRIBUTIONS

P.J.E. conceived and designed the experiments. A.M., A.A.K., H.v.E., E.S., S.K., and P.J.E. performed the experiments. S.J.P. carried out the statistical analysis. P.J.E. wrote the article.

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Regulation of FAD3 by bZIP67

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bZIP67 Regulates the Omega-3 Fatty Acid Content of Arabidopsis Seed Oil by Activating FATTY ACID DESATURASE3
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