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Wrinkled1 Accelerates Flowering and Regulates Lipid Homeostasis between Oil Accumulation and Membrane Lipid Anabolism in *Brassica napus*

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Wrinkled1 (WRI1) belongs to the APETALA2 transcription factor family; it is unique to plants and is a central regulator of oil synthesis in *Arabidopsis*. The effects of WRI1 on comprehensive lipid metabolism and plant development were unknown, especially in crop plants. This study found that BnWRI1 in *Brassica napus* accelerated flowering and enhanced oil accumulation in both seeds and leaves without leading to a visible growth inhibition. BnWRI1 decreased storage carbohydrates and increased soluble sugars to facilitate the carbon flux to lipid anabolism. BnWRI1 is localized to the nucleus and directly binds to the AW-box at proximal upstream regions of genes involved in fatty acid (FA) synthesis and lipid assembly. The overexpression (OE) of Bn*WRI1* resulted in the up-regulation of genes involved in glycolysis, FA synthesis, lipid assembly, and flowering. Lipid profiling revealed increased galactolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and phosphatidylcholine (PC) in the leaves of OE plants, whereas it exhibited a reduced level of the galactolipids DGDG and MGDG and increased levels of PC, phosphatidylethanolamide, and oil [triacylglycerol (TAG)] in the siliques of OE plants during the early seed development stage. These results suggest that BnWRI1 is important for homeostasis among TAG, membrane lipids and sugars, and thus facilitates flowering and oil accumulation in *B. napus*.

Keywords: Wrinkled1 (WRI1), oil accumulation, flowering, lipid homeostasis, transcriptional regulation, *Brassica napus*

INTRODUCTION

Lipids not only serve as storage components of high-density energy, but they also function as essential components of cell membranes and regulators of various cellular processes during growth, development, and stress responses (Wang et al., 2006; Hong et al., 2008, 2009; Phillips et al., 2009; To et al., 2012). Fatty acid (FA) synthesis and lipid assembly involve multiple steps (Li-Beisson et al., 2010). The initial precursors of lipid biosynthesis include acetyl-CoA and glycerol-3-phosphate, which are initially derived from glycolysis and the Calvin–Benson cycle in plants (Kang and Rawsthorne, 1996; Alonso et al., 2007). The acetyl-CoA carboxylase (ACCase) complex is made of three subunits, namely biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), and carboxyltransferase (CT); this complex is encoded by separated

genes in plants and catalyzes acetyl-CoA and CO_2 to produce malonyl-CoA, the first committed step in *de novo* FA synthesis (Slabas and Fawcett, 1992; Ohlrogge and Browse, 1995; Ohlrogge and Jaworski, 1997; Voelker and Kinney, 2001; Sasaki and Nagano, 2004). Malonyl-CoA was then transferred to ACP protein by malonyl-CoA: ACP transferase (MAT) to initiate FA synthesis, and malonyl-CoA provides a two-carbon unit for acyl chain elongation as catalyzed by an FA synthase (FAS) complex (Ohlrogge and Browse, 1995). The synthesized FAs are either retained in the chloroplast for galactolipid synthesis or exported to the endoplasmic reticulum (ER) for membrane phospholipid and storage lipid [triacylglycerol (TAG)] assembly. The final step of TAG assembly as catalyzed by DAG acyltransferase (DGAT), which occurs in the Kennedy pathway, is also regarded as a critical reaction for oil accumulation (Slabas and Fawcett, 1992; Ohlrogge and Browse, 1995; Zou et al., 1999; Voelker and Kinney, 2001). The loss of DGAT1 resulted in reduced seed oil content, whereas *DGAT1* overexpression (OE) enhanced the seed oil content in *Arabidopsis* (Zou et al., 1999; Jako et al., 2001). The OE of maize high-oil *DGAT1-2* also promoted oil accumulation in maize seeds (Zheng et al., 2008). Alternatively, phosphatidylcholine (PC) also provides an acyl chain toward DAG for TAG synthesis, as catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT; Dahlqvist et al., 2000; Tarczynski and Shen, 2008; Zhang et al., 2009).

Given the complicated networks that make up the lipid anabolic process, it would be more efficient to boost oil accumulation by enhancing multiple routes in a coordinated fashion including carbon partitioning, FA synthesis, and lipid assembly. Therefore, the identification of the key enzymes or master regulators involved in multiple steps simultaneously becomes an attractive approach for improving oil production (Ohlrogge and Jaworski, 1997; Ruuska et al., 2002; Cahoon et al., 2007; Mu et al., 2008). Transcriptomic profiling revealed that the genes encoding enzymes involved in FA synthesis is co-regulated to the rate of acyl chain synthesis, suggesting that transcriptional regulation plays an important role in the lipid biosynthesis process (Ruuska et al., 2002; Baud and Lepiniec, 2009; Barthole et al., 2012). Recent studies have identified several transcription factors that are capable of governing multiple oil accumulation steps (Cernac and Benning, 2004; Shen et al., 2010; To et al., 2012). Wrinkled1 (WRI1) belongs to the APETALA2 (AP2)-ethylene-responsive element binding protein family of transcription factors, and it acts as a central regulator in seed oil accumulation by modulating numerous genes simultaneously during late glycolysis and FA biosynthesis. A deficiency mutant of *Arabidopsis WRI1* (At*WRI1*) leads to wrinkled seeds with 80% less seed oil content in *Arabidopsis* (Focks and Benning, 1998; Cernac and Benning, 2004). The loss of AtWRI1 also leads to impaired seed germination and seedling establishment, whereas At*WRI1* OE enhances oil accumulation in *Arabidopsis*, which is accompanied by aberrant seedling development (Stone et al., 2001; Kwong et al., 2003; Cernac and Benning, 2004; Cernac et al., 2006; Baud et al., 2007). AtWRI1 binds to the AW-box consensus [CnTnG](n)$_7$[CG] in the proximal promoter of target genes that are involved in the glycolysis and FA synthesis of *Arabidopsis* (Maeo et al., 2009; To et al., 2012).

The biological significance of WRI1 has been extensively studied in relation to oil accumulation in *Arabidopsis*. The role of WRI1 in comprehensive lipid regulation in other plant species, particularly in crop plants, remains to be elucidated. A recent study showed that the WRI1 homolog from maize is able to compensate for the impaired oil accumulation and seedling establishment of the At*wri1* mutant in *Arabidopsis* (Cernac et al., 2006; Pouvreau et al., 2011). The OE of Zm*WRI1* in maize increased the levels of FAs and some amino acid residues (Pouvreau et al., 2011), suggesting that the role of WRI1 in oil accumulation is highly conserved between monocot and dicot plants. *Arabidopsis* that overexpresses At*WRI1* exhibits undesirable agronomic traits, with retarded growth and reduced biomass (Lotan et al., 1998; Stone et al., 2001; Cernac and Benning, 2004; Wang et al., 2007; Mu et al., 2008), whereas Zm*WRI1* OE in maize promotes oil accumulation without visible side effects on growth and development (Shen et al., 2010). The results suggest that the role of WRI1 in oil synthesis is conserved but distinguishable in different plant species. The WRI1 in different plant species may exhibit a unique role in addition to its effect on oil accumulation. Furthermore, most WRI1 studies have been focused on oil accumulation, and the effects of WRI1 on membrane phospholipids and galactolipids have remained unknown. In the present study, we characterized BnWRI1 (BnaA09g34250D) from *Brassica napus* (*B. napus*). Bn*WRI1* OE in *B. napus* resulted in enhanced lipid anabolism by binding to the *cis*-element CnTnG (n)$_7$CG in the promoter regions of genes involved in FA synthesis and lipid assembly to up-regulate these target genes. BnWRI1 promotes oil accumulation and thylakoid membrane monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and PC biosynthesis to regulate homeostasis among membrane lipids, oils, and carbohydrates. Therefore, Bn*WRI1* OE facilitates flowering, reproduction, and oil production without visible side effects on the growth of *B. napus*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Westar cultivar of canola (*B. napus* L.) was used in this study. Seeds were germinated in either Murashige and Skoog (MS) plates or soil in pots. Three-week-old seedlings were then transferred to pots containing soil. The plants were raised in a growth room under 16 h light (25°C)/8 h dark (20°C), a photosynthetic photon flux density of 200–300 mmol m^{-2} s^{-1}, and 60% relative humidity, or natural conditions during winter-spring seasons in Wuhan, China. For the field growth test, 3-week-old seedlings were transferred to the field at suitable spacing (33 cm × 50 cm) that were arranged in a one-way randomized block design with 30 plants/lines per block, and three replications.

Gene Cloning, Vector Construction, and *B. napus* Plant Transformation

To obtain the full-length BnWRI1 cDNA, total RNA was extracted from the leaves of 4-week-old *B. napus* plants, and it

was subjected to reverse transcription to obtain first strand cDNA according to the manufacturer's instructions (Trans-Gene Biotech, Beijing, China). The full-length Bn*WRI1* cDNA was amplified by PCR with primers Bn*WRI1*F 5′-GGATCCATGAAGAGACCCTTAACCACT-3′ and Bn*WRI1*R 5′-GAGCTCTCAGACAGAATAGTTCCAAGAA-3′, and then it was ligated into binary vector pBI121, which had been digested by *Sac*I and *BamH*I. The resulting construct was transformed into *B. napus* by *Agrobacterium* GV3101 mediation with hypocotyls used as explants for regeneration. The transgenic shoots were first selected on kanamycin (50 μg/ml), and then the kanamycin resistant shoots were transferred to MS medium containing 1-naphthaleneacetic acid for rooting. The transgenic plants were further confirmed by PCR with a pBI121 vector and Bn*WRI1* sequence specific primers (Supplemental Table S1).

Subcellular Localization

The full-length cDNA of Bn*WRI1* was ligated into pCAMB-IA1301 vector that had been digested by the restriction enzymes *Sac*I and *BamH*I. The construct containing Bn*WRI1*-GFP was introduced into *Agrobacterium* GV3101 and infiltrated into tobacco leaves for 24 h to obtain transient protein expression under the control of the 35S promoter. Subcellular localization was visualized under a confocal laser scanning microscope (Leica, Biberach, Germany) with the exciter filter HFT488 and the transmitting optical filter BP505–530 to observe the green fluorescence. The nuclei were labeled with 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) staining.

RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted from various tissues at different stages using TransZol reagent (TransGen Biotech, Beijing, China), and it was then treated with RNase-free DNaseI (NEW ENGLAND Biolabs, Ipswitch, MA, USA) to remove any contaminating DNA. The resulting RNA was used for first strand synthesis by reverse transcriptase with an oligo-d (T) 18 primer (TransGen Biotech, Beijing, China) to obtain cDNA according the manufacturer's protocol. Quantitative real-time PCR was performed with SYBR Green PCR Master Mix (TransGen Biotech, Beijing, China) on a single-color Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). A Bn*Actin* gene was used as the standard control. The quantitative real-time PCR conditions were as follows: 95°C for 1 min; 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and 72°C for 10 min for the final extension. The primers used for real-time PCR are listed in Supplemental Table S2.

Lipid Extraction and Analyses

Lipids were extracted from the leaves, developing siliques, and mature seeds. The lipids were separated on a thin layer chromatography (TLC) plate with developing solvent consisting of petroleum ether, ethyl ether, and acetic acid (80:20:1, v/v). The separated lipids were visualized with iodine vapor and the spots were scraped for measurement by GC analysis (Agilent 7890A, Santa Clara, CA, USA) after a methyl ester reaction with
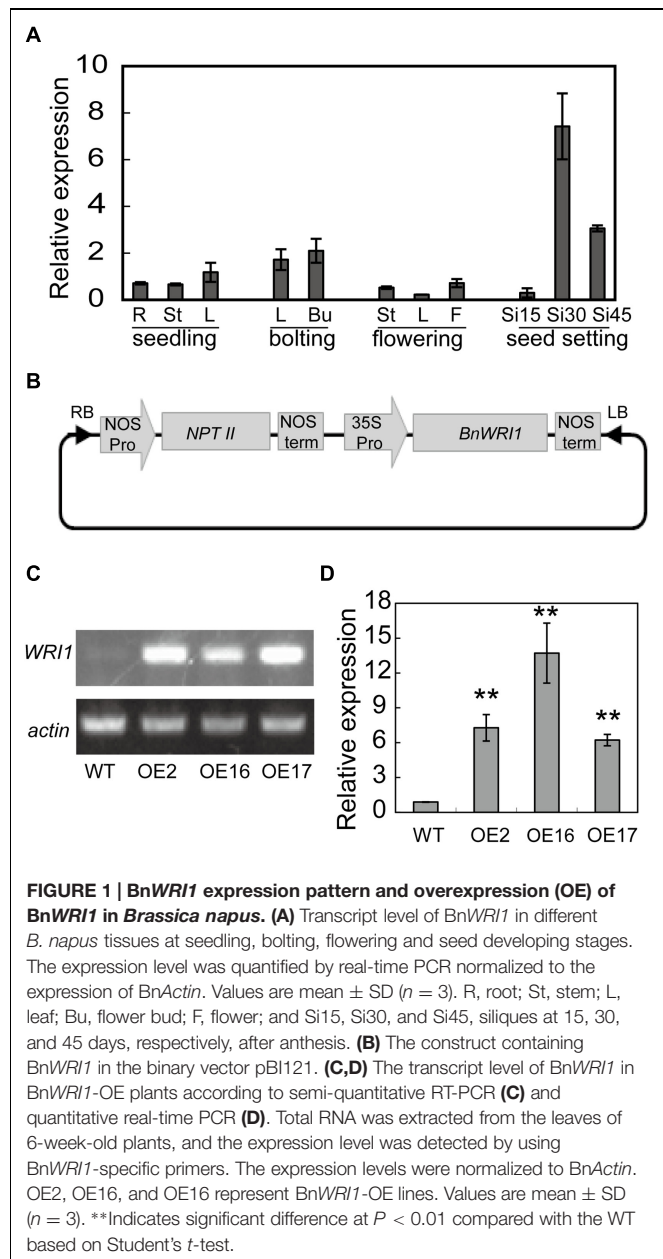
methanol and toluene containing 5% H_2SO_4 at 80°C for 3–4 h. To measure the seed oil contents, oil was extracted from the seeds and tested by GC analysis after the methyl ester reaction as detailed above. The GC running conditions were as follows: the injection port temperature was 180°C, and the oven temperature was set at 180°C for 2 min and was increased by 10°C/min up to 220°C for 5 min. The temperature of the flame ionization detector was 280°C with flow rates of 30, 300, and 25 ml/min for hydrogen, air, and helium, respectively.

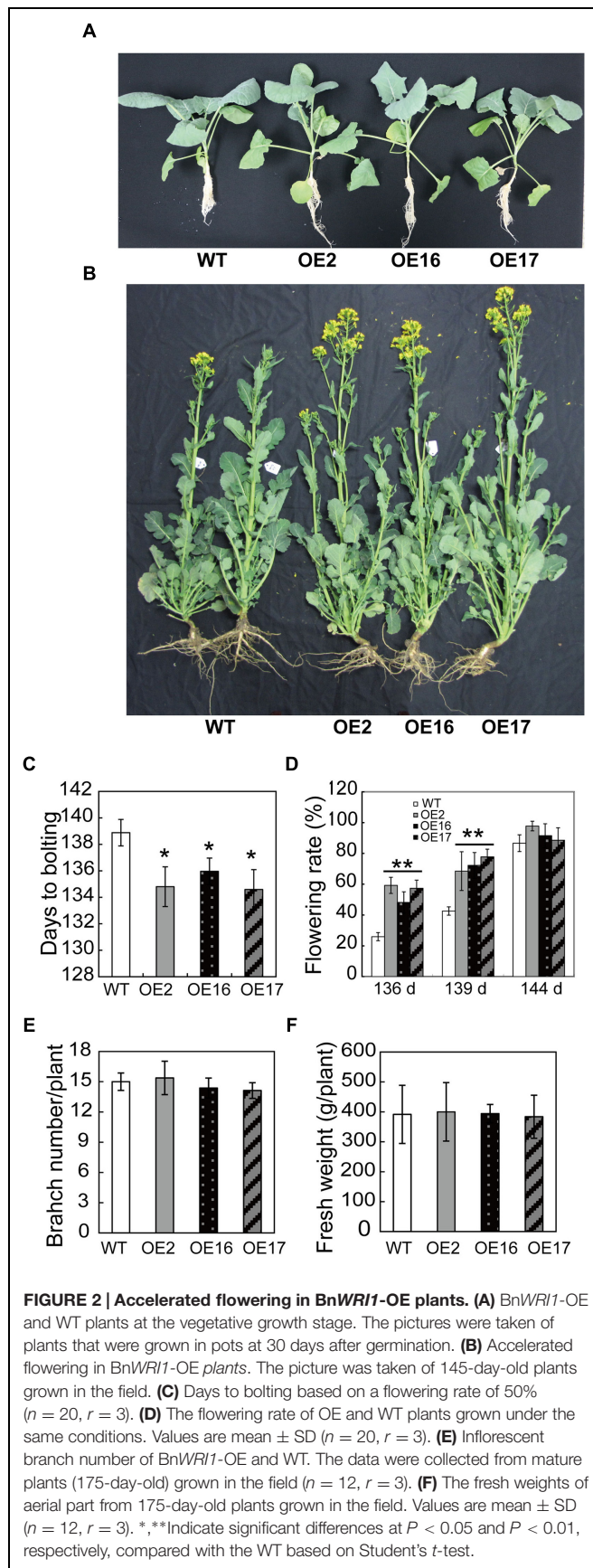## Electrophoretic Mobility-shift Assay

The full-length cDNA of Bn *WRI1* was amplified by using the forward primer 5′-CCCGGGTATGAAGAGACCCTTAACCAC-3′ coupled with the reverse primer 5′-GGATCCCGACAG AATAGTTCCAAGAA-3′, and then it was ligated to pET28a vectors that were digested by *BamH*I and *Sac*I. This construct was transformed into *Escherichia coli* strain Rosetta (DE3), and the BnWRI1 protein was expressed by induction with 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG) while the strain was grown in Luria Bertani (LB) medium overnight at 20°C. The cells were harvested and lysed by sonication in a buffer (300 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10 mM imidazole, 5% glycerol, and 50 mM NaH_2PO_4). The cell lysate was centrifuged at 12,000 r/min for 20 min. The supernatant was incubated with Ni-NTA resin (Shanghai Sangon, http://www.sangon.com) for 3 h at 4°C. The BnWRI1 protein was eluted from the resin after three washes with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8). Protein from *E. coli* cells containing only the pET28a vector was used as a negative control. The DNA sequences that were 300 and 250 bp upstream from the start codon of *KASI* and *GPAT9*, respectively, were amplified from *Arabidopsis* with the *KASI* forward primer 5′-GAATTCTGTTGAGTTACGAATTGGAG-3′, coupled with the *KASI* reverse primer 5′-GAGCTCATTGAG AGAGGTATTGAGAG-3′, and the *GPAT9* forward primer 5′-GAATTCACATAATATGTCCAAGATCATT-3′ coupled with the *GPAT9* reverse primer 5′-GAGCTCCTATTATACTTATA CCACAT-3′. The substitutive nucleotide (C→T, T→C, G→A) mutant at the AW-box [CnTnG](n)$_7$[CG] was amplified by using a similar approach. The amplified DNA fragments containing native or mutant AW-box were incubated with purified BnWRI1 protein in binding buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 2 mM $MgCl_2$, 1% glycerol, 1 mg/ml BSA, 1 mM DTT) for 1 h at 4°C. The resulting mixture was separated on native PAGE (6%) by electrophoresis and was visualized under UV light. The binding activity of BnWRI1 to the AW-box was also determined with biotin labeled DNA probes using a chemiluminescent electrophoretic mobility-shift assays (EMSA) kit (Beyotime, China) according to the manufacturer's instructions.
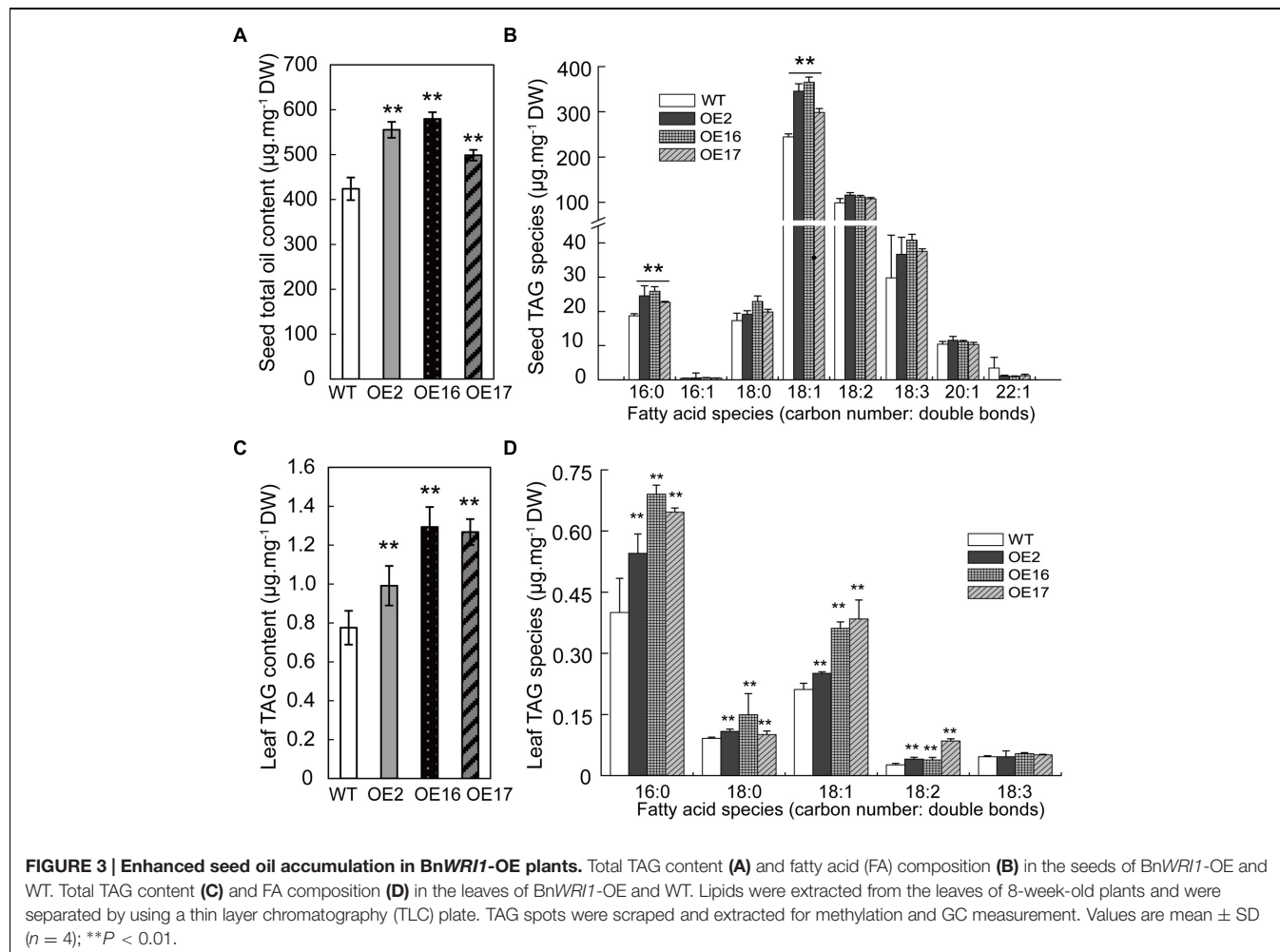
Measurements of Protein, Starch, and Soluble Sugar

Proteins were extracted from leaves and seeds by homogenizing in buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA, and 1% (w/v) SDS, and incubating the mixture for 2 h at 25°C. The homogenate was centrifuged at 16,000 *g* for

FIGURE 1 | Bn*WRI1* expression pattern and overexpression (OE) of Bn*WRI1* in *Brassica napus*. **(A)** Transcript level of BnWRI1 in different *B. napus* tissues at seedling, bolting, flowering and seed developing stages. The expression level was quantified by real-time PCR normalized to the expression of Bn*Actin*. Values are mean ± SD ($n = 3$). R, root; St, stem; L, leaf; Bu, flower bud; F, flower; and Si15, Si30, and Si45, siliques at 15, 30, and 45 days, respectively, after anthesis. **(B)** The construct containing Bn*WRI1* in the binary vector pBI121. **(C,D)** The transcript level of Bn*WRI1* in Bn*WRI1*-OE plants according to semi-quantitative RT-PCR **(C)** and quantitative real-time PCR **(D)**. Total RNA was extracted from the leaves of 6-week-old plants, and the expression level was detected by using Bn*WRI1*-specific primers. The expression levels were normalized to Bn*Actin*. OE2, OE16, and OE16 represent Bn*WRI1*-OE lines. Values are mean ± SD ($n = 3$). **Indicates significant difference at $P < 0.01$ compared with the WT based on Student's *t*-test.

FIGURE 2 | Accelerated flowering in Bn*WRI1*-OE plants. **(A)** BnWRI1-OE and WT plants at the vegetative growth stage. The pictures were taken of plants that were grown in pots at 30 days after germination. **(B)** Accelerated flowering in BnWRI1-OE *plants*. The picture was taken of 145-day-old plants grown in the field. **(C)** Days to bolting based on a flowering rate of 50% ($n = 20, r = 3$). **(D)** The flowering rate of OE and WT plants grown under the same conditions. Values are mean ± SD ($n = 20, r = 3$). **(E)** Inflorescent branch number of BnWRI1-OE and WT. The data were collected from mature plants (175-day-old) grown in the field ($n = 12, r = 3$). **(F)** The fresh weights of aerial part from 175-day-old plants grown in the field. Values are mean ± SD ($n = 12, r = 3$). *,**Indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, compared with the WT based on Student's *t*-test.

10 min, the supernatant was diluted 200 times and the protein concentration was measured by Lowry D protein assay (Bio-Rad). Soluble sugars were measured using phenol-sulfuric acid method (DuBois et al., 1956; Chow and Landhausser, 2004). In brief, leaf samples (1 g fresh weight) were homogenized with deionized water and filtered. The extract (50 µl) was mixed with 450 µl of sulfuric acid containing anthrone (2 mg/ml) at 95°C, and then the absorbance at 625 nm was monitored by spectrometer (Infinite M200 PRD, Untersbergstr, Austria). For starch extraction, the remaining sediment was suspended in a solution containing 0.2 N KOH and incubated at 95°C for 1 h, followed by the addition of 1 N acetic acid and incubation for 15 min. After centrifugation at 16,000 *g* for 5 min, the starch in the supernatant was measured by using a method similar to that of soluble sugars.

FIGURE 3 | Enhanced seed oil accumulation in Bn*WRI1*-OE plants. Total TAG content **(A)** and fatty acid (FA) composition **(B)** in the seeds of Bn*WRI1*-OE and WT. Total TAG content **(C)** and FA composition **(D)** in the leaves of Bn*WRI1*-OE and WT. Lipids were extracted from the leaves of 8-week-old plants and were separated by using a thin layer chromatography (TLC) plate. TAG spots were scraped and extracted for methylation and GC measurement. Values are mean ± SD ($n = 4$); **$P < 0.01$.

RESULTS

Expression Pattern and the Effect of BnWRI1 on Flowering in *B. napus*

To investigate the temporal and spatial distribution of Bn*WRI1* mRNA in *B. napus*, total RNA was extracted from various tissues at different stages and used for analysis by quantitative real-time PCR. During the seedling and bolting stages, the Bn*WRI1* transcript level was higher in leaves and flower buds than it was in roots and stems (**Figure 1A**). During the flowering stage, the Bn*WRI1* expression was higher in flowers than in leaves and stems. The transcript level was rapidly up-regulated in siliques and was highest at 30 days after anthesis (**Figure 1A**).

To explore the biological function of BnWRI1 in *B. napus*, full-length Bn*WRI1* cDNA was cloned by reverse transcription PCR by using mRNA that was extracted from leaves as a template, and the cDNA was ligated into binary vector pBI121. The resulting construct containing Bn*WRI1* was transformed into *B. napus* under the control of the 35S promoter (**Figure 1B**). More than 30 independent transgenic lines were obtained, and the Bn*WRI1* transcript level in transgenic plants was much higher than that of the wild-type (WT; **Figures 1C,D**). Three

representative, independent Bn*WRI1* OE lines, OE2, OE16, and OE17, were selected randomly from 30 transgenic lines, and they were used for further characterization. These plants were grown under natural conditions either in the field or in pots, and no visual growth change was observed between OE and WT plants, which showed a similar leaf size, leaf number, and growth rate during the vegetative growth stage (**Figure 2A**). However, BnWRI1 accelerated flowering; OE plants bolted and flowered 4 to 6 days earlier than WT plants (**Figures 2B,C**). At 136 days after germination, 60% of the OE plants were flowering, whereas only 23% of the WT plants were flowering (**Figure 2D**). The earlier flowering in OE plants did not cause changes in the total number of inflorescent branches and biomass at the mature stage compared with the results for the WT plants (**Figures 2E,F**).

Overexpression of Bn*WRI1* Enhances Oil Accumulation in Seeds and Leaves without Undesirable Agronomic Traits

To investigate the role of BnWRI1 in oil (TAG) synthesis, the oil content was measured in both the seeds and leaves of OE and WT plants. The oil content of Bn*WRI1*-OE seeds was significantly higher than that of the WT, and it was increased by 31, 38,

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