Article

Candidate Gene, *SmCPR1*, Encoding CPR1 Related to Plant Height of the Eggplant Dwarf Mutant *dwf*

Yang Lu ^{1,2,†}, Shuangxia Luo ^{1,†}, Na Li ¹, Qiang Li ¹, Wenchao Du ¹, Weiwei Zhang ³, Ping Yu ¹, Shuxin Xuan ¹, Yanhua Wang ¹, Jianjun Zhao ¹, Xueping Chen ^{1,*} and Shuxing Shen ^{1,*}

- ¹ Key Laboratory for Vegetable Germplasm Enhancement and Utilization of Hebei, Collaborative Innovation Center of Vegetable Industry in Hebei, College of Horticulture, Hebei Agricultural University, Baoding 071000, China; luyangmm@cdmc.edu.cn (Y.L.); yylsx@hebau.edu.cn (S.L.); yyln@hebau.edu.cn (N.L.); lqsdau@163.com (Q.L.); dwchge@163.com (W.D.); yp18233221787@163.com (P.Y.); yyxsx@hebau.edu.cn (S.X.); yywyh@hebau.edu.cn (Y.W.); jjz1971@aliyun.com (J.Z.)
- ² Hebei University R&D Center for Sericulture and Specialty Enabling Technologies, Institute of Sericulture, Chengde Medical University, Chengde 067000, China
- ³ College of Life Sciences, Hebei Agricultural University, Baoding 071000, China; shmzww@hebau.edu.cn
- * Correspondence: chenxueping@hebau.edu.cn (X.C.); shensx@hebau.edu.cn (S.S.)
- † These authors contributed equally to this work.

Abstract: Eggplant is a vegetable crop with high economic value that is cultivated worldwide. The dwarf mutant is an important germplasm material that has been extensively used in crop breeding. However, no eggplant dwarf mutants have been reported, and little is known regarding the genes responsible for dwarfism in eggplant. In this study, we isolated an eggplant dwarf mutant (*dwf*) from an ethyl methyl sulfonate (EMS)-induced mutant library. Genetic analysis revealed that *dwf* was caused by a single recessive gene. A candidate gene *SmCPR1*, encoding cytochrome P450 reductases (CPR1), was identified by bulked segregant analysis (BSA). Mutation from G to A at 8216 bp of *SmCPR1* resulted in mutation of the amino acid from valine to isoleucine. The results of KASP and Sanger sequencing further support the conclusion that *SmCPR1* is a candidate gene responsible for the dwarfism of *dwf*. Moreover, the activity of *SmCPR1* was significantly increased in *dwf*, which might be a response to dwarfism in *dwf*.

Keywords: eggplant; dwarf mutant; BSA; *SmCPR1*

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1. Introduction

Eggplant (*Solanum melongena* L.) is a vegetable crop that is cultivated worldwide, and China is the largest eggplant producer in the world (<http://www.fao.org/faostat/en/>, 10 December 2020). Plant height of eggplant affects the planting density and fruit coloring especially under protected cultivation. Larger plants increase the cost of labor as well as water and fertilizer inputs in production management. Dwarfism is a desirable trait in eggplant breeding that confers higher lodging resistance and allows simplified management and harvest [1–3].

Dwarf mutants have been used as important germplasms in crop breeding since the Green Revolution. The cultivation of dwarf cultivars has been successfully applied in wheat [4,5], maize [6,7], rice [8,9], and other crops [10,11]. However, the use of dwarf germplasms for eggplant breeding has largely been lagging. Therefore, there is a great need to create or discover dwarf materials in eggplant.

Here, we isolated a dwarf mutant, *dwf*, from an ethyl methyl sulfonate (EMS) mutant library in inbred lines ‘14–345’. Genetic analysis showed that the dwarfism of *dwf* was controlled by a signal recessive gene. We discovered by Mutmap and KASP that the mutation responsible for the dwarfism might be a non-synonymous base pair change in *SmCPR1*. As compared with wild type, *dwf* had significantly increased NADPH-cytochrome P450 reductase (CPR1) activity.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The dwarf mutant *dwf* was isolated from a mutant library generated by ethyl methane sulfonate (EMS) mutagenesis from the inbred line ‘14–345’. The plants were grown in the greenhouse of Hebei Agricultural University.

2.2. Genetic Analysis

The *dwf* mutant was crossed with ‘14–345’ reciprocally. F₁ plants were self-pollinated to generate F₂ populations for genetic analysis. The numbers of dwarf plants and plants with normal plant height were counted separately. The chi-square test was used for genetic analysis with SPSS.

2.3. Candidate Gene Identification Using BSA-Seq

The bulked segregation analysis (BSA) strategy was employed for quick identification of molecular markers linked with the dwarf phenotype. Young leaves of WT and *dwf* were sampled for DNA extraction using the CTAB-based method. Equal amounts of DNA from 30 dwarf and 30 wild-type plants were bulked to generate the dwarf bulk (aa) and normal bulk (AA or Aa). Paired-end sequencing was performed on an Illumina HiSeq™ PE150. High-quality clean reads were obtained from raw reads by removing the data ① with $\geq 10\%$ unidentified nucleotides (N), ② with $>50\%$ bases having Phred quality <5 , ③ with >10 nt aligned to the adapter, allowing $\leq 10\%$ mismatches, and ④ putative PCR duplicates generated by PCR amplification during library construction. Then, the clean data were aligned to the reference genome (<https://www.ncbi.nlm.nih.gov/genome/?term=Solanum+melongena>, 28 August 2018) by (Burrows–Wheeler Aligner (BWA) [12]. The alignment files were converted to BAM files using SAMtools, GATK3.8, and ANNOVAR [13–15] and were used for SNP/InDel detection and annotation. We used the wild type as the reference and to determine the read number for the parent’s genotype or the dwarf genotype in the offspring pool. Then, the ratio of the number of different reads to the total number was calculated, which is the SNP/InDel index of the base sites. The average of all SNP/InDel indices in each window was used as the SNP/InDel index for this window. Usually, we applied a window size of 1 Mb and a step size of 1 kb as default settings to reflect the distribution of the SNP index. The difference in the SNP/InDel index of the two pools was calculated as the Δ (SNP/InDel index). At the 95% confidence level, Δ (SNP/InDel index) > 0.5 was used as the condition, and the genes that caused stop/loss, stop/gain, nonsynonymous mutation, or variable splicing were selected as candidate genes. The library preparation and sequencing were conducted by the Novogene Technology Company in Beijing, China.

2.4. Verification of the Candidate SNP Genotype

Sixty-four M₃ plants, 118 F₂ plants, 10 F₁ plants, three *dwf*, and three WT were used in the genotypic assay by Kompetitive Allele-Specific PCR (KASP). Specific primers flanking the candidate SNPs were designed and used for confirmation of the candidate SNPs by Sanger sequencing. A fragment of 601 bp including the candidate SNP in the middle was used to perform PCR amplification (forward primer 5′→3′ AGAGAGATAAAGGATCAATGGAGTC; reverse primer 5′→3′ CTCTCCACTGTCTCACCTACTACC). The PCR product was purified using a kit (Sangon B518141) and sequenced at 3730XL (Life Technologies, South San Francisco, CA, USA). The data were analyzed using sequence analysis software and mapped using SeqMan software 12.2.

2.5. Validation of Selected Genes Using Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

RNA from the leaves and stems of 4-leaf seedlings was analyzed by quantitative real-time RT-PCR. First-strand cDNA was synthesized using RevertAid Premium Reverse Transcriptase (Thermo Scientific™ EP0733, Waltham, MA, USA) according to the manufac-

turer's instructions. *GAPDH* (GenBank JX448342.1) was used as the internal control [16], and primer sequences for the genes analyzed are listed in Table 1. qRT-PCRs were run in a Lightcycler 96 Real-time qPCR detection system (Roche, Indianapolis, IN, USA) with three biological replicates for each type of sample. The program used was as follows: 5 min at 95 °C, 40 cycles of 95 °C (10 s), 58 °C (10 s), and 72 °C (30 s). The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative expression levels of the target genes [17].

Table 1. The primer sequences for qRT-PCR.

Gene ID	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
<i>GAPDH</i>	GTACGACAACGAATGGGGTTA	TCATATCAGCAGCACCAGCA
<i>SmCPR1</i>	CGAGTGGCCCAATCAACAGAT	CCGTCTCTCTCTCTCCAAAACCG
<i>CPR2</i>	GAAAGAACCACTATGCGTATAAACATC	CACCGTGTGTTGTTGTTGTG

2.6. NADPH-Cytochrome P450 Reductase Activity Assay

The activity of NADPH-cytochrome P450 reductase (CPR) in stems was assayed using a Plant CPR ELISA kit (Yuanmu, Shanghai). The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm using a spectrophotometer. To measure the concentration of CPR in the sample, the CPR ELISA Kit includes a set of calibration standards. The calibration standards were assayed at the same time as the samples and allowed the operator to produce a standard curve of optical density versus CPR concentration. Then, the concentration of CPR in the samples was determined by comparing the O.D. of the samples to the standard curve.

2.7. Statistical Analysis

Statistical analyses were performed using Student's *t*-test (SPSS 21.0) [18]. Summary statistics are presented as the mean $\pm$ standard deviation (SD). Asterisks denote significant differences (* $p < 0.05$ and ** $p < 0.01$).

3. Results

3.1. Inheritance of the Dwarf Phenotype in *dwf*

The *dwf* mutant, which showed a significantly dwarfed phenotype due to shortened internodes, was isolated from M₂ populations derived from an EMS-mutagenized eggplant '14-345' (WT) population [19]. To determine the inheritance pattern of dwarfism in *dwfs*, F₁ plants that displayed similar plant heights to WT were obtained from crosses of *dwfs* with WT (Figure 1). The dwarf phenotype was segregated in the F₂ populations. There were 43 plants with a dwarf phenotype and 123 plants with plant height similar or close to that of WT in the F₂ population, which fit a 3:1 segregation ratio by the chi-square test ($\chi^2 < \chi^2_{0.05} = 3.84$) (Table 2). These results suggested that the dwarf phenotype of *dwf* was controlled by a single nuclear recessive gene.

Table 2. The segregation ratio of F₂.

Generation	WT Phenotype	Dwarf Phenotype	Theoretical Segregation Ratio	χ^2
F ₂	123	43	2.86:1	0.823

Figure 1. Comparisons of plant height and width of *dwf*, WT, and F₁ plants. ** Significantly different at $p = 0.01$ by the t test. (A) The plants of *dwf* and WT; (B) The plant height of the *dwf*, WT and F₁; (C) The plant width of the *dwf*, WT and F₁.

3.2. Identification of a Candidate Gene by BSA

BSA-seq was used to identify the candidate gene for *dwf*. Two bulk DNA samples, dwarf bulk (M-bulk) and normal bulk (W-bulk), were constructed by mixing equal amounts of DNAs from 30 dwarf plants and 30 normal plants from the M₃ population, respectively. We obtained clean reads of 26.56 Gb (99.87% coverage) and 26.20 Gb (99.89% coverage) corresponding to the W-bulk and M-bulk, respectively; 97.71% and 96.74% of the reads from W-bulk and M-bulk were aligned to the reference genome (<https://www.ncbi.nlm.nih.gov/genome/?term=Solanum+melongena>, 28 August 2018). A total of 3077032 SNPs were identified between the M-bulk and W-bulk. $\Delta(\text{SNP-index}) > 0.5$ was used as the threshold at the 95% confidence level, 13 SNPs including six nonsynonymous SNPs, a stop gain SNP, a splicing SNP, and seven upstream SNPs were obtained (Table 3).

Table 3. Candidate SNP sites.

Gene ID	Variant	Scaffold	Position	Reference	Alter
<i>Sme2.5_01158.1_g00001.1</i>	Nonsynonymous	<i>Sme2.5_01158.1</i>	8216	G	A
<i>Sme2.5_00670.1_g00001.1</i>	Nonsynonymous	<i>Sme2.5_00670.1</i>	1811	C	A
<i>Sme2.5_06691.1_g00001.1</i>	Nonsynonymous	<i>Sme2.5_06691.1</i>	1988	T	C
<i>Sme2.5_11667.1_g00003.1</i>	Nonsynonymous	<i>Sme2.5_11667.1</i>	28,891	C	T
<i>Sme2.5_16807.1_g00001.1</i>	Nonsynonymous	<i>Sme2.5_16807.1</i>	1476	C	T
<i>Sme2.5_19952.1_g00001.1</i>	Nonsynonymous	<i>Sme2.5_19952.1</i>	2943	G	T
<i>Sme2.5_21615.1_g00001.1</i>	Stop gain	<i>Sme2.5_21615.1</i>	2478	C	T
<i>Sme2.5_08332.1_g00001.1</i>	Splicing	<i>Sme2.5_08332.1</i>	3974	C	T
<i>Sme2.5_01689.1_g00001.1</i>	Upstream	<i>Sme2.5_01689.1</i>	4262	A	T
<i>Sme2.5_01689.1_g00001.1</i>	Upstream	<i>Sme2.5_01689.1</i>	4265	C	G
<i>Sme2.5_06855.1_g00002.1</i>	Upstream	<i>Sme2.5_06855.1</i>	10,623	C	T
<i>Sme2.5_19942.1_g00002.1</i>	Upstream	<i>Sme2.5_19942.1</i>	11,896	T	C
<i>Sme2.5_22728.1_g00001.1</i>	Upstream	<i>Sme2.5_22728.1</i>	4016	G	A

To further confirm the candidate SNP responsible for the dwarf phenotype, the 13 SNPs were used in KASP to design a specific primer for genotyping analysis of the 64 M₃ plants, 118 F₂ plants, 10 F₁ plants, three *dwf*, and three WT. The genotypic assay revealed that SNP 8216 of *Sme2.5_01158.1* cosegregated with the phenotype in the M₃, F₂, F₁ populations and parents. The dwarf plants had a A:A genotype, and the plants with a wild phenotype had a G:G or G:A genotype (Figure 2). Then, the candidate SNP *Sme2.5_01158.1_g00001.1* was used to design a specific primer for genotyping analysis from

ten F₂ individuals by Sanger sequencing. The results showed that the SNP genotype of the dwarf phenotype was A:A, and the genotype of WT was G:A and G:G (Figure 3). Therefore, we confirmed that *Sme2.5_01158.1_g00001.1* was a candidate gene related to dwarfism.

Figure 2. Verification of *Sme2.5_01158.1_g00001.1* by KASP, the four charts represent four plates respectively. Red points indicate genotype A:A, green points indicate A:G, and blue points indicate G:G: (A) 10F₁ plants with the genotype A:G, 20 dwarf plants with genotype A:A of the F₂ population; (B) 4 dwarf plants with the genotype A:A of the F₂ population and 90 wild plants with the genotype A:G and G:G of the F₂ population; (C) 3 WT plants, 3 *dwf* plants, 14 dwarf plants with the genotype A:A of the M₃ population; (D) 16 dwarf plants with the genotype A:A of the M₃ population, 21 wild plants with A:G and 13 with G:G of the M₃ population, and 4 A:G of the F₂ population.

Figure 3. Sanger sequencing revealed the genotype of the *Sme2.5_01158.1_g00001.1* mutation site. The top figure shows the dwarf phenotype in which the genotype was A:A. The middle and bottom figures show the wild-type phenotype, and the genotypes were G:G and A:G.

Sme2.5_01158.1_g00001.1, locating at 12663065–12667066 on chromosome 4 (Figure 4A), contains open reading frame of 3912 bp and encoding NADPH-cytochrome P450 reductase 1 (CPR1) of 1154 amino acids. Thus, we named *Sme2.5_01158.1_g00001.1* as *Sm-CPR1*. In order to understand the effect of base mutation on protein, we used ORF finder (<http://www.ncbi.nlm.nih.gov/orffinder/>, 15 January 2020) to predict domain and MAFFT software to do the amino acid sequence homology alignment. We found the mutation that carried a G to A transition at nucleotide 8216 (Figure 4B) led to a transition at codon 77, resulting in replacement of a valine (Val) with isoleucine (Ile), and the substitution in the fourth site in front of the flavodoxin domain (Figure 4C). These results suggest that the

mutation in *SmCPR1* may cause the dwarf phenotype of mutant *dwf*. Additionally, we found that the amino acid mutation was located in 4 amino acids upstream of flavodoxin domain, which is a conserved domain of NADPH-cytochrome P450 reductase.

Figure 4. Location and structure of the candidate gene *SmCPR1* and its amino acid sequence: (A) *SmCPR1* is located on chromosome 4, and the red frame indicates *SmCPR1*; (B) structure and mutation site of *SmCPR1*, black rectangles indicate exons and gray lines indicate introns, the mutation occurred in the first exon and the mutation base was from G to A; (C) the black line indicates the amino sequence, and the red, yellow, and green rectangles indicate the conserved domain database. FAD, flavin adenine dinucleotide. NAD, nicotinamide adenine dinucleotide.

3.3. Effect of *SmCPR* Mutation on Enzyme Activity

CPRs play a vital role in CYP metabolism in plants. Two CPR genes, *SmCPR1* and *SmCPR2*, were identified in eggplant genome. In order to confirm the physiological function of *SmCPR1*, the relative transcription level of *SmCPR1* and NADPH-cytochrome P450 reductase (CPR) activity were studied in stems of WT and *dwf*. As shown in Figure 5A, *SmCPR2* showed very low expression in both leaves and stems. Although *SmCPR1* was barely expressed in leaves, the expression of *SmCPR1* was highly expressed in stems. The results indicated the important role of *SmCPR1* in regulating the development of stem, which is the main factor determining plant height. The CPR activity was analyzed using CPR ELISA kit. CPR activity was significantly increased in the stems of *dwf* as compared with that of WT (Figure 5B), indicating the negative roles of *SmCPR1* in regulating plant height in eggplant.

Figure 5. Relative expression and enzyme activity: (A) Expression pattern of *SmCPR1* and *SmCPR2* in leaves and stems; (B) CPR enzyme activity assayed in the stems of WT and *dwf*.

4. Discussion

Plant height is an important agronomic trait for crops and is highly associated with agricultural performance. Dwarfism or semi-dwarfism is widely used in plant breeding and is easier to manage and harvest, with considerable cost savings [20,21]. EMS mutagenesis is one of the effective methods to establish a mutant library; the advantages are inducing point mutation and easy to be stably inherited [22–24]. In this study, we established a mutant library by EMS with various phenotypes and isolated a dwarf mutant *dwf* from the mutant library. The F₁ plants which were obtained from the crosses of *dwf* with WT displayed similar plant height with WT, and the F₂ populations showed segregated phenotype and the ratio of wild type and dwarf type fit for 3:1, verifying that the dwarf phenotype was controlled by a single nuclear recessive gene.

BSA is a rapid method used to detect molecular markers associated with target traits in mapping populations [25]. In our study, we isolated the *SmCPR1* gene as a candidate gene that regulate plant growth by BSA and verified this conclusion by KASP. The gene *SmCPR1* carried the mutation at 8216 bp from G to A and resulted in the 77th mutage of Val to Ile, encodes a member of NADPH- cytochrome P450 reductase (CPR), CPR1. In Arabidopsis mutant *cpr1*, CPR1 related to dwarfism has been reported [26].

CPR contains cofactors and can transfer electrons to NAD⁺ or NADP⁺ to diverse cytochrome P450 monooxygenases [27,28] which participate in substance metabolism [29] associated with dwarfism such as endogenous hormone [30,31] and phenylpropanes [32]. We have reported that the GA₃ level, internode length of stems, and the internode cell length of *dwf* were significantly decreased as compared with those of the WT, and confirmed that the dwarfism of *dwf* was associated with gibberellin (GA₃) [19]. The *SmCPR1* played a negative role in regulating the plant height in *dwf*, it might participate in the process of impeding the GA₃ biosynthesis. Further study is needed to provide the direct evidence.

In this study, the expression of the mutant gene *SmCPR1* was not changed, but the enzyme activity of CPR1 was significantly increased in *dwf*. We inferred that the mutant location of the amino acid was very close to the conserved domain, which might alter protein conformation and further affect enzyme activity.

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