Expression of Camelina WRINKLED1 Isoforms Rescue the Seed Phenotype of the Arabidopsis wri1 Mutant and Increase the Triacylglycerol Content in Tobacco Leaves

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Triacylglycerol (TAG) is an energy-rich reserve in plant seeds that is composed of glycerol esters with three fatty acids. Since TAG can be used as a feedstock for the production of biofuels and bio-chemicals, producing TAGs in vegetative tissue is an alternative way of meeting the increasing demand for its usage. The WRINKLED1 (WRI1) gene is a well-established key transcriptional regulator involved in the upregulation of fatty acid biosynthesis in developing seeds. WRI1s from Arabidopsis and several other crops have been previously employed for increasing TAGs in seed and vegetative tissues. In the present study, we first identified three functional CsWRI1 genes (CsWRI1A, B, and C) from the Camelina oil crop and tested their ability to induce TAG synthesis in leaves. The amino acid sequences of CsWRI1s exhibited more than 90% identity with those of Arabidopsis WRI1. The transcript levels of the three Camelina WRI1s were observed in the nuclei of Nicotiana benthamiana leaf epidermal cells. Nile red staining indicated that the transient expression of CsWRI1A, B, or C increased TAG accumulation in leaves. The levels of TAGs were higher by approximately 2.5- to 4.0-fold in N. benthamiana leaves expressing CsWRI1 genes than in the control leaves. These results suggest that the three Camelina WRI1s can be used as key transcriptional regulators to increase fatty acids in biomass.

Keywords: Camelina sativa, fatty acid, leaves, oil, triacylglycerol, WRINKLED1
INTRODUCTION

*Camelina sativa* L. is an underdeveloped oil crop in the family Brassicaceae, which has several advantages in the agronomic and environmental context over the current developed oil crops. *Camelina* has a relatively short growing period (85–100 days to maturity) and can be cultivated twice in 1 year (Putnam et al., 1993). In comparison with other oil crops, it requires lower amounts of fertilizer for growth and is more resistant to various stresses such as cold and drought (Putnam et al., 1993; Kim et al., 2013; Bansal and Durrett, 2016). *Camelina* seed oil is composed of 35–45% triacylglycerol (TAG), which has a high proportion of polyunsaturated fatty acids (PUFAs) (Lu and Kang, 2008; Bansal and Durrett, 2016). Approximately 40% of the total fatty acid (FA) content is composed of linolenic acid (18:3) and linoleic acid (18:2). The considerable amount of PUFAs confers considerable susceptibility to oxidation, which makes it less suitable for biodiesel production and domestic cooking, such as frying vegetables (Soriano and Narani, 2012). However, breeding and genetic engineering programs can generate new varieties of *Camelina* with a lower PUF content for stable oxidation (Kang et al., 2011; Nguyen et al., 2013). *Camelina* can be transformed using the Agrobacterium-mediated flower-dip method, which is a relatively simple and rapid route to generating transgenic plants with superior agronomic traits (Lu and Kang, 2008; Liu et al., 2012). The whole genome sequence and seed transcriptome data present valuable resources for the understanding of the function of genes involved in oil biosynthesis in *Camelina* seeds (Hutcheon et al., 2010; Nguyen et al., 2013; Kagale et al., 2014).

Triacylglycerol is a neutral lipid molecule that stores carbons and hydrogens that are utilized for energy production in the life cycle of plants (Athenstaedt and Daum, 2006). TAGs are mainly observed in seeds, where they are used as energy stores for seed germination; they also occur in chloroplasts of senescent leaves where they aid in fatty acid sequestration and in pollen, where they have been shown to promote pollen germination in *Arabidopsis* (Kaup et al., 2002; Kim et al., 2002; Athenstaedt and Daum, 2006). The first step of fatty acid synthesis in seeds is the formation of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACCase). Malonyl-CoA-ACP malonyltransferase catalyzes the conversion of malonyl-CoA to malonyl-ACP. Then, malonyl-ACP is condensed with acetyl-CoA to make 3-ketoacyl-ACP. After that the series of sequential condensation reactions of malonyl-ACP to 3-ketoacyl-ACP or the growing of acyl-ACP by fatty acid synthase complex results in 16- to 18-carbon fatty acyl-ACPs in the plastids (Chapman and Ohlrogge, 2012). The fatty acyl chains are exported to the cytoplasm and the fatty acid synthase complex results in 16- to 18-carbon fatty acyl-CoAs by long-chain acyl-CoA synthetase (LACS). Fatty acyl-CoAs are pools utilized for the esterification of fatty acids with glycerol-3-phosphate (G3P) sequentially by three acyltransferases called G3P acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT), and diacylglycerol acyltransferase (DGAT) in the endoplasmic reticulum (ER) to synthesize TAG. Oil bodies containing TAG are formed in the ER membrane budding from the TAG synthesizing site as a droplet (Li-Beisson et al., 2010).

The network for AFL master regulators, ABSCISCIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), LEAFY COTYLEDON1 (LEC1), and LEC2 have been studied with the aim of controlling the biosynthesis of *Arabidopsis* seed oil (North et al., 2010; Roscoe et al., 2015; Devic and Roscoe, 2016). This network can regulate the expression of genes encoding enzymes that synthesize the storage lipids and protein reserves in seeds. As a key transcription factor for lipid accumulation, WRINKLED1 (WRI1) is located downstream in this AFL network (Roscoe et al., 2015). Ectopic expression of FUS3 can regulate WRI1 expression (Yamamoto et al., 2010). LEC2 may regulate fatty acid biosynthesis genes through direct regulation of WRI1 (Baud et al., 2007). Although ABI3 is not known to directly regulate the expression of WRI1, it is known that ABI3, FUS3, LEC1, and LEC2 regulate the downstream genes through synergistic interactions with each other during seed maturation (Devic and Roscoe, 2016). The WRI1 gene was first identified in an *Arabidopsis* mutant exhibiting wrinkled seed morphology; it encodes an AP2/EREBP type transcription factor (Focks and Benning, 1998). WRI1 regulates the expression of genes encoding the enzymes involved in catalytic processes such as phosphoglycerate mutase, plastidic pyruvate kinase β subunit 1 (PI-PKβ1), and pyruvate dehydrogenase (PDHE1α) enzymes during glycolysis, wherein it has been observed to increase the amount of pyruvate during acetyl-CoA synthesis (Baud et al., 2007, 2009; Maeo et al., 2009; Fukuda et al., 2013). Furthermore, WRI1 directly binds to the promoters of genes encoding key enzymes for fatty acid biosynthesis during seed maturation, such as biotin carboxyl carrier protein isoform 2 (BCCP2), acyl carrier protein 1 (ACP1), and keto-ACP synthase 1 (KAS1) (Baud et al., 2009; Maeo et al., 2009).

*Arabidopsis wr11* mutants have been shown to exhibit an up to 80% lower TAG content than the wild-type seeds, with correspondingly increased sucrose levels. This implies that WRI1 is a regulator for carbon allocation between sucrose and fatty acids in developing seeds (Focks and Benning, 1998; Ma et al., 2015). Following the discovery of *Arabidopsis* WRI1 (AtWRI1), its homologues have been identified from the seeds of rapeseed (*Brassica napus* L.), corn (*Zea mays* L.), and oil palm (*Elaeis guineensis* Jacq.) (Liu et al., 2010; Shen et al., 2010; Prouvéau et al., 2011; Ma et al., 2013; Wu et al., 2014). In addition to seeds, WRI1 homologs have been identified in non-seed tissues containing oil, such as the stem of poplar (*Populus trichocarpa* L.), tuber parenchyma of nutsedge (*Cyperus esculentus* L.), and leaf blades of *Brachypodium distachyon* L. Beauv. (Grimberg et al., 2015; Yang et al., 2015). Overexpression of the *WRI1* genes contributed to increased TAG levels in the seeds and vegetative tissues. Seed-specific expression of *AtWRI1* transgene increased TAG content to levels 14–30% higher than that in the wild-type seeds in *Arabidopsis, B. napus*, corn, and *Camelina* (*Cernac and Benning, 2004; Liu et al., 2010; Shen et al., 2010; Prouvéau et al., 2011; Wu et al., 2014; An and Suh, 2015). Ectopic overexpression of *WRI1* stimulated oil production in the vegetative tissues (Vanhercke et al., 2013, 2014; Nookaraju et al., 2014; Zale et al., 2016). Five *WRI1* isoforms have been isolated from diverse species such as...
Arabidopsis, potato (Solanum tuberosum L.), oat (Avena sativa L.), and nutsedge. These genes were introduced into leaves using the agroinfiltration method, which resulted in an increase in TAG content from 0.05% to 2.20% in Nicotiana benthamiana Domin. leaves (Grimberg et al., 2015).

In the present study, we first identified three Camelina WR1 isoforms, CsWR1A, CsWR1B, and CsWR1C, that complemented the wrinkled-seed phenotype and partially restored TAG content in Arabidopsis wr1-3 mutants. We then studied the CsWR1 genes to identify a potential candidate to act as a transcriptional regulator for inducing TAG production in vegetative tissues.

MATERIALS AND METHODS

Plant Materials and Growth Conditions
Camelina sativa L. CAME, Arabidopsis thaliana accession Columbia-0, and N. benthamiana plants were grown in long-day growth conditions with 16 h/8 h light/dark photoperiod at 21–24°C, and 50–60% humidity in a sterilized soil mixture (peat moss enriched soil:vermiculite:perlite in 4:2:1 ratio) (An and Suh, 2015). These genes were introduced into leaves using the agroinfiltration method, which resulted in an increase in TAG content from 0.05% to 2.20% in Nicotiana benthamiana (An et al., 2015).

Gene Identification and Isolation
Total RNAs were isolated from developing Camelina seeds 20 days after flowering (DAF) based on the protocol for RNA isolation from seeds (Ohate-Sánchez and Vicente-Carbajosa, 2008). cDNAs were then synthesized by RT-PCR using primers listed in Supplementary Table 1. The PCR protocol was as follows: denaturation at 94°C for 30 s, annealing at 58–62°C for 20 s, and extension at 72°C for 20 s. These cycles were performed 35 times. To control equal cDNA loading in RT-PCR, CsACTIN11 and EIF4A1 (At3g13920) gene-specific primers listed in Supplementary Table 1 were used as controls for transcript levels in various tissues (Hutcheon et al., 2010).

Protein Sequence and Phylogenetic Tree Analysis
Amino acid sequences were aligned using CLUSTALW, and dendrograms for phylogenetic analysis were constructed using the MEGA 6.06 program1 with the maximum likelihood method and a bootstrap value of 500 replicates (Tamura et al., 2013).

RT-PCR Analysis
In Camelina, transcript expression was analyzed in various tissues of the aerial parts such as the flower buds and leaves of 5-week-old plants, stems from 4-week-old plants, roots from 2-week-old plants, open flowers from plants older than 6 weeks, and developing seeds 10, 20, and 30 DAF. To assess the expression level of the transgene in Arabidopsis complementation lines, total RNAs were extracted from developing seeds of transgenic Arabidopsis 6–8 DAF. Total RNAs were isolated from these tissues using QIAGEN RNaseasy® plant mini kit (50), following the manufacturers protocols. In addition, RNAs from developing seeds of Camelina and Arabidopsis were isolated according to the method described in Ohate-Sánchez and Vicente-Carbajosa (2008). First, cDNAs were synthesized from these RNAs by the GoScript™ reverse transcription system (Promega) followed by PCR by the Access Quick™ RT-PCR system (Promega) to analyze the transcript levels of each gene using gene-specific primers (Supplementary Table 1). The PCR protocol was as follows: denaturation at 94°C for 30 s, annealing at 58–62°C for 20 s, and extension at 72°C for 20 s. These cycles were performed 35 times. To control equal cDNA loading in RT-PCR, CsACTIN11 and EIF4A1 (At3g13920) gene-specific primers listed in Supplementary Table 1 were used as controls for transcript levels in various tissues (Hutcheon et al., 2010).

Vector Construction
To express the CsWR1 gene in transgenic plants, each cDNA containing an ORF was inserted under the sequences of CaMV 35S promoter by digestion at the BamHI (5′-terminus) and SacI (3′-terminus) restriction endonuclease sites in the pBA002 vector (Kim et al., 2006). This vector contains the herbicide resistance marker gene PAT (Phosphinothricin acetyltransferase), which enables selection of the transgenic plants. These constructs were transformed into Agrobacterium tumefaciens GV3101 using the freeze-thaw method (An, 1987), which were then used for the transformation of Arabidopsis by the Agrobacterium-mediated floral dip method (Zhang et al., 2006).

CsWR1 cDNAs containing an ORF without the stop codon were inserted at the SacI (5′-terminus) and XmaI (3′-terminus) sites between the CaMV 35S promoter and enhanced yellow fluorescent protein (eYFP) to enable fusion of the eYFP with the in-frame stop codon, and allow expression under the control of the CaMV 35S promoter in the pPZP212 vector (GenBank U10462). These constructs were then used for the detection of fluorescence signals of CsWR1 proteins in the epidermal cells of N. benthamiana infected by Agrobacterium tumefaciens GV3101.

Subcellular Localization
The vectors expressing CsWR1:eYFP under the control of the CaMV 35S promoter were transformed into Agrobacterium tumefaciens LBA4404, followed by the infection of the N. benthamiana leaf epidermis using the agroinfiltration method (Yoo et al., 2007). The N. benthamiana leaves were further grown for 12 h, and the fluorescence was observed using the TCS SP5 AOBS/Tandem laser scanning confocal microscope (Leica Microsystems, Germany). The emission wavelength was 571–617 nm and the excitation wavelengths were 520–554 nm for YFP and 572–618 nm for RFP.

Generation of Transgenic Arabidopsis
Progeny seeds harvested from the transformed Arabidopsis plants were germinated and grown on 1/2 MS medium (1% sucrose, 0.7% phytoagar) containing 5 μg/mL of phosphinothricin (PPT). PPT-resistant T1 plants were tested for the presence of the transgene in the genome by PCR analysis with primers listed in Supplementary Table 1. T2 seeds from T1 transgenic plants were selected for the observation of seed morphology and determination of the fatty acid content.

1http://www.megasoftware.net/
Seed Morphology Analysis
Seed morphology was observed using light and scanning electron microscopes. Dry seeds of the wild-type and the wr1-3 mutant were coated with platinum particles using a Hitachi E1030 coater on aluminum stubs. Their images were scanned using a field emission scanning electron microscope (FE-SEM, Hitachi S-4700, Tokyo, Japan). The ratio of seeds rescued from the wrinkled phenotype to normal in the transgenic lines was recorded from the photographs taken of the T2 seed siblings using an Axiocam, MRc 5 camera equipped with a light microscope (Carl Zeiss: SteREO Lumar. V12).

Nile Red Staining
The leaves of *N. benthamiana* were infiltrated with *Agrobacterium* containing an empty vector (pBA002) or the CsWRI1 vector. These leaves were stained with Nile red solution [10 μg/mL in 0.1 M Tris-HCl buffer (pH 8) Sigma-N3013] at room temperature for 30 min. The stained leaves were washed with 0.1 M Tris-HCl buffer (pH 8) for 10 min, followed by observation of red fluorescence of the oil bodies at wavelengths of 560 nm for excitation and 615 nm for emission using a tandem laser confocal scanning microscope (TCS SP5 AOBS, Leica Microsystems, Germany).

Thin Layer Chromatograph (TLC) Analysis
The fresh leaves of *N. benthamiana* were homogenized and immersed in isopropanol at 65°C for 15 min. Chloroform and water were mixed in same tube, then total lipids were extracted twice using a chloroform:methanol solution (2:1, v/v). The solvents of the lipid phase were collected and evaporated using nitrogen gas. Finally, total lipids were dissolved in chloroform containing tri 17:0-TAG (glyceryl triheptadecanoate; Sigma-T2151) as an internal standard and separated by TLC (Silica gel 60, MERCK) in hexane:diethyl ether:acetic acid (70:30:1, v/v). The solvents of the lipid phase washed with 1 M KCl were mixed in same tube, then total lipids were concentrated by N2 gas and tri 17:0-TAG in toluene were combined and esterified with methanol and H2SO4. The esterified TAGs were analyzed for their fatty acid profile and content, total lipids from developing seeds were collected and evaporated using nitrogen gas. Finally, total lipids were homogenized and immersed in isopropanol at 65°C for 15 min. Chloroform and water were mixed in same tube, then total lipids were extracted twice using a chloroform:methanol solution (2:1, v/v). The solvents of the lipid phase were collected and evaporated using nitrogen gas. Finally, total lipids were dissolved in chloroform containing tri 17:0-TAG (glyceryl triheptadecanoate; Sigma-T2151) as an internal standard and separated by TLC (Silica gel 60, MERCK) in hexane:diethyl ether:acetic acid (70:30:1, v/v). They were then visualized by spraying 0.01% primuline (Sigma-206865) in 80% acetone under UV light (Vanhercke et al., 2013). TAG bands scraped from TLC were dissolved in toluene and esterified with methanol and H2SO4 mixture at 95°C for 90 min. The esterified TAGs were analyzed for their fatty acid content using gas chromatography as described in the following section.

Fatty Acid Analysis
To measure total fatty acid profile and content, total lipids extracted from *Arabidopsis* seeds or *N. benthamiana* fresh leaves and tri 17:0-TAG in toluene were combined and esterified with methanol mixed with H2SO4 at 95°C for 90 min. After the reaction, 0.9% NaCl solution and hexane were added to extract the fatty acid methyl esters. The upper phase containing the fatty acid methyl esters was concentrated by N2 air and separated in a DB23 column (30 m × 0.25 mm, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) using GC-2010 gas chromatography (Shimadzu, Japan). The temperature of the reaction ranged from 160°C to 220°C, increasing at a rate of 2.5°C per min. The peak area on the retention time for each fatty acid was characterized and measured by comparison with the known standard fatty acid profiles, and the concentration of each fatty acid was calculated by comparing its peak area with that of the internal standard.

RESULTS
Camelina Possesses Three Copies of WRI1
In order to identify the factors involved in the accumulation of higher oil content in *Camelina*, we first focused on identifying the homologous genes of *Arabidopsis* WRINKLE1 (AR1) in two different *Camelina* genome databases2,3. Three WRI1 scaffolds and three WRI1 loci were identified in different chromosomes of the hexaploid genome (Supplementary Table 2) (Kagale et al., 2014). Three WRI1 scaffolds showed overall 28–36% nucleotide sequence identity in intron and 5′- and 3′-coding regions (Supplementary Figure 1) (Kagale et al., 2014). Based on the differences in nucleotide sequences we can detect the corresponding three distinct WRI1 transcripts from developing seeds in *Camelina* by cloning their cDNAs. These three *Camelina* cDNAs were designated as CsWRI1A (GenBank KY129795), CsWRI1B (GenBank KY129796), and CsWRI1C (GenBank KY129797) based on the identity with WRI1 genomic DNA sequences in *Camelina* genome scaffolds and *Arabidopsis* WRI1. Excluding the non-coding regions and the intron, the three CsWRI1 genes exhibited a high identity in nucleotide sequences (Supplementary Figure 2). Compared with that of the AtWRI1, the three CsWRI1s exhibited a high identity in the amino acid (aa) sequence levels (~95%) with 4, 1, and 3 amino acids that are longer than that of the 430 aa open reading frame (ORF) of *Arabidopsis*. An AP2/EREBP DNA binding motif and a VYL transcriptional activation motif are conserved between *Camelina* and *Arabidopsis* (Figure 1).

Several WRI1 genes were identified from crop plants following *Arabidopsis* (Figure 2) (Shen et al., 2010; Wu et al., 2014). The majority are single WRI1 genes identified from each species, except for maize, in which two isoforms, ZmWRI1a and ZmWRI1b, are present in the genome. Owing to its hexaploid genome, *Camelina* has three WRI1 isoforms, which is the highest number of copies discovered in any plant species to date. Phylogenetic analysis indicates that the CsWRI1s are grouped in the same clade as these of *Arabidopsis* and *B. napus* among the various WRI1s that are currently identified from plants (Figure 2).

Three WRI1s Are Predominantly Expressed in Developing Seeds
Since *Arabidopsis* WRI1 is known to express during seed development (Baud et al., 2007), the three CsWRI1s were analyzed for the expression pattern of their transcripts in

2 http://camelinagenomics.org/
3 http://www.camelinadb.ca/
developing seeds, which was then compared with that of the various tissues in *Camelina*. To distinguish the transcript of each *CsWRI1* isoform from the three highly homologous genes, transcript-specific primers were designed and their specificity was confirmed by cDNA-PCR. **Figure 3A** shows each set of primers that were able to detect the specific transcript for *CsWRI1* isoform. Using these gene-specific primers, RT-PCR analysis were performed in samples of the root, stems, leaves, flower buds, open flowers and three different stages of developing seeds. The expression levels of *CsWRI1A, CsWRI1B,* and *CsWRI1C* were analyzed using these primers. The results showed that *CsWRI1A* and *CsWRI1C* were predominantly expressed in seeds, while *CsWRI1B* was more expressed in leaves and stems.

![Alignment of the deduced amino acid sequences of WRI1 isoforms from *C. sativa* and *A. thaliana*. Non-conserved and conservatively changed amino acid residues are shaded in black and gray, respectively. Bright green and orange boxes indicate conserved AP2/EREBP DNA binding motifs and the 'VYL' motif for transcriptional activation of WRI1, respectively.](image-url)

| Isoform | Amino Acid Sequence | Accession Number |
|---------|---------------------|------------------|
| CsWRI1A | MKKRTLSTNSSSSSSSSSSTTSPPSETTPKRAKKPSDSDDKKTNPTSPA | 58 |
| CsWRI1B | MKKRTLSTNSSSSSSSSSSTTSPPSETTPKRAKKPSDSDDKKTNPTSPA | 58 |
| CsWRI1C | MKKRTLSTNSSSSSSSSSSTTSPPSETTPKRAKKPSDSDDKKTNPTSPA | 58 |
| AtWRI1  | MKKRTLSTNSSSSSSSSSSTTSPPSETTPKRAKKPSDSDDKKTNPTSPA | 58 |

**FIGURE 1** | Alignment of the deduced amino acid sequences of WRI1 isoforms from *C. sativa* and *A. thaliana*. Non-conserved and conservatively changed amino acid residues are shaded in black and gray, respectively. Bright green and orange boxes indicate conserved AP2/EREBP DNA binding motifs and the 'VYL' motif for transcriptional activation of WRI1, respectively.
and CsWRIIC were predominantly higher in developing seeds than in other organs (Figure 3B). The Csa06g028810 transcript, showing 98% identity with CsWRIIB, represents high expression in developing seeds in the transcriptome analysis (Supplementary Table 3). These results indicate that all three CsWRII isoforms are actively transcribed during seed development.

### Three WRI1 Are Localized in the Nucleus

The amino acid sequences derived from the three CsWRII cDNAs revealed that the protein might be a transcription factor containing the AP2/EREBP DNA binding and VYL activation motifs (Figure 1). In order to detect the subcellular localization of Camelina WRI1, each CsWRII cDNA was fused in-frame with eYFP under the CaMV 35S promoter in the Agrobacterium binary expression vector (Figure 4A). Agrobacteria containing the CsWRIIs:eYFP genes were infiltrated into N. benthamiana leaves and the fluorescent signals were visualized under the confocal scanning microscope. The yellow fluorescent signals emitted by CsWRI1:eYFP were detected throughout the nucleus, except for the nucleolus, in all three WRII isoforms (Figures 4B,E,H). These signals identically overlapped with the blue fluorescent signal (Figures 4C,F,I) emitted by the nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI) under UV light. The subcellular localization of CsWRIIA, CsWRIIB, and CsWRIIC indicate that all three isoforms localize in the nucleus (Figures 4D,G,J).

### Three CsWRIIs Functionally Rescued the Arabidopsis wri1-3 Mutant Phenotype

Arabidopsis has a single WRII gene in its genome. Mutant seeds with T-DNA inserted in the fifth exon of AtWRI1 were obtained from the Arabidopsis Biological Resource Center (ABRC) and we identified the wri1-3 homozygous mutant (Figure 5A). This mutant exhibited the wrinkled phenotype in seeds, in contrast to the round phenotype of wild-type seeds (Figure 5B). To test if the three Camelina WRII genes can work functionally instead of AtWRI1 in the wri1-3 mutant, each of the CsWRII cDNAs was inserted between the CaMV 35S promoter and nos terminator in plant expression vectors (Figure 5C). These vectors were transformed into wrl1-3, and the generated transgenic plants were selected based on the resistance to the herbicide Basta.

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**FIGURE 2** | Phylogenetic tree of WRI1 from higher plants including C. sativa. Phylogenetic tree was generated using MEGA6.06 by the maximum likelihood method. Bootstrap value percentages of 500 replicates are shown at the branching points. AtWRI1, Arabidopsis lyrata (EFH52510.1); AsWRI1, Avena sativa (SRX1079426); BnWRI1, Brassica napus (ABD16282.1); CeWRI1, Cyperus esculentus (SRX1079431); CnWRI1, Cocos nucifera (JQ040545); EgWRI1, Elaeis guineensis; GhWRI1, Gossypium hirsutum (TC200263); JcWRI1, Jatropha curcas (AAS57945.1); OsWRI1, Oryza sativa (CAE00853.1); PtWRI1, Populus trichocarpa (SRX1079428); RcWRI1, Ricinus communis (AB774159.1, AB774160.1); StWRI1, Solanum tuberosum (SRX1079426); VvWRI1, Vitis vinifera (CBI32013.3); ZmWRI1, Zea mays (ACF83189.1, ACF80269.1).
Among the Basta resistant transgenic plants, 9–10 transgenic plants were analyzed for the presence of the transgene insert by the genomic DNA-PCR method with gene-specific primers for each CsWRI1. Transgenes were detected in genome DNA isolated from the leaves of T1 transformants, but not in that of wri1-3 and empty vector transgenic leaves (Figure 5D).

In the transgenic Arabidopsis wri1-3 plants expressing CsWRI1s, the fatty acid content of seeds in the T2 generation in nine to ten independent lines for each Camelina WRI1 isoform was measured and compared with those of the wri1-3, empty vector transformed wri1-3, and wild-type plants (Supplementary Table 4, Figure 6). To detect the fatty acid amount in seeds accurately, the dry weight of seeds and seed number were used for the measurement in three representative lines for each transgene. All three CsWRI1 cDNAs were able to partially complement the wri1-3 phenotype, as evidenced by a greater seed fatty acid content compared to the mutant and the vector-only transformant control irrespective of expression on the basis of seed weight or seed number (Figures 6A–C, Supplementary Table 5). These results suggest that CsWRI1A, B, and C variants are each potentially involved in regulating the level of fatty acid biosynthesis in developing seeds.

We analyzed transcript levels of each CsWRI1 transgene in developing seeds with three best rescued plants for each CsWRI1 gene among all of the transgenic lines and compared them with the wri1-3 host line. RT-PCR analysis showed that each CsWRI1A, B, and C were expressed in developing seeds of transgenic plants and enhanced the expression level of genes involved in glycolysis and fatty acid synthesis, PI-PKβ1 and BCCP2 (Figure 7). These RT-PCR results showed correlation between the expression of CsWRI1 transgene, the partial complementation of seed fatty acid content, and seed morphology.
FIGURE 5 | Isolation of T-DNA inserted wri1-3 knockout mutant and complementation of CsWRI1A, CsWRI1B, and CsWRI1C in wri1-3 mutants.

(A) Genomic organization of the WRI1 gene inserted with T-DNA in wri1-3. (B) SEM image of the seed morphology of WT and wri1-3 mutant seeds. Bar = 10 µm. (C) Schematic diagram of the binary vector constructs for the expression of CsWRI1s in Arabidopsis wri1-3 mutant. (D) Genomic DNA-PCR of CsWRI1s transgenes of WT, wri1-3 mutant, and complementation transgenic lines. w-EV represents transgenic Arabidopsis wri1-3 mutant introducing empty vector. The numbers indicate independent transgenic lines (T1).

In order to determine whether there is a correlation between the induced oil bodies in N. benthamiana leaves and an increased TAG content, N. benthamiana leaves infiltrated by CsWRI1s or the empty vector were measured for the fatty acid content in TAGs fractionated by TLC. Expression of each CsWRI1 enhanced TAG content 2.5- to 4.0-fold in fresh leaves compared to the empty-vector control (Figure 9A). Measurement of the total lipid amount in fresh leaves showed that CsWRI1s produced 17–41% more fatty acids in transformed leaves compared with the control leaves transformed with the empty vector (Figure 9B). The TAG and total lipid content in leaves transformed by CsWRI1s had a fatty acid composition somewhat different to that of the controls. Five fatty acids (18:3 > 16:0 > 18:2 > 18:1 > 18:0) accounted for 97–98% of all TAGs. Transformation with CsWRI1s also induced a subtle change in the fatty acid composition of TAGs, such as an increase in 18:1 and a decrease in 18:0 compared to the empty vector controls. In the total lipid analysis, five fatty acids (18:3 > 16:0 > 18:2 > 18:1 > 18:0) accounted for 94–95% of all lipids. Among major fatty acids in leaves, 18:3 was decreased and 18:2 increased in transformed leaves compared to the control leaves (Table 1). The enhanced leaf TAG content suggests that CsWRI1s can be employed to increase the vegetable oil content in biomass.

DISCUSSION

Camelina has an advantage as an oil crop as it is amenable to metabolic engineering for the manipulation of vegetable oil biosynthesis (Bansal and Durrett, 2016). Metabolic engineering in Camelina is underway to modify the fatty acid composition by blocking the endogenous fatty acid desaturase 2 (FAD) and fatty acid elongase (FAE) 1 pathways, which would make it more suitable for biofuel (Nguyen et al., 2013), and to incorporate new pathways to produce unusual fatty acids for industrial uses (Snapp et al., 2014; Liu et al., 2015; Nguyen et al., 2015), and omega-3 long-chain polyunsaturated fatty acids for human consumption (Petrie et al., 2014). Wax esters replaced 21% of...
the seed oil TAGs in *Camelina* transformed with two genes encoding a fatty alcohol-forming acyl-CoA reductase (FAR) and a wax ester synthase (WS) (Iven et al., 2016). Currently only two key genes that are involved in fatty acid and TAG biosynthesis metabolism, *FAD2* (Kang et al., 2011) and *DGAT1* (Kim et al., 2016), respectively, have been identified to exist in three copies in *Camelina*. In the present study, we show that the gene for *WRI1*, a regulator for fatty acid biosynthesis, also exists in three copies in the *Camelina* genome (Figure 1, Supplementary Table 2). The three copies (CsWRI1A, CsWRI1B, and CsWRI1C).
FIGURE 8 | Transient expression of three CsWRI1 isoforms in N. benthamiana leaves. (A) Schematic diagram of the binary vector constructs for the transient expression of CsWRI1s in N. benthamiana leaves. (B) Oil body (OB) counts in N. benthamiana leaves expressing empty vector (pBA002), CsWRI1A, CsWRI1B, or CsWRI1C. Values are averages and SD of three individual images. Data were statistically analyzed using Student’s t-test (*P < 0.01). (C) Agrobacterium harboring CsWRI1A, CsWRI1B, or CsWRI1C was infiltrated in N. benthamiana leaves and then the leaf disks were stained with Nile red solution. The fluorescent signals were visualized by laser confocal scanning microscopy. The white arrows indicate OBs. Bars = 20 µm.

FIGURE 9 | Fatty acid content present in triacylglycerols (A) and total lipids (B) of N. benthamiana leaves transiently overexpressing three CsWRI1 isoforms. Agrobacterium harboring empty vector (EV), CsWRI1A, CsWRI1B, or CsWRI1C was infiltrated to 5-week-old N. benthamiana leaves and the N. benthamiana plants were further incubated for 5 days. Total lipids were extracted from N. benthamiana leaf disks including the injection region and analyzed by gas chromatography or fractionated on thin layer chromatography. The eluted TAG fractions were transmethylated and the fatty acid methyl esters were analyzed by gas chromatography. Each value is the mean ± SE of three independent measurements. Data were statistically analyzed using Student’s t-test (*P < 0.01).

and CsWRI1C) were very similar in their amino acid sequences to those of A. thaliana (Figure 1). Phylogenetic tree analysis grouped the three CsWRI1s and those of Arabidopsis and Brassica to same clade (Figure 2). The AP2/EREBP DNA binding domain and a key ‘VYL’ motif for controlling the target gene’s transcription involved in fatty acid biosynthesis (Ma et al., 2013) were identically conserved but the N- and C-terminal amino acid sequences were moderately diverse in three Camelina and Arabidopsis WRI1s (Figure 1). A very high homology was observed among the three Camelina WRI1 genes and Arabidopsis WRI1, which may be construed as evidence for a whole-genome triplication event from the ancestral crucifer genome (Kagale et al., 2014). The nuclear localization of WRI1 has not been confirmed in any previous report, although it has been shown that WRI1 binds to the regulatory element of the promoters in fatty acid biosynthesis as a transcription factor (Baud et al., 2009; Maeo et al., 2009). In this report, we demonstrated that WRI1s are localized in the nucleus by using fluorescent fusion proteins (Figure 4).

All three Camelina WRI1s were abundantly expressed in developing seeds (Figure 3). CsWRI1A, the gene with the highest homology with Arabidopsis WRI1 exhibited higher expression levels than those of CsWRI1B and CsWRI1C (Figure 3). Ectopic expression of Camelina WRI1s partially replaced Arabidopsis WRI1 functionality by partially recovering the reduced amount of fatty acids and reversing the wrinkled phenotype in wri1-3 seeds as compared to those of the wild-type (Figures 5 and 6). Each of CsWRI1s transgenic lines upregulated PI-PKβ1 and BCCP2 downstream target genes in developing seeds compared with wri1-3 host plants (Figure 7). The reason that CsWRI1s could not fully recover the fatty acid
amount and reverse the wrinkled phenotype in transgenic seeds may be that the use of the CaMV 35S promoter or the regulation of CsWRI1 by endogenous factors FUS3 and LEC2 may not be optimal for seed oil accumulation (Devic and Roscoe, 2016). The expression of phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT) of flax (Linum usitatissimum) under the control of CaMV 35S promoter in Arabidopsis rod1 mutant could not recover the PUFA content comparable with that observed in wild-type plants (Wickramarathna et al., 2015). In addition, the introduction of a vector combined with CaMV 35S promoter and B. rapa FAD2-1 gene into Arabidopsis fad2-2 loss-of-function mutant did not achieve the level of linoleic acid found in seeds of the wild-type plant (Jung et al., 2011). On the other hand, a recent study showed that the overexpression of WRI1 by Fus3 seed-specific promoter resulted in a stronger effect than that from CaMV 35S promoter alone for the induction of more oil production in seeds (Kanai et al., 2016). These similar results suggest that the CaMV 35S promoter might not be appropriate for metabolic engineering to enhance seed oil accumulation.

Oil production in leaves is an alternative way to increase the production of vegetable oils in limited cultivating lands and under water supply constraints to meet the increasing demand for vegetable oils as foods and biofuels. Oil accumulation in developing seed requires seed maturation controlled by four master regulators: ABI3, Fus3, Lec1, and Lec2. Lec2 is expressed early on in seed development, while Fus3 and ABI3 are more implicated in seed maturation processes (Wang et al., 2007; Wang and Perry, 2013; Roscoe et al., 2015; Zhang et al., 2016). WRI1 is a regulator of lipid accumulation downstream of the above four master regulators (Roscoe et al., 2015). Recently, researchers have attempted to produce vegetable oil (TAG or fatty acids) in leaves with the overexpression of a key regulator WRI1 for fatty acid synthesis as well as senescence-inducible or xylem-specific expression of LEC2, which is a transcription factor involved in the early stages of seed development (Kim et al., 2015). In most cases, since Arabidopsis WRI1 gene has not shown any negative effect on plant growth, it has been used mainly for the production of TAG in the vegetative tissues. Overexpression of Arabidopsis WRI1 alone elevated TAG levels to 2.8-fold in its seedlings (Sanjaya et al., 2011). Co-expression of Arabidopsis WRI1 and DGAT1, involved in the final step of TAG biosynthesis, and oleosin, responsible for the stability of TAG, resulted in an accumulation of 15% TAG of the estimated 17.7% total lipids in N. benthamiana leaves (Vanhercke et al., 2014). Co-expression of WRI1 and DGAT1-2 in a transgenic sugarcane plant, which also possessed suppressed endogenous genes for ADP-glucose pyrophosphorylase (AGPase) and peroximal ABC transport 1, resulted in increased total fatty acid content of up to 4.7 and 1.7% in leaves and stems, respectively (Zale et al., 2015). Since WRI1 is a regulator for lipid accumulation even in non-seed tissues (Kilaru et al., 2015), WRI1 paralogs distinct from seed-specific WRI1 were observed in the tallow layer, the non-seed tissue, in Chinese tallow (Triadica sebifera), which is a valuable oilseed-producing tree (Divi et al., 2016). In yet another study, the ectopic expression of B. distachyon WRI1 isolated from the vegetative tissues induced an increase in TAG content and simultaneously resulted in cell death in the leaf blades (Yang et al., 2015). Ectopic expression of WRI1s from diverse species and tissues in leaves of N. benthamiana has been shown to result in the production of TAG content ranging from 0.05 to 2.2% (Grimberg et al., 2015). In the present study, the overexpression of three Camelina WRI1 isoforms resulted in the formation of oil bodies (Figure 8) and the production of approximately 0.025–0.04% TAG in fresh leaves, which represents a 2.5- to 4.0-fold increase from control leaves (Figure 9A). The amount of total lipids in fresh leaves was increased by a further 17–41% by introducing CsWRI1s (Figure 9B). The fatty acid composition of TAG and leaves was also found to be different (Table 1). Two major fatty acid levels were altered: 18:1 was increased and 18:0 was decreased in the TAG fraction, whereas 18:3 was decreased and 18:2 was increased in the total lipids of CsWRI1s transformants, compared with empty vector control leaves. This suggests CsWRI1s may drive 18:1 deposition for TAG synthesis. In conclusion, three WRI1 genes isolated from the hexaploid

### TABLE 1 | Fatty acid profile of the TAG fraction and total lipids of leaf disks transiently expressing the three CsWRI1 genes.

|                  | EV          | CsWRI1A     | CsWRI1B     | CsWRI1C     |
|------------------|-------------|-------------|-------------|-------------|
| **TAG fraction (mol %)** |             |             |             |             |
| 16:0             | 25.2 ± 0.4  | 28.3 ± 0.2  | 26.5 ± 0.6  | 27.1 ± 0.3  |
| 16:1             | 1.3 ± 0.1   | 0.6 ± 0.0   | 0.7 ± 0.0   | 0.7 ± 0.0   |
| 16:3             | 1.0 ± 0.0   | 0.6 ± 0.0   | 0.5 ± 0.0   | 0.6 ± 0.0   |
| 18:0             | 6.1 ± 0.1   | 5.6 ± 0.0   | 5.3 ± 0.1   | 5.4 ± 0.0   |
| 18:1, 19:0, 11    | 5.1 ± 0.1   | 7.7 ± 0.2   | 8.9 ± 1.1   | 6.0 ± 1.0   |
| 18:2             | 23.5 ± 1.2  | 23.3 ± 0.3  | 26.2 ± 1.4  | 20.8 ± 1.6  |
| 18:3             | 35.9 ± 1.0  | 32.6 ± 0.4  | 30.7 ± 1.7  | 37.9 ± 2.2  |
| 20:0             | 1.1 ± 0.0   | 0.9 ± 0.0   | 0.8 ± 0.0   | 0.9 ± 0.0   |
| 22:0             | 0.4 ± 0.0   | 0.3 ± 0.0   | 0.3 ± 0.0   | 0.3 ± 0.0   |
| 24:0             | 0.3 ± 0.0   | 0.2 ± 0.0   | 0.1 ± 0.0   | 0.2 ± 0.0   |
| **Total lipids (mol %)** |             |             |             |             |
| EV               |             |             |             |             |
| CsWRI1A          | 19.4 ± 0.1  | 21.5 ± 0.3  | 22.5 ± 0.5  | 22.1 ± 0.4  |
| CsWRI1B          | 2.7 ± 0.0   | 1.8 ± 0.1   | 2.1 ± 0.1   | 1.7 ± 0.2   |
| CsWRI1C          | 7.8 ± 0.1   | 6.7 ± 0.2   | 5.9 ± 0.2   | 6.3 ± 0.1   |
| EV               |             |             |             |             |
| CsWRI1A          | 3.1 ± 0.2   | 3.1 ± 0.3   | 3.2 ± 0.0   | 3.3 ± 0.2   |
| CsWRI1B          | 1.4 ± 0.0   | 2.9 ± 0.2   | 3.0 ± 0.1   | 2.4 ± 0.4   |
| CsWRI1C          | 10.4 ± 0.3  | 13.3 ± 0.5  | 14.0 ± 0.2  | 12.3 ± 0.7  |
| **Error**        | ± 3 ± 1     | ± 6 ± 1     | ± 5 ± 1     | ± 8 ± 1     |

Error denotes standard error (SE) with n = 3 for all samples. EV is empty vector.
genome of Camelina, an emerging oil seed crop, can be used for the regulation of transcriptional factors to produce vegetable oils in engineered biomass.

**AUTHOR CONTRIBUTIONS**

MCS and HUK designed the research; DA, HK, SJ, and YSG did experiments; and DA, HK, HUK, and MCS analyzed the data and wrote a manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00034/full#supplementary-material

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