

Overexpression of Avocado (*Persea americana* Mill.) *PaRAP2.1* Promotes Fatty Acid Accumulation in *Arabidopsis thaliana*

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Abstract

Fatty acids in avocado fruit (*Persea americana* Mill.) are vital composition affecting flavour and nutritive value. Hence, horticulturalists are interested in illustrating the functions of transcription factors on fatty acid accumulation in avocado fruit. In the present study, the APETALA2/ethylene-responsive transcription factor gene, *PaRAP2.1*, was cloned from avocado mesocarp, and the subcellular localization demonstrated that *PaRAP2.1* was located in the cytoplasm and nucleus. The *PaRAP2.1* was introduced into *Arabidopsis thaliana* by Agrobacterium-mediated transformation. Furthermore, *PaRAP2.1* were functionally verified its effect on fatty acid biosynthesis. Histological analyses of lipid droplets displayed that the striking difference in the lipid droplets in the mature seeds between *PaRAP2.1*-overexpressing transgenic and wild-type *Arabidopsis thaliana* lines were revealed based on confocal microscopy images. Subsequently, fatty acid analyses of *PaRAP2.1*-overexpressing *Arabidopsis thaliana* lines displayed the significantly higher contents of fatty acids than those in the wild-type plants. Meanwhile, expression amount of ten genes involving in fatty acid biosynthesis dramatically up-regulated in the mature seeds of *PaRAP2.1*-overexpressing lines than those of wild-type plants. These results provide a theoretical basis for future research in regard to the function of *PaRAP2.1* on fatty acid biosynthesis.

Keywords: avocado, *PaRAP2.1*, fatty acids

1. Introduction

Fatty acids are important components in plant (Ge et al., 2017, 2018). The fatty acid biosynthesis has been studied extensively, and these have expounded transcription factors that regulate fatty acid biosynthesis in plants (Ge et al., 2021a, 2021b). Members of many transcription factor families involving APETALA2/ethylene responsive factor (AP2/ERF) superfamily have been found to be involved in regulation in fatty acid biosynthesis in plants (Yeap et al., 2017). The AP2/ERF transcription factors are a multifarious superfamily expressed in plants, and AP2/ERF members have the conserved DNA binding domain, namely the AP2 domain, that binds to the gene's promoter region to regulate expression (Zhang et al., 2020). They are classified into three separate groups: ERF, RAV, and AP2 families according to the repeat number in AP2 domain (Zhang & Li, 2018). Currently, with studies in dicotyledonous plants such as *Ricinus communis* (Xu et al., 2013), *Ziziphus jujuba* (Zhang & Li, 2018), *Arabidopsis thaliana* (Xie et al., 2019), *Dimocarpus longan* (Zhang et al., 2020), monocotyledonous plants such as *Phyllostachys edulis* (Wu et al., 2015), and gymnosperm such as *Taxus chinensis* (Zhang et al., 2019), we present a more in-depth knowledge of the functions and classification of AP2/ERF members.

In our previous study, the 137 *PaAP2/ERF* genes were identified in avocado, and then the expression patterns of them in five developmental stages of avocado mesocarp were presented according to transcriptome data (Ge et al., 2021a). Subsequently, two *PaAP2/ERF* genes (*PaWRI2* and *PaWRI1*) belonging to AP2 subfamily and eight *PaAP2/ERF* genes (*PaRAP2.1*, *PaERF023*, *PaERF102-4*, *PaRAP2.2-2*, *PaERF109-5*, *PaERF082-1*, *PaRAP2.2-3*, and *PaRAP2.4-2*) belonging to ERF subfamily were highly transcribed during five developmental

stages of avocado mesocarp, which might regulate the accumulation of fatty acids in the avocado mesocarp (Ge et al., 2021a). Furthermore, the *PaWR11*, a AP2 subfamily member, was selected to carry out the transgenic functional analysis, and the result implied that *PaWR11* might contribute to fatty acid accumulation (Ge et al., 2021a). Similarly, most of the genes governing fatty acid synthesis are found to be regulated by *WR11* in many plants (Kong & Ma, 2019).

However, neither one of *PaAP2/ERF* genes belonging to ERF subfamily has been found to modulate the vital genes participating in the fatty acid biosynthesis until now. In our previous study, the eight *PaAP2/ERF* genes belonging to ERF subfamily were considered to take part in fatty acid biosynthesis in avocado mesocarp, and the *PaRAP2.1* was more abundantly transcribed than other seven genes (Ge et al., 2021a). Therefore, in this study, we first chose *PaRAP2.1*, and performed the cloning and subcellular localization of *PaRAP2.1*. Second, to further exploit potential function of *PaRAP2.1* on fatty acid accumulation, *PaRAP2.1*-overexpressing transgenic *A. thaliana* were developed, after which gene expression, lipid droplet observation, and targeted fatty acids detection of the transgenic *A. thaliana* and wild-type (WT) lines were carried out to analyse the contents of fatty acids. The data enriches our understanding in regard to the functions of *PaRAP2.1* on fatty acid biosynthesis in the avocado mesocarp.

2. Method

2.1 Plant Materials and Growth Conditions

Avocado fruits (cultivar 'Hass') were collected from six 10-year-old trees in September 2018 at the Chinese Academy of Tropical Agricultural Sciences. *Arabidopsis thaliana* wild-type Col-0 seeds were disinfected surfaces with 70% ethanol for 30 s and 15% sodium hypochlorite for 15 min, and then rinsed with distilled water three times for 20 s. Then, the seeds were removed moisture from the surface, and placed on Murashige and Skoog medium.

2.2 RNA Extraction and cDNA Synthesis

The total RNA was extracted from avocado mesocarps and *A. thaliana* seeds. The mRNA was extracted from total RNA using poly-T oligo-attached magnetic beads. The first-strand cDNA was synthesized based on the sequence of the extracted RNA. The concentration of cDNA was diluted to 12.5 ng/ μ L.

2.3 Cloning of *PaRAP2.1*

The coding sequence of *PaRAP2.1* is 1135 bp, and the amplification primer sequences of *PaRAP2.1* were:

5': TCTGATCAAGAGACAGGATCCATGGAGGGCACC GCCGCTCC

3': CATCGGTGCACTAGTGTGCGACTAAATGCCCATTTGCATCT

PCR amplification system: synthetase 1 μ L, 2 \times PCR buffer 20 μ L, dNTP Mixture 8 μ L, 5' primer 0.3 μ L, 3' primer 0.3 μ L, cDNA 500 ng, plus ddH₂O up to 50 μ L. PCR amplification process: Initialized at 95 °C for 2 min and then 34 repeated cycles at 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 2 min, with a final extension at 68 °C for 7 min. PCR products were purified using an Axygen company Recovery Kit.

2.4 Construction of *PaRAP2.1* Transient Expression Vector and Subcellular Localization

The vector plasmids sequenced correctly were transformed into *Agrobacterium*, spread on the plates including 25 mg/L kanamycin and 25 mg/L rifamycin. The monoclonal shaking bacteria were selected to grow overnight, the bacterial solutions were collected, and then resuspended in infiltration medium. *Agrobacterium* solutions containing vectors were blended in proportion. The liquid mixtures were transfused into leaves of tobacco for 28 days. After 3 days, the leaves of tobacco were scanned through confocal scanning microscope.

2.5 Vector Construction and Plant Transformations

To generate the *PaRAP2.1*-overexpressing (OE) construct, the full-length *PaRAP2.1* CDSs was amplified and transferred into the pCAMBIA1300 vector including the 35S promoter. We introduced the recombinant plasmids into *Agrobacterium tumefaciens* (GV3101). The floral dip method was used for genetic transformation of wild-type *A. thaliana*. Hygromycin-resistant plants were screened from transformed seeds, and then the T₁ generation were obtained. T₁ seeds were sown, and finally T₃ transgenic *A. thaliana* plants were obtained.

2.6 Quantitative Real Time PCR of Gene Expression

The eight genes participating in fatty acid biosynthesis expressed in the seeds of the WT and *PaRAP2.1*-OE *A. thaliana* lines were chosen for qRT-PCR, and *AtActin7* was used as an endogenous control for normalizing data (Table 1). The qRT-PCR amplification process was described by Ge et al. (2019). Relative gene expression

levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). For each sample, the qRT-PCR analysis was completed with three biological replicates and two technical replicates.

Table 1. Primer sequence

Primer	Sequence information 5'-3'
<i>AtPDH (E1α)F</i>	ACTTCGCCAGCTTGTGATTC
<i>AtPDH (E1α)R</i>	AAGATCGCTCCCTCTGACAG
<i>AtACC (Ctα)F</i>	TTCTTTACCACTGGACACCC
<i>AtACC (Ctα)R</i>	CTGCGACCTTAAAGGAACGC
<i>AtACP4F</i>	GAAGGTGTAGGGCGAAGACA
<i>AtACP4R</i>	GTGTAGACAGGCAGTGGACA
<i>AtSADF</i>	GGTATGTCGTCGCTTGTGAA
<i>AtSADR</i>	ACACGCTACTCACCTACACA
<i>AtFATAF</i>	ATGGCAGTTAGATTGGTGGG
<i>AtFATAR</i>	AGAGCCGAGTCGTTATGTCC
<i>AtLACS9F</i>	AGAGGGTTGAGGCGAAGAAC
<i>AtLACS9R</i>	GTTGAAGCGAGCAGTGGAAC
<i>AtGPAT1F</i>	ATCACTCTCTTGAGCTGGCG
<i>AtGPAT1R</i>	ATACTCCTCTGTGACGTGGC
<i>AtFAD2F</i>	TTGCTGGAAAGTGCTGACAA
<i>AtFAD2R</i>	CTATGAGTTGGTCTCGCGAG
<i>AtActin7F</i>	TGCCCAGAAGTTCTATTCCAGC
<i>AtActin7R</i>	CATAGTTGAACCACCACTGAGGAC

2.7 Analysis of Fatty Acid Compositions by Gas Chromatography-Mass Spectrometry

The fatty acid compositions of the *A. thaliana* seeds from the WT and *PaRAP2.1*-OE plants were determined by gas chromatography-mass spectrometry (GC-MS) as described by Ge et al. (2019). The oils extracted from the seeds of the WT and *PaRAP2.1*-OE (20 μ L) were saponified at 80 °C (30 min). After cooling, the solutions were mingled with 3 mL BF₃-MeOH (14%) and incubated at 75 °C (30 min) to generate fatty acid methyl esters (FAMES). The analyses were performed through an Agilent 7890B-7000B GC-MS with a DB-5MS column. The FAMES were identified by comparing the retention times of the peaks with those of commercial standards and comparing the respective ion chromatograms with those in the NIST 2011 library. Methyl nonadecanoate was added as an internal standard and the FAMES were quantified based on the calibration curves for the standards ($R^2 \geq 0.995$). The FAME contents (mg/100 g fresh weight) are herein presented as the mean \pm standard deviation of three biological replicates, each with two technical replicates.

2.8 Histological Analyses

To visualize the lipid droplets in the mature seeds from the WT and *PaRAP2.1*-OE *A. thaliana* plants, the method of sample handling and lipid droplet observation was described by Ge et al. (2019).

3. Results and Discussion

3.1 Cloning and Subcellular Localization Analysis of *PaRAP2.1*

Using the melon cDNA as a template, the fragment of *PaRAP2.1* was amplified and analyzed. Band 1 was about 1131 bp according to the DNA marker (DL2000) (Figure 1), which was consistent with the anticipative result of the present study. Through laser scanning microscopy, it was observed that *PaRAP2.1* was located in the nucleus and cytoplasm of tobacco leaves. However, as shown in Figure 2, the green fluorescent signal of *PaRAP2.1-GFP* was mainly concentrated on the plasma membrane. These results indicated that the *PaRAP2.1* might be a transcription factor that played a role in the nucleus and cytoplasm. However, subcellular localization shows that BnWR11, the same AP2/ERF transcription factor, is only distributed in the nucleus (Wu et al., 2014; Li et al., 2015).

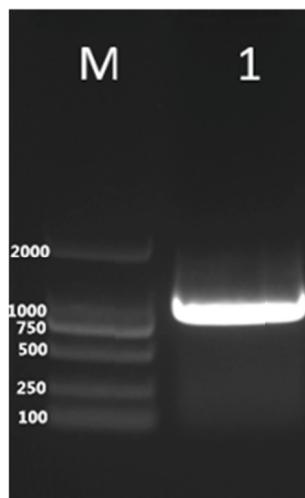


Figure 1. PCR amplification results of *PaRAP2.1*. M: TaKaRa company DL2000; 1: full length of *PaRAP2.1*

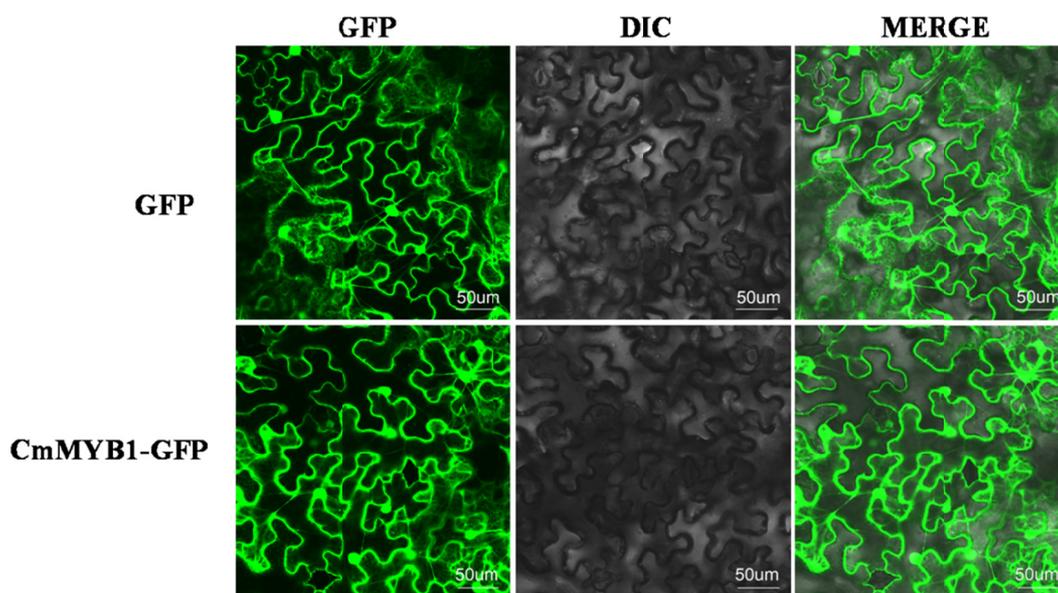


Figure 2. Subcellular localization of *PaRAP2.1* in *N. benthamiana* leaves. *PaRAP2.1* fused to the N-terminal of GFP under the control of the 35S promoter. Bars = 50 μ m

3.2 The Effect of *PaRAP2.1* on the Transcript Level of Ten Genes in Fatty Acid Synthesis and Lipid Assembly

The leaves of transgenic *A. thaliana* were grown for about 4 weeks, the genome was extracted, and the target fragment was amplified. The expression of the *PaRAP2.1* was detected in transgenic *A. thaliana* and positive controls, indicating that the *PaRAP2.1* was integrated into the chromosome of *A. thaliana* (Figure 3). The phenotypic characteristic of transgenic *A. thaliana* T₃ lines and wild-type displayed less variation during growth stage (Figure 4). However, in our previous study, the precocious flowering is simultaneously found in *PaWR11*-OE and *PaWR12*-OE transgenic tomato plants (Ge et al., 20121a). Similarly, *BnWR11* in transgenic *Brassica napus* plants also accelerate flowering and enhance oil accumulation in both seeds and leaves (Li et al., 2015). Besides, all *AtWR11*-OE transgenic potato plants demonstrate alterant tuber morphology, such as extended tuber, deeper eyes, and weight increment (Hofvander et al., 2016). These results suggested that the *PaAP2/ERF* genes presented the diverse biological function although *PaRAP2.1*, *PaWR11*, and *PaWR12* all belong to AP2/ERF superfamily.

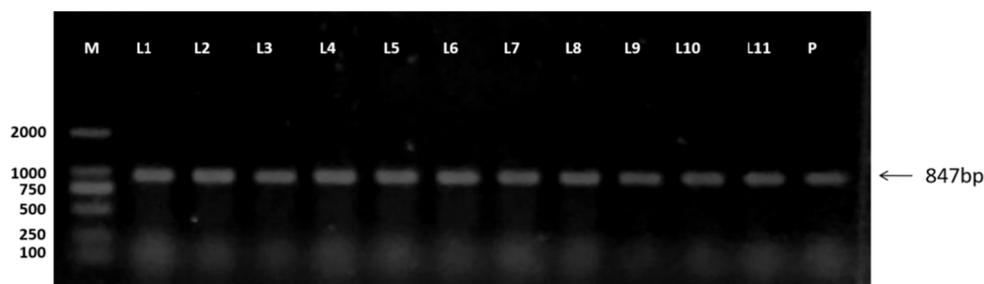


Figure 3. The detection of *PaRAP2.1* expression in transgenic *A. thaliana*. M: DL2000; L1-L11: *PaRAP2.1*-overexpressing transgenic *A. thaliana* T₃ different lines; P: pCAMBIA1300-35s-myc

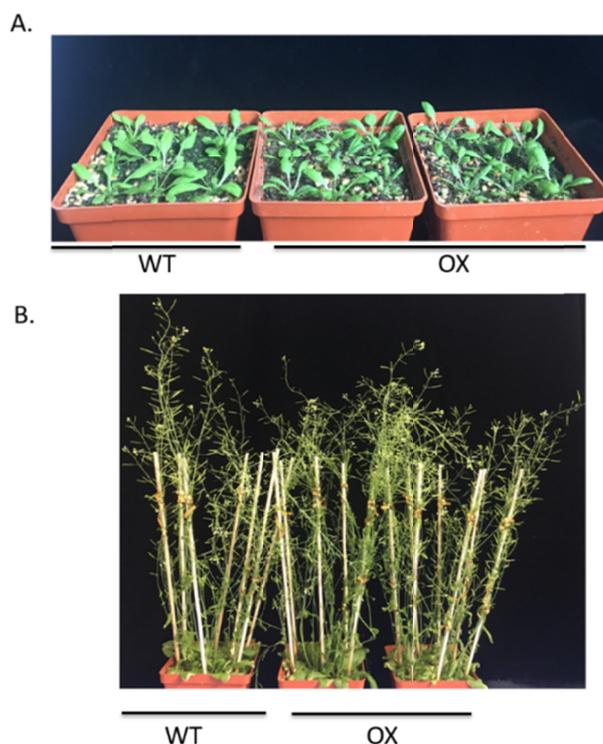


Figure 4. Phenotype analysis of transgenic *A. thaliana*. WT: wild-type Col-0; OX: *PaRAP2.1* overexpression transgenic *A. thaliana* T₃ different lines. A: Wild-type and overexpression *A. thaliana* vegetative growth conditions. B: Growth status of wild-type and overexpressing *A. thaliana* during reproductive growth period

The eight genes participating in fatty acid biosynthesis in *PaRAP2.1*-OE and WT plants were selected for qRT-PCR. The results indicated that the five out of eight genes in fatty acid biosynthesis were all up-regulated in *PaRAP2.1*-OE plants than in the WT, and the expression levels of *AtPDH(E1 α)*, *AtACP4*, *AtSAD*, and *AtFAD2* in *PaRAP2.1*-OE plants were more than twice as much as that of the WT (Figure 5). Recent report demonstrates that many genes in fatty acid biosynthesis are targets of another AP2/ERF transcription factor, WR11 (Kong and Ma, 2019). In our previous study, transcriptome data of *PaWR11*-OE transgenic tomato plants indicates that 12 and five unigenes participating in fatty acid biosynthesis are up-regulated in *PaWR11*-OE plants than in the WT (Ge et al., 2021a). The transcript amounts (> 100 FPKM) of genes in fatty acid biosynthesis including *PDH(E3)*, *ACP1*, two *FatB* paralogs, *SAD*, *ACC(Cta)*, and *FAD2* are up-regulated the most, and the expression amounts in *PaWR11*-2-OE plants are more than twice as much as that of the WT (Ge et al., 2021a). Similarly, several genes contributing to fatty acid biosynthesis, such as *ACP*, *PDH(E1 α)*, *EAR*, *ACC(Cta)*, *LACS9*, and *SAD*, are up-regulated in *WR11*-OE plants than wild plants in some crops, such *Zea mays* (Pouvreau et al., 2011), *B. napus* (Li et al., 2015), *Nicotiana benthamiana* (Grimberg et al., 2015), *Solanum tuberosum* (Hofvander et al., 2016), *Saccharum officinarum* (Zale et al., 2016), and *A. thaliana* (Adhikari et al., 2016; Yeap et al., 2017). Besides, *WR15a*, a AP2-domain transcription factor, is also considered as a master regulator of lipid biosynthesis by

controlling lipid transfer and periarbuscular membrane formation in *Medicago truncatula* (Jiang et al., 2018). It was suggested that *PaRAP2.1* was like *PaWR11* and *WR15a*, and have a role in oil accumulation, which was agreement with the speculative result (Jiang et al., 2018; Ge et al., 2021a).

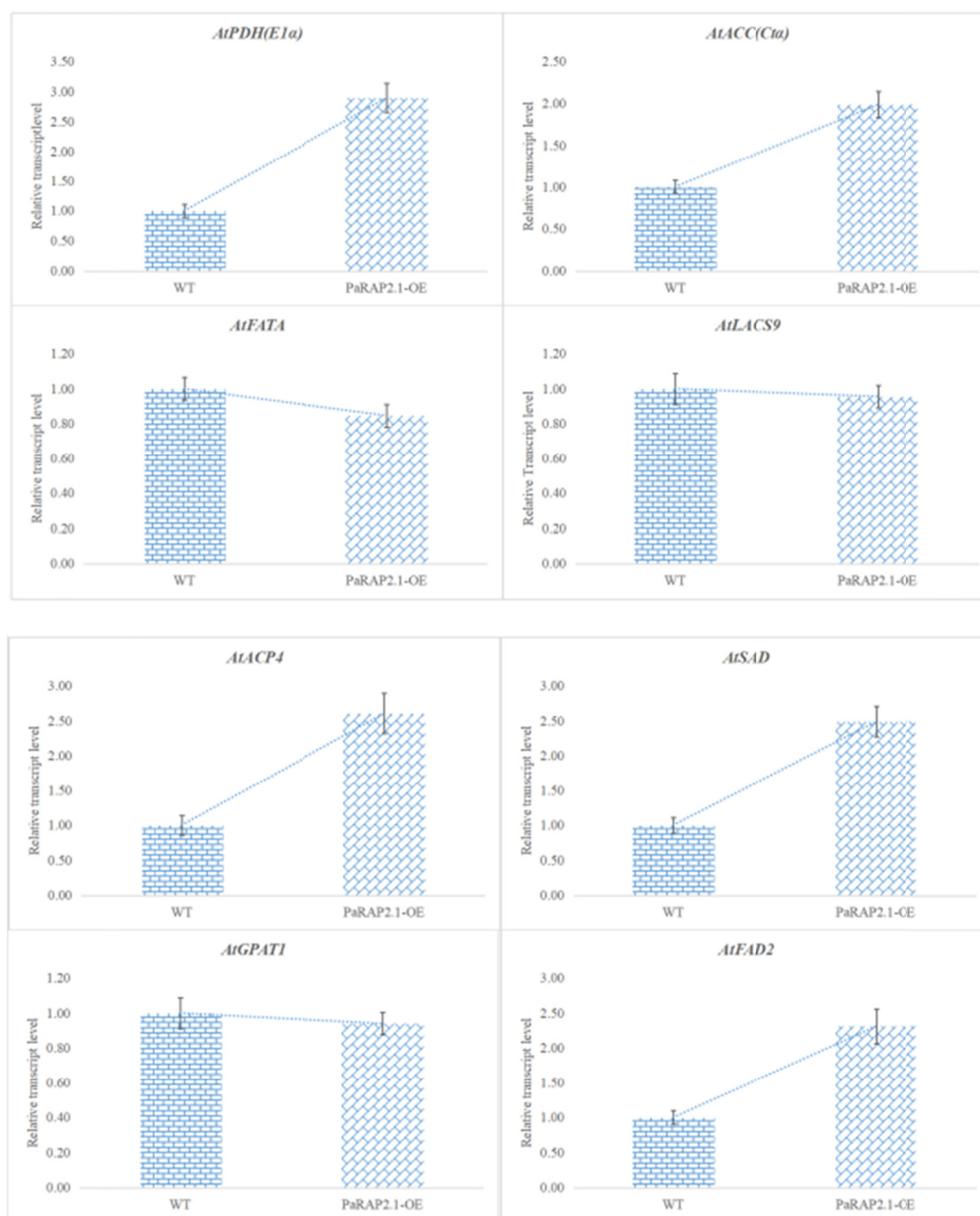


Figure 5. Eight genes were used for qRT-PCR in *PaRAP2.1*-OE and WT plants. The $2^{-\Delta\Delta Ct}$ in WT plants were used as the control for normalization. Results represent the mean of three biological replicates and two technical replicates (mean \pm SD, $n = 6$)

3.3 Effects of *PaRAP2.1* on Fatty Acid Contents

To study the influence of *PaRAP2.1* on fatty acid synthesis, six dominating of fatty acid compositions (palmitic, stearic, oleic, linoleic, Linolenic, and arachidic acids) were detected by GC-MS in *PaRAP2.1*-OE and WT plants. In the present study, the total fatty acid content in the *PaRAP2.1*-OE plants was more than 84% higher than that in the WT plants. All six critical fatty acid compositions in the *PaRAP2.1*-OE lines were higher than those in the WT lines, with the content of linoleic approximately 3-times higher in the *PaRAP2.1*-OE lines than in the WT lines (Figure 6). These results indicated that the expression of *PaRAP2.1* might promote fatty acid accumulation. The complete ion chromatograms for the FAMES of *PaRAP2.1*-OE and WT plants are provided in Figure 7.

Some previous transgenic studies show that another AP2/ERF transcription factor, WRI1, could also induce effectively fatty acid biosynthesis in transgenic plants. In our previous study, the content of total fatty acid in the *PaWRI1-2-OE* transgenic tomato plants is more than 49% higher than that in the untransformed controls, with oleic and linoleic acid contents nearly two fold higher in the *PaWRI1-2-OE* lines than in the WT lines (Ge et al., 2021a). Similarly, a GC-MS analysis of transgenic *Brassica napus* lines displays that the content of total fatty acid in the leaves of *BnWRI1-OE* plants is almost 53% higher than that in the WT plants, with most of this difference due to the greater abundance of oleic, palmitic, linoleic, and stearic acids in the transgenic plants (Li et al., 2015). In a previous study, the total fatty acid content is reportedly 9% higher in *BnWRI1-OE* transgenic *Brassica napus* plants than that in the WT plants, with the contents of oleic, linolenic, stearic, linoleic, and palmitic acids dramatically higher in the *BnWRI1-OE* transgenic plants than that in the untransformed controls (Wu et al., 2014). In another investigation involving a GC-MS analysis, the fatty acid content of transgenic maize lines is approximately one-third fold higher than that in the untransformed controls (Shen et al., 2010).

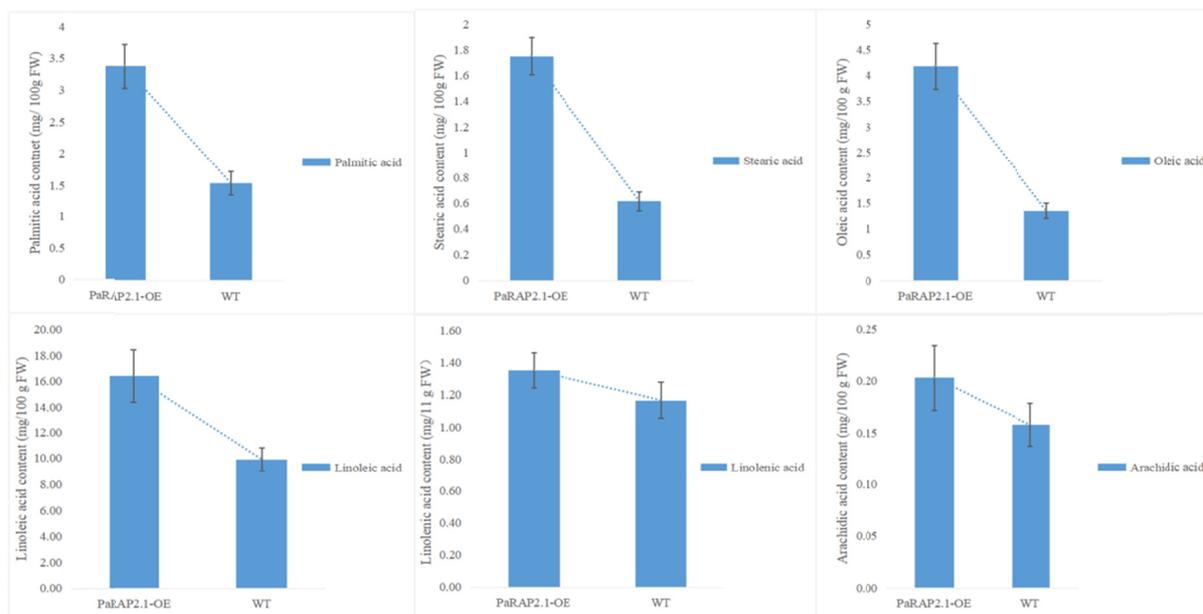


Figure 6. Fatty acid compositions of the mature seeds in the *PaRAP2.1-OE* and WT plants. The fatty acid contents (mg/100g FW) were herein presented as the mean±standard deviation of three biological replicates, each with two technical replicates

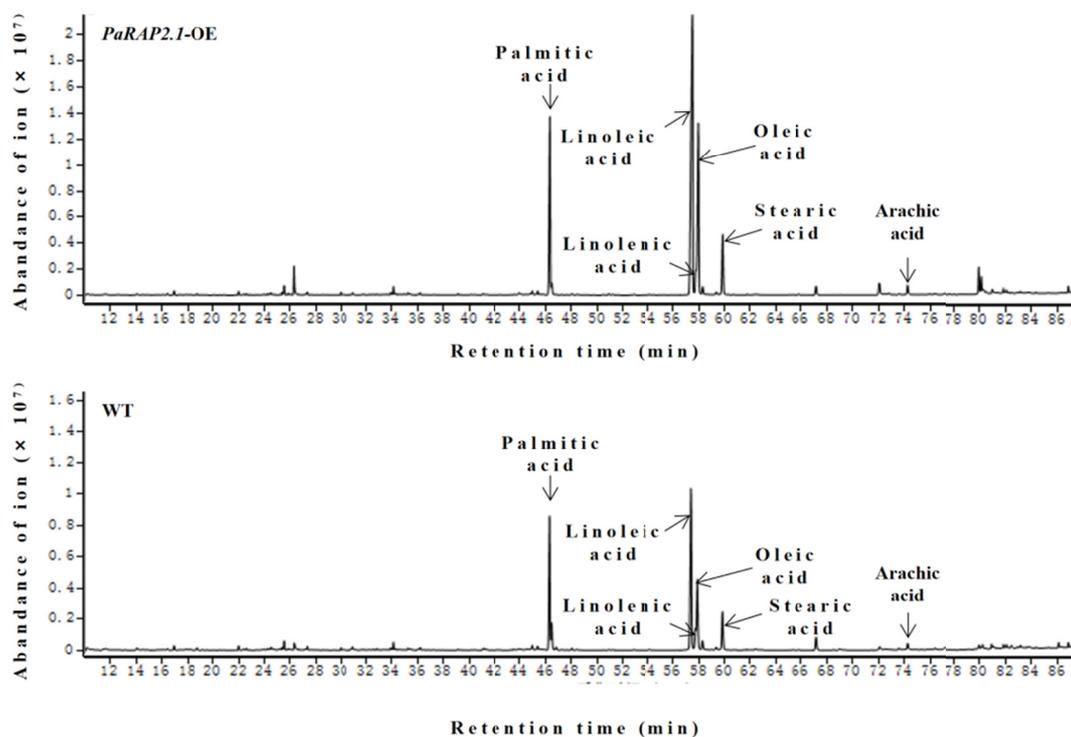


Figure 7. Total ion chromatogram of the fatty acid methyl esters of the *PaRAP2.1*-OE and WT plants

3.4 Histological Analyses of Lipid Droplets in *PaRAP2.1*-OE and WT Plants

In our previous studies, the total fatty acid content is always positively correlated with the area and number of lipid droplets (Ge et al., 2019, 2021b). Therefore, in the present study, the lipid droplets in the mature seeds of *PaRAP2.1*-OE and WT plants were visualized through a histological analysis. A great many lipid droplets (Figure 8B) had formed in the mature seeds of *PaRAP2.1*-OE plants, occupying most of the cell volume. In contrast, a few lipid droplets (Figure 8A) were founded at the edge of the cell wall in the seed of WT plants. Our histological analyses verified the observably difference in total fatty acid content measured through GC-MS between *PaRAP2.1*-OE and WT plants in the present study. Similarly, the accumulation of lipid droplets in *AtWR11*-OE transgenic sugarcane leaf tissue is remarkable relative to WT plants (Zale et al., 2016).

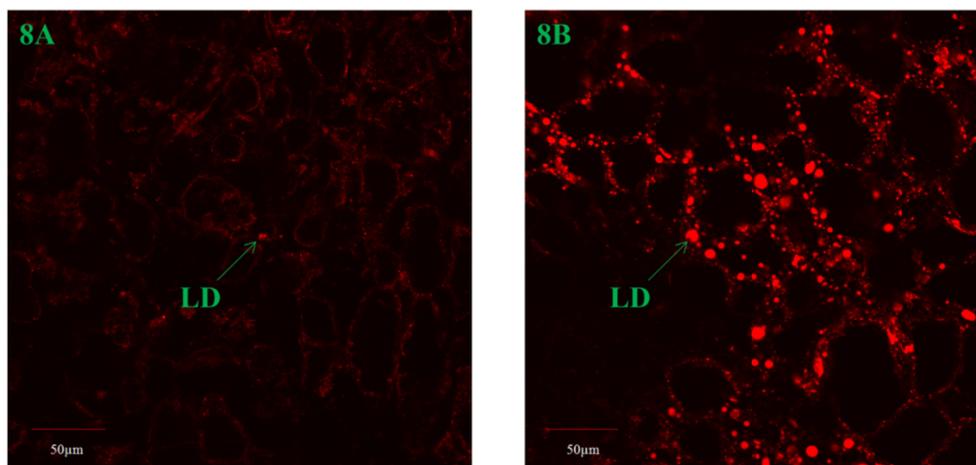


Figure 8. Confocal microscopy images of lipid droplets in WT (A) and *PaRAP2.1*-OE plants (B) using Nile red staining. LD: lipid droplet

4. Conclusion

The functional analyses of transgenic *A. thaliana* lines overexpressing *PaRAP2.1* illustrated the effects of the encoded transcription factors on fatty acid biosynthesis. The data suggested that *PaRAP2.1* might be conducive to fatty acid biosynthesis. The results described herein may help to demonstrate the involvement of *PaRAP2.1* in the fatty acid biosynthetic pathways in plants. The produced data offer worthy clues in regard to the biological functions of AP2/ERF transcription factors in plants.

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