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

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A B S T R A C T

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.

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), Lh*MYB6* and Lh*MYB12* positively regulate anthocyanin biosynthesis and determine organ and tissue specific accumulation of anthocyanin.

The anthocyanin related *MYB*s identified in some plant species are; At*MYB75*, 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^{o}C(Humidity=70g/m^{3})in the incubator for 15 days till seedling stage. The seedlings were subjected to cold stress at 4^{o}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 oligodT 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,

Model-9902). Plant specific GAPDH was used as a housekeeping gene primer to yield product amplicon size of 198bp at annealing temperature of 62^0C.

Primer designing and PCR amplification

Primer designing pertaining to *MYB-6* gene was carried out manually using bio informative 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 $MgCl_2$ (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^oC (3 min), a reputation of 30 cycles comprised of denaturation (45 sec.), annealing (62 ^{o}C), extension (72 ^{o}C) and final extension of 72^oC (10 mins).PCR amplified products were electrophoresed on 1% agarose gel and compared with 1kb DNA ladder (Invitrogen: Cat.No.10488085).

Preparative PCR amplification andgel elution

PCR amplification for anthocyanin gene *MYB-6* was done in a reaction volume of 100 µl in 200 µl PCR tubes consisting of same PCR conditions as above except that cloning primers in the form of *Bgl-II* and *Sal-I* were used to amplify whole MYB-6 gene. The DNA band of *MYB-6* was excised from the gel with a sharp sterilized blade, weighed and put in an autoclaved 2 ml microfuge tube. DNA purification from the excised band was carried out by using MinElute gel purification kit (QIAGEN) according to the manufacturers' instructions.

Cloning

For successful cloning of MYB-6 gene into *E.coli* DH5α, vector *pEGFP-C1* was used targeting *BglII* and *Sal1* restriction sites. Following steps were followed:-

Plasmid isolation

50 ml Liquid LB medium was used to cultivate bacterial cells containing *pEGFPC1* for overnight at 37^oC 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 centrifuging 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 ^{0}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 $CaCl_2$. 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 in1 ml cold 0.1M $CaCl_2$. 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^{o}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^{o}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/m).
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 *Daucuscarota* which were subjected to cold stressed conditions at 4^{o}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^{0}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-S-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 10released the insert of appropriate size, while as rest of the colones 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 effect 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

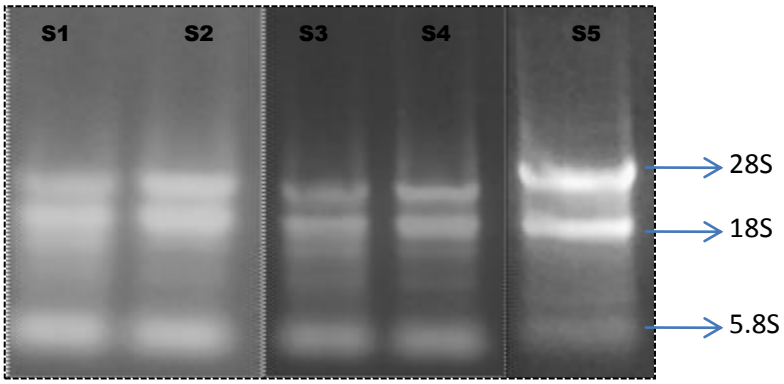


Fig.2 Gel picture of cDNA confirmation through housekeeping gene-GAPDH. Clear amplification of GAPDH band at 198bp reflects successful CDNA preparation. M-100bp DNA ladder

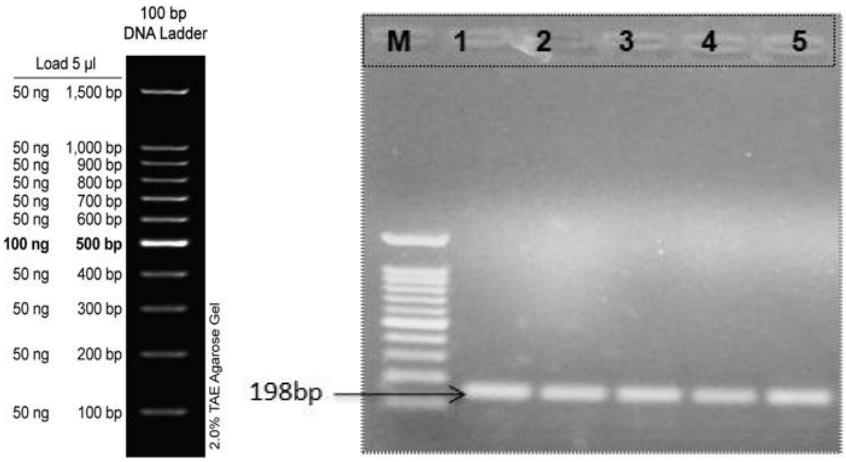


Fig.3 PCR analysis of MYB-6 gene (201bp) in 5 sample cultivars (S1-S5). M- 100bp DNA marker

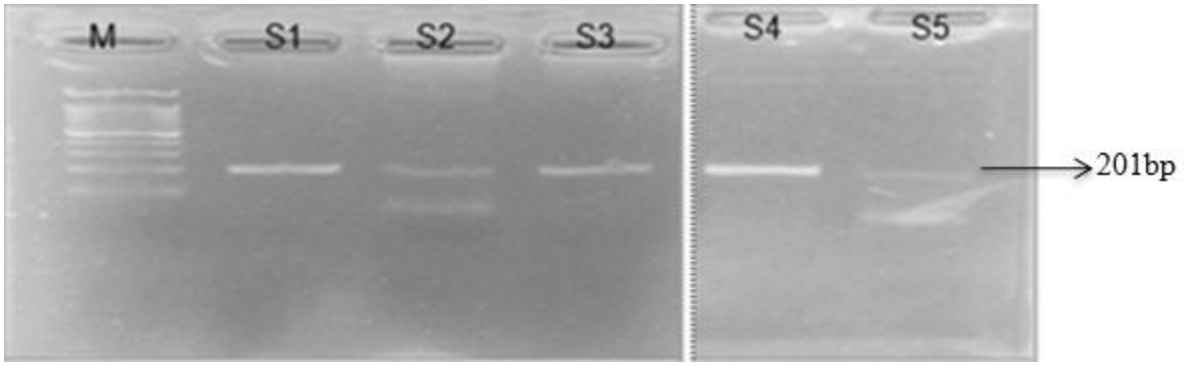


Fig.4 PCR gel profile of whole MYB-6 gene (903 bp) in 5 sample cultivars (S1-S5). M- 100bp DNA marker

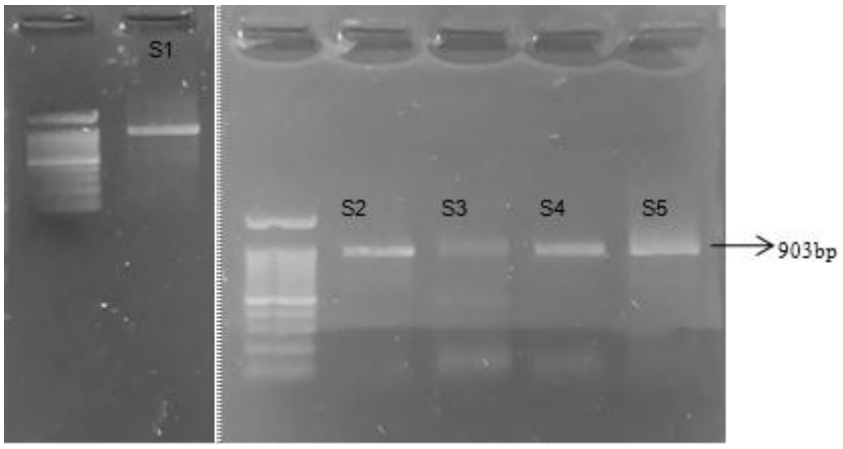


Fig.5 Isolation of pEGFPC1plasmid from harvested bacterial cells, presence of multiple forms of plasmid bands reflect quality of isolated plasmid

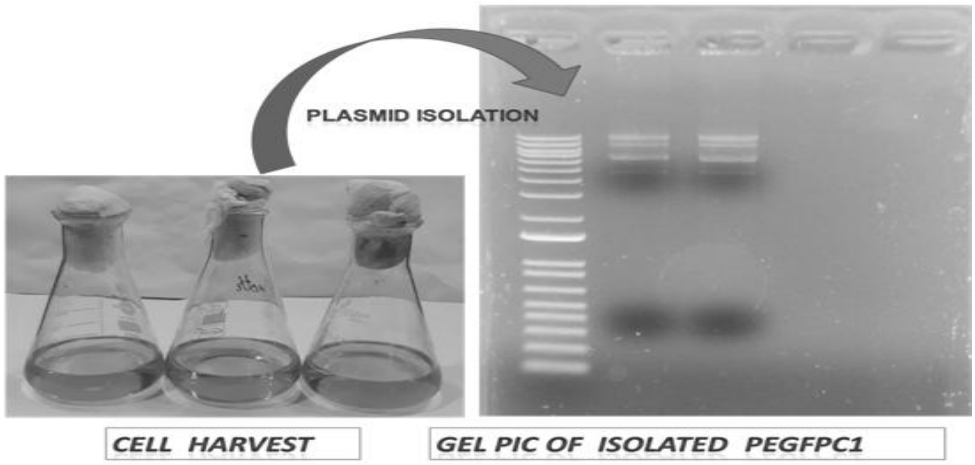


Fig.6 Restriction digestion gel profile of pEGFPC1 vector and insert (MYB-6). M-100bp DNA ladder; 1-Uncut vector; 2-Double digested vector (Bgl-II and Sal-I); 3-Double digested insert

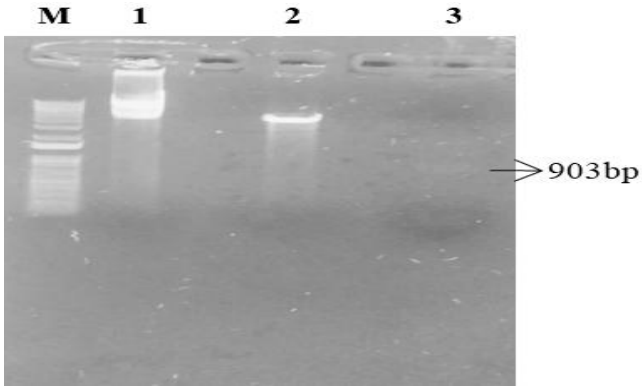


Fig.7 Transformation of MYB-6 gene in *E.coli* DH5α using pEGFPC1 as a vector. A-No DNA control (*E.coli* DH5α); B- Transformed *E.coli* DH5α (Vector) C&D- Transformed *E.coli* DH5α (Vector and insert1 and 2)

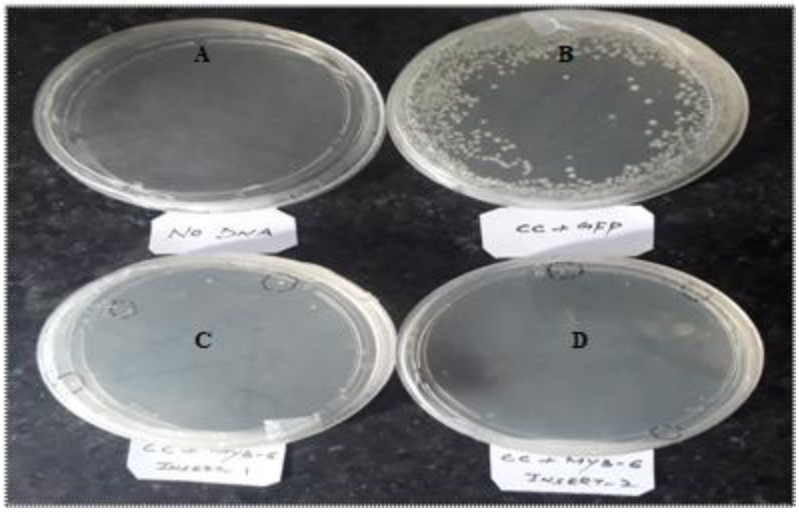


Fig.8 Single digestion gel profile. A) Undigested vector and vector in association with insert B) Single digestion of vector and vector in association with insert. M-100bp marker DNA

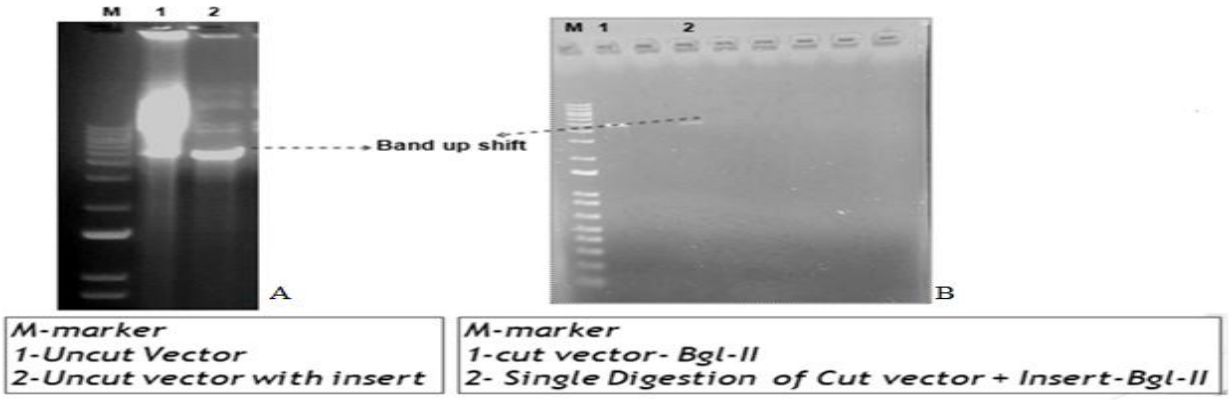


Fig.9 Double digested gel profile of 6 clones, where: 1-Uncut vector with insert, 2-Uncut Vector-1, 3- Uncut Vector-1, 4. Clone 1 (Bgl-II + Sal-I), 5.Clone 2 (Bgl-II + Sal-I), 6.Clone 3 (Bgl-II + Sal-I), 7. Uncut Vector-1, 8. Clone 4 (Bgl-II + Sal-I), 9. Clone 5 (Bgl-II +Sal-I), 10. Clone 6 (Bgl-II +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

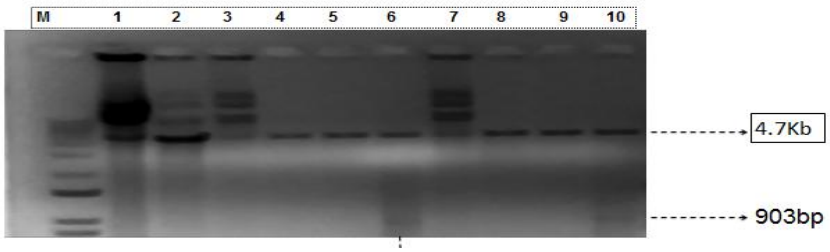
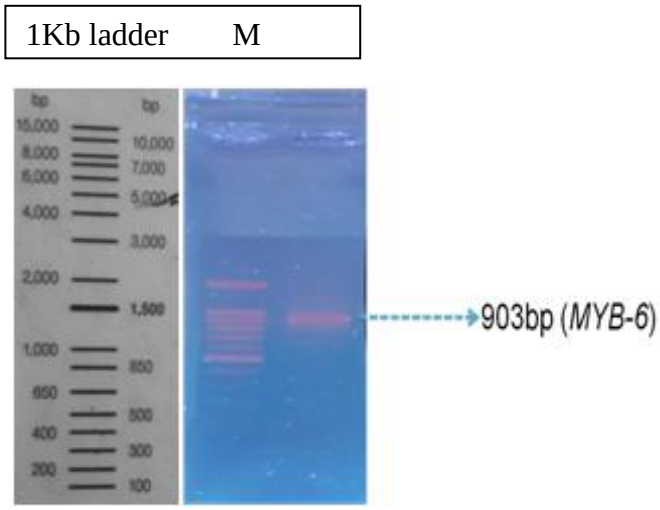


Fig.10 PCR confirmation for MYB-6 full length gene. M-Marker ladder 1Kb; MYB-6 gene



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).

DH5α 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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