Identification of Direct Targets of FUSCA3, a Key Regulator of Arabidopsis Seed Development1[C][W][OA]

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FUSCA3 (FUS3) is a B3 domain transcription factor that is a member of the LEAFY COTYLEDON (LEC) group of genes. The LEC genes encode proteins that also include LEC2, a B3 domain factor related to FUS3, and LEC1, a CCAAT box-binding factor. LEC1, LEC2, and FUS3 are essential for plant embryo development. All three loss-of-function mutants in Arabidopsis (Arabidopsis thaliana) prematurely exit embryogenesis and enter seedling developmental programs. When ectopically expressed, these genes promote embryo programs in seedlings. We report on chromatin immunoprecipitation-tiling array experiments to globally map binding sites for FUS3 that, along with other published work to assess transcriptomes in response to FUS3, allow us to determine direct from indirect targets. Many transcription factors associated with embryogenesis are direct targets of FUS3, as are genes involved in the seed maturation program. FUS3 regulates genes encoding microRNAs that, in turn, control transcripts encoding transcription factors involved in developmental phase changes. Examination of direct targets of FUS3 reveals that FUS3 acts primarily or exclusively as a transcriptional activator. Regulation of microRNA-encoding genes is one mechanism by which FUS3 may repress indirect target genes. FUS3 also directly up-regulates VP1/ABI3-LIKE1 (VAL1), encoding a B3 domain protein that functions as a repressor of transcription. VAL1, along with VAL2 and VAL3, is involved in the transition from embryo to seedling development. Many genes are responsive to FUS3 and to VAL1/VAL2 but with opposite regulatory consequences. The emerging picture is one of complex cross talk and interactions among embryo transcription factors and their target genes.

Seed development is controlled by a network of transcription factors controlling downstream target genes. In Arabidopsis (Arabidopsis thaliana), three B3 domain factors (LEAFY COTYLEDON2 [LEC2], FUSCA3 [FUS3], and ABSCISIC ACID INSENSITIVE3 [ABI3]) and LEC1 (a HEME-ACTIVATED PROTEIN3 subunit of CCAAT-binding factors) are key regulators of zygotic embryo development (Giraudat et al., 1992; Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001). In loss of function, lec1, lec2, and fus3 embryos at least partially lose their embryo identity and enter postgerminative programs (for review, see Harada, 2001). The mutant embryos do not accumulate storage products to the level found in the wild type and, for lec1 and fus3, do not acquire desiccation tolerance properly, resulting in death if the seed is allowed to dry. Under high humidity, they exhibit precocious germination (Raz et al., 2001). When rescued from the developing seed and placed into culture, seedling development occurs and the cotyledons have trichomes, a leaf trait; thus, this group is referred to as LEC genes (for review, see Meinke et al., 1994). ABI3 is not a member of the LEC family but is a major regulator of programs during maturation. All of these genes are expressed specifically or primarily during seed development (Santos-Mendoza et al., 2008). Cross regulation and autoregulation have been demonstrated for the LEC genes and ABI3, both among these genes and in terms of the downstream programs that they control (To et al., 2006).

The LEC genes also promote embryo-specific programs in postgerminative tissues when ectopically expressed. For LEC1 and LEC2, when constitutively expressed via the 35S promoter, upon completion of germination the “seedlings” show ectopic embryo development (Lotan et al., 1998; Stone et al., 2001). For FUS3, the phenotype is milder, with the leaves taking on cotyledon-like traits (Gazzarrini et al., 2004).

Other genes encoding transcription factors also promote embryo development when ectopically expressed, including BABY BOOM (BBM; Boutiller et al., 2002), WUSCHEL (Zuo et al., 2002; Gallois et al., 2004), LEAFY COTYLEDON1-LIKE (LL1; Kwong et al., 2003), PLANT GROWTH ACTIVATOR37/MYB118 and MYB115 (Wang et al., 2009), and AGAMOUS-LIKE15 (AGL15; Harding et al., 2003; Thakare et al., 2008). SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (Hecht et al., 2001) encodes a Leu-rich repeat receptor-like kinase, but it too promotes somatic embryo formation in some systems when expressed via the 35S promoter.

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A number of other genes promote somatic embryogenesis in Arabidopsis when present as loss-of-function alleles, and these include pickle (Ogas et al., 1997), val1 val2 (for vp1/abi3-like; val1 is also known as high level of sugar inducible gene2 [his2], and val2 is also known as his2-like1 [his1]; Suzuki et al., 2007), the curly leaf swenger double mutant (Chanvivattana et al., 2004), histone deacetylase 6 (hdac6) and hdac19 RNA interference plants (Tanaka et al., 2008), and a double knockout/knockdown of AtBMI1 A/B (for B LYMPHOMA Mo-MLV INSERTION REGION1 homolog; in Arabidopsis, this is encoded by At1g30580 and At1g06770; Bratzel et al., 2010). These mutants/RNA interference plants produce embryos on aberrant seedlings, demonstrating defects in the transition from embryo to postgerminative development. This group of genes encode proteins that include domains shown or predicted to be involved in epigenetic control of gene expression and in particular involving the repression of gene expression, indicating an important role for changes in chromatin structure in mediating this developmental phase transition.

Recent work using chromatin immunoprecipitation (ChIP) and expression analysis is revealing how some of these factors control development and how they interact (Braybrook et al., 2006; Kaufmann et al., 2009; Zheng et al., 2009; Mönke et al., 2012). Here, we report the genome-wide identification of in vivo binding sites for FUS3 in an embryonic culture tissue system that allows robust ChIP. The data sets generated by Yamamoto et al. (2010), Chiu et al. (2012), and Lumba et al. (2012) were used to identify FUS3-responsive genes. Yamamoto et al. (2010) documented transcriptomes in developing seed of fus3 compared with the wild type and assessed transcript accumulation in response to an inducible form of FUS3. The native FUS3 promoter was used to drive the expression of FUS3 with a C-terminal 10× c-myc tag (FUS3pro:FUS3-10x-c-myc) and was placed into fus3-3 homozygotes carrying the 35Spro:AGL15 transgene that allows stable production of embryonic tissue. The FUS3 transgene was able to rescue the fus3-3 mutant phenotype, confirming the functionality of the transgene. Specifically, complemented fus3-3 lines showed a decrease in the accumulation of anthocyanin compared with uncomplemented lines (Supplemental Fig. S1B). The mature seed from complemented lines was viable, whereas fus3-3 uncomplemented mature seed was nonviable (Supplemental Fig. S1C). Developing embryos from complemented lines were cultured as described by Harding et al. (2003) and subcultured approximately each 2 to 3 weeks, resulting in stable tissue producing only embryonic organs within a couple of months (Supplemental Fig. S1D). ECT of 35Spro:AGL15 was generated at the same time as a negative control.

Genome-Wide Identification of in Vivo Binding Sites for FUS3

To map binding sites for FUS3, we used a ChIP-chip approach, where anti-c-myc antibody was used to immunoprecipitate FUS3-c-myc-DNA complexes, and the DNA was recovered and used to generate probes to hybridize to tiling arrays. Three independent experiments were performed with controls using anti-c-myc antibody and tissue expressing untagged FUS3. CisGenome (http://www.biostat.jhsph.edu/~hji/cisgenome/; Ji et al., 2008) was used to analyze the data by moving average and hidden Markov model, resulting in 1,218 of the bound genomic regions (false discovery rate [FDR] <
0.05) assigned to a nearby gene (Supplemental Data Set S1).

Sequences corresponding to the peaks of binding were obtained, and the average length was 494 bp. As shown in Table I, nearly all of the sites were intragenic or within 1 kb of the transcription start site and/or the transcription end site, with only two bound regions more distant from an annotated gene. Seventy-two genes had two bound regions associated with them and three had three, resulting in 1,140 different genes associated with FUS3. About one-half of the sites mapped to intergenic regions, with 23% being upstream, 16% downstream, and 8% in a region between two genes. A fairly high percentage of the bound sequences included the coding region (29%). Introns (20%) and 5′ and 3′ untranslated regions were also represented (6% and 4%, respectively). The numbers total somewhat higher than 100% because some sequences span different regions of genes.

Sequences from the bound peaks were analyzed using the Gibbs Motif Sampler in CisGenome (Ji et al., 2008). Binding sites for B3 domain proteins (called an RY motif and of form CATGCA; Mönke et al., 2004) were found to be overrepresented (Fig. 1). Of the 1,218 bound fragments, the majority (75%) had at least one CATGCA motif and 22% had a longer CATGCATG motif. When five separate matched control regions were generated by CisGenome, the average occurrence of CATGCA was 16% (range of 15.4%–16.7%) and that of CATGCATG was 1.3% (range of 1.0%–1.6%). The Gibbs Motif Sampler also found G boxes (CACGTG) that occurred in 12% of the sequences – the average occurrence of CACGTG was 0.3% (range of 0.1%–0.4%). The Gibbs Motif Sampler also found G boxes (CACGTG) that occurred in 12% of the sequences – the average occurrence of CACGTG was 0.3% (range of 0.1%–0.4%).

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Recently, ChIP experiments were reported for ABI3, which, like FUS3, is a B3 domain transcriptional regulator expressed primarily during seed development. Of the 1,140 unique FUS3 directly regulated genes, 57 (5%) were also identified as ABI3 regulated based on evidence of direct association with regulatory regions of the gene on at least one of the two types of arrays and/or expression in response to a dexamethasone-inducible form of ABI3 (Mönke et al., 2012). Of the 320 genes reported to be in the ABI3 regulon, 18% were also associated with FUS3. Potential direct targets have also been identified for the other key B3 domain embryo factor, LEC2 (Braybrook et al., 2006). In this study, a glucocorticoid domain was translationally fused to LEC2, allowing the induction of gene expression by the addition of dexamethasone to cause LEC2-GR (for glucocorticoid receptor) to move into the nucleus. Gene expression changes were assessed at short times (1 and 4 h) after induction in seedlings to focus on direct events. For the 1,140 FUS3 direct targets, there is evidence of potential direct regulation based on increased transcript at 1 and/or 4 h after induction by LEC2 for 73 genes. The genes that overlap with the FUS3-bound data set may be found in Supplemental Data Set S1.

Table I. Locations of sequences coimmunoprecipitated with FUS3-c-myc

Sequences were obtained using CisGenome and mapped using The Arabidopsis Information Resource 9 to intergenic and intragenic regions. For genes bound by and responsive to FUS3, the data set of Yamamoto et al. (2010) was utilized (Supplemental Data Set S2). Totals are more than 100% because some sequences span more than one region. TES, Transcriptional end site; TSS, transcriptional start site; up/down 1 kb, within 1 kb of the TSS and/or TES as indicated; UTR, untranslated region of the transcript 5′ or 3′ of the coding sequences (CDS) that will be translated to protein.

<table>
<thead>
<tr>
<th>Region of Gene</th>
<th>Location of Bound Sites (Percentage of 1,218)</th>
<th>Location of Bound and Responsive Sites (Percentage of 233)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSS up 1 kb</td>
<td>276 (23%)</td>
<td>38 (16%)</td>
</tr>
<tr>
<td>TES down 1 kb</td>
<td>194 (16%)</td>
<td>51 (22%)</td>
</tr>
<tr>
<td>TSS up and TES down 1 kb</td>
<td>110 (8%)</td>
<td>18 (8%)</td>
</tr>
<tr>
<td>Total intergenic</td>
<td>570 (47%)</td>
<td>107 (46%)</td>
</tr>
<tr>
<td>5′ UTR</td>
<td>69 (6%)</td>
<td>3 (1%)</td>
</tr>
<tr>
<td>CDS</td>
<td>356 (29%)</td>
<td>63 (27%)</td>
</tr>
<tr>
<td>Intron</td>
<td>246 (20%)</td>
<td>39 (17%)</td>
</tr>
<tr>
<td>3′ UTR</td>
<td>54 (4%)</td>
<td>33 (14%)</td>
</tr>
<tr>
<td>Total intragenic</td>
<td>725 (59%)</td>
<td>199 (59%)</td>
</tr>
</tbody>
</table>
Veriﬁcation of Select FUS3-DNA Interactions

A number of genes involved in embryogenesis were bound by FUS3 in the ChIP-chip experiment, including genes encoding the MADS domain transcription factor AGL15 (Heck et al., 1995), the LOB domain factor LBD40 (Shuai et al., 2002; Husbands et al., 2007), three LEC genes, LEC1, L1L, and FUS3 (Meinke et al., 1994; Lotan et al., 1998; Kwong et al., 2003), three B3 domain factors, FUS3, ABI3, and VAL1 (Giraudat et al., 1992; Luerssen et al., 1998; Tsukagoshi et al., 2005; Suzuki et al., 2007), the ABI5-like factor ENHANCED EM LEVEL (EEL; Bensmihen et al., 2002), and the AP2 family factor BBM (Boutilier et al., 2002; Supplemental Table S1). Enrichment tests were used to verify that sequences corresponding to these genes were truly occupied in vivo by FUS3. After enrichment of in vivo associated sites by ChIP, the target DNA fragment should be present in greater abundance in the FUS3:c-myc immunoprecipitation than the negative control lacking the c-myc tag. Quantitative PCR was used to assess the amount of DNA fragment recovered by ChIP using three biological replicates of the ChIP experiment that were independent from those used for ChIP-chip. The "fold change" is the amount of target recovered from the FUS3:c-myc tissue compared with the untagged FUS3 negative control and normalized to the nonbound TUA3 fragment. As shown in Figure 3A, the fold-change values of all genes tested were indeed signiﬁcantly higher when ChIP was performed using c-myc antibody and FUS3:c-myc tissue than with untagged FUS3 tissue, with the exception of L1L, where the P value was 0.059. However, the fold change was signiﬁcantly higher than the control in all three individual experiments looking at technical replicates for L1L. Differential site occupancy was also calculated by comparing the amplicon of the targets in the immunoprecipitation using FUS3:c-myc tissue with that of the control that was not expected to be bound by FUS3 in the same immunoprecipitation (TUA3). All of the targets tested were enriched in the FUS3-c-myc tissue but not in the untagged FUS3 tissue compared with the nonbound control (Fig. 3B).

Gene Expression in Response to FUS3: Direct and Indirect Targets

Several microarray experiments have been performed to assess transcriptomes in response to FUS3. Yamamoto et al. (2010) reported on the transcriptomes of fus3 developing seeds (8 and 12 DPA) compared

![Figure 1. Overrepresented motifs identified by the Gibbs Motif Sampler found within the sequences bound by FUS3. Motifs included an RY motif that is a binding site for B3 domain proteins (A) and a G box that is recognized by bZIP transcription factors (B). [See online article for color version of this figure.]](https://www.plantphysiol.org/)

![Figure 2. Functional categorization of FUS3 directly bound and responsive genes using AmiGO GO term enrichment. Genes with regulatory regions identified as bound by FUS3 by CisGenome when all three replicates of the ChIP-chip experiment are analyzed together were characterized for GO biological process and compared with the whole genome (white bars compared with black bars within each category). Expression data from Yamamoto et al. (2010) were integrated with the binding data. In Yamamoto et al. (2010), the transcriptome of fus3 developing seed was compared with the wild type, and genes responsive to an estrogen-inducible (ESTR) form of FUS3 were documented. Genes significant in these data sets with regulatory regions bound by FUS3, as analyzed with AmiGO, are shown in gray. Only a subset of GO terms are illustrated. P values are indicated above the bars, and GO terms and accession number categories are shown below the bars. Not significantly different from the percentage in category for the whole genome.)
with wild-type seeds and found 785 genes for which transcripts are decreased in fus3-3 seed and 644 genes for which transcripts are increased. In addition, they generated transgenic plants with a form of FUS3 (ER-FUS3-DH) in the pER8 system that is inducible by estrogen (Zuo et al., 2000) and measured transcript accumulation in seedlings. The list from their data sets (significantly responsive in fus3 developing seeds at 8 and/or 12 DPA [q < 0.05] and/or responsive to ER-FUS3) was integrated with the binding data and is available in Supplemental Data Set S2. Of the genes associated with regions bound by FUS3, 19% (220 unique genes) showed a significant response in the loss- or gain-of-function data from Yamamoto et al. (2010). Data for a select set is shown in Supplemental Table S1. When bound and responsive genes were analyzed with AmiGO, an even greater percentage of the genes than in the bound data set were in categories associated with seed processes. Of the 220 unique genes, 56 (25%) were seed specific (Zhang et al., 2012a). Genes involved in abscisic acid (ABA) response and GA metabolism were also enriched (Fig. 2). There was evidence for 17% and 12% of these FUS3 direct response targets also being regulated by ABI3 (Mönke et al., 2012) and LEC2 (Braybrook et al., 2006), respectively (Supplemental Data Set S2).

The sequences of DNA fragments bound by FUS3 and responsive in the Yamamoto et al. (2010) data set were obtained and analyzed for location. The results were generally similar to the locations of all binding sites, with more than one-quarter of sites encompassing the coding sequences (Table I).

Interestingly, when the data set of genes directly bound by FUS3 was compared with the expression data from Yamamoto et al. (2010), nearly all of the directly bound genes were up-regulated in response to FUS3 at 8 and/or 12 DPA or in response to an estrogen-induced FUS3 construct. The exceptions include four genes (At1g16390, At3g05600, At1g12900, and At3g08740) for which transcript was significantly reduced in 8-DPA fus3-3 seed compared with the wild type but significantly increased (P < 0.05) at 12 DPA. The only other discrepancies were two genes that were induced by ectopic FUS3 and thus were on the FUS3-expressed list, but one of these genes (At1g04250) showed a significant increase in transcript at 12 DPA and a decrease at 8 DPA, although this later did not meet the cutoff of q < 0.05. The other induced gene, At4g39130, showed fus3-3:wild-type ratios of 5.1 at 8 DPA and 0.4 at 12 DPA, with both being significant. Thus, for all of these, there is evidence for activation by FUS3 at least at one stage/context of development, indicating that FUS3 may act primarily or exclusively as a transcriptional activator.

Chiu et al. (2012) recently reported a role for FUS3 in delaying germination at supraoptimal temperatures, thereby protecting the next generation. They also reported a time-course analysis of the transcriptome of imbibed seeds at the supraoptimal temperature of 32°C compared with the optimal temperature of 21°C. Although differential expression was not conclusively tied to FUS3 accumulation in this experiment, because FUS3 is involved in protection against high-temperature imbibition, some genes may be directly controlled by FUS3. Therefore, we integrated the data set from Chiu et al. (2012) with the Arabidopsis Genome Initiative loci to identify genes that may be directly regulated by

Figure 3. Confirmation of the in vivo association of FUS3 with select DNA fragments associated with genes involved in embryogenesis. A, Fold enrichment measures the amount of the suspected target fragment recovered in the immunoprecipitation using FUS3-c-myc tissue compared with the same fragment recovered from ChIP on control tissue expressing FUS3 without a c-myc tag. Data shown are normalized to results for TUA3. Significant differences for the suspected targets in tagged compared with untagged tissue are shown with asterisks: *P < 0.05, **P < 0.01. B, Differential site occupancy measures the amount of the suspected target recovered compared with a nonbound control fragment (TUA3) in the same immunoprecipitation. Significant differences from the control tissue with FUS3 lacking the c-myc tag are shown with asterisks: * P < 0.05, ** P < 0.01. [See online article for color version of this figure.]
FUS3 to protect the next generation during supraoptimal temperature imbibition. The list of 241 binding sites (223 unique genes) for which there was a significant change in response to imbibition temperature and for which FUS3 shows in vivo association in ECT may be found in Supplemental Data Set S3. A subset of these genes (26, 12%) respond to a posttranslationally inducible form of LEC2 (Braybrook et al., 2006), and 35 (16%) may be direct targets of ABI3 (Mönke et al., 2012). Forty-nine (22%) are expressed specifically in seeds (Zhang et al., 2012a).

The analysis by Chiu et al. (2012) indicated that, in response to high-temperature imbibition, seed-specific and ABA biosynthesis/signaling genes are up-regulated, whereas genes involved in germination, growth, and GA biosynthesis are down-regulated. To determine if these types of genes may be directly controlled by FUS3, we analyzed the bound and temperature up-regulated genes using AmiGO. As shown in Supplemental Figure S2, genes assigned to GO terms involved in dormancy, maturation, and other seed processes such as lipid localization were overrepresented, indicating that the effect Chiu et al. (2012) describe on seed-specific genes at high temperature may be mediated by direct regulation by FUS3. Interestingly, genes involved in water response, and in particular water deprivation, and genes involved in response to freezing were also overrepresented, possibly indicating the up-regulation of genes involved in protective functions (Supplemental Fig. S2). Those involved in response to freezing include three oleosins that promote freezing tolerance of seeds (Shimada and Hara-Nishimura, 2010). When the same type of analysis was performed for the high-temperature repressed and directly FUS3-associated list (116 genes), genes encoding growth (0040007, 1.64E-02) and cell wall organization and modification (0071555, 3.60E-02; 0042545, 4.89E-02) were enriched using AmiGO.

FUS3 has also been reported to be involved in vegetative phase transitions, in part via control of the ethylene response (Lumba et al., 2012). Genes responsive to ectopic activation of FUS3 (via an AtML1pro:FUS3-GR construct) resulted in a list of 19 genes up-regulated and 37 genes down-regulated by FUS3-GR. Ten of the 19 up-regulated genes (52%) were associated with regions directly bound by FUS3, while five down-regulated genes (14%) may be direct FUS3 targets (Supplemental Data Set S4).

Verification of the Response of Select Genes to Loss and Gain of Function of FUS3

Quantitative reverse transcription (qRT)-PCR was performed to confirm whether the key regulators of embryo-genes that were repressed and directly bound by FUS3 were significantly up-regulated and bound by FUS3-GR. Ten of the 19 up-regulated genes (52%) were associated with regions directly bound by FUS3, while five down-regulated genes (14%) may be direct FUS3 targets (Supplemental Data Set S4).

FUS3 Regulates miRNA Genes

The ChiP-chip data revealed that FUS3 was associated with potential regulatory regions of eight genes.

Figure 4. Transcripts of embryo-associated genes are reduced in fus3 loss-of-function developing seeds. qRT-PCR to assess transcript accumulation from select genes from fus3 loss-of-function developing seeds was performed and compared with the Columbia wild type (Col wt) that is set to 1. Means and so for three independent biological replicates are shown (two replicates for LEC1 at 11–12 DPA). *Significant at P < 0.05; **significant at P < 0.01. [See online article for color version of this figure.]
encoding miRNAs that regulate various developmental programs (Supplemental Data Set S1). A recent study showed that miR156-mediated SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE10 (SPL10) and SPL11 gene repression is important for normal embryogenesis as well as proper timing of the juvenile-to-adult vegetative phase transition (Nodine and Bartel, 2010), developmental processes in which FUS3 also has roles (Lumba et al., 2012). MIR156A and MIR156C were on the FUS3 target list (Supplemental Data Set S1), with the bound region for both being at the 5' end and into/through the gene and containing potential RY motifs. We confirmed an in vivo association of FUS3 for both miRNA-encoding genes (Fig. 6, A and B). MIR156C was a stronger target of FUS3 compared with MIR156A, as it showed much higher fold change and differential site occupancy, and this difference was consistent with the CisGenome peak numbers (28 and 429, respectively). Transcript accumulation of primary-microRNA156A (pri-miRNA156A) and pri-miR156C were significantly reduced in the fus3-3 mutant compared with the wild type at both seed stages (Fig. 6C), indicating that FUS3 not only binds but also positively regulates the expression of these genes. Both SPL10 and SPL11 transcript amounts were up-regulated significantly in fus3-3 mutant compared with the wild type in the 7- to 8-DPA stage seed.

Interaction between FUS3, ABI3, and LEC2 in the Control of Downstream Targets

A list of the 392 regions bound by FUS3 (366 unique genes) and associated with a significant change in transcript accumulation in the Yamamoto et al. (2010), Chiu et al. (2012), and/or Lumba et al. (2012) data sets was generated (Supplemental Data Set S5). Of these genes, 72 (18%) were seed specifically expressed, 46 (12%) showed evidence of direct regulation by ABI3 (Mönke et al., 2012), and 34 (9%) showed evidence of potential direct regulation by LEC2 (Braybrook et al., 2006). Twelve genes showed evidence of direct regulation by all three of these B3 domain proteins (Supplemental Table S2). These genes mainly encode proteins involved in storage protein and lipid accumulation but also include two unknown proteins and two transcriptional regulators that are expressed predominantly if not specifically in seeds. ATAF1 is a NAC domain transcription factor that is involved in the negative regulation of ABA-mediated signaling (Jensen et al., 2008), whereas LBD40 is an uncharacterized transcription factor of the LOB domain family. Recently, direct targets of LEC1 were identified using ChIP on 35Spro:LEC1:GR seedlings treated with dexamethasone for 1 d and using a promoter macroarray (Junker et al., 2012). One gene in Supplemental Table S2 was also associated with LEC1 based on these data (At1g01720).

DISCUSSION

Numbers and Types of Genes Regulated by FUS3

Hundreds to thousands of binding sites identified by ChIP appear to be relatively common for transcription factors, with generally only a small percentage of the associated genes showing significant changes in expression, at least under conditions assessed in the studies (Lee et al., 2007; Kaufmann et al., 2009; Oh et al., 2009; Zheng et al., 2009). FUS3 was found to associate with 1,218 genomic regions that were annotated to 1,140 different genes identified as bound by FUS3 included previously described targets At2S3, SDH2-3 (At5g65165), and an aspartic protease-encoding gene (At1g62290; Kagaya et al., 2005a, 2005b; Roschztardtz

Figure 5. Direct targets of FUS3 respond to ectopic induction of FUS3 in seedlings. AGL15 (top), LBD40 (middle), and VAL1 (bottom) show increased transcript accumulation as measured by qRT-PCR in response to dexamethasone treatment of seedlings carrying a FUS3-GR construct (see text). All data points are normalized to the untreated Columbia wild type (Col wt) at the zero time point. *Significant difference at P < 0.001 from the Columbia wild-type control tissue. A representative experiment from three biological replicates is shown. [See online article for color version of this figure.]
transcript accumulation in response to FUS3. Using the data set of Yamamoto et al. (2010), 19% of the genes associated with bound regions showed significant responses to gain and/or loss of function of FUS3. The fraction of genes responsive to FUS3 binding is higher than that reported for AGL15, HY5, or PIL5/PIF1 (Lee et al., 2007; Oh et al., 2009; Zheng et al., 2009). Even so, why are the majority of genes nonresponsive, with only 19% showing perturbations in transcript accumulation in response to FUS3 accumulation? This could reflect the sensitivity of the technique used to measure expression or the time point/developmental context examined. As reviewed by Wyrick and Young (2002), binding without obvious regulation appears common and might indicate a need for particular cofactors or signals to trigger a response.

Surprisingly, a relatively large percentage of sequences associated in vivo with FUS3 encompass coding regions of genes even when only the subset of genes that are responsive to FUS3 are considered (Table I). While generally, promoter regions are considered to contain the important elements to control gene expression, other regions such as introns have also been documented as important (for review, see Rose, 2008). More recently, exons that not only encode protein but also function as enhancers are being discovered. These elements are sometimes referred to as eExons. One recent paper (Birnbaum et al., 2012) found that four of seven potential eExons tested functioned to control the expression of nearby genes. Another paper found that enhancers in coding regions target their own gene (Ritter et al., 2012).

When bound regions were analyzed in CisGenome with the Gibbs Motif Sampler, not only were RY motifs [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) with the Gibbs Motif Sampler, not only were RY motifs [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) with the Gibbs Motif Sampler, not only were RY motifs [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well. Binding sites for ABI3 were recently reported, [CATGCA(TG)] overrepresented but G boxes (CACGTG) as well.

**FUS3 and Genes Regulated during Seed Development**

Yamamoto et al. (2010) assessed the transcriptomes of fus3-3 seeds compared with the wild type at 8 and 12 DPA and reported 785 genes for which transcripts are decreased in fus3-3 seed and 644 genes for which transcripts are increased. When the data set of genes directly bound by FUS3 is compared with the expression data, nearly all of the directly bound genes are up-regulated in response to FUS3 accumulation (e.g., transcript accumulation is reduced in fus3-3 compared with wild-type developing seed and/or transcript accumulates in response to an estrogen-induced FUS3 construct). These results indicate that FUS3 association with DNA leads to gene expression, consistent with prior findings that FUS3 has a region that functions as a transcriptional activator (Kagaya et al., 2005a).

Genes involved in fatty acid biosynthesis were overrepresented in the list of genes with reduced transcript abundance in fus3 compared with the wild et al., 2009). A cruciferin that is thought to be indirectly regulated by FUS3, based on the fact that transcript from this gene did not accumulate until 24 h after induction of FUS3 expression in seedlings (Kagaya et al., 2005a), was not bound in the ChIP-chip study, supporting indirect control of this gene. A relatively large number of the associated genes showed changes in
type that would include direct and indirect targets (Yamamoto et al., 2010). Likewise, when the subset of this list that also has putative regulatory regions bound by FUS3 is assessed for GO categories, genes involved in “nutrient reservoir activity” (GO:00045735) as well as lipid localization, storage, metabolic processes, and seed oil-body biogenesis (GO:0010876, GO:0019915, GO:0006629, and GO:0010344, respectively) are overrepresented. Not only was WRINKLED1 (WRI1; At1g54320), which encodes a transcription factor that regulates fatty acid biosynthesis genes, in this category, but also some of the WRI1-regulated genes, including ACP1 (At1g05020), KCS18/FAE1 (At4g34520), and LACS4 (At4g23850; Maeo et al., 2009), indicating that FUS3 controls lipid biogenesis not only at a top level of the regulatory network but also via the regulation of genes further down in the network.

Yamamoto et al. (2010) reported on a number of genes involved in hormone biosynthesis as responsive to FUS3. With the exception of At1g25410 (ATTIP6, involved in cytokinin biosynthesis), none of their genes were associated with bound regions in the ChIP-chip experiments. However, a number of other genes involved in hormone biosynthesis were in our data set as well as genes involved in response to ABA and GA metabolic processes (GO:0009737 and GO:0009685). Yamamoto et al. (2010) also found genes involved in photosynthesis and secondary metabolic processes overrepresented on the responsive list. However, these categories were not identified in the bound responsive list as overrepresented by AmiGO or by GOToolBox (http://genome.crg.es/GOToolBox/; with Bonferroni correction; Martin et al., 2004) but were overrepresented on the list of indirect genes (e.g. not found as bound by ChIP-chip but responsive at 8 and/or 12 DPA in the Yamamoto et al. [2010] data set).

Transcriptional regulators were significantly overrepresented among the bound and responsive list in GOToolBox (GO:0003700; P < 0.05 by hypergeometric statistical test with Bonferroni correction). These genes are discussed further below.

**FUS3 and Genes Regulated in Response to Supraoptimal Temperature Imbibition**

Although we mapped FUS3-associated sites in a somatic embryo tissue culture and the Chiu et al. (2012) transcriptome involved the imbibition of mature seeds, it is reasonable to expect that a subset of FUS3 sites would overlap. In prior work with AGL15, also determining in vivo DNA-binding sites in embryonic tissue, we found genes relevant for AGL15’s role in the control of flowering time (Zheng et al., 2009). Likewise, Yant et al. (2010) found much overlap for the binding of AP2 in inflorescence and leaf tissue. Although the data set of Chiu et al. (2012) documents transcriptomes in response to supraoptimal imbibition, rather than to the accumulation of FUS3 specifically, they present evidence that FUS3 may be involved in protection from high-temperature imbibition. They also document that FUS3 is expressed and the protein accumulates in response to high-temperature imbibition (Chiu et al., 2012). While it is possible that sites bound by FUS3 in ECT and genes responsive during high-temperature imbibition may not be regulated by FUS3 in the context of the imbibed seed, FUS3 is active during this phase of the life cycle, and it is quite possible that the genes shared between the FUS3-bound data set and the data reported by Chiu et al. (2012) are direct FUS3 targets. Therefore, we have integrated their data set with genes bound by FUS3 to present targets that may be regulated by FUS3 in the imbibed seed, with the caveat that any subsequent work on verifying targets of interest should include ChIP from imbibed seed.

Very few genes that are bound by FUS3 (our data) responded to supraoptimal temperatures during imbibition within 1 h (Chiu et al., 2012). This would be consistent with FUS3 first being up-regulated by heat and downstream effects observed at later time points. Our FUS3 ChIP data support a direct role for FUS3 in up-regulating seed genes, including those involved in maturation and dormancy (Supplemental Fig. S2). The various annotation tools indicated that bound and high-temperature imbibition-repressed genes were largely involved in cell wall processes as well as hormone stimulus. If FUS3 truly functions as a transcriptional activator, these genes may be indirect and the in vivo association of FUS3 with the loci has no consequence. FUS3 is fairly rich in acidic and Asn residues in the C-terminal end, with a stretch of eight Asn residues, and these are features of some transcriptional activation domains. Indeed, the C-terminal region was found to be necessary for the activation of target genes (Kagaya et al., 2005a). However, it is possible that this factor acts as both an activator and a repressor, possibly depending on tissue context and interacting factors.

**FUS3, Phase Transitions, and the Regulation of pri-miRNA-Encoding Genes**

Lumba et al. (2012) reported a role for FUS3 in the control of phase transitions via the repression of ethylene-regulated genes when FUS3 is activated after the completion of germination. Furthermore, fus3 loss of function led to shortened juvenile vegetative development, and ethylene is involved in this process. The changes in transition from juvenile to adult vegetative development were influenced by both embryonic and postembryonic expression of FUS3 (Lumba et al., 2012). Two of the genes that they identified as down-regulated and involved in ethylene response have associated regions bound by FUS3 (At5g25190 and At1g25560). Whether FUS3 is able to directly repress gene expression is unknown. However, FUS3 directly up-regulates VAL1 (Figs. 3–5), also a B3 domain protein but with a repression domain, and genes involved in ethylene response are overrepresented in both the lists up- and down-regulated in the val1 val2 mutant compared with the wild-type (data set of...
regulation of miR156A and miR156C, which are involved in the genes. We have found that FUS3 directly increased transitions is by the regulation of miRNA-encoding development, and this is discussed further below.

Another mechanism by which FUS3 may slow phase transitions is by the regulation of miRNA-encoding genes. We have found that FUS3 directly increased miR156A and miR156C, which are involved in the regulation of SPL10 and SPL11. The genomic regions around/containing SPL10 and SPL11 do not show any indication of binding by FUS3, with the closest peak being approximately 1.7 kb away and associated with the upstream gene. Thus, SPL10 and SPL11 appear indirectly regulated by FUS3 via the miRNAs.

Interactions between FUS3, miR156-encoding genes, and SPL genes are more complicated during embryogenesis. The loss of function of DICER-LIKE1 (DCL1) that is required for miRNA biogenesis (the presumed null allele dcl1-5 [Nodine and Bartel, 2010] and dcl1-15 that contains a point mutation that affects a conserved residue within one of the two active sites of DCL1 [Willmann et al., 2011]) leads to the premature accumulation of genes involved in embryo maturation as well as other embryo defects, including arrest. Nodine and Bartel (2010) provided evidence that miRNA regulation of SPL transcripts prevents premature entry into maturation programs. However, FUS3 directly regulates genes involved in maturation, and Willmann et al. (2011) found that premature expression of maturation genes can be partially rescued by fus3-3. It is possible that the net effect of FUS3 (to prevent maturation via miR156 regulation of SPL10/SPL11 or to express maturation-related genes) depends on the developmental time point, but the inconsistencies could also be due to defects in other miRNAs and consequent misregulation of their targets. In fact, FUS3 is itself regulated directly or indirectly by miRNAs, based on significant increases in FUS3 transcript in dcl1-15 (Willmann et al., 2011).

Although much is known about the processing of and subsequent gene regulation by miRNAs, little is known about how the pri-miRNA-encoding genes are themselves regulated. Very few direct interactions between transcriptional regulators and pri-miRNA-encoding genes have been reported in plants or other organisms. In animals, there are some reports of transcription factors directly associating with pri-miRNA genes and resulting in the expression or repression of the associated genes (Hino et al., 2008; Xiong et al., 2009; Zhou et al., 2010; Zhang et al., 2012b). In Arabidopsis and other angiosperms, miR156 and miR172 are regulators of phase transitions, with miR156 delaying phase transitions while miR172 promotes the progression through the life cycle (for review, see Huijser and Schmid, 2011). Several direct regulators of miR172-encoding genes have been identified, including the MADS factor SHORT VEGETATIVE PHASE, which is involved in the repression of pri-miR172a (Cho et al., 2012), and SPL9, which leads to an accumulation of miR172 (Wu et al., 2009). miR172 regulates AP2 that, in a feedback loop, directly expresses miR156e and directly represses miRNA172b, thereby contributing to the reinforcement of repression of flowering (Yant et al., 2010). Genes encoding miR156 are repressed by PENNYWISE and POUND-FOOLISH, two BEL1-like homeodomain proteins, and this regulation may be direct, based on the presence of binding sites for these transcription factors (Lal et al., 2011). Factors that function upstream of miR156 to establish the accumulation of this miRNA early are less well understood. Here, we provide evidence that FUS3 contributes to the direct expression of pri-miR156-encoding genes in the developing seed, thereby reducing SPL10/SPL11 transcript accumulation and delaying the juvenile-to-adult vegetative phase transition (Fig. 7).
FUS3, Embryo Transcription Factors, and the AFL/VAL Network

Transcription factors were overrepresented among the directly bound by and responsive to FUS3 list, and these included genes encoding key regulators of embryogenesis, LEC1, LIL, and ABI3. FUS3 may also autoregulate itself. In addition, FUS3 directly associated with AGL15 and BBM, which, like LEC1 and LIL, can promote somatic embryogenesis. LIL has been reported to interact with bZIP67, the gene for which is also potentially regulated by FUS3, and these factors together control the expression of CRC (At4g28520) and SLS2 (At15g49190; Yamamoto et al., 2009). FUS3 also directly up-regulates LBD40, which may be a direct target of ABI3 (Mönke et al., 2012) and LEC2 (Braybrook et al., 2006) as well. PEI1, which encodes a transcription factor essential for embryogenesis, was reported as a target of ABI3 and was found as directly bound (this study) and responsive to FUS3 (Yamamoto et al., 2010). Meanwhile, EEL appears to be a direct target of LEC2 (Braybrook et al., 2006) and FUS3. IAAD30 was previously identified as an AGL15 direct target (Zheng et al., 2009) and may also be a direct target of LEC2 (Braybrook et al., 2006), and this gene was also bound by FUS3. While there was a reduction in transcript from this gene at 8 and 12 d in fus3 compared with the wild type that was significant at P < 0.05 in the Yamamoto et al. (2010) data set, it was not significant at q < 0.05. The interactions between some of these transcription factors and the genes encoding them are shown in Figure 7. A simplified model focusing only on interactions among the AFL B3 domain factors is shown in Supplemental Figure S3.

The so-called AFL genes include ABI3, FUS3, and LEC2, all three of which encode B3 domain proteins. The VAL1/HSI2, VAL2/HSI1, and VAL3 genes also encode B3 domain proteins that act to promote the transition from embryo to seedling development by shutting down the AFL network prior to germination (Suzuki et al., 2007; Suzuki and McCarty, 2008; Veerappan et al., 2012). In the val1 val2 double mutant, somatic embryos form on the seedlings, in part because the AFL genes are not properly repressed. Therefore, the switch from embryonic to postembryonic development does not occur as it should (Suzuki et al., 2007). Here, we show that FUS3 leads to direct activation of VAL1. While FUS3 appears to function perhaps exclusively as an activator, VAL1 (and VAL2) are repressors. Interestingly, Suzuki et al. (2007) reported on genes at least 4-fold up-regulated or 4-fold down-regulated in val1 val2 compared with the wild type, resulting in 824 nuclear genes on the up-regulated (and, therefore, normally repressed by VAL1/VAL2) list compared with 635 nuclear genes on the down-regulated list (Suzuki et al., 2007). Chloroplast and mitochondrial genes responsive to val1 val2 were removed because, as expected, FUS3 did not associate with any genes in these organelles. Only five genes down-regulated in val1 val2 but 88 genes up-regulated in val1 val2 were on the FUS3 bound and responsive list (Supplemental Data Set S6).

More than one-half of these were expressed specifically in seeds (Zhang et al., 2012a). This would be consistent with this set of genes being bound by FUS3 and possibly also by VAL1 and/or VAL2, with opposite consequences (expression or repression, respectively) for regulatory control (Fig. 7). However, it remains to be determined if the VAL proteins associate with RY motifs via the B3 domain (Suzuki and McCarty, 2008).

CONCLUSION

We report on direct targets of FUS3, revealing that FUS3 functions primarily or exclusively as a transcriptional activator during embryogenesis. Many genes relevant for embryogenesis are FUS3 targets, and the network between key transcriptional regulators has much cross regulation. FUS3 also directly regulates two genes encoding transcription factors (AGL15 and LBD40) expressed primarily during embryonic development as well as VAL1, which is involved in the transition to postgerminative development. Finally, FUS3 is able to directly regulate miRNA-encoding genes, with molecular consequences consistent with developmental roles for FUS3.

MATERIALS AND METHODS

Plant Material

Arabidopsis (Arabidopsis thaliana) wild-type, fus3-3, and 35Spro:FUS3-GR plants (all Columbia ecotype) were sown on germination medium (Murashige and Skoog, 1962; supplemented with 10 g L−1 Suc, 0.5 g L−1 MES, and 7 g L−1 agar, pH 5.8), with 50 μg mL−1 kanamycin for 35Spro:FUS3-GR seed, and kept at 4°C for 3 d, then transferred to a growth room under long-day light conditions (16 h of light/8 h of dark). Seedlings were transferred to a potting mix (ProMix BX; Premier Brands) and grown in a chamber with a 16-h-light (20°C)/8-h-dark (18°C) cycle. To stage seed, flowers were tagged on the day that they opened, and seeds were collected and flash frozen in liquid nitrogen for RNA extraction.

To generate ECT accumulating FUS3-c-myc, fus3-3 knockouts were crossed with 35Spro:AGL15 and bred to recover the homozygous fus3-3 allele, and the line was maintained by embryo rescue prior to desiccation. These plants were transformed with the FUS3pro:FUS3-c-myc construct, and transgenics were recovered by rescue of the embryo-lethal phenotype and by demonstration of hygromycin resistance. Developing embryos were cultured as described (Harding et al., 2003) to initiate ECT. The control tissue was derived from embryos containing the 35Spro:AGL15 transgene and expressing the endogenous FUS3 gene that lacks the epitope tag.

Transgene Constructs

To generate a posttranslationally inducible form of FUS3, a GR domain was added to the C-terminal end (Sablowski and Meyerowitz, 1998). The FUS3 coding region was amplified from complementary DNA and cloned into Xhol and SpeI sites of pBLScript SK+ (pBS; Stratagene). PCR-amplified DNA corresponding to codons 508 to 795 of the glucocorticoid receptor were amplified from CD3-444 (Arabidopsis Biological Resource Center) and were cloned into SpeI and SalI of pBS-FUS3. FUS3-GR was excised with Xhol and SalI and cloned into pBMC vector (a derivative of pBluescript II EMBL4 for which the GUS gene is replaced by a cloning site; a gift of Dr. D. Falcone).

For the FUS3pro:FUS3-c-myc construct, about 2 kb of promoter of FUS3 and 2 kb of genomic region were amplified and cloned into pENTR/D-TOPO vector (Invitrogen). The insert was moved into destination vector pGW819 (Nakagawa et al., 2009; obtained from Dr. T. Nakagawa, Shimane University) following the manufacturer’s instructions for Gateway LR Clonase II Enzyme Mix (Invitrogen).
ChIP-Chip and Data Analysis

ECT with the FLU3pro::FLU3-c-myc and ECT expressing the endogenous FLU3 (no tag) were fixed in MC buffer as described previously (Zheng and Perry, 2011). ChIP was performed as described (Wang et al., 2004; Zheng et al., 2009; Zheng and Perry, 2011) except that a modified sonication buffer with lower sarkosyl was used (10 ms potassium phosphate, pH 7.5, 0.1 M NaCl, 0.25% sarkosyl, 10 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride). The immuno precipitation buffer from Zheng and Perry (2011) was also modified to lower ionic detergents (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1.2 mM EDTA, pH 8.0), and 4 volumes of immunoprecipitation buffer was added to 1 volume of solubilized chromatin in modified sonication buffer. Antibody (2.5 μL of myc-tag [9B11] mouse monoclonal antibody; Cell Signaling) was allowed to mix with the diluted solubilized chromatin (from 10 g of ECT) overnight at 4°C. Protein A-Sepharose (Invitrogen; 50 μL of 50% slurry) was added the next day and incubated for 3 h at 4°C with rotation. The rest of the procedure was as described previously (Wang et al., 2004; Zheng et al., 2009; Zheng and Perry, 2011) except the modified immunoprecipitation buffer was used to wash the beads.

Linear amplification and incorporation of dUTP was performed as detailed in the Affymetrix Chromatin Immunoprecipitation Assay Protocol (http://www.affymetrix.com/products/arrays/specific/arab_tiling.affx). Targets were tested using multiplex enrichment tests to confirm maintenance of the enrichment in the immunoprecipitated sample compared with the controls after amplification, and DNA was sent to the UK Microarray Core Facility for fragmentation, labeling, hybridization of the Affymetrix GeneChip Arabidopsis Tiling 1.0R array, and scanning.

CisGenome (Ji et al., 2008) was used as described previously (Zheng et al., 2009) to analyze the data. Perfect match was used to compute probe intensity, and peak detection was performed using a moving-average cutoff 2.5 over a minimal window of five probes. Hidden Markov model was also used to detect bound regions and to generate FDR (Ji et al., 2008). The intersect between the two lists with FDR < 0.05 is reported in this paper.

Enrichment Test and Quantitative PCR

For enrichment tests, the reactions were run and data were analyzed as described (Zheng et al., 2009), and Student’s t test was used to determine significance. Oligonucleotides used for these experiments are listed in Supplemental Table S5.

qRT-PCR

Total RNA from seeds or seedlings was prepared using the RNeasy Plant Mini Kit (Qiagen) with 1% polyethylene glycol added to the RLC buffer for seeds as described by Gehrig et al. (2000). One microgram of total RNA was treated with Dnase I (Invitrogen) and transcribed into complementary DNA (Reverse Transcription System; Promega). qRT-PCR was performed in triplicate on 96-well plates using SsoAdvanced SYBR Green Supermix (Bio-Rad) and a CFX Connect real-time PCR system (Bio-Rad). The cycling conditions were as follows: initial DNA denaturation step (95°C for 30 s), 40 cycles (95°C for 5 s, 55°C for 30 s), and melt curve of 65°C to 95°C (in 0.5°C increments, 5 s per step). Data were processed using CFX Manager software. Relative transcription via direct binding to the pri-miR172a promoter in Arabidopsis. Plant Cell 14: 1391–1403


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