



Article Phenotypic Characterization and Differential Gene Expression Analysis Reveal That Dwarf Mutant dwf Dwarfism Is Associated with Gibberellin in Eggplant

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Abstract: Dwarfism is a desirable trait in eggplant breeding, as it confers higher lodging resistance and allows simplified management and harvest. However, a few dwarf mutants have been reported, and the molecular mechanism underlying dwarfism in eggplant is completely unknown. Here, we report a dwarf mutant (dwf) isolated from an ethyl methyl sulfonate (EMS)-induced mutant library. The hypocotyl length, plant height, and length of internode cells of *dwf* were significantly decreased compared to those of the wild-type parent '14-345' (WT). Differential gene expression analysis revealed that GA-related genes, including GA2ox and DELLA, were up-regulated whereas the gibberellin (GA₃) content decreased in *dwf*. Moreover, exogenous GA₃ treatment significantly increased the relative growth rate of dwf compared to WT, further indicating the important roles of GA in regulating the dwarf phenotype of dwf. Collectively, our findings shed light on GA-mediated dwarfism in *dwf* plants and offer a good germplasm that could be used for eggplant dwarfism breeding in the future.

Keywords: eggplant; dwarfism; plant height; gibberellin; differentially expressed gene

1. Introduction

Eggplant (Solanum melongena L.) is a vegetable crop that is cultivated worldwide and belongs to the genus Solanum in the Solanaceae family. Currently, China is the largest producer of eggplant, with 39.2 million tons and 783,000 ha in 2019 [1]. The vast majority of eggplant varieties have an indeterminate in growth habit and are not suitable for planting at high density. Moreover, the production of indeterminate eggplants is labor intensive.

Dwarfism is an important agronomic trait for crops and is highly associated with agricultural performance, such as higher lodging resistance, yield and harvest index [2–4]. Dwarfism or semidwarfism is widely used in plant breeding, such as wheat, rice, and woody species [3–5]. The determinate or dwarf varieties make easier management and harvest, with considerable cost savings [6,7]. Therefore, dwarfism is also an important breeding trait in eggplant.

Dwarfism is tightly regulated by plant hormones, among which gibberellin is a crucial signaling molecule involved in dwarfism that has been extensively studied in plants [8–11]. Genes involved in GA biosynthesis and degradation, including ent-kaurene oxidase (KO) [12],



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GA 20-oxidase (*GA*200x) [13], *GA* 3-oxidase (*GA*30x) [14,15] and *GA* 2-oxidase (*GA*20x) [16], play important roles in determining dwarfism by regulating endogenous bioactive GA contents. One remarkable example is the loss-of-function of *GA*200x-2 leading to the rice 'Green Revolution' [13]. GIBBERELLIN INSENSITIVE DWARF1 (GID1), which acts as the GA receptor in the signaling pathway, has been demonstrated to control the dwarf phenotype in wheat [17], peach [18] and rice [19]. DELLA is a negative regulator that controls repressors of GA signaling [20,21]. Previous studies showed that if DELLA combines with GAs and GID1 to form a DELLA-GA-GID1 complex, the inhibition of DELLA can be eliminated, and the GA signal can be transmitted downstream [22]. Mutations of DELLA proteins, which impair GA-promoted protein degradation and plant growth, have been documented for many crops including rice [23], barley [24] and tomato [25].

To date, dwarf and semidwarf mutants with short internode lengths have been widely reported for many crops [26,27]. However, few of the dwarf mutants have been documented for eggplant. The creation of new dwarf germplasms and identification of new dwarf genes that can be used for dwarfism breeding in eggplant is a great need. The completion and improvement of eggplant genome sequencing [28], as well as the rapid development of gene cloning, provides convenience for research on the dwarfing mechanism of eggplant.

In this study, we isolated a dwarf mutant dwf obtained from a mutant library in the inbred line '14-345' treated with EMS. We characterized the dwf by performing the plant height and histological comparisons between dwf and WT. We analyzed RNA-seq data and endogenous levels of indole-3-acetic acid (IAA), brassinosteroid (BR), zeatin riboside (ZR) and GA₃ from young leaves as well as the effects of exogenous GA₃ on plant growth of the dwf and WT. Our results imply a tight relationship between the dwarf phenotype of dwf and GA metabolism in eggplant. The objective of this work is to clarify the dwarfing mechanism of dwf and accelerate dwarf breeding of eggplant.

2. Materials and Methods

2.1. Plant Materials and Growth Condition

The dwarf mutant dwf was isolated from a population generated by EMS mutagenesis from the inbred line '14-345'. The dwf mutant was selected at the M₂ generation. All seedlings of the dwf mutant and WT were grown in the greenhouse of Hebei Agricultural University in the fall of 2018.

2.2. Plant Height Measurement

The plant height of *dwf* and WT was measured at the stage when the first true leaf was fully expanded, as well as 30 and 50 days after transplanting (DAT). Measurement was performed using a ruler. Three biological replicates were conducted, each with five plants per genotype.

2.3. Histological Analysis

Longitudinal sections of stems were analyzed by paraffin sectioning. Briefly, the stem samples between the 3rd and 4th leaves were collected when the fourth true leaf was fully expanded and then fixed in FAA (70% ethyl alcohol: glacial acetic acid: formaldehyde = 16:1:1, v/v/v) for more than 24 h. After dehydration and infiltration, all tissues were embedded in paraffin, and 8 µm sections were prepared (Leica RM2145). The sections were affixed to microscope slides, stained with fast green, and observed under a stereomicroscope (DP74, OLYMPUS). Three sections of one sample and approximately 100 cells were observed in our study.

2.4. Hormone Assay

Approximately 0.5 g of leaves at the six-leaf stage was used to determine the contents of GA₃, IAA, BR and ZR. Sample treatment and hormone measurement were performed using an enzyme-linked immunosorbent assay (ELISA) as described previously [29].

2.5. RNA-seq Analysis

Young leaves of *dwf* and WT were collected, frozen quickly in liquid nitrogen and then stored at -80 °C. Total RNA was extracted using an EASTEP Super Total RNA Kit (Promega, Shanghai, China) according to the manufacturer's instructions, and three biological replicates were designed for each genotype. The quality of RNA was checked by determining the RNA integrity and concentration using RNase-free 1% agarose gel electrophoresis, an RNA Assay Kit in a Qubit2.0 Fluorometer (Life Technologies, South San Francisco, CA, USA) and RNA Nano6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). cDNA library preparation and sequencing were conducted by the Novogene Technology Company in Beijing, China. Sequencing libraries were generated using the NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, lpswich, MA, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample [30,31]. After generating 125 bp/150 bp paired-end reads sequencing on an Illumina Hiseq platform, clean data (clean reads) were obtained by removing reads containing adapter, reads containing poly-N and low quality reads from raw data aligned to the eggplant reference genome [28] using Hisat2 v2.0.5 [30]. The gene expression level was normalized by the fragments per kilobase of exon per million mapped reads (FPKM) value using the feature Counts v1.5.0-p3 [32]. Differential gene expression analysis was performed using the DESeq2 R package (1.16.1) [33]. Genes with adjusted padj < 0.05 and $|\log_2 foldchange| > 1$ were determined to be significantly differentially expressed genes (DEGs). Gene Ontology (GO) enrichment was performed using the cluster Profiler R package [34]. GO terms with corrected *p*-value less than 0.05 were considered significantly enriched.

2.6. Validation of Selected DEGs Using Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

The RNA samples used for RNA-Seq were also used for qRT-PCR analysis. Firststrand cDNA was synthesized using RevertAid Premium Reverse Transcriptase (Thermo ScientificTM EP0733) according to the manufacturer's instructions. *GAPDH* (GenBank: JX448342.1) was used as the internal control [35], and primer sequences for all genes analyzed are listed in Table S1. The qRT-PCRs reactions 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: 5 min at 95 °C; 40 cycles of 95 °C (10 s); 58 °C (10 s); 72 °C (30 s). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of the target genes [36].

2.7. Exogenous GA₃ Treatments

The shoot tips of *dwf* and WT at four-true leaf stage were both sprayed with 100 and 200 mg/L GA₃. The treatments were continued for seven days with one application per day. The plants treated with water were used as control. The measurement of plant height was performed before treatments and at the 2nd day of the last application of GA₃ and water. Increment (plant height after treatment-plant height before treatment) and relative growth rate (Increment/plant height before treatment) were investigated. Three biological replicates were performed to evaluate the plant height, each with five plants per genotype.

2.8. Statistical Analysis

Statistical analyses were performed using Student's *t*-test (SPSS21.0) [37]. All data represent mean \pm SD. Asterisks denote significant differences (* *p* < 0.05; ** *p* < 0.01) as determined by Student's *t*-tests.

3. Results

3.1. Phenotypic Comparison of the dwf Mutant and WT

The dwf plant was found in the M₂ generation, which showed a dwarfism phenotype (Figure 1A). To further characterize the dwf mutant, we compared the plant height of the

dwf mutant and WT at three developmental stages, which were all significantly decreased compared to that of the WT (Figure 1B). Moreover, the plant height difference between *dwf* and WT increased with the development of plants (Figure 1B). In addition, we investigated the cell length of the internode, which was significantly reduced by 18.82% in *dwf* (Figure 1C,D).



Figure 1. Phenotypic analysis of WT and *dwf*. (**A**) Plants of WT and *dwf* at the fruiting stage. (**B**) Plant height of WT and *dwf* at the three developmental stages. I, II and III represent the first true leaf stage, at 30 and 50 DAT. (**C**) Representative longitudinal sections of stems of WT and *dwf*. Scale bars = 100 μ m. (**D**) Cell length comparison of WT and *dwf*. * indicates significant differences, compared with the WT (Student's *t*-test, *p* < 0.05), ** indicates Student's *t*-test, *p* < 0.01.

3.2. Analysis of the Contents of GA₃, IAA, BR, and ZR

Given that phytohormones play essential roles in regulating plant height, we further investigated the contents of IAA, BR, GA₃ and ZR in dwf and WT. A significant reduction in GA₃ content was observed in dwf (Figure 2A), whereas the contents of IAA, BR and ZR did not significantly differ between dwf and WT (Figure 2B–D). These results suggested that GA₃ might be associated with dwarfism in dwf plants.

3.3. DEGs and GO Enrichment Analysis

To further characterize the *dwf* mutant, we performed RNA-seq experiments using total RNA isolated from young leaves of the *dwf* mutant and WT. The samples were analyzed in three independent biological replicates (six RNA-Seq libraries in total). Summary statistics for each of the RNA-Seq libraries are shown in Table 1. A total of 171.12 and 146.31 million raw reads were obtained from the *dwf* mutant and WT, respectively. After filtering and removing adapter sequences, low quality reads, and ribosomal reads, 167.54 and 143.52 million clean reads were obtained for the *dwf* mutant and WT, respectively.

More than 90% of the clean reads were mapped to the reference genome (Table 1). The Pearson correlation coefficient analysis showed high reproducibility between the three replicates, ranging from 0.907 to 0.988 (Table S2).



Figure 2. The contents of GA₃, IAA, BR and ZR in WT and *dwf*. (A) The GA3 content. (B) The IAA content. (C) The BR content. (D) The ZR content. The four hormones are measured in ng/g·FW. * indicates significant differences, compared with the WT (Student's *t*-test, p < 0.05).

Table 1. Summary of read numbers	in,	W1	l and	dw	f
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Sample Name	dwf-1	dwf-2	dwf-3	WT-1	WT-2	WT-3
Raw reads	58,570,416	56,724,560	55,828,514	48,299,032	48,653,906	49,359,746
Clean reads	57,610,860	54,994,770	54,936,474	47,392,084	47,418,858	48,711,482
Mapped reads	52,295,231	49,904,306	49,978,934	42,883,337	42,975,644	44,637,625
Ratio of mapped reads (%)	90.77	90.74	90.98	90.49	90.63	91.64

DEG analysis indicated that there were 2682 DEGs between the *dwf* mutant and WT. Of these, there were 1226 down-regulated and 1456 up-regulated genes in the *dwf* mutant (Figure 3A; Table S3).

To explore the functions of the DEGs, GO enrichment analysis of biological process (BP) was performed. The top twelve up-regulated GO pathways indicated that DEGs were significantly enriched in pathways related to pollen and reproductive (Figure 3B), whereas the top twenty GO pathways indicated that the down-regulated DEGs were enriched for many cellular-related processes, such as 'microtubule-based processes', 'nuclear division', 'cell wall macromolecule catabolic/metabolic process' and 'cell cycle process' (Figure 3C). These results implied that the mutant gene, which is responsible for the dwarfism phenotype of the *dwf* mutant, had a significant impact on the global gene expression profile in eggplant, especially for genes related to cellular processes.

3.4. DEGs in GA Biosynthesis and Signal Transduction Pathways

Considering that the endogenous GA₃ level in *dwf* was significantly decreased compared to that in WT, we examined the DEGs involved in GA biosynthesis and signal transduction pathways. Four DEGs involved in GA biosynthesis and signal transduction pathways were found. Of these, three genes (*Sme2.5_04516.1_g00001.1, Sme2.5_00108.1_g00003.1* and *Sme2.5_01907.1_g00015.1*) encode GA2oxs and one gene (*Sme2.5_00538.1_g00012.1*) encodes the DELLA protein. The function of GA2ox is to decrease the levels of active GAs by catalyzing 2 β -hydroxylation, and DELLA proteins act as negative regulators in GA signal transduction [38]. Interestingly, the four genes were all significantly up-regulated in *dwf*, and the expression of these genes showed similar expression patterns in qRT-PCR (Figure 4). The data suggest that the three genes *GA2ox* and DELLA might play important roles in regulating dwarfism in the *dwf* mutant.



Figure 3. Comparisons and biological process GO enrichment of DEGs between WT and *dwf*. (**A**) Numbers of up-regulated and down-regulated DEGs. (**B**) Up-regulated DEGs enriched in thirteen significant BP pathways. (**C**) Down-regulated DEGs enriched in the top twenty significant BP pathways.



Figure 4. qRT-PCR verified the results of RNA-seq. The gene expression levels were normalized using the relative expression level of the internal control GAPDH, and wild-type expression levels were normalized to 1. The data presented are the means from three biological replicates. * indicates significant differences, compared with the WT (Student's *t*-test, p < 0.05).

3.5. The Effects of Exogenous GA₃ on dwf and WT Plant Growth

Based on the aforementioned findings, we speculated that the plant height of dwf could be restored by exogenous GA₃. Under control conditions, dwf displayed a significantly reduced plant height and increment compared to WT. Interestingly, under both 100 mg/L and 200 mg/L GA₃ treatment, dwf significantly increased the relative growth rate compared to WT, whereas the plant height increment of dwf and WT was not different (Figure 5). These findings indicated that exogenous GA₃ can partially restore the plant height of dwf.



Figure 5. Effect of exogenous GA₃ treatment on plant growth. (**A**) Treatment with water. (**B**) Treatment with 100 mg/L GA₃. (**C**) Treatment with 200 mg/L GA₃. In each figure of Plant height, blue indicates plant height before treatment, and yellow indicates the increment. * indicates significant differences, compared with the WT (Student's *t*-test, p < 0.05).

4. Discussion

Dwarf mutants generally show internode shortening, which is closely correlated with the cell number, cell length and intercellular space of internodes [26,39–41]. In the present study, internode cell length of dwf was significantly decreased compared to WT, which could account for, at least partially, the dwarfism of dwf.

Previous studies showed that GA is one of the most important plant hormones in regulating plant height [42–44]. GA2ox plays a key role in GA homeostasis by converting active GAs to inactive forms; modification (mutation or overexpression) of *GA2ox* can decrease GA levels [45] and lead to a dwarf phenotype in many crops, such as Arabidopsis [46], rice [47] and tomato [48]. In the *dwf* mutant, several genes encoding GA2oxs were up-regulated (the left three of Figure 4) and the content GA₃ decreased significantly in *dwf* compared to WT (Figure 2), indicating that the increased expression of *GA2ox* promoted GA deactivation in *dwf*, leading to a low level of GA₃ which inhibited internode elongation.

GA-related dwarf mutants are divided into two classes based on their phenotypic response to exogenous GA. One class is GA-deficient mutants, in which the GA content is significantly lower than that of the wild type, and the phenotype of this class of mutants can be rescued by exogenous application of GAs [49]. Several GA-deficient mutants have been found in rice [50], cotton [51] and maize [14]. In our study, GA₃ was significantly reduced in the *dwf* compared to WT. Meanwhile, we found that the dwarf phynotype of *dwf* could partially restore by exogenous GA₃ treatment, and that the plant height increment of *dwf* after GA treatment increased significantly. These results indicated that the mutant *dwf* was responsive to GA₃.

The other class of GA dwarf mutants is GA-insensitive mutants, the phenotype of which cannot be rescued by exogenous GAs. This class of mutants is mainly caused by mutations in DELLA proteins that act as suppressors of the GA signaling pathway [52]. Gain-of-function or overexpression of DELLA genes gives rise to dwarfism [53]. Studies have shown that mutations or modifications of genes involved in GA synthesis and signaling pathways can lead to dwarfism or semidwarfism [54,55]. However, the up-regulation

Sme2.5_00538.1_g00012.1 gene encoding DELLA in *dwf* seemed to provide evidence that *dwf* is a GA-insensitive mutant. When the GA level decreased, DELLA will bound the transcription factor (TF) and maintained inhibition, which affected stem growth [56,57], proving the phenomenon that occurred in *dwf* plants.

As discussed above, *dwf* is a GA-related dwarf mutant, and the genes *Sme*2.5_04516.1_ g00001.1, *Sme*2.5_00108.1_g00003.1, *Sme*2.5_01907.1_g00015.1 encode GA2oxs and *Sme*2.5_ 00538.1_g00012.1 encodes DELLA were mainly responsible for dwarfism. The mutant *dwf* which may offer a good germplasm that could be used for eggplant dwarfism breeding in future.

5. Conclusions

In this study, we isolated a dwarf mutant (dwf), the hypocotyl length, plant height, and length of internode cells of dwf were significantly decreased compared to those of the WT. The GA-related genes, including *GA2ox* and *DELLA* were up-regulated expression whereas the GA₃ content decreased in the dwf. We treated the seedlings of the WT and dwf with exogenous GA₃, and found that the relative growth rate of dwf significantly increased compared to that of WT, indicated that the dwf was a GA-mediated mutant. Our findings offer a good germplasm that could be used for eggplant dwarfism breeding in the future.

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