PLANT SIGNALING & BEHAVIOR
2023, VOL. 18, NO. 1, e2213937 (5 pages)
https://doi.org/10.1080/15592324.2023.2213937

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SHORT COMMUNICATION

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Characterization of *fab2* T-DNA insertion mutants in terms of fatty acid composition and plant phenotype

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ABSTRACT

Fatty acid biosynthesis 2 (FAB2) is an essential enzyme responsible for the synthesis of unsaturated fatty acids in chloroplast membrane lipids found in leaves and triacylglycerols (TAG) in seeds. FAB2 functions at the junction of saturated to unsaturated fatty acid conversion in chloroplasts by converting 18:0-ACP to 18:1-ACP. In the present study, plant growth and seed phenotypes were examined in three Arabidopsis T-DNA mutants (*fab2–1*, *fab2–2*, and *fab2–3*). The three *fab2* T-DNA mutants exhibited increased 18:0 fatty acid content in both the leaves and seeds. The degree of growth inhibition of the *fab2* mutant was proportional to the increase in 18:0 and decrease in 18:3 fatty acids present in the leaves. The *FAB2* mutation affected seed yield but not the seed phenotype. This result indicates that FAB2 affects the fatty acid composition of the leaf chloroplast membrane more than seed TAG. In summary, the characteristics of these three *fab2* mutants provide information for studying leaf membrane lipid and seed oil biosynthesis.

ARTICLE HISTORY
Received 25 January 2023
Revised 25 April 2023
Accepted 27 April 2023

KEYWORDS
Arabidopsis; chloroplast; FAB2; fatty acid; phenotype; T-DNA

Plant lipids constitute the chloroplast membrane in the leaf, a photosynthetic tissue, and the cell membranes of various organelles. In addition, it is a component of the cuticle of the epidermis, and neutral lipids are used as an energy source in seeds.[1] Fatty acids consist of carbon, hydrogen, and oxygen, with a methyl group on one side and a carboxyl group on the other side.[2] Fatty acids were biosynthesized from acetyl-CoA in chloroplasts.[3] Furthermore, 16:0-acyl carrier protein (ACP) and 18:0-ACP, saturated fatty acids, were synthesized by fatty acid synthase.[4,5]

The gene of interest in this study is *FAB2* (*Fatty Acid Biosynthesis 2*), which encodes an enzyme that synthesizes 18:1-ACP from 18:0-ACP.[6–8] Within chloroplasts, 18:1-ACP is produced and serves as a precursor to polyunsaturated fatty acids. These polyunsaturated fatty acids are integral components of glycolipids in chloroplast membranes, phospholipid in cell membranes, and seed triacylglycerols (TAGs).[9,10] The loss of function of the *Arabidopsis FAB2* gene induced by EMS resulted in growth defects, in which the average value of the leaf area and fresh weight of the *fab2* mutant was reduced to less than 2% of that of the wild type.[11] In the *fab2* mutant, the leaf epidermal and mesophyll cells did not expand, forming a brick-wall, and the chloroplasts were smaller than those in the wild type. In addition, the thylakoid stacks of chloroplasts were less developed in *fab2* mutant.[11] When the *fab2* mutant was incubated at high temperatures, the leaf phenotype was restored to some extent, and the leaf palisade and spongy parenchyma were organized.[11] In addition, *fab2*, a *ssi2* (*suppressor of SA insensitivity 2*) mutant, is related to JA and SA defense signaling to increase the resistance of several pathogen.[12,13]

In the present study, we analyzed the fatty acid composition of the leaves and seeds of three *fab2* T-DNA insertion mutants. T-DNA lines inserted into the *FAB2* gene region were referred to as SALK_039852, SAIL_209_D07, and SALK_036854 lines and were obtained from The Arabidopsis Information Resource (TAIR). T-DNA insertion sites differed in each line. The T-DNA was inserted into intron 1 of Salk_039852, exon 2 of SAIL_209_D07, and exon 3 of Salk_036854 (Figure 1a). These lines were named *fab2–1*, *fab2–2*, and *fab2–3* in order from the 5′ direction of the gene (Figure 1a). The *fab2–1* line has also been known as *ssi2–3* in previous studies.[14] The LP, RP, and BP primers were designed, and the BP primers were located in the T-DNA for genotyping (Table S1). As a result of PCR analysis using genomic DNA, PCR bands were detected in all LP+RP combinations in the wild type, but no band was detected in the BP+RP combinations. In the case of the three mutants, the PCR band was not observed in the LP+RP combination. However, it was confirmed at 500 bp and 800 bp, which were expected to be the size of the PCR fragment in the BP+RP combination; therefore, it can be regarded as a *fab2* T-DNA insertion mutant (Figure 1b).

RNA was extracted from the leaves of the homozygous T-DNA insertion mutants for RT-PCR analysis (Figure 1c). *fab2–1* with T-DNA in the intron expressed weak *FAB2* compared to the wild type, whereas *fab2–2* and *fab2–3* lines with T-DNA in the exon did not express *FAB2* (Figure 1c). In addition, the growth phenotypes of the T-DNA mutants were observed (Figure 1d). Growth problems occurred in all types of *fab2* mutant lines compared to the wild type five weeks after implantation in the soil (Figure 1d). All three mutants

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/15592324.2023.2213937

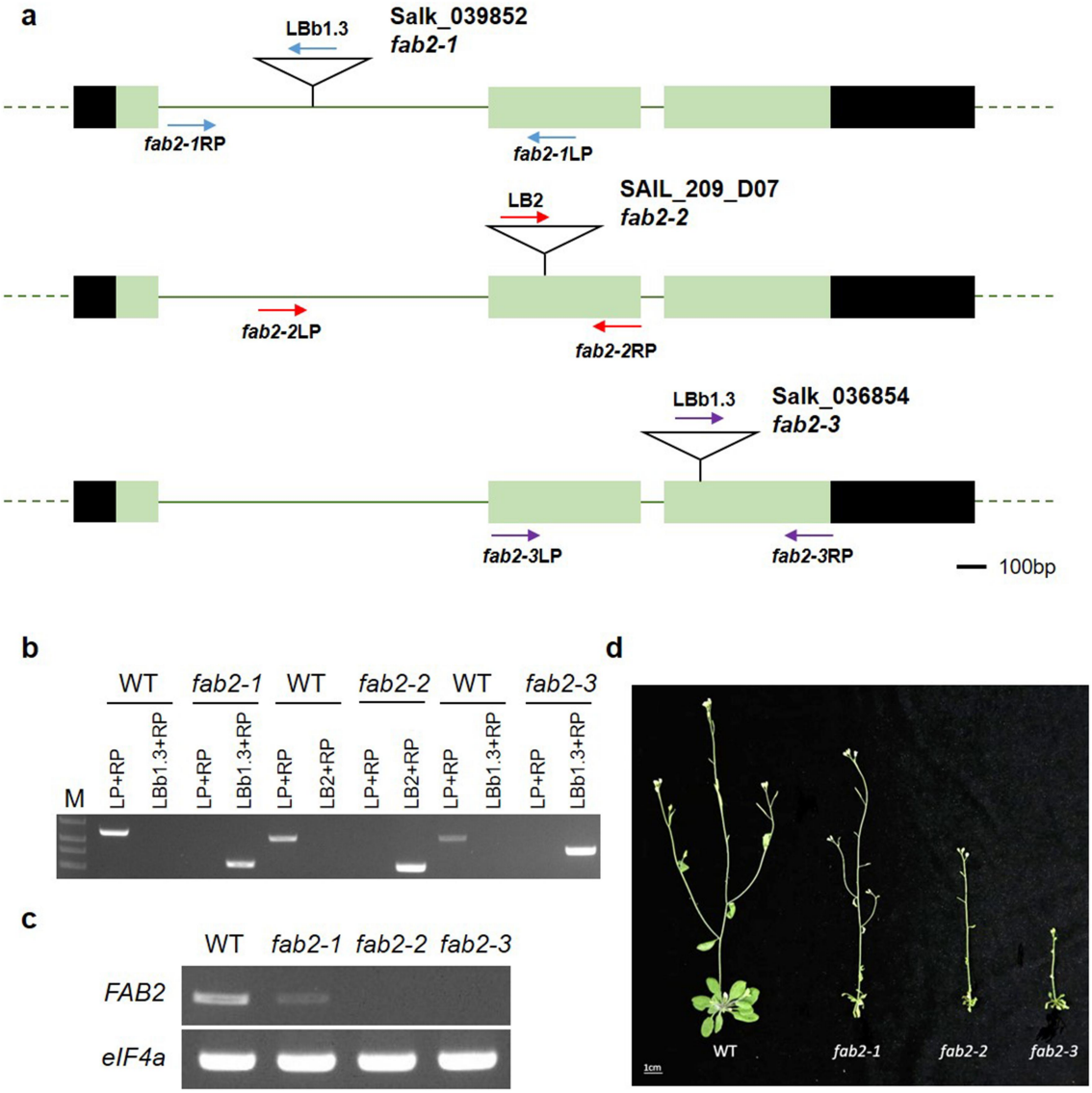

Figure 1. Identification of *fab2* T-DNA mutant alleles (*fab2–1*, *fab2–2*, and *fab2–3*) and growth phenotype. (a) Location of T-DNA insertion in *FAB2* gene structure. Arrow indicates the primer to check the T-DNA mutant. (b) Genotyping of *fab2* T-DNA mutants in genomic DNA genotype compared to wild type. (c) *FAB2* gene expression between wild type and *fab2* T-DNA mutants by RT-PCR. (d) Growth phenotype of *fab2* T-DNA mutants and wild type.

exhibited small rosette leaves, delayed shoots, and small siliques (Figure 1d). Among the three mutants, the phenotype of the *fab2–1* line, in which *FAB2* was weakly expressed because of a T-DNA insertion into the intron, appeared similar to that of the wild type, whereas the *fab2–2* and *fab2–3* lines exhibited a very distinct growth problem (Figure 1d).

The fatty acid composition was examined for *fab2–1, fab2–2*, and *fab2–3* mutants in the leaves and seeds (Table 1). In leaf fatty acid analysis, *fab2* mutants exhibited a 9- to 14.7-fold increase in 18:0 content compared to that in the wild type. The 16:0 content, a precursor of 18:0 fatty acids, increased by 17–46% compared to that in the wild type. However, the product of the FAB2 reaction, 18:1, increased by 3.6 to 11.3-fold in the *fab2* mutants compared to that in the wild type. The reaction products 16:3, 18:2, and 18:3 produced by chloroplast desaturases (FAD5, FAD6, and FAD7) were significantly reduced compared to that in the wild type (Table 1). In particular, the reduction in 18:3 in *fab2* mutants was 73–34% compared to that in the wild type (Table 1). To determine whether the expression of the three chloroplast desaturases was affected in *fab2* mutants, gene expression in leaves was measured by RT-qPCR. The expression of *FAD5*, *FAD6*, and *FAD7* was lower than that in the wild type (Figure 2). This suggests that the transcription level of chloroplast membrane desaturases is suppressed in *fab2* mutants, and the resulting reduction in polyunsaturated fatty acids adversely affects plant growth (Figure 1d).

Analysis of seed fatty acids revealed that the 18:0 fatty acid content increased 2.7 to 3.1-fold in *fab2* mutants. However, 16:0 was not significantly different from that of wild type. 18:1, synthesized from 18:0, decreased by 18–33% in *fab2* mutants. In contrast to the observed changes in the leaves, alterations in the levels of 18:2 and 18:3 polyunsaturated fatty acids were not significant and remained similar to those in the wild type (Table 1). This was due to the action of AAD1, AAD5, and AAD6, which are stearoyl-ACP

Table 1. Fatty acid composition of leaf and seed in *fab2* T-DNA mutants.

Fatty acid (mol %)	Leaf				Seed			
	WT	*fab2–1*	*fab2–2*	*fab2–3*	WT	*fab2–1*	*fab2–2*	*fab2–3*
16:0	19.7 ± 1.1	23.2 ± 2.3	28.8 ± 2.2	28.2 ± 3.2	8.8 ± 0.3	8.3 ± 0.4	9.3 ± 0.8	9.0 ± 0.4
16:1	3.9 ± 0.4	4.1 ± 0.6	1.7 ± 0.3	2.3 ± 0.5	-	-	-	-
16:3	13.8 ± 1.0	8.9 ± 1.3	2.6 ± 0.2	2.5 ± 1.2	-	-	-	-
18:0	1.7 ± 0.3	15.4 ± 3.5	25.1 ± 4.0	24.0 ± 3.9	3.2 ± 0.2	8.6 ± 0.4	9.8 ± 0.9	9.0 ± 0.4
18:1	1.9 ± 0.2	7.0 ± 2.0	16.0 ± 2.8	21.5 ± 2.0	17.6 ± 0.2	14.6 ± 0.3	14.2 ± 0.5	11.8 ± 0.8
18:2	13.1 ± 1.2	7.5 ± 0.9	6.0 ± 0.9	5.8 ± 2.0	32.5 ± 0.5	29.4 ± 0.6	28.5 ± 1.2	29.0 ± 0.6
18:3	45.9 ± 2.0	33.9 ± 2.0	19.9 ± 0.2	15.7 ± 5.5	15.2 ± 0.1	15.4 ± 0.1	14.6 ± 0.4	16.2 ± 0.6
20:0	-	-	-	-	2.1 ± 0.04	5.3 ± 0.1	5.7 ± 0.1	6.4 ± 0.3
20:1	-	-	-	-	17.4 ± 0.1	15.5 ± 0.3	14.9 ± 0.5	15.0 ± 0.2
20:2	-	-	-	-	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.9 ± 0.03
22:1	-	-	-	-	1.5 ± 0.01	1.3 ± 0.03	1.3 ± 0.1	1.6 ± 0.03
SFAs	21.4 ± 1.0	38.6 ± 2.5	53.9 ± 2.2	52.2 ± 7.1	14.1 ± 0.2	22.2 ± 0.3	24.8 ± 0.6	24.4 ± 0.4

Note: All data are mol %. SFAs indicate saturated fatty acids (16:0 + 18:0 or 16:0 + 18:0 + 20:0) in leaves and seeds. Leaf and seed ($n = 3$), – = not detected.

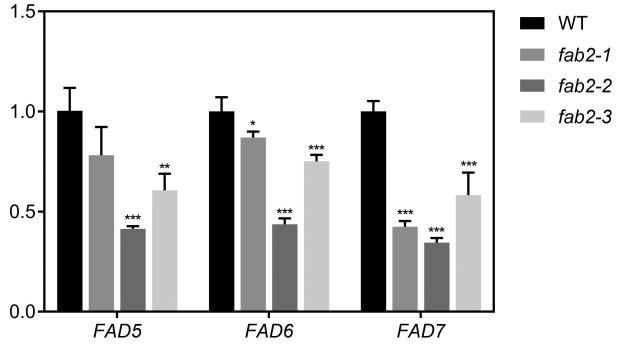

Figure 2. RT-qPCR analysis of *FAD5*, *FAD6*, and *FAD7* in wild type and *fab2* mutant leaves. Statistical significance is indicated by asterisk using one-way ANOVA test with Tukey's multiple comparison tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

desaturases with redundant functions with FAB2 in the seeds.[15] The content of 20:1 fatty acid synthesized solely in the seeds was reduced up to 15% compared to that in the wild type (Table 1). The loss-of-function mutation of *FAB2* exhibited a shared characteristic of significant elevation in the total saturated fatty acid content, both in the leaves and seeds, in comparison to that in the wild type (Table 1).

Seeds were harvested from wild type and *fab2* mutants, and their size and shape were observed (Figure 3). All *fab2* mutants exhibited significantly reduced seed yield per plant. Compared with that in the wild type, seed yield of *fab2–1* was reduced by 59%, that in *fab2–2* by 94%, and that in *fab2–3* by 98%, respectively (Figure 3a). *Fab2–1* and *fab2–2* had slightly larger seed sizes than the wild type, whereas *fab2–3* exhibited smaller seed sizes than the wild type (Figure 3b). There were no significant differences in the appearance of seeds among the *fab2* mutants (Figure 3c).

The saturated fatty acid contents of the *fab2–1, fab2–2*, and *fab2–3* lines increased with growth problems (Figure 1d). This result suggests that the degree of fatty acid unsaturation in leaf chloroplast membranes is important for plant growth. However, the morphology of the *fab2* seeds was similar to that of the wild type; therefore, the difference in saturated fatty acid content did not significantly affect seed development (Figure 3). Changes in *fab2* seed size may be a secondary consequence of seed yield. In the case of *fab2–1* with T-DNA inserted into the first intron, plant growth inhibition was weaker than that in *fab2–2* and *fab2–3* with T-DNA inserted into the exon. Although the growth inhibition in *fab2–1* was not as significant as that in the wild type, it can be attributed to the weak expression of *FAB2* (Figure 1c). The reason why *fab2–3* with a T-DNA insertion in exon 3 had a more severe growth defect than *fab2–2* with a T-DNA insertion in exon 2 is unknown. The degree of growth inhibition by *fab2* correlated with changes in leaf unsaturated fatty acids. An increase in saturated fatty acids, including 16:0 and 18:0 fatty acids, and a decrease in 16:3 and 18:3 fatty acid contents further impeded plant growth (Table 1, Figure 1d). This suggests that FAB2 plays a more important role in the synthesis of 18:1 fatty acid in leaves than in seeds.

According to a previous study, when the phenotype was observed in Arabidopsis *fab2* mutants *ssi2–1, ssi2–2*, and *ssi2–3* (*fab2–1*), *ssi2–1* and *ssi2–3* mutants exhibited severe growth defects. In the *ssi2–2* mutant, the phenotype was better than that of the other two mutants (*ssi2–1* and *ssi2–3*), but disease resistance showed an indeterminate phenotype that was less strong than the other two mutants.[14] Analysis of several types of *fab2* mutants showed that the loss of *FAB2* adversely affects plant growth but can enhance saturated fatty acids in seed oil. Therefore, FAB2 should be specifically suppressed in the seeds, but not in the leaves, to enhance industrially useful saturated fatty acids in oil crops. For instance, Cas13a or RNAi techniques may be effective strategies. Because both Cas13a and RNAi can specifically target RNA and suppress target gene expression, seed-specific expression of Cas13a or RNAi may increase the saturated fatty acid content of seeds without affecting plant growth.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana plants used Col-0 as the wild type. All *fab2* T-DNA insertion mutants were obtained from The Arabidopsis Information Resource (TAIR). To grow the wild type and mutants, the seeds were sterilized using 70% EtOH and 0.5% NaOCl and washed 10 times with distilled water. Furthermore, stratification was performed for three days at 4°C in the dark. Subsequently, seeds were plated in half-strength MS medium containing 1% sucrose and cultured in a culture chamber at 23°C under 100 μmol $\cdot m^{-2}s^{-1}$ and 16 h light/8 h dark conditions. The seedlings

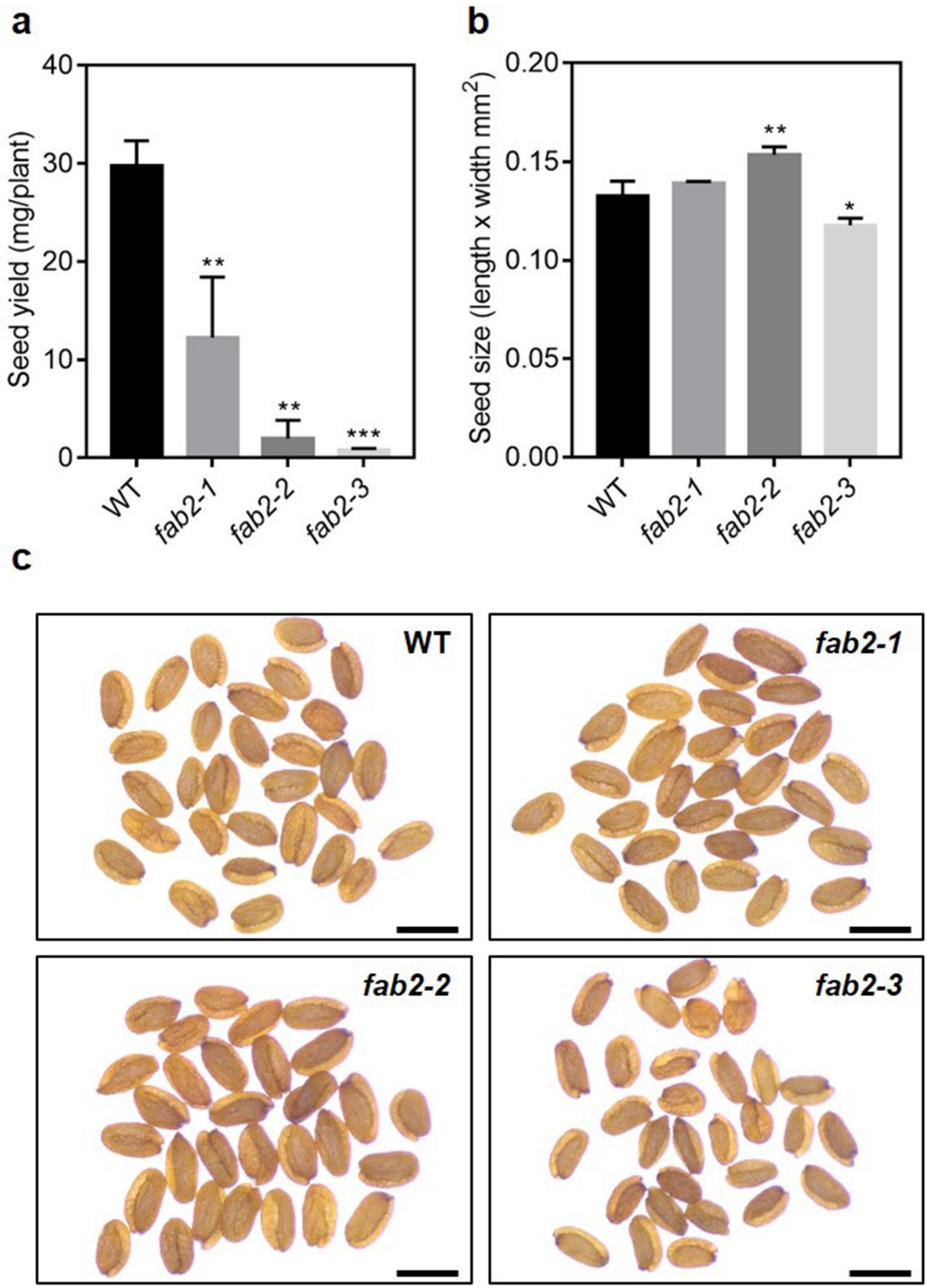

Figure 3. Analysis of seed phenotype in wild type and *fab2* T-DNA mutants. Measurement of seed yield per plant (a) and seed size (b). Image of wild-type seeds and *fab2* T-DNA mutants seeds (c). Scale bars = 0.5 mm. Error bars represent SD of the mean. Statistical significance is indicated by asterisk using one-way ANOVA test with Tukey's multiple comparison tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

were cultured for 10 days, transplanted into the soil, and grown in a growth chamber under the conditions described above.

Identification of T-DNA insertion mutants

To determine whether the T-DNA insertion was homozygous, *FAB2* gene-specific LP and RP primers were designed, and

PCR was performed using genomic DNA extracted from the rosette leaves of the wild type and *fab2* T-DNA insertion mutants. The *fab2* mutants were selected based on differences in PCR size compared to the wild type. Primers for identifying T-DNA insertion mutants were designed using T-DNA Primer Design Site (http://signal.salk.edu/tdnaprimers.2.html) (Table S1).

RT-qPCR analysis

Total RNA was isolated from the leaves using TRIzol reagent (Invitrogen) and treated with DNase I (Thermo Fisher Scientific). cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara). Primers targeting *FAD5*, *FAD6*, and *FAD7* were designed using Primer3Plus (Table S1). RT-qPCR analysis was performed using the TB Green Premix Ex Taq™ II (Takara) reagent in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The ΔCT value was calculated by subtracting the CT values between the target gene and the endogenous control. eIF4a (AT3G13920) was used as a control gene. The two ΔCT values were subtracted, and the value of $2^{(-\Delta\Delta CT)}$ was obtained to calculate the relative expression level.

Fatty acid analysis

Fatty acids were isolated and analyzed from approximately 20 mg of leaves (10 days after imbibition) and 30 mature seeds. Each sample was placed in a glass tube, and 500 µl of 5% H_2SO_4 containing an internal standard (15:0) and 500 µl toluene were added. After boiling for approximately 2 h in an 85°C water bath, the mixture was cooled for approximately 10 min, and 1 ml of 0.9% NaCl and 1 ml n-hexane were added. The mixture was centrifuged to separate the supernatant containing fatty acid methyl esters (FAME). FAME was transferred to a 6 ml tube and purged using nitrogen gas. FAME was dissolved in 200 µl of n-hexane and transferred to a GC vial for gas chromatography analysis. The extracted FAMEs were analyzed using GC-2030 (Shimadzu) and the DB-23 column (30 m × 0.25 mm, 0.25 µm film, Agilent). The temperature of the GC oven was increased from 190 to 230°C at a rate of 3°C/min.

Analysis of seed size and weight

The size of the seeds was randomly repeated three times with 20 seeds from the same line, and images were captured using an SMZ745T microscope (Nikon). To measure the seed size, the width and length of the seeds were calculated using ImageJ, and the length and width were multiplied to compare the size of the seeds. The weight of 100 seeds was measured using an electronic scale (OHAUS), with five replicates.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by grants from the Mid-Career Researcher Program of the National Research Foundation of Korea (NRF-2020R1A2C2008175) and New Breeding Technologies Development Program (Project No. PJ016533), and the Program of the Rural Development Administration, Korea

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