Transparent Testa16 Plays Multiple Roles in Plant Development and Is Involved in Lipid Synthesis and Embryo Development in Canola[1][W][OA]

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Transparent Testa16 (TT16), a transcript regulator belonging to the B_{sister} MADS box proteins, regulates proper endothelial differentiation and proanthocyanidin accumulation in the seed coat. Our understanding of its other physiological roles, however, is limited. In this study, the physiological and developmental roles of TT16 in an important oil crop, canola (*Brassica napus*), were dissected by a loss-of-function approach. RNA interference (RNAi)-mediated down-regulation of *tt16* in canola caused dwarf phenotypes with a decrease in the number of inflorescences, flowers, siliques, and seeds. Fluorescence microscopy revealed that *tt16* deficiency affects pollen tube guidance, resulting in reduced fertility and negatively impacting embryo and seed development. Moreover, *Bntt16* RNAi plants had reduced oil content and altered fatty acid composition. Transmission electron microscopy showed that the seeds of the RNAi plants had fewer oil bodies than the nontransgenic plants. In addition, *tt16* RNAi transgenic lines were more sensitive to auxin. Further analysis by microarray showed that *tt16* down-regulation alters the expression of genes involved in gynoecium and embryo development, lipid metabolism, auxin transport, and signal transduction. The broad regulatory function of *TT16* at the transcriptional level may explain the altered phenotypes observed in the transgenic lines. Overall, the results uncovered important biological roles of TT16 in plant development, especially in fatty acid synthesis and embryo development.

The plant MADS box family genes, which are named after the characterization of four members in this group, MINICHROMOSOME MAINTENANCE1, AGAMOUS, DEFICIENS and SERUM RESPONSE FACTOR, encode transcription factors that share a common DNA-binding domain (the MADS box) and play multiple roles in flower pattern formation, gametophyte cell division, and fruit wall differentiation (Ng and Yanofsky, 2001; Dinneny and Yanofsky, 2005; Colombo et al., 2008). A number of MADS domain proteins from vascular plants share a conserved structural organization, the so-called MIKC-type domain structure, where the MADS (M) domain is followed by an Intervening (I), a Keratin-like (K) and a C-terminal domain (Theissen et al., 1996). MIKC-type MADS box genes are involved in important aspects of plant reproductive development, such as flower initiation, specification of floral meristem and organ identity, and ovule and fruit development (Becker and Theissen, 2003).

MADS box genes are the major members of plant floral organ identity genes, which have been divided into five classes according to the ABCDE model (Theissen, 2001; Krizek and Fletcher, 2005). The ABCDE model explains flower formation by the interaction of five classes of homeotic genes (A–E), with A controlling sepal, A+B+E controlling petal, B+C+E controlling stamen, C+E controlling carpel, and D controlling ovule development (Theissen, 2001; Krizek and Fletcher, 2005). Therefore, MADS box genes appear to have a central role in flower development. The functions of floral organ identity genes are highly correlated to their phylogenetic relationships in that these genes are members of well-defined gene clades termed *SQUAMOSA* (class A), *DEFICIENS* or *GLOBOSA* (class B), *AGAMOUS* (classes C and D), and *SEPALLATA* (class E; Becker and Theissen, 2003; Melzer et al., 2010).

Recently, a group of MADS box proteins, closely related to the B class floral homeotic proteins, were identified as the B_{sister} subfamily (Becker et al., 2002). It has been suggested that the B and B_{sister} gene lineages were generated by a duplication of an ancestral gene

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before the divergence of gymnosperm and angiosperm lineages 300 million years ago but after the separation of the fern lineage 400 million years ago (Becker et al., 2002; Stellari et al., 2004). B genes are predominantly expressed in petal and stamen primordia; however, the expression of B_{sister} genes is found almost exclusively in female reproductive structures or is even restricted to the ovules (Theissen et al., 1996, 2000; Becker et al., 2002; de Folter et al., 2006). It has been hypothesized that the B class proteins are important for male reproductive organ development, whereas the B_{sister} proteins are important for female reproductive organ development (Becker et al., 2002).

Transparent Testa16 (*TT16*) is a B_{sister} gene that acts as a transcription factor and appears to play a role in seed coat pigmentation and proanthocyanidin (PA) accumulation in the endothelium of developing seeds. A *tt16* mutant, in which the *TT16/ABS* gene encoding a MIKC-type MADS box protein was disrupted, showed altered seed pigmentation and PA accumulation, but *TT16/ABS* alone does not appear to be crucial for female reproductive development in Arabidopsis (*Arabidopsis thaliana*; Nesi et al., 2002). Interestingly, the recently characterized B_{sister} MADS box *Floral Binding Protein24* (*FB24*) from petunia (*Petunia hybrida*) was unable to complement the Arabidopsis *tt16* mutant, despite having similar developmental roles to *TT16/ABS* (de Folter et al., 2006). The only other B_{sister} MADS box gene in Arabidopsis is *GORDITA* (*GOA*; formerly known as *AGL63*), which like *TT16/ABS* arose from duplication during the diversification of the Brassicaceae (Erdmann et al., 2010). Functional studies suggest that *GOA* not only contributes to integument development but also regulates fruit growth (Erdmann et al., 2010; Prasad et al., 2010); however, up to now, the detailed physiological functions of the B_{sister} transcription factors have not been extensively studied.

In developing oilseeds, transcription factors not only govern fruit and seed development but also storage lipid metabolism, including fatty acid (FA) and triacylglycerol synthesis, which is of interest for biotechnological applications in seed oil modification. WRINKLED1 (WRI1) is a member of the APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEINS belonging to the A class in the ABCDE model, and overexpression of *WRI1* enhanced the transcription of FA biosynthetic genes, leading to increased triacylglycerol accumulation in both seeds and leaves (Cernac and Benning, 2004; Baud and Lepiniec, 2009). LEAFY COTYLEDON1 (LEC1) is an NFY-B-type or CCAAT-binding factor-type transcription factor, and LEC2 belongs to the plant-specific B3 transcription factor family (Lotan et al., 1998; Stone et al., 2001). LEC2 and LEC1 can control the expression of FA biosynthesis genes (Baud et al., 2007; Mu et al., 2008). To date, however, there is no report on the function of B_{sister} proteins in FA synthesis or triacylglycerol accumulation, despite their importance in other aspects of seed development.

This study aimed to determine the physiological functions of four recently identified canola (*Brassica napus*) *TT16* (*BnTT16*) homologs in plant development and seed oil synthesis. Using an RNA interference (RNAi) approach, we found that down-regulation of *TT16* in canola affects pollen tube guidance, reduces fertility, and influences FA synthesis and embryo development. The results strongly suggest that *TT16* plays multiple physiological roles beyond endothelial development and PA accumulation and that *TT16* may be a suitable biotechnological target for seed oil modification.

RESULTS

Down-Regulation of *Bntt16s* Alters Canola Development

We identified four *TT16* homologs from canola based on the analysis of ESTs and genomic DNA sequences, in which two shared the identical sequences of the

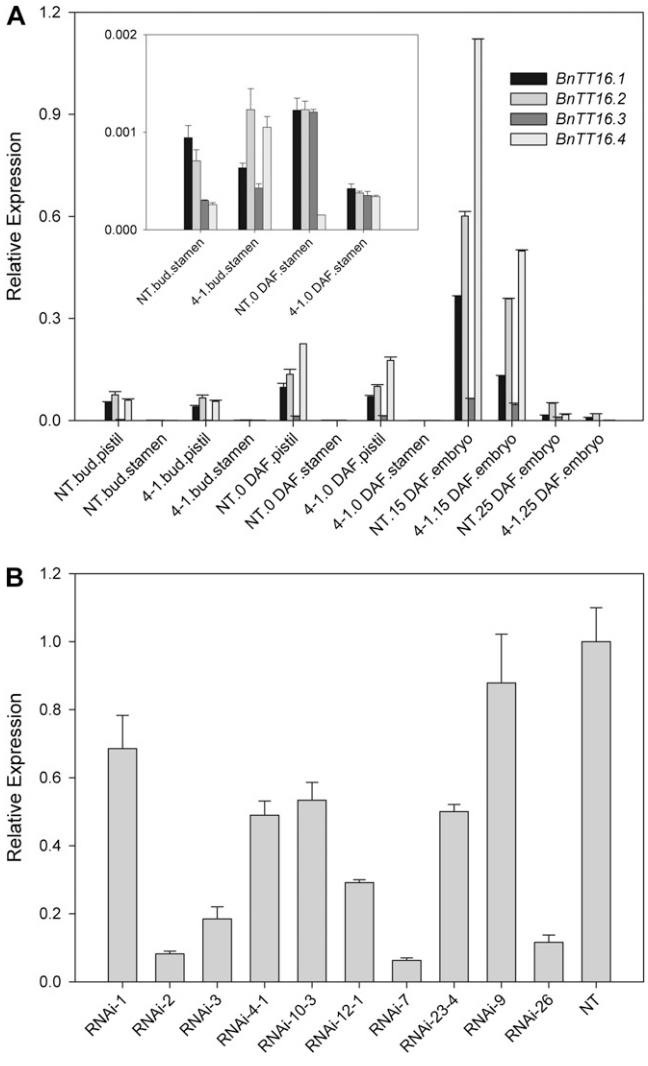

Figure 1. RNAi-mediated silencing of *Bntt16* expression. A, Gene expression patterns ($2^{-\Delta CT}$) of *BnTT16s* in canola tissues. *BnTT16* expression levels in the stamen are also shown in the inset. B, Overall expression levels of *BnTT16s* in samples 2 DAF were down-regulated in all RNAi lines. *Bntt16* expression level in NT plants was set as 1 for comparison. 4-1, *tt16* RNAi transgenic line 4-1.

reported BnTT16.1 (EU192028) and BnTT16.2 (EU192029). The other two were named *BnTT16.3* (HM449990) and *BnTT16.4* (HM449989). Similar to *TT16* in Arabidopsis, the *BnTT16s* regulate endothelium development and PA accumulation (G. Chen, W. Deng, F. Peng, M. Truksa, S. Singer, C. Snyder, and R. Weselake, unpublished data). As shown in Figure 1A, *TT16s* have much higher expression levels in female organs, consistent with other plant B_{sister} genes (Chen et al., 2012). Although the expression levels of the four *TT16s* varied, they all have the highest expression level in the embryo of early developing seeds (15 d after flowering [DAF]). To assess the effects of the loss of function of four *TT16s* on canola growth and development, a 146-bp conserved complementary DNA fragment was cloned into pHellsgate 12 under the control of a cauliflower mosaic virus 35S promoter for subsequent generation of canola RNAi transgenic plants. Over 40 transgenic lines (*TT16s* RNAi) were generated, and 10 were used for further study. All 10 transgenic lines showed lower accumulation of *TT16* transcripts (Fig. 1B). In line RNAi-4-1, the overall TT16 expression level decreased about 50% (Fig. 1B) and the expression of each *TT16* was down-regulated (Fig. 1A). Therefore, this line was selected as a representative for further study.

Multiple phenotypes related to vegetative growth and development were identified in transgenic plants. Nontransgenic (NT) plants flowered earlier than the transgenic plants, indicating that down-regulation of *TT16s* delayed the transition from vegetative growth to reproductive stage in canola. As shown in Figure 2, A and B, the 40-d-old transgenic plants exhibited dwarf stature and smaller, wrinkled leaves compared with the NT plants. Dwarf phenotypes were also identified in 70-d-old *TT16* RNAi transgenic plants (Fig. 2C). Regarding the influence of *tt16* down-regulation on the reproductive organs, the first phenotype is that the RNAi plants had larger flowers and floral organs with stigmas protruding out of the unopened buds (Fig. 2D; Supplemental Table S2). Moreover, the number of inflorescences and total flowers in transgenic plants significantly decreased compared with the NT plants (Fig. 2, E and F; $P < 0.01$). In addition, the transgenic plants produced shorter siliques and fewer seeds (Supplemental Fig. S1; $P < 0.01$).

Down-Regulation of *Bntt16s* Affects Pollen Tube Guidance

To investigate the cause(s) for the reduced seed set of the canola *tt16* lines, pollen viability was first tested using iodine-potassium iodide staining. Pollen in both *tt16* RNAi and NT lines stained as normal dark brown color, indicating that pollen viability was not affected

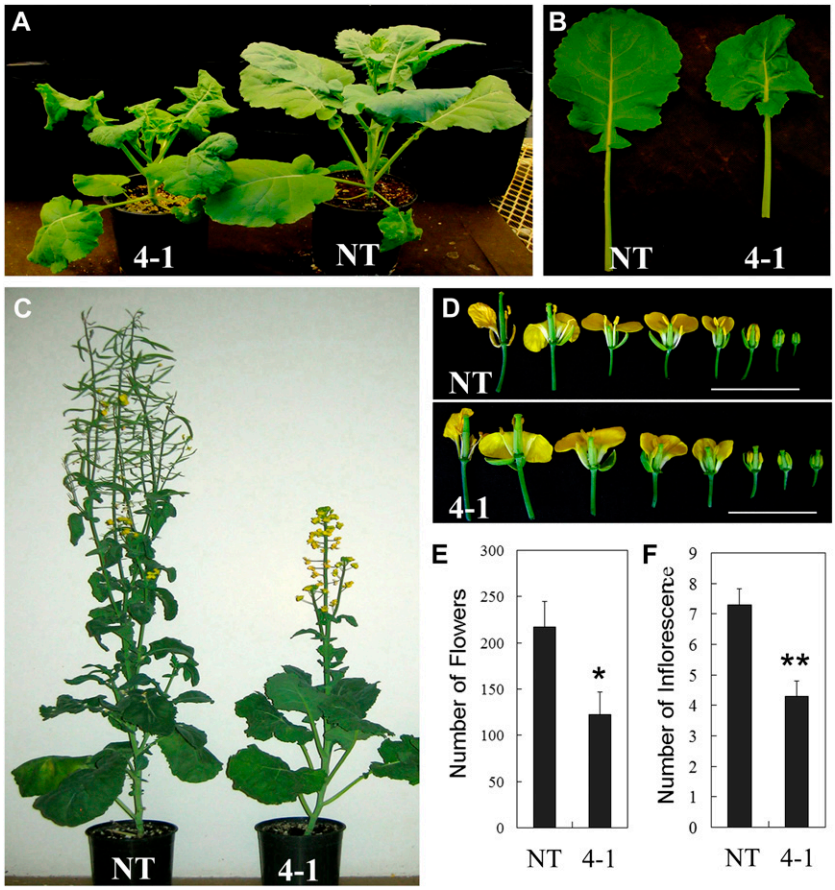

Figure 2. Morphological alteration exhibited by the *tt16* down-regulated canola line. A, Dwarf phenotypes of 40-d-old *tt16* RNAi transgenic lines. B, Leaves of 40-d-old transgenic lines. C, Delayed flowers of 70-d-old *tt16* RNAi transgenic lines. D, Flowers of 70-d-old transgenic lines. E, Number of flowers and inflorescences in 70-d-old *tt16* RNAi transgenic lines. 4-1, *tt16* RNAi transgenic line 4-1. SD ($n = 3$) is indicated by vertical bars. Single and double asterisks indicate significant differences between transgenic and NT plants with $P < 0.05$ and $P < 0.01$, respectively, as determined by *t* test.

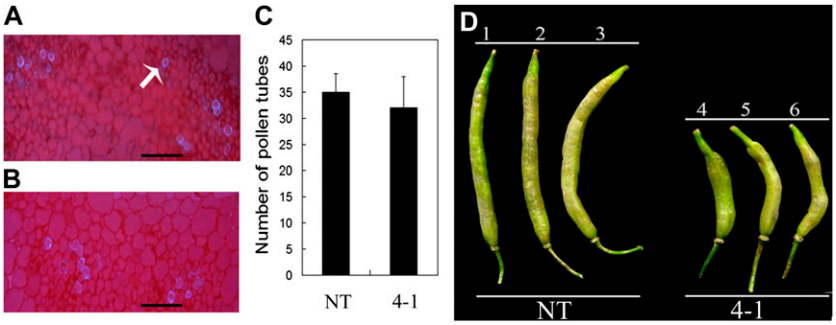

Figure 3. Pollen tubes development in a *tt16* RNAi transgenic line and artificial pollination. A, Style section of a NT plants. The arrow indicates a pollen tube. B, Style section of the *tt16* RNAi transgenic line 4-1. Bars = 8 μm. C, Number of pollen tubes in style sections. SE values (n = 3) are indicated by vertical bars. D, Artificial pollination. 1, NT plants without artificial pollination. 2, NT plants with artificial pollination (self-pollination). 3, NT plants pollinated with line 4-1 (cross-pollination). 4, Line 4-1 without artificial pollination. 5, Line 4-1 with artificial pollination (self-pollination). 6, Line 4-1 pollinated with NT plants (cross-pollination).

(data not shown). Second, to determine if pollen tube development in style was affected by *tt16* down-regulation, styles were stained with aniline blue and pollen tubes were counted by fluorescence microscopy. The pollen tube numbers in RNAi and NT plants showed no statistical difference, indicating that the pollen tube developed normally in RNAi lines (Fig. 3, A–C). RNAi and NT lines were manually cross-pollinated to examine pollen development. As shown in Figure 3D, transgenic lines pollinated with pollen from NT lines produced shorter siliques and fewer seeds, whereas NT lines pollinated with pollen from RNAi plants produced normal siliques and seeds. Therefore, the reduced fertility in RNAi plants was not due to pollen development. Finally, we examined pollen tube extension by fluorescence microscopy. Most of the pollen tubes can extend into the ovules in 2-DAF siliques of self-pollinated NT plants and NT plants pollinated with line 4-1 (Fig. 4, A and B), whereas only a few pollen tubes can extend into the ovules in siliques (2 DAF) of self-pollinated line 4-1 and line 4-1 pollinated with NT plants (Fig. 4, C and D). Overall, these results revealed that *tt16* down-regulation affected pollen tube guidance, thus resulting in reduced fertility.

Down-Regulation of *Bntt16s* Alters Seed Development and Lipid Synthesis

The *Bntt16* RNAi plants also produced abnormal mature seeds in terms of seed morphology. In order to characterize this phenotype in detail, we first examined embryos at 8, 12, 20, 24, and 34 DAF. As shown in Figure 5A, the embryos of *tt16* RNAi transgenic plants developed normally at 8 and 12 DAF but abnormally at 20, 24, and 34 DAF. We compared seed morphology between RNAi and NT lines. The seeds of the *tt16* RNAi lines can be classified into two types. Type 1 seeds were deflated and flattened, whereas type 2 seeds were wrinkled and irregular in shape (Fig. 5B). Type 1 seeds had only seed coats with no embryo. Type 2 seeds contained defective embryos with irregular single cotyledons. Some embryos of type 2 seeds turned dark brown at maturity (Fig. 5C). Finally, we compared the seeds by microscopy. As shown in Figure 5, D and E, the transverse sections of mature type 2 seeds illustrate the defective embryo with single cotyledon and remaining endosperm. The transverse sections of mature type 1 seeds had seed coats but no embryo, demonstrating that embryo abortion occurred in type 1 seeds. Collectively, these results strongly indicate that down-regulation of *tt16s* affects the development of embryos and seeds.

The FA content and composition in mature seeds were analyzed using gas chromatography-mass spectrometry. The FA content was dramatically decreased, which was consistent with transmission electron microscopy observations showing fewer oil bodies in RNAi

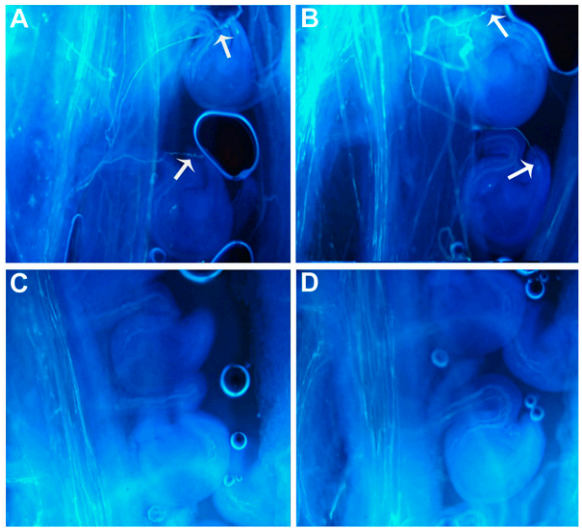

Figure 4. Guiding of pollen tubes to ovules in self- and cross-pollinated plants. A, Self-pollinated NT plants. B, NT plants pollinated with *tt16* RNAi transgenic line 4-1. C, Self-pollinated line 4-1. D, Line 4-1 pollinated with NT plants. Arrows indicate pollen targeted into ovules.

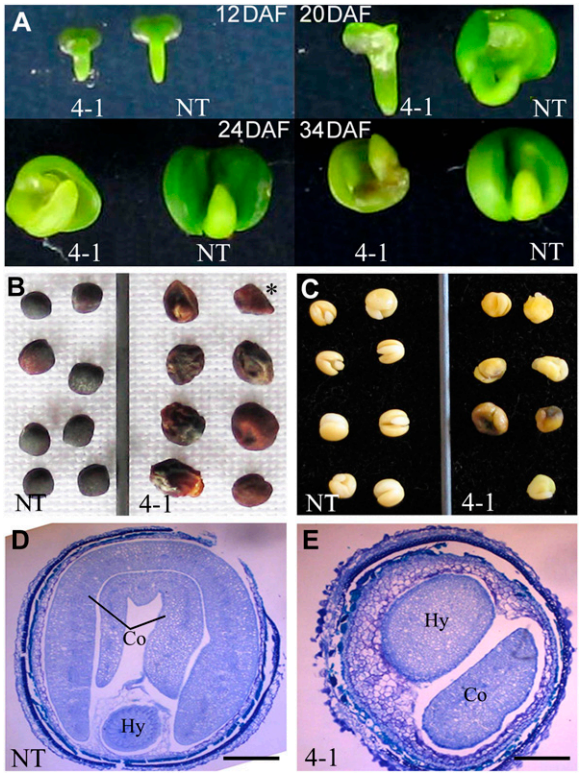

Figure 5. Embryo and seed development in *tt16s* RNAi transgenic lines. A, Embryo development in line 4-1. B, Mature seeds (43 DAF) of transgenic lines. The single asterisk indicates a type 1 seed with no embryo. The rest are type 2 seeds. C, Embryos in mature seeds (43 DAF). D, Seed transverse sections (34 DAF) of NT plants. E, Seed transverse sections (34 DAF) of line 4-1. Co, Cotyledon; Hy, hypocotyl. Bar = 40 mm.

seeds compared with the NT (Fig. 6). The FA composition of RNAi seeds was also significantly different from the NT (Table I). The percentage of 16-carbon FA and very-long-chain FA (C20-22) was higher in the RNAi lines. The transgenic seeds also showed an increase in linoleic acid (18:2$^{cis\Delta 9,12}$) at the expense of oleic acid (18:1$^{cis\Delta 9}$) compared with NT seeds.

Down-Regulation of *Bntt16s* Alters the Expression of Genes Involved in Gynoecium and Embryo Development, Lipid Metabolism, and Auxin Signaling

To further understand why the down-regulation of transcription factor *tt16s* caused the above phenotypes, we compared the gene expression profiles of RNAi plants (2-DAF siliques) with the NT line by microarray. In order to validate the results from the microarray analyses, 20 genes were selected and further studied by real-time PCR. Nineteen out of 20 genes showed similar expression patterns by microarray assay (Fig. 7). The results indicated that the data from the microarray were reproducible and reliable.

Analysis of the microarray data revealed that genes involved in lipid metabolism, gynoecium and embryo development, and auxin transport and signal transduction were affected by *tt16* down-regulation (Tables II–IV). For lipid metabolism, nine genes were down-regulated and 18 genes were up-regulated. For example, *3-Oxoacyl-ACP reductase5* (*KAR5*) and *Chloroplastic acetyl-coenzyme A carboxylase1* (*BCCP1*), both involved in FA synthesis, were down-regulated in RNAi plants (Table II; Harwood, 1988; O'Hara et al., 2007). For gynoecium and embryo development, 50 genes were down-regulated and 15 genes were up-regulated. *GAMETOPHYTE FACTOR1* (*GFA1*)/*MATERNAL EFFECT EMBRYO ARREST5* (*MEE5*), which regulates egg cell differentiation in embryo sacs, was down-regulated in transgenic plants (Table III; Liu et al., 2009). Interestingly, microarray analysis showed that the expression of many auxin-related genes was altered as well (Table IV).

Down-Regulation of *tt16s* Alters the Response to Auxin

As shown in Table IV, eight genes related to auxin transport and signal transduction were down-regulated and nine were up-regulated in the microarray data. We hypothesized that down-regulation of *TT16s* may have changed canola's response to auxin. To further investigate this, we examined the auxin dose response on adventitious root formation of hypocotyl segments. The results showed that the promotion of root organ

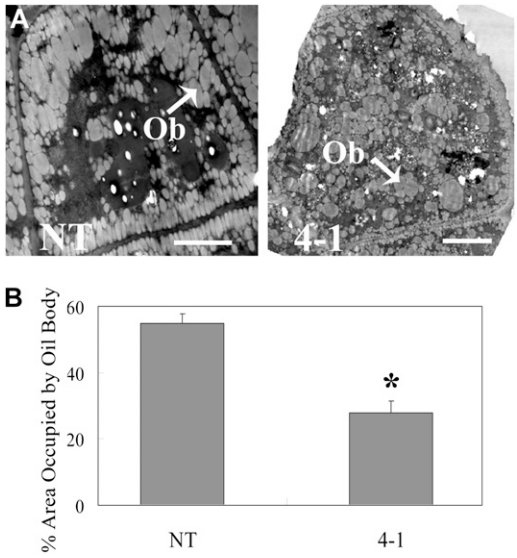

Figure 6. Transmission electron microscopy observation of hypocotyl cells (34 DAF) in *tt16* RNAi transgenic lines. A, Transmission electron microscopy observation of hypocotyl cells. Ob, Oil bodies. Bars = 2.5 μm. B, Quantitation of the area occupied by oil bodies. The percentage area occupied by oil bodies was determined using ImageJ software (http://rsbweb.nih.gov/ij/). SE values (n = 3) are indicated by vertical bars. The single asterisk indicates a significant difference between transgenic and NT plants with $P < 0.05$ as determined by t test. 4-1, *tt16* RNAi transgenic line 4-1.

Table I. *Total FA and FA compositions in NT and tt16 RNAi plants*

Asterisks denote significant differences (*$P < 0.05$ and **$P < 0.01$) between transgenic and NT plants by *t* test. The data are means ± SE corresponding to three independent experiments.

FA	Composition of Fatty Acids	
	NT Plant	RNAi Plant
	%	
16:0	4.4 ± 0.0	7.6 ± 0.0**
16:1	0.1 ± 0.0	0.4 ± 0.0**
18:0	2.8 ± 0.1	4.9 ± 0.2**
18:1	73.4 ± 0.2	60.1 ± 0.1**
18:2	11.8 ± 0.0	19.4 ± 0.3**
18:3	4.9 ± 0.0	4.6 ± 0.1*
20:0	0.9 ± 0.1	1.3 ± 0.0*
20:1	0.8 ± 0.0	0.3 ± 0.1**
22:0	0.9 ± 0.2	1.3 ± 0.1**
Total FA (mg g^{-1})	413.0 ± 4.4	261.7 ± 6.2**
Total FA (mg $seed^{-1}$)	1.9 ± 0.0	0.9 ± 0.0**

regeneration from hypocotyl explants was auxin dose dependent in both NT and *tt16* RNAi plants (Fig. 8). In NT seedlings, however, the synthetic auxin, naphthaleneacetic acid, promoted adventitious root regeneration at concentrations above 0.1 mM, while in *tt16* RNAi seedlings, the critical concentration was 10-fold lower (0.01 mM). At 0.1 and 0.5 mM, there were more adventitious roots in *tt16s* RNAi lines than in NT plants. These results indicated that down-regulation of *tt16s* conferred increased sensitivity to auxin.

DISCUSSION

The function of transcription factor TT16 in regulating endothelial differentiation and PA accumulation has been reported in Arabidopsis and petunia (Nesi et al., 2002; de Folter et al., 2006). Our knowledge of its other physiological functions, however, is very limited. Here, we generated *Bntt16* RNAi plants and comprehensively studied the physiological function of canola TT16s, including possible mechanisms at the transcription level. Currently, the RNAi method is broadly applied to study gene functions. However, several reports have shown that off-target gene silencing can occur during RNAi and result in misleading conclusions in RNAi experiments (Jackson et al., 2003; Xu et al., 2006). In order to minimize the potential off-target gene silencing, we selected the 146-bp *TT16*-specific fragment by a BLAST search against the *Brassica* genome database (http://brassicadb.org). In addition, a publicly Web-based computational tool called siRNA Scan was developed to identify potential off-targets (Xu et al., 2006). Using this tool, we further analyzed the designed fragment to check the risk of potential nonspecific effects (off-target effects), and no potential off-target candidates against the canola mRNA database were detected in our designed fragment.

The *tt16* RNAi transgenic plants displayed reduced fertility, and fluorescence microscopy observation indicated impaired guidance of pollen tubes to the ovule

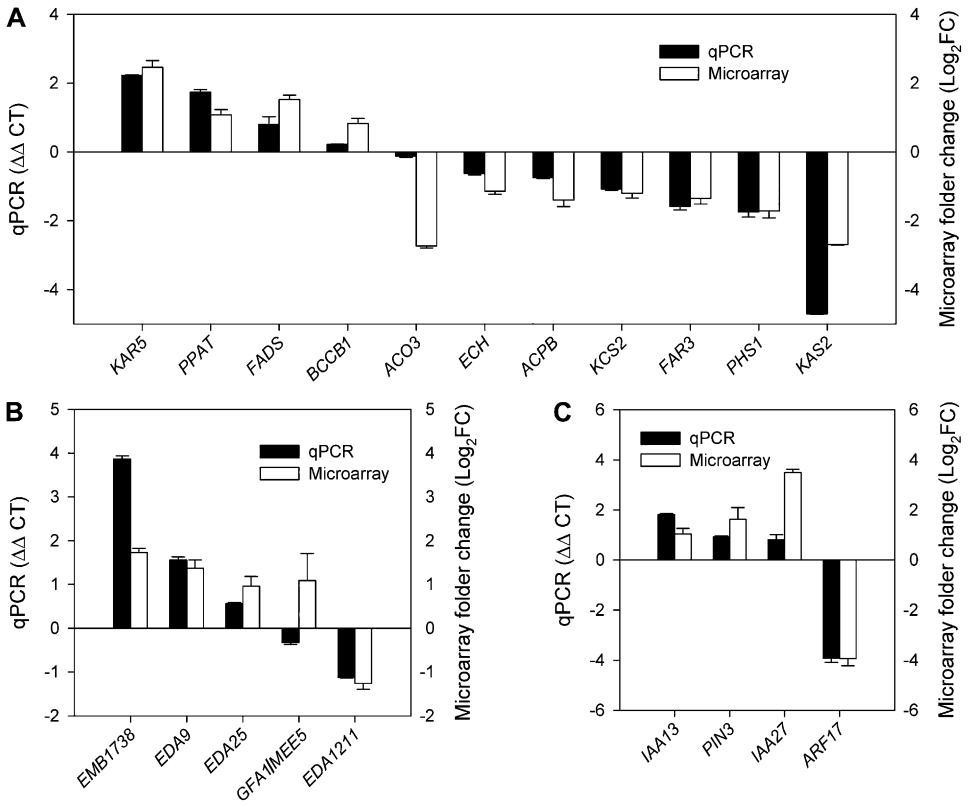

Figure 7. Verification of microarray data using real-time PCR. A, Genes involved in lipid metabolism. B, Genes involved in gynoecium and embryo development. C, Auxin-related genes.

Table II. *Microarray analysis of tt16 RNAi plants: genes involved in lipid metabolism in siliques (2 DAF) of tt16 RNAi plants*

Microarray data were analyzed using the open-source R statistical programming language and Bioconductor packages with $P < 0.01$ (Gentleman et al., 2004; R Development Core Team 2010). GO, Gene Ontology categories.

Gene Name	Gene Identifier	Arabidopsis Genome Initiative Locus Identifier	Ratio	*P* Value	Description
LTPG1	EV178367	AT1G27950	2.73	0.0004	Glycosylphosphatidylinositol-anchored lipid protein transfer1 (LTPG1); GO: lipid transport
KAR5	CN830460	AT1G24360	2.46	0.0004	3-Oxoacyl-acyl-reductase5 (KAR5); GO: fatty acid biosynthesis process
ACOX3	TA34635_3708	AT1G06290	2.16	0.0014	Acyl-CoA oxidase3 (ACOX3); GO: fatty acid β-oxidation, medium-chain fatty acid metabolic process
FADS	ES267177	AT1G06090.1	1.52	0.0023	Fatty acid desaturase family protein (FADS); GO: lipid metabolic process, oxidation-reduction process
FAR4	TA27656_3708	AT3G11980	1.22	0.0012	Fatty acid reductase2 (FAR4); GO: oxidation-reduction process, pollen exine formation
PPAT	TA25260_3708	AT2G18250	1.08	0.0024	4-Phosphopantetheine adenylyltransferase (PPAT); GO: lipid metabolic process, lipid storage
LTP12	TA20970_3708	AT3G51590	0.91	0.0029	Lipid transfer protein12 (LTP12); GO: lipid transport, bind fatty acids and acyl-CoA esters and can transfer several different phospholipids
ACO2	DY023994	AT5G65110	0.85	0.0021	Acyl-CoA oxidase2 (ACO2); GO: fatty acid β-oxidation, long-chain fatty acid metabolic process, long-chain fatty acid biosynthesis
BCCP1	CB331865	AT5G16390	0.83	0.0027	Chloroplastic acetyl-CoA carboxylase1 (BCCP1); GO: fatty acid biosynthetic process, encodes for the biotin carboxyl-carrier subunit of the multienzyme plastidial acetyl-CoA carboxylase complex
PLC	EE464144	AT1G49740	−3.41	0.0001	PLC-like phosphodiesterase superfamily protein (PLC); GO: intracellular signal transduction, lipid metabolic process
SGNH	EV135949	AT2G38180	−2.95	0.0001	Hydrolase-type esterase superfamily protein (SGNH); GO: lipid metabolic process, metabolic process
ACO3	ES911417	AT1G06290	−2.73	0.0004	Acyl-CoA oxidase3 (ACO3); GO: fatty acid β-oxidation, medium-chain fatty acid metabolic process
KAS2	TA34448_3708	AT1G74960	−2.69	0.0005	β-Ketoacyl-ACP synthetase2 (KAS2); GO: unsaturated fatty acid biosynthetic process, involved in fatty acid elongation from 16:0-ACP to 18:0-ACP
PHS1	TC105857	AT4G14440	−1.72	0.0018	3-Hydroxyacyl-CoA dehydratase1 (PHS1); GO: fatty acid catabolic process, involved in unsaturated fatty acid degradation
ACPB	TA21354_3708	AT1G31812	−1.4	0.0016	Acyl-CoA-binding protein6 (ACBP); GO: lipid transport, response to absence of light, response to cold, response to freezing
PLC	BQ704400	AT3G08510	−1.4	0.0013	Phospholipase C (PLC); GO: lipid metabolic process, metabolic process, signal transduction
GLIP1	DY024637	AT3G48460	−1.39	0.0031	GDSL-like lipase (GLIP1); GO: lipid metabolic process, metabolic process
ECH	TA26530_3708	AT4G29010	−1.36	0.001	Enoyl-CoA hydratase/isomerase family (ECH); GO: fatty acid β-oxidation, flower development, jasmonic acid biosynthetic process
FAR3	CB686175	AT4G33790	−1.35	0.0007	Fatty acid reductase3 (FAR3); GO: microsporogenesis, oxidation-reduction process, wax biosynthetic process
FADS2	TA21960_3708	AT3G12120	−1.34	0.0011	Fatty acid desaturase2 (FADS2); GO: lipid metabolic process, oxidation-reduction process, responsible for the synthesis of 18:2 fatty acids
ECHIb	CX278875	AT4G14430	−1.28	0.0026	Enoyl-CoA hydratase/isomerase b (ECHIb); GO: fatty acid catabolic process, involved in unsaturated fatty acid degradation
LTP	CD843685	AT5G64080	−1.23	0.0025	Lipid-transfer protein (LTP); GO: lipid transport
KCS2	TC95262	AT1G04220	−1.2	0.0035	3-Ketoacyl-CoA synthase2 (KCS2); GO: biosynthesis of very-long-chain fatty acids
ACPD	TA19551_3708	AT3G02630	−1.06	0.001	Stearoyl-acyl-carrier-protein desaturase family protein (ACPD); GO: fatty acid biosynthetic process, oxidation-reduction process
HSP	CD822549	AT3G49050	−1.06	0.0019	α/β-Hydrolase superfamily protein (HSP); GO: lipid catabolic process, lipid metabolic process
LTP	EV148422	AT1G36150	−1.02	0.0016	Lipid-transfer protein (LTP); GO: lipid transport
LTP	CX192351	AT1G18280	−0.83	0.0025	Lipid-transfer protein (LTP); GO: lipid transport

Table III. *Microarray analysis of tt16 RNAi plants: genes involved in gynoecium and embryo development in siliques (2 DAF) of tt16 RNAi plants*

Microarray data were analyzed using the open-source R statistical programming language and Bioconductor packages with $P < 0.01$ (Gentleman et al., 2004; R Development Core Team 2010). GO, Gene Ontology categories.

Gene Name	Gene Identifier	Arabidopsis Genome Initiative Locus Identifier	Ratio	*P* Value	Description
Pepper	ES902629	AT4G26000	3.26	0.0018	Pepper; GO: gynoecium development, shoot development
EMB1865	EE453093	AT3G18390	2.46	0.0015	Embryo defective1865 (EMB1865); GO: embryo development ending in seed dormancy
LEA	TC104467	AT2G44060	2.32	0.0004	Late embryogenesis abundant protein (LEA); GO: embryo development ending in seed dormancy
EMB1745	EV057385	AT1G13120	1.78	0.0005	Embryo defective1745 (EMB1745); GO: embryo development ending in seed dormancy
EMB1738	TA24074_3708	AT1G11680.1	1.73	0.0012	Embryo defective1738 (EMB1738); GO: embryo development ending in seed dormancy
L19e	TC101398	AT1G02780	1.7	0.0027	Ribosomal protein L19e family protein (L19e); GO: embryo development ending in seed dormancy
EDA9	TA29939_3708	AT4G34200.1	1.37	0.0004	Embryo sac development arrest9 (EDA9); GO: megagametogenesis
EMB161	EG019088	AT5G27740	1.23	0.0009	Embryo defective161 (EMB161); GO: embryo development ending in seed dormancy
GFA1/MEE5	TA30692_3708	AT1G06220.1	1.09	0.0035	Gametophyte factor1 (GFA1)/maternal effect embryo arrest5 (MEE5); GO: embryo development ending in seed dormancy, regulation of embryo sac egg cell differentiation
LEA4-1	EE407756	AT1G32560	1.07	0.0016	Late embryogenesis abundant4-1 (LEA4-1); GO: embryo development ending in seed dormancy, seed development
EDA2474	TA33458_3708	AT3G46560	0.98	0.0019	Embryo defective2474 (EDA2474); GO: embryo development ending in seed dormancy
EDA25	EE414397	AT1G72440	0.96	0.0019	Embryo sac development arrest 25 (EDA25); GO: embryo sac development, polar nucleus fusion
OVA6	TC98067	AT5G52520	0.96	0.0027	Ovule abortion6 (OVA6); GO: embryo sac development, metabolic process, ovule development
MEE49	DY017410	AT4G01560	0.82	0.0033	Maternal effect embryo arrest49 (MEE49); GO: embryo development ending in seed dormancy
EDA3004	TC98127	AT3G06350	0.77	0.0032	Embryo defective3004 (EDA3004); GO: embryo development ending in seed dormancy
EDA1637	TA21841_3708	AT3G57870	−4.52	0.0001	Embryo defective1637 (EDA1637); GO: embryo development ending in seed dormancy
EDA2759	EV176859	AT5G63050	−3.25	0.0004	Embryo defective2759 (EDA2759); GO: embryo development ending in seed dormancy
PRD3	EE505648	AT1G01690	−2.8	0.0003	Putative recombination initiation defects 3 (PRD3); GO: embryo sac development
EDA1624	TA23462_3708	AT3G55620	−2	0.0007	Embryo defective1624 (EDA1624); GO: embryo development ending in seed dormancy
EDA1144	EE467621	AT1G48850	−1.78	0.0005	Embryo defective1144 (EDA1144); GO: embryo development ending in seed dormancy
LEA	TA23013_3708	AT3G17520	−1.75	0.0012	Late embryogenesis abundant protein family protein (LEA); GO: embryo development ending in seed dormancy
EDA1303	CD832555	AT1G56200	−1.73	0.0030	Embryo defective1303 (EDA1303); GO: embryo development ending in seed dormancy
EDA60	TC82547	AT1G01040	−1.63	0.0026	Embryo defective60 (EDA60); GO: regulation of seed maturation
EDA140	TC100011	AT4G24270	−1.32	0.0007	Embryo defective140 (EDA140); GO: embryo development ending in seed dormancy
EDA1401	EV093340	AT5G20920	−1.26	0.0017	Embryo defective1401 (EDA1401); GO: embryo development ending in seed dormancy
EDA1211	EE474572	AT5G22640	−1.26	0.0022	Embryo defective1211 (EDA1211); GO: embryo development, embryo development ending in seed dormancy
EDA1138	TC108258	AT5G26742	−1.11	0.0013	Embryo defective1138 (EDA1138); GO: embryo development ending in seed dormancy
LEA	TA24711_3708	AT3G53040	−1.07	0.0013	Late embryogenesis abundant protein (LEA); GO: embryo development ending in seed dormancy
EDA1401	BQ704950	AT5G20920	−1.01	0.0022	Embryo defective1401 (EDA1401); GO: embryo development ending in seed dormancy
LEA	DY002842	AT4G21020	−0.89	0.0030	Late embryogenesis abundant protein family protein (LEA); GO: embryo development ending in seed dormancy

Table IV. *Microarray analysis of tt16 RNAi plants: genes involved in auxin signal transduction and transport in 2-DAF siliques of tt16 RNAi plants*

Microarray data were analyzed using the open-source R statistical programming language and Bioconductor packages with $P < 0.01$ (Gentleman et al., 2004; R Development Core Team 2010). GO, Gene Ontology categories.

Gene Name	Gene Identifier	Arabidopsis Genome Initiative Locus Identifier	Ratio	*P* Value	Description
IAA27	EV128512	AT4G29080	3.49	0.0005	IAA inducible27 (IAA27); GO: regulation of translation, response to auxin stimulus
WXR1	TA34132_3708	AT2G31190	2.95	0.0002	Weak auxin response1 (WXR1); GO: auxin polar transport, response to UV-B
PIN3	TC103218	AT1G70940	1.62	0.0031	Pin-formed3 (PIN3); GO: auxin polar transport
TIR3	EV025801	AT3G02260	1.57	0.0015	Transport inhibitor response3 (TIR3); GO: auxin polar transport, response to auxin stimulus
ILR1	TC102010	AT3G02875	1.34	0.0026	IAA-Leu resistant1 (ILR1); GO: auxin metabolic process, metabolic process, proteolysis
ARF8	TC64476	AT5G37020	1.14	0.0029	Auxin response factor8 (ARF8); GO: regulation of transcription, response to auxin stimulus
IAA13	CX187822	AT2G33310.3	1.04	0.0007	Auxin-induced protein13 (IAA13); GO: regulation of transcription, response to auxin stimulus
MYB15	TC91892	AT3G23250	0.84	0.0025	Myb domain protein15 (MYB15); GO: regulation of transcription, response to auxin stimulus
ARF17	EE455835	AT1G77850	−3.94	0.0003	Auxin response factor17 (ARF17); GO: auxin-mediated signaling pathway, regulation of transcription
RBX1	TC92162	AT5G20570	−2.57	0.0002	RING box1 (RBX1); GO: protein ubiquitination, response to auxin stimulus
Auxin homeostasis gene	CD818157	AT2G32410	−2.07	0.0026	Auxin homeostasis; GO: auxin homeostasis, auxin-mediated signaling pathway
BRX	EE465134	AT1G31880	−1.76	0.0018	Brevis radix (BRX); GO: auxin-mediated signaling pathway
ETA3	DY002982	AT4G11260	−1.26	0.0016	Enhancer of tir1-1 auxin resistance3 (ETA3); GO: auxin-mediated signaling pathway
AXR1	TC75767	AT1G05180	−1.21	0.0007	Auxin resistant1 (AXR1); GO: auxin homeostasis, auxin-mediated signaling pathway
CNX1	CD836602	AT5G20990	−1.16	0.0022	Molybdopterin biosynthesis protein (CNX1); GO: auxin-mediated signaling pathway
GH3	EE455254	AT4G03400	−1.1	0.0015	Auxin-responsive GH3 family protein (GH3); GO: response to auxin stimulus, response to light stimulus
TIR1	ES900600	AT3G62980	−0.94	0.0018	Transport inhibitor response1 (TIR1); GO: auxin-mediated signaling pathway, response to auxin stimulus

in transgenic plants (Fig. 4). The fertilization process begins when the pollen grain germinates on the female stigmatic cells. Then, pollen tubes emerge at the base of the stigmatic papillae and grow down to the style and through the transmitting tissue of the style and septum (Hill and Lord, 1987; Lennon et al., 1998). When the pollen tube emerges from the septum, it grows over the surface of the septum to a funiculus and then grows along the funicular surface to the micropylar opening of the ovule, where it enters to release the sperm cells (Yadegari and Drews, 2004). Pollen tubes are guided through female tissues until they turn toward an available ovule. The two synergid cells in the female gametophyte have important roles in pollen tube guidance (Shimizu and Okada, 2000; Higashiyama et al., 2001; Okuda et al., 2009). The egg cells and central cells also play a role in pollen tube guidance (Chen et al., 2007; Alandete-Saez et al., 2008). *GFA1/MEE5*, which regulates egg cell differentiation in the embryo sac, was down-regulated in transgenic plants (Table III; Pagnussat et al., 2005; Liu et al., 2009). Based on our study, the down-regulation of *GFA1/MEE5* in *tt16* RNAi transgenic plants may have affected the development of egg cells, resulting in impaired guidance of pollen tubes to the ovule.

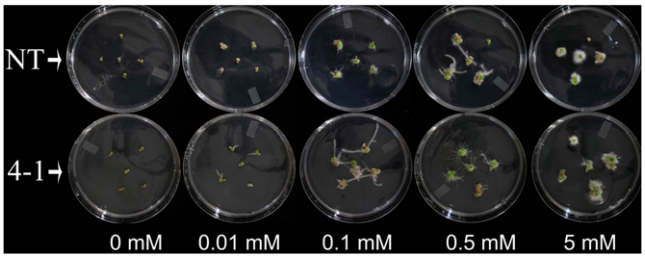

Figure 8. Auxin dose response of *tt16* RNAi transgenic lines. Hypocotyl explants of 9-d-old canola seedlings were incubated on one-half-strength Murashige and Skoog medium containing the indicated naphthaleneacetic acid concentrations for 20 d. 4-1, *tt16* RNAi transgenic line 4-1.

Lipids are essential for the growth and development of plants. Although many of the reactions in storage lipid metabolism have been well studied in plants, less is known about their regulatory mechanisms, especially at the transcriptional level. A few transcription factors have been identified as potential regulators of storage lipid biosynthesis in plants. *WRI1*, for example, can enhance the transcription of FA biosynthetic genes, including *BCCP2* (Baud et al., 2009). *LEC2* directly regulates *WRI1*, which, in turn, controls the expression of a subset of genes involved in late glycolysis and FA biosynthesis as well as the biosynthesis of biotin and lipoic acids (Baud et al., 2007). Overexpression of the Arabidopsis *LEC1* gene causes globally increased expression of FA biosynthetic genes, encoding products involved in key reactions of condensation, chain elongation, and desaturation of FA biosynthesis (Mu et al., 2008). In our study, we found that down-regulation of four *TT16* genes resulted in decreased FA content (Table I) and oil body abundance (Fig. 6). Consistent with this observation, *tt16* RNAi altered the expression of some genes encoding enzymes and/or proteins involved in the lipid metabolism pathway.

Microarray analysis showed that transcripts of two FA synthesis genes, *KAR5* and *BCCP1*, accumulated at lower rates than in NT plants (Table II). The KAR enzyme catalyzes the first reduction step in FA biosynthesis (Harwood, 1988). O'Hara et al. (2007) reported that antisense expression of the canola *KAR* gene results in reduced seed oil content and seed yield. BCCP1 is an essential subunit for a heteromeric acetyl-CoA carboxylase, which catalyzes the ATP-dependent formation of malonyl-CoA in the first committed step of the FA biosynthetic pathway (Ohlrogge and Browse, 1995). Down-regulation of *BCCP1* decreases FA accumulation in seeds and severely affects normal vegetative plant growth (Li et al., 2011). The down-regulation of *KAR5* and *BCCP1* expression in siliques (2 DAF) may explain the decreased FA accumulation in *tt16* RNAi transgenic plants. We speculate that *TT16s* may directly or indirectly interact with the cis-regulatory elements of *KAR5* and *BCCP1* genes, thereby regulating FA synthesis in canola seeds. Down-regulation of these genes in the *tt16* RNAi lines is consistent with the reduced seed oil content in mature seeds.

The changes observed in seed FA composition in *tt16* RNAi plants (Table I) may also be explained by our microarray data. The down-regulation of *β-Ketoacyl-ACP Synthetase2* (*KAS2*) in transgenic seeds is consistent with the observed increase in 16-carbon FA in the RNAi lines, which occurred primarily at the expense of the total 18-carbon FA. KAS2 is involved in the elongation of FA from palmitic acid (16:0) to stearic acid (18:0). The up-regulation of *Fatty Acid Desaturase2* (*FADS2*), encoding a Δ12 desaturase activity, is in agreement with the observed increase in $18{:}2^{cis\Delta 9,12}$ in the transgenic lines. Moreover, the content of very-long-chain FA (20- and 22-carbon FA) in transgenic plants is higher than that in NT plants (Table I). This result may be explained by the up-regulation of *3-Ketoacyl-CoA Synthase2* (*KCS2*), which is required for the biosynthesis of these longer FA (Lee et al., 2009).

We also found that the embryos of *tt16* RNAi transgenic lines develop abnormally beyond 20 DAF (Fig. 5). In canola, the synthesis and accumulation of storage lipids in the embryo occurs between about 3 and 6 weeks after flowering (Weselake et al., 2009). The abnormal embryo development may be due, at least partly, to the decreased synthesis of storage oil, although we could not rule out other possibilities. Indeed, other studies have shown that some genes encoding components of FA synthesis play important roles in embryo and seed development in Arabidopsis and canola (O'Hara et al., 2007; Wu and Xue, 2010; Li et al., 2011).

The phytohormone auxin regulates essential aspects of plant growth and developmental processes (Friml, 2003). Auxin regulates gene expression through a ubiquitin-dependent proteolytic signal transduction system (Dharmasiri and Estelle, 2004). The auxin/indole-3-acetic acid (Aux/IAA) proteins are able to repress the activity of Auxin Response Factor (ARF) proteins (Tiwari et al., 2001, 2004). Increased auxin reduces the levels of Aux/IAA proteins by accelerating their degradation (Zenser et al., 2001), such that ARF activity is derepressed and numerous auxin-mediated transcriptional changes occur (Tiwari et al., 2001, 2004). Aux/IAA proteins act as negative regulators of the auxin response (Wang et al., 2005). ARF proteins can be either activators or repressors of auxin-related gene transcription (Vanneste and Friml, 2009). In this study, it is interesting that down-regulation of *tt16s* confers increased sensitivity to auxin (Fig. 8). Microarray data showed that two AUX/IAA genes, *IAA27* and *IAA13*, are down-regulated in transgenic plants, which may explain why the transgenic plants show more sensitivity to auxin. Also, the fact that *tt16* RNAi transgenic lines had fewer inflorescences and flowers suggests that the plants are more sensitive to auxin (Mockaitis and Estelle, 2008; Deng et al., 2012), which further confirms the conclusion that down-regulation of *tt16s* confers increased sensitivity to auxin. However, analysis of the root growth of transgenic plants showed that there was no obvious difference between the *tt16* RNAi and NT plants. This result indicates a complex mechanism of *tt16* and auxin signal transduction in the regulation of root development in canola.

In summary, our data demonstrate that TT16 plays roles in pollen tube guidance, FA synthesis, and embryo development in addition to its known role in endothelium development in the seed coat. These results expand our knowledge about the physiological functions of B_{sister} MADS proteins in plant development and provide valuable information for improving canola quality by using modern breeding biotechnologies.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Canola (*Brassica napus* double haploid line DH12075) plants were grown in the greenhouse or growth chamber. The growth conditions were set as

follows: 16-h-day/8-h-night cycle, 25°C/20°C day/night temperature, 60% relative humidity, 250 μmol m^{-2} s^{-1} light intensity. Canola plants were transformed as described by Bondaruk et al. (2007).

Light Microscopy

Mature seeds were fixed in formaldehyde:acetic acid:ethanol (5:5:90) for 24 h. Fixed tissues were dehydrated in a series of ethanol-toluene concentrations and embedded in paraffin wax at 56°C. Sections of 8 μm thickness were cut with a rotary microtome and fixed on glass slides. Sections were dewaxed with toluene and then stained with 0.01% toluidine blue. Observations were made with a light microscope.

Pollination Assay and Fluorescence Observation of Pollen Tube Development

For hand pollination, large flower buds (1 d before anthesis) were emasculated and covered with pollination bags. Tagged pistils were hand pollinated 1 d later, covered for 5 to 7 d for seed production, and then left uncovered to maturity. The pistils (2 DAF) were embedded in paraffin, and sections were made with a rotary microtome. Style sections were stained with aniline blue and observed with a Leica DMRXA epifluorescence microscope. For fluorescence observation of pollen tube extension to ovules, pistils (2 DAF) were fixed with 3:1 95% ethanol:glacial acetic acid overnight and softened with 8 M sodium hydroxide for 2 d. Then, the pistils were stained for 3 h with 0.05% aniline blue and mounted in a drop of 50% glycerin. Samples were observed with a Leica DMRXA epifluorescence microscope.

Electron Microscopy

Mature embryos were fixed in 2% glutaraldehyde in 75 mM phosphate buffer, pH 7.2, and were postfixed in 2% osmium in the same buffer. An ethanol dehydration series was performed, and the samples were embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate followed by lead citrate. Micrographs were taken using a Philips/FEI Morgagni transmission electron microscope.

Microarray Analysis

Total RNA was isolated from the siliques (2 DAF) using an RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. Quantity and purity of RNA were measured with a NanoDrop 1000 (PEQLAB Biotechnologie). Microarray analysis was performed using the Agilent 4x44k Brassica Gene Expression Microarray (Agilent Technologies). Cy3-labeled complementary RNA was produced with the Quick Amp Labeling Kit, one-color (Agilent Technologies), and hybridized to the microarrays according to the manufacturer's instructions. Hybridized and washed slides were scanned at 5-μm resolution with a GenePix 4000B scanner (Molecular Devices). Image processing was performed with Feature Extraction Software 10.5.1.1 (Agilent Technologies). Microarray data were analyzed using the open-source R statistical programming language and Bioconductor packages (Gentleman et al., 2004, R Development Core Team 2010).

Quantitative Reverse Transcription-PCR

RNA was extracted using the RNeasy plant mini kit (Qiagen). DNase-treated RNA was then reverse transcribed using the QuantiTect Rev Transcription kit (Qiagen). Quantitative reverse transcription-PCR was performed to confirm microarray results according to the method reported previously by Chen et al. (2010). The primers used in this assay are listed in Supplemental Table S1.

Auxin Dose-Response Experiments

Hypocotyl explants of 9-d-old seedlings from NT and *tt16* RNAi were incubated on one-half-strength Murashige and Skoog medium containing the indicated naphthaleneacetic acid concentrations in growth chamber conditions described as above for 20 d.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU192028, EU192029, HM449989, and HM449990.

Supplemental Data

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

Supplemental Figure S1. Short siliques of the *tt16* RNAi transgenic line.

Supplemental Table S1. Primers used in this study.

Supplemental Table S2. The lengths of sepals, stamens, and pistils of open flowers and buds in RNAi plants (line 4-1) and NT plants.

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