TRANSPARENT TESTA GLABRA1 Regulates the Accumulation of Seed Storage Reserves in Arabidopsis

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Seed storage reserves mainly consist of starch, triacylglycerols, and storage proteins. They not only provide energy for seed germination and seedling establishment, but also supply essential dietary nutrients for human beings and animals. So far, the regulatory networks that govern the accumulation of seed storage reserves in plants are still largely unknown. Here, we show that TRANSPARENT TESTA GLABRA1 (TTG1), which encodes a WD40 repeat transcription factor involved in many aspects of plant development, plays an important role in mediating the accumulation of seed storage reserves in Arabidopsis (Arabidopsis thaliana). The dry weight of ttg1-1 embryos significantly increases compared with that of wild-type embryos, which is accompanied by an increase in the contents of starch, total protein, and fatty acids in ttg1-1 seeds. FUSCA3 (FUS3), a master regulator of seed maturation, binds directly to the TTG1 genomic region and suppresses TTG1 expression in developing seeds. TTG1 negatively regulates the accumulation of seed storage proteins partially through transcriptional repression of 2S3, a gene encoding a 2S albumin precursor. TTG1 also indirectly suppresses the expression of genes involved in either seed development or synthesis/modification of fatty acids in developing seeds. In addition, we demonstrate that the maternal allele of the TTG1 gene suppresses the accumulation of storage proteins and fatty acids in seeds. Our results suggest that TTG1 is a direct target of FUS3 in the framework of the regulatory hierarchy controlling seed filling and regulates the accumulation of seed storage proteins and fatty acids during the seed maturation process.

Plant seeds contain abundant storage reserves, which not only provide energy for seed germination and subsequent seedling establishment, but also form an integral part of human and animal diet. These storage compounds usually consist of starch, oil stored as triacylglycerols (TAGs) and storage proteins, and their relative proportions vary among different plant species. Accumulation of these compounds in Arabidopsis (Arabidopsis thaliana) seeds is mediated during the generation of three distinct tissues, including embryo, endosperm, and seed coat, in two major stages of seed development, namely embryo morphogenesis and seed maturation (Jenik et al., 2007; Baud and Lepiniec, 2009). During embryo morphogenesis, double fertilization results in a fertilized embryo sac that subsequently develops into the zygote (2n) and endosperm (3n). The zygote divides asymmetrically to give rise to the embryo and its suspensor, with the former progressively acquiring the basic plan of the plant after a series of cell divisions (Baud and Lepiniec, 2009; Boscá et al., 2011), while the development of the triploid endosperm occurs through a coenocytic stage followed by a cellularized and differentiated stage (Olsen, 2001; Berger et al., 2006). Embryo morphogenesis ends until the formation of a torpedo-shaped embryo with a clear polarity along an apical-basal axis at approximately 6 d after pollination. At the end of this stage, the endosperm usually occupies more than one-half of the total seed volume (Baud et al., 2002).

During seed maturation from 7 to 20 d after pollination, there is an accumulation of seed storage reserves and an acquisition of dormancy and desiccation tolerance (Goldberg et al., 1994; Fait et al., 2006). At the early maturation stage from 7 to 10 d after pollination, the embryo expands into a fully developed embryo that fills almost the entire seed space, whereas the endosperm is degraded to one cell layer surrounding the embryo. This rapid increase in embryo volume is accompanied by elevated starch concentrations and...
initiation of the synthesis and accumulation of TAGs and storage proteins (Hills, 2004; Berger et al., 2006). Both storage proteins and the components of TAGs, fatty acids, increase in seeds steadily at the midmaturation stage from 10 to 16 d after pollination and reach the maximal levels approximately at the end of the seed maturation stage (Baud et al., 2002; Baud and Lepiniec, 2009). In mature seeds, TAGs and storage proteins account for approximately 30% to 40% of the seed dry weight each, whereas only trace amounts of starch are detectable (Focks and Benning, 1998; Baud et al., 2002).

The Arabidopsis seed coat is derived from the integuments of an epidermal origin that surround a mature ovule and consists of five cell layers, including the innermost endothelial layer enclosed by inner and outer integuments, each containing two cell layers (Lepiniec et al., 2006). The outer cell layer of the outer integument secretes mucilage, which mainly consists of a group of acidic polysaccharides and forms a gelatin-like coating covering the mature seed (Windsor et al., 2000). The seed coat protects the embryo against adverse external conditions and provides the means of balancing seed dormancy and germination in response to environmental signals.

Previous studies have shown that TRANSPARENT TESTA GLABRA1 (TTG1), which encodes a WD40 repeat transcription factor, plays multiple roles in embryogenesis and postembryonic development in Arabidopsis (Koornneef, 1981; Shirley et al., 1995; Walker et al., 1999; Debeaujon et al., 2000; Western et al., 2001; Tsuchiya et al., 2004). Consistently, TTG1 is expressed in all plant tissues examined, particularly in cauline leaves, seed coats, and embryos. TTG1 acts postembryonically as an epidermal morphogenesis regulator that controls the formation of hairs on leaves, stems, and roots. During embryogenesis, TTG1 affects synthesis of anthocyanin pigments and seed coat mucilage as well as seed dormancy. TTG1 expression during embryogenesis is negatively affected by a B3-domain family transcription factor FUSCA3 (FUS3) that is a master regulator of seed maturation and regulates a wide range of seed-specific characters (Keith et al., 1994; Luerssen et al., 1998; Tsuchiya et al., 2004). In addition, loss of function of TTG1 partially suppresses a number of phenotypes of fus3 mutants, such as defects in the accumulation of anthocyanin and seed storage proteins (Tsuchiya et al., 2004). These results suggest that FUS3 regulates embryogenesis at least partially through TTG1.

In this study, we show that TTG1 negatively regulates the accumulation of seed storage reserves, including storage proteins and fatty acids during embryogenesis. We present evidence that 2S3, which encodes a precursor of a predominant class of seed storage proteins, is an early target of transcriptional repression by TTG1. TTG1 also indirectly suppresses the expression of a group of genes that either act as master regulators of seed development or are involved in synthesis or modification of fatty acids during seed development. We also demonstrate that FUS3 is associated with the TTG1 genomic region and directly suppresses TTG1 expression in developing seeds. Our findings establish TTG1 as an important regulator that modulates the accumulation of seed storage reserves, particularly storage proteins and fatty acids, downstream of FUS3 during the seed maturation processes.

RESULTS

**ttg1 Mutants Exhibit Increased Accumulation of Seed Storage Compounds**

To further understand the function of TTG1 in seed development, we performed a detailed analysis on seed characters of a previously reported loss-of-function mutant *ttg1-1* in the Landsberg erecta (Ler) background (Koornneef, 1981; Walker et al., 1999). The *ttg1-1* mutation results from a single base change from C to T in the TTG1 coding region, which generates a termination code in place of the code for Gln (Q)-317, resulting in C-terminal truncation of 25 amino acids (Fig. 1A; Walker et al., 1999). In agreement with previous reports (Koornneef, 1981; Walker et al., 1999), *ttg1-1* seeds exhibited the transparent testa phenotype because of reduced anthocyanin synthesis in seed coats (Fig. 1B).

There was no obvious difference in several seed morphological traits, such as embryo size (Fig. 1C), embryo cell size (Fig. 1C; Supplemental Table S1), seed size (Fig. 1D), and seed dry weight (Fig. 1D), in mature seeds of *ttg1-1* and wild-type (Ler) plants. However, the dry weight of *ttg1-1* embryos significantly increased compared with that of wild-type embryos (Fig. 1D), implying that TTG1 may affect the accumulation of certain storage compounds in embryos. In developing wild-type seeds, TTG1 expression remained stable at the embryo morphogenesis stage and the early seed maturation stage from 4 to 10 d after pollination but progressively increased afterward at the mid-seed maturation stage, until it reached the maximal level at 14 d after pollination (Fig. 1E). This expression pattern also indicates that dynamic regulation of TTG1 could be relevant to the accumulation of seed storage reserves, which mainly occurs at the seed maturation stage (Fait et al., 2006; Baud et al., 2008).

To test whether TTG1 plays a direct role in the accumulation of seed storage compounds, we measured the contents of starch, total protein, and fatty acids in developing seeds (6 and 16 d after pollination) and mature seeds (22 d after pollination) of *ttg1-1* and wild-type (Ler) plants. Developing seeds of *ttg1-1* accumulated more starch than those of the wild type at 6 d after pollination, which was roughly at the end of the embryo morphogenesis stage (Fig. 2A). The starch content dramatically decreased during the later phase of seed development in both wild-type and *ttg1-1* seeds (Fig. 2A).

By contrast, both the contents of total protein and fatty acids in wild-type and *ttg1-1* seeds exhibited a reversely increasing trend, with the highest levels in mature seeds at 22 d after pollination (Fig. 2, B and C). Notably, the levels of total protein and fatty acids were significantly higher in *ttg1-1* than in wild-type seeds almost at all
stages of seed development examined (Fig. 2, B and C). These results demonstrate that TTG1 negatively affects the accumulation of seed storage compounds.

TTG1 Expression Is Directly Regulated by FUS3 in Developing Seeds

It has been shown that TTG1 expression during embryogenesis is negatively affected by FUS3 and that loss of function of TTG1 partially suppresses the defect in the accumulation of seed storage proteins of fus3 mutants (Tsuchiya et al., 2004). These results, together with our observation of the negative effect of TTG1 on the levels of seed storage compounds, prompted us to investigate whether FUS3 directly regulates TTG1 expression in developing seeds.

We first created a steroid-inducible version of FUS3 in the background of fus3-3 (Keith et al., 1994), in which the FUS3 gene was fused to the hormone-binding domain of a rat glucocorticoid receptor (GR) under the control of a Cauliflower mosaic virus 35S promoter. Induced FUS3 activity by dexamethasone treatment of the siliques from one selected fus3-3/ + 35S:FUS3-GR line partially rescued the purple-red embryo phenotype of fus3-3, which was mainly due to the anthocyanin accumulation in cotyledons (Supplemental Fig. S1, A and B; Keith et al., 1994). This indicates that the FUS3-GR fusion protein expressed in this transgenic line is biologically functional in a hormone-dependent manner. By using this line, we found that treatment with dexamethasone of fus3-3 35S:FUS3-GR siliques at 12 d after pollination for 2 or 4 h resulted in a reduction of TTG1 RNA levels relative to mock-treated controls (Fig. 3A). A combined treatment of dexamethasone and cycloheximide, an efficient inhibitor of protein synthesis, for 2 or 4 h resulted in a similar reduction of TTG1 RNA levels as observed with dexamethasone treatment alone (Fig. 3A), indicating that TTG1 may be an immediate target of transcriptional repression by FUS3 in developing seeds.

To examine whether FUS3 directly binds to the TTG1 genomic region to control its expression, we performed
chloroplasts to the cell wall (Zrenner and Schopfer, 2005). The presence of a functional ER stress response is crucial for the folding of membrane proteins and the transport of proteins into the ER lumen (Hansen and Wallick, 2003). The ER stress response is therefore important for the efficient secretion of proteins, as well as for the assembly of protein complexes such as those found in the ER lumen. The ER stress response is also regulated by the unfolded protein response (UPR), which is activated in response to an accumulation of unfolded or misfolded proteins in the ER (Hansen and Wallick, 2003). The UPR can be activated by the accumulation of unfolded proteins in the ER, which leads to the activation of the transcription factor ATF6 (Abeliovich et al., 2001). The UPR is also activated by the accumulation of misfolded proteins in the ER, which leads to the activation of the transcription factor XBP1 (Abeliovich et al., 2001). The activation of the UPR leads to the induction of genes that are involved in the folding and transport of proteins, as well as the degradation of misfolded proteins (Hansen and Wallick, 2003). The UPR is therefore important for the efficient processing of proteins and the prevention of protein aggregation in the ER. The ER stress response is also important for the efficient secretion of proteins, as well as for the assembly of protein complexes such as those found in the ER lumen.
has similar biological function as the wild-type TTG upon steroid induction.

Using this established steroid-inducible activation of TTG1, we further examined whether the expression of 2S2, 2S3, 2S4, and 2S5 is repressed by TTG1 activity. Dexamethasone treatment of ttg1-1 35S:TTG1-GR siliques at 12 d after pollination for 2 or 4 h caused a reduced expression of all these genes relative to mock-treated controls (Fig. 5C; Supplemental Fig. S3). However, a combined treatment of dexamethasone and cycloheximide for 2 or 4 h only significantly reduced the expression of 2S3 (Fig. 5C; Supplemental Fig. S3), suggesting that only 2S3 could be an immediate target of transcriptional repression by TTG1 in developing seeds, whereas repression of 2S2, 2S4, and 2S5 by TTG1 is dependent on other intermediate proteins.

To study how TTG1 affects the expression patterns of 2S3 in developing seeds, we transcriptionally fused a 1.752-kb 2S3 5'-upstream sequence to the GUS reporter gene and generated more than 20 p2S3:GUS-independent lines in the wild-type (Ler) background. All of these lines show similar GUS staining patterns during seed development, and one representative line was crossed with ttg1-1. Intense GUS staining was observed in the whole embryos of developing wild-type seeds at 12 and 14 d after pollination, and the levels of p2S3:GUS activity, rather than the expression domain, significantly increased in the ttg1-1 background (Fig. 5, D and E). These results are consistent with those from gene expression analysis (Fig. 4), further substantiating that TTG1 suppresses the expression of 2S3 in developing seeds.

Figure 3. TTG1 is directly suppressed by FUS3 during seed development. A, Induced FUS3 activity transcriptionally represses TTG1 expression during seed development. fus3-3 35S:FUS3-GR siliques at 12 d after pollination were mock treated (Mock) or treated with 10 μM dexamethasone (Dex), 5 μM cycloheximide (Cyc), or 5 μM cycloheximide plus 10 μM dexamethasone (Cyc+Dex). TTG1 expression was examined after 2 or 4 h of treatment by quantitative real-time PCR analyses of three independently collected replicates. Results were normalized against the expression of EF1α4. Asterisks indicate significant differences in TTG1 expression in dexamethasone-treated samples compared with their respective controls (two-tailed paired Student’s t test, P < 0.05). Error bars denote so of triplicate assays. B, Western analysis of FUS3-6HA in nuclear extracts or immunoprecipitated fractions of wild-type or fus3-3 35S:FUS3-6HA siliques at 12 d after pollination. Nuclear extracts served as the input (I), while immunoprecipitated fractions by anti-HA antibody were used as the eluate (E). Immunoblot analysis was performed using anti-HA antibody. C, Schematic diagram of the TTG1 genomic region. The coding region and other genomic regions are represented by black and white boxes, respectively. Arrowheads indicate the putative binding sites (CATG [CA/TG]) of FUS3. Four DNA fragments (P1–P4) spanning these sites were examined by ChIP enrichment test as shown in D. D, ChIP analysis of FUS3-6HA binding to the TTG1 genomic region. To detect FUS3-6HA binding during seed development, ChIP analysis was performed using the input and eluate described in B. Similar ChIP analysis was also performed on rosette leaves. A TUB2 fragment was amplified as a negative control. Asterisks indicate significant differences in the enrichment fold at P3 compared with that of other DNA fragments tested (two-tailed paired Student’s t test, P < 0.05). Error bars denote so of triplicate assays. E, List of the putative FUS3-binding element (native probe) in the TTG1 promoter and its mutated version (mutated probe). These probes are part of the 60-bp TTG1 sequences used for EMSA assays shown in F. F, EMSA assay shows the binding of GST-FUS3 to the conserved CATGTG motif in the TTG1 promoter. GST-FUS3 protein was incubated with biotinylated native or mutated probe. Nonlyabeled native or mutated probes in 100-fold molar excess relative to the biotinylated native probe were used as cold competitors. A supershift band was observed when anti-GST antibody was added. Col-0, Ecotype Columbia.
TTG1 Indirectly Regulates Genes Involved in Seed Development and Accumulation of Fatty Acids in Developing Seeds

To further understand how TTG1 affects seed development and accumulation of fatty acids in seeds, we compared the dynamic changes in the expression of three groups of genes involved in either seed development or synthesis/modification of fatty acids in developing wild-type and ttg1-1 seeds (Fig. 6; Supplemental Fig. S4). The genes in the first group are important transcription factors regulating different aspects of the seed mature process, including LEAFY COTYLEDON1 (LEC1), LEC2, FUS3, ABSCISIC ACID3 (ABI3), and WRINKLED1 (WRI1). The second group includes the genes encoding various enzymes involved in the synthesis of fatty acids, such as BIOTIN CARBOXYL CARRIER PROTEIN ISOFORM2 (BCCP2), ACETYL COENZYME A CARBOXYLASE CARBOXYLTRANSFERASE subunit (CAC2), MOSAIC DEATH1 (MOD1), CYTIDINE-DIPHOSPHATE DIAC- YLGLYCEROL SYNTHASE2 (CDS2), β-KETOACYL-ACYL CARRIER PROTEIN SYNTHETASE2, FATTY ACID BIOSYNTHESIS2, and ACETYL COENZYME A CARBOXYLASE CARBOXYLTRANSFERASE α SUBUNIT. The third group of genes, including those encoding FATTY ACID ELONGATION1 (FAE1), FATTY ACID DESATURASE2 (FAD2), and FAD3, participates in the modification of fatty acids. While the expression of six of these genes was not greatly changed in ttg1-1-developing seeds compared with that in wild-type plants (Supplemental Fig. S4), the expression of the other nine genes increased significantly at least 1-fold in ttg1-1-developing seeds at certain day(s) after pollination (Fig. 6). Notably, the maximal difference in the expression of these nine genes in wild-type and ttg1-1 seeds usually appeared at the midmaturation stage from 10 d after pollination (Fig. 6), during which storage proteins and fatty acids increased steadily in seeds (Baud et al., 2002; Baud and Lepiniec, 2009). These results show that TTG1 also negatively regulates the expression of both major regulators of seed development and the genes encoding the enzymes involved in the synthesis or modification of fatty acids.

To test whether these genes are directly regulated by TTG1, we further examined the expression of these genes upon induction of TTG1 activity using the established steroid-inducible ttg1-1 35S:TTG1-GR system. Although dexamethasone treatment of ttg1-1 35S:TTG1-GR siliques at 12 d after pollination for 4 h decreased the expression of all these genes relative to mock-treated controls to different extents, there were no significant differences in the expression of these genes by a combined treatment of dexamethasone and cycloheximide for 4 h relative to cycloheximide-treated controls (Supplemental Fig. S5). These results indicate that TTG1 could indirectly regulate these genes through other unknown intermediates.

The Maternal Allele of ttg1-1 Affects Accumulation of Storage Proteins and Fatty Acids in Seeds

To understand whether TTG1 acts maternally or zygotically in regulating the accumulation of seed storage reserves, we performed reciprocal crosses between wild type and ttg1-1 plants (Fig. 7A) and examined the contents of fatty acids and seed storage proteins in different F1 mature seeds. The levels of fatty acids and seed storage proteins, particularly 2S albumins, were consistently higher in H3 and H4 F1 seeds from the crosses using ttg1-1 as the female parent than in H1 and H2 F1 seeds from the crosses using wild-type plants as the female parent (Fig. 7, A–C). By contrast, the changes in the genotypes of the pollen donors, such as H1 versus H2, did not affect the levels of fatty acids and seed storage proteins in F1 seeds (Fig. 7, B and C). These results suggest that the maternal allele of the TTG1 gene suppresses the accumulation of storage proteins and fatty acids in seeds.

DISCUSSION

TTG1 encodes a WD40 repeat transcription factor that has been shown to influence many developmental...
processes in Arabidopsis, including synthesis of anthocyanin pigments and seed coat mucilage and seed dormancy during embryogenesis (Koornneef, 1981; Shirley et al., 1995; Walker et al., 1999; Debeaujon et al., 2000; Western et al., 2001; Tsuchiya et al., 2004). In this study, we have presented several pieces of evidence that suggest an important role of TTG1 in mediating the accumulation of seed storage proteins and fatty acids during the seed maturation process (Fig. 8).

First, the levels of total protein and fatty acids are consistently higher in ttg1-1 than in wild-type seeds during the seed maturation process (Fig. 2, B and C), which is characterized by a dramatic accumulation of seed storage reserves, including storage proteins and fatty acids (Baud et al., 2008). This result, together with the observation of an increased expression of TTG1 in developing seeds at the mid-seed maturation stage (Fig. 1E), suggests that TTG1 is an important player in the regulatory network that suppresses the accumulation of seed storage reserves. Second, as the embryo fully expands to fill almost the entire seed space at the early seed maturation stage, most of the seed storage proteins and fatty acids are accumulated in the embryo of a mature Arabidopsis seed. Consistent with a negative effect of TTG1 on the accumulation of seed storage reserves, the dry weight of ttg1-1 embryos is much heavier than that of wild-type ones (Fig. 1D). Third, four out of the five genes encoding 2S precursors are

Figure 5. 2S3 is an immediate target of transcriptional repression by TTG1 in developing seeds. A, A biologically active TTG1-GR fusion. Individual seedlings of ttg1-1 35S:TTG1-GR plants were mock-treated (Mock) or treated with 10 μM dexamethasone (Dex) every other day after germination. Mock-treated plants generate leaves without trichomes as ttg1-1, whereas dexamethasone treatment rescues this phenotype. Bottom sections show enlarged views of the leaves shown on the top sections. B, Induced TTG1 activity rescues the seed phenotypes of ttg1-1. ttg1-1 35S:TTG1-GR siliques at 4 d after pollination were mock-treated or treated with 10 μM dexamethasone every other day until 20 d after pollination. The seeds from mock-treated plants exhibit the same transparent testa phenotype as ttg1-1 seeds, whereas dexamethasone-treated plants produce normal-looking seeds. C, Induced TTG1 activity transcriptionally represses 2S3 expression during seed development. ttg1-1 35S:TTG1-GR siliques at 12 d after pollination were mock treated or treated with 10 μM dexamethasone, 5 μM cycloheximide (Cyc), or 5 μM cycloheximide plus 10 μM dexamethasone. 2S3 expression was examined after 2 or 4 h of treatment by quantitative real-time PCR analyses of three independently collected replicates. Results were normalized against the expression of EF1aA4. Asterisks indicate significant differences in 2S3 expression in dexamethasone-treated samples compared with their respective controls (two-tailed paired Student’s test, P < 0.05). Error bars denote SD of triplicate assays. D, GUS expression in seeds of p2S3:GUS and ttg1-1 p2S3:GUS at 12 and 14 d after pollination. E, Quantitative analysis of GUS activity in seeds of p2S3:GUS and ttg1-1 p2S3:GUS at 12 and 14 d after pollination. GUS activity values were measured from at least 120 seeds for each genotype. MU, 4-Methyl umbelliferone. Asterisks indicate significant differences in GUS activity between two types of seeds. Error bars denote SD.
greatly up-regulated in ttg1-1 seeds at the midmaturation stage (Fig. 4). In particular, induced TTG1 activity immediately suppresses 2S3 mRNA expression independently of protein synthesis (Fig. 5C), indicating that 2S3 could be a direct target of transcriptional repression by TTG1 in developing seeds. Consistently, we have found that accumulation of seed storage proteins, including 2S albumins, is relatively low in F1 seeds from the crosses using ttg1-1 as the female parent (Fig. 7C). Last, the expression of seven genes involved in synthesis or modification of fatty acids is up-regulated by at least 1-fold in ttg1-1-developing seeds at certain time point(s) during the midmaturation stage (Fig. 6), although TTG1 regulation of these genes may be mediated by other intermediate proteins (Supplemental Fig. S5). Taken together, all these results support that TTG1 serves as an important regulator that negatively controls the expression of the genes contributing to the accumulation of seed storage proteins and fatty acids during the seed maturation process.

Figure 6. Comparison of the expression of genes involved in synthesis or modification of fatty acids or seed development in developing seeds of wild-type (Ler) and ttg1-1 plants by quantitative real-time PCR analysis. Results were normalized against the expression levels of EF1aA4 as an internal control. Asterisks indicate significant differences in gene expression levels in ttg1-1 compared with those in wild-type plants (two-tailed paired Student’s t test, P < 0.05). Error bars denote sd.

Figure 7. The maternal allele of ttg1-1 affects the accumulation of storage proteins and fatty acids in seeds. A, Genotypes of embryos, endosperms, and seed coats of the F1 seeds from different hybrid combinations (H1–H4). B, Comparison of fatty acid levels in mature F1 seeds from different hybrid combinations (H1–H4). Data represent mean ± sd of three independent biological repeats. C, Comparison of storage protein levels in mature F1 seeds from different hybrid combinations (H1–H4). The same volume of protein extracts from each genotype was loaded onto the 15% SDS polyacrylamide gel and stained. The locations of the major storage proteins, including 12S globulins and 2S albumins, are indicated on the left. DW, Dry weight.
Previous studies have shown that TTG1 expression in the embryo is negatively affected by FUS3, which is a master regulator of seed maturation (Keith et al., 1994; Luerssen et al., 1998; Tsuchiya et al., 2004). Loss of function of TTG1 partially suppresses a number of phenotypes of fus3 mutants, such as ectopic trichome production, desiccation intolerance, and defects in the accumulation of anthocyanin and seed storage proteins (Tsuchiya et al., 2004). We have revealed that induced FUS3 activity could immediately repress TTG1 expression independently of protein synthesis and that the functional FUS3-6HA fusion protein binds directly to the TTG1 genomic region in developing seeds (Fig. 3, A–D). In addition, GST-FUS3 binds in vitro to the conserved CATGTG motif in the TTG1 promoter. These observations indicate that FUS3 directly controls TTG1 expression to regulate the accumulation of seed storage reserves during the seed maturation process.

Regulation of TTG1 by FUS3 could serve as an important link that mediates the feedback regulation of the complex regulatory network governing the accumulation of seed storage proteins. Four transcription factors, namely FUS3, LEC1, LEC2, and ABI3, act as master regulators of seed maturation and regulate the expression of many other players in the metabolic networks leading to the synthesis of storage compounds, such as storage proteins and fatty acids (To et al., 2006; Baud et al., 2008). FUS3, LEC2, and ABI3 are similar transcription factors of the B3-domain family and have been shown to directly induce the expression of storage protein genes (Ezcurra et al., 1999; Reidt et al., 2000; Kroj et al., 2003; Braybrook et al., 2006; Wang and Perry, 2013). FUS3 directly suppresses TTG1 expression, which could further promote the expression of at least four out of the five genes encoding 25 precursors that are otherwise suppressed by TTG1 (Figs. 4 and 8). This incorporates another level of regulation governed by FUS3 on the expression of storage protein genes in developing seeds. It is noteworthy that the expression of LEC2 and ABI3 in developing seeds is also greatly affected by TTG1 (Fig. 6). Because both LEC2 and ABI3 promote FUS3 expression (Kroj et al., 2003; To et al., 2006), a negative feedback effect of TTG1 on LEC2 and ABI3 could play a role in mediating the expression levels of FUS3 in seeds, thus controlling an appropriate pace of accumulation of seed storage proteins required for postgerminative seedling growth. Therefore, in contrast to most of the other positive regulators in the model for the control of storage protein accumulation (Baud et al., 2008), TTG1 serves as a unique negative regulator that not only directly suppresses the expression of storage protein genes, but also restricts the expression of other master regulators of storage protein accumulation in maturing Arabidopsis seeds (Fig. 8).

The underlying mechanisms by which the master regulators of seed maturation and TTG1 control the accumulation of fatty acids still remain largely elusive. Among those known master regulators, LEC2 acts as a key player that regulates the accumulation of fatty acids in two different manners. While it directly induces the expression of oleosin genes, it also indirectly regulates many other genes, such as BCCP2 and MOD1, encoding enzymes required for the synthesis of fatty acids through WRI1, a secondary transcription factor of the APETALA2-ethylene-responsive element-binding protein family (Santos Mendoza et al., 2005; Braybrook et al., 2006; Baud et al., 2007). Interestingly, although up-regulation of LEC2 in ttg1-1 seeds is expectedly associated with up-regulation of other genes involved in fatty acid synthesis (BCCP2, CAC2, CDS2, and MOD1; Fig. 6), the expression of WRI1 is almost not altered in ttg1-1 (Supplemental Fig. S4), demonstrating that the role of TTG1 in suppressing these genes could be mostly independent of WRI1. These observations imply that in addition to WRI1, other redundant factor(s) exist to mediate the effect of TTG1 and LEC2 on fatty acid synthesis.

Notably, two genes, BCCP2 and CAC2, encoding the subunits that contribute to the assembly of a biologically active acetyl-CoA carboxylase complex are prominently suppressed by TTG1 in developing seeds (Fig. 6). Acetyl-CoA carboxylase catalyzes the ATP-dependent formation of malonyl-CoA from the precursor acetyl-CoA, which is the first step of de novo fatty acid biosynthesis (Konishi et al., 1996; Thelen and Ohlrogge, 2002). Thus, suppression of BCCP2 and CAC2 by TTG1 allows TTG1 to influence the overall flux of fatty acids in seeds at the very early stage of the biosynthetic pathway. Furthermore, TTG1 also greatly affects the expression of the genes encoding enzymes involved in modification of fatty acids, such as FAE1, FAD2, and FAD3 (Fig. 6). FAE1 is a condensing enzyme that contributes to the generation of long-chain fatty acids through elongation of the fatty acid carbon chain from C18 to C20 and C22 (James and Dooner, 1990; Lemieux et al., 1990), while FAD2 and FAD3 are desaturases that are responsible for the generation of desaturated fatty acid species (Okuley et al., 1994; Shah et al., 1997). Thus, TTG1 seems to participate in multiple
regulatory events that mediate both fatty acid levels and profiles in seeds (Fig. 8).

Comparison of the contents of fatty acids and seed storage proteins in different F1 mature seeds from reciprocal crosses between wild-type and ttg1-1 plants (Fig. 7) has demonstrated a maternal effect of TTG1 in suppressing the accumulation of fatty acids and storage proteins in seeds. In addition to its effect in the embryo, TTG1 has been found to promote the production of anthocyanin pigments and seed coat mucilage (Koornneef, 1981; Walker et al., 1999), both of which could confer a maternal effect on the accumulation of seed storage reserves, such as fatty acids, in embryos. Anthocyanins belong to a parent class of flavonoids, which could permeate from the seed coat to the adhering embryo, thus inhibiting the accumulation of fatty acids in the embryos through repressing the expression of the genes encoding important reductases involved in the elongation of the fatty acid carbon chain (Zhang and Rock, 2004; Wang et al., 2014). Furthermore, biosynthesis of fatty acids and seed coat mucilage shares the same carbon source, thus competing sinks for photosynthetic in seeds. Thus, a role of TTG1 in mediating the production of anthocyanin pigments and mucilage in the seed coat could also contribute to its maternal effect on an overall level of fatty acids in seeds.

CONCLUSION

Accumulation of seed storage reserves is regulated by intricate regulatory networks that also coordinate the growth of various seed tissues of different origins during the seed maturation processes. Our results demonstrate that TTG1 plays an important role in mediating the accumulation of seed storage reserves, including storage proteins and fatty acids, during seed development. TTG1 acts as a unique negative regulator that suppresses the expression of the genes encoding both the precursors of storage proteins and other master regulators of storage protein accumulation in maturing seeds. It also participates in multiple regulatory events in maternal and embryonic tissues that mediate fatty acid levels and profiles in seeds. Therefore, the interaction between TTG1 and other master regulators of seed maturation, such as FUS3, can engage TTG1 as an important negative regulator that ensures to maintain an appropriate rate of accumulation of seed storage proteins required for subsequent seed germination and subsequent seedling establishment.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The various genotypes of Arabidopsis (Arabidopsis thaliana) plants were grown under long-day (16-h-light/8-h-dark) conditions at 22°C. ttg1-1 and fus3-3 are in the Ler and Columbia background, respectively. All transgenic plants were generated through Agrobacterium tumefaciens-mediated transformation. Transformants harboring various constructs were selected by Basta on soil.

Plasmid Construction

To construct 35S:FUS3-6HA, the FUS3 complementary DNA (cDNA) was amplified with primers FUS3-Xho1-F and FUS3-Spe1-R1. The PCR products were digested by XhoI and SpeI and cloned into pGreen-35S-6HA (Li et al., 2008) to obtain an in-frame fusion of FUS3-6HA under the control of the 35S promoter. To construct 35S:FTTG3-GR, the FUS3 cDNA was amplified with primers FUS3-Xho1-F and FUS3-ClaI-R2. The resulting PCR products were digested by XhoI and ClaI and cloned into pGreen-35S-GR (Yu et al., 2004) to obtain an in-frame fusion of FUS3-GR driven by the 35S promoter. Similarly, the TTG1 cDNA was amplified with primers TTG1-Xho1-F and TTG1-ClaI-R. After enzyme digestion, the PCR products were cloned into pGreen-35S-GR to obtain 35S:TTG1-GR. To construct pZS3:GUS, a 1.72-kb 5′ upstream sequence was amplified with pZ5S-Hind3-F and pZ5S-XmaI-R and cloned into pHY107 (Liu et al., 2007). The primers used for plasmid construction are listed in Supplemental Table S2.

Expression Analysis

Total RNA from siliques (Figs. 3A and SC) or developing seeds (other expression studies) was extracted using the RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using the SuperScript reverse-transcription-PCR system (Invitrogen). Quantitative real-time PCR was performed in triplicate, each on three independently collected samples using the CFX96 Real-Time PCR Detection System (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). The ubiquitously expressed Arabidopsis EF1a4 gene was used as an internal control. Primers used for quantitative real-time PCR analysis are listed in Supplemental Table S3. GUS staining and quantitative analysis of GUS activity were carried out as previously described (Jefferson et al., 1987).

Light Microscopy

Observation of embryos was carried out as described previously (Ohto et al., 2005; Chen et al., 2014). Mature dry seeds were imbibed in 1 h in tap water and dissected under the microscope to isolate mature embryos. Embryos were incubated in a buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 1% [v/v] Triton X-100, and 1% [v/v] dimethyl sulfoxide) at 37°C overnight, fixed with the 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 series of ethanol (50%, 70%, 80%, 90%, 95%, and 100% [v/v]) for approximately 5 min, respectively. Embryos were then treated for 1 h in Hoyer’s solution (chloral hydrate:water:glycerol [3:0.8:0.4]). The cleared embryos were observed using a light microscope (Olympus S2 61). Cell size of embryos was measured using NIH IMAGE analysis software (http://rsb.info.nih.gov/nih-image/index.html).

Measurement of Fatty Acid, Starch, and Protein

Total fatty acids were converted into fatty acid methyl esters in the methanol solution containing 1 M HCl for 2 h at 80°C. Measurement of fatty acids in seeds was subsequently performed using the gas chromatograph (GC-2014, Shimadzu) as previously described (Poirier et al., 1999; Chen et al., 2012a). Starch and protein in seeds were measured as described before (Chen et al., 2012b).

Storage Protein Analysis by SDS-PAGE Analysis

Analysis of seed storage proteins was conducted as previously reported (Chen et al., 2014). Briefly, 1 mg of mature dry seeds was homogenized with 50 µL of extraction buffer (100 mM Tris-HCl, pH 8.0, 0.5% [v/v] SDS, 10% [v/v] glycerol, and 20% [v/v] 2-mercaptoethanol) using a microglass pestle and mortar. After boiling for 5 min, the extract was centrifuged and the supernatant was used for SDS-PAGE analysis.

ChIP Assay

ChIP assay was performed as previously described (Shen et al., 2011). Siliques or leaves were fixed on ice in the buffer (10 mM potassium phosphate, pH 7.0, 50 mM NaCl, and 0.1 mM Suc) with 1% (v/v) formaldehyde under vacuum for 45 min. The fixed materials were homogenized in liquid nitrogen. The chromatin was extracted and sonicated to produce DNA fragments between 200 and 500 bp. The solubilized chromatin was incubated with anti-HA agarose
were repeated with three biological replicates. Nondenatured fragment of DNA was recovered using the QIAquick PCR purification kit (Qagen). A genomic fragment of TUBULIN2 (TUB2) was amplified as a control. ChIP assays were repeated with three biological replicates.

EMSA

EMSA assays were performed using LightShift Chemiluminescent EMSA Kit (Pierce). The full-length FLU53 coding sequence was cloned to pGEX-4T-1, which was used to produce the GST-FUS3 recombinant protein. Biotin-labeled or unlabeled single-strand DNA oligos were annealed to produce the probes or competitors, respectively.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Microscopic observation of mature seeds of Arabidopsis ecotype Columbia plants in various genetic backgrounds.

Supplemental Figure S2. Quantitative real-time PCR analysis of the expression of the genes that encode various precursors of seed storage proteins in developing seeds of wild-type (Lev) and ttg1-t plants.

Supplemental Figure S3. 252, 254, and 255 are not immediate targets of transcriptional repression by TTG1 in developing seeds.

Supplemental Figure S4. Comparison of the expression of genes involved in seed development or synthesis of fatty acids in developing seeds of wild-type (Lev) and ttg1-t plants by quantitative real-time PCR analysis.

Supplemental Figure S5. The genes involved in synthesis or modification of fatty acids or seed development are not immediate targets of transcriptional repression by TTG1 in developing seeds.

Supplemental Table S1. Quantitative comparison of cell size of epidermal cell layers of the cotyledons from wild-type (Lev) and ttg1-t mature embryos.

Supplemental Table S2. Primers used for plasmid construction.

Supplemental Table S3. Primers used for quantitative real-time PCR analysis.

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