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INVESTIGATING THE STABILITY OF PHENOTYPE, SEED COMPONENTS AND POWDERY MILDEW RESISTANCE OF THE *GmMLO* EDITED SOYBEAN PLANTS

Phuong Thao Bui[1,2], Quyen Phan[1], Thi Mai Huong To[2] and Tien Phat Do[1,3,✉]

[1]*Institute of Biotechnology- Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, 10000, Vietnam.*

[2]*University of Science and Technology of Hanoi- Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, 10000, Vietnam.*

[3]*Graduate University of Science and Technology- Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, 10000, Vietnam.*

✉To whom correspondence should be addressed. E-mail: dtphat@ibt.ac.vn

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ABSTRACT

Previously, utilizing the CRISPR/Cas9 system, we had successfully induced targeted mutations in the *GmMLO* genes of the Vietnamese elite soybean cultivar ĐT26 for the first time. The mutant lines carried homozygous mutations of four or three *GmMLO* genes (including *GmMLO02*, *GmMLO19*, *GmMLO23* and/or *GmMLO20*) with no pleiotrophic effects and increased powdery mildew resistance in the T3 generation. In this study, we selected two representative lines and evaluated them in the T4 generation to see if their phenotype and resistance were maintained stably. The analysis results of agronomic and yield parameters under the net-house conditions indicated that there were no undesired data in the two mutant lines in comparison with the wild-type. In addition, the major nutritional compositions of the seeds including the fatty acids, free amino acids and crude protein content of the mutant lines were similar to the control line. Importantly, the response to *Erysiphe diffusa* challenge of the quadruple mutant line was still maintained at a moderate resistance level (grade 2.5) as compared to the moderate infection level of the wild-type and the triple mutant line (approximately grade 4). These results again demonstrate that inducing targeted mutations of four tested *GmMLO* genes via the CRISPR/Cas9 system is not accompanied with undesired traits, and the quadruple mutant line is the potential one with increased powdery mildew resistance maintained stably through generations. This soybean mutant line will be valuable material for further breeding programs as well as being able to be propagated for production.

Keywords: CRISPR/Cas9, edited soybean lines, GmMLO, phenotype, powdery mildew resistance, seed components

INTRODUCTION

Powdery mildew is a major disease that causes great losses for soybean production. This disease is caused by an obligate biotrophic fungus known as *Erysiphe diffusa* (Cooke & Peck) (syn. *Microsphaera diffusa* (Cooke & Peck) (McTaggart *et al.*, 2012; Tam *et al.*, 2016). The disease initiates with white spots of mycelium forming on cotyledons, stems, fruits and especially on the upper leaf surfaces. When the fungus develops at a high level, the infected soybean leaves lose chlorophyll, turn yellow, necrosis and fall off (Grau, 1975). In addition, the fungus can grow through the infected pods to the seeds. Previous studies have recorded an average yield loss of 13%. In favorable weather conditions for the fungal growth, the yields of some susceptible varieties could be reduced by up to 35-60% (Gonçalves *et al.*, 2002). In Vietnam, powdery mildew spreads and damages all soybean cultivation regions, especially in the North. The soybean yield loss caused by powdery mildew can be up to 60% in the spring growing season (Tran *et al.*, 2015).

The use of resistant varieties is considered the most effective method that has been widely applied for controlling plant powdery mildew. However, natural mutant sources for soybean powdery mildew resistance are limited and challenged to transfer to elite cultivars. Engineering susceptibility genes (S-genes) of host plants is now considered as an alternative approach for disease resistant breeding. The *Mildew Locus O (MLO)* genes are well-studied S-genes responsible for powdery mildew susceptibility in various plant species. *MLO* genes encode for proteins with seven trans-membrane domains, an extra-cellular N-terminal and a cytosolic C-terminal (Devoto *et al.*, 1999; Acevedo-Garcia *et al.*, 2014). They are required for the entering of powdery mildew fungus through the epidermal cells of host plants. For years the phylogenetic analysis of the MLO family grouped the proteins in six clades. Interestingly, all dicots MLO proteins associated with powdery mildew susceptibility group in clade V (Pépin *et al.*, 2021), whereas in monocots, they group in clade IV (Acevedo-Garcia *et al.*, 2014; Kusch and Panstruga, 2017).

Previous studies demonstrated that recessive mutations in *MLO* genes confer complete or enhanced resistance to powdery mildew in various crop species such as barley, tomato, bread wheat, etc. (Kusch and Panstruga, 2017). Besides using different mutagenic approaches, such as chemical, RNAi, TALLEN and TILLING, CRISPR/Cas9 system has been recently proved to be the most precise one to induce targeted mutations of *MLO* genes. The success in increasing powdery mildew resistance through this method had been reported in tomato (Nekrasov *et al.*, 2017; Pramanik *et al.*, 2021), wheat (Li *et al.*, 2022), grapevine (Wan *et al.*, 2020), and cucumber (Tek *et al.*, 2022). However, most of those studies just displayed the results at T1 generation and the time for getting resistance data post inoculation was quite short.

In soybean, the involvement of four *GmMLO* genes, including *GmMLO02*, *GmMLO19, GmMLO20* and *GmMLO23* in powdery mildew susceptibility had been indicated for the first time in our previous study (Bui *et al.*, 2023). Moreover, the *Gmmlo02/Gmmlo19/Gmmlo20/Gmmlo23* quadruple knockout mutants and the *Gmmlo02/Gmmlo19/Gmmlo23* triple mutant lines had been successfully created by utilizing CRISPR/Cas9 system. They all

exhibited immunity to powdery mildew without growth and yield penalties in T3 generation, especially the quadruple ones. With the purpose of generating resistant soybean varieties that can be used for production in the future, strict and careful assessments are necessary. In this study, we selected two representative mutant lines inherited from our last research, which are line 3.1-3-41 carried quadruple mutations and line 15.1-6-4 carried triple mutations, to examine the maintenance of powdery mildew resistance in the T4 generation. The growth and yield parameters as well as the seed nutritional components of mutant lines were also compared to the wild-type to check for abnormal phenotypes. The mutant lines, which retained crop growth and yields while conferring robust powdery mildew resistance, obtained from this study will be potential lines for further soybean breeding programs.

MATERIALS AND METHODS

Materials

T4 seeds of the two *GmMLO* soybean mutant lines 3.1-3-41 (carrying quadruple mutants), 15.1-6-4 (carrying triple mutants), and the seeds of the control soybean cultivar, ĐT26, were provided by the Plant Cell Biotechnology Laboratory, Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology (VAST).

The specific primers used to identify induced mutations in four targeted *GmMLO* genes (*GmMLO02, GmMLO19, GmMLO20* and *GmMLO23*) in two soybean mutant lines were inherited from our previous study (Bui *et al.*, 2023).

E. diffusa fungus causing soybean powdery mildew was maintained at the greenhouse of Co Nhue Experimental Biology Station, VAST.

Methods

Plant cultivation and assessment of morphological and productivity characteristics of soybean lines

The T4 mature soybean seeds were imbibed on moist paper for 48h and sown in plastic pots. The soybean plants were regularly watered and fertilized for three times, the first time at V3 stage with NPK (15:5:20), the second and third times at 40 and 65 days-old-stage with NPK (16:16:16). Experiment was carried out in the net-house condition at Co Nhue Experimental Biology Station, VAST. Growth parameters such as stem length (cm) and internode number were measured when each soybean plant achieved maturity (R8 stage), while yield parameters such as the number of pods, the ratio of pods with 3 and 4 seeds, and the weight of 100 seeds (grams) were collected after harvesting the plants. Harvested mature seeds were dried and stored at 4°C and 40% humidity for further studies.

Mutant identification and characterization

Total genomic DNA was extracted from young leaves of the T4 soybean mutant lines and the wild-type plant ĐT26 using the CTAB protocol modified from the method of Doyle and Doyle (1990). The induced mutations in four *GmMLO* genes were identified by PCR using specific primers for each gene and analyzed by 1% agarose gel electrophoresis. The PCR amplicons were then purified, Sanger sequenced as described in detail in the study of Le *et al.* (2020), and analyzed by the FinchTV chromatogram

viewer program (Geospiza) and MEGA X (Kumar *et al.*, 2018).

Nutritional components analysis of soybean seeds

The soybean seeds were dried at 30°C for 24 hours, then milled and sieved through an 840-µm sieve using a stone mortar. After milling, powder samples will be kept at -20°C until soybean seed component analysis.

Fatty acid (FA) analysis of soybean seeds using Gas Chromatography - Mass Spectroscopy (GC - MS)

FA composition in soybean seeds was determined according to the method of (Bligh and Dyer, 1959) with some modifications. Particularly, the entire oil of approximately 150 mg of ground soybean seeds was extracted by adding 2 mL of chloroform/methanol solution (1:2) and shaken every 15 minutes for 1 hour. Next, each tube was added 0.1 mL of standard which was pentadecanoic acid (C15:0) for quantitative analysis. Then, 0.67 mL of chloroform and 0.67 mL of saturated NaCl were added, and the mixture was vortexed for 1 min. Using a system of two glass pipettes to collect the lower layer after centrifuging at 3000 rpm for 5 minutes. The extracted solution was added to 1 mL of saturated NaCl, and then again centrifuged for 5 minutes at 3000 rpm. The lower layer was collected, then blast nitrogen gas into the vials to evaporate the solvent. After the evaporation, the residue was dissolved in 500 µL BF3-Methanol, incubated at 80°C in a water bath for an hour, and then cooled for five minutes to produce fatty acid methyl esters (FAMEs) derivatives. The FAMEs were extracted using 2 mL of hexane and 2 mL of saturated NaCl, followed by centrifuging at 1300 rpm for 3 minutes. The upper layer was transferred to 2 mL vials and analyzed with GC-MS. FAMEs were analyzed using a GC-MS system using a Thermo Scientific GC (TRACE 1300 series GC) and an MS (ISQ series system). For chromatographic separation, a capillary gas chromatograph (GC) was performed using an Agilent HP-5MS-UI capillary column (30 m × 0.25 mm × 0.25 µm, Agilent, Palo Alto, CA, USA). For the chromatographic analysis, 1 µL of the extracted lipid sample was employed. The elution process with hexane takes place five times before injecting the FAMEs sample into the GC-MS instrument for analysis. The oven's initial temperature was set at 110°C and maintained there for 2 minutes, then ramped up to 200°C at a rate of 10°C/min. The temperature was then raised to 250°C at a rate of 4°C/min, then raised to 280°C at a rate of 20°C/min and maintained there for three minutes. The total run time lasted 28 minutes.

Quantitative analysis of free amino acids (FAA) using ion exchange chromatography – Biochrom 30+

The FAAs in soybean seed were extracted according to the protocol described by Jia *et al.* (2018). Briefly, 1.5 mL of sodium loading buffer (pH 2.2) was used to homogenize 200 mg of the powder sample, then vortexed for 1 min. The mixture was carefully transferred into an Eppendorf tube and shaken on a microtube mixer at 1000 rpm for an hour before being centrifuged at 15,000 rpm for 10 minutes. The supernatant was collected, filtered through a 0.22 µm nylon syringe filter, and then put into a 2 mL glass vial for a Biochrom 30+ analysis. A Biochrom + 30 system with a high-pressure PEEK (Poly Ethyl Ketone) column

filled with Ultropac 8 cation exchange resin was used to inject about 20 µL of this mixture. The dedicated Biochrom 30+ Amino Acid Analyzer was established for post-column detection. The multistep elution process was carried out using six buffers. When ninhydrin and amino acids react, colorful molecules are produced that may be detected photometrically at 440 nm (primary amines) and 570 nm (secondary amines). Data collection and analysis were performed using EZChrom Elite data handling software and BioSys control software. On the basis of peak area of the chromatography and standard concentration, each amino acid was identified.

The following formula was used to determine the free amino acid content:

$$FAA = 2 \times concentration\ of\ amino\ acid \times molecular\ mass\ of\ amino\ acid \times 10^{-5}\ \ (\frac{mg}{g\ sample})$$

Measurement of total N/crude protein using the Kjeldahl method

The protein content was determined by the Kjeldahl method as described in a previous study (Thiex *et al.*, 2002). Briefly, about 0.5 g of powdered whole seed sample was digested in 15 mL of concentrated sulfuric acid and sulfate salts at 420ºC. After 90 minutes of digestion, 70 mL of deionized water was used to dilute the sample. Before distillation takes place, a flask containing 25 mL of boric acid was put on the platform. The glass tube was attached to the rubber holder, and the safe door was closed. The flask of boric acid received a drop of ammonia distillate. Next, the titration with hydrochloric acid happened. The flask was placed on a magnetic stirrer, and the distillate was added slowly with 0.1 N HCl. The titration was finished when the color turned a pale pinkish.

The content of Nitrogen in the sample can be calculated:

$$\%N = \frac{concentration\ of\ HCl \times volume\ of\ HCl\ consumed\ during\ titration\ (mL) \times 14.01\ (g) \times 100}{Weight\ of\ a\ sample\ (g) \times 1000}$$

The amount of crude protein was determined:

$$\%\ crude\ protein\ = \%\ N\ \times 6.25$$

Powdery mildew resistance assessment

Artificial infection was carried out at the net-house conditions when the T4 mutant plants were at the V2 growth stage. On the day before infection, the soil was irrigated to ensure adequate moisture. *E. diffusa* spore solution (density 5.10^4 spores/mL) was sprayed at a dose of 100 mL/m^2 in the evening. The infection level of each line was observed and evaluated at V3, V5 and V7 stages. At each growth stage, the plants were divided into 3 leaf layers (including lower, middle and upper layers) and scored based on the most severely diseased leaf of those leaf layers. The infection level was assessed according to the symptom scales (0 to 5 grades) described by Tran *et al.* (2015).

Statistical analysis

All experiments were conducted in three replicates. Data were collected and analyzed by Microsoft Excel 2019 and SPSS software (Version 20, Chicago, IL, USA). Values are mean ± SD. Statistical significance was conducted using one-way ANOVA followed by a post hoc Turkey's test at $P < 0.05$.

RESULTS AND DISCUSSION

Inheritance analysis of *GmMLO* mutants

The seeds of the two T4 soybean mutant lines were tested before conducting the experiments to ensure that they were homozygous for the desired induced mutations and were not mixed ones. The results of characterization of *GmMLO* induced mutations in the T4 generation via agarose gel electrophoresis and Sanger sequencing showed that both tested lines carried homozygous mutations (Figures 1, 2). In particular, line 3.1-3-41 carried simultaneously homozygous mutations in four *GmMLO* genes, including small deletions ofΔ-2 bp in the *GmMLO02* gene, Δ-6/-2 bp in the *GmMLO19* gene, Δ-8 bp in the *GmMLO20* gene, and Δ-1 bp in the *GmMLO23* gene. Meanwhile, line 15.1-6-4 contained triple mutations in 3 genes, including an insertion of Δ+11 bp in the *GmMLO02* gene, a small deletion of Δ-2/-1 bp in the *GmMLO19* gene, and a large indel of Δ-428 bp in the *GmMLO23* gene. These results were also consistent to the induced mutation forms of these lines at T2 generation (Bui *et al.*, 2023). Thus, the *GmMLO* mutations continued to be stably inherited at this generation. The seeds of these two T4 lines will be used for phenotyping, analyzing nutritional compositions as well as evaluating powdery mildew resistance in subsequent experiments.

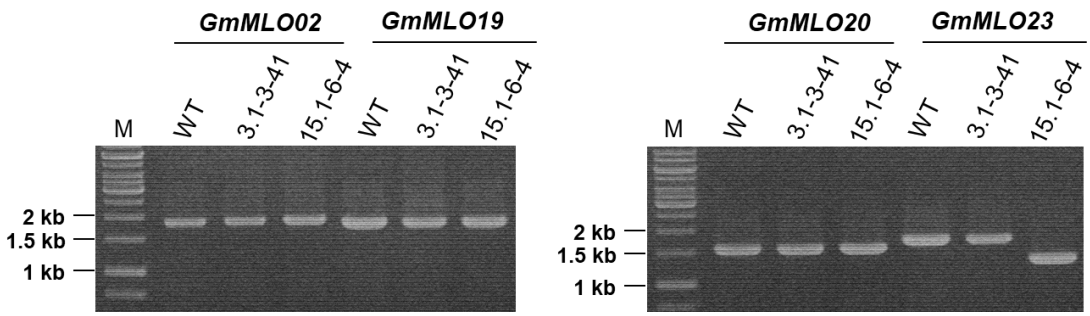

Figure 1. Gel electrophoresis of PCR amplicons with specific primers to identify mutations in four *GmMLO* genes (*GmMLO02*, *GmMLO19*, *GmMLO20* and *GmMLO23*) in T4 soybean mutant lines. M: 1 kb DNA ladder; WT: non-transgenic wild-type plant; 3.1-3-41, 15.1-6-4: T4 *GmMLO* soybean mutant lines.

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GmMLO02                    (      Target 1      )      1294bp      (      Target 2      )              Δ      Genotype
MLO02-WT    ACATATTGCAGATCCTGAGAGGTTCAGGTTTGCAAGGGACACAA  .....  TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGATCTG
3.1-3-41    ACATATTGCAGATCCT--GAGGTTCAGGTTTGCAAGGGACACAA  .....  TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGATCTG      -2/0      Homo
15.1-6-4    GCAGATCCTGAGATATATATCAAAGGTTCAGGTTTGCAAGGGACACAA 1294bp  TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGAT     +11/0      Homo

GmMLO19                    (      Target 1      )      1304bp      (      Target 2      )              Δ      Genotype
MLO19-WT    CATATTACAGATCCTGAGAGGTTCAGGTTTGCAAGGGACACAA  .....  GTTGTCAAAGGTGCACCTGTGGTTGAGCCAGGCGATGATCTG
3.1-3-41    CATATTACAGATCCTGAG------AGGTTTGCAAGGGACACAA  .....  GTTGTCAAAGGTGCACCTGTGGTTGA--CAGGCGATGATCTG      -6/-2     Homo
15.1-6-4    CATATTACAGATCCT--GAGGTTCAGGTTTGCAAGGGACACAA  .....  GTTGTCAAAGGTGCACCTGTGGTTG-GCCAGGCGATGATCTG      -2/-1     Homo

GmMLO20                    (      Target 1      )      1196bp      (      Target 2      )              Δ      Genotype
MLO20-WT    CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA  .....  GTCGTCAAGGGTGCACCTGTGGTTGAGCCAGGAGATGGATTG
3.1-3-41    CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA  .....  GTCGTCAAGGGTGCACCT--------GCCAGGAGATGGATTG      0/-8      Homo
15.1-6-4    CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA  .....  GTCGTCAAGGGTGCACCTGTGGTTGAGCCAGGAGATGGATTG      0/0       WT

GmMLO23                    (      Target 1      )      1353bp      (      Target 2      )              Δ      Genotype
MLO23-WT    CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA  .....  ATTCAAGGGTGCACCTGTGGTTGAGCCAGGAGATGACCTG
3.1-3-41    CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA  .....  ATTCAAGGGTGCACCTGTGGTTGA-CCAGGAGATGACCTG      0/-1      Homo
15.1-6-4    CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA 967bp  TAACAGA----------------------CAACCGTCCACGC     0/-428    Homo
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Figure 2. Inheritance of induced mutations in *GmMLO02*, *GmMLO19*, *GmMLO20* and *GmMLO23* genes in the T4 generation of mutant lines. Target sequences and PAMs are indicated in red and blue color, respectively. Each target sequence contains 20 nucleotides and is on the exon region of four studied genes. Inserted nucleotides are shown in yellow. Δ indicates targeted sequence changes: no change (0), deletion (-), insertion (+). Homo: homozygous genotype.

Phenotyping and yield performance of *GmMLO* soybean mutant lines

The morphological and agronomic traits of the two homozygous mutant lines were first evaluated in the T3 generation (Bui *et al.*, 2023). Accordingly, there was no statistically significant difference in phenotype among the studied lines, with stem length ranging around 56 cm, 12 internodes in each line, and seed weight ranging from 18-20 grams. In this study, we wanted to examine the above traits in the T4 generation to see if there were any changes. The results showed no statistically significant difference in the stem length among the tested lines (Figure 3A). Specifically, the mutant and wild-type plants had similar stem length, which fluctuated around 59 cm. Meanwhile, there was a significant increase in the number of internodes in the two mutant lines compared to the wild-type (Figure 3B).

Agronomical traits related to yield were also considered and evaluated. The seed weight and the total pod had small variation between the wild-type and the mutant lines, but once again, these differences were not statistically significant (Figure 3C, D). In addition, line 3.1-3-41 had the highest percentage of pods with 3 and 4 seeds compared to the other lines (Figure 3E). Moreover, it was observed that all the phenotyping data obtained in the T4 generation were slightly higher than those in the T3 generation. However, the important thing is that in both growing seasons, the two mutant lines, especially the quadruple mutant one (3.1-3-41), had similar agronomic traits to the wild-type line, or even slightly better.

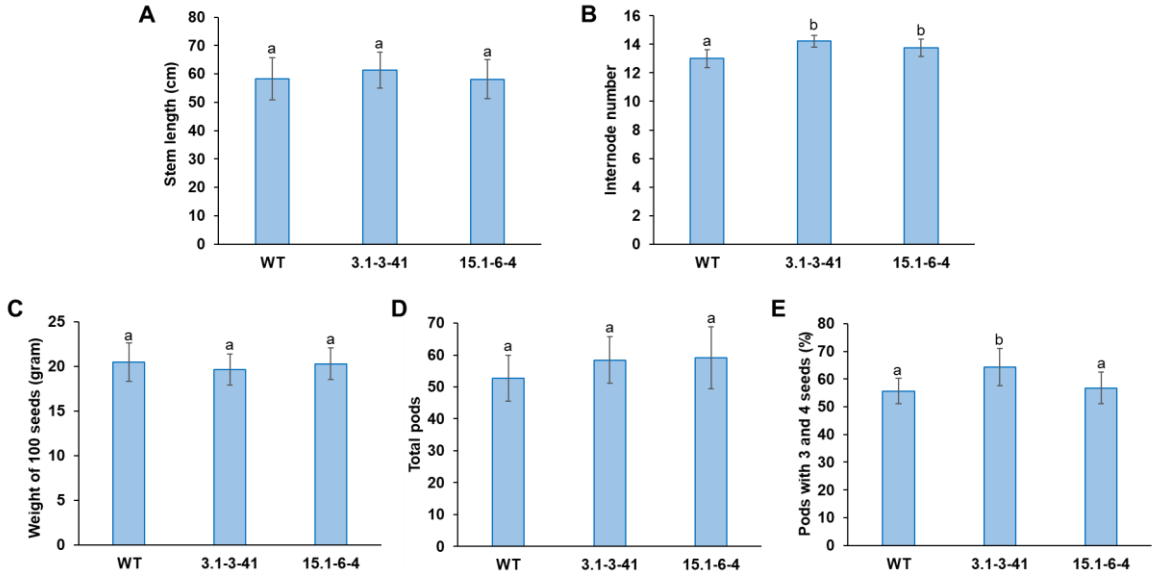

Figure 3. Growth and yield performance of *GmMLO* soybean mutant lines under the net-house conditions. A: Stem length (cm); B: Internode number; C: Weight of 100 seeds (gram); D: Total pods; E: The percentage of pods with 3 and 4 seeds. WT: wild-type plant; 3.1-3-41, 15.1-6-4: T4 *GmMLO* soybean mutant lines. Mean value ± SD for n = 12-17 are shown. Statistical analysis was done using one-way ANOVA followed by a post hoc Turkey's test. A significant difference was considered at $P < 0.05$.

MLO genes are S-genes in plants and involve in many physiological processes of plants (Acevedo-Garcia *et al.,* 2014). Loss-of-function mutations of the *MLO* genes could help enhance resistance to powdery mildew, but could also be accompanied by undesirable phenotypes, such as senescence-like chlorosis and necrosis in grapevine (Wan *et al.,* 2020), slower growth and development in *A. thaliana* (Consonni *et al.,* 2006) and reduced plant size in pepper (Zheng *et al.,* 2013). In our study, synthesizing data in the T3 and T4 generations, it could be confirmed that the targeted mutations of the *GmMLO* genes did not cause negative effects on the morphology, growth and development of soybean plants under net-house conditions.

Nutritional composition of the seeds of the *GmMLO* soybean edited lines

Soybean seeds are important protein and oil sources that are essential for human health. Therefore, in addition to agronomic paremeters, we also considered and evaluated the nutritional composition of the seeds, including FAs, FAAs and total protein to see whether induced mutations of the *GmMLO* genes would affect these factors.

Fatty acid composition

Fatty acids in soybean oil were transformed into FAMEs as determined by GC-MS. Four major FAs had been found in mutant soybean seeds including palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) (Figure 4). The absence of linolenic acid (18:3) in this analysis may be due to the less sensitivity of our system. The two unsaturated FAs (oleic acid and linoleic acid) were the most prominent component of the total seed FAs, which accounted for more than 65% in comparison with the two saturated FAs (palmitic acid and stearic acid) in both the wild-type and mutant lines. Moreover, there was a slight increase in the oleic acid (18:1) and a decrease in the linoleic acid (18:2) content in *GmMLO* mutant soybean seeds as compared to the wild-type. The increased amount of monosaturated FA, especially oleic acid (18:1), in the mutant soybean lines indicates that the oil's oxidative stability and shelf-life have been enhanced (Goffman *et al.,* 2003). Because oleic (18:1) and linoleic acid (18:2) concentrations were inversely connected, the linoleic acid (18:2) content in the *GmGOLS* mutant lines was decreased, as seen in Figure 4. Linoleic acid (18:2) is reduced as desired because it has lower oxidation stability than oleic acid (Fehr, 2007). However, the minor changes in FA contents had no significant difference between soybean mutant and wild-type control seeds ($P > 0.05$).

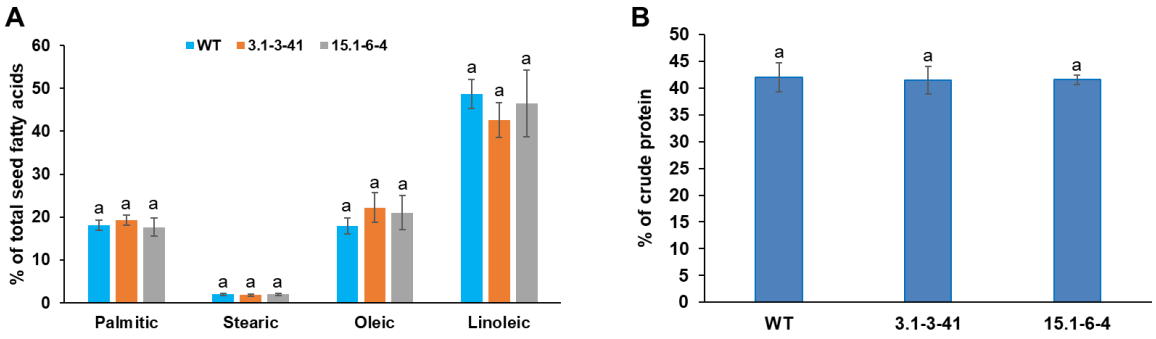

Figure 4. The percentage of total seed fatty acids (A) and the percentage of crude protein (B) of soybean seeds from the wild-type and T4 *GmGOLS* mutant lines. Mean value ± SD for n = 5-6 are shown. Statistical analysis was done using one-way ANOVA followed by a post hoc Turkey's test ($P < 0.05$).

Free amino acid composition

The individual amino acid content and total FAA detected from the seeds of two mutant soybean lines and the wild-type were shown in table 1. The FAAs in relatively high concentrations in all three tested lines were proline, serine, glutamic acid, and arginine. Among them, proline was the most abundant FAA. In which, the 3.1-3-4 line had the greatest mean concentration (1,331.82 mg/100 g), whereas the wild-type had the lowest one (1,181.15 mg/100 g). In addition, histidine, alanine, arginine, glycine, proline and serine contents rose slightly in the *GmMLO* mutant seeds, while other FAA contents observed small decreases in the seeds of two *GmMLO* mutant lines as compared to the wild-type. However, all of these changes had no significant differences, except the content of cysteine. Importantly, we also found no significant change in the total FAA among the two mutant and the wild-type soybean seeds ($P > 0.05$).

Table 1. The free amino acid content in seeds of *GmMLO* soybean edited lines and the control.

No	AA (mg/100g)	WT	3.1-3-41	15.1-6-4
1	Histidine	182.46 ± 47.91^a	228.66 ± 35.70^a	186.55 ± 34.12^a
2	Isoleucine	171.72 ± 40.00^a	135.14 ± 21.72^a	144.54 ± 31.42^a
3	Methionine	88.54 ± 15.47^a	69.25 ± 13.99^a	75.44 ± 18.72^a
4	Phenylalanine	97.96 ± 19.95^a	80.87 ± 15.73^a	88.25 ± 23.74^a
5	Valine	80.09 ± 18.69^a	72.03 ± 15.81^a	73.17 ± 22.10^a
6	Alanine	152.14 ± 44.33^a	167.56 ± 32.02^a	175.09 ± 36.67^a
7	Arginine	556.48 ± 148.08^a	637.17 ± 127.16^a	565.81 ± 61.84^a
8	Cysteine	233.93 ± 39.09^b	190.06 ± 17.54^a	179.62 ± 18.62^a

9	Glutamic acid	549.28 ± 56.34[a]	507.02 ± 27.38[a]	543.39 ± 96.69[a]
10	Glycine	57.08 ± 13.07[a]	66.45 ± 12.97[a]	65.59 ± 14.51[a]
11	Proline	1181.15 ± 203.71[a]	1331.82 ± 121.09[a]	1188.24 ± 277.80[a]
12	Serine	780.98 ± 166.69[a]	1106.43 ± 182.91[a]	769.68 ± 209.27[a]
13	Tyrosine	83.95 ± 21.49[a]	70.52 ± 24.20[a]	74.40 ± 26.46[a]
	Total FAA	4215.76 ± 615.50[a]	4662.98 ± 336.70[a]	4098.59 ± 601.12[a]

Seed protein content

The crude protein content of soybean seeds was evaluated by measuring the quantity of nitrogen present in the samples. The percentage of crude protein in the seed of WT, 3.1-3-41, and 15.1-6-4 lines was 42.06%, 41.44%, and 41.55%, respectively (Figure 4B). Because protein is composed of amino acids, the protein content of soybean seed is significantly consistent with the number of free amino acids available (Wang *et al.*, 2019). In line with the total free amino acid content, we found no significant change in the protein content of mutant soybean seeds as compared to the wild-type control.

As mentioned above, the application of the CRISPR/Cas9 system in inducing mutations of *MLO* genes might accompany with some undesirable traits, which are mainly related to morphology and yield. However, there has been no publication on the effects of loss-of-function mutations of *MLO* genes on fruit and seed quality of the mutants. The most important thing was that, in this study, we did not observe statistically significant differences in the seed nutritional composition of the mutant lines compared to the wild-type variety. This proved that inducing mutations of the *GmMLO* genes using the CRISPR/Cas9 system did not result in undesirable seed quality traits in soybean.

Powdery mildew resistance analysis

The resistance of the T4 mutant lines to powdery mildew was also further assessed under net-house conditions. Using a symptom scale of 0 to 5 corresponding to complete resistance to severe infection, we obtained the evaluation results of the experimental soybean lines (Figure 5A). The score recorded for line 3.1-3-41 was 2.5, corresponding to moderate resistance. Meanwhile, this score significantly increased up to 3.8 for the wild-type and 3.5 for line 15.1-6-4, which is equal to moderate infection. The differences in response to *E. diffusa* infection among tested lines could be observed in figure 5B with representative leaves for each leaf layer. After 24 days of infection, the fungus causing powdery mildew spread and developed to V6 leaf in line 3.1-3-41 with milder disease symptoms, while in the two other lines, this fungus spread to V7 leaf with more severe disease symptoms, especially in the WT. Therefore, the quadruple mutant line 3.1-3-41 showed the highest resistance to powdery mildew infection.

E. diffusa challenge was also performed in the T3 generation under net-house conditions. The infection scores recorded in three lines 3.1-3-41, 15.1-6-4 and WT were 2.6, 4.6, and 4.7, respectively (Bui *et al.*, 2023). In comparison with the infection

results in the T3 generation, although the recorded scores changed due to the influence of weather conditions, which was increased in temperature and decreased in humidity in the second testing year, the trend in response to powdery mildew of the two mutant lines did not change. Thus, powdery mildew resistance was maintained stably through the generations of tested soybean cultivars.

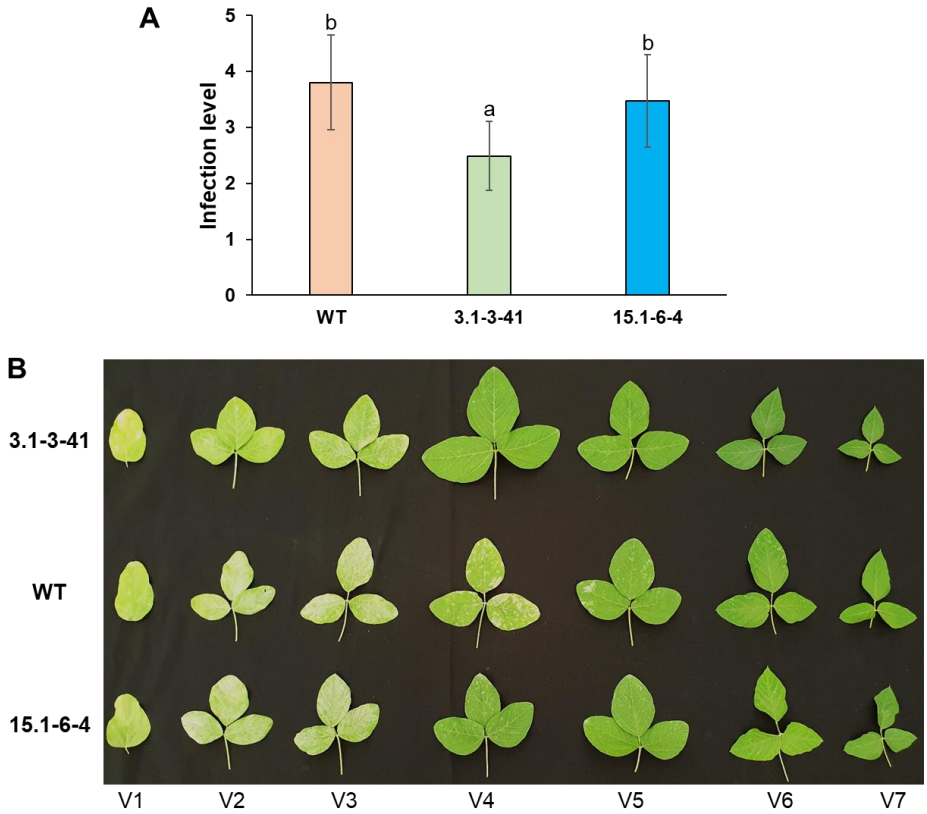

Figure 5. *E. diffusa* susceptibility assessment at the net-house conditions. A: Infection level of two *GmMLO* mutant lines compared to wild-type plants. Mean values ± SD for n = 15 are shown. Statistical analysis was done using a one-way ANOVA followed by a post hoc Turkey's test ($P < 0.05$). B: The representative leaves were collected 24 days after inoculation. WT: wild-type plant; 3.1-3-41, 15.1-6-4: T4 *GmMLO* soybean mutant lines; V1-V7: different vegetative growth stages of soybean.

In addition, line 3.1-3-41 carrying simultaneous mutations of four genes (*GmMLO02*, *GmMLO19*, *GmMLO20* and *GmMLO23*) expressed the lowest susceptibility to powdery mildew infection. Meanwhile, line 15.1-6-4 carrying triple mutations (except *GmMLO20* gene) showed a similar susceptibility level to the wild-type when being infected for a long time. This might indicate an important role of *GmMLO20* gene in the response to powdery mildew, however, further analysis should be performed to clarify this contribution.

CONCLUSION

This study indicated that the nutritional compositions of seeds were not significantly different among the mutant lines and the wild-type. In addition, the morphological

traits, yield and powdery mildew resistance were maintained stably through generations of the *GmMLO* soybean mutant lines, of which the quadruple mutant line 3.1-3-41 still showed better performance as compared to the triple one 15.1-6-4. This soybean mutant line will be potential material for further soybean breeding programs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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