Cloning of the AP3 Gene and Construction of Gene Editing Vectors in Hydrangea 'Duli' Post-print

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Abstract

Hydrangea macrophylla is a landscape plant whose primary ornamental part is the inflorescence, widely used for cut flower decoration and landscape construction, and extensively cultivated in Asia, America, and Europe. To investigate the function of the AP3 gene in hydrangea sepal formation and accelerate the breeding process of new double-flowered hydrangea varieties, this study used Hydrangea 'Duli' as material to clone its MADS-box class B gene HmAP3 and predict gene function using bioinformatics methods; based on the HmAP3 sequence information, highly specific editing target sites were screened and CRISPR/Cas9 gene editing vectors were constructed, which were integrated into the hydrangea genome via Agrobacterium-mediated transformation. The results showed that: (1) The full length of HmAP3 was 546 bp, encoding a total of 181 amino acids, and sequencing results indicated that its amino acid sequence had 100% identity with the reference sequence and 58.8% similarity with Arabidopsis thaliana AtAP3; (2) AP3 amino acid sequences differed significantly among plants from different genera, while the main structure of AP3 protein was relatively conserved among different species within the same genus, with differences only in a few motifs; (3) Two highly specific target sites were identified in HmAP3, and two single-target CRISPR/Cas9 gene editing vectors were successfully constructed; (4) This study obtained a total of 5 resistant shoots containing Cas9 sequences in their genomes, but none of their target sites were mutated, and Cas9 expression was not detected in the resistant shoots. This study explored the value of the AP3 gene in double-flowered hydrangea breeding, conducted a preliminary exploration of CRISPR/Cas9 gene editing technology in hydrangea, and laid a foundation for the breeding of superior hydrangea varieties.

Full Text

Preamble

AP3 Gene Cloning and CRISPR/Cas9 Vector Construction in Hydrangea macrophylla 'Dooley'

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Abstract: Hydrangea macrophylla is a garden plant widely cultivated in Asia, America, and Europe for its ornamental inflorescences, commonly used in interior decoration and landscape design. To investigate the role of the AP3 gene in sepal formation and accelerate the breeding process of double-flowered hydrangea varieties, this study cloned the MADS-box class B gene HmAP3 from H. macrophylla 'Dooley' and predicted its function through bioinformatics analysis. Based on the HmAP3 sequence, highly specific editing targets were screened and CRISPR/Cas9 gene-editing vectors were constructed. The vectors were integrated into the hydrangea genome via Agrobacterium-mediated transformation. The results showed: (1) HmAP3 has a full length of 546 bp, encoding 181 amino acids. Sequencing revealed 100% identity with the reference sequence and 58.8% similarity with Arabidopsis thaliana AtAP3. (2) AP3 amino acid sequences differ significantly among different genera, but the main structural features are relatively conserved among species within the same genus, with variations occurring only in a few motifs. (3) Two highly specific targets were identified in HmAP3, and two single-target CRISPR/Cas9 gene-editing vectors were successfully constructed. (4) Five resistant shoots containing Cas9 sequences were obtained, but none showed mutations at the target sites, and Cas9 expression was not detected in these shoots. This study explored the value of AP3 gene in double-flowered hydrangea breeding and provided a preliminary investigation of CRISPR/Cas9 technology in hydrangea, laying a foundation for elite variety development.

Keywords: Hydrangea macrophylla, MADS-box family, AP3, CRISPR/Cas9, vector construction

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Hydrangea macrophylla (Saxifragaceae family, Hydrangea genus), also known as "Baxianhua" in Chinese, has a long history of use in garden landscapes and represents a highly ornamental plant species popular as a cut flower worldwide. Currently, hydrangea inflorescences are classified into two main types: lacecap and mophead. The sterile flowers in these inflorescences feature large, showy, petaloid sepals that constitute the primary ornamental tissue. Sterile flowers

can be either single-flowered (with one whorl of ornamental sepals) or double-flowered (with multiple whorls). Double-flowered hydrangeas possess higher ornamental and economic value, representing an important direction for new variety breeding (Suyama et al., 2015). Current hydrangea breeding programs primarily rely on hybridization, which suffers from low efficiency and long breeding cycles, making it difficult to meet growing market demands (Wu et al., 2021). More rapid and efficient breeding methods need to be explored.

CRISPR/Cas9 technology is an emerging gene-editing tool capable of directionally modifying ornamental traits such as flower form, color, and longevity, offering tremendous potential and economic value for new variety development in ornamental plants (Kaur et al., 2021). The CRISPR/Cas9 system consists of Cas9 nuclease and single guide RNA (sgRNA) (Jinek et al., 2012). After transcription in plant cells, these components form a complex that recognizes and binds to approximately 20 nucleotides upstream of the protospacer adjacent motif (PAM) in the plant genome, where Cas9 creates DNA double-strand breaks (DSBs). This triggers the plant' s DNA damage repair mechanism, resulting in random base deletions (Hsu et al., 2013). Compared with other horticultural crops, CRISPR/Cas9 application in ornamental plants remains limited, with successful reports only in Populus tomentosa (Fan et al., 2015), Petunia (Zhang et al., 2016; Sun & Kao, 2018; Xu et al., 2020; Yu et al., 2021), Chrysanthemum (Kishi-Kaboshi et al., 2017), Dendrobium officinale (Kui et al., 2017), Lilium (Yan et al., 2019), Ipomoea (Shibuya et al., 2018; Watanabe et al., 2018), Torenia (Nishihara et al., 2018), and Phalaenopsis (Tong et al., 2020; Semiarti et al., 2020).

Flower organs consist of four parts: petals, sepals, stamens, and carpels, with their developmental regulation explained by the ABCDE model. In this model, class B genes primarily work with class A genes to regulate petal formation and with class C genes to control stamen formation (Coen & Meyerowitz, 1991). Except for AP2 in class A genes, which belongs to the AP2/ERF family, all other genes in this model are MADS-box family members (Wang et al., 2021).

MADS-box class B gene subfamily members are widely present in plant genomes, expressed in both gymnosperm microsporangiate strobili and angiosperm petals and stamens, playing crucial roles in plant development (Albert et al., 1998). Class B genes include two lineages: APETALA3 (AP3) and PISTILLATA (PI), with the AP3 lineage primarily regulating petal and sepal formation (Jaramillo & Kramer, 2004). AP3 proteins contain a conserved K-box domain that guides AP3 to form tetramers with PI, SEP3, and AP1 proteins, inducing petal primordium formation (Melzer & Theißen, 2009; Theißen et al., 2016). In ornamental plants, AP3 gene silencing has been shown to cause homeotic transformation from petals to sepals in Petunia (van der Krol, 1993), orchids (Mondragón-Palomino & Theißen, 2009), and Aquilegia (Zhang et al., 2013).

Therefore, this study cloned and performed bioinformatics analysis of the MADS-box class B gene HmAP3 from H. macrophylla 'Dooley' . Building upon our previous establishment of a regeneration system for this cultivar,

we constructed two single-target CRISPR/Cas9 vectors targeting HmAP3 and obtained resistant shoots through transformation. The study aimed to address: (1) characterization of conserved domains and protein structure of HmAP3; (2) phylogenetic relationships and functional prediction of HmAP3; and (3) investigation of factors affecting CRISPR/Cas9 editing efficiency in hydrangea. These efforts provide practical references and technical support for trait improvement and new variety breeding in hydrangea.

1.1 Plant Materials and Reagents

Hydrangea macrophylla 'Dooley' plants were grown at the Beijing Botanical Garden (116°28 E, 40°N). In April, healthy, pest-free green leaves were selected as experimental material. Leaves with petioles attached were cut and transferred to the laboratory in clean distilled water.

Reagent kits used in this study included: Plant Genomic DNA Extraction Kit (Tiangen Biotech, DP350), Plant Total RNA Extraction Kit (Tiangen Biotech, DP432), cDNA Reverse Transcription Kit (TaKaRa, RR047A), DNA Gel Extraction Kit (Beijing Tsingke Biotech, GE0101), One-step ZTOPO-Blunt/TA Zero Background Quick Cloning Kit (Beijing Zoman Biotech, ZC206), SE Seamless Cloning and Assembly Kit (Beijing Zoman Biotech, ZC231), and restriction endonuclease BsaI (New England Biolabs).

1.2 Cloning of HmAP3 Gene from H. macrophylla 'Dooley'

Based on the CDS sequence of the AP3 gene from H. macrophylla 'Blue Sky' (GenBank: AF230702.1) retrieved from NCBI, highly specific primers were designed (Table 1) and synthesized. Total DNA and RNA were extracted from leaf materials following kit protocols, and RNA was reverse-transcribed into cDNA. Using cDNA and DNA as templates, PCR amplification was performed with KOD One high-fidelity DNA polymerase (TOYOBO, KMM-101) and primers HmAP3-F1/R1 (Table 1). Amplification products were purified by agarose gel electrophoresis, with target bands excised and recovered using the DNA gel extraction kit. Purified PCR products were ligated into T-vector and transformed into DH5α *E. coli* competent cells, which were plated and cultured for 12 h. Single colonies were selected and sent to a sequencing company (Beijing Tsingke) for plasmid extraction and sequencing to obtain the CDS and genomic DNA sequences of HmAP3.

1.3 Bioinformatics Analysis of HmAP3

Subcellular localization of HmAP3 was predicted using Cell-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) (Chou & Shen, 2010). NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare HmAP3 amino acid sequence similarity. Top-ranking AP3 amino acid sequences from other plants and the Arabidopsis AtAP3 sequence were downloaded, and a phylogenetic tree was constructed using MEGA-X with the neighbor-joining method (Zhang et al., 2019). Conserved motifs in

HmAP3 were predicted using MEME (http://meme-suite.org/tools/meme/). Physicochemical properties of HmAP3 protein were analyzed using ProtParam (https://web.expasy.org/protparam/) (Li et al., 2020). Secondary structure was predicted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_{automat}.pl?page=npsa_{sopma}.html/) and tertiary structure using Swiss Model (https://swissmodel.expasy.org/).

1.4 CRISPR/Cas9 Vector Construction and Transformation

Using the CRISPRdirect website (http://crispr.dbcls.jp/), two highly specific targets were selected based on the HmAP3 gene sequence. Using the pCAMBIA1300-sgRNA/Cas9 plasmid as template and primers HmAP3-F2/R2 and HmAP3-F3/R3 (Table 1), PCR amplification was performed to obtain target fragments with sticky ends. The pCAMBIA1300-sgRNA/Cas9 linear vector was obtained by BsaI digestion and ligated with target fragments using a seamless cloning kit (Beijing Zoman Biotech, ZC231) to generate recombinant plasmids. Constructed plasmids were transformed into DH5α competent cells, plated and cultured for 12 h. Single colonies were selected for plasmid extraction and sequencing (Beijing Tsingke), and successfully constructed vectors were recovered. The pCAMBIA1300::HmAP3 vectors were introduced into GV3101 *Agrobacterium* competent cells by freeze-thaw method and cultured for 2 days at 28°C on LB medium with double antibiotics (50 $mg \cdot L^{-1}$ kanamycin + 50 $mg \cdot L^{-1}$ rifampicin). Single colonies were selected and expanded in liquid LB medium. *Agrobacterium* cells were collected by centrifugation and resuspended in infection solution (MS + 30 $g \cdot L^{-1}$ sucrose + 200 $mol \cdot L^{-1}$ acetosyringone) to $OD_{600} = 0.4$.

H. macrophylla 'Dooley' leaves were cut into 1 cm × 1 cm pieces, with 3-4 cuts made on the abaxial surface. Leaf pieces were immersed in the infection solution for 10 min, transferred to co-cultivation medium (MS + 2.0 $mg \cdot L^{-1}$ 6-BA + 0.1 $mg \cdot L^{-1}$ IBA) and cultured in darkness for 2 days, then moved to selection medium (MS + 2.0 $mg \cdot L^{-1}$ 6-BA + 0.1 $mg \cdot L^{-1}$ IBA + 2 $mg \cdot L^{-1}$ hygromycin + 200 $mg \cdot L^{-1}$ cefotaxime) until resistant shoots regenerated.

1.5 Detection and Identification of Resistant Shoots

DNA was extracted from resistant shoot leaves using a genomic extraction kit, followed by total RNA extraction and cDNA reverse transcription. Using leaf DNA and cDNA as templates, Cas9 sequences were amplified with primers Cas9-F/R (764 bp fragment). HmAP3 sequences from resistant shoots were amplified using DNA template and primers HmAP3-F1/R1, with products sent for sequencing (Beijing Tsingke). Sequencing results were aligned with wild-type sequences using DNAMAN.

Table 1 Primer sequences used in this study

Primer name	Sequence (5 $→$3) Annealing temperature	Purpose
HmAP3-F1	5 - ATGTTCTCCACTACCAACAAACT- 3	HmAP3 full-length amplification
HmAP3-R1	5 - CTAATCGAGCAATGCATACGTAG- 3	
HmAP3-F2	5 - ACAGCTAGAGTCGAAGTAGTGATTGGATCTCATAGAGACGACAATGTTTTAGAGC 3	CRISPR/Cas9 vector construction- Target 1
HmAP3-R2	5 - TTCTGCAGACAAATGGCCCCCATTCGGAGTTTTTGTATCT- 3	
HmAP3-F3	5 - ACAGCTAGAGTCGAAGTAGTGATTGGTACTCGATACTTTCGTTCAGTTTTAGAGC 3	CRISPR/Cas9 vector construction- Target 2
HmAP3-R3	5 - TTCTGCAGACAAATGGCCCCCATTCGGAGTTTTTGTATCT- 3	
Cas9-F	5 - CAAGTTCATCAAGCCCATCC- 3	Cas9 sequence detection in resistant buds
Cas9-R	5 - GTCCTCGTTTTCCTCATTGTC- 3	

2.1 Cloning and Sequence Analysis of AP3 Gene from H. macrophylla 'Dooley'

Based on the AP3 gene CDS sequence from H. macrophylla 'Blue Sky' , primers HmAP3-F1/HmAP3-R1 (Table 1) were used to amplify two identical nucleotide sequences from both cDNA library and genomic DNA of H. macrophylla 'Dooley' , indicating the gene contains no introns. The cloned gene was 546 bp in length, encoding 181 amino acids. NCBI analysis revealed a K-BOX conserved domain between positions 30–123 bp [Figure 1: see original paper]. The C-terminus contained PI and euAP3 motifs, characteristic of MADS-box family genes, and was designated HmAP3. The HmAP3 amino acid sequence showed 58.8% similarity with Arabidopsis AtAP3 and 100% DNA sequence similarity

with H. macrophylla 'Blue Sky' AP3, confirming its identity as the AP3 gene in H. macrophylla 'Dooley' . Subcellular localization prediction indicated nuclear expression.

2.2 Phylogenetic and Motif Analysis of HmAP3 Protein

The HmAP3 amino acid sequence was submitted to NCBI BLAST, and highly similar AP3 sequences from other plants were selected for phylogenetic tree construction using MEGA-X with the neighbor-joining method [Figure 2: see original paper]. Overall, hydrangea and other Asterid species from the Rosid subclass clustered in the same major clade, demonstrating AP3 protein conservation during evolution. At the sub-clade level, AP3 sequences showed some variation among different species but high similarity within the same species. Hydrangea showed closest relationships with Synsepalum dulcificum, Aucuba japonica var. borealis, and Ilex aquifolium. Among model plants, hydrangea was more closely related to Nicotiana tabacum than Arabidopsis thaliana, suggesting tobacco genome would be more suitable as a reference for predicting hydrangea gene-editing targets.

MEME-motif suite analysis of these sequences identified 15 motifs and their relative positions [Figure 2: see original paper]. Most plant AP3 sequences contained 7 motifs, with 8 sequences containing 8 motifs and 1 containing 9 motifs. The C-terminus was highly conserved across all AP3 sequences, containing motifs 2, 4, 5, 6, and 7, while the N-terminus primarily contained motif 3. Notably, hydrangea AP3 contained a unique motif 12, also found only in Aucuba japonica var. borealis AP3, suggesting HmAP3 may have additional functions compared to other plant AP3 proteins. For homologous genes, motif composition was generally similar, but interspecific differences contributed to functional variation.

2.3 Physicochemical Properties and Structural Analysis of HmAP3 Protein

The HmAP3 protein from H. macrophylla 'Dooley' has a molecular formula of $C_{927}H_{1462}N_{268}O_{287}S_7$ and molecular weight of 21,177.85 Da. The 181-amino-acid protein has an instability coefficient of 38.79, classifying it as stable. It contains 28 negatively charged residues (Asp + Glu) and 25 positively charged residues (Arg + Lys), with a theoretical pI of 6.17. The aliphatic index is 79.12, and the GRAVY score is -0.791, indicating a hydrophilic protein. Secondary structure analysis showed α-helices as the dominant conformation (64.09%), followed by random coils (22.65%), extended strands (8.84%), and β-sheets (4.42%) [Figure 3: see original paper].

Using Arabidopsis MADS-box protein tertiary structure as a template, HmAP3 protein tertiary structure was predicted, revealing two long α-helices twisted at 90°. The model showed high reliability with a GMQE (Global Model Quality

Estimation) value of 0.32 and QMEAN score of 0.74 ± 0.05 [Figure 4: see original paper].

2.4 CRISPR/Cas9 Vector Construction

Two highly specific targets were selected on HmAP3 using CRISPRdirect: HmAP3-Target1 (5 -GATCTGTACCAGACGACAATGGG-3) and HmAP3-Target2 (5 -TGAACGAAAGTATCGAGTACCGG-3), with GC contents of 45% and 40%, respectively. The 12 bp adjacent to the PAM site in each target matched only one site in the reference genome (tobacco), confirming high specificity. Primers HmAP3-F2/R2 and HmAP3-F3/R3 were used to amplify fragments with sticky ends, which were ligated to linearized vector and transformed into *E. coli*. Sequencing confirmed successful insertion [Figure 5: see original paper].

Hydrangea showed high sensitivity to hygromycin. After screening approximately 2,000 infected leaves with 2 $mg \cdot L^{-1}$ hygromycin, only nine resistant shoots were obtained. Using DNA from resistant shoots as template, amplification with target gene primers HmAP3-F1/R1 and Cas9 primers Cas9-F/R identified five shoots containing Cas9 sequences in their genomes, but none showed target site mutations [Figure 6B: see original paper]. Total RNA extraction and cDNA synthesis from resistant shoots failed to amplify Cas9 sequences, indicating that although vector sequences were integrated into the genome, Cas9 protein was not successfully transcribed or expressed, leaving target sequences unmodified.

3 Discussion and Conclusion

This study cloned and performed bioinformatics analysis of HmAP3 from H. macrophylla 'Dooley' . The K-box domain of HmAP3 showed similar length and structure to those in model plant Arabidopsis (Yang et al., 2003) and horticultural crops such as bamboo (Zhu, 2013), grape (Hu et al., 2021), and pineapple (Zheng et al., 2021), indicating high conservation across species. Physicochemical analysis revealed HmAP3 as a stable, hydrophilic protein, consistent with Zheng et al. (2021). In the tertiary structure model, the K-box formed long α-helices characteristic of plant MADS-box family proteins, playing a crucial role in tetramer formation with other proteins (Yang & Jack, 2004).

Phylogenetic analysis demonstrated AP3 gene conservation during plant evolution, with hydrangea clustering in the same major clade as Antirrhinum, tobacco, and tomato AP3 genes, similar to Viaene et al. (2009). Studies by Martino et al. (2006) and Liu et al. (2004) showed that silencing of tomato SlAP3 and tobacco NtAP3 resulted in increased sepal whorls and petal loss. Through homology comparison, we infer that HmAP3 likely functions similarly to its orthologs SlAP3 and NtAP3, regulating sepal and petal formation in hydrangea floral organs.

We constructed two single-target vectors for HmAP3 and detected vector integration in resistant shoots, but failed to detect Cas9 expression or target site mutations. This resembles findings by Ren et al. (2013), who suggested gene-editing efficiency relates to promoter activity: mutation rates increased from 0 to 3.2% when the promoter changed from nos-mini to U6b. In ornamental plants, Kishi-Kaboshi et al. (2019) compared promoter activities and found CaMV 35S and Ubiquitin promoters had lower activity than the chrysanthemum CmActin2 promoter in chrysanthemum callus. Our study used the Ubiquitin promoter for Cas9, which we hypothesize has extremely low activity in hydrangea tissues, preventing Cas9 expression. Future work should replace the promoter with a hydrangea-native promoter to investigate promoter activity effects on editing success.

High hygromycin sensitivity in hydrangea also significantly affected CRISPR/Cas9 efficiency. Using 2 $mg \cdot L^{-1}$ hygromycin for selection yielded only 0.45% regeneration rate, consistent with reports in apple (Jia et al., 2013), suggesting hydrangea wild-type genome lacks hygromycin resistance genes. Gan et al. (2018) proposed gradually increasing hygromycin concentrations during regeneration to improve resistant shoot survival. Subsequent hydrangea screening conditions could be optimized by adjusting hygromycin concentrations at different regeneration stages to enhance gene-editing efficiency.

In conclusion, we cloned a 546 bp HmAP3 gene from H. macrophylla 'Dooley' encoding a stable, hydrophilic protein of 181 amino acids. Sequence analysis confirmed MADS-box class B subfamily characteristics. Phylogenetic analysis revealed close relationships with tobacco, tomato, and Antirrhinum, with conserved motif composition. Two CRISPR/Cas9 vectors targeting HmAP3 were successfully constructed and integrated into the hydrangea genome. These results provide a theoretical foundation for further functional studies of HmAP3 and technical support for gene-editing assisted breeding of double-flowered hydrangeas.

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Note: Figure translations are in progress. See original paper for figures.