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Expression Vector Construction and Genetic Transformation of *Paeonia lactiflora* Gibberellin 20-Oxidase Gene

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Abstract

GA20-oxidase (*GA*20*ox*) gene encodes a key enzyme in gibberellins (GAs) biosynthesis pathway. Previously, we have cloned a *PlGA*20*ox* gene (GeneBank accession number: KU886552) from *Paeonia lactiflora*. To further reveal its function, we constructed an expression vector in the present study and then transformed it into *Arabidopsis thaliana* plants by floral dip method. The transgenic plants exhibited an early bolting, increased height and improved vegetative growth. These results provided efficient vector tool and functional information of *PlGA*20*ox* for future gene engineering in peony.

Keywords

*PlGA*20*ox*, Expression Vector, *Arabidopsis thaliana*, Transformation

1. Introduction

Gibberellins (GAs) are important phytohormones and influence almost every aspect of plant growth and development [1] [2]. GA20-oxidase gene catalyzes the bioactive GAs and controls the levels of bioactive GAs [3] [4]; therefore the study about GA20ox characterization and its function has been extensively performed in GAs-related field.

So far, *GA*20*ox* has been isolated from many plants including *Arabidopsis thaliana* [5], tomato [6], cucumber [7] and tree peony [8]. And it was found that overexpression or downexpression of *GA*20*ox* produced different effects on plant growth and development. For example, overexpression of *AtGA*20*ox* improved the growth of the transgenic populous by enlarging leaves, thickening stems and elongating the trunks [9]; and silencing of *GA*20*ox* from tomato pro-

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duced vegetative defect and retarded the growth [10]. For another, overexpression of *GA*20*ox* from cotton improved the stem growth and reduced the growth of leaves and roots of transformed poplar [11]. These results implied that *GA*20*ox* from different plant species might alter plant growth in different orientation.

In herbaceous peony, we have isolated a full length cDNA clone, designated as *PlGA*20*ox* (GeneBank accession number: KU886552). The present study is a further attempt to reveal its function. In this study, we constructed an expression vector, and then transformed it into *Arabidopsis thaliana* plants by the floral dip method. Based on the PCR detection and phenotypic analysis of transgenic plants, we investigated the function of *PlGA*20*ox.*

2. Material and Methods

2.1. Material

The wild type of *Arabidopsis thaliana* (*A. thaliana*) was Columbia ecotype (Col-0). Incubation conditions were 23˚C and 180 μmol·m^{-2}·s^{-1} with 16 h in light/8 h in dark. *Escherichia coli* DH5α, Agrobacterium strain GV3101 and the expression vector pROKII-GFP contained GFP, 35S promoter and kanamycin resistance gene were stored in our laboratory.

A variety of incision enzyme and T4 DNA ligase were purchased from Thermo company; kanamycin (Kan), ampicillin (Amp), rifampicin (Rif) were purchased from Sigma company; Kits for rapid extraction of plasmid DNA, gel extraction kit and tissue DNA kit were purchased from Kangwei biotechnology company.

The primers were synthesized by Sangon. The sequencing was determined by BGI and the sample is bacteria liquid.

2.2. Construction of pROKII-*PlGA*20*ox*-GFP Vector

The primers without the terminator and adding the restriction site (*PlGA*20*ox*-F: 5'-CGGGATCCATGTCTCTCCTACTGGACTCAAG-3', *PlGA*20*ox*-R: 5'-GGGGTACCGAATTTTGATGGTTTAGATGATAG-3') were designed and synthesized according to the open reading frame (ORF) sequence of the known *PlGA*20*ox* gene. The bacteria liquid of *PlGA*20*ox* as template, the PCR amplification procedure was as follow: predenature at 94˚C for 5 min, then 94˚C 1 min, 52˚C 30 s, 72˚C 45 s, for 35 cycles, finally extended at 72˚C for 10 min. Purified the target segments and then linked to pMD19-T vector. The ligated plasmid was then used to transform the competent *E. coli* DH5α. The positive clones were selected using PCR and sequenced.

The plasmid DNA of pMD19-T-*PlGA*20*ox* and an empty expression vector pROKII-GFP was cleaved with XbaI and BamHI by 37˚C for 2 h respectively. The target fragment of *PlGA*20*ox* was fused with pROKII-GFP fragment using T4 DNA ligase by 16˚C for 12 h. The product was transformed into *E.coli* DH5α, The positive clones were selected using PCR and the products after enzyme digestion were sequenced by BGI. The recombinant was named pROKII-*PlGA*20*ox*-GFP (**Figure 1**).

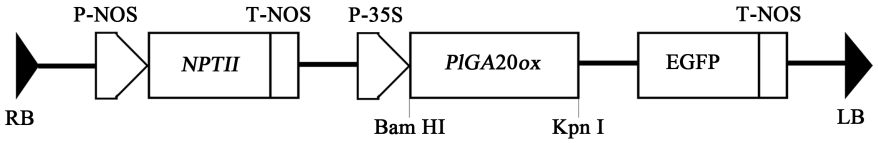


Figure 1. The diagram of constructing pROKII-*PlGA*20*ox*-GFP vector.

2.3. Transformation of *A. thaliana* with the Recombinant Vector

The constructed pROKII-*PlGA*20*ox*-GFP plasmid was transformed into Agrobacterium strain GV3101 and the positive clones were selected by PCR. The products were cultured in YEP medium (50 mg·mL^{-1} Km, 50 mg·mL^{-1} Rif) to OD600 for 0.8 - 1.2 at 28˚C with shaking at 200 r·min^{-1}. The thallus collected were resuspended in the infection of liquid (5% of sucrose, 0.03% of Silwet L-77) and adjusted OD600 to 0.6-0.8 to infect the half-opened flowers of *A. thaliana* which grows well. Method is as follows: infection lasted 5 - 10 s, a total of 10 - 15 times, cultured 24 h with keeping moisture and protecting form light. Then, according to plant growth, 5 - 7 d can be repeated infection. Cultured in light incubator (23˚C, 180 μmol·m^{-2}·s^{-1}, 16 h in light/8 h in dark) until the seeds are collected.

2.4. Screening and Detection of Transgenic Plants

2.4.1. Screening Test of Resistance to Kanamycin

The transformed *A.thaliana* seeds (T1) were harvested and then germinated on 1/2 MS solid media with kanamycin after surface sterilization. The seeds were incubated at 23˚C with 16 h L/8 h D photoperiod for one week. The *A.thaliana* plants with transformed pROKII-*PlGA*20*ox*-GFP recombinant vector were selected and their seeds (T2) were harvested. The strong transgenic T2 plants were screened with kanamycin for PCR identification and phenotypic analysis.

2.4.2. PCR Detection of Transgenic Plants

Extracted the genomic DNA of transgenic T2 plants by tissue DNA kit. Used the genomic DNA and F1 (5'-ACAAAATGTCTCTCCTACTGG-3')/R1 (5'-AAGTGACTAGAATTTTGATGG-3') as template and specific primer, respectively. PCR amplification with wild-type plants as negative control and the pROKII-*PlGA*20*ox*-GFP vector as positive control. PCR products were tested on a 1% agarose gel and sequenced, to test the integration of foreign genes.

2.5. Phenotype of Overexpression of *PlGA*20*ox*

2.5.1. Bolting Time and Plant Height

Recording the bolting time of wild and transgenic *A. thaliana*, respectively. To measure the main stems height by tape regard as plants height when each line of *A. thaliana* began bolting , and once every 2 d, a total of 6 periods.

2.5.2. Vegetative Growth

The wild and transformed *A. thaliana* seeds were vernalized on 1/2 MS solid media after surface sterilization for 3 d before incubated vertically in a light incubator for 14 d, and then to measure the root length of *A. thaliana*; The whole

leaves of typical *A. thaliana* cultured for 20 d were scanned Epson Perfection V330 Photo scanner; To measure the diameter of main stem at the end of growth by vernier caliper.

3. Results and Analysis

3.1. Construction of *pROKII-PlGA20ox-GFP* Vector

Previously, we have cloned a *PlGA20ox* gene from *Paeonia lactiflora* and the gene is 1351 bp in length, containing 1146 bp open reading frame (ORF) encoding 381 amino acids. Then, the plasmids of expression vector pROKII-GFP and *PlGA20ox* were extracted. After double restriction enzyme digestion (**Figure 2(a)**), ligation and sequencing test, the pROKII-*PlGA20ox*-GFP vector was successfully constructed. The recombinant plasmid was then transferred into agrobacterium GV3101 for positive screening. PCR results showed the band size was consistent with the target sequence (**Figure 2(b)**), indicating the pROKII-*PlGA20ox*-GFP vector was successfully transferred into agrobacterium.

3.2. Screening and Detection of Transgenic Plants

3.2.1. Screening Test of Resistance to Kanamycin

After 7 days of culture, the growth of most T1 generation *A. thaliana* seedlings was inhibited. And the regenerated seedlings resistant to Km were then transplanted for subculture. T2 generation seedlings were further screened. As shown in **Figure 3**, most seedlings grew well and the regenerated seedlings were transplanted for further molecular detection.

3.2.2. PCR Detection of Transgenic Plants

The transgenic T2 plants were detected by PCR amplification with wild-type plants as negative control and the GFP-*PlGA20ox* vector as positive control. The results showed 90% transgenic plants produced specific band of about 1.1 kb (**Figure 4**), and the sequence detection verified the existence of the target gene,

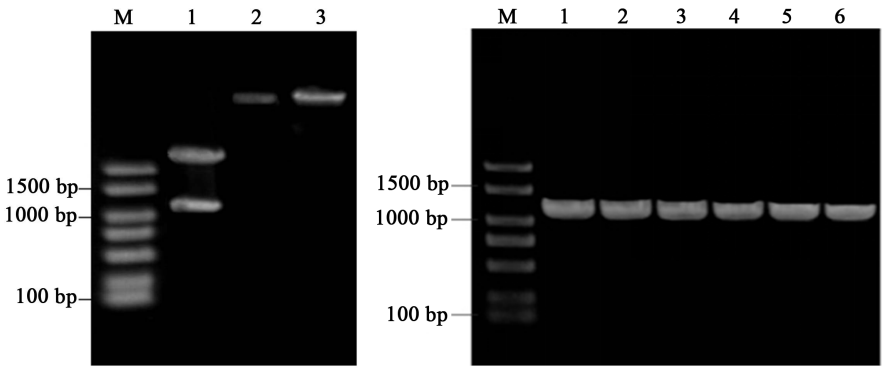
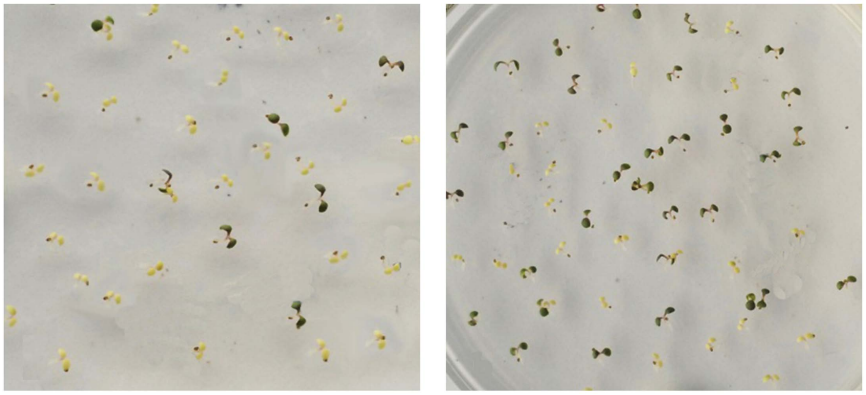


Figure 2. Construction of pROKII-*PlGA20ox*-GFP vector. (a) Identification of digested *PlGA20ox* expression vector by *Bam*HI and *Kpn*I; Notes: M: DL2000 DNA marker; 1: Digesting detection of *PlGA20ox*; 2: Digesting detection of vector pROKII-GFP; 3: pROKII-GFP vector plasmid; (b) Identification of *Agrobacterium* transformants by plasmid PCR. Notes: M: DL2000 DNA marker; 1-6: PCR result of *PlGA20ox* plasmid.



(a) (b)

Figure 3. Kanamycin response of the *PlGA20ox*-transgenic. Notes: (a) T1 seedlings; (b) T2 seedlings.

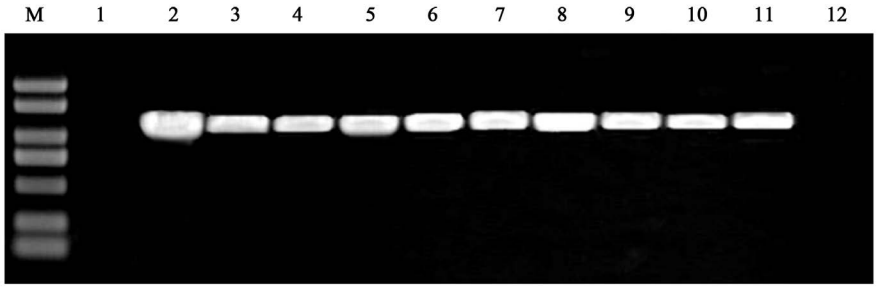


Figure 4. PCR detection results of transgenic plants. Notes: 1: wild-type lines; 2: GFP-*PlGA20ox* vector; 3-12: transgenic plants.

which suggested that the vectors had been successfully transformed into *A. thaliana*.

3.3. Phenotype of Overexpression of *PlGA20ox*

3.3.1. Bolting Time and Plant Height

As shown in **Figure 5**, overexpression of *PlGA20ox* advanced early bolting of *A.thaliana*. Transgenic plants bolted at 15d-16d after transplanting, whereas the wild-type lines bolted at 19 d after transplanting. It indicated overexpression could accelerate bolting and flowering.

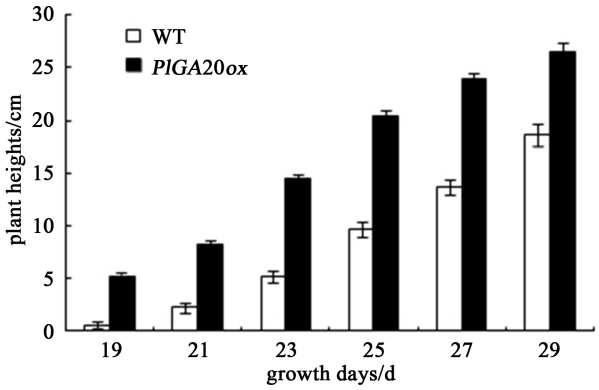
Since the wild-type lined came to bolt, the plant height of both lines had been examined. At the 19th day after transplanting, the transgenic plants reached 5 - 7 cm high when the wild-type lines finished rosette stage (**Figure 5(a)**). At the 29th day after transplanting, the transplanted plants grew to 25 - 29 cm, while the wild-type plants were 16 - 20 cm high (**Figure 5(b)**). The comparison during the whole growth cycle was showed in **Figure 5(c)**, which suggested the transgenic plants significantly surpassed the wild-type plants all the way they grew in light of plant height.

3.3.2. Vegetative Growth

At the end of the growth cycle, the transgenic plant produced 1.075 mm of shoot thickness, ranging from 1.0 mm to 1.2 mm; whereas that of wild-type lines was

(a)

(b)



(c)

Figure 5. Plant growth and flowering performance of *PlGA20ox*-overexpressed *A. thaliana* plants. (a) 19 days after transplanting; (b) 29 days after transplanting; (c) plant height dynamics during stem elongation.

0.8 mm, ranging from 0.7 mm to 0.9 mm. In terms of leaf growth, the transgenic plants and wild-type lines got the total area of 1368.53 and 645.66, respectively. The results indicated that overexpression of *PlGA20ox* enhanced shoot thickness and strongly enlarged the leaf growth.

In addition to the aboveground growth performance, overexpression of *PlGA20ox* also promoted the underground growth. As shown in Figure 6, at day 14, the transgenic T2 plants got about 6.08 cm, 1.6 multiple long as the wild-type lines 3.82 cm.

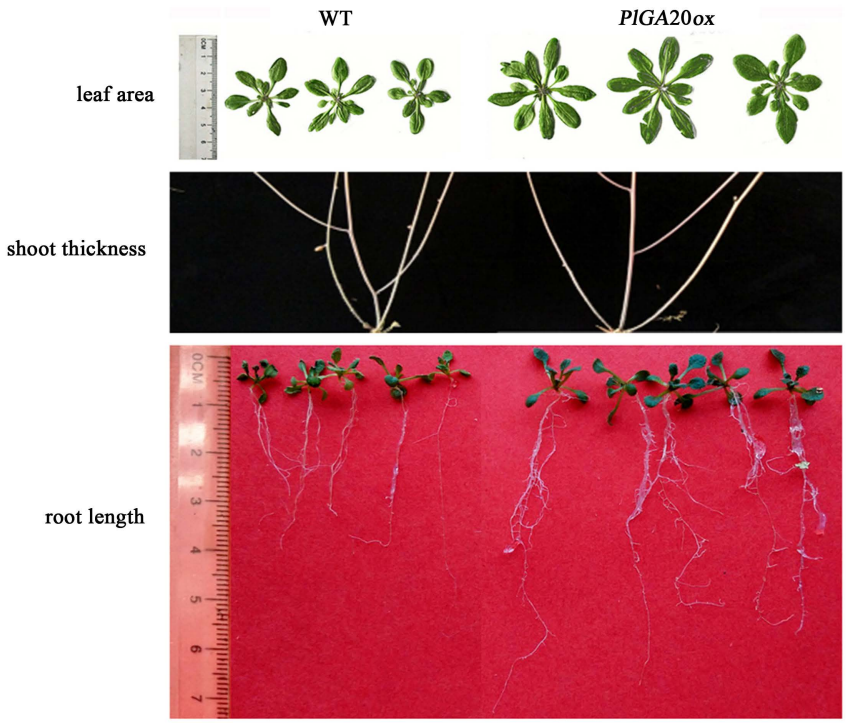


Figure 6. The growth performance of different vegetative organs of transgenic plants and wild-type plants.

4. Discussion

The sensible and antisense expression of gene is a common method to investigate its function, and it is also one of the most direct and effective methods [12]. GA20-oxidase belongs to dioxygenase. It not only plays an important role in the later period of gibberellin biosynthesis, but also regulates multiple developmental and physiological processes of plants, such as plant height, stem diameter and so on [13]. Based on the *PlGA20ox* sequence information, the expression vector pROKII-*PlGA20ox*-GFP was constructed and successfully transferred into *A. thaliana* to verify its function. This is the first report on the heterologous transformation of *PlGA20ox* gene, which lays the foundation for future gene engineering of *Paeonia lactiflora*.

Previous studies have shown that overexpression of *GA20ox* gene would accelerate growth and elongate internodes of plants, etc [14] [15]. For example, overexpression of *OsGA20ox* gene can accelerate the growth of rice and antisense expression caused "dwarf" [16] [17] [18]. *ClGA20ox1* and *ClGA20ox2* gene were transformed into tobacco, the heights and internodes of transgenic plants increased significantly [19]. However, there are some contrary results; for instance, the overexpression of *GA20ox* gene in zucchini reduced the plant height, and deepened leaf color [20] [21]. In this study, compared with the wild-type *A. thaliana*, the overexpression of *PlGA20ox* gene could advance the florescence, increase the leaf area and plant height and promote elongation of roots. These results indicated that *PlGA20ox* gene has an important function in regulating plant height, leaf size and flowering of *A. thaliana*. It was speculated that

overexpression of gene might enhance the *GA*20*ox* enzymatic activity, which therefore lead to accumulationt of GAs in the plants and promote the growth of *A. thaliana* plants.

In conclusion, the overexpression vector of the *PlGA*20*ox* gene was successfully constructed and the function was explored in this study, which will undoubtedly facilitate the genetic transformation of herbaceous peony. However, the genetic transformation system of *Paeonia lactiflora* has not been established yet, so whether overexpression of *PlGA*20*ox* gene can cause changes in the phenotype of peony remains need to be studied. In future, complete transformation system of peony should be constructed for achieving molecular breeding of the famous plant, other members of *PlGA*20*ox* gene family has not been isolated and the functions have been known completely, so the interaction among family members also needs to be studied. In addition, the key technical obstacles of peony genetic transformation system need to be further solved, which not only helps us have a deeper cognition on the mechanism of *PlGA*20*ox*, but also improve ornamental value and application prospect by genetic engineering.

Acknowledgements

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