

# The Effect of *TRANSPARENT TESTA2* on Seed Fatty Acid Biosynthesis and Tolerance to Environmental Stresses during Young Seedling Establishment in *Arabidopsis*<sup>1</sup>[W][OA]

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In plants, fatty acids (FAs) and FA-derived complex lipids are major carbon and energy reserves in seeds. They are essential components of cellular membranes and cellular signal or hormone molecules. Although *TRANSPARENT TESTA2* (*TT2*) is well studied for its function in regulating proanthocyanidin biosynthesis in the seed coat, little attention has been given to its role in affecting seed FA accumulation and tolerance to environmental stresses. We demonstrate that the *tt2* mutation remarkably increased the seed FA content, decreased seed weight, and altered the FA composition. The increase in FA content in the *tt2* seeds was due to the relative decrease of seed coat proportion as well as the more efficient FA synthesis in the *tt2* embryo. Microarray analysis revealed that *tt2* mutation up-regulated a group of genes critical to FA biosynthesis and embryonic development. The mutation also altered the gene expressions that respond to stress. The microarray analysis discovered that the increase in FA accumulation of the *tt2* seeds were accompanied by the significant up-regulation of *FUSCA3*, a transcriptional factor for embryonic development and *FATTY ACID ELONGASE1*, which catalyzes the elongation of FA chains. Moreover, lower seed protein accumulation during seed maturation also contributed to the increased seed FA accumulation in *tt2* mutants. This study advances the understanding of the *TT2* gene in seed FA accumulation and abiotic stresses during seed germination and seedling establishment.

In *Arabidopsis* (*Arabidopsis thaliana*), mature seeds consist of three major components, namely, the embryo, endosperm, and seed coat. Fatty acids (FAs) and FA-derived complex lipids are among the major energy reserves predominantly stored in the seed embryo, which develops into the vegetative plant. FAs and FA-derived lipids facilitate successful seed germination and seedling establishment (Li et al., 2006). Seed development can be divided into two main steps, embryonic morphogenesis (EM) and maturation (Baud and Lepiniec, 2009). EM is initiated by double fertilization within anatropous bitegmic ovules. Through a series of programmed cell divisions, the embryo acquires the basic architecture of the plant (Mayer and

Jürgens, 1998; Jenik et al., 2007). At 6 d after pollination (DAP), the embryo of *Arabidopsis* becomes torpedo shaped, and EM is accomplished. The maturation phase from 7 to 20 DAP is characterized by the accumulation of storage compounds, such as seed FA and proteins, and the acquisition of dormancy and desiccation tolerance (Goldberg et al., 1994; Baud et al., 2002; Fait et al., 2006). Finally, during the last few days of seed maturation, storage compound synthesis ends and the embryo becomes metabolically quiescent. The accumulation of seed FA sharply increases between 7 and 17 DAP with a maximum level of 18 DAP. A slight decrease in seed lipid content has been observed in several oilseed species, including *Arabidopsis*, at the very end of the maturation process (Turnham and Northcote, 1983; Eastmond and Rawsthorne, 2000; Baud et al., 2002). During the embryonic maturation phase of *Arabidopsis*, the endosperm is almost degraded and reduced to one cell layer surrounding the embryo (Baud and Lepiniec, 2009). As a tissue system, the endosperm serves as nutrition for the developing embryo and/or germinating seedling (Stone et al., 2008). Around 10% of the FAs present in whole seeds are accumulated in the endosperm (Penfield et al., 2004). In contrast, the seed coat that surrounds the embryo and the endosperm is derived from ovular tissue and is therefore strictly of maternal origin. The mature seed coat of *Arabidopsis* is composed of four

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cell layers (from outside to inside the seed): An epidermis and a palisade layer composes the outer integument, whereas a crushed layer and an endothelial layer form the inner integument (Debeaujon et al., 2000). The seed coat plays an important role in embryonic nutrition during seed development and in protecting the embryo against detrimental agents from the environment (Mohamedyasseen et al., 1994; Weber et al., 1996; Debeaujon et al., 2000).

Seed coats are consisted of some important compounds like flavonoids, which are unique to higher plants as secondary metabolites involved in physiological functions, such as dormancy or viability. Flavonoids also serve as beneficial micronutrients in human and animal diets. Three major classes of flavonoids, namely, anthocyanins (red to purple pigments), flavonols (colorless to pale yellow pigments), and proanthocyanidins (colorless pigments that turn brown), also known as condensed tannins, are present in Arabidopsis. They give the red, purple, and brown pigmentation to flowers, fruits, and seeds (Koes et al., 1994; Shirley, 1996; Mol et al., 1998; Nesi et al., 2001; Lepiniec et al., 2006). Proanthocyanidins specifically accumulate in the innermost cell layer of the seed coat, called the endothelium (Devic et al., 1999; Nesi et al., 2001). The defect in flavonoid pigmentation results in mutants such as the *transparent testa* (*tt*) and the *tt glabra* (Shirley et al., 1995), different from another kind of seed coat mutations with abnormal testa structure, such as the *glabra2* mutant (Rerie et al., 1994). Coordinated growth and the development of the seed coat, endosperm, and embryo is essential for the reserve accumulation of FAs and proanthocyanidins in mature seeds. Although the mechanisms of FA and

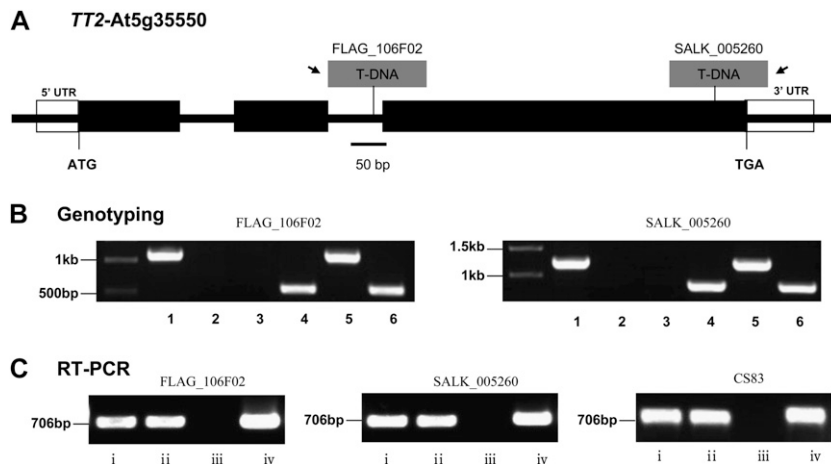
proanthocyanidins biosynthesis have been extensively studied, little attention has been given to their relationship in determining the final seed storage compounds.

To understand this relationship, the seed FA content and composition of *tt2* mutant plants were investigated. *TT2* features an R2R3 MYB domain protein located mostly in the nucleus, which is consistent with its regulatory function. It is a major limiting regulator in the proanthocyanidin regulatory network and predominantly expressed during early seed development. The loss of *TT2* function specifically affects seed pigmentation and dramatically reduces the expression of several structural genes involved in tannin metabolism. Gain-of-function experiments have demonstrated that *TT2* induces ectopic *BAN* expression, which is regarded as the first enzyme committed to proanthocyanidin biosynthesis (Nesi et al., 2001). Despite knowledge regarding the function of *TT2* in proanthocyanidin biosynthesis, the role of *TT2* in affecting seed FA storage has been poorly understood. In this research, previously uncharacterized functions of *TT2*, i.e. the negative effect on seed FA accumulation and the positive effect on stress tolerance during seed germination and seedling establishment were identified.

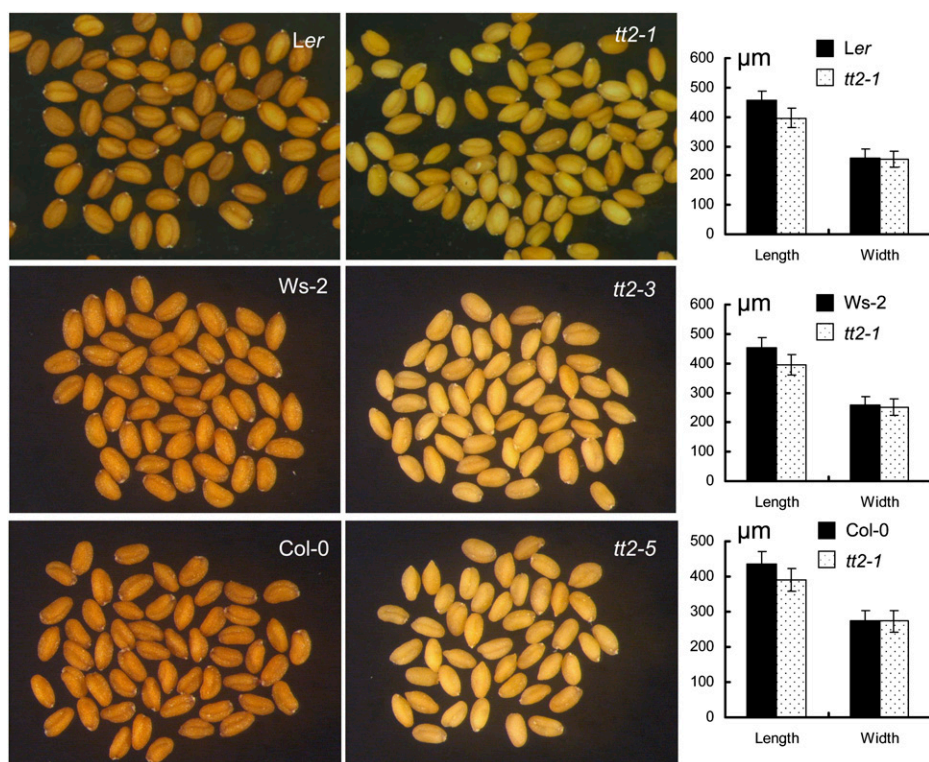
## RESULTS

### Increase in FA Content and Alteration of FA Composition in Mature *tt2* Seeds

Two mutant lines of the *TT2* gene, CS83 (*tt2-1*; Nesi et al., 2001) and SALK\_005260 (*tt2-5*), a newly



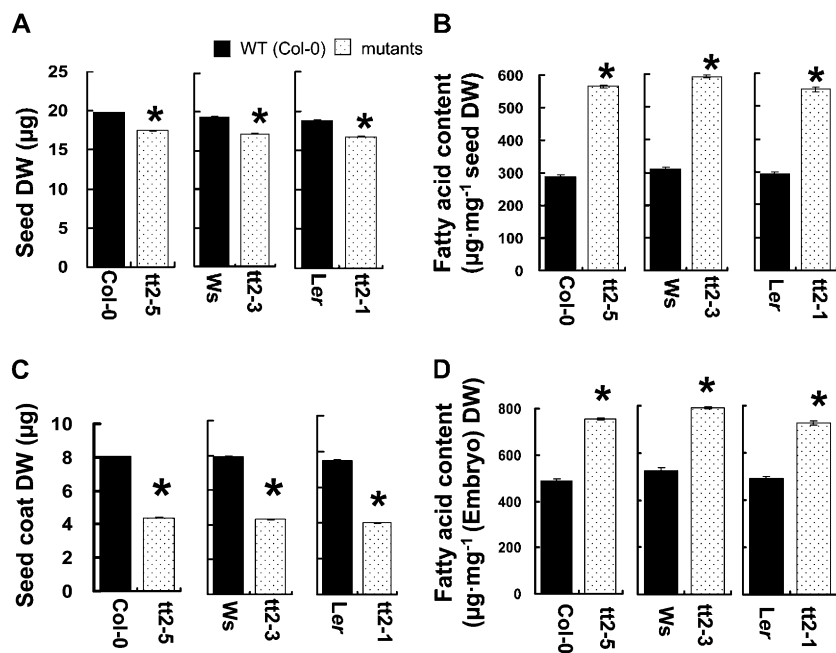
**Figure 1.** Identification of *tt2* mutant lines using the primers given in Supplemental Tables S1 and S2. A, Molecular characterization of the *tt2* mutation. Structure of the *TT2* gene showing the position of the T-DNA insertions in FLAG\_106F02 (*tt2-3*) and SALK\_005260 (*tt2-5*) mutants. Black boxes represent exons while open boxes stand for untranslated regions (UTRs). The arrows indicate the left border on T-DNA. B, PCR-based genotyping of the SALK mutants. 1, 3, and 5 stand for bands of LP and RP primers for genotyping wild types and homozygous and heterozygous SALK mutants, respectively; 2, 4, and 6 stand for bands of RP and BP primers for genotyping wild types and homozygous and heterozygous SALK mutants, respectively; LP and RP refer to the *TT2* gene-specific primers and BP refer to T-DNA right-border primer given in Supplemental Table S1. C, RT-PCR was used to measure the *TT2* gene expression in the SALK mutants. i and ii represent bands of *EF1α4* for homozygous SALK mutants and the wild type, respectively; iii and iv represent bands of primers specific for the *TT2* gene for homozygous SALK mutants and the wild type, respectively.



**Figure 2.** Microscopic observation of *tt2* mature seeds. Quantitative comparisons of the length and width of a seed between *tt2* mutant lines and their respective wild-type controls are shown in a graph neighboring each pair of the wild type and mutant.

nomenclature allele in this article, were provided by the Arabidopsis Biological Resources Center in the United States. Another mutant line, FLAG\_106F02 (*tt2-3*; Nesi et al., 2001), was obtained from the National Institute for Agricultural Research, France. Figure 1A shows the structure of the *TT2* gene and the respective insertion sites of the two allelic mutants. To investigate

the effects of the *tt2* mutations on seed FA accumulation, the mutant lines (*tt2-3* and *tt2-5*) were backcrossed with their respective wild-type parents in case other mutations were present in the mutant lines. Genotyping by PCR (Fig. 1B) indicated the presence of the homozygous *tt2-3* and *tt2-5* mutants, which completely lack the transcription of *TT2* gene, as detected



**Figure 3.** Comparison between the *tt2* mutant lines and their respective wild types. A, Seed DW. B, FA content. C, Seed coat DW (including endosperm). D, FA of embryo. Data presented are mean values of three independent experiments (three biological repeats)  $\pm$  SE. The asterisks (\*) indicate significant difference ( $P \leq 0.05$ ) compared with the wild type.

by reverse transcriptase (RT)-PCR (Fig. 1C). On the other hand, the mutant *tt2-1* was created by x-ray irradiation and was difficult to genotype by PCR. Thus, the yellow seed coat trait (Fig. 2) was used as an indicator for genotyping and the mutant plants were further confirmed by RT-PCR for null *TT2* transcripts (Fig. 1C). The *tt2* mutant seeds are smaller in size than their respective wild-type controls (Fig. 2). In terms of dry weight (DW), the mutant seeds upon maturation were approximately 12% lighter than their corresponding wild-type seeds (Fig. 3A). In contrast, the mutant embryos were approximately 11% to 13% heavier than those of the respective wild types (Fig. 3, A and C).

The seed FA content and FA composition in mature *tt2* seeds were analyzed using gas chromatography. As shown in Table I and Figure 3B, the *tt2* seeds demonstrated 86% to 95% higher FA content than the wild-type seeds. The degree of increase depended on the allelic variations at the *TT2* locus (Fig. 3B). Regardless of the mutated allele, the increase in FA content of the mutant seeds was attributed to the increased proportion of the embryo and the increase in FAs in the embryos (Fig. 3, C and D). Moreover, the increase in FA content was accompanied by the alteration of FA composition. In the mature mutant seeds, the proportions of 16-C and 18-C FAs, with an exception of 18:1, decreased significantly, whereas the proportions of 20-C, 22-C, and 24-C FAs (very-long-chain FAs [VLCFAs];  $C \geq 20$ ) increased significantly (Table I). Subcellular structure of the *tt2* mutant lines (*tt2-3*, *tt2-5*, and *tt2-1*) was presented in comparison with that of their respective wild-type controls (Fig. 4). The average number of oil bodies that were contained in a cell as well as the average lengths of long and short axis of an oil body were also shown. It is obvious that the *tt2* cells had much greater number and larger sizes of oil bodies contained.

These results demonstrate that the *tt2* mutation led to defective proanthocyanidin biosynthesis in the seed coat, significant increases in the embryo weight and FA content, and alternation of the FA composition, and changes in oil body number and size within a mature seed cell.

#### Differentially Expressed Genes between the *tt2-5* and the Wild Type in Developing Seeds at 6 and 16 DAP

We elaborately selected two critical developing stages for comparison of expressional profiles between a *tt2* mutant (*tt2-5*) and its wild-type control (Columbia-0 [Col-0]). At 6 DAP, EM is completed and seed FA accumulation starts (Baud and Lepiniec, 2009), whereas, at 16 DAP, large differences in seed FA content and FA composition between the mutant and the wild type were identified. As shown in Table II, the total FA content in the *tt2-5* seeds was 420.99  $\mu\text{g}/\text{mg}$ , significantly higher than 252.45  $\mu\text{g}/\text{mg}$  in the wild-type seeds. The FA content of developing seeds at 16

**Table I.** Comparison of FA composition and total FA content at mature seed stage between *tt2* mutant lines and their respective wild types

	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:0	Sum
Col-0	27.78 ± 0.80	8.04 ± 0.27	26.13 ± 0.52	107.51 ± 2.44	77.62 ± 1.26	3.85 ± 0.09	34.87 ± 0.41	0.71 ± 0.05	2.96 ± 0.32	0.45 ± 0.05	289.92 ± 5.66
<i>tt2-5</i>	48.92 ± 0.92	14.69 ± 0.46	60.97 ± 0.76	201.79 ± 3.17	135.24 ± 2.18	9.68 ± 0.11	83.97 ± 0.68	1.54 ± 0.23	7.31 ± 0.11	1.13 ± 0.05	565.24 ± 4.29
Ws-2	28.09 ± 1.12	9.96 ± 0.61	26.59 ± 0.98	113.26 ± 2.27	82.50 ± 1.85	5.13 ± 0.12	38.39 ± 1.08	0.78 ± 0.07	3.59 ± 0.15	0.76 ± 0.07	309.05 ± 6.99
<i>tt2-3</i>	50.24 ± 0.64	17.21 ± 0.11	59.41 ± 0.55	206.21 ± 1.15	142.30 ± 2.66	12.56 ± 0.13	91.24 ± 1.42	1.92 ± 0.12	9.91 ± 0.31	1.89 ± 0.11	592.89 ± 4.19
Ler	26.81 ± 0.39	9.79 ± 0.39	27.22 ± 1.29	108.91 ± 1.32	77.91 ± 1.18	4.67 ± 0.11	36.13 ± 0.85	0.75 ± 0.15	3.01 ± 0.28	0.55 ± 0.05	295.75 ± 5.27
<i>tt2-1</i>	46.06 ± 1.09	16.44 ± 0.58	63.85 ± 1.42	180.26 ± 2.22	138.96 ± 1.47	10.88 ± 0.17	84.87 ± 1.32	1.77 ± 0.05	6.98 ± 0.12	1.29 ± 0.05	551.36 ± 6.63
<b>Col-0</b>	<b>9.58 ± 0.79</b>	<b>2.77 ± 0.31</b>	<b>9.01 ± 0.33</b>	<b>37.08 ± 3.27</b>	<b>26.77 ± 3.68</b>	<b>1.33 ± 0.13</b>	<b>12.03 ± 0.56</b>	<b>0.24 ± 0.07</b>	<b>1.02 ± 0.31</b>	<b>0.16 ± 0.03</b>	
<i>tt2-5</i>	<b>8.65 ± 0.86</b>	<b>2.60 ± 0.47</b>	<b>10.79 ± 0.39</b>	<b>35.70 ± 4.12</b>	<b>23.93 ± 4.15</b>	<b>1.71 ± 0.15</b>	<b>14.86 ± 0.75</b>	<b>0.27 ± 0.03</b>	<b>1.29 ± 0.23</b>	<b>0.20 ± 0.05</b>	
Ws-2	9.09 ± 1.23	3.22 ± 0.53	8.60 ± 0.52	36.65 ± 3.15	26.69 ± 3.95	1.66 ± 0.17	12.42 ± 1.23	0.25 ± 0.06	1.16 ± 0.18	0.25 ± 0.07	
<i>tt2-3</i>	8.47 ± 0.86	2.9 ± 0.15	10.02 ± 0.31	34.78 ± 2.07	24.00 ± 4.62	2.12 ± 0.21	15.39 ± 1.56	0.32 ± 0.08	1.67 ± 0.19	0.32 ± 0.09	
Ler	9.07 ± 0.42	3.31 ± 0.38	9.20 ± 0.67	36.83 ± 2.98	26.34 ± 3.17	1.58 ± 0.15	12.22 ± 0.98	0.25 ± 0.05	1.02 ± 0.26	0.19 ± 0.03	
<i>tt2-1</i>	8.35 ± 1.13	2.98 ± 0.51	11.58 ± 0.73	32.69 ± 3.01	25.20 ± 3.02	1.97 ± 0.31	15.39 ± 1.63	0.32 ± 0.07	1.27 ± 0.35	0.23 ± 0.06	

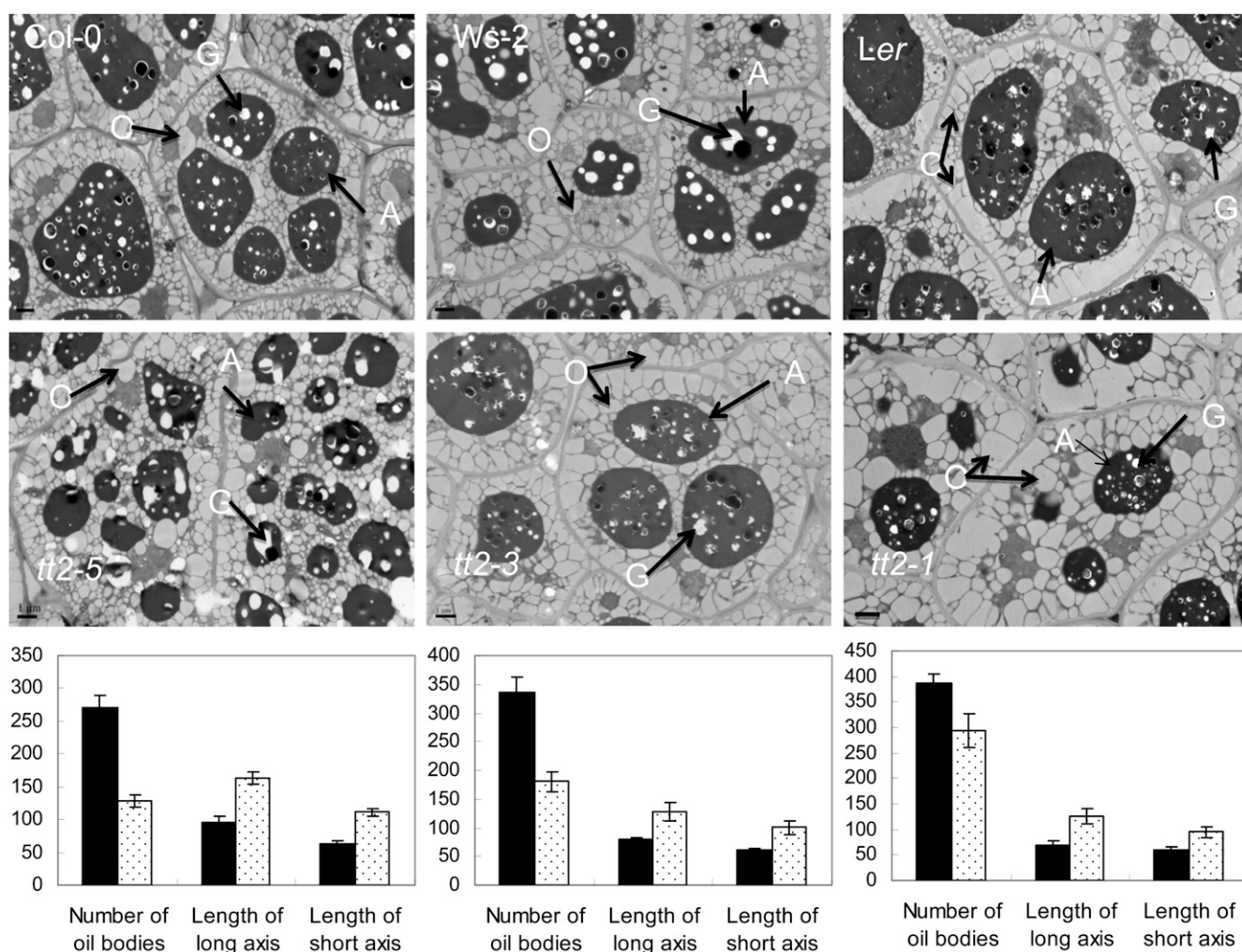
Values are expressed in  $\mu\text{g mg}^{-1}$  seed DW (in roman) and mol/% (in boldface). Data reported are mean values of five independent experiments (five biological repeats)  $\pm$  st. Arrows indicate the significant differences ( $P \leq 0.05$ ).

DAP accounted for more than 75% of the final FA content in mature seeds in both genotypes (Tables I and II). The accumulation of seed FAs increased sharply during the late embryonic maturation stages from 16 to 18 DAP and reached the final seed FA content at the end of EM maturation, indicating an active involvement of the genes that contribute to seed FA accumulation. Therefore, molecular analysis of developing seeds at 6 and 16 DAP could provide clues to figure out the downstream targets of *TT2* involved in FA biosynthesis and understand the molecular mechanism underlying *TT2*-regulated seed FA accumulation.

Microarray analysis revealed that 503 differentially expressed genes (DEGs) were identified, among which 447 were up-regulated (Supplemental Table S3) and 56 were down-regulated in the *tt2* mutant (Supplemental Table S4) at the seed developmental stage 6 DAP.

Functional analysis of these 503 DEGs discovered that 51 (11.4%) and 59 (13.2%) of the up-regulated genes are related to carbohydrate metabolism and general protein synthesis, respectively. However, only 13 (2.9%) of the up-regulated genes are involved in lipid metabolism and embryogenesis. Many of the DEGs are related to stress/defense response (Table II; Supplemental Tables S3 and S4). Therefore, the mutation may promote seed starch and protein synthesis and alters the responses to environmental stresses during EM.

At 16 DAP, 746 DEGs were identified. Of these, 289 were up-regulated (Supplemental Table S5) and 457 were down-regulated (Supplemental Table S6) in the developing mutant seeds. Functional analysis unveiled that the expression of genes involved in lipid metabolism and embryogenesis was significantly altered in the *tt2-5* seeds. Relative to the 6 DAP stage, a greater



**Figure 4.** Comparison of the number and size of oil bodies in mature seed cells between the wild type and *tt2* mutant lines in pairs. Arrows in TEM images point to oil bodies (O), aleurone grains (A), and globoids (G). Bar = 1 μm. The quantitative data are shown in a graph under each pair of mutant and wild type. The number of oil bodies are mean value of 120 cells from six seeds. The length of long axis and short axis were calculated based on all oil bodies that were contained in 120 cells. The y axis of the graphs stands for numbers as well as 1% μm.

**Table II.** Comparison of FA composition and total FA content at 16 DAP between *tt2-5* and *Col-0*

Data reported are mean values of five independent experiments (five biological repeats) ± se. Values are expressed as  $\mu\text{g mg}^{-1}$  DW (in roman) and mol/% (in boldface).

Genotypes	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:0	Sum
<i>Col-0</i>	24.34 ± 0.59	6.66 ± 0.24	21.29 ± 0.83	92.05 ± 0.74	68.09 ± 1.51	3.34 ± 0.12	33.68 ± 0.36	0.28 ± 0.02	2.38 ± 0.12	0.35 ± 0.01	252.46 ± 3.26
<i>tt2-5</i>	36.37 ± 0.38	10.74 ± 0.21	48.40 ± 1.02	145.65 ± 1.48	104.29 ± 0.72	6.30 ± 0.20	63.34 ± 1.05	0.64 ± 0.03	4.63 ± 0.23	0.62 ± 0.01	420.98 ± 3.05
<b><i>Col-0</i></b>	<b>9.64 ± 0.35</b>	<b>2.64 ± 0.23</b>	<b>8.43 ± 0.53</b>	<b>36.46 ± 0.52</b>	<b>26.97 ± 0.65</b>	<b>1.32 ± 0.09</b>	<b>13.34 ± 0.27</b>	<b>0.11 ± 0.01</b>	<b>0.94 ± 0.09</b>	<b>0.14 ± 0.01</b>	
<b><i>tt2-5</i></b>	<b>8.64 ± 0.29</b>	<b>2.55 ± 0.19</b>	<b>11.50 ± 0.63</b>	<b>34.60 ± 1.09</b>	<b>24.77 ± 0.39</b>	<b>1.50 ± 0.13</b>	<b>15.05 ± 0.91</b>	<b>0.15 ± 0.02</b>	<b>1.10 ± 0.16</b>	<b>0.15 ± 0.01</b>	

number of DEGs were involved in lipid metabolism and embryogenesis at 16 DAP. More than 17% of the down-regulated genes, particularly the ribosomal protein genes, were involved in general protein synthesis (Supplemental Table S6). Up to 48 down-regulated genes (10.5%) and 24 up-regulated genes (8.4%) were related to carbohydrate metabolism. A greater number of DEGs involved in carbohydrate metabolism were identified in the *tt2-5* seeds at 16 DAP than at 6 DAP. The largest class of DEGs at 6 DAP and at 16 DAP between the mutant and the wild type was related to stress/defense response (Tables III and IV; Supplemental Tables S3–S6). Therefore, the *tt2-5* mutation may decrease the rate of protein and starch syntheses during embryonic maturation, and gave rise to a different response to environmental stresses.

According to Beisson et al. (2003) and Baud and Lepiniec (2009), 33 genes encode key enzymes or enzyme subunits that catalyze FA synthesis (FAS) in the plastids and endoplasmic reticulum during seed development. Most of these genes were slightly up-regulated in the *tt2-5* mutant at 6 and 16 DAP, with  $\log_2$  ratios lower than 1.00 (Supplemental Tables S7 and S8). Notably, *FATTY ACID ELONGASE1* (*FAE1*; or *KCS1*; At4g34520), which encodes condensing enzymes for VLCFA synthesis (James et al., 1995; Millar and Kunst, 1997) was significantly up-regulated in the *tt2-5* developing seeds at 16 DAP (Supplemental Tables S3 and S5). Only 2.9% of all up-regulated genes ( $\log_2$  ratios  $\geq 1.00$ ,  $P$  values  $\leq 0.5$ ) in the *tt2-5* seeds are related to lipid metabolism and embryonic development at 6 DAP (Table II). In contrast, the proportion of the up-regulated genes involved in lipid metabolism and embryonic development in the *tt2* seeds increased to 10.1% at 16 DAP ( $\log_2$  ratios  $\geq 1.00$ ,  $P$  values  $\leq 0.5$ ; Table IV).

FA biosynthesis is regulated by several key transcription factors (TFs), including *WRINKLED1* (*WR11*; At3g54320), *LEAFY COTYLEDON1* (*LEC1*; At1g21970), *LEC2* (At1g28300), *ABSCISIC ACID INSENSITIVE3* (*ABI3*; At3g24650), and *FUSCA3* (*FUS3*; At3g26790). No significant differences in TF expression between the *tt2-5* mutant and the wild type (*Col-0*) at 6 and 16 DAP were observed, except for *FUS3* expression, which was significantly increased at 16 DAP in *tt2-5* relative to the wild type (*Col-0*; Supplemental Tables S3–S8).

#### Confirmation of the Up-Regulated Genes for FA Synthesis and Embryonic Development at Different Developmental Stages in the *tt2* Seeds

To confirm the up-regulation of genes for FA biosynthesis and embryonic development in the *tt2-5* mutant, quantitative (q)RT-PCR was performed. As shown in Figure 5, the relative *FUS3* expression remained unchanged at 6 DAP, then increased dramatically from 6 to 16 DAP, and reached its peak at 10 DAP in the *tt2-5* mutant compared with the wild type. The expression of the genes that encode the critical FA biosynthetic enzymes, such as *BCCP2* (*At5g15530*),

**Table III.** Functional classification of DEGs in developing seeds of *tt2-5* plants at 6 DAP

Functional classification of the DEGs was performed using the biological process category of Arabidopsis Gene Ontology (<http://www.geneontology.com>). Percentage (far-right column) refers to the ratio of genes of each functional category relative to total up-regulated or down-regulated DEGs identified in the microarray experiment. The DEGs with log<sub>2</sub> ratios greater than 1.00 or less than -1.00 (only GO Slim IDs with *P* values equal to or less than 0.5) were listed.

Category	Up-Regulated DEGs				Down-regulated DEGs			
	≥2	1-2	Total	Percentage	≤-2	-2 to -1	Total	Percentage
	<i>Log<sub>2</sub> ratio</i>							
Metabolism								
Flavonoid metabolism	0	1	1	0.2	3	0	3	5.4
Lipid metabolism	1	6	7	1.6	0	2	2	3.6
Carbohydrate metabolism	15	36	51	11.4	1	5	6	10.7
Nucleic acid	0	5	5	1.1	0	1	1	1.8
Amino acid and protein	7	52	59	13.2	2	4	6	10.7
Growth and development								
Leaf and root development	0	1	1	0.2				
Embryo and seed development	1	5	6	1.3	1	0	1	1.8
Flower development	2	1	3	0.7				
Cell growth	2	14	16	3.6	1	1	2	3.6
Cytoskeleton structure					1	0	1	1.8
Hormone	4	25	29	6.5	2	1	3	5.4
Stress/defense response	27	123	150	33.6	5	13	18	32.1
Cell regulation								
Transcriptional regulation	11	37	48	10.7	1	1	2	3.6
Signaling transduction	5	19	24	5.4	1	7	8	14.3
Electron transport	0	6	6	1.3				
Transport facilitation	4	14	18	4.0				
Other	5	18	23	5.1	0	3	3	5.4

*CAC2* (*At5g35360*), *KASII* (*At1g74960*), *MOD1* (*At2g05990*), *FAB2* (*At2g43710*), *FatA* (*At3g25110*), *CDS2* (*At4g22340*), *PIS2* (*At4g38570*), *FAD2* (*At3g12120*), *FAD3* (*At2g29980*), and *FAE1* (*At4g34520*), which catalyze various steps in the FA biosynthetic pathway, was also investigated. As detailed in Figure 5, except for *CDS2* and *FAE1*, the expression of the 11 genes in the *tt2-5* mutant were up-regulated slightly at 16 DAP and peaked at the 10 DAP stage by about 2-fold that of the wild type (Col-0). The relative *CDS2* expression peaked at 6 DAP and decreased at 10 and 16 DAP. However, it was always higher than that of the wild type. The relative *FAE1* expression in the *tt2-5* mutant was less than 2-fold that of the wild type at 6 DAP, but it increased dramatically from 6 to 16 DAP and peaked at 16 DAP.

Taken together, the *tt2-5* mutation promotes the expression of *FUS3*, a TF for embryonic development, and the genes encoding critical enzymes catalyzing FA biosynthesis.

#### Dynamic Comparison of Seed Starch and Protein Accumulation between *tt2-5* and the Wild Type (Col-0)

Indicated by the microarray analysis, the loss of *TT2* function alters the expression of genes related to carbohydrate and protein biosynthesis (Tables II and III; Supplemental Tables S4–S7). The *tt2-5* and Col-0 were, therefore, compared at 6 DAP, 16 DAP, and the mature stage for the starch and protein contents. As shown in Figure 6A, the starch content of the wild type (Col-0;

205.86 μg/mg DW) and *tt2-5* (234.75 μg/mg DW) exhibited the highest levels in the developing seeds at 6 DAP. The mutant seeds contained significantly higher starch than the wild-type seeds. The starch content decreased markedly later during EM in both genotypes. In the seeds at 16 DAP and mature stage, only traces of starch could be detected in both the wild type and the mutant. Figure 6B shows the increase of seed total protein and storage protein contents from 6 DAP to the mature stage in the wild type and *tt2-5* mutant. The seed protein contents in the *tt2-5* seed were significantly higher than that of the wild type at 6 DAP (Fig. 6B), which is consistent with the finding that the up-regulated genes outnumbered the down-regulated genes for carbohydrate and protein metabolism in the *tt2-5* mutant (Tables III and IV; Supplemental Tables S3 and S4). The expression of the genes involved in protein metabolism significantly changed at 16 DAP and the number of up-regulated genes was much less than those of down-regulated genes (Tables III and IV; Supplemental Tables S5 and S6). This may give rise to the lower content of seed total and storage proteins in the *tt2-5* seeds at 16 DAP as well as at mature stage. This was helpful to the FA accumulation in *tt2-5* mutant line on the other hand.

#### Alteration of Responses to Stress in the *tt2* Mutant Lines

To determine the effect of the *tt2* mutation on stress responses, seed germination and seedling establishment

**Table IV.** Functional classification of DEGs in developing seeds of *tt2-5* plants at 16 DAP

Functional classification of the DEGs was performed using the biological process category of Arabidopsis Gene Ontology (<http://www.geneontology.com>). Percentage (far-right column) refers to the ratio of genes of each functional category relative to total up-regulated or down-regulated DEGs identified in the microarray experiment. The DEGs with log<sub>2</sub> ratios greater than 1.00 or less than -1.00 (only GO Slim IDs with *P* values equal to or less than 0.5) were listed.

Category	Up-regulated DEGs				Down-regulated DEGs			
	≥2	1-2	Total	Percentage	≤-2	-2 to -1	Total	Percentage
	<i>Log<sub>2</sub> ratio</i>							
Metabolism								
Flavonoid metabolism	0	4	4	1.3				
Lipid metabolism	2	21	23	8.0	1	6	7	1.5
Carbohydrate metabolism	0	24	24	8.4	9	39	48	10.5
Nucleic acid	0	7	7	2.4	4	28	32	7.0
Amino acid and protein	1	30	31	10.7	6	75	81	17.7
Growth and development								
Leaf and root development	0	1	1	0.3	0	9	9	2.0
Embryo and seed development	2	4	6	2.1	0	23	23	5.0
Flower development	0	6	6	2.1	1	11	12	2.6
Cell growth	1	19	20	6.9	2	18	20	4.4
Cytoskeleton structure	1	9	10	3.5	2	18	20	4.4
Hormone	0	11	11	3.8	4	13	17	3.7
Stress/defense response	5	66	71	24.6	13	65	78	17.1
Cell regulation								
Transcriptional regulation	0	2	2	0.7	2	40	42	9.2
Signaling transduction	0	15	15	5.2	1	27	28	6.1
Transport facilitation	0	39	39	13.5	1	12	13	2.8
Other	0	19	19	6.6	2	25	27	5.9

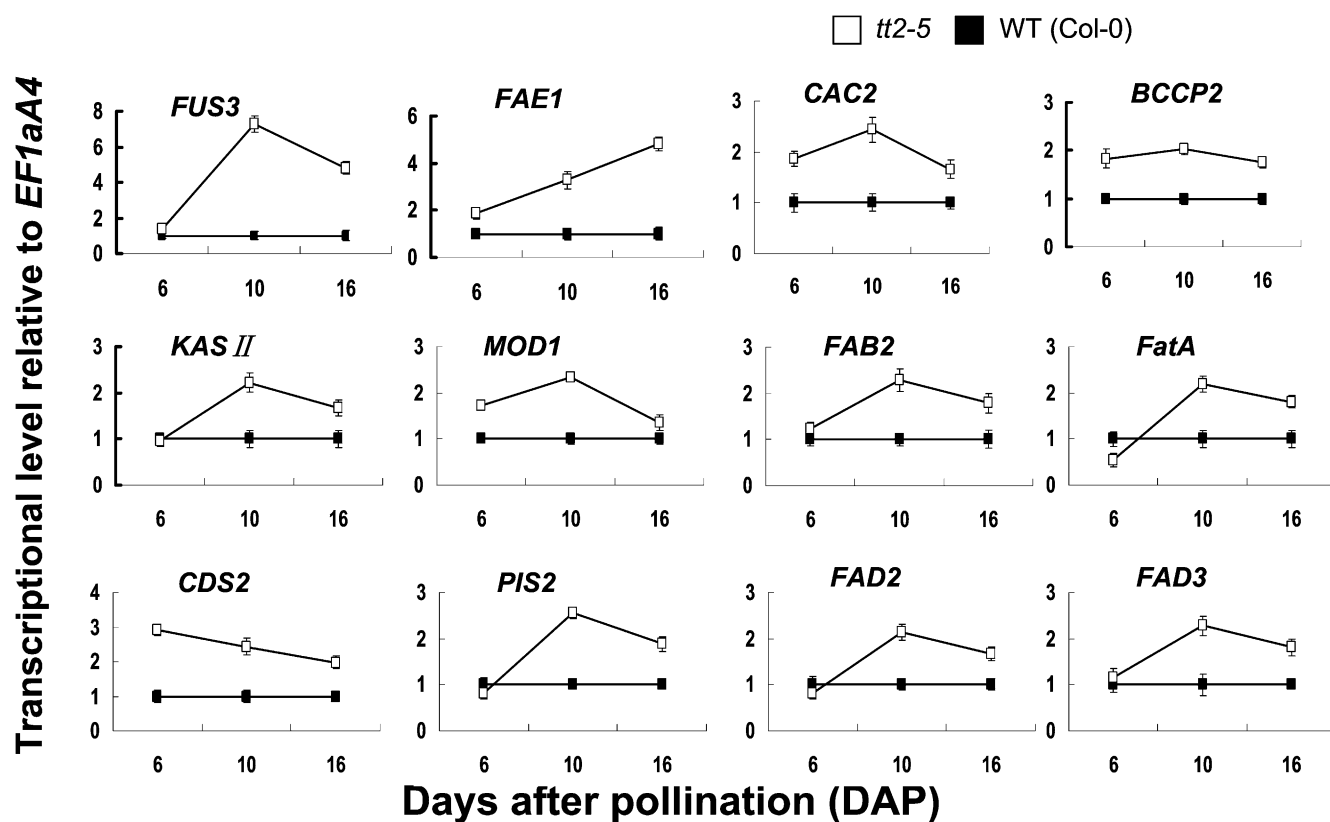
were investigated in medium containing 3% (w/v) Glc, 100 mM NaCl, or 1 μM abscisic acid (ABA). Seed germination rate and seedling growth on the medium without any stress treatment were similar between the *tt2* mutant lines and their respective wild-type controls. In the medium containing 3% (w/v) Glc, the rate of seed germination and vigor of the *tt2* mutant lines were significantly lower than that of their respective wild-type controls. Seven days after sowing, only about three-fourth of the *tt2* mutant seeds germinated. In contrast, more than 97% of the wild-type seeds germinated (Fig. 7). All wild-type plants reached the two-leaf stage 10 d after sowing (DAS) unlike the *tt2* plants that failed to develop normal cotyledons and green leaves in the medium (Fig. 8). Moreover, a comparison of the expression of genes related to osmotic stress and ABA between the wild type (Col-0) and the *tt2-5* plants through semiquantitative RT-PCR unveiled that Glc stress distinctly represses *NCED3* and induces the expression of *ABA3* and *CBL9* in the *tt2-5* mutant plants. In contrast, the expression of *ABA2*, *ABI1*, *ABI2*, *CBL1*, and *RD29A* in the *tt2-5* seedlings was similar to that in the wild-type plants (Fig. 9B). Under the 100 mM NaCl treatment, the seed germination vigor of the *tt2-5* plants 7 DAS was lower than that of the wild type (Fig. 7). The cotyledons and green leaves developed poorly in the *tt2* mutant lines, which indicates that the loss of *TT2* function induces sensitivity to salinity stress (Fig. 8). Semiquantitative RT-PCR analysis shows that *NCED3* is significantly down-regulated, whereas *ABA3* and *Rab18* were up-regulated in the *tt2-5* seedlings 10 DAS (Fig. 9C).

The *tt2-5* mutants were also sensitive to exogenous ABA (1 μM) during seed germination and seedling establishment. Compared with the wild type, the *tt* mutants exhibited low seed germination rates (Fig. 7) and badly developed seedlings in the Murashige and Skoog (MS) medium containing 1 μM ABA (Fig. 8). The expression of *ABI1*, *ABI2*, and *NCED3* was distinctly repressed. However, the expression of *CBL9*, *ADH*, and *Rab18* was obviously induced in the *tt2-5* mutant plants (Fig. 9D). The experiments with the *tt2-3* and *tt2-1* mutant lines obtained similar results (data not shown), which indicate that *TT2* may directly or indirectly confers tolerance to Glc, salinity, and ABA stresses during seed germination and seedling establishment.

## DISCUSSION

Factors that control the overall amount of FA stored in the seeds are still largely unknown. In this article, the novel function of the *TT2* in affecting seed FA accumulation and abiotic stress tolerance was demonstrated. *TT2*, which encodes a R2R3 MYB domain protein, is a key regulator of proanthocyanidin biosynthesis in the Arabidopsis seed coat (Nesi et al., 2001). We found that the *tt2* mutation resulted in considerably increased seed FA content (86%–95% depending on mutated allele; Table I; Fig. 3B), decreased seed size (Fig. 2), and seed weight (Fig. 3A), and altered FA composition (Table I). The increase in FA content in the *tt2* seeds accompanied by the relative decrease of seed coat proportion (Fig. 3C), more sufficient FAS, and reduced protein





**Figure 5.** Time-course analysis of the up-regulated genes for FAS and embryo development at different developing stages in *tt2* seeds. RNA samples were extracted from developing seeds at three different developmental stages, including 6, 10, and 16 DAP, and gene expression level was estimated by qRT-PCR using *EF1aA4* as the internal standard. Values are the means of two replicates carried out on cDNA dilutions obtained from two independent RNA extractions.

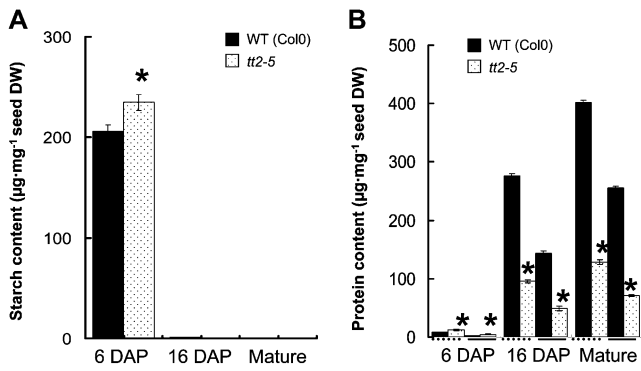
accumulation in the *tt2* embryo (Figs. 3D and 6). On the other hand, the *tt2* mutant lines demonstrated poor tolerance to stressful environments (Fig. 7–9). Microarray analysis unveiled that *TT2* regulates genes critical to lipid metabolism, embryonic development, and stress/defense responses (Tables III and IV; Supplemental Tables S3–S8).

#### Loss of *TT2* Function Results in More Sufficient FA Synthesis

In *Arabidopsis*, *TT2*, together with *TT GLABRA1* and *TT8*, synergistically control the expression of biosynthetic genes in the proanthocyanidin biosynthetic pathway, including *TT12*, *DFR*, *LDOX*, and *BAN* (Pelletier et al., 1997; Nesi et al., 2000, 2001; Debeaujon et al., 2003; Lepiniec et al., 2006). Consistent with previous studies, the *tt2* mutation significantly down-regulated the expression of *DFR*, *LDOX*, and *BAN* in the developing seeds at 6 DAP (Supplemental Table S5) and slightly down-regulated *TT12* (data not shown). The loss of *TT2* gene function up-regulated the *TT10* and the *RNS1* in the developing seeds at the 16 DAP stage (Supplemental Table S6). The reduction of proanthocyanidins in the seed coat caused the *tt2* seeds to appear yellow (Debeaujon et al., 2000) and

have smaller sizes and reduced weights (Figs. 2 and 3A). Their embryos were, however, much heavier than those of the wild type (Fig. 3, A and C).

FAs, a class of prominent metabolites in seeds, are predominantly stored in embryo and play essential roles during the growth and development of all living organisms. Although the biochemistry of FA biosynthesis and the regulatory mechanism of FA metabolism have been well studied in *Arabidopsis*, little attention has been given to the regulation of *TT2* in the metabolism of seed FA and other seed metabolites. Malonyl-CoA formed from acetyl-CoA catalyzed by ACCase is the precursor for seed FA biosynthesis (Lepiniec et al., 2006; Baud and Lepiniec, 2009). ACCase is the key switch that controls FA flux, which must be highly dynamic in responding to variable signals and kept at a low threshold to allow tight control. Therefore, ACCase acts as a sensor or a gating system to monitor the overall flux of FA biosynthesis (Mu et al., 2008). As ACCase subunits, *BCCP2* and *CAC2* were up-regulated during *tt2-5* seed EM (Fig. 5). More active ACCase should lead to increased FA accumulation in the *tt2* seeds. Moreover, total protein and storage protein contents in the developing seeds at the 16 DAP and the mature seeds of *tt2-5* were much lower than that of the wild



**Figure 6.** Evaluation of starch and protein content in developing Arabidopsis seeds between Col-0 and *tt2-5*. A, Starch. B, Protein contents were analyzed at three different developmental stages, including 6, 16 DAP, and mature stage, on the DW basis. The dash underlines of B indicate total protein content, and the solid underlines show seed storage protein content. Data presented are mean values of three independent experiments (biological repeats) ± SE. The asterisks (\*) indicate significant difference ( $P \leq 0.05$ ) compared with the wild type.

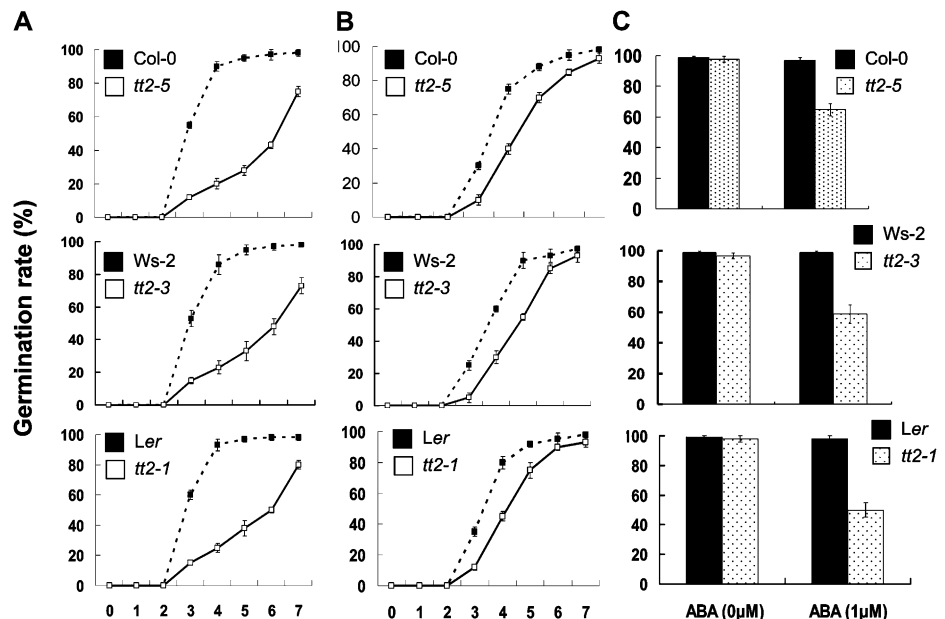
type (Col-0; Fig. 6), which may be helpful for seed FA accumulation in the mutant. Therefore, *TT2* could be a reasonable choice for genetic engineering toward higher seed oil content in oil crops, such as *Brassica* spp. oilseeds.

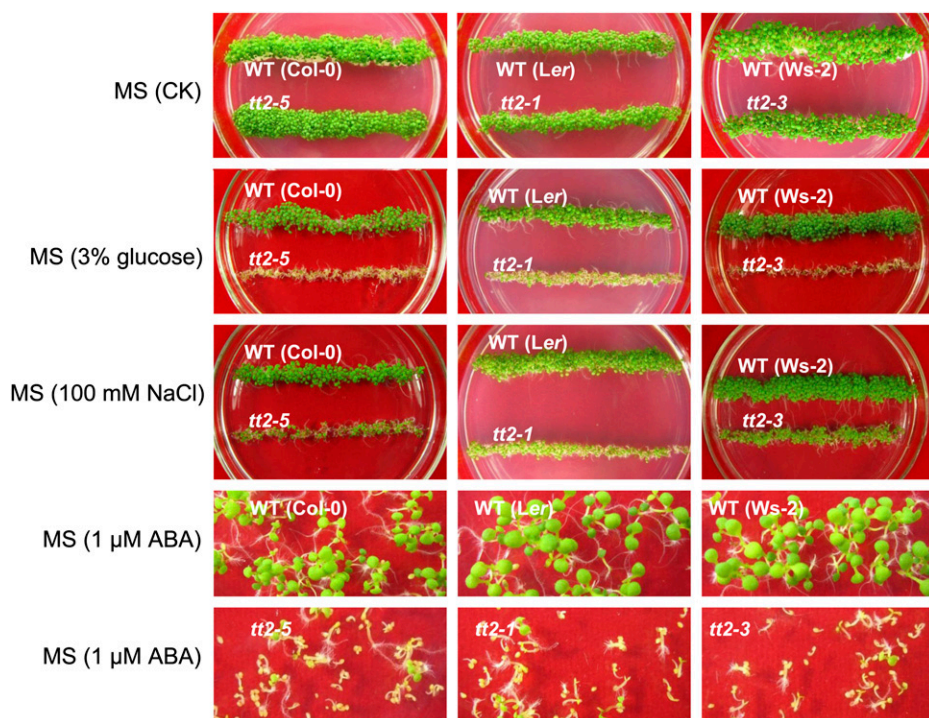
Expression of the genes critical for FAS is coordinately regulated (Ruuska et al., 2002; Baud et al., 2003). Loss of function of the *TT2* gene up-regulated many FA biosynthetic genes in the plastid and endoplasmic reticulum. Of these enzymes, the BCCP2 and CAC2, subunits of ACCase, catalyze the initial synthesis step, the formation of malonyl-CoA from acetyl-CoA. A key reaction is to transfer acetyl-CoA and malonyl-ACP

to 3-ketoacyl-ACP, which is catalyzed by 3-ketoacyl-ACP synthase. The enzyme MOD1 functions as an enoyl-ACP reductase, whereas *FAB2* (*SSI2*) encodes a stearoyl-ACP desaturase as the *ssi2* mutants increase 18:0 and reduce 18:1 FA. In the endoplasmic reticulum, CDP-diacylglycerol is formed from 1,2-diacylglycerol-3-phosphate catalyzed by CDS2 and transferred to phosphatidylinositol by PIS2. FAD2 is essential for polyunsaturated lipid synthesis (Okuley et al., 1994), and FAD3 is responsible for the synthesis of 18:3 FA from phospholipids, which uses cytochrome b5 as an electron donor (Shah et al., 1997). *FAE1* is essential for the synthesis of VLCFAs in seeds, extending the chain length of FAs from C18 to C20 and C22. In this study, most FA synthetic genes were only moderately up-regulated, including the genes for ACCase. In contrast, *FAE1* was up-regulated remarkably (Fig. 5; Supplemental Table S6). As a consequence of this increased expression, the biosynthesis of VLCFAs, such as C20:0, C20:1, and C22:1 was much more efficient than other FAs, such as C16:0, C18:0, C18:2, and C18:3 in the *tt2* seeds at 16 DAP (Table I; Supplemental Table S3). The synthesis of C18:1 in the *tt2* seeds was more sufficient than that in the wild-type seeds (Table I). This could be caused by the remarkably increased expression of *KASII* and *FAB2*, which are essential in transferring 16:0-ACP into 18:1-ACP in plastids (Fig. 5).

FA biosynthesis is regulated at multiple levels, of which transcriptional control is important for regulating the FA biosynthesis pathway (Ohlrogge and Jaworski, 1997; Millar et al., 2000). The key TFs regulating FA biosynthesis may include *WR11*, *LEC1*, *LEC2*, *FUS3*, and *ABI3*. *WR11* encodes a transcriptional regulator of the AP2/EREB family that targets enzymes in late glycolysis and in the plastidial FA

**Figure 7.** Comparison of seed germination rate under stressed environments between *tt2* mutants and their respective wild-type controls. Seed germination was scored daily after the radical tips had fully emerged from the seed coats. Data are the means ± SE from three independent experiments evaluating 100 seeds. A, Time courses of seed germination on 1× MS agar medium containing 3% (w/v) Glc. B, Time courses of seed germination on 1× MS agar medium containing 100 mM NaCl. C, Seed germination rate of 7 DAS on 1× MS agar medium containing 1 µM ABA.

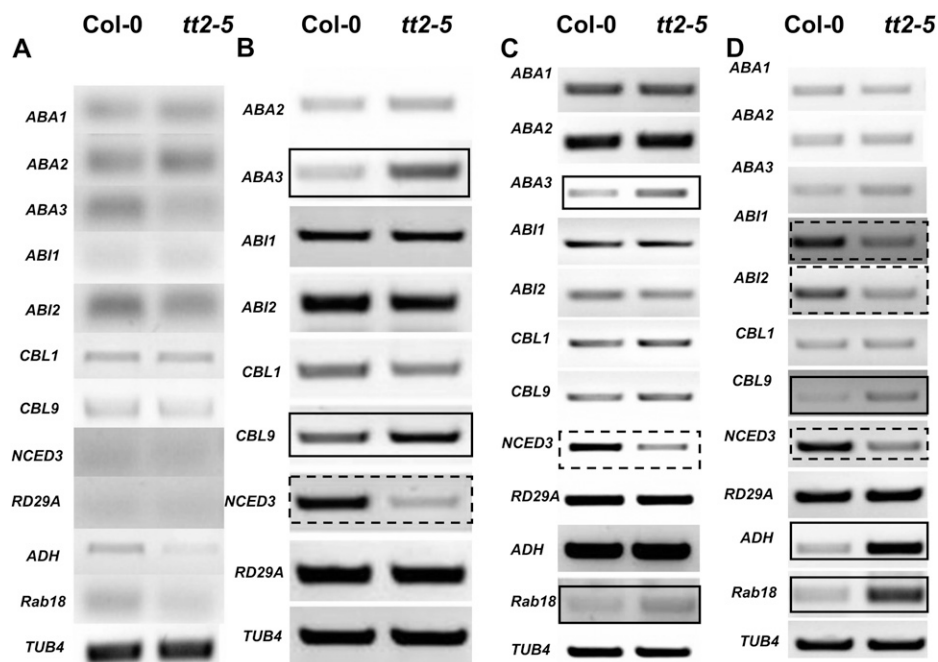




**Figure 8.** Comparison of seedling establishment under stressed environments between *tt2* mutant lines and their respective wild-type controls. The pictures were taken 10 DAS, showing the situations on the control medium (without stress), 1× MS agar medium containing 3% (w/v) Glc, 1× MS agar medium containing 100 mM NaCl, and 1× MS agar medium containing 1 μM ABA.

biosynthetic network (Focks and Benning, 1998; Cernac and Benning, 2004). *LEC1* encodes the HAP3 subunit of the CCAAT binding factor and plays essential roles in FA biosynthesis (Lee et al., 2003; Mu et al., 2008). *LEC2*, *FUS3*, and *ABI3* encode B3 domain TFs of functional importance for the regulation of seed maturation (Luerssen et al., 1998; Stone et al., 2001; To et al., 2006). These five TFs have unique and

overlapping functions in regulating embryonic development and FA biosynthetic pathway in Arabidopsis (To et al., 2006; Wang et al., 2007; Santos-Mendoza et al., 2008; Baud and Lepiniec, 2009). In this study, the *tt2* mutation did not cause significant changes in the expression levels of *WR11*, *LEC1*, *LEC2*, and *ABI3* (Supplemental Tables S8 and S9). However, the *FUS3* expression level increased remarkably (Fig. 5;



**Figure 9.** Comparison of osmotic-stress- and ABA-related gene expressions between the wild type (Col-0) and *tt2-5* under stressed environments. Total RNA was isolated from the whole seedling 10 DAS. RT-PCR was performed with osmotic-stress- and ABA-related gene primers. The cDNA samples were normalized by RT-PCR using a tubulin probe as an internal control. A, 1× MS agar medium without stress. B, 1× MS agar medium containing 3% (w/v) Glc. C, 1× MS agar medium containing 100 mM NaCl. D, 1× MS agar medium containing 1 μM ABA. Boxes of solid lines indicate an up-regulation of a gene in the *tt2-5* mutant; boxes of dashed lines show a down-regulation of a gene in the *tt2-5* mutant.

Supplemental Table S6). *FUS3* is expressed in seeds mostly during seed filling, and promotes oil deposition by synchronizing genes in the FA biosynthetic pathway to increase the transcript levels of *CAC2*, *KASI*, *FAB2*, *FAD3*, *BCCP2*, *ACP1*, and *ACP5*, etc. (Wang et al., 2007; Yamamoto et al., 2010). On the other hand, it also plays an active role in positively regulating the expression of photosynthetic genes during early seed development (Yamamoto et al., 2010). Therefore, it was possible that the increased expression of *FUS3* in the *tt2* mutant could have resulted in more photosynthates, which might be further partitioned into seed FA deposits.

According to Debeaujon et al. (2003), *TT2* expression is limited to the testa of early developing seeds. However, FA is mainly synthesized in embryos. It is not likely that *TT2* directly regulates the FAS in embryos. Possibly, the deficiency of flavonoids, the precursors of proanthocyanidins, in *tt2* mutant causes more sufficient FA biosynthesis in the embryo, because flavonoids, permeable from testa to embryo, are potential inhibitors of FA biosynthesis. They suppress the expression of *FabG* and *FabI*, that encode important reductases involved in chain elongation (Zhang and Rock, 2004). The *tt2* mutation, therefore, indirectly promoted the FA accumulation due to the removal of the flavonoid repression. In nature, there are two basic types of FAS architectures. The prototypical FAS type I is found in mammals and consists of a single gene that produces a polypeptide, which contains all of the reaction centers required to produce a FA (Smith et al., 2003). In contrast, the dissociated FAS type II system, in which each single step on the FAS pathway is catalyzed with a unique protein encoded by a separate gene, is found in bacteria, plants, and parasites (White et al., 2005).

### Loss of *TT2* Function Leads to Reduced Tolerance to Environmental Stress during Seedling Establishment

FAS is a primary metabolic pathway essential for the functioning of plant cells. FAs or FA derivatives act as signal molecules or hormones, carbon and energy storage, and surface layer protecting the plant from environmental stress (Ohlrogge and Jaworski, 1997; Hong et al., 2008; Mu et al., 2008). The seed coat plays an important role in the protection against harmful microorganisms and unfavorable environmental conditions (Mohamedyassen et al., 1994; Debeaujon et al., 2000). This study shows that the *tt2* mutant line were much more sensitive to abiotic stresses caused by high concentrations of Glc, NaCl, or ABA in the growth medium (Fig. 7–9). The transcription levels of genes related to osmotic stress and ABA were different between the *tt2* and the wild type under stressful environments. The *NCED3* expression in the *tt2* significantly decreased under a stressful environment. *NCED3* encodes 9-cis-epoxycarotenoid dioxygenase, which functions as a

key enzyme in ABA biosynthesis (Fig. 7–9). The microarray analysis revealed that the largest class of DEGs between the *tt2* mutant and the wild type was related to stress/defense response (Tables II and III; Supplemental Tables S4–S7).

It is also possible that the deficiency of flavonoids gave rise to the alternation of the expression pattern of stress-responsive gene during young seedling establishment. On the other hand, the stress-sensitive phenotypes of *tt2* mutant lines are because of increased permeability of stressful elements through the *tt2* seed coats.

This work advances the understanding of *TT2* functions in seed FA accumulation and abiotic stresses during seed germination and seedling establishment. However, the mechanisms by which *TT2* regulate FA accumulation in seeds and stress responses during young seedling establishment are not completely clear.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

*Arabidopsis* (*Arabidopsis thaliana*) accessions Col-0, Landsberg *erecta* (*Ler*), and Wassilewskija-2 (*Ws-2*) were used as the wild-type controls according to the background of the mutant. The seeds of these lines were prechilled at 4°C for 2 to 4 d and then surface sterilized with 6% (w/v) NaClO solution for 6 to 8 min. Subsequently, the seeds were washed five to seven times with distilled, deionized water and then sown onto solid MS stock medium. After about 2 weeks, the healthy seedlings were transferred into autoclaved soil in a flowerpot 7 cm in diameter. The plants were fertilized three times at two true leaf stages, the rosette leaf stage and the bolting stage. The fertilizer (Shanghai Wintong Chemicals Company) contained 20% total nitrogen (5.97% nitrate nitrogen, 3.92% ammonia nitrogen, and 10.11% urea nitrogen), 20% water-soluble phosphorus (P<sub>2</sub>O<sub>5</sub>), 20% water-soluble potassium (K<sub>2</sub>O), 0.07% water-soluble magnesium, 0.009% boron, and 0.02% copper, 0.1% iron, 0.05% manganese, 0.002% molybdenum, 0.02% zinc, and 0.0005% cobalt (all chelated). The plants were grown under long daytime conditions (16 h) in an air-conditioned growth room, at 24°C and 18°C day and night temperatures, respectively. The light intensity for day was 100 μmol m<sup>-2</sup> s<sup>-1</sup>, as measured on the base of a plant.

### Analysis of FAs, Seed Starch, and Protein

The extraction and analysis of FAs were carried out as follows (Poirier et al., 1999; Mu et al., 2008). About 10 mg of intact seeds were heated at 80°C in methanol solution containing 1 M HCl for 2 h. The FA methyl esters were extracted with 2 mL hexane and 2 mL of 0.9% (w/v) NaCl, and the organic phase was used for analysis by gas chromatography, using methyl heptadecanoate as an internal standard. The machine (Shimadzu, GC-2014) was equipped with a flame ionization detector and a 30 m (length) × 0.25 mm (i.d.) × 0.5 μm (liquid membrane thickness) column (Supelco wax-10, Supelco, Cat. no. 24079). The initial column temperature was maintained at 160°C for 1 min, then increased by 4°C min<sup>-1</sup> to 240°C, and held for 16 min at the final temperature. After the run, the peaks corresponding to each FA species were identified by their characteristic retention times. Concentrations of each sample were normalized against the internal control.

To analyze the FA content and FA composition in the embryo, the seed envelopes that are composed of the integuments plus the endosperm layer were left on the MS medium when the seeds germinated, and then were carefully collected. Up to 100 seed envelopes for each of the three replicates were collected, dried at the similar condition as the mature seeds, and then weighed until the weight was unchanged. The weight of the seed envelopes was subtracted from that of the whole seed, leaving the weight of the embryo. The FA content of the seed embryo was calculated using the FA content of the whole seed divided by the net weight of the seed embryo.

The seed starch content was determined as follows (McCleary et al., 1994; Baud et al., 2002). Up to 20 seeds were ground twice in 2 × 500 μL of 80%

(v/v) ethanol at 4°C. After centrifugation (15,000g, 10 min, 4°C), the resulting pellet was dried at 50°C for 30 min and then homogenized in 150  $\mu$ L of 50 mM MOPS buffer (pH 7.0) containing 15.4 units of heat-stable  $\alpha$ -amylase. The suspension was incubated at 100°C for 6 min. Then, 200  $\mu$ L of 0.2 M sodium acetate buffer (pH 4.8) containing 26.1 units of amyloglucosidase were added, and the suspension was incubated at 50°C for 30 min with regular shaking. After centrifugation, the Glc in the supernatant liquid was used for starch quantification.

The quantification of seed storage and total protein was adapted from Baud et al. (2002). Twenty seeds were homogenized in 200  $\mu$ L of extraction buffer containing 50 mM HEPES, 5 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 10% (v/v) ethylene glycol (pH 7.5) in the presence of Polyclar. After centrifugation (17,530g, 10 min, 4°C), the supernate was used for storage protein measurements according to Bradford (1976). The remaining proteins insoluble in the extraction buffer were recovered by treatment with 1 M sodium hydroxide and measured as described above. In both assays, gammaglobulin (Bio-Rad) was used for calibration.

### Morphologic Observations of Mature Seeds

The seeds for observation were selected from the siliques harvested from the basal part of a major inflorescence in this study. The mature seeds of the wild type and the *tt2* mutant lines were randomly selected and were photographed using an OLYMPUS SZ 61 stereomicroscope. Six seeds of each genotype were randomly selected for sectioning. Perpendicular transections were produced and the sections with the largest oval-shaped surface area were selected randomly for the oil body observation. The oil bodies were observed under transmission electron microscopy (TEM). A number of 20 cells from one seed were observed for the oil bodies contained. The average number of oil bodies within a cell was calculated based on six seeds. TEM observation was performed as follows. The main steps, double fixation, dehydration, infiltration and embedding, and ultrathin sectioning are described in detail as follows. Double fixation: The Arabidopsis seeds were first fixed with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) for about 16 h and then washed three times in the phosphate buffer (0.1 M, pH 7.0) for 25 min. The seeds were then postfixed with 1% OsO<sub>4</sub> in phosphate buffer (0.1 M, pH 7.0) for 2 h and washed three times in phosphate buffer (0.1 M, pH 7.0) for 25 min, respectively. Dehydration: The specimen was dehydrated using a graded series of ethanol (50%, 70%, 80%, 90%, 95%, and 100%) for about 20 min, respectively, and then transferred to absolute acetone for 20 min. Infiltration: The specimen was placed in a 1:1 mixture of absolute acetone and the final Spurr resin mixture for 2 h at room temperature and then transferred to a 1:3 mixture of absolute acetone and the final Spurr resin mixture for 5 h and to final Spurr resin mixture overnight. Embedding and ultrathin sectioning: The specimen was placed in Eppendorf tubes containing embedding medium and heated at 70°C for about 9 h. The specimen sections were stained with uranyl acetate and alkaline lead citrate for 15 min, respectively, and observed under a TEM (JEM-1230).

### Microarray Hybridization and Data Analysis

The flowers of the wild type (Col-0) and *tt2-5* were tagged with different-colored threads to indicate the DAP. Only the seeds from the siliques on the primary shoots were utilized in this study. Three independent biological replicates were conducted for the wild type (Col-0) and *tt2-5* in the microarray experiment. Probe labeling and chip hybridization were carried out through the Affymetrix custom service (CapitalBio) following the standard protocol ([http://www.affymetrix.com/support/technical/manual/expr-ession\\_manual.affx](http://www.affymetrix.com/support/technical/manual/expr-ession_manual.affx)). Normalization was performed according to the standard Affymetrix protocol to allow the comparison of the samples for each set of experiments. The Excel add in for significance analysis of microarrays was used to identify DEGs between the control and the T-DNA insertion mutant line *tt2-5*. Functional classification of the DEGs was performed using the biological process category of Arabidopsis Gene Ontology (<http://www.geneontology.com>). The percentage (far-right column) refers to the ratio of genes relative to the total up-regulated or down-regulated DEGs in each functional category. For the microarray experiment, three independent biological replicates were conducted, and the DEGs with log<sub>2</sub> ratios  $\geq 1.00$  or  $\leq -1.00$  (only GO Slim IDs with *P* values  $\leq 0.5$ ) were analyzed.

### Analysis of Gene Expression by RT-PCR and qRT-PCR

The RNA samples were isolated using the Invisorb spin plant RNA mini kit (Invitex; microarray experiments) following the manufacturer's instructions.

The RNA samples were treated with RNase-free DNase I (New England Biolabs) to remove any trace genomic DNA. For semiquantitative RT-PCR and qRT-PCR templates, first-strand complementary DNA (cDNA) were synthesized in a 20- $\mu$ L reaction solution containing approximately 0.5 and 3  $\mu$ g total RNA, respectively, using PrimerScript RT (Takara) and oligo (dT) 12-18 as a primer. *EF1aA4* and *UBIQUITIN4* (AT5G44340) were used as internal controls in the RT-PCR and qRT-PCR. All primer pairs used in the RT-PCR and qRT-PCR analyses are listed in Supplemental Table S1. The real-time amplification reactions were performed using the SYBR Green kit (Takara) and iCycler iQ thermocycler (Bio-Rad) according to the manuals from the manufacturers.

### Analysis of the Seed Germination Rate and Seedling Establishment of *tt2-5* Mutant Lines under Stressed Conditions

The seeds used for the germination assays were harvested at nearly the same time from plants grown under the same growth conditions as previously described, allowed to mature for 5 weeks at room temperature, and stored in a dry cabinet (approximately 15% moisture level). The Arabidopsis seeds were surface sterilized and then placed on MS medium containing 3% (w/v) Glc, 100 mM NaCl, or 1  $\mu$ M ABA as previously described. The seed germination frequency (defined as radicle emergence) was scored daily (Cernac et al., 2006). Arabidopsis plants at 10 DAS were photographed and harvested for RNA analysis.

### Statistical Analysis

A completely randomized block design with at least three biological replicates was conducted for each experiment in this study. Data were classified with Win-Excel and analyzed via an ANOVA using the SPSS statistical package (version 8.0). Comparisons between the treatment means were made via a Tukey's test at a probability level of  $P \leq 0.05$ .

### Supplemental Data

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

**Supplemental Table S1.** Primers used for RT-PCR and qRT-PCR analyses.

**Supplemental Table S2.** Primers used for the genotyping of the *tt2* mutants.

**Supplemental Table S3.** A list of up-regulated genes in the developing seeds of SALK\_005260 plants at 6 DAP.

**Supplemental Table S4.** A list of down-regulated genes in the developing seeds of SALK\_005260 plants at 6 DAP.

**Supplemental Table S5.** A list of up-regulated genes in the developing seeds of SALK\_005260 plants at 16 DAP.

**Supplemental Table S6.** A list of down-regulated genes in the developing seeds of SALK\_005260 plants at 16 DAP.

**Supplemental Table S7.** A list of TFs and genes encoding key enzymes for FA biosynthesis at 6 DAP.

**Supplemental Table S8.** A list of TFs and genes encoding key enzymes for FA biosynthesis at 16 DAP.

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### LITERATURE CITED

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