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Full Length Research Paper

Characteristics of chalcone isomerase promoter in crabapple leaves (*M.*cv. 'royalty') and transient expression assay modified in onion epidermal cell

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Anthocyanins are secondary metabolites found in higher plants that contribute to the colors of plants and chalcone isomerase (CHI) is one of the key enzymes in anthocyanin biosynthetic pathway. What characteristic is CHI promoter known as the regulation sequence of CHI gene, has been rarely investigated. We isolated A 329 bp 5'-upstream sequence of McCHI promoter sequence from Malus crabapple cultivar 'royalty'. According to PLACE database, we found all kinds of cis-elements which contained light, hormone, stress, Ca^{2+} and MYB responsive factor in McCHI promoter. Transient expression assay showed that the McCHI promoter fragments had the promoter activity and can drive GUS expression. So it is presumed that McCHI promoter had a promising application prospect in the genetic transformation to modify the color of plant and anthocyanins production. We also compared the effect of GUS gene expression in different bombardment distances and in different onion epidermis culture patterns. The results show that 6 cm was the best bombardment distance and sorbitol was better to conduct plasmolysis than mannitol and that 4 to 5 layers onion epidermis was suitable for transient expression assay. As is in the foregoing, the GUS gene expression is sufficient and is the best state to analyze promoter activity.

Key words: Chalcone isomerase, promoter, cis-element, transient expression assay*, Malus crabapple.*

INTRODUCTION

In higher plants, flavonoids are ubiquitous secondary products that have diverse functions in the physiology and ecology process. Most remarkably, flavonoids can protect plants against UV-B radiation and pathogen attack, attract pollinating insects and act as signal molecules for initiating plant-microbe symbiotic associations (Katsumoto et al., 2007). Many aspects of how flavonoids are synthesized through the phenylpropanoid pathway have been extensively studied (Dixon and Steele, 1999; Grotewold and Peterson, 1994; Koes et al., 2005), in which a large number of the structure genes as well as some regulatory genes in the flavonoid pathway have been isolated from many species (Winkel-Shirley, 2001).

Chalcone isomerase (CHI), as one of the key enzymes in flavonoid biosynthetic pathway, can convert chalcone to (2S)-naringenin in the occurring spontaneously and at more than 10^7 fold efficiency (Bednar and Hadcock, 1988). After the first chalcone isomerase gene was cloned by antibody technology from *Vicia narbonensis* (Mehdy et al.,1987) and the second from *Petunia hybrida* (van Tunen et al.,1988), in next year, CHI cDNAs have been cloned from many plants, such as *Citrus sinensis*, *Zea mays* (Grotewold and Peterson, 1994), *Phaseolus vulgaris* (Blyden et al.,1991), *Medicago sativa* (McKhann and Hirsch, 1994), *Pisum safivum* (Wood and Davies, 1994), *P. hybrida* (van Tunen et al., 1988) and *Saussurea*

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Abbreviations: CHI, Chalcone isomerase; **GUS,** b-glucuronidase; **CaMV,** cauliflower mosaic virus; **MS,** murashige and skoog; **hi-TAIL PCR,** high-efficient thermal asymmetric interlaced PCR; **X-Gluc,** 5-bromo-4-chloro-3- indolyl-b-D-glucuronide.

#These authors contributed equally to this work.

medusa (Li et al.,2006). The characteristics of function and structure have been research.

For the regulation sequence in CHI gene promoter, chiA and chiB promoter sequences was isolated from *P. hybrida* and analyzed the temporal and spatial expression model of these promoters. The results showed thatchiA promoter region was a tandem structure connected by two independent promoter (P_{A1}, P_{A2}) and these promoters had their dependent function which the downstream promoter P_{A1} drove chiA expression in corolla tissue, but the upstream promoter P_{A2} drove chiA expression in pollen and later periods of anther development. Furthermore, chiB only had one promoter and the promoter specifically drove chiB gene expression in early stage of anther (van Tunen et al., 1990). However, the studies of CHI are still few and mainly focus on gene function. It is necessary to make a further research in the regulation sequence, the specific factor and the function of CHI promoter sequence and the promoter regulation mode to the gene expression.

Malus crabapple is an important ornamental and economic germplasm resource and it does not only provides us abundant plant landscape resources, but also provides favorable research material in exploiting the mechanism of color formation due to diversity in color presented in leaves, flowers and fruits. Here, we analyzed the CHI promoter of *M.* crabapples in order to know the major regulation factor of CHI gene and to understand the specific regulate mechanism of CHI gene in ever-mauve-leaf varieties. We also look forward to providing favorable foundation in improvement color of ornamental plants.

MATERIALS AND METHODS

Plant materials

The leaves of *M. crabapple* cultivar ‘Royalty’ was collected from shoots of 6 years old trees in BUA Crabapple Germplasm Resource Garden (Beijing University of Agriculture, Beijing, PR China) in 2008. ‘Royalty’ is an ever-mauve-leaf cultivar that has high pigmented tissues throughout plant present in leaves, cortex, fruit skin and flesh. The leaves were immediately frozen in liquid nitrogen and then were stored at -80℃ until they could be used for DNA isolation. Onion cultured in water until it has new roots for transient expression assay.

Cloning of the McCHI promoter

Genomic DNA was isolated from leaf tissue of young *M. crabapple* cultivar ‘Royalty’ using the CTAB (cetyltrimethyl ammonium bromide) method. 5’ upstream sequence were amplification by hi-TAIL PCR (Liu and Chen, 2007), the primer, reaction program and amplification (Tables 1, 2 and 3). The primer for hi-TAIL was designed by McCHI (FJ817485).

Construction of expression vectors with McCHI promoter fragment

Using the primer pBICHIF and pBICHIR to amplified McCHI promoter by PCR performed for 35 cycles under the following standard conditions: denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s and extension at 72℃ for 1 min using LA Taq DNA polymerase (Takara, Ohtsu, Japan). PCR-amplified fragment was gel purified and the resulting fragment were cloned and sequenced. The fragment was inserted into a HindIII+BamHI site in pBI121 by replacing the CaMV 35S promoter. The constructs were generated: pBICHI (containing the McCHI promoter, –1 to -329 bp). The vector pBI121 harboring the CaMV 35S promoter was used as a control. The primer for vectors construction is shown in the Table 4.

Transient expression assay

Onion epidermis was cultured in 1/2 MS with 0.4M osmotic solution for 3 h. Each sample was bombarded three times with tungsten particles coated with plasmids. In all experiments, an equimolar amount of each construct’s DNA (5 µg) was mixed with 1 mg of tungsten particles and suspended in 60 µl of ethanol. Each plant’s material was bombarded with a 12 µl aliquot of the suspension per shot using a helium-driven Biolistic PDS-1000/He system (Bio-Rad) with a 28 mmHg vacuum. The distances between the rupture disc (1100 psi) and macrocarrier and the macrocarrier and samples were 6.0 cm. After bombardment, the tissues were incubated in the dark for 24 h at 25℃ in ½ MS. All constructs used in the transient assay were repeated three times.

Histochemical staining of GUS expression

Histochemical staining for GUS activity was performed as described by Meisel and Lam (1996) and Jefferson et al. (1987), with the following modifications. Onion epidermal cell and transgentic tobacco were used directly for vacuum infiltration in 50 mM NaH2PO4, pH 7.0, 0.1 mM X-Gluc,10 mM EDTA, 0.1% Triton X-100. Samples were incubated at 37℃ until sufficient staining had developed (24–72 h). Plant material was then fixed in 4% formaldehyde, 4% acetic acid, 28.5% ethanol for 30 min. Subsequent incubation in 70% ethanol for 1 h, 100% ethanol for 1 h, 70% ethanol for 1 h, followed by incubation in distilled water. Stained tissues were analyzed by bright or dark field microscopy and were imaged with a digital camera AXIOIMAGER A1 (Carl Zeiss, German).

RESULTS

Isolation McCHI promoter and sequence analysis in PLACE

Using the leaves of *M.* crabapple cultivar ‘royalty’ as template, we cloned the 743 bp sequence of McCHI by means of hi-TAIL PCR, and found 394 bp McCHI gene sequence and 329 bp promoter in this fragment. According to searching the homologous cis regulation elements of McCHI 5’ upstream sequence with the known elements in PLACE database (Higo et al., 1999; Prestridge, 1991) (Table 5), we determined that the McCHI promoter sequence contained several consensus eukaryotic regulatory domains such as TATA box and CAAT box-like sequences in which he TATA box was present -62 bp upstream of the ATG. A CAAT box sequence, CAAT, was present -18 bp upstream of the ATG.

In the McCHI 5’ upstream sequence, there were light response elements, for example, that the -10PEHVPSBD

elements was activated by blue, white or UV-A light and

Table 1. hi-TIAL PCR reaction system.

Reagent	Amount in ready reaction (μl)	Amount in primary reaction (μl)	Amount in secondary reaction (μl)
ddH_2O	14.4	17.9	17.9
10×PCR buffer	2	2.5	2.5
dNTPs(10mM each)	0.4	0.5	0.5
$MgCl_2$(25mM)	1.2	1.5	1.5
SP(20uM)	0.3	0.3	0.3
LAD(20uM)	1	—	—
AC1	—	1	1
Taq	0.2	0.25	0.25
DNA template	0.5	1(40×ready reaction product)	1(10×primary reaction product)

Table 2. hi-TIAL PCR reaction program.

Reaction	Number	Reaction condition	Cycle number
Ready reaction	1	93℃ 2 min; 95℃ 1 min	1
	2	94℃ 30 s; 60℃ 1 min; 72℃ 3 min	10
	3	94℃ 30 s; 25℃ 2 min ramping to 72℃ over 2 min ;72℃ 3 min	1
	4	94℃ 20 s ;60℃ 1 min; 72℃ 3 min	25
	5	72℃ 5 min	1
	6	94℃ 2 min	1
Primary reaction	7	94℃ 20 s; 66℃ 1 min; 72℃ 3 min; 94℃ 20 s; 66℃ 1 min 72℃ 3 min; 94℃ 20 s; 50℃ 1 min; 72℃ 3 min	12
	8	72℃ 5 min	1
Secondary reaction	9	94℃ 20 s; 66℃ 1 min; 72℃ 3 min; 94℃ 20 ; 66℃ 1 min 72℃ 3 min; 94℃ 20 s; 50℃ 1 min; 72℃ 3 min	10
	10	72℃ 5 min	1

Table 3. hi-TIAL Primer sequence.

Primer name	Primer sequence
LAD1	5’-ACGATGGACTCCAGAGCGGCCGCVNVNNNGGAA-3’
LAD2	5’-ACGATGGACTCCAGAGCGGCCGCBNBNNNGGTT-3’
LAD3	5’-ACGATGGACTCCAGAGCGGCCGCHNVNNNCCAC-3’
LAD4	5’-ACGATGGACTCCAGAGCGGCCGCVVNVNNNCCAA-3’
LAD5	5’-ACGATGGACTCCAGAGCGGCCGCBDNBNNNCGGT-3’
AC1	5’-ACGATGGACTCCAGAG-3’
CHI1	5’-TTTCCCACGA TAGACTCTAG AACTGTC-3’
CHI2	5’-AGAGTATTGC TGGCCCGTCA GTGGCAA-3’
CHI3	5’- GAAACGCAGTGGCTCCGACCTGT-3’

Table 4. The primer for vectors construction.

Primer name	Primer sequence
pBICHIF	5’-ATT AAGCTT CGATGGACTC CAGAGCG-3’

pBICHIR	5'-ATT GGATCC TTGACAATTT TGTTGTTATT GTG-3'

Table 5. The putative cis-regulatory elements in the *McCHI* promoter (a) (-),DNA sequence of the antisense strand; (+), DNA sequence of the sense strand. (b) W=A+T;M=A+C;Y=C+T;R=A+G;N=A+T+G+C;V=G+A+C.

Element type	Trans-factor	Position	Sequence	Reference
Light response element	-10PEHVPSBD	-188 to -193	TATTCT	
	GATABOX	-186 to -189	GATA	
	REALPHALGLHCB21	-298 to - 303、 +42 to +47	AACCAA	
	BOXCPSAS1	+136 to +142	CTCCCAC	
Stress response element	CBFHV	+87 to +92	RYCGAC	
	CCAATBOX1	-300 to -304	CCAAT	
	LTRE1HVBLT49	+46 to +51	CCGAAA	
	MYB1AT	+43 to +48	WAACCA	
	ABRELATERD1	-268 to -272	ACGTG	
Ca+ response element	ABRERATCAL	-267 to -273	MACGYGB	
	CGCGBOXAT	-162 to -167 +162 to +167	VCGCGB	
Hormone response element	ASF1MOTIFCAMV	-97 to -101	TGACG	
	GCCCORE	+158 to +163	GCCGCC	
	T/GBOXATPIN2	-267 to -272	AACGTG	
	WBOXATNPR1	-1 to -5	TTGAC	Chen and Chen (2002)
	WBOXNTERF3	+291 to +295	TGACY	Nishiuchi et al. (2004)
	WRKY71OS	+292 to +296、 -97 to -100、 -2 to -6	TGAC	Eulgem et al. (1999)
MYB related element	MYBCORE	-131to-136	CNGTTR	Solano et al. (1995)
	MYBPLANT	+40 to +46	MACCWAMC	
	MYBPZM	-201 to -206	CCWACC	Grotewold et al., (1994)

REALPHALGLHCB21 elements was required for phytochrome regulation. There existed some stress response elements in which ABRELATERD1 and CBFHV were responsible for dehydration, LTRECOREATCOR15 was involved in cold induction. There were Ca^{2+}-responsive cis-element ABRERATCAL and CGCGBOXAT and a lot of hormone response activated elements where WRKY71OS was responsible for GA induction, GCCCORE and T/GBOXATPIN2 were responsible for Jasmonate, WBOXNTERF3 were responsible for ethylene by wounding, ASF1MOTIFCAMV and WBOXATNPR1 were responsible for auxin and salicylic acid, respectively. McCHI 5' upstream sequence had some elements in relation to anthocyanin metabolism, such as MYBCORE, MYBPZM and MYBPLANT. We also found there were some special elements in McCHI 5' upstream sequence, such as CCAATBOX1activated by heat, OSE1ROOTNODULE activated in infected cells of root nodules, POLLEN1LELAT52 being responsible for pollen specific activation. The properties of those elements are consistent with the function characteristics of anthocyanins in the induction and stress-resistance (Figure 1).

Transient expression of McCHI promoter in onion epidermal cell

To determine the activity of McCHI promoter, a 329 bp McCHI promoter fragment (from –1 to -329) was cloned to pBI121 by replacing the CaMV 35S promoter. The McCHI promoter fragment fused with the GUS gene were introduced into onion epidermal cell and subjected to a transient expression assay. A schematic map of the vector is presented in Figure 2.

We did a particle bombardment assay to examine the expression of chimeric gene of 5'upstream sequence of McCHI promoter with GUS gene. The result of transient expression assay showed that pBI121 and pBICHI can drive GUS expression and made onion epidermal cell turn into blue (Figure 3). It was proved that the McCHI promoter fragment had the promoter activity and it can

drive GUS expression, but the activity of McCHI promoter was lower than pBI121.


```
-329  CGATGGACTC CAGAGCGGCC GCTGTATTGG TTGATGACCG TTTAAGTAGT AGCTGTCCAC
                              REALPHALGLHCB21              ABRELAT
-269  GTTACCTCTT TGCCCTTTAA CCTTAAAATG AATCTTTTAC TCTCTCTCTC TCTCTCTTTC
      ERD1
-209  TGGGGTAGGT AGTTCCAGAA TATCGGTTTG TGGACACTTC CCCCGCGCCG CCCGCATGCC
         MYBPZM        -10PEHVPSBD                   CGCGBOXAT
-149  TTTAAGGCTC CCACGCAACC GCCTCTTCTT ACTCATCAAA CCCTCCCCCG TCACTCAATC
                 MYBCORE                                  CBFHV
-89   GACCCACCAC TTTCCATCTC TATTTAAACC CACCGTTTCC GAAACCAAAC CCGATCTTTC
                     TATA box   +1        LTRE1HVBLT49
-29   TCTCTACACA ATAACAACAA AATTGTCAA ATG GCTCCAAC GCCATCGCTC GCCGGACTCC
               CAAT box          WBOXATNPR1
+32   AGGTCGAGAC GACTGCGTTT CCACCGTCCG CCAAACCTCC GGGCTCCTCC AACACTCTGT

+92   TCCTCGGCGG CGCAGGTTTG TAAACTTCGC ATCTCCGGCG CTTTTATCTG CGATTCTGAT

+152  TGTTTGAATG TTTGTTGCTT GCTTTTGCTT CCGCTTGACG GTATTTTGGT TGCTAACGTG

+212  CACAGGGATG AGGGGGTTGG AGATTCAGGG GAACTTCGTG AAGTTCACGG CGATCGGAGT

+272  GTACTTGGAG GATAACGCCG TGCCTCTGCT CGCCGTTAAG TGGAAGGGTA AGACGGCCGA

+332  GGAGTTGACG GAGTCCGTTG AGTTCTTCAG GGACATAGTT ACAGGTCGGA GCCACTGCGT

+392  TTC
```

Figure 1. The DNA sequence of the promoter region of `chalcone isomerase (CHI). A 723 bp fragment (–329 to +394) including the part of the 5’-encoding region. Nucleotides are numbered on the left side, with the position of the translation start codon designated as +1 and indicated in red with dark. cis-elements are indicated in pane, The dark sequence is the intron.

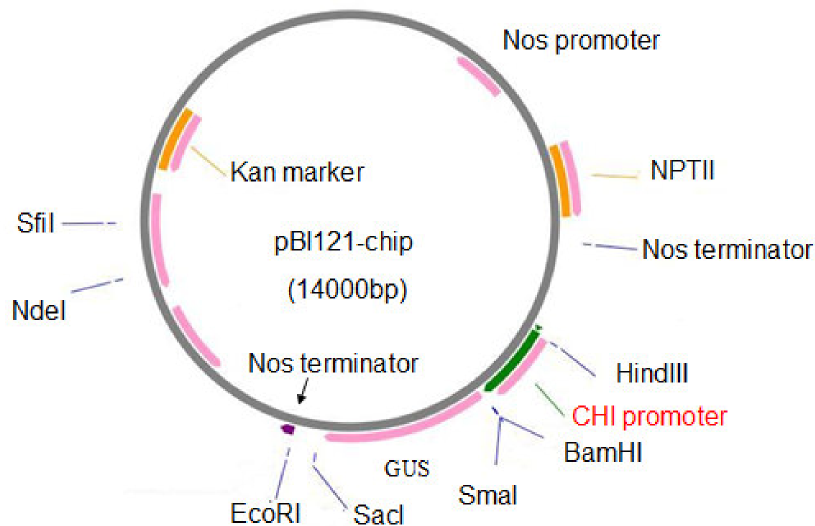

Figure 2. The T-DNA region of the binary vector, pBI121. The promoter fragments were cloned in the HindIII-BamHI sites. The *GUS* gene was driven by the *McCHI*

promoter (coordinates -329 to -1 bp).

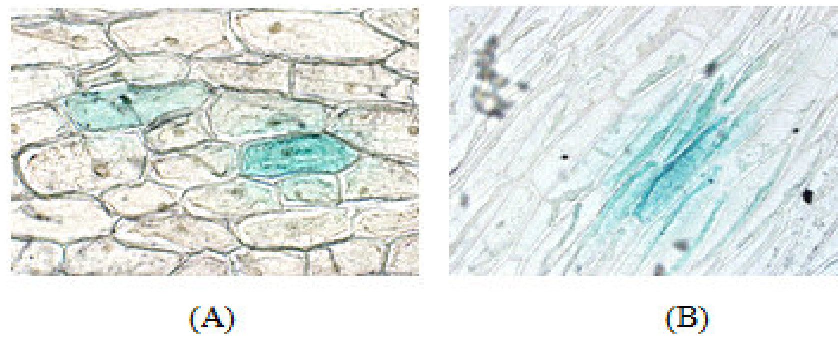

Figure 3. The schematic map of GUS staining in the transient expression vector pBI121 with CaMV 35S promoter-driven *GUS* and *McCHI* promoter-driven GUS in onion epidermal cells (A). pBICHI. (B) pBI21 as a control.

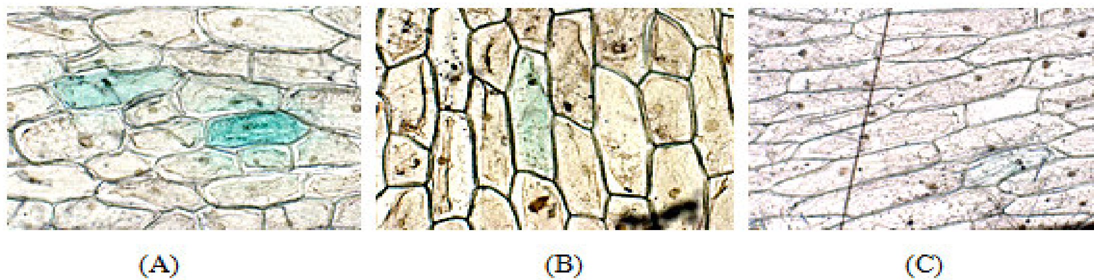

Figure 4. The schematic map of GUS staining in the transient expression with different bombardment distance. (A) 6 cm bombardment distance. (B) 9 cm bombardment distance. (C) 12 cm bombardment distance.

The comparison of different bombardment distance in onion epidermal cell

In order to conduct better research in the activity of McCHI promoter and particle bombardment, we compared the different bombardment distance with 6, 9 and12 cm by McCHI promoter in onion epidermal cell (Figure 4). The results show that 12 cm bombardment distance was not suitable for transient assay because we hardly observe the color change in the cell for the weaker promoter and we cannot distinguish whether this result was caused by the low promoter activity or by the inappropriate bombardment distance. The GUS expression in 9 cm bombardment distance was better than that in 12 cm bombardment distance where we were able to detect some cell become blue, but the effect was worse than that in 6 cm bombardment distance in which we can more clearly observe the expressed cell and stronger GUS expression. So we suggest that the 6 cm distance is the best bombardment distance in the research of McCHI promoter or other promoters.

The comparison of different culture pattern in transient expression assay

To compare the effect of sorbitol and mannitol to conduct plasmolysis in transient expression assay, we did McCHI particle bombardment assay compared with these two solutions (Figure 5). The results show that both bombarded cells were changed to blue after 3 h cultured in 1/2 MS plus sorbitol or mannitol, but the cell cultured with mannitol was damaged severely and cytoplasm became long and narrow and the cell cultured with sorbitol had ordinary shape and the expression of GUS was more sufficient.

We also compared the influence of different part of onion epidermal cell in particle bombardment. We observed that the GUS gene was hardly expressed in the 2 to 3 lateral layers epidermal cell in onion and the 4 to 5 layers cell had a strong expression of GUS. The 7 to 8 layers cell was too young to conduct bombardment so that many particles were penetrated into medium and the expression of GUS was low like as 2 to 3 layers cell.

DISCUSSION

The red color of plant, as an important horticultural trait, is mainly produced by the flavonoids pigments, anthocyanins. Chalcone isomerase is thought as a key

enzyme to increase the content of flavonoids in relation to anthocyanin biosynthesis. It is clear that CHS is the first

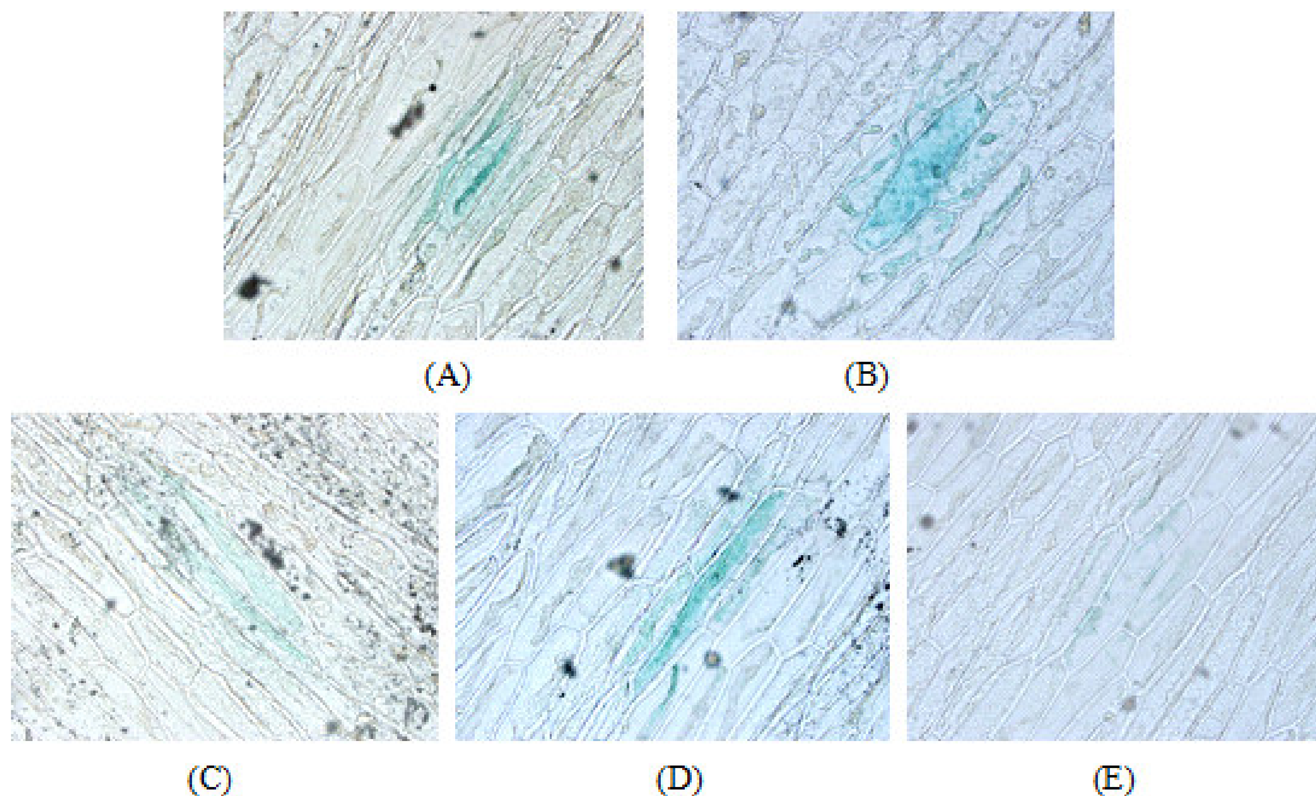

Figure 5. The schematic map of GUS staining in the transient expression with different cultured pattern. (A) plasmolysis with 1/2+mannitol. (B) plasmolysis with 1/2+ sorbitol. (C) 2 to 3 layer onion epidermis. (D) 4 to 5 layer onion epidermis. (E) 7 to 8 layer onion epidermis.

enzyme of flavonoid biosynthesis and it converts malonyl CoA and 4- coumaroyl -CoA into narigenin chalcone and CHI, the consecutive enzyme, isomerizes the chalcone into the corresponding flavanone. The chalcone isomerase is identified in many plants, such as *P. hybrida, Z. mays, M. sativa, C. sinensis* and so on. The researchers has been made many achievements about function analysis and genetic transformation in *CHI* , but the active group , regulation mechanism and effective factor are still unclear yet (Li et al.,2006).

In this study, we isolated a 329bp 5'-flanking region of McCHI gene from ever-mauve-colored *M.* crabapple cultivar 'royalty' genomic DNA that has universal characteristics of plant promoters with a TATA box (TATA) and CAAT box (CAAT). After blasting, we found that there was no similar sequence in NCBI and it should be special CHI prompter sequences in *M*. crabapple plants. Cis-elements analysis was made by PLACE database (Table 1) and the result showed that the McCHI 5' upstream sequence can be activated by the stress condition including dehydration, heat, cold, can be responsive to the blue, white, UV light, can be induced by hormone such as auxin, GA, JA, ethylene and salicylic acid and can also regulate anthocyanin biosynthesis by all kinds of transcription factor binding. The McCHI promoter also had some special cis-elements which can be activated in infected cells of root nodules and control guard cell-specific gene expression. Anthocyanins is known to be accumulated in stress, light, hormone conditions, so the activated feature of McCHI promoter is consistent with the induced characteristics of anthocyanins.

To determine the specific expression characteristic of the promoter, the full-length of *McCHI* promoter was isolated, fused with GUS and subjected to a transient expression assay in onion epidermal cell. The result shows that the McCHI promoter had the activity of promoter and a relatively strong activity. But we can see that the activity of McCHI promoter was lower than that of CaMV35S (Figure 3).

Promoter transient expression assay is an approach for determination the activity of promoter, which has been widely applicated in promoter research (Ghosh-Biswas, 2006). We carried out different bombared mode to find the perfect result of transient expression assay.

First, we compared different effects among 6, 9 and 12 cm bombard distance of GUS staining in particle bombarded. The result shows that 6 cm was the best bombardment distance in transient expression assay. This result is consistent with former research in different bombardment distance with japonica rice seeds callus

(Alfonso-Rubi et al., 1999). Secondly, we compared different effects of sorbitol and mannitol solutions which were normal to conduct osmotic treatment in transient expression assay, because these two solutions can decrease the turgor pressure to avoid breaking by bombardment and increase the survival chance of receptor cell (Rosillo et al., 2003).

However, the difference of these solutions was little studied before. In this study, we added sorbitol and mannitol into ½ MS medium, respectively and observed that the cell cultured in mannitol had a severe plasmolysis, in which cytoplasm became very small and appeared a long and narrow bands, so it was hard to distinguish promoter activity in the GUS staining, However, the shape of onion epidermis cells were cultured in sorbitol; the cell was integrated and the plasmolysis was complete, consequently, the effect of GUS staining was better suitable for comparing of promoter activity. These results were opposite to the experiment of rice embryogenesis suspension cells, in which the effect of mannitol is better than that of sorbitol (Nandadeva et al., 1999), this difference, may be due to the different species. So, we suggest that for the different receptor tissue we should select different osmotic solution. Finally, our results also show that the different layers of onion epidermis had different activity for expressing GUS gene. The 2 to 3 layers and 7 to 8 layers onion epidermis were not suitable for particle bombardment, due to their low activity and poor hardness, respectively and the onion epidermis in 4 to 5 layers had a suitable activity and flexibility to suffice the requirement of transient expression assay.

Anthocyanins are an effective and universal protection substance in plants and can improve the resistance to light injury, drought, low temperature and are also good for human health. As the key biosynthetic enzyme in the anthocyanin and flavonoid biosynthetic pathway, over expression of the CHI gene in plants can increase the content of anthocyanins and flavonoids; an excellent way to modify a plant. In the process of genetic improvement, a suitable promoter is critical for the expression of exogenous genes, Necessarily, further research of the regulation mechanism and specific expression in different tissue of McCHI promoter might provide new insights into the mechanisms of anthocyanin biosynthesis and will give a way to modify plant anthocyanin metabolism.

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