MYB89 Transcription Factor Represses Seed Oil Accumulation[1][OPEN]

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In many higher plants, seed oil accumulation is precisely controlled by intricate multilevel regulatory networks, among which transcriptional regulation mainly influences oil biosynthesis. In Arabidopsis (*Arabidopsis thaliana*), the master positive transcription factors, *WRINKLED1* (*WRI1*) and *LEAFY COTYLEDON1-LIKE* (*L1L*), are important for seed oil accumulation. We found that an R2R3-MYB transcription factor, MYB89, was expressed predominantly in developing seeds during maturation. Oil and major fatty acid biosynthesis in seeds was significantly promoted by *myb89-1* mutation and *MYB89* knockdown; thus, MYB89 was an important repressor during seed oil accumulation. RNA sequencing revealed remarkable up-regulation of numerous genes involved in seed oil accumulation in *myb89* seeds at 12 d after pollination. Posttranslational activation of a MYB89-glucocorticoid receptor fusion protein and chromatin immunoprecipitation assays demonstrated that MYB89 inhibited seed oil accumulation by directly repressing *WRI1* and five key genes and by indirectly suppressing *L1L* and 11 key genes involved in oil biosynthesis during seed maturation. These results help us to understand the novel function of MYB89 and provide new insights into the regulatory network of transcriptional factors controlling seed oil accumulation in Arabidopsis.

Seed storage reserves in many higher plants usually constitute starch, oil stored as triacylglycerols (TAGs), and storage proteins. Seed TAGs not only serve as the main source of nutrients for humans and livestock but also facilitate postgerminative growth and subsequent seedling establishment (Li et al., 2006; Graham, 2008). They are also widely utilized by various industries (Lu et al., 2011) and serve as feedstock for the production of biofuels (Durrett et al., 2008). Thus, understanding the mechanisms underlying seed oil accumulation is of great interest, particularly in terms of its social and economic significance.

Arabidopsis (*Arabidopsis thaliana*) is considered an excellent model system for investigating oil biosynthesis in seeds (Baud and Lepiniec, 2009). Several previous studies have shown that a complex regulatory network of master positive transcription factors (TFs), including *WRINKLED1* (*WRI1*), *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON1-LIKE* (*L1L*), *LEC2*, *FUSCA3* (*FUS3*), and *ABSCISIC ACID INSENSITIVE3* (*ABI3*), regulate the accumulation of storage reserves during seed maturation (Santos-Mendoza et al., 2008). WRI1, which is the TF of the APETALA2-ethylene-responsive element-binding protein family, directly or indirectly targets some enzymes involved in the late glycolysis and plastidial fatty acid (FA) biosynthetic network (Cernac and Benning, 2004; Baud et al., 2007; Maeo et al., 2009). Mutations in *wri1* cause an 80% reduction in Arabidopsis seed oil content (Focks and Benning, 1998), and overexpression of *WRI1* leads to a significant increase in the oil content (Cernac and Benning, 2004; Baud et al., 2009; Maeo et al., 2009; Sanjaya et al., 2011; Grimberg et al., 2015; Adhikari et al., 2016). *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 closely related plant-specific TFs of the conserved B3 DNA-binding domain family (Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001). Activation of LEC1 or LEC2 induces ectopic embryogenesis in the vegetative tissues of Arabidopsis (Lotan et al., 1998; Santos Mendoza et al., 2005; Mu et al., 2008). Induced expression of *LEC1* causes a global increase in the expression of genes involved in FA biosynthesis, thereby substantially promoting the accumulation of oil and major FA species (Mu et al., 2008). L1L is closely related to LEC1 and genetically suppresses the *lec1*

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mutation when driven by the *LEC1* promoter (Kwong et al., 2003). L1L also plays an important positive role in seed FA biosynthesis (Mu et al., 2008; Tan et al., 2011) and has been implicated in storage oil biosynthesis (Baud and Lepiniec, 2010). The ectopic expression of *LEC2* activates the expression of genes encoding seed storage proteins, enzymes required for oil biosynthesis, and oil body-associated proteins, which, in turn, trigger oil deposition in rosette leaves (Santos Mendoza et al., 2005; Braybrook et al., 2006). FUS3 promotes oil deposition by positively regulating the expression of genes involved in photosynthesis and FA biosynthesis (Wang et al., 2007; Yamamoto et al., 2010; Zhang et al., 2016). Both LEC1 and LEC2 function as positive regulators upstream of *WRI1*, *ABI3*, and *FUS3*, which jointly control the expression of genes that contribute to the accumulation of seed oil and storage proteins (Kroj et al., 2003; Kagaya et al., 2005; Mu et al., 2008; Pan et al., 2010). In addition, ABI3 and FUS3 positively regulate themselves and each other, thereby forming feedback loops (To et al., 2006). *L1L* induction also is dependent on both LEC1 and FUS3 (Mu et al., 2008).

Recently, important progress regarding TFs in the regulation of seed oil accumulation has been made in Arabidopsis. Loss of GL2 activity in the seed coat, rather than the embryo, produces the high seed oil phenotype, partially by influencing *MUCILAGE MODIFIED4* expression, which promotes mucilage biosynthesis in the seed coat (Shi et al., 2012). bZIP67 regulates seed α-linolenic acid content by binding G-boxes in the *FATTY ACID DESATURASE3* (*FAD3*) promoter (Mendes et al., 2013). TRANSPARENT TESTA8 (TT8) inhibits seed FA accumulation by targeting *LEC1*, *LEC2*, and *FUS3* in the seeds (Chen et al., 2014). The negative feedback effect of TTG1 on *LEC2* and *ABI3* plays a role in mediating *FUS3* expression in seeds, thereby regulating the rate of seed storage reserve accumulation (Chen et al., 2015). An R2R3-MYB protein, MYB123 (TT2), was found to be a key regulator of not only proanthocyanidin deposition but also oil accumulation in seeds (Nesi et al., 2001; Chen et al., 2012; Wang et al., 2014). Knockout of MYB118, which also is a member of the R2R3-MYB TFs, caused the derepression of maturation-related genes, thus stimulating storage processes in endosperm (Barthole et al., 2014). Another R2R3-MYB TF, MYB96, directly regulates *FATTY ACID ELONGATION1* (*FAE1*) to stimulate the accumulation of very-long-chain fatty acids (VLCFAs; C $\geq$ 20) during seed maturation (Lee et al., 2015). However, TFs and the regulatory network controlling oil biosynthesis in plant seeds remain largely unknown and, thus, require further investigation.

In this study, we found that an R2R3-MYB TF, MYB89, is highly expressed in developing seeds during seed maturation in Arabidopsis. We found that MYB89 directly and indirectly targets some key genes that contribute to oil accumulation during seed maturation. Our results suggest that MYB89 functions as a negative regulator of seed oil accumulation during maturation in Arabidopsis seeds.

RESULTS

MYB89 Is Expressed Predominantly in Developing Seeds

MYB89 encodes an unknown MYB protein that belongs to the R2R3-MYB family TFs (Dubos et al., 2010). According to the Arabidopsis eFP Browser, *MYB89* is localized predominantly in mature pollen and developing seeds, particularly at the green cotyledon stages, suggesting that it regulates seed-related traits. Therefore, MYB89 was selected as the potential target in investigating the role of MYB TFs in the regulation of seed oil accumulation.

We investigated the subcellular localization of MYB89 in *Nicotiana benthamiana* leaves by using the GFP fusion construct, *35S:MYB89-GFP*. MYB89-GFP was specifically localized in the nucleus (Fig. 1A), indicating that MYB89 functions as a TF.

We further investigated *MYB89* expression in various tissues of wild-type plants by using quantitative real-time (qRT)-PCR and found the highest expression in developing seeds (Fig. 1, B and C). During seed development, *MYB89* expression remained relatively low at the early seed maturation stage, from 6 to 10 d after pollination (DAP), but increased progressively afterward at the mid seed maturation stage, until it reached the maximal level at 16 DAP (Fig. 1C). To monitor the detailed expression pattern of *MYB89*, we generated a *pMYB89:GUS* reporter construct, in which a 1.199-kb *MYB89* 5′ regulatory region upstream of the ATG start codon was fused to the *GUS* reporter gene. No GUS staining signal was observed in the nontransgenic embryo (data not shown). Among the 17 *pMYB89:GUS* independent lines in the wild-type background, most lines showed similar GUS staining patterns in developing seeds (Fig. 1D), which is highly consistent with the results of qRT-PCR analysis (Fig. 1C). Positive GUS staining also was observed in other tissues, including root tips and true leaves of young seedlings (Supplemental Fig. S1). Furthermore, in situ hybridization revealed high *MYB89* expression in developing seeds at 12 DAP (Fig. 1E). This expression pattern indicated that the dynamic regulation of *MYB89* was relevant to the accumulation of seed oil, which occurs mainly at the seed maturation stage (Fait et al., 2006; Baud et al., 2008).

MYB89 Represses Seed Oil Accumulation in Arabidopsis

To investigate its biological function on seed oil accumulation, we isolated a corresponding T-DNA insertion mutant (SALK_109375) in the Col-0 background in the *MYB89* exon from the Arabidopsis Biological Resource Center (Fig. 2A). The SALK mutant was designated *myb89-1*, which was backcrossed three times with Col-0, in case other mutations were present in this mutant. qRT-PCR showed that *MYB89* was hardly expressed in developing seeds of *myb89-1* homozygous plants but was highly expressed in the wild-type plants (Fig. 2, B and C).

Arabidopsis seed oil contains six major FA species, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1^{n9}), α-linoleic acid (C18:2^{n6}), α-linolenic acid

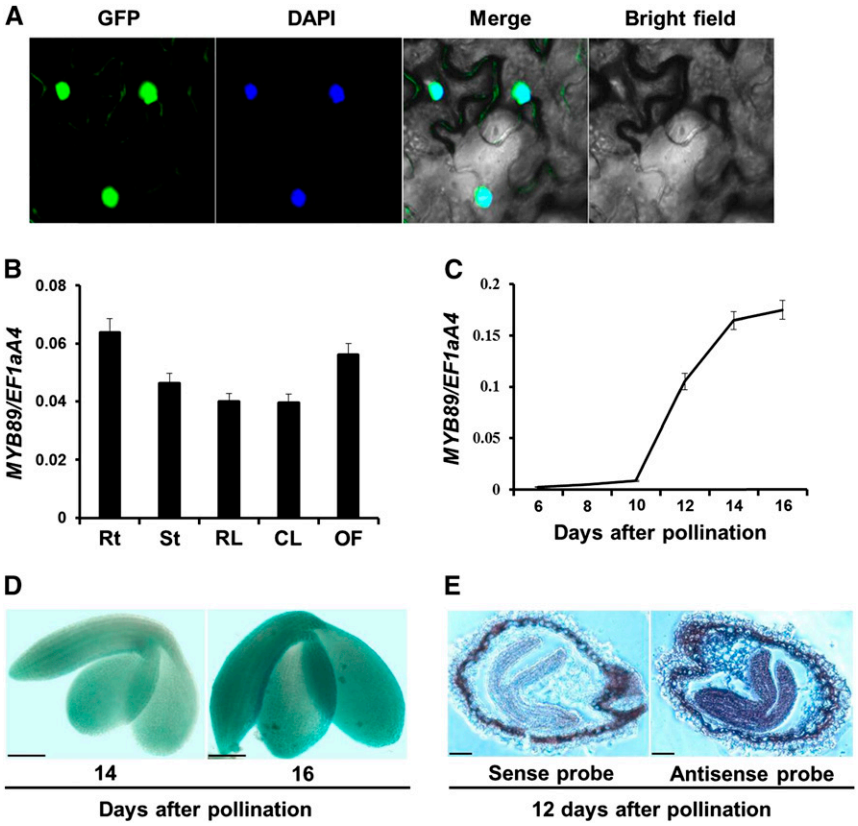

Figure 1. Analysis of *MYB89* expression pattern. A, Subcellular localization of the MYB89 protein fused with GFP (*35S:MYB89-GFP*) in *N. benthamiana* leaves. DAPI, Fluorescence of 4′,6-diamino-2-phenylindole; Merge, merge of GFP, DAPI, and bright-field images. B, qRT-PCR analysis of *MYB89* expression in various tissues of the wild type Columbia-0 (Col-0). Rt, Roots; St, stems; RL, rosette leaves; CL, cauline leaves; OF, open flowers. Results were normalized against the expression of *EF1aA4* as an internal control. Values are means ± SD ($n = 3$). C, qRT-PCR analysis of *MYB89* expression in developing seeds of the wild type (Col-0). Results were normalized against the expression of *EF1aA4* as an internal control. Values are means ± SD ($n = 3$). D, Representative GUS staining of *pMYB89:GUS* transgenic plants shows *MYB89* expression in developing embryos at 14 and 16 DAP in the wild type (Col-0). Bars = 100 μm. E, mRNA in situ localization of *MYB89* in wild-type (Col-0) developing seeds at 12 DAP. These seeds were hybridized with the antisense or sense *MYB89* probe as indicated. Bars = 100 μm.

(C18:3^{n3}), and eicosenoic acid (C20:1^{n11}), and many minor species (less than 3 mol % each). To test whether MYB89 affects the accumulation of seed oil, we measured the contents of these major FAs in developing seeds at 12, 14, and 16 DAP and in mature seeds (22 DAP) of *myb89-1* and wild-type plants. The contents of all FA species detected in wild-type and *myb89-1* seeds increased, with the highest levels in mature seeds at 22 DAP (Fig. 2G; Supplemental Table S2). Notably, the levels of total FAs and each FA composition were uniformly considerably higher in *myb89-1* than in the wild-type seeds at all stages of seed development investigated. We also found no obvious differences in several seed morphological traits, such as seed coat color (Fig. 2D), seed size and dry weight (Fig. 2E), embryo weight and size (Fig. 2, E and F), and embryo cell size and number (Fig. 2F; Supplemental Table S1), between mature seeds of *myb89-1* and wild-type plants. Furthermore, the loss of MYB89 activity did not alter plant growth size (Supplemental Fig. S2) and the responses to the abiotic stresses caused by high concentrations of NaCl and Glc during seedling establishment (Supplemental Fig. S3). These results indicated that MYB89 negatively regulates seed oil accumulation without affecting other aspects of plant growth and development that influence seed oil deposition.

To verify that the loss of MYB89 function is responsible for the high oil phenotype, we introduced the *MYB89* double-stranded RNA interference construct (*35S:MYB89-RNAi*) into wild-type Arabidopsis using *Agrobacterium tumefaciens*-mediated transformation. We obtained a total of 19 transformants, of which four independent *35S:MYB89-RNAi* T3 homozygous transgenic lines with the strongest effect on *MYB89* expression were selected (Fig. 3B). No obvious differences in seed coat color (Fig. 3A), seed size (Fig. 3A; Supplemental Fig. S4A), and dry weight (Supplemental Fig. S4B) were observed between mature seeds of the *35S:MYB89-RNAi* and wild-type plants. This result showed that the contents of total FAs and each FA composition per seed or milligram of seed were uniformly considerably higher in *35S:MYB89-RNAi* plants than in wild-type plants (Fig. 3, C and D; Supplemental Table S2). Furthermore, a *MYB89-6 HEMAGGLUTININ* (*6HA*) fusion gene driven by the 35S promoter was transformed into *myb89-1* plants. Four selected independent *myb89-1 35S:MYB89-6HA* T3 homozygous transgenic lines with the strongest effect on *MYB89* expression (Fig. 3F) exhibited comparable total FA quantity per seed or milligram of seed to that of the wild-type plants (Fig. 3, G and H; Supplemental Table S2). In addition, the content of each FA detected also was almost restored to the wild-type level (Supplemental Table S2). It is worth mentioning that *myb89-1 35S:MYB89-6HA* plants produced significantly larger and heavier seeds (Fig. 3E; Supplemental Fig. S4, C and D). Lower seed production often is correlated with larger seeds. Consistently, the total number of seeds per *myb89-1 35S:MYB89-6HA* plant was reduced markedly, whereas that of the *myb89-1* and *35S:MYB89-RNAi* plants did not

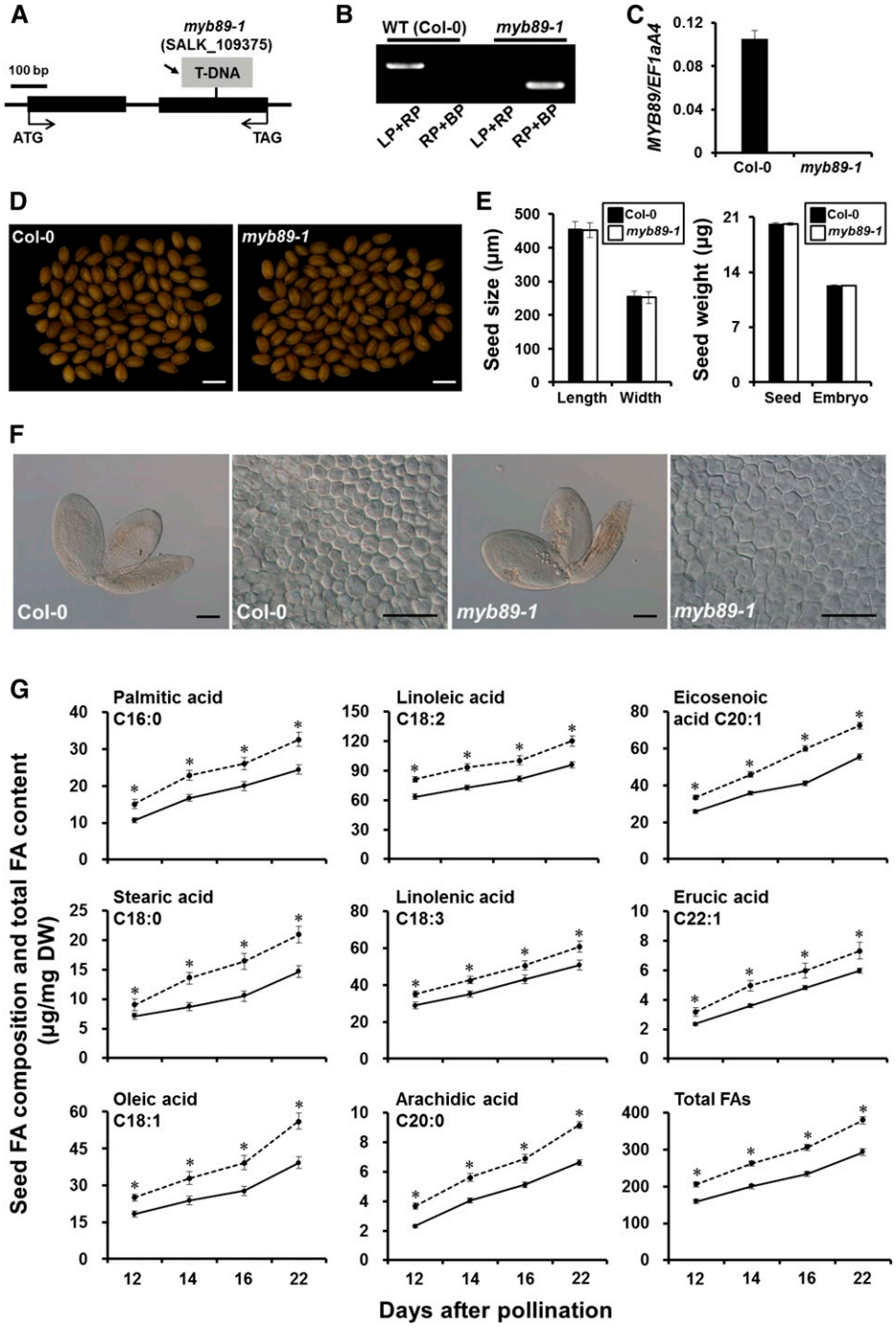

Figure 2. Comprehensive characterization of *myb89-1* seeds. A, Molecular identification of the *myb89-1* mutation. The structure of the *MYB89* gene shows the position of the T-DNA insertion in the *myb89-1* (SALK_109375) mutant. Black boxes represent exons, whereas black lines stand for the intron and other genomic regions. The arrow indicates the left border of the T-DNA. B, PCR-based genotyping of the wild type (WT; Col-0) and the homozygous *myb89-1* mutant. LP and RP refer to the *MYB89* gene-specific primers, and BP refers to the T-DNA right-border primer. C, qRT-PCR analysis of *MYB89* expression in the wild type (Col-0) and the *myb89-1* mutant. RNA samples were extracted from developing seeds at 12 DAP. Results were normalized against the expression of *EF1aA4* as an internal control. Values are means ± SD ($n = 3$). D, Microscopic observation of mature seeds randomly selected from wild-type (Col-0) and *myb89-1* plants. Bars = 500 μm. E, Quantitative comparisons of seed size (length and width) and dry weight of seeds and embryos between wild-type (Col-0) and *myb89-1* plants. Values are means ± SD ($n = 5$), and each of the three assays for each biological replicate contained 200 seeds from 12 individual plants grown in different pots arranged randomly within one of three blocks. F, Microscopic observations of mature embryos and epidermal cell layers of the central region of cotyledons from wild-type (Col-0) and *myb89-1* plants. Bars = 100 μm (embryos) and 20 μm (epidermal cell layers). G,

change relative to the wild-type plants (Supplemental Fig. S5A). We also found that the seed yield per plant was not changed, but the oil yield per plant was increased significantly in *myb89-1* and *35S:MYB89-RNAi* lines compared with the wild-type control (Supplemental Fig. S5, B and C). However, the seed yield and oil yield per plant were decreased significantly in *myb89-1 35S:MYB89-6HA* lines (Supplemental Fig. S5, B and C). Taken together, our findings suggest that MYB89 represses seed oil accumulation during seed maturation.

Differentially Expressed Genes in Developing Seeds at 12 DAP between Wild-Type and *myb89-1* Plants

We carefully selected one critical stage of seed oil accumulation to compare the expression profiles between *myb89-1* and the wild-type control. At 12 DAP, large differences in the contents of total FAs and major FA composition in seeds between the mutant and wild-type plants were identified (Fig. 2G; Supplemental Table S2). At this stage, the total FA content in the *myb89-1* seeds was 205.94 μg mg^{-1}, which was significantly higher than that in the wild-type seeds (159.15 μg mg^{-1}), and the FA content of developing seeds accounted for about 54% of the final FA content in mature seeds in both genotypes (Supplemental Table S2). The accumulation of seed oil increased markedly during the late embryonic maturation stages from 12 to 16 DAP and reached the final seed FA content at the end of embryo maturation (Fig. 2G), thereby indicating an active involvement of the genes that contribute to seed oil accumulation. Therefore, transcriptome analysis of developing seeds at 12 DAP could provide useful information on the downstream targets of MYB89 that contribute to oil biosynthesis as well as facilitate a better understanding of the regulatory networks underlying MYB89-mediated seed oil accumulation.

RNA sequencing (RNA-seq) analysis identified 1,171 differentially expressed genes (DEGs), among which 649 were up-regulated (Supplemental Table S3) and 522 were down-regulated (Supplemental Table S4) in *myb89-1* developing seeds at 12 DAP. Functional analysis indicated that 71 (10.9%) and 45 (6.9%) of the up-regulated genes were related to carbohydrate metabolism and general protein biosynthesis, respectively (Table I). Notably, numerous genes (34) involved in oil metabolism, accounting for 5.2% of the total up-regulated genes, were significantly up-regulated in *myb89-1* seeds (Table I; Supplemental Table S3). However, the expression of some genes that play major roles in oil accumulation did not change in *myb89-1* seeds relative to the wild-type seeds (Supplemental Table S5). Obviously, the number of up-regulated genes involved in the metabolism of carbohydrates, proteins, and oil was significantly higher than that of the down-regulated genes in *myb89-1* seeds (Table I).

In all, 20 genes involved in transcriptional regulation were regulated by MYB89, of which 15 genes were up-regulated and five genes were down-regulated in *myb89-1* seeds (Table I). Interestingly, *WRI1* and *L1L*, the master regulators of oil accumulation, were significantly up-regulated in *myb89-1* seeds, whereas no significant differences in the expression of the other TFs, including *LEC1*, *LEC2*, *ABI3*, *FUS3*, *bZIP67*, *TT2*, and *TTG1*, were observed between wild-type and *myb89-1* developing seeds at 12 DAP (Supplemental Tables S3–S5). Therefore, MYB89 inhibits seed oil accumulation by repressing two master regulators, *WRI1* and *L1L*, and a range of genes in the oil biosynthetic pathway during seed development.

Verification of Regulated Master Regulators and Genes Contributing to Oil Biosynthesis at Different Developmental Stages in *myb89-1* Seeds

Seed oil content is a complex quantitative trait controlled by multiple genes. TAG biosynthesis could be briefly classified into three phases in plant cells (Baud and Lepiniec, 2009; Itabe, 2010; Chapman and Ohlrogge, 2012). First, pyruvate and other substances produced during glycolysis are catabolized into acetyl-CoA, which is the precursor for FA biosynthesis, and then C16-18 FAs are synthesized in the plastid. Subsequently, most of these are exported to the cytoplasm, and, after chain elongation and desaturation, they form various FA derivatives at the acyl chains. Finally, oil is formed and stored in the oil body in the form of TAGs (Fig. 4A).

To confirm the regulation of the master regulators, *WRI1* and *L1L*, and genes involved in oil biosynthesis in *myb89-1* developing seeds at 12 DAP, and to extensively explore potential genes involved in the oil biosynthetic pathway that is regulated by MYB89, we performed qRT-PCR to compare the expression patterns at the seed maturation stages (6–16 DAP) between the wild-type and *myb89-1* plants. First, we selected 13 highly up-regulated TFs or genes, *WRI1*, *L1L*, *FBA2*, *ENO1*, *BIO2*, *BCCP1*, *KASI*, *HD-L*, *SAD* (AT3G02630 and AT5G16240), *KCS11*, *KCS17*, and *PLA2α*, and one significantly down-regulated gene, *CYFBP*, in *myb89-1* developing seeds at 12 DAP (Fig. 4B). The expression of *WRI1* was significantly higher at the midmaturation stage from 10 DAP in *myb89-1* developing seeds than in the wild-type seeds. Relative to that of the wild type,

Figure 2. (*Continued.*)
Dynamic accumulation of major FA species and total FAs in developing seeds (12, 14, and 16 DAP) and mature seeds (22 DAP) of wild-type (Col-0) and *myb89-1* plants. Solid and dotted lines indicate the content of FAs in wild-type (Col-0) and *myb89-1* seeds, respectively. Values are means ± SD ($n = 5$). Asterisks indicate significant differences in the contents of FA composition and total FAs between wild-type and *myb89-1* plants (two-tailed paired Student's *t* test, $P \leq 0.05$). DW, Dry weight.

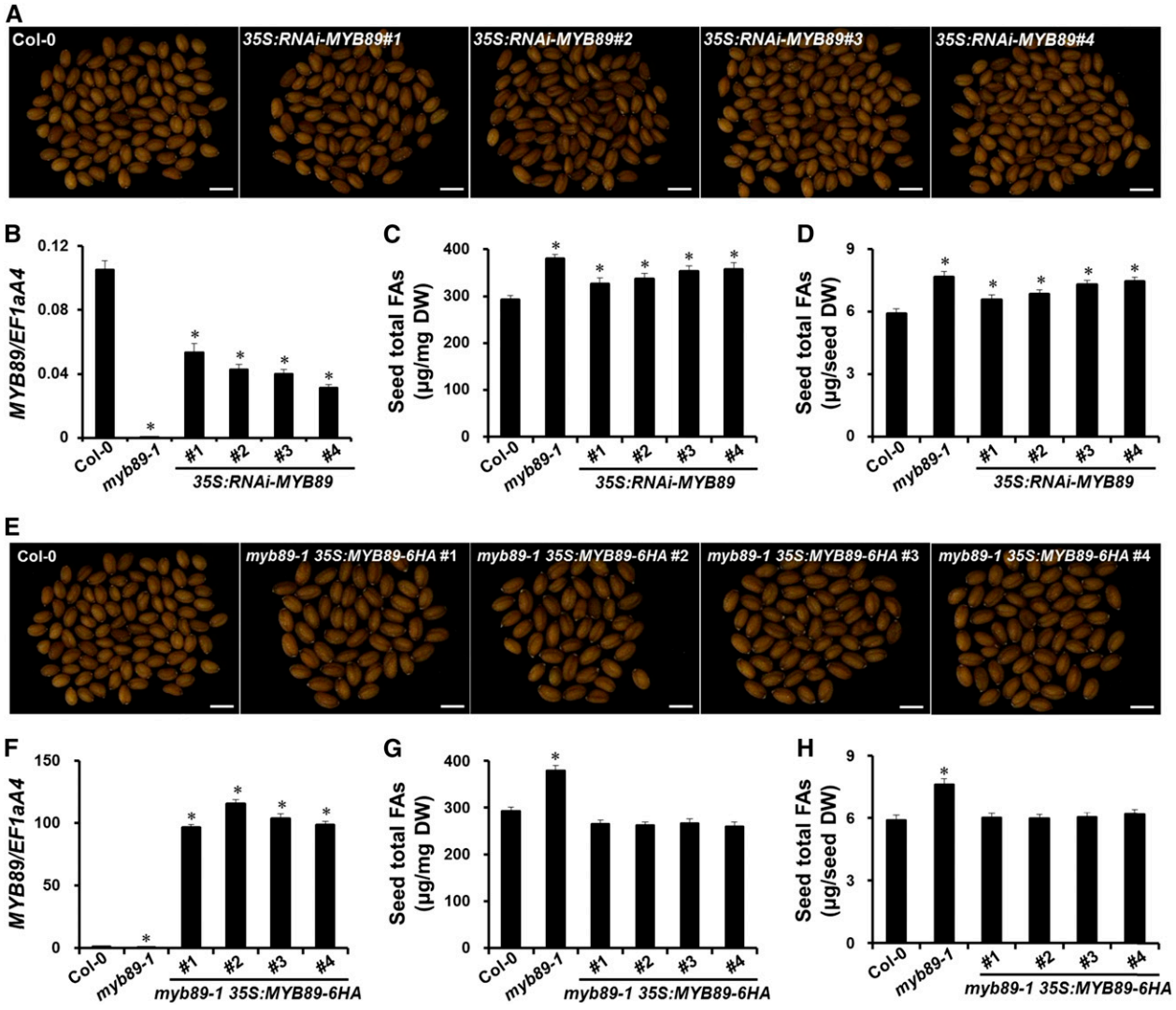

Figure 3. Effects of *MYB89* knockdown in the wild-type (Col-0) background and *MYB89* overexpression in the *myb89-1* mutant background on seed traits. For each construct (*35S:RNAi-MYB89* and *35S:MYB89-6HA*), four independent homozygous T3 transgenic lines with the strongest effect on *MYB89* expression were selected and analyzed. A, Microscopic observation of mature seeds randomly selected from the wild type (Col-0) and *35S:RNAi-MYB89* transgenic plants. Bars = 500 μm. B, qRT-PCR analysis of *MYB89* expression in wild-type (Col-0), *myb89-1*, and four selected independent *35S:RNAi-MYB89* transgenic plants. RNA samples were extracted from developing seeds at 12 DAP. Results were normalized against the expression of *EF1aA4* as an internal control. Values are means ± SD ($n = 3$). C, Comparisons of seed total FA content (μg mg^{-1}) among wild-type (Col-0), *myb89-1*, and *35S:RNAi-MYB89* transgenic plants. Values are means ± SD ($n = 5$). Asterisks indicate significant differences in the seed total FA content compared with that in the wild type (two-tailed paired Student's *t* test, $P \leq 0.05$). DW, Dry weight. D, Comparisons of seed total FA content (μg seed^{-1}) among wild-type (Col-0), *myb89-1*, and *myb89-1 35S:MYB89-6HA* transgenic plants. Values are means ± SD ($n = 5$). Asterisks indicate significant differences in the seed total FA content compared with that in the wild type (two-tailed paired Student's *t* test, $P \leq 0.05$). E, Microscopic observation of mature seeds randomly selected from wild-type (Col-0) and *myb89-1 35S:MYB89-6HA* transgenic plants. Bars = 500 μm. F, qRT-PCR analysis of *MYB89* expression in wild-type (Col-0), *myb89-1*, and four selected independent *myb89-1 35S:MYB89-6HA* transgenic plants. RNA samples were extracted from developing seeds at 12 DAP. Results were normalized against the expression of *EF1aA4* as an internal control. Values are means ± SD ($n = 3$). G, Comparisons of seed total FA content (μg mg^{-1}) among wild-type (Col-0), *myb89-1*, and *myb89-1 35S:MYB89-6HA* transgenic plants. Values are means ± SD ($n = 5$). Asterisks indicate significant differences in the seed total FA content compared with that in the wild type (two-tailed paired Student's *t* test, $P \leq 0.05$). H, Comparisons of seed total FA content (μg seed^{-1}) among wild-type (Col-0), *myb89-1*, and *myb89-1 35S:MYB89-6HA* transgenic plants. Values are means ± SD ($n = 5$). Asterisks indicate significant differences in the seed total FA content compared with that in the wild type (two-tailed paired Student's *t* test, $P \leq 0.05$).

Table I. *Functional classification of DEGs in developing seeds of myb89-1 plants at 12 DAP*

Functional classification of DEGs was performed using the biological process category of Arabidopsis Gene Ontology (http://www.geneontology.com). Percentage refers to the ratio of genes of each functional category relative to total up-regulated or down-regulated DEGs identified in the RNA-seq experiment. The DEGs with $\log_2$ ratios greater than 1 or less than −1 (only GO Slim identifiers with $P \leq 0.05$ and false discovery rate ≤ 0.05) are listed.

Category	Up-Regulated DEGs				Down-Regulated DEGs			
	≥2	1 to 2	Total	Percentage	≤−2	−2 to −1	Total	Percentage
	log₂ ratio				*log₂ ratio*			
Metabolism								
Photosynthesis	3	14	17	2.6	3	2	5	1.0
Cell wall	4	11	15	2.3	1	3	4	0.8
Oil metabolism	4	30	34	5.2	4	17	21	4.0
Carbohydrate metabolism	4	67	71	10.9	16	38	54	10.3
Nucleic acid	3	16	19	2.9	3	24	27	5.2
Amino acid and protein	8	37	45	6.9	5	11	16	3.1
Growth and development								
Leaf and root development	0	12	12	1.8	1	9	10	1.9
Shoot development	2	2	4	0.6	0	0	0	0
Embryo/seed development	1	11	12	1.8	1	9	10	1.9
Flower development	5	7	12	1.8	2	3	5	1.0
Cell growth	5	42	47	7.2	5	6	11	2.1
Hormone	5	15	20	3.1	13	12	25	4.8
Stress/defense response	9	40	49	7.6	27	76	103	19.7
Cell regulation								
Transcriptional regulation	0	15	15	2.3	1	4	5	1.0
Signaling transduction	6	34	40	6.2	10	23	33	6.3
Transport facilitation	3	28	31	4.8	8	27	35	6.7
Others	30	176	206	31.7	45	113	158	30.3

myb89 mutation resulted in at least a 2-fold higher level of *L1L* transcripts in developing seeds at 12 and 14 DAP. The expression of the other genes was significantly higher at the seed maturation stage between wild-type and *myb89-1* plants at 12 DAP and other examined stages, except for *CYFBP* expression, which was decreased significantly from 6 to 12 DAP and was always lower during seed maturation in *myb89-1* than in the wild type. The expression of nearly all of these up-regulated genes was higher than in the wild-type seeds from 6 to 16 DAP (Fig. 4).

Furthermore, another 28 genes (Fig. 4B; Supplemental Fig. S6), the expression of which was not altered significantly in *myb89-1* developing seeds at 12 DAP (Supplemental Table S5), were selected to investigate whether these are regulated by MYB89 during seed maturation. No significant differences in the expression of 24 genes were noted at the seed maturation stage between wild-type and *myb89-1* plants (Supplemental Fig. S6); however, the expression of *FAD2*, *FAD3*, *FAE1*, and *ROD1* was significantly up-regulated in *myb89-1* developing seeds at 14 and/or 16 DAP (Fig. 4B).

These significantly up-regulated genes, except for the two master regulators, *WRI1* and *L1L*, are marked in blue with an orange box, and the down-regulated gene, *CYFBP*, is indicated in green with an orange box, in the oil biosynthetic pathway (Fig. 4A). Taken together, these findings suggest that MYB89 regulates the expression of *WRI1* and *L1L*, the TFs critical for oil accumulation, and a class of genes in the oil biosynthetic pathway, which is concomitant with a steady increase in FA levels in seeds at the midmaturation stage (Fig. 2G; Supplemental Table S2), indicating that MYB89 mediates seed oil accumulation by affecting the expression of these genes.

MYB89 Directly Targets *WRI1*, *ENO1*, *BCCP1*, *KASI*, *KCS11*, and *PLA2α* in Arabidopsis Developing Seeds

To determine how MYB89 regulates the mRNA expression of downstream targeted genes, we generated a steroid-inducible version of MYB89 in the background of *myb89-1*, in which the *MYB89* gene was fused to the rat glucocorticoid receptor (GR) and driven by the 35S promoter. We isolated a *myb89-1 35S:MYB89-GR* transgenic line, which rescued the higher oil content phenotype of *myb89-1* seeds after dexamethasone treatment every alternate day after germination, whereas the mock-treated *myb89-1 35S:MYB89-GR* accumulated higher oil like *myb89-1* (Supplemental Fig. S7). This showed that the MYB89-GR fusion protein has a biological function like that of the wild-type MYB89 upon steroid induction.

By using this established steroid-inducible activation of MYB89, we further determined whether the expression of these genes is repressed by MYB89 activity (Fig. 4). Dexamethasone treatment of *myb89-1 35S:MYB89-GR* siliques at 12 or 14 DAP for 2 or 4 h caused a reduction in the expression of all these genes, except for *CYFBP*, relative to that of the mock-treated controls (Fig. 5; Supplemental Fig. S8). However, the combined

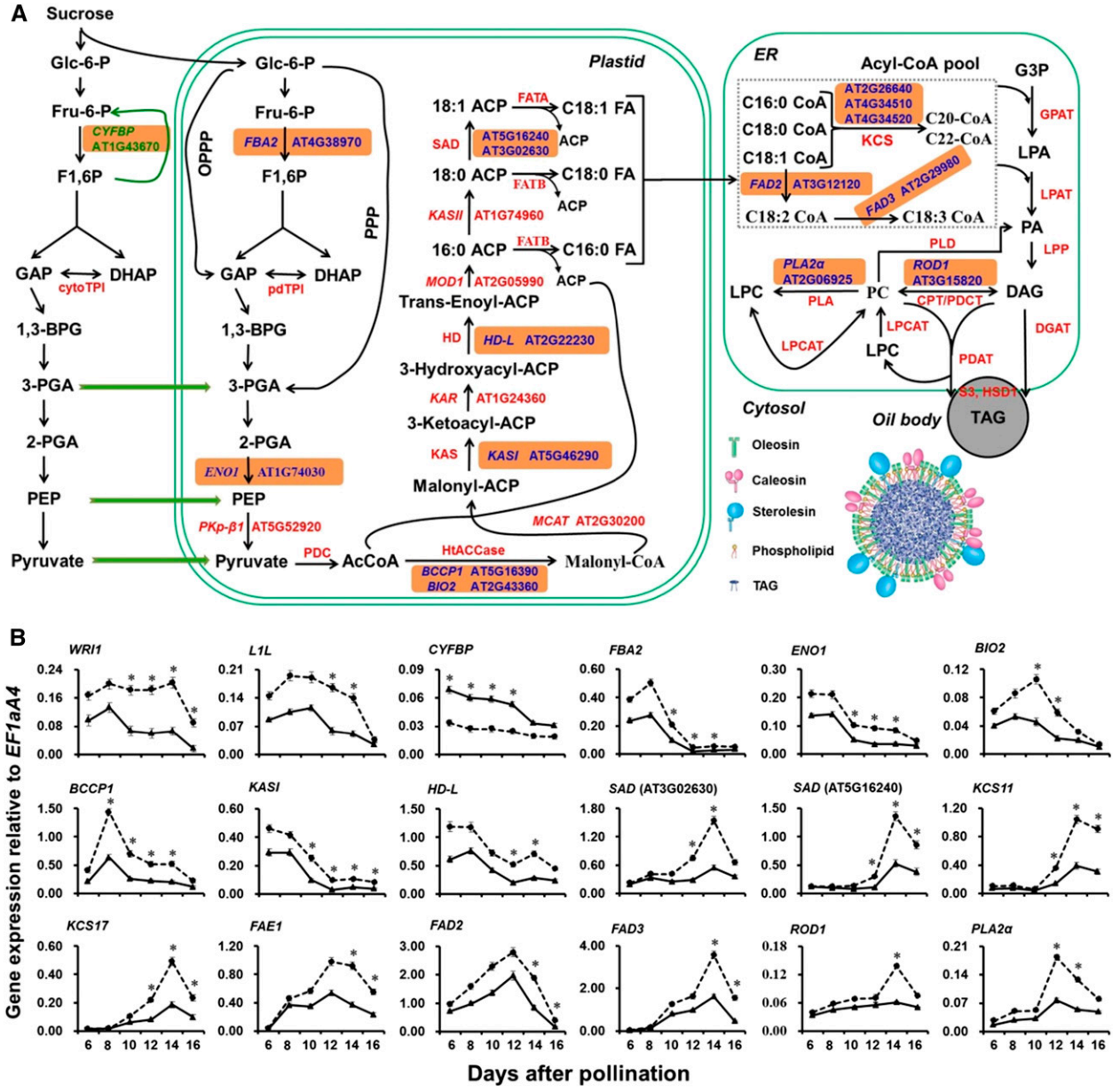

Figure 4. Dynamic expression analysis of genes involved in the processes of glycolysis, FA biosynthesis and modification, and TAG accumulation in developing seeds of wild-type (Col-0) and *myb89-1* plants. A, Simplified scheme showing the altered expression levels of genes involved in the oil biosynthetic pathway in developing seeds between wild-type (Col-0) and *myb89-1* plants. The seed oil body generated from the endoplasmic reticulum (ER) accumulates TAG inside and is surrounded by a layer of phospholipids and proteins, including oleosin, caleosin, and steroleosin. The genes marked by blue color with an orange box were significantly up-regulated, and the gene *CYFBP* indicated by green color with an orange box was down-regulated in *myb89-1* developing seeds. F1,6P, Fru-1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxy-acetone-phosphate; cytoTPI, cytosolic triose phosphate isomerase; pdTPI, plastid TPI; 1,3-BPG, 1,3-bisphosphoglycerate; 3PGA, glycerate-3-phosphate; 2PGA, glycerate-2-phosphate; PEP, phospho*enol*pyruvate; PDC, pyruvate dehydrogenase complex; AcCoA, acetyl-CoA; HtACCase, heteromeric acetyl-CoA carboxylase; ACP, acyl carrier protein; KAS, 3-ketoacyl-ACP synthase; HD, hydroxyacyl-ACP dehydratase; SAD, stearoyl-ACP desaturase; FAT, fatty acyl-ACP thioesterase; KCS, 3-ketoacyl-CoA synthase; G3P, glycerol 3-phosphate; GPAT, G3P acyltransferase; LPA, lysophosphatidic acid; LPAT, LPA acyltransferase; PA, phosphatidic acid; LPP, lipid phosphate phosphatase; DAG, diacylglycerol; PC, phosphatidylcholine; PDCT, PC:DAG phosphocholine transferase; CPT, choline-phosphotransferase; PLA, phospholipase A; PLD, phospholipase D; LPCAT, lyso-PC acyltransferase; LPC, lyso-PC; DGAT, DAG acyltransferase; PDAT, phospholipid:DAG acyltransferase; TAG, triacylglycerol; HSD, hydroxysteroid dehydrogenase; *CYFBP, CYTOSOL FRU-1,6-BISPHOSPHATASE*; *FBA2, FRU-1,6-BISPHOSPHATE ALDOLASE2*; *ENO1, PLASTID-LOCALIZED PHOSPHOENOLPYRUVATE ENOLASE1*; *PKp-β1, PLASTIDIC PYRUVATE KINASE β-SUBUNIT1*; *BCCP1, BIOTIN CARBOXYL-CARRIER PROTEIN1*; *BIO2, BIOTIN AUXOTROPH2*; *MCAT, MALONYL-COA:ACP TRANSACYLASE*; *KAR, 3-KETOACYL-ACP*

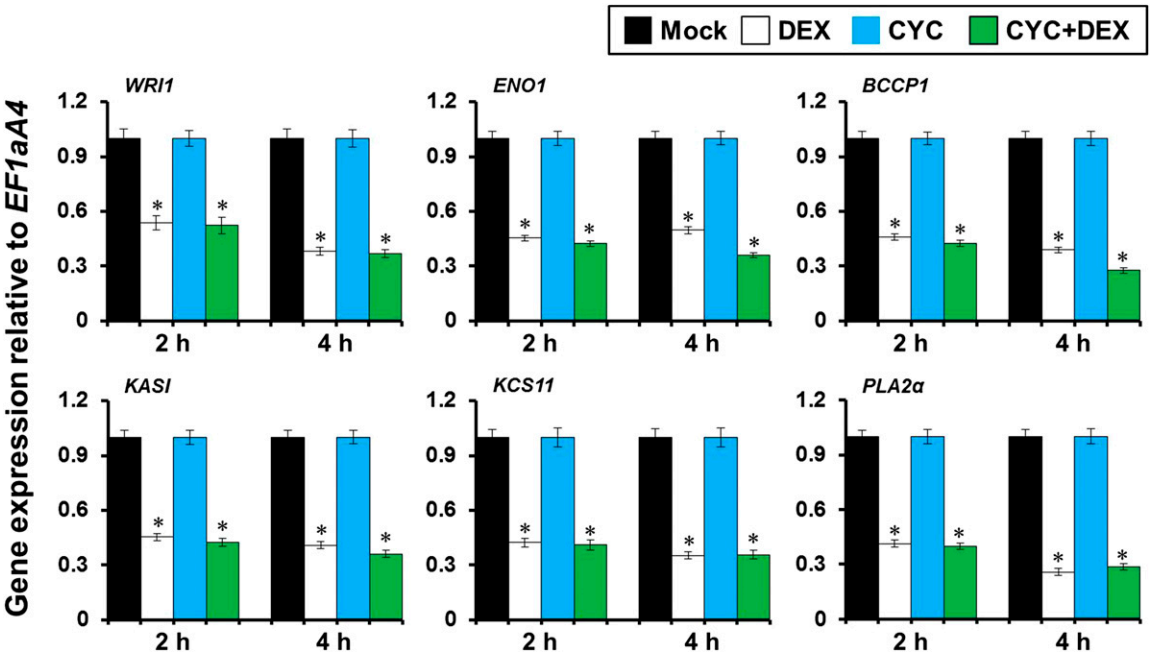

Figure 5. Induced MYB89 activity transcriptionally represses the expression of *WRI1*, *ENO1*, *BCCP1*, *KASI*, *KCS11*, and *PLA2α* during seed development. The *myb89-1 35S:MYB89-GR* siliques at 12 DAP were mock treated (Mock) or treated with 10 μM dexamethasone (DEX), 10 μM cycloheximide (CYC), or 10 μM CYC plus 10 μM DEX (CYC+DEX). The expression of these genes was examined after 2 or 4 h of treatment using qRT-PCR analyses. Results were normalized against the expression of *EF1aA4* as an internal control. Values are means ± SD ($n = 3$). Asterisks indicate significant differences in gene expression in dexamethasone-treated samples compared with their respective controls (two-tailed paired Student's *t* test, $P \leq 0.05$).

treatment of dexamethasone and cycloheximide for 2 or 4 h only significantly reduced the expression of *WRI1*, *ENO1*, *BCCP1*, *KASI*, *KCS11*, and *PLA2α* (Fig. 5; Supplemental Fig. S8), suggesting that these six genes are immediate targets of transcriptional repression by MYB89 in developing seeds, whereas the induction of *CYFBP* and repression of the other 12 genes by MYB89 are dependent on other intermediate proteins.

To investigate whether MYB89 binds directly to the promoter regions of *WRI1*, *ENO1*, *BCCP1*, *KASI*, *KCS11*, and *PLA2α* to control its expression, we performed chromatin immunoprecipitation (ChIP) assays using a functional transgenic line that expressed a *MYB89-6HA* fusion gene driven by the 35S promoter. *35S:MYB89-6HA* completely rescued the higher oil content phenotype of *myb89-1* in the *myb89-1 35S:MYB89-6HA* lines (Fig. 3, G and H; Supplemental Table S2), indicating that the fusion protein of MYB89-6HA at least retains the same biological function as MYB89 in developing seeds. We designed sufficient pairs of primers to cover all the possible cis-elements bound by MYB89 in the promoter regions of these six genes (Fig. 6). ChIP assays performed using a representative line, *myb89-1 35S:MYB89-6HA#1*, showed that MYB89-6HA was associated with the promoter regions near fragments 6 and 7 of *WRI1*, fragment 1 of *ENO1*, fragments 2 and 5 of *BCCP1*, fragments 5 and 9 of *KASI*, fragments 4 and 6 of *KCS11*, and fragments 5 and 8 of *PLA2α* (Fig. 6). These results collectively suggested that MYB89 binds directly to the loci of *ENO1*, *BCCP1*, *KASI*, *KCS11*, and *PLA2α* to suppress their expression.

DISCUSSION

Seed oil accumulation in many higher plants is precisely controlled by intricate regulatory networks that coordinate various environmental and developmental signals and by multilevel regulation, among which

Figure 4. (*Continued.*)
REDUCTASE; *MOD1*, *MOSAIC DEATH1*; *FAD2*, *FATTY ACID DESATURASE2*; *ROD1*, *REDUCED OLEATE DESATURATION1*; *PLA2α*, *PHOSPHOLIPASE A2α*; OPPP, oxidative pentose phosphate pathway; PPP, nonoxidative pentose phosphate pathway. B, qRT-PCR analysis of the expression of genes involved in the oil biosynthetic pathway in developing seeds between wild-type (Col-0) and *myb89-1* plants. Solid and dotted lines indicate the dynamic expression of genes in wild-type and *myb89-1* plants, respectively. Results were normalized against the expression of *EF1aA4* as an internal control. Values are means ± SD ($n = 3$). Asterisks indicate significant differences in gene expression levels in *myb89-1* plants compared with those in wild-type plants (two-tailed paired Student's *t* test, $P \leq 0.05$).

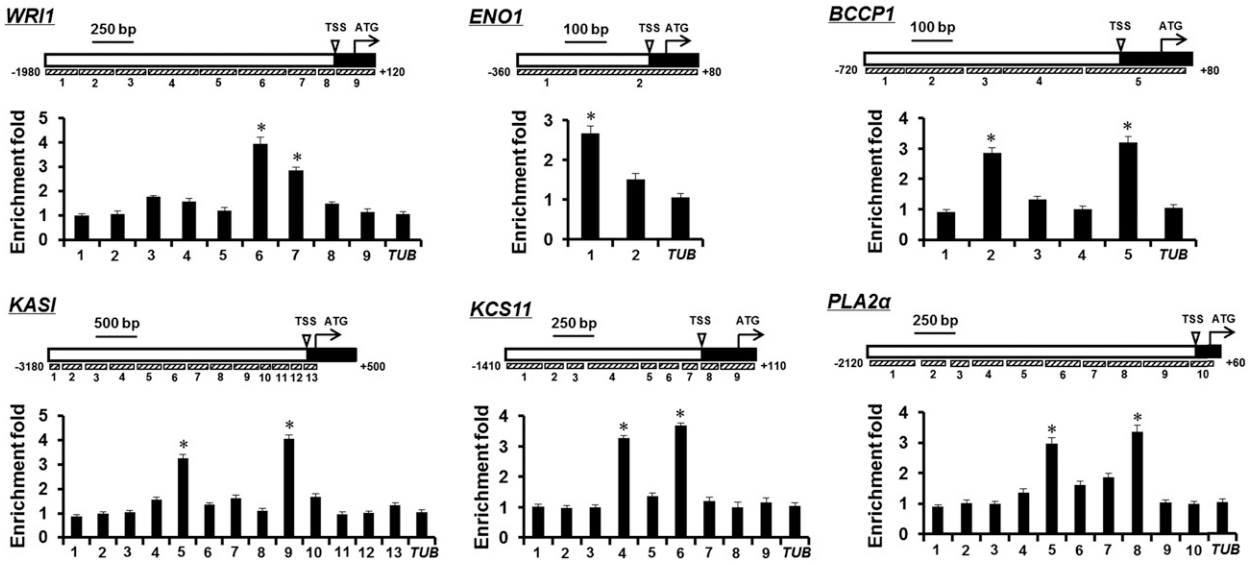

Figure 6. Schematic diagrams show the promoter regions of *WRI1*, *ENO1*, *BCCP1*, *KASI*, *KCS11*, and *PLA2α*, and ChIP analysis shows MYB89-6HA binding to their promoter regions in developing siliques at 12 DAP. The transcriptional start site (TSS) and exon are represented by black boxes, whereas promoter regions are represented by white boxes. Gray boxes represent the DNA fragments amplified in ChIP analysis for each gene. The enrichment fold of each fragment was calculated first by normalizing the amount of a target DNA fragment against a genomic fragment of *EF1aA4* as an internal control and then by normalizing the value for *myb89-1 35S:MYB89-6HA* against that for *myb89-1*. A *TUB2* (*TUB*) fragment was amplified as a negative control. Values are means ± SD ($n = 3$). Significant differences in comparison with the enrichment of the *TUB* fragment are indicated with asterisks (two-tailed paired Student's *t* test, $P \leq 0.05$).

transcriptional regulation is a major factor influencing FA supply for TAG biosynthesis. However, the mechanism underlying how TFs and the regulatory network control the overall amount of oil stored in plant seeds is not completely understood to date. The MYB family includes numerous proteins and is functionally involved in the regulatory networks that control the development, metabolism, and responses to biotic and abiotic stresses (Dubos et al., 2010). We used the reverse genetic approach and found that *myb89* mutation causes a significant increase in the levels of total FAs and each major FA composition during seed maturation (Fig. 2G; Supplemental Table S2), which leads to the remarkable accumulation of seed FAs (Baud et al., 2008). In contrast, knockdown of *MYB89* resulted in a significant increase of total FAs and each FA composition per seed or milligram of seed (Fig. 3, B–D; Supplemental Table S2). Furthermore, the ectopic expression of *MYB89* in the *myb89-1* background could completely restore the higher seed oil content of *myb89-1* plants to the wild-type level (Fig. 3, F–H; Supplemental Table S2). These results, together with the observation of the increased expression of *MYB89* in developing embryos at the mid seed maturation stage (Fig. 1, B–E), suggest that MYB89 plays an important role in the regulatory network that suppresses the accumulation of seed oil in Arabidopsis.

The coordinated expression of genes involved in the oil biosynthetic pathway is essential for seed oil accumulation (Ohlrogge and Jaworski, 1997; Ruuska et al., 2002; Graham, 2008). Loss of function of *MYB89* regulated the expression of many genes involved in various important metabolic processes, including glycolysis, FA biosynthesis and modification, and TAG deposition in the plastids or endoplasmic reticulum, which, in turn, contribute to oil accumulation (Fig. 4; Supplemental Tables S3 and S4). Of these enzymes, FBA2, which is localized in the plastids, is an essential enzyme that generates metabolites for starch biosynthesis, and *fba2* seedlings accumulate less starch (Sonnewald et al., 1994; Lu et al., 2012). CYFBP, which is expressed in the cytoplasm and nucleus, catalyzes the formation of Fru-6-P for Suc biosynthesis, whereas its disruption elevates the levels of starch and glycerate-3-phosphate in the leaves during the day (Fig. 4A; Rojas-González et al., 2015). *ENO1* encodes the plastid-localized phospho*enol*pyruvate enolase that functions metabolically in the conversion of glycerate-2-phosphate into phospho*enol*pyruvate (Fig. 4A; Prabhakar et al., 2009), which mediates the production of acetyl-CoA, the precursor for FA biosynthesis. Previous studies have proposed that starch serves as a carbon source for the accumulation of seed storage reserves such as oil and storage proteins (Norton and Harris, 1975; daSilva et al., 1997; Periappuram et al., 2000; Andriotis et al., 2010). The master regulator, *WRI1*, plays an essential role in the developmental regulation of carbohydrate metabolism, particularly in the incorporation of Suc and Glc into TAGs during seed filling (Focks and Benning, 1998). Notably, *WRI1* is significantly up-regulated in *myb89-1* developing seeds from 10 to 16 DAP (Fig. 4; Supplemental

Table S3-2). Thus, the decreased expression of *CYFBP* (Fig. 4; Supplemental Table S4-2) and the subsequent elevated expression of *WRI1*, *FBA2*, and *ENO1* (Fig. 4; Supplemental Table S3-2) might assist in the partitioning of carbons for oil accumulation in *myb89-1* developing seeds (Fig. 2G).

The ATP-dependent carboxylation of acetyl-CoA to yield malonyl-CoA is a primary reaction that occurs during de novo FA biosynthesis (Baud and Lepiniec, 2009). This process is mediated by HtACCase, which also acts as a sensor or gating system that controls the overall flux of FA biosynthesis (Reverdatto et al., 1999; Mu et al., 2008). Our results showed that *BCCP1*, which encodes an HtACCase subunit and *BIO2*, was significantly up-regulated in *myb89-1* developing seeds (Fig. 4; Supplemental Table S3-2). Reduced BCCP1 activity severely affects normal vegetative growth and also markedly decreases seed FA accumulation (Li et al., 2011). BIO2 converts dethiobiotin to biotin in the biotin biosynthetic pathway (Picciocchi et al., 2001), and the loss of BIO2 reduces the content of seed oil (Pommerrenig et al., 2013). Thus, more active HtACCase and more biotin might promote oil accumulation in *myb89-1* developing seeds at the very early stage of the biosynthetic pathway (Figs. 2G and 4A; Supplemental Table S2). Three separate condensing enzymes, or 3-ketoacyl-ACP synthases (KASI–KASIII), are necessary for the production of C18 FAs. Among these, KASI is responsible for producing six- to 16-carbon chains (Fig. 4A; Pidkowich et al., 2007), and its deficiency markedly reduces seed FA accumulation (Wu and Xue, 2010). Furthermore, 3-hydroxyacyl-ACP dehydratase (HD) is required after each condensation step to obtain a saturated and two-carbon-longer FA (Fig. 4A; Mou et al., 2000), and some 16:0-ACP molecules are elongated to C18:0-ACP, which is efficiently desaturated by a stromal SAD (Fig. 4A; Browse and Somerville, 1991). Hence, the suppression of *KASI*, *HD-L*, and *SADs*, including AT5G16240 and AT3G02630, by MYB89 (Fig. 4; Supplemental Table S3-2) influences the accumulation of FA composition such as C16:0, C18:0, and C18:1 in seeds at the middle stage of the biosynthetic pathway (Figs. 2G and 4; Supplemental Table S2). The biosynthesis of long-chain FAs in the plastids is terminated when the acyl group is removed from ACP by fatty acyl-ACP thioesterase (Fig. 4A; Salas and Ohlrogge, 2002; Bonaventure et al., 2003). Once activated to CoA esters and transported to the endoplasmic reticulum, these FA species can be modified (i.e. desaturated and elongated). FAD2 is essential for polyunsaturated lipid synthesis (Fig. 4A; Okuley et al., 1994), and FAD3 is responsible for the biosynthesis of C18:3 from phospholipids using cytochrome b_5 as an electron donor (Fig. 4A; Shah et al., 1997). Thus, the highly up-regulated expression of the genes *FAD2* and *FAD3* would accelerate the accumulation of C18:2 and C18:3, respectively, in *myb89-1* developing seeds (Fig. 2G; Supplemental Table S2). The *KCS* genes are essential for the biosynthesis of VLCFAs and facilitate the extension of the chain length of FAs from C18 to C20 and C22 (Fig. 4A). Notably, MYB89 largely suppresses the expression of genes involved in the elongation of FAs, such as *KCS11*, *KCS17*, and *KCS18* (*FAE1*; Fig. 4; Supplemental Table S3-2). This could explain why *myb89-1* seeds accumulated considerably more VLCFAs than wild-type seeds (Fig. 2G; Supplemental Table S2). Furthermore, the expression of *PLA2α* and *ROD1*, which participate in TAG deposition at the late stage of the biosynthetic pathway, was increased significantly in the *myb89* developing seeds (Fig. 4; Supplemental Table S3-2). PLA2α, a secretory phospholipase A2 enzyme, specifically hydrolyzes the *sn*-2 position of phospholipids and has a preference toward the linoleoyl acyl chain (Ryu, 2004; Ryu et al., 2005). *ROD1* encodes a phosphatidylcholine:diacylglycerol cholinephosphotransferase that is involved in a major reaction for the transfer of C18:1 to phosphatidylcholine for desaturation as well as for the reverse transfer of C18:2 and C18:3 to the TAG synthesis pathway (Fig. 4A; Lu et al., 2009). Therefore, MYB89 participates in multiple regulatory events at the transcriptional level that mediate oil accumulation in seeds (Fig. 4A).

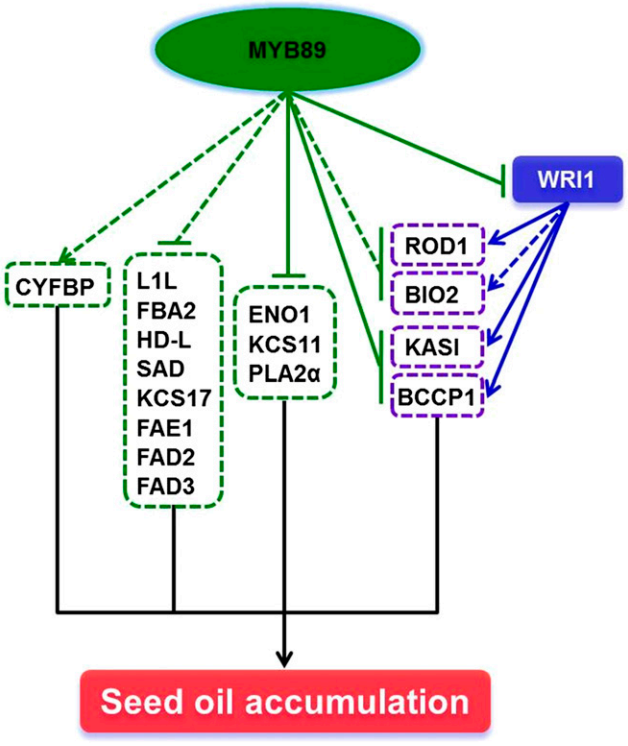

Figure 7. A simplified model shows that MYB89 directly and indirectly regulates the expression of key regulators and genes that control the accumulation of seed oil in Arabidopsis. Arrows and T bars, respectively, indicate promoting and inhibitory effects, whereas solid and dotted lines indicate direct and indirect transcriptional regulation, respectively.

RNA-seq and qRT-PCR analyses showed that *LEC2* expression was not altered (Supplemental Fig. S6; Supplemental Table S5), whereas *WRI1* expression was increased significantly, in *myb89-1* developing seeds (Fig. 4; Supplemental Table S3-2). By using the

GR-inducible system and the ChIP assay, we found the novel result that *WRI1* is repressed directly by MYB89 in developing seeds (Figs. 5 and 6). A previous study showed that the genes encoding enzymes required for FA biosynthesis are not direct targets of LEC2 in leaves (Santos Mendoza et al., 2005). Further genetic analyses indicated that *WRI1* is a direct target of LEC2 and functions as a global regulator, specifying the regulatory action of LEC2 toward FA biosynthesis, and fine-tunes the expression of lipogenic genes during seed maturation in Arabidopsis (Baud et al., 2007). We reasoned that MYB89 represses *WRI1* expression directly, not via LEC2 at the transcriptional level. These findings indicated that MYB89 serves as a novel negative regulator in directly suppressing the expression of *WRI1*, the master regulator of oil accumulation, in maturing Arabidopsis seeds (Fig. 7).

Molecular analyses have revealed that *ENO1*, *BCCP1*, *KASI*, *KCS11*, and *PLA2α* are repressed directly (Figs. 5 and 6), whereas *L1L* and the remaining 11 key genes, *CYFBP*, *FBA2*, *BIO2*, *HD-L*, *SAD* (AT3G02630 and AT5G16240), *KCS17*, *FAE1*, *FAD2*, *FAD3*, and *ROD1*, are regulated indirectly (Supplemental Fig. S8), by MYB89 during seed maturation. Previous studies indicated that WRI1 acts as the master regulator of FA biosynthesis by directly promoting the expression of *SUS2*, *PI-PKβ1*, *BCCP1*, *BCCP2*, *KASI*, *GLB1*, and *ROD1* and indirectly inducing *ACP1*, *CAC2*, *CAC3*, *BIO2*, *PDH E1α*, *KASIII*, *MOD1*, and *LAS* during Arabidopsis seed development (Focks and Benning, 1998; Cernac and Benning, 2004; Masaki et al., 2005; Baud et al., 2007; Maeo et al., 2009; To et al., 2012). *ROD1* also was demonstrated to be up-regulated by overexpressing Arabidopsis *WRI1* in *N. benthamiana* leaves (Grimberg et al., 2015) and castor (*Ricinus communis*) seeds (Adhikari et al., 2016). Thus, alterations in the FA profile of *myb89* seeds become complicated and cannot be attributed to specific genes. The loss of MYB89 and the elevated expression of *WRI1* together might promote the expression of *KASI* and *BCCP1*, and the other two genes, *ROD1* and *BIO2*, might be induced via the up-regulation of *WRI1* in *myb89-1* developing seeds, thereby accelerating seed oil accumulation (Fig. 7). These four genes might be regulated by multiple forms during seed development. Theoretically, except for *KASI*, *BCCP1*, *ROD1*, and *BIO2*, the up-regulation of *WRI1* induces the expression of all the other genes that are positively regulated by WRI in *myb89-1* developing seeds. However, no changes in transcriptional levels of the genes, including *SUS2*, *PI-PKβ1*, *BCCP2*, *GLB1*, *ACP1*, *CAC2*, *CAC3*, *PDH E1α*, *KASIII*, *MOD1*, and *LAS*, in *myb89-1* seeds were noted compared with those in the wild-type seeds (Supplemental Fig. S6; Supplemental Table S5). These genes up-regulated by WRI1 (Fig. 7) are likely promoted by MYB89 or regulated by unknown TFs, and other unknown TFs might independently act or interact with MYB89 and/or master regulators described here to regulate those genes that were targeted by MYB89. A previous study showed that the loss of BTB/POZ-MATH (BPM) proteins widely affects plant development and causes altered FA contents in Arabidopsis mutant seeds, and BPM proteins function as negative regulators of WRI1 activities by mediating assembly with the CULLIN3-based REALLY INTERESTING NEW GENE E3 ligases core and ultimately causing its degradation via the 26S proteasome (Chen et al., 2013). However, the expression of the *BPM* genes is not changed in *myb89-1* developing seeds compared with wild-type seeds (Supplemental Fig. S9; Supplemental Table S5), suggesting that MYB89 controls the expression of *WRI1* not via *BPM* genes at the transcriptional level to affect seed oil accumulation in Arabidopsis but possibly via a distinct regulatory pathway. These interesting questions need to be investigated further.

In summary, to our knowledge, this study is the first to show that an R2R3-MYB TF, MYB89, inhibits Arabidopsis seed oil accumulation by directly repressing the expression of the master regulator, *WRI1*, and that of five key genes, *ENO1*, *BCCP1*, *KASI*, *KCS11*, and *PLA2α*, and indirectly suppressing another master regulator, *L1L*, and various other key genes in the oil biosynthetic pathway during seed maturation (Fig. 7). In addition, the deletion of MYB89 does not lead to the formation of impaired phenotypes in plants and alter the responses to abiotic stresses during seedling establishment (Fig. 2, D–F; Supplemental Figs. S2 and S3). These factors ensure the appropriate manipulation of seed oil production in oil-producing crops and plants by antagonizing MYB89 activity at the late stages of seed development to promote the expression of *WRI1* and other various key genes that contribute to oil accumulation, thereby improving both the quantity and the quality of seed oil.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 was used as the wild-type control. The mutant *myb89-1* (SALK_109375) was in the Col-0 background, and its genotyping primers are listed in Supplemental Table S6. All transgenic plants were generated in the Col-0 background through *Agrobacterium tumefaciens*-mediated transformation and selected by Basta on soil. Since the growth conditions can markedly affect the total oil accumulation in seeds (Li et al., 2006), all plants for FA measurements were grown at the same time in the same chamber under long-day conditions (16 h of light/8 h of dark) at 22°C. The overhead light intensity was 160 μmol m^{-2} s^{-1}, as detected at the middle region of the plant.

Light Microscopy

Embryo observation was performed as described previously (Ohto et al., 2005; Chen et al., 2014). Isolated embryos were incubated in a buffer (50 mM sodium phosphate [pH 7], 10 mM EDTA, 1% [v/v] Triton X-100, and 1% [v/v] dimethyl sulfoxide) at 37°C overnight, fixed in a formaldehyde-acetic acid solution (10% [v/v] formalin, 5% [v/v] acetic acid, 45% [v/v] ethanol, and 0.01% [v/v] Triton X-100) for 45 min, and then rehydrated using a graded ethanol series. Embryos were then cleared in Hoyer's solution (chloral hydrate:water:glycerol, 3:0.8:0.4) and observed with a light microscope (Olympus SZ 61). The cell size of embryos was measured using NIH Image software (http://rsb.info.nih.gov/nih-image/index.html).

Measurement of Seed FAs

The seeds for FA measurement were harvested from the lower part of the main stem of 16 individual plants grown in different pots arranged randomly

within one of three blocks. Seed FAs were extracted and analyzed as reported previously in detail (Poirier et al., 1999; Chen et al., 2012). Briefly, total FAs were converted to FA methyl esters in methanol solution containing 1 M HCl for 2 h at 80°C. FAs in seeds were subsequently measured using a gas chromatograph (GC-2014; Shimadzu).

Plasmid Construction

The plasmids *35S:MYB89-6HA* and *35S:MYB89-GR* were constructed by amplifying the full-length coding region of MYB89 and cloning it into pGreen-35S-6HA (Liu et al., 2008) and pGreen-35S-GR (Yu et al., 2004), respectively. To construct *35S:RNAi-MYB89*, the amplified antisense and sense fragments were digested with restriction enzymes into the pGreen-HY104 vector (Yu et al., 2004) before and after the GUS fragment, respectively. The GUS reporter construct *pMYB89:GUS* was constructed by amplifying its 5′ regulatory region upstream of the ATG start codon and cloning it into pHY107 (Liu et al., 2007). The primers used for plasmid construction are listed in Supplemental Table S6.

RNA-Seq and Data Analyses

The flowers of wild-type (Col-0) and *myb89-1* plants were tagged with different colored threads to indicate DAP. Only developing seeds from the siliques on the primary shoots of 80 individual plants for each genotype in one biological replicate, which were grown in different pots arranged randomly, were used for the RNA-seq experiment. Two independent biological replicates from two different plantings were conducted for the wild type and *myb89-1* in the RNA-seq experiment. The following analysis was performed using the services of Gene Denovo (http://www.genedenovo.com/) following the standard protocol (http://www.genedenovo.com/product/41.html). The Excel add-in for significance analysis of RNA-seq was used to identify DEGs between the wild type and *myb89-1*. The DEGs were functionally classified using the biological process category of Arabidopsis Gene Ontology (http://www.geneontology.com). The DEGs with $\log_2$ ratios greater than 1 or less than -1 (only GO Slim identifiers with $P \leq 0.05$ and false discovery rate ≤ 0.05) are listed in Supplemental Tables S3 and S4.

Expression Analysis

Total RNA from siliques or developing seeds was extracted using the MiniBEST Plant RNA Extraction Kit (TaKaRa) and reverse transcribed using PrimerScript RT (TaKaRa). qRT-PCR was performed for three biological replicates using SYBR Green Master Mix (TaKaRa). The siliques or developing seeds were from at least 12 individual plants grown in different pots arranged randomly, and three independent biological replicates from three different plantings were used for the expression analysis. qRT-PCR is the preferred method for targeted gene expression measurements because of its sensitivity and reproducibility. However, normalization using the internal control, which is necessary to correct for sample input and reverse transcriptase efficiency, is a crucial step to obtain reliable qRT-PCR results. *EF1aA4* was regarded as the internal control in this study. However, the ubiquitously expressed genes *AP2M* (AT5G46630) and *MON1* (AT2G28390) during seed development (Czechowski et al., 2005; Dekkers et al., 2012) might be more appropriate internal controls for gene expression measurements in Arabidopsis developing seeds. GUS staining were performed as described previously (Jefferson et al., 1987). In situ hybridization experiments were performed as described elsewhere (Yu et al., 2002). GR induction and RNA analysis were performed as reported previously (Xu et al., 2014). Primers used for in situ hybridization and qRT-PCR analyses are listed in Supplemental Tables S6 and S7, respectively.

ChIP Assay

The ChIP assay was performed as reported previously (Chen et al., 2015). Siliques were fixed on ice in a buffer (10 mM potassium phosphate [pH 7], 50 mM NaCl, and 0.1 M Suc) with 1% (v/v) formaldehyde under vacuum for 1 h. Chromatin was extracted and sonicated to produce DNA fragments of sizes about 250 bp. MYB89-6HA was immunoprecipitated using an anti-HA agarose conjugate (Sigma-Aldrich). The relative enrichment of each fragment was determined by qRT-PCR, and ChIP assays were repeated using three biological replicates. The siliques for the three independent biological replicates were from at least 50 individual plants grown in different pots arranged randomly and not a resample from the same 50 plants. Primer pairs used in the ChIP assays are listed in Supplemental Table S8.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: MYB89 (AT5G39700), WRI1 (AT3G54320), LEC1 (AT1G21970), LEC2 (AT1G28300), L1L (AT5G47670), FUS3 (AT3G26790), ABI3 (AT3G24650), CYFBP (AT1G43670), SUS2 (AT5G49190), FBA2 (AT4G38970), ENO1 (AT1G74030), PI-PKβ1 (AT5G52920), BCCP1 (AT5G16390), BCCP2 (AT5G15530), CAC2 (AT5G35360), CAC3 (AT2G38040), PDH E1α (AT1G01090), ACP1 (AT3G05020), KASI (AT5G46290), KASII (AT1G74960), KASIII (AT1G62640), KAR (AT1G24360), HD-L (AT2G22230), MOD1 (AT2G05990), SAD (AT3G02630), SAD (AT5G16240), LAS (AT5G08415), KCS11 (AT2G26640), KCS17 (AT4G34510), FAD2 (AT3G12120), FAD3 (AT2G29980), FAE1 (AT4G34520), SSI2 (AT2G43710), CDS2 (AT4G22340), PIS2 (AT4G38570), BIO2 (AT2G43360), LPAT1 (AT4G30580), LPAT2 (AT3G57650), LPAT3 (AT1G51260), LPAT4 (AT1G75020), LPAT5 (AT3G18850), LPP1 (AT2G01180), LPP2 (AT1G15080), LPP3 (AT3G02600), ROD1 (AT3G15820), PLA2α (AT2G06925), DGAT1 (AT2G19450), DGAT2 (AT3G51520), PDAT1 (AT5G13640), PDAT2 (AT3G44830), CCT1 (AT2G32260), and CCT2 (AT4G15130).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Representative GUS staining of *pMYB89:GUS* transgenic plants shows MYB89 expression in true leaves and a root tip of a 15-d-old seedling.

Supplemental Figure S2. Comparison of plant size between wild-type (Col-0) and *myb89-1* plants.

Supplemental Figure S3. Comparison of wild-type (Col-0) and *myb89-1* young seedlings under stressed environments.

Supplemental Figure S4. Effect of *MYB89* knockdown in the wild-type (Col-0) background and *MYB89* overexpression in the *myb89-1* mutant background on seed size and seed weight.

Supplemental Figure S5. Effect of altering *MYB89* expression on seed number, seed yield, and oil yield.

Supplemental Figure S6. qRT-PCR analysis of the expression of genes involved in FA biosynthesis and TAG deposition in developing seeds of wild-type (Col-0) and *myb89-1* plants.

Supplemental Figure S7. Confirmation of the biologically active MYB89-GR fusion.

Supplemental Figure S8. The genes involved in glycolysis, FA biosynthesis and modification, and TAG deposition in developing seeds are not immediate targets of transcriptional regulation by MYB89 in developing seeds.

Supplemental Figure S9. qRT-PCR analysis of the expression of six *BPM* genes in developing seeds of wild-type (Col-0) and *myb89-1* plants.

Supplemental Table S1. Quantitative comparison of the cell size of epidermal cell layers of the cotyledons from wild-type (Col-0) and *myb89-1* mature embryos.

Supplemental Table S2. Seed major FA compositions of the mutants and transgenic plants in this study.

Supplemental Table S3. List of up-regulated genes in developing seeds of *myb89-1* plants at 12 DAP.

Supplemental Table S4. List of down-regulated genes in developing seeds of *myb89-1* plants at 12 DAP.

Supplemental Table S5. List of TFs and genes encoding key enzymes for oil biosynthesis whose expression was not altered in developing seeds of *myb89-1* plants at 12 DAP.

Supplemental Table S6. Primers used for various constructs, in situ hybridization, and genotyping of SALK mutants in this study.

Supplemental Table S7. Primers used for qRT-PCR analysis in this study.

Supplemental Table S8. Primers used for ChIP analysis in this study.

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