

## ORIGINAL ARTICLE

# TRANSPARENT TESTA 4-mediated flavonoids negatively affect embryonic fatty acid biosynthesis in *Arabidopsis*

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## Abstract

Flavonoids are involved in many physiological processes in plants. TRANSPARENT TESTA 4 (TT4) acts at the first step of flavonoid biosynthesis, and the loss of TT4 function causes a lack of flavonoid. Flavonoid deficiency is reportedly the main cause of increased fatty acid content in pale-coloured oilseeds, but details regarding the relationship between seed flavonoids and fatty acid biosynthesis are elusive. In this work, we applied a genetic strategy combined with biochemical and cytological assays to determine the effect of seed flavonoids on the biosynthesis of fatty acids in *Arabidopsis thaliana*. We showed that TT4-mediated flavonoids negatively affect embryonic fatty acid biosynthesis. A crossing experiment indicated that seed flavonoid biosynthesis and the impact of this process on fatty acid biosynthesis were controlled in a maternal line-dependent manner. Loss of TT4 function activated glycolysis in seed embryos, thereby enhancing fatty acid biosynthesis, but did not improve seed mucilage production. Moreover, loss of TT4 function reduced *PIN-FORMED 4* expression and subsequently increased auxin accumulation in embryos. Pharmacologically and genetically elevated auxin levels enhanced seed fatty acid biosynthesis. These results indicated that flavonoids affect fatty acid biosynthesis by carbon source reallocation via regulation of *WRINKLE1* and auxin transport.

## KEYWORDS

*Arabidopsis*, auxin, fatty acids, flavonoids, *PIN4*, TRANSPARENT TESTA4

## 1 | INTRODUCTION

Flavonoids, which consist of flavonols, anthocyanin, and proanthocyanidins (PAs), are mainly present as pigments in the seeds and flowers of *Arabidopsis*. Flavonoids perform a range of biological functions, such as protection against microbes and UV light, defense against environmental stress, and allelopathy (Lotkowska et al., 2015; Pollastri & Tattini, 2011; Zhang, Abrahan, Colquhoun, & Liu, 2017). TRANSPARENT TESTA (TT) family members function as either transcription factors or enzymes in flavonoid biosynthesis pathways in *Arabidopsis*. Among these members, CHALCONE SYNTHASE (CHS;

encoded by the *TT4* gene) catalyses the first step of flavonoid biosynthesis to produce naringenin chalcone, a common precursor of various flavonoids (Burbulis, Iacobucci, & Shirley, 1996; Pollastri & Tattini, 2011; Shirley et al., 1995). Mutation of *tt4* causes abnormal CHALCONE SYNTHASE activity, and seed coats of *tt4* mutants exhibit a pale yellow colour, resulting from flavonoid deficiency, whereas wild-type (WT) seed coats exhibit a normal brown or dark brown colour due to the oxidation of PAs (Burbulis et al., 1996; Debeaujon, Leoon-Kloosterzie, & Koornneef, 2000; Koornneef, 1990; Lepiniec et al., 2006; Shirley et al., 1995). TT2, TT8, and TTG1 are the important transcriptional factors regulating the biosynthesis of PAs in *Arabidopsis* seeds. The mutation of these genes results in defect of PA production, but flavonols are still detectable (Routaboul et al., 2006).

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In *Arabidopsis* seeds, 33% to 43% of the storage compounds are fatty acids (FAs) and FA-derived complex lipids that serve as carbon and energy reserves. *Arabidopsis* is a member of the *Brassicaceae* family and is a close relative of *Brassica* oilseeds, which are important sources of vegetable oil worldwide; thus, *Arabidopsis* serves as a good model to study the mechanism of seed lipid formation. Lipid biosynthesis in *Arabidopsis* starts with acetyl-CoA, which is a glycolytic intermediate. FA synthases catalyse the transfer of a malonyl moiety to the acyl carrier protein (ACP) by adding two carbons to the elongating chain. The formation of C16:0-ACPs and C18:0-ACPs as well as the successful elongation of unsaturated FAs occur in the plastids. The formation of very long-chain FAs (VLCFAs;  $C \geq 20$ ) occurs at the endoplasmic reticulum (ER), where malonyl-CoA from the cytosol is used for VLCFA elongation (Baud & Lepiniec, 2009). Malonyl-CoA also serves as a substrate for flavonoid biosynthesis and reacts with 4-coumaroyl-CoA, under the activity of TT4, in the cytosol (Bharti & Khurana, 2003; Sasaki, Konishi, & Nagano, 1995). Genes such as *CARBOXYLASE* subunits (*CAC2* and *CAC3*), *BIOTIN CARBOXYL CARRIER PROTEIN2* (*BCCP2*), *MOSAIC DEATH1* (*MOD1*), *CYTIDINEDIPHOSPHATE DIACYLGLYCEROL SYNTHASE2* (*CDS2*), *FATTY ACID BIOSYNTHESIS2* (*FAB2*), and *FATTY ACID DESATURASE 2* (*FAD2*) play critical roles in the FA biosynthesis pathway (Baud & Lepiniec, 2009).

The development of an *Arabidopsis* seed involves two steps, namely, embryonic morphogenesis (EM) and maturation. A torpedo-shaped embryo indicates the accomplishment of EM, which involves a series of programmed cell division steps starting from double fertilization. Seed maturation follows EM and starts 7 to 20 days after pollination (DAP), leading to the accumulation of storage compounds and acquisition of dormancy induction and desiccation tolerance (Baud, Boutin, Miquel, Lepiniec, & Rochat, 2002; Jenik, Gillmor, & Lukowitz, 2007). Seed embryogenesis is regulated by five key transcription factors, namely, *LEAFY COTYLEDON2* (*LEC2*), *LEC1*, *FUSCA3* (*FUS3*), *WRINKLE1* (*WRI1*), and *ABSCISIC ACID INSENSITIVE3* (*ABI3*); together, these factors induce more than 90% of the downstream genes for seed development (Baud et al., 2007; Baud & Lepiniec, 2009; Cernac & Benning, 2004; Delmas et al., 2013; Elahi, Duncan, & Stasolla, 2015; Horstman, Berner, & Boutilier, 2017; Stone et al., 2001; Stone et al., 2008). Although *LEC1* and *LEC2* are expressed at the EM stage and the expression levels of these genes peak at 5 DAP, these genes remain detectable during the seed maturation phase (Stone et al., 2001; Stone et al., 2008). *WRI1* specifies the regulatory action of *LEC2* toward FA metabolism during seed maturation and regulates the genes involved in late glycolysis and early FA biosynthesis (Baud et al., 2007; Baud & Lepiniec, 2009). The overexpression of *WRI1* resulted in increased oil accumulation in both seeds and developing seedlings (Cernac & Benning, 2004). In addition, *FUS3* induces numerous genes involved in photosynthesis and in FA biosynthesis pathways. *ABI3* functions as a regulator of seed desiccation tolerance, seed longevity, and chlorophyll degradation via the abscisic acid (ABA) response pathway (Delmas et al., 2013; Wang et al., 2014). Recently, *TT2* was found to regulate embryonic FA biosynthesis by targeting *FUS3* during the early seed developmental stage (Wang et al., 2014). *TT8* inhibits seed FA accumulation by targeting several seed developmental regulators, such as *LEC2*, *LEC1*, *FUS3*, and *CDS2*.

These studies indicate a close relationship between flavonoids and FA biosynthesis in seeds (Chen et al., 2014; Wang et al., 2014) and suggest that different TT members regulate and/or impact FA accumulation in seeds via different mechanisms.

A mature *Arabidopsis* seed comprises the seed coat, endosperm, and embryo. These components of the seeds have distinct genetic origin and have different effects on seed development. The seed coat is a sporophytic tissue that surrounds and protects the embryo and endosperm (Debeaujon et al., 2000). The endosperm supplies nutrients to the embryo and degrades into a monolayer in mature seeds (Stone et al., 2008). The embryo is a major organ for the synthesis and accumulation of storage compounds (Baud & Lepiniec, 2009). Unlike embryo and endosperm that have both parent origins, seed coat has a maternal origin (Debeaujon et al., 2000). Flavonols and PAs are the two main types of flavonoids in *Arabidopsis* seeds. Rhamnose and glucose are substrates for flavonoid synthesis. Quercetin-3-O-rhamnoside (Q-3-O-R) is a main type of flavonol in seeds, and an overwhelming majority of Q-3-O-R is localized in seed coats (Routaboul et al., 2006). Mucilage, which is mainly composed of cell wall polysaccharides, is deposited in the outer integument layer attached to the seed coat (Western, Skinner, & Haughn, 2000). Rhamnose is a substrate for both mucilage and flavonoid biosynthesis (Oka, Nemoto, & Jigami, 2007; Shi, Katavic, Yu, Kunst, & Haughn, 2012). In *Arabidopsis*, sugars are first delivered to the maternal seed coat via the funicular phloem, which is symplastically connected to the outer integument and then reach the embryo (Stadler, Lauterbach, & Sauer, 2005).

Auxin, in the form of indole-3-acetic acid (IAA), plays an important role in various aspects of plant growth and development, from promoting root formation to inhibiting bud outgrowth and delaying senescence (Mueller-Roeber & Balazadeh, 2014). IAA moves through the shoot from tip to base and regulates growth in a concentration- and tissue-specific manner. Auxin transport is facilitated by auxin efflux carriers, including PIN-FORMED (PIN) proteins, which have polar distributions on the cell membranes (Heisler et al., 2005).

Pale-coloured seeds are of interest to oilseed breeders owing to the elevated total FA content of these seeds (Shirzadegan & Röbbelen, 1985; Tang, Li, Zhang, Chen, & Wang, 1997). However, details regarding the mechanism via which flavonoids affect FA formation in seeds remain elusive. In this study, we show that *TT4*-mediated flavonoids negatively affect embryonic FA biosynthesis. The effects of flavonoids on FA biosynthesis were regulated in a maternal line-dependent manner. Loss of *TT4* function accelerated the seed glycolysis pathway, thereby enhancing FA biosynthesis, but did not benefit seed mucilage production. The loss of *TT4* function reduced *PIN4* expression and subsequently gave rise to embryonic auxin accumulation, which enhanced the expression of seed developmental regulators such as *WRI1* and a variety of enzymes important for FA biosynthesis.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant materials and growth conditions

The *tt4* mutants, namely, CS85 (*tt4-1*), with a *Ler* background, and CS66120 (*tt4-4*) and CS66121 (*tt4-5*), with a *Col-0* background, were

ordered from the Arabidopsis Biological Resource Center (<https://www.arabidopsis.org/>). The Col-0 seeds carrying the *DR5::GUS* construct were donated by Dr. Junhui Wang of Zhejiang University. The *tt4* mutant seeds harbouring *DR5::GUS*, based on the donated seeds, were generated by us. The *gl2 tt4-5* double mutant was previously produced in our own lab (Wang et al., 2014). The transformants harbouring *35S::iaaM* and *35::iaaL* were given by Dr. Hong-Quan Yang (Zhang, He, Li, & Yang, 2014). The primers used for genotyping are listed in Table S2. Pretreatment of seeds for sowing and growth conditions for the plants followed our previous descriptions (Chen et al., 2012; Chen et al., 2014; Wang et al., 2014).

## 2.2 | Morphological observations of mature seeds

Siliques and seeds were randomly collected from the basal part of the main inflorescence of a plant. A Leica stereomicroscope (Leica DFC300 FX, Wetzlar, Germany) was used for observation and photography.

## 2.3 | Determination of FA, soluble sugar, and starch content in seeds

The extraction of seed FAs was conducted as our previous description (Wang et al., 2014). In brief, 10 mg of seeds was extracted with 4 ml of extraction solution (or 100 seeds were extracted with 1 ml of extraction solution). The total FA content of the seeds was analysed using gas chromatography (GC; GC-2014 Gas Chromatograph, Shimadzu, Japan). The GC instrument was equipped with a flame ionization detector. Methyl heptadecanoate was used as the internal standard. The temperature programme used with a Supelcowax 10 column was as follows: 160 °C for 1 min, 4 °C/min till 240 °C, and hold for 16 min. Determination of the soluble sugar and starch content was performed as previously described (Clegg, 1956).

## 2.4 | RNA isolation and real-time quantitative PCR

The total RNA from *Arabidopsis* siliques at 6, 9, 12, and 15 DAP and of developing seeds collected from in vitro culture media (ICM) were extracted using an RNA extraction kit (cat. no. R6827-1, Omega Biotek Inc., USA). First-strand cDNA was synthesized using a PrimeScript™ First Strand cDNA Synthesis Kit (cat. no. 6110A, TaKaRa, Japan). EF1aA4 was used as an internal control, and SoFast™ EvaGreen® Supermix (cat. no. 1725201, Bio-Rad, USA) was used for the real-time quantitative PCR (RT-qPCR) experiments. The primer pairs used for the RT-qPCR experiments are listed in Tables S3–S6. Threads with different colours were used for tagging flowers, and seeds were collected at different DAP.

## 2.5 | Subcellular localization of TT4

Subcellular localization was conducted as previously described (Liu et al., 2016). The pCambia1300 (*35S::YFP-NOS*) plasmid and tobacco seedlings carrying the *35S::RFP-H2B* nuclear indicator were provided by the author of the abovementioned study. The coding sequence of *TT4* was amplified and inserted into the pCambia1300 (*35S::YFP-NOS*) vector to generate the *35S::YFP-TT4-NOS* construct,

which was verified by DNA sequencing. Primers used for amplification of the fragments are listed in Table S7. *Agrobacterium tumefaciens* strain GV3101 carrying the *35S::YFP-TT4-NOS* construct was propagated to infect healthy tobacco leaves that were approximately 1 month old, wherein *35S::RFP-H2B* was harboured as a nuclear indicator. The fluorescence signals were observed and photographed using a confocal microscope (Zeiss LSM510, Jena, Germany).

## 2.6 | Construction of the *TT4<sub>Promoter</sub>::GUS* molecular cassette and plant transformation

To generate the *TT4<sub>Promoter</sub>::GUS* construct, we amplified approximately 2,000 bp of the upstream fragment of the *TT4* coding sequence from Col-0 genomic DNA using the primers listed in Table S8. The PCR-amplified fragment was cloned into a modified pCambia1300 vector carrying the  $\beta$ -glucuronidase (GUS) reporter (<http://www.cambia.org/daisy/cambia/585.html>). Construction was verified by DNA sequencing. *A. tumefaciens* strain GV3101 carrying the above construct was used to transform Col-0 plants by the floral dip method (Clough & Bent, 1998). The expected transgenic seedlings, which survived on Murashige and Skoog medium (Murashige & Skoog, 1962) containing 30 mg/ml hygromycin, were further validated by PCR. T<sub>3</sub> transgenic plants were verified and used for GUS staining.

## 2.7 | GUS histological assay

The GUS histological assay was performed as described by Shi et al. (2012). Seeds were collected from *Arabidopsis* siliques and ICM. Soft pressing of the seeds was necessary for permeation of the GUS staining buffer. After overnight culturing, embryos were observed and photographed with a compound light microscope (Olympus, Tokyo, Japan).

## 2.8 | Grafting experiment

The grafting experiment was conducted after seedling bolting when the stem was about 4 cm (3–5 cm) in height. Healthy seedlings at similar growth stages were selected for grafting. Stem connecting the base of the rosette leaves was cut with a sharp knife. A transparent silicone tube (internal diameter: 0.5 mm) was used to connect and fix the scion and stock. Transparent plastic gloves were used to cover the combination of tissues for approximately 1 week to ensure a suitable environment for successful grafting. There were obvious inflated nodules at the joint.

## 2.9 | Flavonoid observation

Seeds were dipped in saturated (0.25%, w/v) diphenylboric acid 2-aminoethyl ester (DPBA; cat. no. D9754, Sigma, USA) containing 0.005% (v/v) Triton X-100 (cat. no. T8787, Sigma, USA) overnight. DPBA fluorescence was visualized and photographed under a confocal microscope (Zeiss LSM510, Jena, Germany) according to a previously described method (Buer, Muday, & Djordjevic, 2007).

## 2.10 | Ruthenium red staining of seed coat mucilage

Ruthenium red staining of seed coat mucilage was performed by following our previous described method (Wang et al., 2014). *Arabidopsis* seeds were randomly collected from the main branches of mature plants. After shaking in water for 1 hr, the seeds were dipped in 0.01% (w/v) ruthenium red (cat. no. 84071, Sigma, USA) solution for observation and photography with a compound light microscope (Olympus, Tokyo, Japan).

## 2.11 | Determination of chlorophyll and auxin content

Chlorophyll from *Arabidopsis* siliques was extracted with a mixed solution of acetone and ethanol ( $V_{\text{acetone}}:V_{\text{ethanol}} = 1:1$ ). Absorbance was detected at wavelengths of 645 and 663 nm with a spectrophotometer (UV-2450, Shimadzu, Japan). Auxin extraction was conducted in accordance with the method described by Zhao et al. (2015). Approximately 100 mg of siliques was ground into a powder with liquid nitrogen. Five hundred microliters of an extraction buffer ( $V_{\text{isopropanol}}:V_{\text{water}}:V_{\text{HCl}} = 2:1:0.005$ ) were added to each sample, and indole-3-acetic-2,2-d<sub>2</sub> acid (cat. no. 492817, Sigma, USA) was used as an internal standard. Liquid chromatography–tandem mass spectrometry (Thermo Scientific, San Jose, USA) was used to analyse the auxin content.

## 2.12 | In situ hybridization

In situ hybridization was performed as previously described (Lefebvre et al., 2006). Probes were synthesized by PCR amplification. The primers used for the PCR were as follows: PIN4-Forward (SP6), 5'-GAATTGATTAGGTGACACTATAGTTATTTCTCGGTAACGAGGA AATAAGCAAAGCCTTTATTT-3'; PIN4-Reverse (T7), 5'-GAATTG TAATACGACTCACTATAGGAGGAAGAGAGATTTTCTTCTTGGC GTCTAATGAAGACA-3'. The PCR product, which included the ~200 bp PIN4 cDNA sequence and the SP6/T7 promoter, was used as a template for probe synthesis.

## 2.13 | In vitro culture of *Arabidopsis* embryos

For observation of the polar distribution of auxin, the Col-0 and *tt*-5 seeds were collected at 7 DAP. The experiment was performed according to a previously described protocol (Sauer & Friml, 2004). ICM were prepared with or without the 50  $\mu\text{M}$  IAA. The ovules were cultured in the dark at 24 °C. Both the *tt*-5 and WT seeds were collected at the 12-DAP stage and cultured in different media, including (a) ICM (as a mock control); (b) ICM plus 50- $\mu\text{M}$  naphthylphthalamic acid, an auxin efflux inhibitor (Fujita & Syono, 1996); (c) ICM plus 25- $\mu\text{M}$  IAA; and (d) ICM plus 50- $\mu\text{M}$  IAA. After culturing for 3 days, the seeds were harvested for FA level measurement.

## 2.14 | Metabolite extraction and profiling

The experiment was performed by following the method described by Lisec, Schauer, Kopka, Willmitzer, and Fernie (2006). Approximately 100-mg siliques were ground into powder by using liquid nitrogen,

and the samples were transferred into 2-ml centrifuge tubes. A volume of 1.4 ml of pre-cooled 100% methanol was added, and the mixture was vortexed for 10 s. In addition, 60 ml of ribitol (0.2 mg/ml stock solution in ddH<sub>2</sub>O) was used as an internal standard. After all the extraction steps were completed, the metabolites in the samples were analysed with a 7890A GC/5975C MS system (Agilent, California, USA). The temperature programme was set as follows: 70 °C for 2 min; heat at 10 °C/min to 140 °C, 4 °C/min to 240 °C, and at 10 °C/min to 300 °C; hold at 300 °C for 8 min. After normalization, the data were imported into SIMCA-P software (version 11.5; <http://www.umetrics.com/simca>) for analysis of differentially expressed metabolites. Information on the metabolites was obtained from databases such as Kyoto Encyclopedia of Genes and Genomes (<http://www.genome.jp/kegg/>) and National Institute of Standards and Technology (<http://www.nist.gov/index.html>).

## 2.15 | Statistical analysis

After data collection, the data were classified with Microsoft Excel and analysed via one-way analysis of variance using the SPSS software package (version 8.0). The treatment means were compared by Tukey's test. Significance was indicated by  $p \leq 0.05$ .

# 3 | RESULTS

## 3.1 | Genetic interruption of *TT4* reduced flavonoid biosynthesis and increased seed total FA content

To determine whether flavonoids affect seed FA biosynthesis, we isolated three distinct alleles of *tt4* mutants (Figure S1a), namely, *tt4*-1 (Ler ecotype), *tt4*-4 (Col-0 ecotype), and *tt4*-5 (Col-0 ecotype). DNA sequencing results showed that the three alleles contained non-synonymous changes in amino acid codes (Figure S1b,c). To exclude mutations at unknown loci, we backcrossed the mutants with the corresponding WT parents for three generations. Consistent with previously described *tt4* mutant seed phenotypes (Debeaujon et al., 2000), the seeds of the three *tt4* allelic mutations exhibited a pale yellow colour (Figure S2). Although the *tt4* mutants showed reduced seed length (Figure S2), the embryo cotyledon and root sizes of these mutants were similar to those of the WT (Figure S3). Notably, all three *tt4* allelic mutants exhibited significantly increased total FA content and embryo weights of mature dry seeds (Table 1). The *tt4*-1, *tt4*-4, and *tt4*-5 seeds contained 45%, 48%, and 49% more FAs, respectively, than the corresponding WT seeds of the same dry weight, and single *tt4*-1, *tt4*-4, and *tt4*-5 seeds contained 45%, 46%, and 49% more FAs, respectively, than a single WT seed (Table 1). However, no significant changes in the ratios of various FA species were observed in the *tt4* mutant seeds (Table 1). Comparison of the dynamics of FA accumulation between *tt4*-5 and Col-0 revealed that *tt4*-5 and Col-0 had similar seed FA content at the EM stage. However, the seed FA content of *tt4*-5 seeds significantly surpassed that of Col-0 seeds at 15 DAP. We further compared the reduction in FA content between the mutant and WT seeds and found that the reduction was less significant in *tt4*-5 seeds (2.5  $\mu\text{g}/\text{mg}$ ) than in WT seeds (4.0  $\mu\text{g}/\text{mg}$ ). The *tt4*-5 seeds exhibited more FA biosynthesis but less FA



**TABLE 1** Comparisons of FA contents, compositions, and embryo weight of mature dry seeds between WT and *tt4* mutants

	Col-0	<i>tt4-4</i>	<i>tt4-5</i>	<i>Ler</i>	<i>tt4-1</i>
C16:0	0.61 ± 0.03	0.82 ± 0.02	0.93 ± 0.03	0.62 ± 0.03	0.85 ± 0.02
C18:0	0.15 ± 0.01	0.22 ± 0.01	0.22 ± 0.02	0.21 ± 0.02	0.29 ± 0.01
C18:1	0.87 ± 0.07	1.28 ± 0.06	1.31 ± 0.02	1.06 ± 0.03	1.51 ± 0.01
C18:2	1.84 ± 0.07	2.77 ± 0.11	2.64 ± 0.14	1.67 ± 0.03	2.53 ± 0.05
C18:3	1.54 ± 0.07	2.22 ± 0.17	2.34 ± 0.11	1.44 ± 0.04	2.02 ± 0.02
C20:0	0.09 ± 0.01	0.14 ± 0.02	0.14 ± 0.01	0.14 ± 0.01	0.19 ± 0.01
C20:1	0.59 ± 0.26	0.91 ± 0.13	0.99 ± 0.10	0.93 ± 0.05	1.40 ± 0.11
C22:0	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
C22:1	0.10 ± 0.02	0.13 ± 0.03	0.12 ± 0.02	0.10 ± 0.02	0.18 ± 0.02
C24:0	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.03 ± 0.00
Total FA (μg/mg)	283.21 ± 15.83	419.20 ± 12.61*	422.30 ± 11.34*	328.47 ± 12.56	477.90 ± 14.25*
Total FA (μg/seed)	5.85 ± 0.55	8.56 ± 0.59*	8.73 ± 0.46*	6.24 ± 0.22	9.02 ± 0.25*
Embryo weight (μg)	11.58 ± 0.26	13.39 ± 0.26*	13.25 ± 0.53*	10.50 ± 0.14	12.35 ± 0.65*

Note. The asterisks (\*) show significant differences from the respective wild type. *tt4-4* and *tt4-5* are mutants of Col-0 background, and *tt4-1* is of *Ler* background. The values are means of three biological replicates. Following ± are standard deviations (SD). FA: fatty acid.

degradation (Figure S4), indicating that genetic impairment of flavonoid biosynthesis favours FA accumulation in seeds.

In our previous work, we reported that TT2 negatively regulates embryonic FA biosynthesis by targeting *FUS3* during the early developmental stage of *Arabidopsis* seeds (Wang et al., 2014), and TT8 inhibits seed FA accumulation by targeting several seed developmental regulators such as *LEC1*, *LEC2*, and *FUS3* (Chen et al., 2014). To examine whether TT4 affects embryonic FA production via the known mechanism, we investigated the expression of TT2 and TT8 in the *tt4-5* siliques. The expression of TT2 at 6 DAP was high, but this expression decreased at 9 DAP and was absent at 12 DAP. On the other hand, the expression of TT8 was high at 6 DAP and was low at 12 DAP (Figure S5a). TT2 and TT8 expressions were slightly increased in the *tt4-5* mutant at 6 DAP, and the expression of TT8 was significantly decreased at 12 DAP in *tt4-5* seeds (Figure S5b).

### 3.2 | Flavonoids appear at the early and late embryo developmental stages, and the subcellular distribution of flavonoids is consistent with TT4 localization

To determine the spatial and temporal expression patterns of TT4 during embryo development, we conducted RT-qPCR analysis at 6, 9, 12, and 15 DAP during WT seed development. The TT4 transcripts were abundant during EM and gradually reduced after EM until the levels were almost undetectable at 15 DAP (Figure 1a). We also used the *GUS* reporter gene driven by the TT4 native promoter to confirm the expression pattern of TT4 during embryogenesis. Strong *GUS* staining was observed in the whole embryos at 6 DAP, whereas the 15-DAP embryos exhibited weakened staining at the tips of the cotyledons (Figure 1b).

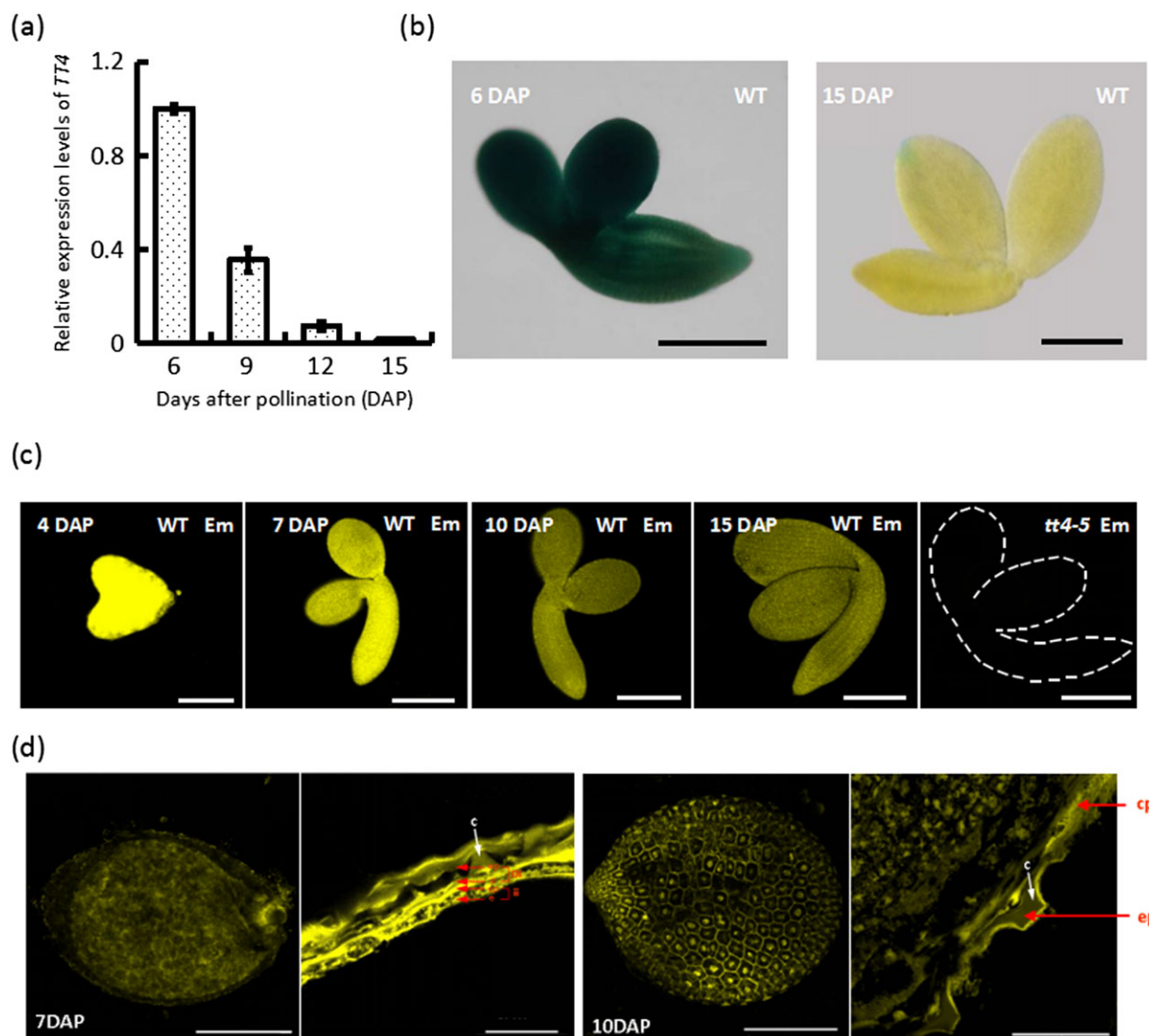
Flavonols are the prevalent form of flavonoids in seed embryos. To investigate the subcellular distribution of flavonols in embryos, we used DPBA, a fluorescent flavonol stain, to visualize flavonoids during embryo development. Flavonols were detected in both the embryos and seed coats of WT (Figure 1c). As shown in Figure 1c, relatively clear fluorescence signals were observed in the 4- and 7-DAP

WT embryos; at 7 DAP, the intensities of the fluorescence signals gradually decreased in the embryos (10 and 15 DAP). In contrast, no fluorescence signals were detectable in the *tt4-5* embryo. We also performed DPBA staining for the immature seed coat section. Unlike previous study conducted with seeds at 4 DAP (Pourcel et al., 2005), our experiment were performed with seeds at 7 and 10 DAP. We observed strong signals at the epidermis of out integument (ep) and the crushed parenchymatic layer of inner integument (cp; Figure 1d). Overall, Figure 1 showed that flavonols were present throughout seed development. Although the flavonol signal had a higher intensity in the early stage than in the late stage, total flavonol production could be higher in the late stage than in the early stage considering the volumes of developing embryos. The position for flavonols deposit in a seed coat was not fixed but varied depending on development stages.

In addition, flavonols were found to be localized in the nuclei, cytoplasm, and plasma membranes of various tissue cells, including embryo, seed coat, leaf, and root cells (Figures 1c, 2a, and S6a–d). Consistent with the subcellular distribution of flavonols, the YFP-fused TT4 protein was localized in the nuclei, cytoplasm, and plasma membranes of tobacco (*Nicotiana benthamiana*) epidermal leaf cells (Figure S6e). Taken together, these results suggest that the subcellular location of flavonoids was consistent with TT4 localization.

### 3.3 | Embryonic flavonoid biosynthesis and seed FA production are maternally controlled

To further explore the details of flavonoid biosynthesis in embryos, we used DPBA staining to examine flavonoid levels in the embryos and maternal tissues (carpel and funicular) from WT, *tt4-5*, WT (♀) × *tt4-5* (♂) F1, and *tt4-5* (♀) × WT (♂) F1 plants. In WT and WT (♀) × *tt4-5* (♂) F1 plants, normal fluorescence signals were detectable in the embryos as well as carpels and funiculars (Figure 2a–b), and brown seed colour was observed (Figure 2a–b, bottom). In contrast, in the *tt4-5* (♀) × WT (♂) F1 and *tt4-5* plants, no or extremely dim fluorescence signals were observed in embryos and maternal tissues (Figures 2c and S2c), and yellow seeds were observed (Figure 2c,

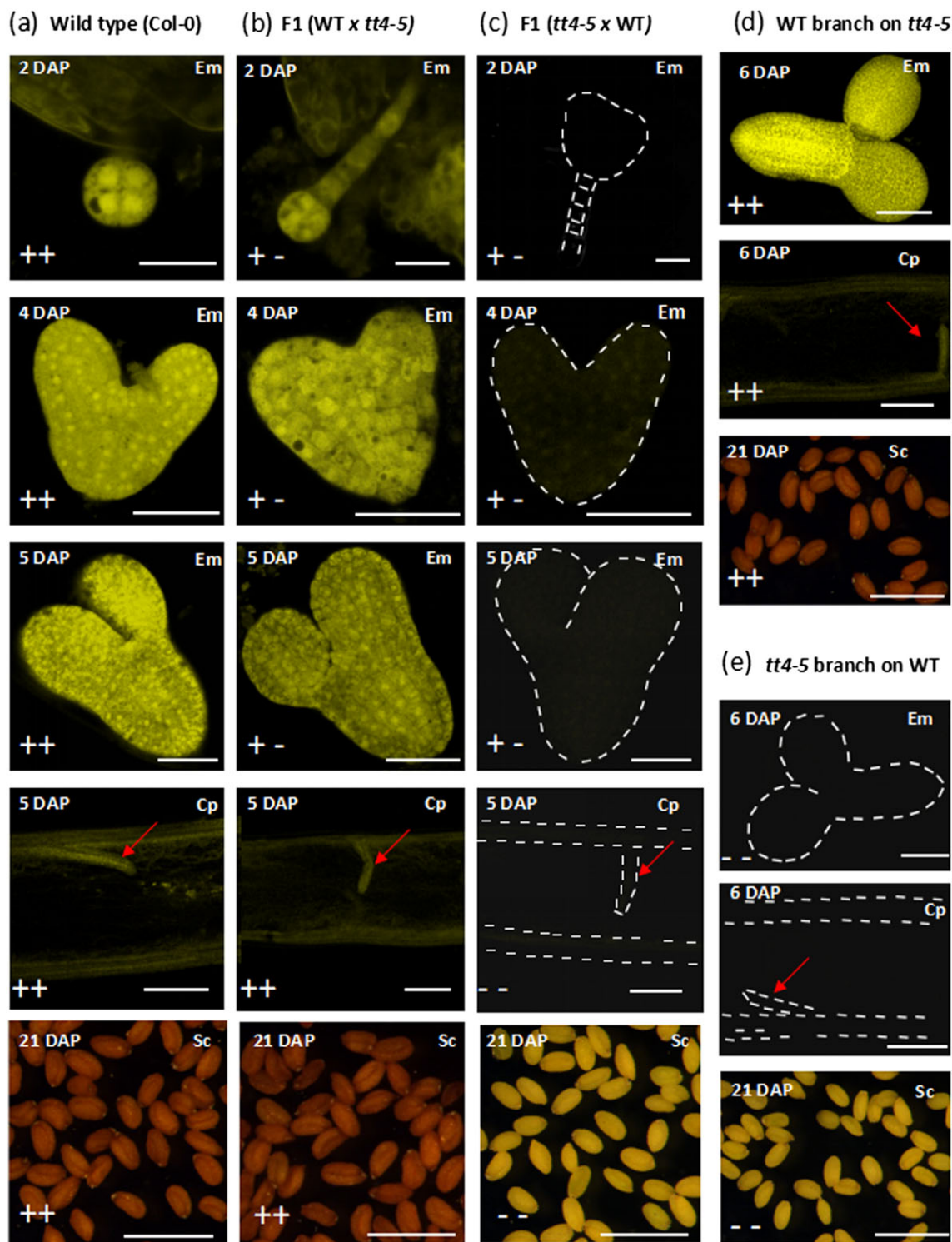


**FIGURE 1** Expression of *TT4* and presence of flavonoids in developing wild-type (WT) embryos. Flavonoids are produced not only in the seed coat (SC) but also in the embryo (Em) throughout the various Em developmental stages in WT plants. (a) Expression of *TT4* in WT seeds, as indicated by real-time quantitative PCR analysis, where *EF1aA4* was used as an internal control and the expression of *TT4* at 6 days after pollination (DAP) was set to 1. The values of the bars are means of three biological replicates. (b) GUS expression driven by the *TT4* promoter in developing cotyledons at the 6- and 15-DAP seed development stages. (c) Flavonol signal visualized by DPBA yellow fluorescence emitted by WT embryos at the heart (4 DAP), torpedo (7 and 10 DAP), and bent cotyledon stages (15 DAP); no visible flavonol signal was emitted by mature *tt4-5* embryos at 15 DAP. (d) Observation of flavonol in WT SC (7 and 10 DAP) by diphenylboric acid 2-aminoethyl ester staining. SC consists of outer integument (oi) and inner integument (ii). Strong signal was observed at the crushed parenchymatic layers (cp) and outline of epidermis (ep); “e” represents endothelium layer and “p” represents palisade layer. The scale bar for the embryo at the heart stage (4 DAP) represents 50  $\mu$ m, while the scale bars for other embryos represent 200  $\mu$ m. The scale bars for SC at 7 DAP represent 200  $\mu$ m, while the scale bars for SC at 10 DAP represent 200 and 40  $\mu$ m, respectively (from left to right)

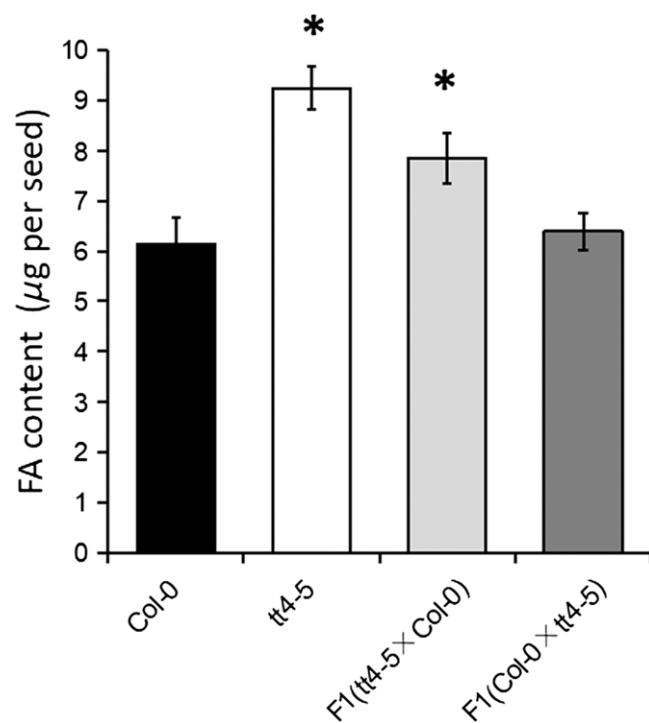
bottom and Figure S2a). Above all, this result indicated the importance of maternal tissues in controlling seed flavonoid biosynthesis. To examine whether flavonoids can be transported remotely, we grafted a *tt4-5* scion on a WT stock (*tt4-5* branch on WT) as well as a WT scion on a *tt4-5* stock (WT branch on *tt4-5*) at the seedling bolting stage. Fluorescence signals were detected in the 6-DAP embryos and the maternal tissues of the WT on *tt4-5* plant but not in those of the *tt4-5* on WT plant, indicating that flavonoids cannot be transported from the remote stock into the grafting carpels and embryos (Figure 2d–e). Consistent with flavonol accumulation, brown colour was observed for the seeds of the WT branch on *tt4-5*

(Figure 2d, bottom), and a pale colour was observed for the *tt4-5* branch on WT (Figure 2e, bottom).

Considering that loss of *TT4* function enhances the total FA content in seeds (Table 1), we next investigated whether the embryo FA content was affected by maternal tissues. Therefore, we examined the total FA content in mature seeds from WT, *tt4-5*, WT ( $\varnothing$ )  $\times$  *tt4-5* ( $\sigma$ ) F1, and *tt4-5* ( $\varnothing$ )  $\times$  WT ( $\sigma$ ) F1 plants. As expected, the total FA content of the *tt4-5* or *tt4-5* ( $\varnothing$ )  $\times$  WT ( $\sigma$ ) F1 seeds was significantly higher than that of the WT or WT ( $\varnothing$ )  $\times$  *tt4-5* ( $\sigma$ ) F1 seeds (Figure 3). Taken together, these results suggest that embryonic flavonoid biosynthesis and, consequently, seed FA formation were maternally controlled.



**FIGURE 2** Maternal control of embryonic flavonoid biosynthesis. Flavonoid signals were observed in (a, b, c) 2-, 4-, and 5-DAP embryos; 5-DAP carpels; and 21-DAP seed coats of (a) WT; (b) F1 (WT (♀) × *tt4-5* (♂)); F1 (*tt4-5* (♀) × WT (♂)); (d) 6-DAP embryos and 6-DAP carpels, 21-DAP seed coats of a WT branch grafted on *tt4-5* plant; (e) and 6-DAP embryos and 6-DAP carpels, 21-DAP seed coats of a *tt4-5* branch grafted on WT plant. There were no visible flavonoids from 2-, 4-, and 5-DAP embryos and 5-DAP carpels of *tt4-5* (♀) × WT (♂) F1 plant (c), from 6-DAP embryos and 6-DAP carpels of the *tt4-5* branch grafted on a WT plant (e); + or – indicates the presence or absence of a dominant *TT4* allele in embryos (Em), carpels (Cp) and seed coats (Sc). Red arrows point to the presence of flavonoids in the funiculars, which are maternal tissues. The scale bars for the embryos at 2 DAP represent 20  $\mu$ m, the scale bars for the embryos at 4 and 5 DAP represent 50  $\mu$ m, the scale bars for the embryos at 6 DPA represent 100  $\mu$ m, the scale bars for the siliques represent 20  $\mu$ m, and the scale bars for the seeds at 21 DAP represent 1 mm. The values are means of three biological replicates. DAP: days after pollination; WT: wild type

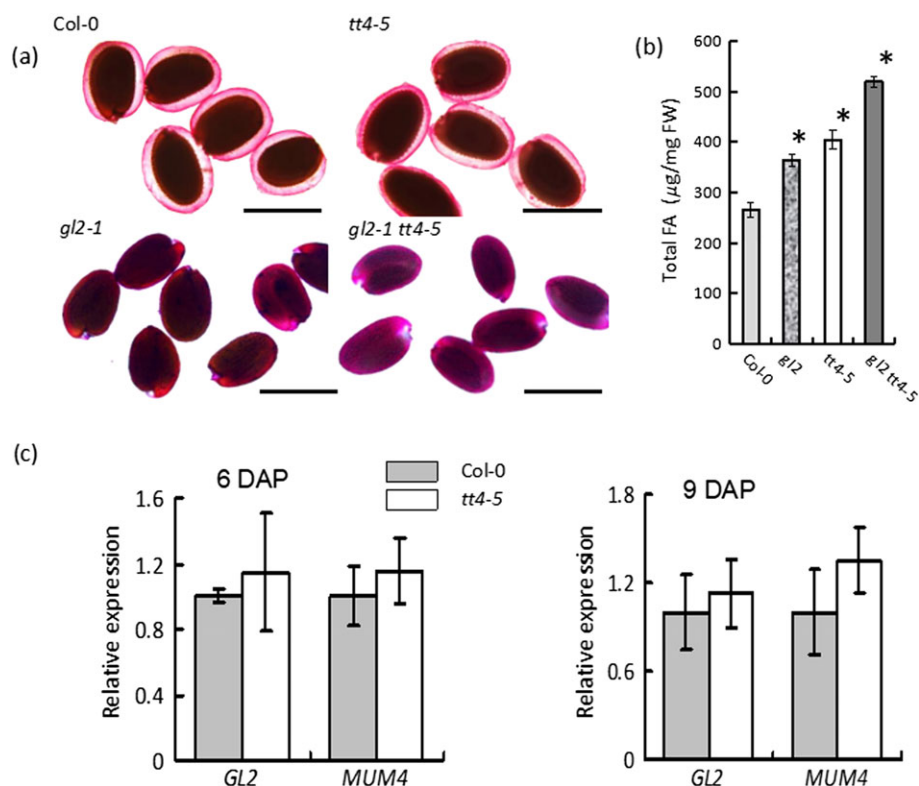


**FIGURE 3** Comparison of fatty acid (FA) content among wild type (Col-0), *tt4-5*, F1 (*tt4-5*(♀) × Col-0(♂)) and F1 (Col-0(♀) × *tt4-5*(♂)). Error bars indicate standard variations. Asterisk (\*) indicates statistical significance relative to control ( $p \leq 0.05$ )

### 3.4 | Mucilage formation was not noticeably altered in *tt4-5* seeds

Sugars (rhamnose, glucose, etc.) can be used for flavonoids and mucilage biosynthesis, and previous studies have shown that rhamnose functions as a cosubstrate for flavonoid as well as mucilage biosynthesis in seed coats (Oka et al., 2007; Routaboul et al., 2006; Shi et al., 2012). To test whether rhamnose remaining after flavonoid production in *tt4-5* seed coats was used for *tt4-5* mucilage layer formation, ruthenium red staining was performed to visualize the mucilage layers. The mucilage layers in the *tt4-5*, *tt4-1*, and *tt4-4* mutants were found to be similar to those of the corresponding WT in terms of thickness (Figures 4a and S7).

*GLABRA2* (*GL2*) and *MUCILAGE MODIFIED 4* (*MUM4*) are required for complete mucilage synthesis (Western et al., 2001). Loss of function of *GL2* and/or *MUM4* led to loss of mucilage (Shi et al., 2012). According to Western et al. (2000), mucilage biosynthesis mainly occurs in "Stage 3," which is approximately the same as the 6–9 DAP developmental stages. We showed that *GL2* and *MUM4*, a rhamnose synthase directly targeted by *GL2*, were expressed at the same level in the *tt4-5* seeds as in WT seeds at 6 and 9 DAP (Figure 4c). For further confirmation, *tt4-5* was introgressed into a *glabra2-1* (*gl2-1*) mutant, which was defective in mucilage biosynthesis but exhibited enhanced FA accumulation in seeds (Shi et al., 2012). Similar to the *gl2-1* mutant, the *tt4-5 gl2-1* double mutant did not exhibit any mucilage formation (Figure 4a). In contrast, the *tt4-5 gl2-*



**FIGURE 4** Comparison of the (a) mucilage layer, (b) fatty acid (FA) content, (c) and expression of the genes associated with mucilage layer formation among Col-0 and the *tt4-5* and *gl2* single and/or double mutants. The mucilage layers of the seed coats were visualized by ruthenium red staining. Bars represent 500 μm. The values of the bars are means of three biological replicates (b and c). Asterisk (\*) indicates statistical significance relative to the control ( $p \leq 0.05$ ) (b). *EF1a4* was used as an internal standard for the real-time quantitative PCR experiment, where the expression level of wild type was set to 1 (c). DAP: days after pollination [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



1, *tt4-5*, and *gl2-1* mutant seeds exhibited 91.4%, 49.0%, and 36.0% higher FA content, respectively, than that of the WT seeds (Figure 4 b). The increase in FA content in the *gl2-1 tt4-5* double mutant was nearly equal to the sum of the increase in FA content in the *gl2-1* and *tt4-5* mutants individually, indicating that *TT4* and *GL2* affected seed FA biosynthesis via different pathways. These results suggested that the loss of *TT4* function enhanced seed FA formation but not mucilage formation.

### 3.5 | Consumption of soluble sugars was enhanced during *tt4-5* seed development

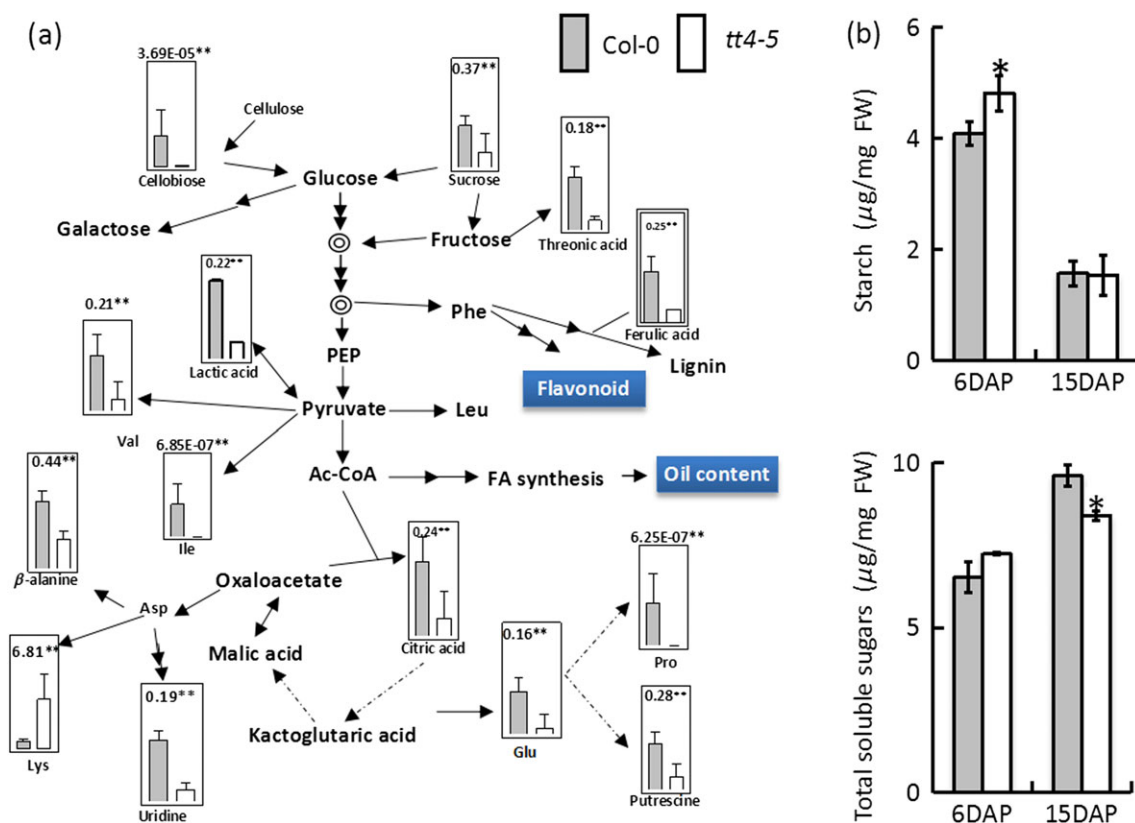
The difference in total FA content between WT and *tt4-5* was observed starting at 12 DAP (Figure S4). To explore the detailed mechanism via which flavonoid deficiency affects seed FA biosynthesis in *tt4* mutants, we examined the metabolite profiles of the 15-DAP siliques in WT and *tt4-5*. As shown in Figure 5a, the concentrations of sugars (such as cellobiose and sucrose) and metabolites associated with galactose, pyruvate, and phenylalanine metabolism were significantly lower in *tt4-5* than in WT, leading to a significant decrease in the levels of soluble sugars and their downstream metabolites, such as threonic acid, Ile, Val, and ferulic acid, in *tt4-5*. In addition, the tricarboxylic acid cycle and its related pathways (such as Asn metabolism) in *tt4-5* were significantly downregulated relative to the same pathways in WT, resulting in marked

decrease in the levels of amino acids such as Glu, Pro, and  $\beta$ -alanine in *tt4-5*. On the other hand, more soluble sugars and glycolytic intermediates were consumed for FA biosynthesis, but not for lignin, flavonoid, and amino acid production, in the *tt4-5* siliques than in the WT siliques.

To exclude the possibility that *tt4-5* siliques might yield less soluble sugars than WT siliques, we examined the starch and total soluble sugar content in the 6- and 15-DAP WT and *tt4-5* siliques. As shown in Figure 5b, *tt4-5* contained higher amounts of starch and soluble sugars than WT at 6 DAP. However, a significant reduction in the total soluble sugar content of *tt4-5* compared with that of WT was observed at 15 DAP, indicating greater consumption of soluble sugars in *tt4-5* than in WT during seed development. Taken together, Figure 5a,b show that the greater consumption of soluble sugars in the *tt4-5* siliques than in the WT siliques benefited the biosynthesis of FA rather than that of other metabolites.

### 3.6 | *WRI1*, *ABI3*, and the genes involved in glycolysis and FA biosynthesis were activated in developing *tt4-5* seeds

To further explore the mechanism via which seed FA biosynthesis increased in *tt4* mutants, we examined the transcription levels of critical genes for seed development, glycolysis, and FA biosynthesis in WT and *tt4-5*. RT-qPCR analysis showed that the levels of *WRI1* and



**FIGURE 5** Comparison of the (a) changes in the metabolic profile in 15-DAP siliques between *tt4-5* and WT. The numbers indicate fold changes in metabolite concentrations in *tt4-5* relative to WT. The solid black arrows point to direct downstream products, whereas the dashed arrows indicate indirect downstream products. (b) Starch and soluble sugar content in 6- and 15-DAP siliques. The values are means of three biological replicates. Error bars indicate standard variations. Single and double asterisks indicate significant differences from WT at 5% and 1% levels, respectively. DAP: days after pollination; WT: wild type [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

*ABI3* expression at 12 and 15 DAP were significantly elevated in *tt4-5* relative to the WT, whereas the expression levels of *LEC1*, *LEC2*, and *FUS3* exhibited only a slight increase at these two stages (Figure 6a). To analyse the expression of genes important for glycolysis, *PKp2* and *PDH-E1 $\alpha$*  were examined by RT-qPCR. The result showed that the transcription levels of *PKp2* and *PDH-E1 $\alpha$*  at 9, 12, and/or, 15 DAP were significantly higher in *tt4-5* than those in WT (Figure 6b). Next, the genes encoding key enzymes of the FA biosynthesis pathway were analysed for relative expression. As shown in Figure 6c, the relative expression levels of *BCCP2*, *CAC2*, *MOD1*, *KASII*, *CDS2*, *FAB2*, *FatA*, *PIS2*, *FAD2*, and *FAD3* in the *tt4-5* mutants were significantly enhanced at 9 and/or 12 DAP relative to the expression levels in WT (Figure 6c).

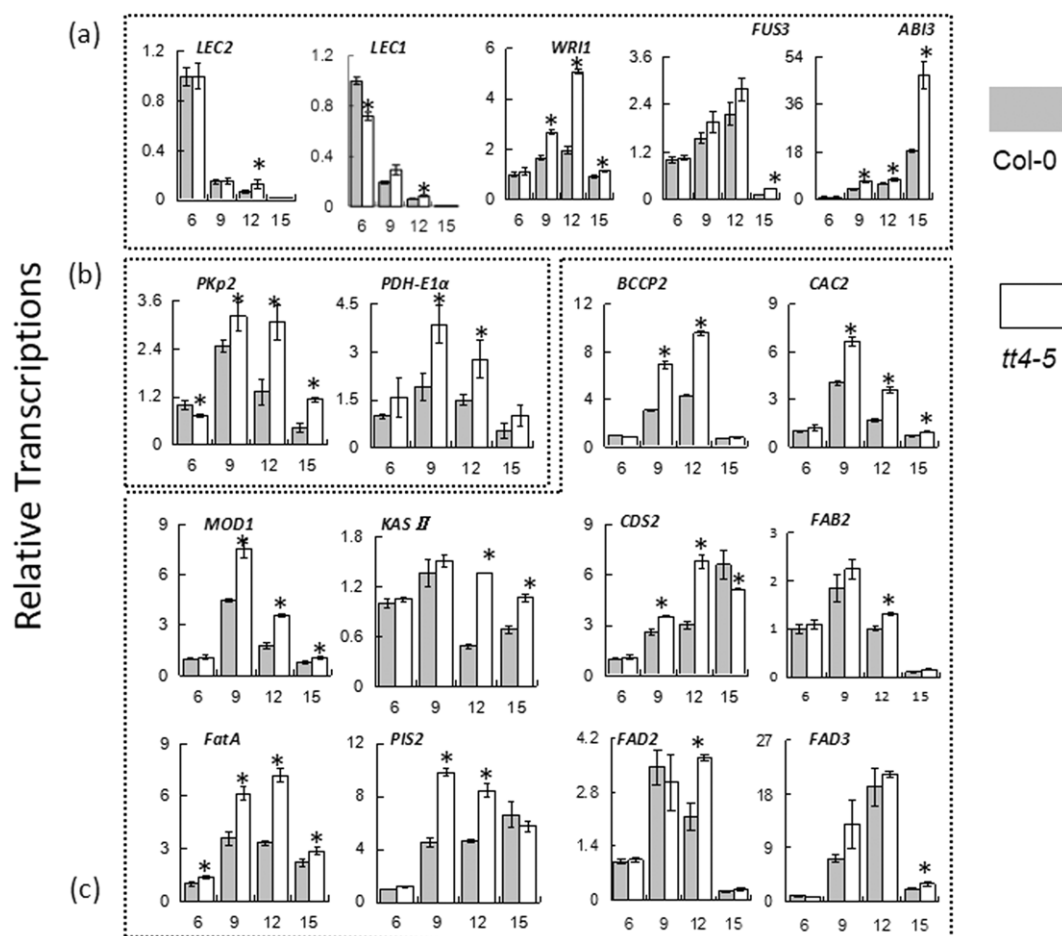
Furthermore, the siliques were separated into seeds and silique walls. *WRI1* and the genes involved in glycolysis were specifically upregulated in the seeds rather than in the silique walls. In addition, *SUCROSE SYNTHASE 2* (*SUS2*) and *SUS3*, which are responsible for sugar metabolism (Baud, Vaultier, & Rochat, 2004), exhibited enhanced expression in the 9-DAP silique walls (Figure S8). Taken together, the results suggested that most of the examined genes that were critical for seed development, glycolysis, and FA biosynthesis pathways had higher activity at 9 to 12 DAP in the *tt4-5* mutant than in WT, suggesting that the increased FA synthesis in *tt4-5* seeds could

benefit from the elevated expression of *WRI1* and the other genes (Figures 6 and S8).

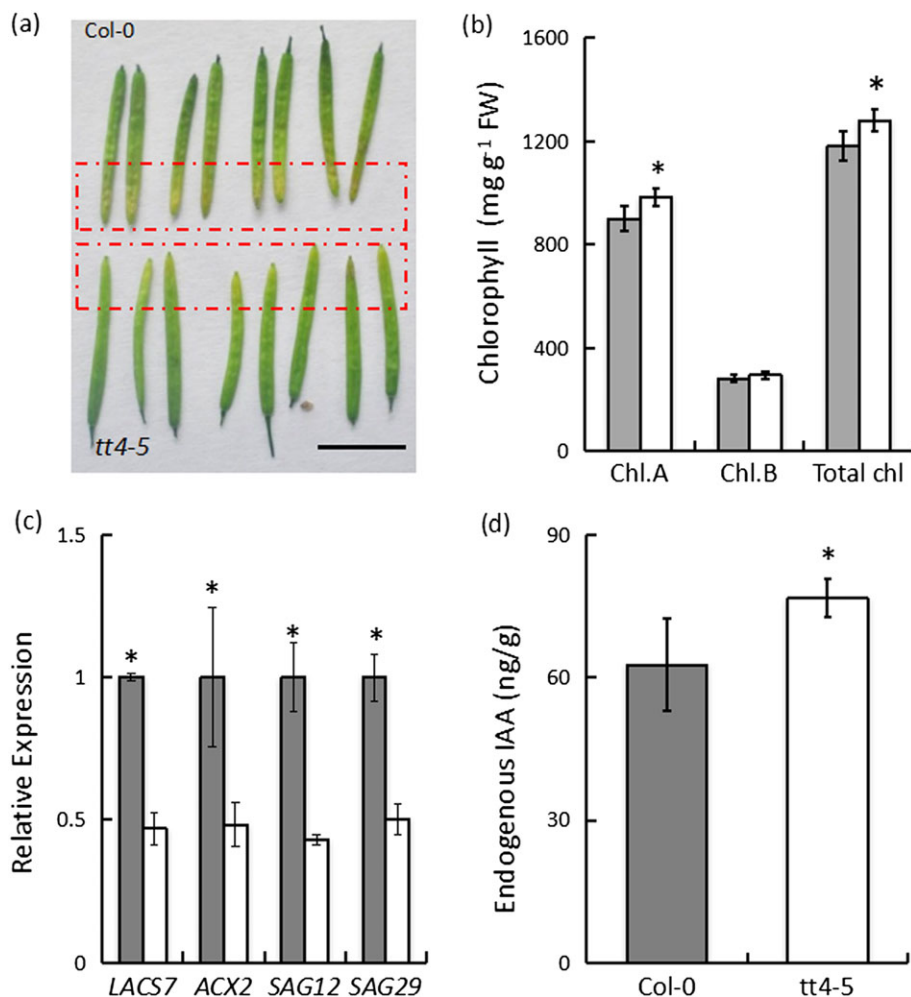
### 3.7 | Accumulation of auxin was enhanced, and *PIN4* transcription was reduced in *tt4-5* embryos

Upon observing that all *tt4* plants exhibited greener siliques at the late stage of seed development than the WT plants did, as represented in Figure 7a, we examined the chlorophyll content and transcription levels of genes involved in beta-oxidation, such as *LACS7* and *ACX2*, and senescence indicator genes, such as *SAG12* and *SAG29*, in the WT and *tt4-5* siliques. Higher total chlorophyll and chlorophyll A content was observed in *tt4-5* than in WT (Figure 7b). In contrast, the transcription levels of *LACS7*, *ACX2*, *SAG12*, and *SAG29* in *tt4-5* were markedly lower than those in WT, suggesting delayed senescence, occurring at a late stage of seed development, in the *tt4-5* siliques (Figure 7c).

Previous studies have demonstrated that auxin delays plant senescence (Wolters & Jürgens, 2009). Thus, the delayed senescence phenotype of *tt4-5* siliques prompted us to examine whether increased accumulation of endogenous auxin in siliques delays silique senescence in *tt4* mutants. As expected, the endogenous levels of IAA in *tt4-5* siliques ( $76.55 \pm 4.03$  ng/g fresh weight) were significantly



**FIGURE 6** Comparison of the expression of (a) major seed development regulators; (b) the enzymes *PKp2* and *PDH-E1 $\alpha$* , which are involved in glycolysis; and (c) the genes encoding key enzymes in the fatty acid biosynthesis pathway between *tt4-5* and wild type. The values of the bars are means of three biological replicates. Asterisk (\*) indicates statistical significance relative to the control ( $p \leq 0.05$ ). *EF1aA4* was used as an internal standard in the real-time quantitative PCR experiment, where the expression level of wild type at 6 days after pollination was set to 1



**FIGURE 7** Comparison of (a) the colour of the silique tips at 12 DAP; (b) chlorophyll content in 12-DAP siliques; (c) expression levels of senescence indicators such as *LACS7*, *ACX2*, *SAG12*, and *SGA29*; and (d) endogenous IAA content in 12-DAP siliques between WT and *tt4-5*. The values of the bars are means of three biological replicates. Asterisk (\*) indicates statistical significance relative to the control ( $p \leq 0.05$ ). *EF1aA4* was used as an internal standard in the RT-qPCR experiment, where the expression level of WT was set to 1 (c). The bar represents 1 cm in (a). DAP: days after pollination; IAA: indole-3-acetic acid; WT: wild type; RT-qPCR: real-time quantitative PCR

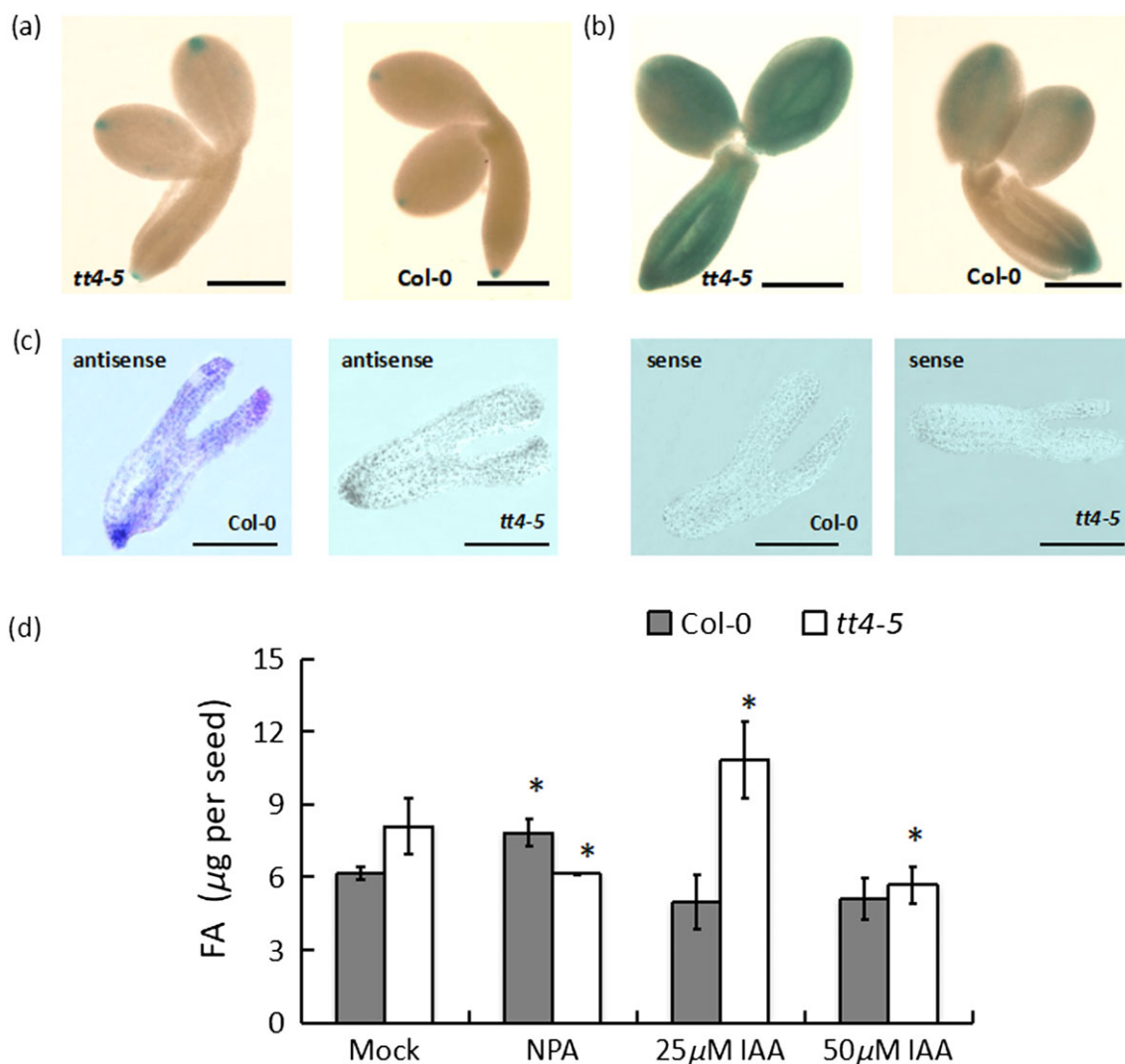
higher than those in the WT siliques ( $62.60 \pm 9.75$  ng/g fresh weight; Figure 7d).

Considering that the auxin gradient is essential for embryogenesis (Friml et al., 2003), we used *DR5::GUS* (Sabatini et al., 1999), an auxin response marker, to examine whether endogenous auxin distribution was altered in the *tt4-5* embryos. Upon exposure to exogenous auxin treatment, the observed GUS staining of the embryos was markedly stronger in *tt4-5* than in WT (Figure 8a). Approximately 12% (9/74) of the *tt4-5* embryos exhibited whole-embryo distribution of the GUS signal after exogenous auxin treatment. In contrast, all the WT embryos exhibited polar distribution of GUS signals at the tips of cotyledons and suspensors, although the WT embryo treated with exogenous IAA had stronger GUS signals than the mock control (Figure 8b). This result indicated that the polar auxin transport in *tt4-5* embryos was somehow impaired. Impairment of polar auxin transport was also observed when the *tt4-5* roots were treated with exogenous auxin; GUS staining was observed throughout the *tt4-5* roots, whereas the GUS signal was limited to the root tips in WT roots (Figure S9).

Previous studies in *Arabidopsis* have shown that PIN4 is an auxin efflux transporter and that loss of PIN4 function promotes auxin accumulation in embryos (Friml et al., 2002). To determine whether the enhanced endogenous auxin levels in *tt4-5* embryos was due to reduced PIN4 capacity, we conducted RNA in situ hybridization to examine *PIN4* transcription levels in 6-DAP WT and *tt4-5* embryos. As expected, the torpedo-shaped WT embryos exhibited stronger RNA hybridization signals for *PIN4* than the *tt4-5* embryos at the same stage (Figure 8c). Together, these results suggested that loss of TT4 function was associated with reduced *PIN4* expression and thereby with enhanced auxin accumulation in embryos during embryogenesis.

### 3.8 | Pharmacologically and genetically elevated auxin levels enhanced seed FA biosynthesis

To determine the role of auxin in elevating the total FA content in *tt4* seeds, we searched the *Arabidopsis* cis-regulatory element database and found that several genes involved in glycolysis and FA biosynthesis pathways contain auxin response factor-binding sites (Ulmasov,



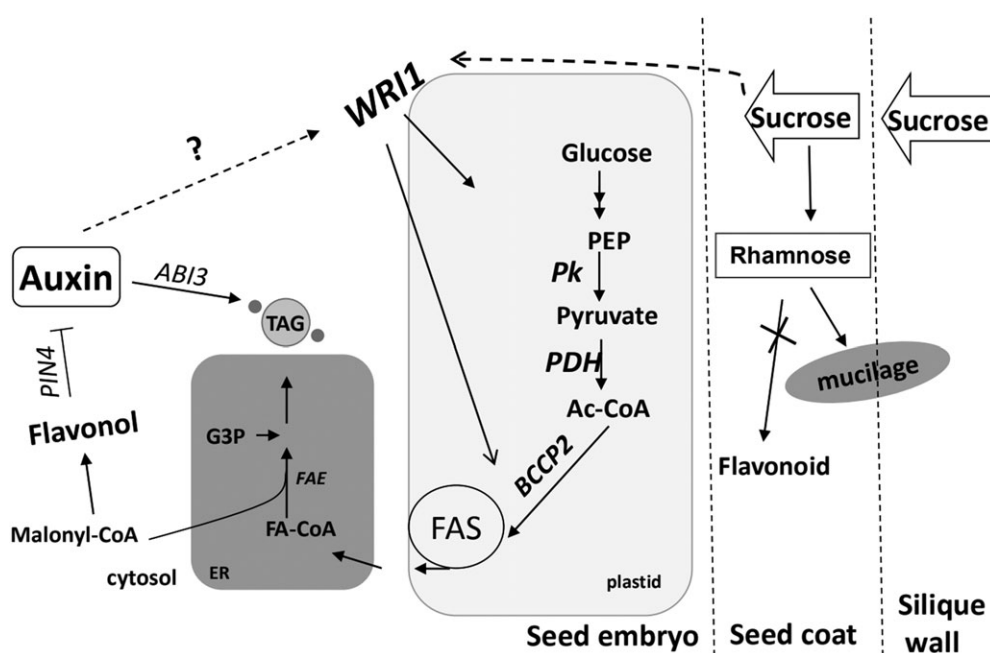
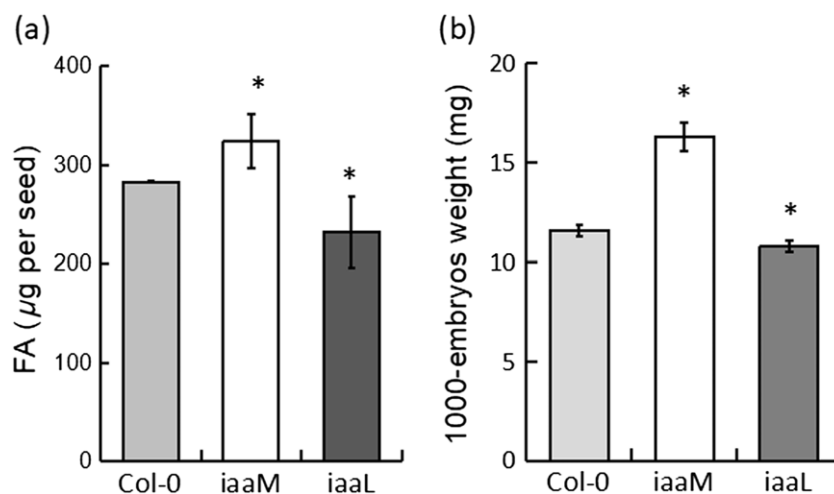
**FIGURE 8** Comparison of (a) expression of the DR5 auxin responsive reporter in embryos without exogenous auxin treatment; (b) expression of DR5 auxin responsive reporter in 7-DAP (by harvest) embryos that were treated with exogenous IAA (50  $\mu$ M) in culture medium for 3 days; and (c) *PIN4* mRNA levels of 6-DAP developing embryos visualized by in situ hybridization between WT and *tt4-5*. (d) FA content in 12-DAP seeds that were treated with 50- $\mu$ M naphthylphthalamic acid and 25- or 50- $\mu$ M IAA in the culture medium. Bars represent 200  $\mu$ m in (a) and (b) and 100  $\mu$ m in (c). Error bars represent standard deviations. Asterisks indicate statistical significance of the values of the various treatments compared with the value of the mock control ( $p \leq 0.05$ ). DAP: days after pollination; IAA: indole-3-acetic acid; WT: wild type; FA: fatty acid

Murfett, Hagen, & Guilfoyle, 1997; Table S1). The regulatory regions of *WRI1* also possessed auxin responsive elements (AuxREs and TGA elements), and upregulation of *WRI1* under auxin treatment was detected (Figure S10). Thus, we first investigated the effects of pharmacologically or exogenously altered auxin levels on seed FA content. Upon treatment with naphthylphthalamic acid (50  $\mu$ M), an auxin efflux inhibitor (Fujita & Syono, 1996), the FA content in the WT seeds grown on ICM increased but that of the *tt4-5* seeds decreased in comparison with the corresponding mock controls. In contrast, exogenous IAA (25  $\mu$ M) treatment elevated the seed FA content of *tt4-5* but not WT compared with the corresponding mock controls. However, treatment with a higher auxin concentration (50  $\mu$ M) decreased the seed FA content of *tt4-5* but not WT relative to the corresponding mock controls (Figure 8d).

To further confirm the role of auxin in embryonic FA biosynthesis, we compared the transgenic lines harbouring the 35S::*iaaM* or 35S::*iaaL* constructs. Previous studies have shown that the 35S::*iaaM* plants produced higher levels of auxin by promoting endogenous IAA biosynthesis, whereas the 35S::*iaaL* plants reduced endogenous auxin levels via an auxin-lysine conjugation mechanism (Romano, Hein, & Klee, 1991; Romano, Robson, Smith, Estelle, & Klee, 1995; Zhang et al., 2014). As shown in Figure 9, the 35S::*iaaM* plants exhibited increased FA levels per unit of seeds as well as 1,000-embryo weight relative to the WT control. In contrast, the 35S::*iaaL* plants exhibited a slight but significant reduction in seed FA content and 1,000-embryo weight relative to the WT control (Figure 9). Based on the results of our study, a simple model was constructed to summarize the factors responsible for the increase in FA content of *tt4-5* seeds (Figure 10).



**FIGURE 9** Comparison of (a) FA content ( $\mu\text{g}/\text{mg}$ ) and (b) 1,000-embryo weight between the *35S::iaaL* transformants, with reduced free auxin levels, and *35S::iaaM* transformants, with elevated auxin levels. Error bars represent standard deviations. The values of the bars are means of three biological replicates. Asterisks indicate statistical significance of the *tt4-5* values from compared with those of WT ( $p \leq 0.05$ ). WT: wild type; FA: fatty acid



**FIGURE 10** A model summarizing the factors responsible for the high fatty acid (FA) content in *tt4-5* seeds. Rhamnose is one main common substrate for both Q-3-O-R and mucilage biosynthesis in seed coats. The defective flavonoid synthesis in *tt4-5* seed coats led to the influx into the embryos of substrates that favoured glycolysis. The activated glycolysis reactions led to accumulation of glycolytic intermediates, which were favourable for various downstream pathways involved in the synthesis of various metabolites such as proteins, flavonoids, lignins, and FAs. However, the increased glycolytic intermediates were not allocated proportionally to all these downstream pathways but preferentially to FA biosynthesis; the expression of *WRI1* in *tt4-5* embryos was enhanced, and *WRI1* targets the enzymes involved in glycolysis pathway and FA biosynthesis. The upregulation of *WRI1* in *tt4-5* might be induced by increased embryonic auxin signaling. *ABI3* could be induced by auxin, and the increased *ABI3* expression level should correspond to the less FA degradation at late development stage. The abnormal accumulation of auxin in *tt4-5* embryos was likely due to the weakened *PIN4* efflux pumping capacity. On the other hand, the increasing influx of soluble sugar into the *tt4-5* embryos due to the inhibition of flavonoid synthesis in seed coats may cause higher *WRI1* expression as a mechanism to adapt to enhanced glycolysis. The solid black arrows point to direct downstream products, whereas the dashed arrows indicate possible activation

## 4 | DISCUSSION

### 4.1 | The mechanism by which TT4 affected embryonic FA synthesis

For several decades, yellow-coloured seeds have been of particular interest to oilseed breeders because of the increased oil content of

these seeds, which is considered to be a consequence of thinner seed coats, which contain decreased levels of pigments, melanin, polyphenols, lignin, and crude fiber (Wang et al., 2016; Yu, 2013). However, the mechanisms via which pale-coloured seeds generate high seed FA content are not completely understood. In our previous studies, we reported that *TT2* regulates embryonic FA biosynthesis by targeting *FUS3* during the early seed developmental stage, whereas

TT8 inhibits FA accumulation by targeting several seed developmental regulators, such as *LEC1*, *LEC2*, and *FUS3* (Chen et al., 2014; Wang et al., 2014). To investigate whether different mechanisms are present in the *tt4-5* mutant, we measured the expression of *TT2* and *TT8* in *tt4-5* seeds (Figure S5). The elevation of FA production in the *tt4-5* seeds was unlikely to have been caused by the removal of *TT2* and/or *TT8* repression, as both genes exhibited higher expression in *tt4-5* than in WT at 6 DAP, which is the most important stage for the activity of *TT2* and *TT8* (Chen et al., 2014; Wang et al., 2014). We showed that loss of *TT4* function leads to a significant increase in *WRI1* expression at 12 DAP and a significant increase in *ABI3* expression at 15 DAP. Increased expression of *WRI1* at 12 DAP could be the upstream factor that led to the upregulation of genes involved glycolysis, such as *PKp2* and *PDH-E1 $\alpha$*  (Figure 6b), and FA biosynthesis, such as *BCCP2*, *CAC2*, *MOD1*, *KASII*, *CDS2*, *FAB2*, *FatA*, *PIS2*, *FAD2*, and *FAD3* (Figure 6c; Baud & Lepiniec, 2009; Ruuska, Girke, Benning, & Ohlrogge, 2002). *ABI3* plays an important role in the regulation of ABA signalling. Increased expression of *ABI3* at 15 DAP in *tt4-5* indicated an elevated ABA response, which favoured seed dormancy by inhibiting storage compound decomposition (Delmas et al., 2013; Giraudat et al., 1992). The FA content of the *tt4-5* seeds was significantly higher than that of Col-0 seeds starting from 12 DAP. The difference, possibly due to the lower FA degradation in maturing seeds in *tt4-5* than in WT plants, was larger at 15 and 18 DAP (Figure S4). *TT4*, unlike *TT2* and *TT8*, was not a transcription factor. The mechanism via which *TT4* affected embryonic FA synthesis was different from previously known mechanisms.

#### 4.2 | The alteration of flavonol synthesis in seeds presumably leads to a selective rechanneling of available carbon source to lipids

The flavonoids in seeds mainly include both flavonols (mainly Q-3-O-R) and PAs. Here, we reported the negative effect of flavonoids on embryonic FA biosynthesis. In the *tt4* mutants, the reduction in flavonoids in seeds led to a marked increases in total FA content and embryo weight, but the composition of the FA species was not significantly changed (Table 1). The synthesis of flavonoid requires malonyl-CoA as a cytosolic substrate (Baud & Lepiniec, 2009). Malonyl-CoA is transported from the cytosol to ER for the synthesis of VLCFAs. Apparently, given the similar VLCFA ratios in the WT and *tt4* mutant, the malonyl-CoA remaining after flavonol production in *tt4* mutants did not improve VLCFA synthesis in the ER (Table 1).

Before being transported to the embryo, sucrose is unloaded in the seed coat (Shi et al., 2012; Stadler et al., 2005). Glycolysis, which provides acetyl-CoA for FA biosynthesis, occurs in the embryo (Baud et al., 2007). In *Arabidopsis* seeds, 90% of the Q-3-O-R, the prevalent form of flavonol, accumulates in the seed coat (Routaboul et al., 2006), where rhamnose, which is substrate for both flavonol and mucilage production, is generated from sucrose. The *gl2* mutant, which lacks the seed mucilage layer, exhibited increased FA content due to the reallocation of the carbon source to the embryo (Shi et al., 2012). MUM4, a rhamnose synthase directly targeted by *GL2*, was expressed at the same level in the *tt4-5* seed as in the WT

seeds at 6 and 9 DAP, suggesting that the mucilage should have been normally produced in the *tt4-5* seeds (Figure 4c). Indeed, the increase in FA content in the *gl2-1 tt4-5* double mutant was nearly equal to the sum of the increase in FA content in the *gl2-1* and *tt4-5* mutants individually, indicating that *GL2* and *TT4* do not act via the same pathway. Therefore, rhamnose, which is used for flavonoid production in the WT seed coat, was used for embryonic FA biosynthesis in the *tt4-5* mutant. On the other hand, the *tt4* mucilage layer did not benefit from the absence of flavonoid production in the seed coat, possibly because Q-3-O-R and the mucilage are not synthesized simultaneously. The formation of the mucilage layer occurs in Stage 3, which is approximately the 6–9 DAP developmental stages (Western et al., 2000). The production of Q-3-O-R in the seed coat occurs mainly after 12 DAP (Routaboul et al., 2006). Comparison of the dynamics of FA accumulation between WT and *tt4-5* revealed that 12 DAP is the approximate time point at which the FA content of *tt4-5* exceeded that of WT (Figure S4). FA synthesis in *tt4-5* embryos represented an increased carbon flux after 12 DAP, which would have been directed to Q-3-O-R production in seed coats in WT embryos.

Although the flavonol signal appeared with higher intensity in embryos at the early stage than those at the late stage (Figure 1c), the total flavonol production at the late stage could be higher than that at the early stage considering the volumes of the embryos and seeds. Quantitative analysis of DPBA staining must be performed with care because the staining may depend on flavonol composition, stain penetration, and probable interactions with other compounds. *TT4* transcripts were abundant in the embryos during EM, gradually reducing after EM but still detectable at the 15 DAP stage (Figure 1a–b). *TT4* catalyses the first step of flavonoid biosynthesis to produce naringenin chalcone, and *TT5*, *TT6*, *TT7*, and *FLAVONOL SYNTHASE* further catalyse the remaining steps of flavonol production (Burbulis et al., 1996; Pollastri & Tattini, 2011; Shirley et al., 1995). Therefore, the expression of *TT4* could occur much earlier than the stage at of highest flavonol production in seeds. Although flavonol can be transported to pollen, it cannot be transferred from root to seed coat (Buer & Djordjevic, 2009). In our study, we did not detect flavonol signal in embryos and siliques of the *tt4-5* branch grafted on WT plant. In both the WT (♀) × *tt4-5* (♂) F1 plants and the *tt4-5* (♀) × WT (♂) F1 plants, seeds and siliques had the same pattern of flavonol accumulation, either with or without flavonol accumulation. There could exist some kind of nourishing tissue linking seeds to the silique so that flavonols in embryos could have been both transported from silique and synthesized locally. As the flavonol signal in the *tt4-5* (♀) × WT (♂) F1 embryo was rather dim (Figure 2c), we suggest that there was more flavonol transported than synthesized locally in WT embryos. In addition, despite possible signals coming from maternally inherited seed coat tissue, there was a cascade of events that blocked flavonoid synthesis within the embryo and presumably led to selective rechanneling of available carbon source to lipids.

#### 4.3 | Flavonoids and FA biosynthesis in seeds is maternally controlled

Flavonoids and FA biosynthesis in seeds were seen to be maternally controlled (Figure 2 and Figure 3). The fact that *tt4-5* seeds contained

high FA content, from the perspective of maternal control, could be explained by the following reasons: First, the seed coat is a maternal tissue. The preliminary photosynthates remaining after flavonoid production in *tt4* seed coats could possibly enter the *tt4* embryos, thereby promoting glycolysis, which is an intermediate step of FA synthesis. In *Arabidopsis*, sugars are transported to maternal seed coat via funicular phloem and then reach the embryo (Stadler et al., 2005). In our study, both embryo development and mucilage production were not significantly affected, suggesting that the flow of carbon through seed coat was not affected (Figures 4 and S3). Second, silique walls are maternal tissues and are important for seed FA accumulation. Genes such as *SUS2* and *SUS3*, which are involved in sucrose metabolism, were significantly upregulated in the *tt4-5* silique walls, indicating the enhanced photoassimilation capacity of the walls, which could potentially favour FA biosynthesis (Tan et al., 2011; Figure S8). Third, in the absence of inner integument and pigment deposition, the *tt4-5* seed coat becomes thinner (Debeaujon et al., 2000), allowing the growing *tt4-5* embryo to adapt to the enlarged physical space with a heavier embryo and increased FA accumulation (Table 1). A study in oilseed rape showed that in vivo metabolic flux was locally regulated and associated with seed architecture, driving the embryo toward efficient use of available photosynthetic compounds and physical space (Borisjuk et al., 2013).

Moreover, the upregulation of the transcription factors *WRI1* and *ABI3* in the *tt4* maturing seeds might have caused the increase in FA content. Genes involved in glycolysis (*Pkp2* and *PDHE1α*) and FA biosynthesis (*BCCP2*, *MOD1*, etc.) pathways were upregulated in plants harbouring the *35S:WRI1* construct and *tt4* plants (Figure 6; Baud et al., 2007). On the contrary, these genes were downregulated in the *wri1* mutant (Ruuska et al., 2002). Consequently, the change in sucrose and oil content in *wri1* was opposite to that in *tt4* (Ruuska et al., 2002; Baud et al., 2007; Figure 5; Table 1). *abi3* seeds had lower seed FA content than WT (Cernac, Andre, Hoffmann-Benning, & Benning, 2006). Seeds with elevated auxin concentration showed an increase in FA content (Figure 9), which might have been partly due to the upregulation of *ABI3*, because *ABI3* could be activated by auxin (Liu et al., 2013). The increased *ABI3* expression should have led to lower FA degradation at late development stages and eventually higher FA contents in *tt4-5* seeds (Figure 6; Supporting Information Figure S4).

#### 4.4 | The *tt4-5* mutation increased embryonic auxin concentration

*tt4-5* siliques contained more IAA than WT siliques (Figure 7d). Twelve percent of the *tt4-5* embryos exhibited abnormal polar transportation of auxin. Our finding is consistent with those of previous studies, in which a proportion of, but not all, the observed samples lost the polar distribution of auxin due to the presence of excessive auxin (Weijers et al., 2005). The auxin concentration of a seed may depend on the position of the seed in a silique. The results of the GUS staining experiment (Figure 8b) were consistent with those of FA measurement, as shown in Figure 8d; in regard to FA biosynthesis, *tt4-5* was much more sensitive to the pharmacologically elevated IAA levels than WT.

PIN family genes specify the location of the auxin maxima, which is necessary for embryonic patterning. Embryonic auxin distribution is largely dependent on auxin transporters, especially PIN efflux carriers such as PIN1, PIN4, and PIN7, which are present in embryos (Friml et al., 2003). PIN4 is an important efflux carrier, functioning during EM from the late globular stage onward (Friml et al., 2002; Weijers et al., 2005). PIN7 is mainly expressed at the preglobular stage and is required for the establishment of the preglobular auxin gradient. Both PIN1 and PIN4 are expressed at and after the globular stage. However, PIN4 is overwhelmingly distributed at the suspensor region, where efflux pumping occurs (Friml et al., 2003; Weijers et al., 2005). Therefore, we selected PIN4 as an indicator of auxin efflux capacity.

The roots and embryos of *tt4-5* responded to auxin treatment in a manner similar to the roots and embryos of *pin4* (Friml et al., 2002; Figures 8 and S9). We hypothesized that the *tt4-5* embryos could have weakened auxin transporter capacity due to reduced PIN4 function. To verify this hypothesis, RNA in situ hybridization was carried out to compare the expression of PIN4 between *tt4-5* and WT embryos. The expression of PIN4 was indeed much lower in *tt4-5* than in WT (Figure 8c). The reduced PIN4 expression in the *tt4-5* embryos was evident at 6 DAP; however, the accumulation of auxin could be a gradual process in which the embryonic auxin concentration may peak at a later stage.

Flavonoids have been recognized as potential signal molecules regulating auxin transportation (Jacobs & Rubery, 1988). However, the mechanism via which flavonoids affect auxin distribution is not completely understood. In this study, we showed that the flavonoids were localized in the cytoplasm as well as nuclei (Figures 2a and S6). Flavonoids can bind directly to auxin transporters, such as P-glycoprotein 1 (PGP1), PGP4, and PGP19, in the cytoplasm (Li & Zachgo, 2013; Peer et al., 2004; Peer & Murphy, 2007; Terasaka et al., 2005). However, the effect of flavonoids on PIN4 may not be similar, as the removal of flavonoids (and not the synthesis of excess flavonoids) decreased PIN4 expression. Nevertheless, the effect of flavonoids on auxin response factors in the nucleus cannot be ruled out.

To obtain direct evidence that auxin can promote seed FA biosynthesis, we compared total FA content between the seeds of the *35::iaaM* and *35::iaaL* transformants. The *35::iaaM* transformants, which produce elevated levels of auxin in vivo, exhibited significantly higher total seed FA content and heavier 1,000-embryo weight than the WT control and the *35::iaaL* transformants (Figure 9). High seed FA content and heavy 1,000-embryo weight were common features of all the *tt4* allelic mutants (Table 1). We performed an in vitro experiment comparing the effect of auxin dosage on seed FA synthesis between *tt4-5* and WT. The result suggested that whether auxin plays a positive or negative role in FA synthesis is dependent on auxin concentration. The *tt4-5* seeds were much more sensitive to exogenous auxin in terms of changes in FA content, likely due to the reduced embryonic efflux pumping capacity of these seeds (Figure 8d).

We constructed a model summarizing the factors responsible for the high FA content in *tt4-5* seeds (Figure 10). Rhamnose is the common substrate for both Q-3-O-R and mucilage biosynthesis. The defective flavonoid synthesis in *tt4-5* seed coats added soluble sugar substrates to the embryos, favouring glycolysis. The activated

glycolysis reactions led to increased levels of glycolytic intermediates, which are favourable for downstream pathways involved in the synthesis of various metabolites, such as proteins, flavonoids, lignins, and FAs. However, the increased glycolytic intermediates were not allocated proportionally to all these downstream pathways but preferentially to FA biosynthesis, because the expression of *WRI1* in *tt4-5* embryos was enhanced, and *WRI1* targets enzymes involved in FA biosynthesis. The upregulation of *WRI1* in *tt4-5* might be induced by increased embryonic auxin signalling. The abnormal accumulation of auxin in *tt4-5* embryos was likely due to the weakened PIN4 efflux pumping capacity. On the other hand, the increased influx of soluble sugars into *tt4-5* embryos due to the inhibition of flavonoid synthesis in seed coats may also lead to increased *WRI1* expression as a mechanism to adapt to enhanced glycolysis. The elevated auxin concentration could also have increased *ABI3* expression, which could lead to lower FA degradation in *tt4-5* seeds.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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