Original Research Article

Cloning of GFP Tagged MYB-6 Gene: An Important Transcription Factor in Regulating Anthocyanin Biosynthesis of Daucus carota

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Abstract

MYB-6 gene modulates the biosynthetic pathway of anthocyanins in plants in response to cold stress. In the present study, the full length version of this gene was identified and characterized from black carrot (Daucus carota L.) through PCR amplification using specific primers. The size of amplified MYB-6 gene was found to be 903bp which was confirmed through sequencing. Post double digestion of both vector (pEGFP-C1) and insert (MYB-6 gene), the ligated product was subjected to transformation using bacterial host (E.coli DH5α). Confirmation of successful transformation has revealed no growth of cells on Kanamycin enriched LB-plates, while as clear colonies were found on vector and vector-insert LB-plates. Further, analysis via PCR, restriction digestion and gene sequencing has confirmed successful cloning of carrot derived MYB-6 gene in E.coli DH5α. In the current study, we aimed to clone GFP tagged MYB-6 gene that could act as easy to use gene pool candidate for amelioration of cold susceptible crops and for sustainable agricultural development through various high-throughput transgenic studies. The GFP tagged MYB-6 can be used for localization studies as well.

Keywords
Anthocyanin, Cold stress, Daucus carota, MYB-6 and Restriction digestion

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Introduction

Carrot (Daucus carota L.) a root crop belonging to Apiaceae family, is considered as economically important at the global level. The taproot of carrots exhibit a range of colours including orange, yellow, red, white and purple (Xu et al., 2017). Anthocyanins are secondary metabolites present in carrots and are responsible for enhancing cold, drought and salt tolerance. Anthocyanins also contribute towards health benefits, such as the reduction in the risk of coronary heart diseases, reduced risk of stroke, antitumor properties, anti-inflammatory effects and improved cognitive behavior (Algarra et al., 2014). Although the biological effects of anthocyanins and flavonoids are attributed to their antioxidant activity, it is also proposed that they may affect signaling pathways in
animal cells. **MYB** transcription factors (TFs) are one of the most abundant among transcription factors responsible for biosynthesis of anthocyanins. They were first identified from avian myeloblastosis virus known as v-**MYB**, while as *Zea mays* is the first plant from which **MYB** was identified (Martin and Paz-Ares et al., 1997).

**MYB** proteins are the key components determining the variation in anthocyanin production (Xu et al., 2015). It has been reported that transcription factors involved in anthocyanin biosynthesis are **LDOX2** (Mapped to chromosome 2A and 2B), **MYB3** (mapped to chromosome 8A and 8B), **MYB 5** (Mapped to Chromosome 7A and 7B), **LhMYB6** and **LhMYB12** positively regulate anthocyanin biosynthesis and determine organ and tissue specific accumulation of anthocyanin.

The anthocyanin related **MYBs** identified in some plant species are; **AtMYB75**, **AtMYB90**, **AtMYB113** and **AtMYB114** in *Arabidopsis thaliana* (Yildiz et al., 2013; Dubos et al., 2010); **VvMYB1a** in *Vitis vinifera* and **MdMYB10**, **MdMYB1/MdMYBA** in *Malus × domestica* (Sadilova et al., 2009). It is important to clone and characterize relevant cold induced genes in important plant species. **MYB10**, **PabHLH3**, **PabHLH33** and **PaWD40** TF’s have been cloned in different families like *Rosaceae P. avium*, *P. persica* and other members of the *Prunus* genus (Cultrone et al., 2010; Zou et al., 2018; Yildiz et al., 2013). In order to find out presence of **MYB-6** gene in *Daucus carota*, it is important to screen out more and more number of carrot cultivars located in various geographical locations.

Therefore, the current study has investigated presence of **MYB-6** gene in 5 carrot cultivars grown in Kashmir Himalayas and its subsequent cloning using bacterial host system (*E.coli* DH5α).

**Materials and Methods**

**Plant material and cold stress**

Plant seed material of Black carrot (*Daucus carota* L.) was collected from five different sources within J&K (S1-S4), using Orange carrot (S5) as a negative control (Table 1). The collected seed material was placed in portrays sown in coco peat plus vermicompost at 28°C(Humidity=70g/m³) in the incubator for 15 days till seedling stage. The seedlings were subjected to cold stress at 4°C.

**RNA extraction**

Total RNA was extracted at seedling stage from 5 cultivars of *Daucus carota* including both control as well as cold stressed samples using Trizol method (Chomczynski and Sacchi, 1987) as per manufacturer’s instructions (Invitrogen, CA, USA). The extracted RNA was subjected to electrophoresis using 1.5% gel made in DEPC treated TAE (1X) buffer.

**DNase treatment of RNA samples**

DNase kit (Invitrogen cat.no.18068015) was used for removal of traces of DNA in the extracted RNA. The DNase treatment was given following the manufacturer’s protocol.

**First strand cDNA synthesis**

cDNA synthesis was carried out using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Cat.no.K1621) using oligoT primers.

**Validation of cDNA synthesis using PCR**

To validate cDNA synthesis polymerase chain reaction (PCR) was carried using Veriti 96-well thermal cycler (Applied Biosystems,
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Model-9902). Plant specific GAPDH was used as a housekeeping gene primer to yield product amplicon size of 198bp at annealing temperature of 62°C.

**Primer designing and PCR amplification**

Primer designing pertaining to MYB-6 gene was carried out manually using bioinformative tool (Untergasser et al., 2012). PCR amplification for anthocyanin geneMYB-6) and reference gene (GAPDH) was done in a reaction volume of 25 µl in 200 µl PCR tubes consisting of 2.5 µl PCR buffer (1 X), 0.5 µl MgCl₂ (25 mM), 0.5 µl dNTPs (25 mM), 2 µl Primer, 0.25 µl Taq Polymerase (5 U/µl), 0.5 µl cDNA sample (70 ng/µl) and 18.75 µl dist.water.

The amplification reaction was carried out in a thermal cycler (Applied Biosystems, Model-9902) using initial denaturation at 94°C (3 min), a reputation of 30 cycles comprised of denaturation (45 sec.), annealing (62 °C), extension (72°C) and final extension of 72°C (10 mins).PCR amplified products were electrophoresed on 1% agarose gel and compared with 1kb DNA ladder (Invitrogen: Cat.No.10488085).

**Cloning**

For successful cloning of MYB-6 gene into E.coli DH5α, vector pEGFP-C1 was used targeting BglIII and SalI restriction sites. Following steps were followed:-

**Plasmid isolation**

50 ml Liquid LB medium was used to cultivate bacterial cells containing pEGFP-C1 for overnight at 37°C in a shaker. Overnight grown culture was used for plasmid isolation. 5 ml of an overnight recombinant E. coli was centrifuged at ≥ 12,000g for 1 minute and the supernatant was discarded. The bacterial pellet was resuspended in 200µL of the resuspension solution by vortex and pipette up and down to thoroughly resuspend the cells until homogeneous. The resuspended cells were lysed by adding 200 µL of the lysis solution. The contents were mixed by gentle inversion (6-8 times) until the mixture becomes clear and viscous. The cell debris was precipitated by adding 350 µL of the neutralization / binding solution. The tubes were inverted 4-6 times. The cell debris was centrifuged at ≥ 12000×g for 1 minute. The cell debris was centrifuged at ≥ 12000×g for 10 minutes. Cell debris, proteins, lipids, SDS and chromosomal DNA was observed falling out of solution as a cloudy, viscous precipitate. Genelute miniprep binding column was inserted into a provided micro centrifuge tube, 500 µL of the column preparation solution was added to each miniprep column and centrifuged at ≥ 12000g for 1 minute and the flow through liquid was discarded.750 µL of the diluted wash solution was added to the column and then centrifuged at ≥ 12000g for 1 minute. The column wash step removes residual salt and other contaminants introduced during the column load. The flow through liquid was discarded and centrifuged again at 12000g speed for 2 minutes without any additional wash solution to remove excess ethanol. 100 µL of elution solution
was transferred to the column and was centrifuged at ≥ 12000×g for 1 minute. The DNA so obtained was stored at -20°C.

**Restriction digestion of the PCR fragments and Vector**

The eluted fragments of MYB6 gene and pEGFPC1 Vector were double digested simultaneously in 10 µl with the specific restriction enzymes (Table 1).

The above constituents were gently mixed and the tubes were spun briefly and incubated at 37°C for 4 hours. The products were run on a 1% agarose TAE gel and visualized on a UV-transilluminator and photographed using a gel documentation system. 1Kb ladder was used as a molecular weight marker. The restricted fragments were gel purified using MinElute gel purification kit (QIAGEN) according to the manufacturers’ instruction.

**Ligation**

The ligation reaction of digested DNA (Vector and Insert) was carried out in 20µl reaction. The samples were incubated at 15°C overnight in an incubator. The recombined plasmid was transformed into *E.coli* DH5α as a host (Table 2).

**Competent cell preparation**

**Day-1:** Frozen glycerol stock of *E.coli* DH5α was streaked on LB plate (Without antibiotics) and allowed to grow at 37°C overnight.

**Day-2**

*a*) 100ml of LB, 100ml of 100mM CaCl2 and 100mM of MgCl2 were autoclaved  
*b*) A single colony of *E.coli* DH5α was inoculated in 10ml of fresh LB and allowed to grow at 37°C overnight in a shaker.

**Day-3**

a) 1ml of the above grown culture was used to inoculate 100ml of LB in 250ml flask and kept in an incubator shaker at 37°C for 4h with constant shaking (250rpm) and continued till the absorbance of above suspension culture was done till OD$_{600}$ reaches above 0.4

b) The culture was kept on ice for 10 min and transferred to 50ml falcon tube and centrifuged at 5000 rpm for 5 min at 4°C.

c) After centrifugation the supernatant was decanted and the cells were resuspended in 1ml cold 0.1M CaCl2. The cells were vortexed and again centrifuged at 5000 rpm for 5 min at 4°C. After centrifugation the supernatant was decanted and the cells were resuspended in 1 ml cold 0.1M CaCl2. The cells were vortexed and incubated on ice for 20 min to make them competent.

d) The competent cells were dispensed in 2ml microfuge tubes (200µl/tube) and stored at -80°C for further use.

**Transformation**

The following steps were performed for transformation:-

1. The competent cells were thawed on ice (90 µl)
2. Ligated product (1 µl) was added into competent cells (90 µl) and maintained another vial of 90 µl competent cells as no DNA control (Negative control)
3. The cells were incubate on ice for 20-30 minutes
4. Heat shock was provided at 42°C for 90 seconds
5. The cells were shifted immediately on ice and kept for 2 minutes
6. 1ml of LB-broth was added to each vial and kept for 1 hour at 37°C in a shaking incubator
7. The cells were spun at 10,000rpm for 5 minutes and supernatant was discarded.
8. The pellet was resuspended in 100 μl of LB broth and the cells were plated on LB-agar plate containing Kanamycin (50mg/ml).
9. The plates were incubated at 37°C for overnight in an incubator.

**Confirmation of cloning by restriction digestion and sequencing**

**Restriction digestion**

Plasmid DNA was isolated from clones using plasmid purification kit (Sigma) following manufacturers’ instructions (Table 3). Restriction digestion of both vector and insert (MYB-6) was carried out by protocol: The above constituents were gently mixed and the tubes were spun briefly and incubated at 37°C for 4 hours. The products were run on a 1% agarose TAE gel and visualized on a UV-transilluminator and photographed using gel documentation system. 1Kb ladder was used as a molecular weight marker.

**Sequencing**

30 μl of cloned plasmid DNA was put in 1.5ml microfuge tubes along with 50μl cloning primers (10μ M) and were outsourced for sequencing to Agri. Genomics Lab. Kerala.

**Results and Discussion**

**RNA isolation, cDNA preparation and PCR amplification**

High quality total RNA was isolated from leaf samples of Daucus carota which were subjected to cold stressed conditions at 4°C. The intactness, size and quality of RNA extracted was checked on 1.5% agarose gel electrophoresis and shown as figure 1. The RNA gel showed distinctly separated sharp ribosomal RNA bands (28S, 18S and 5.8S) with thickness of 28S rRNA twice than that of 18S, further ration of absorbance at 260 and 280 was found closer to 2.0, this indicates integrity and good quality of isolated RNA. cDNA synthesis confirmation has been observed as GAPDH gene band was amplified in 198bp region (Fig. 2). PCR amplification reaction for screening of presence of MYB-6 gene in 5 sample cultivars showed amplified PCR product at 201bp using annealing temperature of 62°C after running samples on 1% of agarose gel (Fig. 3).

**PCR amplification of full length MYB-6 gene and gel elution**

Two combinations of cloning primers which were used to amplify whole MYB-6 gene through PCR i.e. K-Lab– MYB6-DC-F–Bgl2 (Forward primer) and K-Lab-MYB6-DC–R-Sall (Reverse Primer) has indicated presence of this gene in all 5 sample cultivars (Fig. 4). Further, for preparative PCR amplification, only S3 variety was chosen for further analysis. A 100 ul PCR reaction was carried out and DNA was successfully eluted from the gel before subjecting to further use. The sequencing has revealed full length size of MYB-6 gene as 903bp which was published in NCBI database (MK086024.1).

**Cloning of MYB-6 gene (903bp)**

**Isolation of Plasmid DNA (pEGFPC1)**

The overnight grown culture bacterial cells containing pEGFPC1 were subjected to isolation of plasmid which resulted in isolation of intact form of plasmid (Fig. 5).

**Restriction digestion**

The eluted fragments of MYB6 gene and pEGFPC1 Vector were double digested simultaneously in 10 µl with the specific
restriction enzymes (Bgl-II and Sal-I) whose sites were embedded in the primers used for gene amplification. The reaction used uncut vector as control, while as formation of a single band in double digested vector reflects successful digestion of vector (Fig. 6). Further, double digested insert band of MYB-6 gene was found matching with 900bp size of marker DNA and is thus matching with the full length size of MYB-6 gene (Table 4).

**Ligation and transformation**

The ligation product was transformed into E.coli DH5α host. The absence of bacterial colonies on Kanamycin based LB-agar plates inoculated with plain E.coli DH5α cells and presence of colonies in plates containing vector (E.coli DH5α) and insert (MYB-6) reflects successful transformation (Fig. 6).

**Confirmation of Cloning**

**Restriction digestion**

Upon isolation of plasmid from colonies that grew on kanamycin LB agar plates, single digestion (sd) using Bgl-II has resulted appearance of a single band that showed clear up-shift when compared with vector DNA. The presence of single bands in digested products indicates successful digestion of both vector and vector in association with insert (Fig. 7).

The suitable restriction enzymes in the form of Bgl-II and Sal-I were used to digest different plasmids isolated from 6 clones. The digestion profile has demonstrated that only clone 6 and clone 10 released the insert of appropriate size, while as rest of the colonies have shown only single band of 4.7kb. Double digestion of vector PEGFP-C1 using Bgl-II and Sal-I restriction enzymes have released 903bp insert and 4.7kb vector band (Fig. 8).

**PCR amplification**

The plasmid isolated from transformed cells upon PCR amplification using cloning primers embedded with Bgl-II and Sal-I restriction sites has resulted amplification of MYB-6 full length gene (903bp) and thus confirmed successful cloning (Fig. 7).

*Daucus carota* L. (*Apiaceae*) is an economically important root crop in the world. Black carrots are rich in anthocyanins, phenols, flavonols, carotenoids, calcium, iron, and zinc. Black carrot contains anthocyanins, whereas the orange, red, and yellow pigmentation of carrot is due to carotenoids (Akhtar et al., 2017; Wang et al., 2017; Algarra et al., 2014). In this study, we have identified full-length cDNA of MYB-6 gene corresponding size of 903bp, the same was published in NCBI database (MK086024.1). When similarity search for MYB-6 was performed using BLAST, it was observed that our query sequence showed 98% similarity with database sequence (KY020445.1) confirming identification of right target gene for further cloning studies. As per previous reports, MYB transcription factors play an important role in abiotic stress signaling including cold (Zou et al., 2018). The study of major MYB transcription factor is reported to be MYB-6 that is involved in biosynthesis of anthocyanin synthesis (Xu et al., 2017; Li et al., 2015; Zou et al., 2018). Therefore, isolation of MYB-6 gene along with its cloning studies was taken up by the current study; we reported successful cloning of this gene in pEGFPC1 as a cloning vector. Plants show differential response towards various stress conditions including temperature, drought, cold, microbial attack, salt etc. The dynamic changes which takes place at molecular level involves altered expression of genes. It is imperative to study gene expression patterns in response to different stress conditions that will provide the basis
for effective strategies towards management of stress tolerance. The novel stress responsive genes that are expressed in plants could be of paramount importance as their expression markedly affect growth and metabolic composition of particular plant species. Transcription factors (TFs) which are natural master regulators of cellular processes play an essential role in signaling pathways during stress related conditions. Understanding the behavior of transcription factors under different stress conditions could help to modify traits of various crop species through biotechnological interventions (Fig. 9 and 10).

**Table.1** Source and specimen ID of collected carrot samples (*Daucus carota* L.)

| S.No. | Source                                                                 | Variety        | Sample ID |
|-------|------------------------------------------------------------------------|----------------|-----------|
| 1     | JK-KrishiVikas Cooperative Ltd. LalMandhi Srinagar.                   | IMP            | S1        |
| 2     | Amity CR seeds, Court Road, Srinagar.                                  | Black Kashmiri | S2        |
| 3     | Nahvison Seeds, NursinGarh, Srinagar.                                  | Scarlet Globe  | S3        |
| 4     | Division of Vegetable Science, SKUAST-K, Shalimar, Srinagar.           | Cheman         | S4        |
| 5     | Genei-next, Seeds Company, 23, Court Road Srinagar Kashmir.            | Early Nantes   | S5        |

**Table.2** Restriction digestion reaction of Vector (*pEGFPC1*) and insert (*MYB-6*)

| Constituents | *MYB6* | *pEGFPC1* |
|--------------|--------|-----------|
| H_2O         | 5µl    | 5µl       |
| Buffer (5X) Orange | 1 µl | 1 µl    |
| DNA          | 3µl (100ng) | 3µl (50ng) |
| Restriction Enzyme (Thermo) (1U) | BglII and Sal1-0.5 µl | BglII and Sal1-0.5 µl |

**Table.3** Ligation reaction of vector (*pEGFPC1*) and insert (*MYB-6*)

| Constituents                     | Volume             |
|----------------------------------|--------------------|
| 10X ligase buffer minus ATP       | 2µl                |
| Vector DNA                       | 1 µl (50ng)        |
| Insert DNA                       | 1µl (100ng)        |
| 10mM ATP                         | 1µl                |
| T4 DNA Ligase                    | 1µl                |
| Distilled Water                  | 14 µl              |
| H_2O                             | 5 µl               |

**Table.4** Restriction digestion of clone confirming successful cloning of *MYB-6* gene

| Constituents                      | *MYB6* | Empty Vector (*pEGFPC1*) |
|-----------------------------------|--------|--------------------------|
| H_2O                              | 5µl    | 5µl                      |
| Buffer (5X) Orange                | 1 µl   | 1 µl                      |
| Plasmid clone (0.5µg)             | 3µl    | 3µl                      |
| Restriction Enzyme (Thermo) (1U)  | BglII and Sal1-1.0 µl | BglII and Sal1-1.0 µl |
**Fig. 1** Gel picture of Total RNA isolated from *Daucus carota* cultivars. 28S, 18S and 5.8 S rRNA and intactness of bands depicts high quality of isolated total RNA.

**Fig. 2** Gel picture of cDNA confirmation through housekeeping gene-GAPDH. Clear amplification of GAPDH band at 198bp reflects successful CDNA preparation. M-100bp DNA ladder.

**Fig. 3** PCR analysis of MYB-6 gene (201bp) in 5 sample cultivars (S1-S5). M- 100bp DNA marker.
**Fig. 4** PCR gel profile of whole MYB-6 gene (903 bp) in 5 sample cultivars (S1-S5). M- 100bp DNA marker

**Fig. 5** Isolation of pEGFPC1 plasmid from harvested bacterial cells, presence of multiple forms of plasmid bands reflect quality of isolated plasmid

**Fig. 6** Restriction digestion gel profile of pEGFPC1 vector and insert (MYB-6). M-100bp DNA ladder; 1-Uncut vector; 2-Double digested vector (Bgl-II and Sal-I); 3-Double digested insert
**Fig. 7** Transformation of MYB-6 gene in *E. coli* DH5α using pEGFPC1 as a vector. A-No DNA control (*E. coli* DH5α); B- Transformed *E. coli* DH5α (Vector) C&D- Transformed *E. coli* DH5α (Vector and insert1 and 2)

![Transformation of MYB-6 gene in E.coli DH5α using pEGFPC1 as a vector. A-No DNA control (E.coli DH5α); B- Transformed E.coli DH5α (Vector) C&D- Transformed E.coli DH5α (Vector and insert1 and 2)](image)

**Fig. 8** Single digestion gel profile. A) Undigested vector and vector in association with insert B) Single digestion of vector and vector in association with insert. M-100bp marker DNA

![Single digestion gel profile. A) Undigested vector and vector in association with insert B) Single digestion of vector and vector in association with insert. M-100bp marker DNA](image)

**Fig. 9** Double digested gel profile of 6 clones, where: 1-Uncut vector with insert, 2-Uncut Vector-1, 3- Uncut Vector-1, 4. Clone 1 (Bgl-II + Sal-I), 5.Clone 2 (Bgl-II + Sal-I), 6.Clone 3 (Bgl-II + Sal-I), 7. Uncut Vector-1, 8. Clone 4 (Bgl-II + Sal-I), 9. Clone 5 (Bgl-II +Sal-I), 10. Clone 6 (Bgl-II + Sal-I). M-100 bp marker DNA

![Double digested gel profile of 6 clones, where: 1-Uncut vector with insert, 2-Uncut Vector-1, 3- Uncut Vector-1, 4. Clone 1 (Bgl-II + Sal-I), 5.Clone 2 (Bgl-II + Sal-I), 6.Clone 3 (Bgl-II + Sal-I), 7. Uncut Vector-1, 8. Clone 4 (Bgl-II + Sal-I), 9. Clone 5 (Bgl-II +Sal-I), 10. Clone 6 (Bgl-II + Sal-I). M-100 bp marker DNA](image)
These transcription factors could remove the genetic barrier to modulate the biological pathways of particular species using superior transcription factors from another species. Different transcription factors like AP2/EREBP/ERF, bZIP, Zinc-finger, MYB, CBF/DREB1, MYC are reported to play major roles to sustain a particular stress for growth and development in various plant species (Chen et al., 2010; Abe et al., 2003; Chen et al., 2005;). Cold stress, being an important limiting factor for larger agricultural production, identification of genes associated with cold tolerance in different agricultural crops is an important step towards amelioration of cold susceptible crops and for sustainable agricultural production (Irulappan et al., 2017).

DH5ɑ associated clone containing MYB-6 gene could act as a gene pool candidate to transfer MYB-6 gene in other plant species which are susceptible to cold stress and also could help to understand signaling mechanism involved in the biosynthesis of anthocyanins under different cold stress conditions. Taken together, the current study has laid foundation to clone carrot based anthocyanin biosynthetic gene (MYB-6) that can be further used in amelioration of different crops.

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