**The WRKY6 transcription factor affects seed oil accumulation and alters fatty acid compositions in *Arabidopsis thaliana***

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In rapeseed, the oil content of the seed not only supplies energy for seed germination and seedling development, but also provides essential dietary nutrients for humans and livestock. Recent studies have revealed that many transcription factors regulate the accumulation of fatty acids (FAs) during seed development. *WRKY6*, a WRKY6 family transcription factor, was reported to serve a function in the plant senescence processes, pathogen defense mechanisms and abiotic stress responses. However, the precise role of *WRKY6* in influencing FA accumulation in seeds is still unknown. In this study, we demonstrate that *WRKY6* has a high expression level in developing seeds and plays an essential role in regulating the accumulation of FAs in developing seeds of *Arabidopsis*. Mutation of *WRKY6* resulted in significant increase in seed size, accompanied by an increase in FA content and changes in FA composition. Ultrastructure analyses showed that the absence of *WRKY6* resulted in more and higher percentage of oil body in the cell of mature seeds. Quantitative real-time PCR analysis revealed changes in the expression of several genes related to photosynthesis and FA biosynthesis in *wrky6* mutants at 10 or 16 days after pollination. These results reveal a novel function of *WRKY6* influencing seed oil content and fatty acids compositions. This gene could be used as a promising gene resource to improve FA accumulation and seed yield in *Brassica napus* through genetic manipulation.

*Abbreviations* **–** DAP, days after pollination; FA, fatty acid; GC, gas chromatography; qRT-PCR, quantitative real-time PCR; TEM, transmission electron microscopy; TAG, triacylglycerol.

**Introduction**

Plants seeds contain abundant storage reserves, which not only serve as a nutrient source for human beings and livestock but also facilitate seed germination and subsequent seedling development (Shahid et al. 2019).Fatty acids (FAs), as the main storage reserve in the seed, can be widely used in industries as well as the raw material for biofuels production (Fadhil et al. 2019). Therefore, understanding the roles of key functional genes in the regulation of seed FAs accumulation will be very important to provide gene resources for molecular crop breeding.

More and more studies have reported that many transcription factors (TFs) can regulate seed FA accumulation in Arabidopsis. *FUSCA3* (*FUS3*) promotes the FA accumulation by modulating the expression of genes involved in the biosynthesis of FAs (Wang et al. 2007, Zhang et al. 2016a). Overexpression of *LEAFY COTYLEDON1* (*LEC1*) increases the expression of fatty acid synthesis genes and also significantly elevates the level of major fatty acids in seeds by activating the expression of *WRI1*, *FUS3* and *ABI3* (Mu et al. 2008). WRINKLED1 (WRI1) is a key transcription factor for the synthesis of FAs, whereas *WRI1* is the target gene of LEAFY COTYLEDON2 (LEC2) and is indispensable for LEC2 to regulate the metabolism of FAs (Baud et al. 2007). *GLABRA2* (*GL2*) is reported to prevent seeds to produce more oil by producing seed coat mucilage which will decreases carbon allocation to the embryo (Shi et al. 2015). BASIC LEUCINE ZIPPER TF 67 (bZIP67) modulates the omega-3 polyunsaturated fatty acid α-linolenic acid (ALA) by binding to the G-box domain in the promoter of *FATTY ACID DESATURASE3* (*FAD3*) (Mendes et al. 2013). TRANSPARENT TESTA2 (TT2) regulates numerous genes expression level in the pathway of FA biosynthesis by directly binding to the promoter of *FUS3* (Wang et al. 2014). TRANSPARANT TESTA8 reduces FA accumulation by inhibiting the activities of *LEC1*, *LEC2* and *FUS3* (Chen et al. 2014). TRANSPARENT TESTA GLABRA1 (TTG1) suppresses the expression of FA synthesis related genes in developing seed by being direct target of *FUS3 (*Tsuchiya et al. 2004*)*. SPATULA (SPT) elevates the accumulation of FA by inhibiting the expression of seed storage protein genes and promoting the expression of FA synthesis genes during seed development (Liu et al. 2017). Phospholipid:diacylglycerol acyltransferase 1 (PDAT1) and acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) are two key enzymes that catalyze the last acylation step for the production of triacylglycerol (TAG) (Zhang et al. 2009).

WRKY protein family is plant-specific transcription factors which have 74 members reported in Arabidopsis and many of them have multiple functions during the whole plant life cycle (Dong et al. 2003). WRKY6, one of the members of the WRKY protein family is a multi-functional gene in plants. AtWRKY6 (At1g62300) was first reported to be associated with triggering plant senescence processes as well as plant defense responses (Robatzek and Somssich 2010). WRKY6 positively influences the activity of the *PR1* promoter which is associated with the senescence and pathogen defense and act the receptor-like protein kinase SIRK as a target in plants (Robatzek 2002, Choi et al. 2015). Overexpression and mutation of *WRKY6* accelerates and delays dark-induced senescence and chlorophyll degradation, respectively, by interaction with DELLA proteins in Arabidopsis (Zhang et al. 2018). WRKY6 is also involved in the reactions of different stress conditions. WRKY6 responses to low-Pi stress act mainly through downregulating the expression of *PHOSPHATE1*(*PHO1*) by binding to the *PHO1* promoter and WRKY6 will be degraded during Pi deficiency through modulation of a ubiquitin E3 ligase, PHOSPHATE RESPONSE UBIQUITIN E3 LIGASE1(PRU1) in Arabidopsis (Chen et al. 2009, Ye et al. 2018). In addition, the absence of *WRKY6* in Arabidopsis increases root hair density in response to low phosphorus condition (Stetter et al. 2017). Under boron deficiency, the expression of *WRKY6* is induced in the part of root tip and the *WRKY6* mutants exhibits growth defect compare to the wild-type (Kasajima et al. 2010). WRKY6 is proved as an arsenate-responsive transcription factor that regulates the expression of arsenate/phosphate transporter gene and limits arsenate-induced transposon activation (Castrillo et al. 2013).However, whether WRKY6 plays a key role in regulating the accumulation of the FA in Arabidopsis is still unknown.

In this study, we showed that WRKY6 affects the seed oil content and alters fatty acid compositions in developing *Arabidopsis* seeds. Mutation of *WRKY6* significantly increased the accumulation of the FA in Arabidopsis developing seeds, and resulted in bigger seed size, heavier seed weight, more FA total content and increased proportion of C18:3 and C20:0 FA compositions. WRKY6 also suppressed the expression of genes that act as master regulators in seed development process and are involved in FA synthesis or modification during seed development. WRKY6 also suppressed the expression of genes which are involved in FA biosynthesis and modification during seed development. These results uncovered a novel function of *WRKY6* and it could be used as a new genetic resource to improve FA accumulation and seed yield by molecular breeding in oilseed crops.

**Materials and methods**

**Plant materials and growth conditions**

Two *WRKY6* function-deficient mutants *wrky6-1* and *wrky6-2* in the background of Col-0 and wild type control (Col-0) were used in all experiments in this study (Huang et al. 2016). All the *Arabidopsis thaliana* plants were grown in a controlled growth chamber with the following conditions: 20-23℃, 16/8 h photoperiod, 95-120 μmol m−2 s−1 and 70-81% humidity as we described before (Wakeel et al. 2018). All *Arabidopsis* seeds were sterilized with 5% (w/v) NaClO solution for 15 min and washed four times with distilled deionized water and vernalized at 4℃ for 2 days. Subsequently, the seeds were gently sown on solid half MS medium and after 10 days the seedlings were transferred into 5 cm in diameter flowerpot filled with autoclaved soil as we reported before (Zhou et al. 2013).

**Mutants phenotype characterized in related seed development traits**

Seed developmental traits of *WRKY6* function-deficient mutants *wrky6-1*, *wrky6-2* and wild type plants were checked from 25 individual plants for each genotype. Siliques and seeds used for analyses were obtained from the first ten basal siliques of the main inflorescence. Plant height was measured from the rosette leaf to the tip of the plant after maturation and the silique length, silique number, seed number for each silique and 1000 seed weight were calculated at the same time. The mature seeds were photographed by using an OLYMPUS SZ 61 stereomicroscope (Tokyo, Japan). The seed length and width were measured with Image-Pro Plus software (version 6.0).

**Gene expression analysis by quantitative RT-PCR**

The RNA extraction from vegetative tissues and developing seeds were performed by using Omega Plant RNA Extraction Kit (Omega) and reverse transcription reaction was conducted by using oligo d(T) 18 primers and M-MLV transcriptase (Takara). All the tissues used for RNA isolation were from at least 20 individual plants grown in different flowerpots, and three independent biological replicates were conducted for the expression analysis. Quantitative RT-PCR was performed for three biological replicates using ChamQ SYBR Color qPCR Master Mix (Vazyme) as we described before (Khan et al. 2019, Liu et al. 2018). *Arabidopsis* *ACTIN7* (At5g09810) was used as the internal control. Primers used for quantitative RT-PCR are listed in Table S1.

**Measurement of FAs**

FAs were extracted and analyzed according to Poirier and Caldelari (1999) and Mu (2008).For the extraction of fatty acid methyl esters, 10 mg mature seeds were heated at 80℃ in 1 M HCL/CH3OH for 2.5 h by following the addition of 1 ml hexane and 2 ml 0.9% (w/v) NaCl and vortex for 60s to mix it well. The organic phase obtained after centrifuging at 600 g for 5 min was used for FA analysis by gas chromatography (GC) and methyl heptadecanoate was used as the internal standard. GC analysis was conducted with machine (SHIMADZU, Kyoto, Japan, GC-2014) equipped with a flame ionization detector and a capillary polar column (Supelcowax-10, Sigma-Aldrich, Schnelldorf, Germany). The GC procedure runs as the initial column temperature held at 160℃ for 1 min, then followed by increasing to 240℃ with a 4℃ min-1 growth rate, and maintained at the final temperature for 16 min. After the whole procedure, each of FA compositions was identified by their corresponding retention times and the quantity for each species was calculated by area determination of corresponding peak with GC solution software (SHIMADZU, Kyoto, Japan). Concentrations of each FA compositions were normalized against internal control.

**Ultrastructure analysis of mature seeds**

The oil body in the cells were recorded by transmission electron microscopy (TEM) as we described before (Liu et al. 2017). Eight seeds of each genotype were sectioned and ten cells from each seed were selected for the oil body observation. TEM observation was conducted as the follow main steps: double fixation, dehydration, infiltration, embedding. The seeds were sectioned by LEICA EM UC7 ultratome and sections were stained with uranyl acetate and alkaline lead citrate for 10 min, respectively, and then observed and recording by TEM (Hitachi Model H-7650).

**Statistical analysis**

All experiments in this study were performed in randomized complete block design with at least three biological replicates. All of the Data were classified with Microsoft-Excel and analyzed via an ANOVA using GraphPad Prism statistical package (version 8.0). Student’s *t*-test was performed to judge significance level at either 5% (\**P* < 0.05 significant level) as previously described (Bao et al. 2011, Zhang et al. 2016b).

**RESULT**

**Analysis of *WRKY6* expression pattern**

The expression level of *WRKY6* was measured in various tissues from Col-0 wild type plants by using quantitative RT-PCR. As shown in Fig.1, *WRKY6* was expressed widely in different tissues. It is mainly expressed in developing seeds, open flowers, cauline leaves and stems, and to a lesser extent in roots and rosette leaves (Fig. 1A). During the seed development stages, the expression of *WRKY6* was increased gradually from 8 days after pollination (DAP), reached to the peak at 14 DAP and descended slightly afterwards (Fig. 1B). These results indicate that WRKY6 might play a role in seed related traits in Arabidopsis. Therefore, the transcription factor of WRKY6 was selected to investigate the function related to FAs accumulation in Arabidopsis seeds.

**Characterization of *wrky6* mutants phenotypes related to the seed traits**

The seed yield traits depend on the allocations of resources and nutrition from vegetative development to reproductive development (Kozłowski 1992). As shown in Fig. 2A, a WRKY functional domain was found in the protein sequence. Two *wrky6* mutants in the background of the Columbia (Col-0) were used to study the physiological function of *WRKY6* in the accumulation of FA in Arabidopsis seeds, one was an En-1 insertion line, named *wrky6-1* and another was a T-DNA insertion line, named *wrky6-2* (Fig. 2B), provided by Prof. Chen Yifang from China Agricultural University (Huang et al. 2016).The quantitative RT-PCR results revealed that *WRKY6* expression were significantly reduced in both *wrky6-1* and *wrky6-2* mutants compared with wild type (Fig. 2C).

In order to study the effect of *WRKY6* on seed development, we first analyzed plant height, silique length and number in both wild type and mutant plants. The results showed that the average height of both *wrky6-1* and *wrky6-2* mutant lines was significantly greater than that of Col-0 (Fig. 3B), but the average silique number per plant had no significant change (Fig. 3C). However, the average silique length of *wrky6-1* and *wrky6-2* mutants was 19.51 and 12.93% longer than Col-0, respectively (Fig. 3D). For the seed traits, the average seed number for per silique was significantly increased in both *wrky6-1* and *wrky6-2* mutants (Fig. 3E). For the average of thousand seed weight, *wrky6-1* and *wrky6-2* showed increases of 22.56 and 21.79%, respectively, compared to Col-0 (Fig. 3F). The length and width of the seeds were also measured. The *wrky6-1* seeds were 13.37 longer and 25.38% wider than Col-0, while *wrky6-2* seeds were 9.82% longer and 18.21% wider compared with Col-0. (Fig. 3A and 3G). These findings indicate that the absence of *WRKY6* function results in larger and heavier seeds in Arabidopsis.

**WRKY6 affects FAs biosynthesis in mature seeds**

To explore *WRKY6* function on seed FA biosynthesis, gas chromatography was used to analyze the seed FA content and major FA compositions in *wrky6* mutants and Col-0 mature seeds. As shown in Fig. 4, *wrky6-1* and *wrky6-2* mutants showed 27.21 and 25.26% higher FA content than that of Col-0 seeds (Fig. 4A). However, the seed total protein has no significant changes (Fig. 4B). Furthermore, the rise of FA was accompanied by the change of FA composition. In both *wrky6* mutant mature seeds, the FA compositions of C18:3 and C20:0 were significantly higher than that in Col-0 mature seeds (Fig. 4C), while the proportion of C18:2 was decreased in *wrky6* mutants.

In dicots, the seed storage reserves were mainly restored in the seed embryo (Chen et al. 2012).In order to investigate whether *WRKY6* influences the FA contents in Arabidopsis seeds, the transmission electronic microscopy (TEM) was performed to analyze the ultrastructure differences of seed embryo cell between *wrky6* mutants and wild type. The TEM results showed that the absence of *WRKY6* led to a slight rise of aleurone grain number and the significant increase of oil body number (Fig. 5A and 5B). Percentages of total area of oil bodies and aleurone grains were also calculated to have a better understanding of compositional changes in seed embryo cells. As shown in Fig. 5C, the oil body area proportion in seed embryo cells of *wrky6* mutants was significantly higher than that of Col-0. In contrast, the percentage of aleurone grain area was lower in *wrky6* mutants compared with that of Col-0. However, the size of oil body was significantly smaller in *wrky6* mutants than wild type (Fig. 5A). These findings illustrated that WRKY6 affects seed oil content in Arabidopsis.

***WRKY6* affects the expression of FAs accumulation genes during seed development**

For the FA biosynthesis during the seed development, FA is synthesized from 6 DAP and reaches its fastest rate of synthesis at 10 DAP, whereas the accumulation of FA almost reaches its peak at 16 DAP (Kanai et al. 2016).Our results showed that the mutation of *WRKY6* promoted FA accumulation in Arabidopsis seeds and raised oil body proportion in seed embryo cells (Figs 4 and 5). In order to have a clear understanding of the mechanism of the functions of WRKY6 in seed oil accumulation process, qRT-PCR was performed to examine the expression level of key genes involved in seed development with RNA extracted from seeds at 6, 10 and 16 DAP, respectively.

We first checked the expression of *FUS3*, *LEC1*, *LEC2*, *WRI1* and *ABI3* which encode the master transcription factors and which all were reported to be key genes for FA biosynthesis in Arabidopsis (Focks and Benning 1998, Giraudat et al. 1992, Keith et al. 1994, Lotan et al. 1998, Stone et al. 2001). As shown in Fig. 6, in the absence of *WRKY6*, the expression of *ABI3* was remarkably induced, especially at the stage of 16 DAP. The expression of *ABI3* in *wrky6-1* and *wrky6-2* were 10.6- and 11.2-fold higher than in wild-type plant. The expression of *FUS3* in both *wrky6* mutants had a slight increase at 10 DAP as compared with Col-0. However, the expression of *LEC1*, *LEC2* and *WRI1* showed no significant difference between *wrky6* mutants and Col-0 at all three different stages. In summary, WRKY6 regulates FA accumulation and biosynthesis by influencing the expression of *FUS3* and *ABI3*.

The expression of *BCCP1*, *CAC2*, *CAC3*, *CDS2*, *FAB2*, *FAD2*, *FAD3*, *FAE1*, *FatA*, *KASII* and *MOD1*, which encoded the vital FA biosynthetic enzymes were also examined in both *wrky6* mutants and wild type plants (Fig. 6). *FAE* transcript level at 10 DAP in both *wrky6-1* and *wrky6-2* mutants was significantly decreased by 10.6- and 9.0-fold, respectively, compared to wild type plant. Meanwhile, *FAD2* expression also slightly declined in both mutants at 10 DAP. On the contrary, in the absence of *WRKY6*, *CDS2* expression level continually increased from 6 to 16 DAP in comparison to Col-0. *FAD3* expression level was much higher than that in wild type at all three growth stages, which increased gradually from 6 DAP and reached to the peak at 10 DAP, after that, it sloped down until 16 DAP. However, the other genes, *BCCP1*, *CAC2*, *CAC3*, *FAB2*, *FatA*, *KASII* and *MOD1*, had no significant expression difference between *wrky6* mutants and wild type (Fig. 6). In conclusion, WRKY6 mediates FA biosynthesis mainly through regulating the expression of *FAE1*, *FAD2*, *FAD3* and *CDS2*.

**Discussion**

FA, a class of prominent metabolites in seed, is primarily stored in embryo and plays a vital role during the growth and development of the living organism (Chen et al. 2012).Furthermore, it also serves as a nutrient sources, especially unsaturated FA compositions, which are beneficial for the health of human beings and livestock. Despite the regulatory mechanism of FA metabolism and the biochemistry of FA biosynthesis have been studied well in Arabidopsis, the transcription factors that regulate FA biosynthesis and accumulation are still largely unknown.

*WRKY6* encodes a WRKY transcription factor that has been revealed to influence many developmental processes in Arabidopsis, including senescence processes (Robatzek and Somssich 2010, Zhang et al. 2018), plant pathogen defense (Robatzek 2002, Choi et al. 2015) and the reaction to different stress conditions (Castrillo et al. 2013, Chen et al. 2009, Ye et al. 2018, Kasajima et al. 2010). However, whether *WRKY6* plays a role in FA biosynthesis is still unknown. In this study, we have shown several pieces of evidence that suggest a novel function of WRKY6 in influencing FA biosynthesis and accumulation during the *Arabidopsis* seed development.

**Loss functions of *WRKY6* increases total FA content in Arabidopsismature seeds**

We found that two leaky mutants, *wrky6-1* and *wrky6-2*, resulted in bigger and more heavy seeds in Arabidopsis (Fig.3E-G), as well as a significant increase in the amount of total FA content in mature seeds (Fig. 4A). The described phenotype may be caused by knocking out *WRKY6* as two independent En-1 and T-DNA alleles showed the same phenotype, although genetic complementation is not performed yet. These results together with the observation of gradual increased expression of *WRKY6* in developing seeds at 6, 10 and 16 DAP (Fig. 1B), indicates that *WRKY6* affects FA accumulation in Arabidopsis seeds.

Five transcription factors, namely LEC1, LEC2, FUS3, ABI3 and WRI1 are the master regulators of seed development and regulate the expression of many other genes in the metabolic networks resulting in the synthesis of storage compounds such as FAs and storage proteins in developing seeds (To et al. 2006, Baud and Lepiniec 2009). *LEC1* plays vital role in the biosynthesis of FA and encodes the HAP3 subunit of the CCAAT binding factor (Lee et al. 2003, Mu et al. 2008). *LEC2*, *FUS3* and *ABI3* are similar functional transcription factors, which encode B3-domain family and have shown important functions for the regulation of seed maturation (Braybrook et al. 2006, Ezcurra et al. 1999, Kroj et al. 2003, Reidt et al. 2010, Wang and Perry 2013). *WRI1* encodes the AP2/EREB family that acts on enzymes in the late glycolysis process and the plastidial FA biosynthesis network (Cernac and Benning 2004, Focks and Benning 1998).These five transcription factors have similar and unique functions in regulating seeds development and FA accumulation network in Arabidopsis(Baud et al. 2009, Santos-Mendoza et al. 2008, To et al. 2006, Wang et al. 2007). In this study, the expression level of *LEC1*, *LEC2* and *WRI1* did not show any striking changes in *wrky6* mutants compared to wild type. Nevertheless, *FUS3* and *ABI3* expression levels increased dramatically at 10 and 16 DAP, respectively (Fig. 6). FUS3 functions mainly in the seed filling process and promotes the expression of FA biosynthesis genes such as *ACP1*, *ACP5*, *BBCP2*, *CAC2*, *FAB2*, *FAD3* and *KASI* etc. (Wang et al. 2007, Yamamoto et al. 2010). Furthermore, FUS3 plays an essential role in affecting the expression of photosynthetic genes especially during the early seed development (Yamamoto et al. 2010). Hence, it was possible that the increased expression level of *FUS3* in the *wrky6* mutants lead to more photosynthate for further accumulation of seed FA deposit. ABI3, the same as FUS3 is a key regulator during the process of seed development which interacts genetically with *FUS3* in controlling the accumulation of chlorophyll (Mönke et al. 2004). As shown in Fig. 6, the significant increased expression level of *ABI3* at 10 and 16 DAP in *wrky6* mutant seeds can regulate *FUS3* expression, thus producing more chlorophyll and photosynthate, which results in more FA accumulation in Arabidopsis developing seeds. CDS2 is a synthase that can catalyze 1,2-diacylglycerol-3-phosphate to CDP-diacylglycerol and is an essential intermediate in the biosynthesis of phosphatidylglycerol, cardiolipin and phosphatidylinositol, which are essential for the synthesis of FAs (Blunsom et al. 2018). Seeds contain a large quantity of oil, most of which are composed of triacylglycerol (TAG). Three fatty acids can be esterified into TAG. *DGAT1* and *PDAT1*, encode two key enzymes that catalyze the last acylation step for the production of TAG were also analyzed during seed development (Zhang et al. 2009). The expression of *DGAT1* significantly increased from 10 to 16 DAP than that of wild type (Fig. 6), but the expression level of *PDAT1* has no obvious change from 10 to 16 DAP stages (Fig. 6). Therefore, the mutation of *WRKY6* significantly increase total FA content and oil body in the mature seeds may result from the raise expression level of *CDS2* and *DGAT1*. In this study, we analyzed the compositions of FA and oil body percentage in mature Arabidopsis seed. To figure out a better molecular mechanism between *WRKY6* and seed oil quantity and quality, we will perform the measurement of the TAG content and the percentage of FA composition in seed oil composition in the future.

The WRKY6 protein contains a conserved WRKY domain, which is mirrored by a conserved binding site, the W-box (T)(T)TGAC(C/T) (Eulgem et al. 2000). The promoter of *FUS3* and *ABI3* were analyzed and the results shown that there are five and three W-box sites in the promoter of *FUS3* and *ABI3*, respectively (Fig. 7). Taken together, our results demonstrated that WRKY6 could affect FA content accumulation may mainly by regulating the expression of *FUS3* and *ABI3*.

**WRKY6 influents the expression of FAs biosynthesis genes during Arabidopsis seed development**

The critical gene expression for FA composition biosynthesis are coordinately regulated (Baud et al. 2003, Ruuska et al. 2002). *FAE1* encodes a condensing enzyme which elongates the FA carbon chain length from C18 to C20 and C22; *FAD2* encodes a desaturase which is essential for the synthesis of polyunsaturated lipid C18:2 from C18:1 (James and Dooner 1990, Lemieux et al. 1990, Okuley et al. 1994). The absence of *WRKY6* gene remarkably down-regulated the expression level of *FAE1* and *FAD2*(Fig. 7). FAD3 uses cytochrome b5 as an electron donor to synthesize C18:3 from phospholipids and loss function of *FAD3* results in a decrease level of C18:3 and increase level of C18:2 in developing seeds (Shah et al. 1997). In our study, the absence of *WRKY6* resulted in high expression of *FAD3* from 6 to 16 DAP in Arabidopsisdeveloping seed. Some other genes related to the biosynthesis of FAs were analyzed as well, such as *BCCP1*, *CAC2*, *CAC3*, *FAB2*, *FatA*, *KASII* and *MOD1* (Fig. 7). However, there was no obvious expression variation of these genes in *wrky6* mutant seeds in compared to that of wild type. In addition, there are several W-box sites in the promoter of *FAD2*, *FAD3* and *FAE1* (Fig. 7). Thus, WRKY6 may also regulates FA biosynthesis through mediating the expression of FAs biosynthesis genes (*FAE1*, *FAD2*, *FAD3*) during *Arabidopsis* seed development. However, whether WRKY6 directly acts on these genes remains to be further studied.

In summary, this study uncovered the novel function of *WRKY6* in affecting FA accumulation in developing seeds by changing the expression of genes related to photosynthesis, FA synthesis and modification in Arabidopsis. Together with previous discoveries that the introduction of heterologous genes, miRNAs and transcription factors into *A. thaliana* and *Brassica napus* could enhance FA content (Anna et al. 2015, Belide et al. 2012, Hashmi et al. 2012, Wang et al. 2016),WRKY6 can be used as a new gene resource to improve FA accumulation and seed yield in *Brassica napus* through genetic manipulation.

**Author contributions**

G.S.designed the research, performed the experiments, analyzed the data and wrote the manuscript; X.P.L. performed the experiments and provided data analysis; R.M. provided experiment assistance; A.R.K. and W.A. helped to plant the Arabidopsismaterials; M.U.Y. helped to check the writing of the manuscript; Q.N.J. helped to sample the tissues for qRT-PCR; I.B. helped to correct the writing and design of the manuscript; Y.B.G. designed the research and corrected the manuscript.

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**Supporting information**

Additional supporting information may be found in the online version of this article:

**Table S1.** the primers used for quantitative real-time PCR

[**Data sharing and data accessibility**](http://physiologiaplantarum.org/instructions-for-manuscrip/#Data%20sharing%20and%20data%20accessibility)

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Figure legends**

**Fig. 1.** Analysis of *WRKY6* expression level in Arabidopsis different tissues. (A) qRT-PCR analysis of *WRKY6* expression in different tissues of the wild type (Col-0). RT, roots; ST, stem; RL, rosette leave; CL, cauline leave; OF, open flower. (B) qRT-PCR analysis of *WRKY6* expression in different seed development stages. All of the qRT-PCR data were normalized against the expression of *AtACTIN7* as an internal control. All the expression analysis for each tissue were repeated three times (n=3) and the error bars denote SD.

**Fig. 2.** Identification of the *A. thaliana* *wrky6* mutation. (A) the protein sequence of WRKY6 and the red sequence is the conserved WRKY domain. (B) Structure of the *WRKY6* gene indicating the position of the T-DNA and En-1 insertions of *wrky6-1* and *wrky6-2* mutants. The untranslated regions and UTR regions of *WRKY6* are represented by dark lines and white boxes, respectively, and coding sequences are represented by dark grey boxes. Translation start site (ATG) and stop codon (TAG) are shown. (C) The expression level of *WRKY6* in *wrky6-1* and *wrky6-2* mutants. The transcript level in (C) was performed by quantitative RT-PCR analyses of three independently collected tissues. The expression results were normalized against the expression of *AtACTIN7* as an internal control. Error bars indicate the SD.

**Fig. 3.** Plant, silique and seed morphology among the *wrky6* mutants and wild type (Col-0). (A) The seed morphology of *wrky6* mutants and wild type (Col-0). The seeds observed under the light microscopy were selected randomly from the mature seeds. (B) Plant height was measured from the rosette leave to the tip of the plant after maturation. (C, D, E) Silique number, silique length and seed number were calculated from the basal ten siliques at the plant totally mature stage. (F, G) The seeds used to measure thousand seed weight and seed size were selected randomly after collecting all of the mature seeds. The error bar means SD, asterisks indicate significant difference (*P* < 0.05) as compared with the wild type.

**Fig. 4.** Characterization of *WRKY6* function on seed FAs accumulation. (A) Comparison of seed total FA content (ug/mg) between the *wrky6* mutants and the wild type. (B) the seed total protein comparison of *wrky6* mutants and Col-0. (C) Comparison of contents of major seed FA compositions between the *wrky6* mutants and the wild type. The error bars denote SD and the asterisks indicate significant difference (*P* < 0.05) as compared with the wild type. DW, dry weight.

**Fig, 5.** Characterization of *wrky6* mutants and wild type embryo cells.(A) TEM pictures of embryo cells in dry mature seeds of *Arabidopsis* from *wrky6* mutants and Col-0. (B, C) The number and area proportion of oil body and aleurone grain were measured from 80 cells of embryo. The error bars denote SD and the asterisks indicate significant difference (*P* < 0.05) as compared with the wild type.

**Fig. 6.** Comparison of the relative expression levels of genes that regulate seed development, synthesis and modification of FAs in developing seeds of the *wrky6* mutants and wild type by quantitative real-time PCR analysis. The x-axis represents the day after pollination (DAP). All the expression analysis was repeated three independent times. The expression levels were normalized against *AtActin7* as an internal control. Error bars denote SD.

**Fig. 7.** The promotor structures of *FUS3*, *ABI3*, *FAD2*, *FAD3* and *FAE1*. The upstream promotor region of these five genes are represented by the white box, while the genomic DNA sequence is represented by the grey boxes. The black arrowheads at the top indicate the putative W-box on the promotor of these five genes above.