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Isolation and characterization of a chalcone isomerase gene promoter from potato cultivars

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ABSTRACT. Chalcone isomerase (CHI) is a key enzyme involved in anthocyanin metabolism. Previous research on CHI has mainly focused on cDNA cloning and gene expression. In the current study, the 1425-bp potato *CHI* promoter (PCP) was isolated from four potato cultivars (Heijingang, Zhongshu 7, Désirée, and Favorita) using PCR and DNA sequencing. The PCP contained many *cis*-regulatory elements (CREs) related to anthocyanin metabolism, tissue specificity, light response, stress, and hormone induction. Of the PCP CREs identified, 19 were common to those found in the higher plants examined, based on plant CRE databases. Multiple sequence alignment showed six single nucleotide variation sites in PCP among the potato cultivars examined, resulting in changes in the number of CREs connected with tissue specificity, anthocyanin metabolism, and light response. The 665-bp PCP fragments from Favorita and 1425-bp PCP fragments from Heijingang were used to construct plant expression vectors, which may be a useful tool for biological engineering. A transient expression assay demonstrated that the two PCP fragments from Heijingang could direct the expression of a green

fluorescent protein gene in onion epidermis and a β-glucuronidase gene in all potato tuber tissues with different colors, suggesting that the single nucleotide variation in the PCP did not affect its activity, and that silencing of the *CHI* gene in Favorita may be attributed to other regulatory factors.

Key words: Potato; *CHI* promoter; *Cis*-regulatory elements; Single nucleotide variation; Activity

INTRODUCTION

Anthocyanins are a type of flavonoid, and they play a role in many biological processes, such as plant color formation (Koes et al., 2005), pollen development, UV protection (Bieza and Lois, 2001), and stress response (McKhann and Hirsch , 1994). Moreover, anthocyanins have an extensive medicinal value to human health, including inhibition of the proliferation of two types of human cancer cells (HL-60 promyelocytic leukaemia and HT-29 colorectal adenocarcinoma) in a dose-dependent manner (Netzel et al., 2007), significant reduction in tert-butyl hydroperoxide-induced oxidative injury, and the proliferation index of intestinal adenoma (Cai et al., 2010; Hwang et al., 2011). Therefore, anthocyanins have received increasing attention over the past decade.

Chalcone isomerase (CHI) is an important enzyme in the phenylalanine metabolism pathway, which controls tissue-specific flavonoid biosynthesis in plants. The CHI gene was first isolated from pea using an antibody technique (Mehdy and Lamb, 1987). To date, cDNA sequences encoding CHI have been cloned from many higher plants, such as *Petunia hybrida* (van Tunen et al., 1988), *Zea mays* (Grotewold and Peterson, 1994), *Saussurea medusa* (Li et al., 2006), and *Arachis hypogaea* (Liu et al., 2015). *CHI* shows high expression levels in plant tissues that contain abundant flavonoids or anthocyanins, and overexpression of the CHI gene has been shown to increase the accumulation of flavonoids in transgenic tomato fruits and the hull of mutant rice (Muir et al., 2001; Wang et al., 2010; Hong et al., 2012). In contrast, *CHI* suppression by RNAi has been reported to reduce pigmentation and change flavonoid components in flower petals of tobacco (Nishihara et al., 2005).

Promoter sequences control transcription of genes in conjunction with RNA polymerase and transcription factors. Many important *cis*-regulatory elements (CREs) are located within promoter sequences, and these are the binding sites for corresponding transcription factors. Analysis of promoter sequences can further our understanding of gene expression and regulation. To date, limited research has been undertaken on the *CHI* promoter, which was first isolated from the *Petunia hybrida* inbred line V30 in 1989 (van Tunen et al., 1989).

Potato is an important crop worldwide and its tubers are unique parts that are used in our diet. Interest in potatoes with pigmented flesh has grown, partly because of increased antioxidant content in pigmented tubers with abundant anthocyanins (Naito et al., 1998; Rodriguez-Saona et al., 1998; Eichhorn and Winterhalter, 2005). In previous studies, we cloned anthocyanin structural genes, including *CHS*, *F3H*, *DFR*, *ANS*, and *3GT* from wild potato (Lu and Yang, 2006; Wang et al., 2011), but studies on their promoters remains elusive. In this study, we isolated *CHI* promoters from 4 potato cultivars with different colored flesh, compared their structures, and analyzed their activity by assessing transient expression of a green fluorescent protein gene (*GFP*) and a β-glucuronidase gene (*GUS*).

MATERIAL AND METHODS

Plant material and growth conditions

The potato cultivars used in this study were Zhongshu 7 (yellow skin and white flesh), Hui-2 (yellow skin and yellow flesh), HQ (red skin and yellow flesh), Ziyun 1 (purple skin and purple flesh), Heijingang (purple skin and purple flesh), Favorita (yellow skin and yellow flesh), and Désirée (red skin and yellow flesh). These cultivars were grown under natural light and temperature in spring in Nanjing, China. Genomic DNA was extracted from the leaves of the potato plants and 60-day-old tubers were harvested for RT-PCR analysis and transient expression assay.

RNA extraction and RT-PCR analysis

Total RNA was isolated from the peeled flesh of the seven cultivars using Trizol reagent (Invitrogen, USA), according to the manufacturer protocol. RNA was then converted into cDNA using random primers and reverse transcriptase (Promega, USA), following treatment with DNase (Promega). To analyze expression of the CHI gene, two primers - were designed, CHI-F and CHI-R, and primers 18S-F and 18S-R were used to amplify potato 18S ribosomal RNA, as an internal control (Table 1). All PCR reactions were performed under the following conditions: 5 min at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at 54°C, and 1 min at 72°C, with a final extension step for 10 min at 72°C.

Isolation of the *CHI* promoter

Genomic DNA was extracted from four of the seven potato cultivars, Zhongshu 7, Favorita, Heijingang, and Désirée, using a Plant Genomic DNA kit (TIANGEN Biotech, Beijing, China).Two potato *CHI* promoter (PCP) sequences were amplified from each of the four cultivars using promoter-specific primers, CHIP1-F, CHIP2-F, and CHIP1-R (Table 1), designed according to the potato CHI gene (accession No. HQ659497) and potato genome data (http://solgenomics.net/). CHIP1-F and CHIP1-R were used to amplify a 1425 bp promoter fragment upstream of the CHI initiation codon ATG in each of the four cultivars, and CHIP2-F and CHIP1-R were employed to amplify a 665 bp promoter fragment upstream of the CHI initiation codon in the Favorita cultivar. The PCR conditions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 58°C for 30 s, and 72°C for 45 s, with a final extension step at 72°C for 10 min. PCR products were then cloned into the pMD-19T vector (TaKaRa Biotech, Dalian, China) for sequencing (Beijing Genomics Institute, China).

Table 1. Sequences of oligonucleotide primers used to analyze expression of the chalcone isomerase gene (*CHI*) in potato and to amplify two *CHI* promoter sequences.

Primer	Sequence (5'-3')	Amplified fragment (bp)
CHI-F	TGCCTATAAACAATAATCAGTG	1152
CHI-R	ACCATGCTTTTATTGAGCTATT	
18S-F	TGCCAGTAGTCATATGCTTGTCTC	454
18S-R	AGCCCGGTATTGTTATTTATTGTC	
CHIP1-F	CGGGGTACCTAGATAGTCACATGCGAAAT	1425
CHIP1-R	CATGCCATGGTTTTACACTGATTATGGTTATAGGC	
CHIP2-F	CGGGGTACCACTGGATGACAATATGTACTTACG	665

Underlined sequences indicate restriction enzyme sites (*Kpn*I and *Nco*I) engineered for construction of binary vectors.

Sequence analysis

The PCP sequences were analyzed for the presence of *cis*-regulatory sequence motifs and related transcriptional factor binding sites (TFBs) using the plant *cis*-acting regulatory element (PlantCARE) database (Lescot et al., 2002) and plant *cis*-acting regulatory DNA elements (PLACE) database (Higo et al., 1999). The neural network promoter prediction (NNPP) server (Reese, 2001) was used to predict the transcription start site (TSS). Nucleotide sequence comparisons were performed using NCBI BLAST tools (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was conducted using DNAMAN software (version 5.2.2, Lynnon Biosoft, Canada).

Transient expression assay

StCHIP1 which obtained 1425 bp PCP isolated from Heijingang and StCHIP2 which obtained 665 bp PCP from Favorita. The two PCP fragments were inserted into the binary vector pCMBIA1304, using the restriction enzymes *Kpn*I and *Nco*I, in place of the CaMV 35S promoter upstream of the GFP-GUS fusion. The recombinant vectors, named p1304-StCHIP1 and p1304-StCHIP2, were transformed into the *Agrobacterium tumefaciens* strain GV3101 using the freeze-thaw method (Hofgen and Willmitzer , 1988). Onion epidermis and fresh potato chips (1 to 2 mm thick) from the Heijingang, Desiree, HQ, Favorita, and Hui-2 cultivars were placed on Murashige and Skoog (MS) solid medium at 25°C for 48 h, after infiltration in a suspension solution containing the *Agrobacterium* cells (Murashige and Skoog, 1962).

GFP protein was visualized using a fluorescence microscope (Zeiss Axio Imager A1, Germany). A histochemical GUS assay was carried out as described previously (Jefferson et al., 1987). Samples were incubated in the GUS assay buffer at 37°C overnight, washed with 70% ethanol, and then photographed under a stereomicroscope (Zeiss Stemi 2000-C, Germany).

RESULTS

Expression of CHI genes in different potato cultivars

CHI has previously been cloned from potato cultivar tubers (Wei et al., 2012). In this study, the expression level of *CHI* in the peeled flesh of seven potato cultivars was examined using a semi-quantitative RT-PCR assay. The results show that *CHI* was only expressed in cultivars with colored tuber flesh, Heijingang and Ziyun1, but was not expressed in the other cultivars (Zhongshu 7, Hui-2, Favorita, HQ, and Désirée; Figure 1).

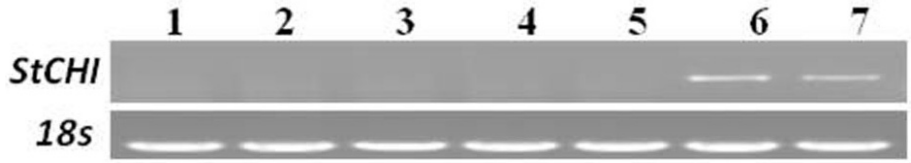

Figure 1. Semi-quantitative RT-PCR analysis of the anthocyanin structural gene *StCHI* in tuber flesh during tuber formation. *Lane 1* = potato cultivar with yellow skin and white flesh (Zhongshu 7); *lanes 2* and *3* = cultivars with yellow skin and yellow flesh (Hui-2 and Favorita); *lanes 4* and *5* = cultivars with red skin and yellow flesh (HQ and Désirée); *lanes 6* and *7* = cultivars with purple skin and purple flesh (Ziyun1 and Heijingang). Expression of 18S rRNA was evaluated as an internal control.

Isolation and sequence analysis of PCP

To determine why the CHI gene is silenced in the tissue of potato cultivars with non-colored flesh, a 1425 bp PCP sequence, including the CHI 5'-UTR, was isolated from four potato cultivars (Heijingang, Zhongshu 7, Favorita, and Désirée; Figure 2). The sequences were enriched in bases A and T (~70%) along the whole sequence and contained a transcription start site (TSS) at the 1356th position. A total of 71 CREs, including core elements, such as the TATA-box and CAAT-box, anthocyanin gene CREs (30 copies), tissue-specific CREs (100 copies), environmental CREs (47 copies), and hormone-inducted CREs (16 copies), were isolated from the *CHI* promoter of the Heijingang cultivar (Table 2).

Comparison of PCP CREs between potato and other plants

To reveal the characteristics of the *CHI* promoter among higher plants, we identified the *CHI* promoter region in tomato, tobacco, cacao, *Arabidopsis*, corn, petunia, barley, and rice using the GenBank and Solanaceae genomics databases (NM001247492, KJ730247, CM001882, CP002686, AC196033, X14589, AF474923, AF474922, respectively). There were 73 CREs in tomato, 75 CREs in tobacco, 74 CREs in cacao, 98 CREs in *Arabidopsis*, 67 CREs in corn, 83 CREs in petunia, 97 CREs in barley, 83 CREs in rice, and 71 CREs in potato, found by blasting the PLACE database, of which 19 CREs were common in the *CHI* promoters of all these plants (Figure 3). These CREs contained environmental CREs, such as dehydration-responsive CREs (CANNTG, WAACCA, and YAACKG) and light-responsive CREs (GATA, GRWAAW, and GATAA), tissue-specific CREs, such as root-specific CREs (ATATT), pollen-specific CREs (AGAAA and GTGA), seed-specific CREs (CAAT and RTTTTTR), and mesophyll-specific CREs (YACT and TAAAG). They also contained anthocyanin gene CREs (GRWAAW, CANNTG, and CNGTTR), which are the sites binding the MYB and MYC factors involved in tissue-specific anthocyanin biosynthesis genes, such as *CHS* and *CHI* (Solano et al., 1995; Stålberg et al., 1996; Hartmann et al., 2005), and TGACY connected with wounding. Compared with other plants assessed, potato has the largest number of the mesophyll-specific element YACT (35 copies) and the MYB binding site WAACCA (3 copies; Table 2). In addition to the 19 motifs, 2 specific motifs were only found in PCP, the GT-1 motif KWGTGRWAAWRW for light response and the DNA sequence TGTATATAT, which is over-represented in the light-repressed promoter (Table 2).

Nucleotide variation in PCP among different potato cultivars

Comparison of PCP nucleotide sequences among different potato cultivars was performed and the results are shown in Figure 2. Compared with the Heijingang cultivar, six nucleotide variation sites were detected in the PCP, located at the 162nd site (G>A in Désirée, Favorita, and Zhongshu 7), the 178th site (A>G in Favorita), the 573rd site (T>C in Favorita), the 1007th site (T>C in Zhongshu 7), the 989th site (A>G in Désirée), and the 1211st site (T>A in Désirée). These nucleotide variations caused the loss of several CREs in the non-pigmented-flesh cultivars Désirée, Favorite, and Zhongshu 7. For example, the G>A mutation at the 162nd site caused the loss of the mesophyll-specific element YACT and the MYB core element CNGTTR, connected with anthocyanin biosynthesis genes, in Désirée, Favorita, and Zhongshu 7; the A>G mutation at the 178th position caused the loss of the light response element TGTATATAT in Favorita; the

Table 2. Putative *cis*-regulatory elements (CREs) of the chalcone isomerase gene (*CHI*) promoter in the Heijingang potato cultivar, identified from the PlantCARE and PLACE databases.

cis-acting element	Position	Sequence	Description
TATA-box	Many	Many	Core promoter element around -30 of transcription start
CAAT-box	Many	CAAT*	Common cis-acting element in promoter and enhancer regions
Element related to anthocyanin gene			
GT1 consensus	Many	GRWAAW*	Consensus GT-1 recognition site in many light-regulated genes, including bean *CHS15* (anthocyanin biosynthetic gene); involved in SA-inducible gene expression; R = A/G; W = A/T
MYB core	(+)510, (-)157, (-)1064	CNGTTR*	Binding site for MYB proteins connected with water stress and flavonoid biosynthesis gene
MYC consensus	Many	CANNTG*	E-box; MYC binding site in anthocyanin biosynthetic genes; dehydration-responsive gene promoter sequence
PAL promoter element	(-)706	TATTTAA	DNA element in the promoter of phenylalanine ammonia-lyase (PAL) gene
Tissue-specific element			
Mesophyll-specific element	Many	YACT*	Core component of mesophyll-specific gene expression in the C4 plant *Flaveria trinervia*; Y = T/C
Seed-specific element	(+)792, (-)232, (-)1206	TAAAG*	Target site binding of Dof protein regulating guard Cell-specific gene expression
	(+)1130	AACAAAC	Endosperm-specific response element
	(+) 309	CNAACAC	Core element in storage protein genes; seed specificity
	(-) 267	CATGCAY	DNA element in seed-storage protein genes
	(+)721, (+)991, (+)1045, (+)585, (+)1085	RTTTTR*	Seed-specific element; R = A/G
	(+)1100	ACACNNG	Core sequence of Dc3 gene promoter in the carrot (D.c.); Dc3 expression is normally embryo-specific, and can also be induced by ABA
GATABOX	(-)766	GTCAT	Cis-acting regulatory element required for endosperm expression
L1BOX	Many	GATA*	Light response element; tissue-specific element
Dof binding site	(-)360	TAAATGYA	L1 box involved in L1 layer-specific expression
Organ-specific element	(-)792	ACTTTA	Dof protein binding site; required for tissue-specific expression and auxin induction
Pollen-specific element	Many	AAAGAT/CTCTT	Organ-specific elements (OSE)
Root-specific element	Many	AGAAA*	Pollen-specific element
Light response element	Many	ATATT*	Root-specific element
I box	(+)430, (-)305, (-)506, (-)1208	GATAA*	I box; light response element
Inr elements	(+)90, (+)949, (-)210, (-)1147, (-)329	YTCANTYY	Light response element
PRE	(+)734	SCGAYNRNNNNNNNNNNNNNNHD	Plastid response element; light response element
SORLIP	(+)45/(-)217, (-)256/(-)170	GCCAC/GGGCC/TGTATATAT**	Sequences Over-Represented in Light-Induced Promoters (SORLIPs)
T box	(-)130, (-)922	ACTTTG	T box; light response element
Box 4	(+)916	ATTAAT	Part of a conserved DNA module involved in light responsiveness
Box I	(+)127, (+)297, (-)326	TTTCAAA	Light responsive element
GAG-motif	(+)1239	AGAGATG	Part of a light responsive element
TCCC-motif	(+)1320	TCTCCCT	Part of a light responsive element

Continued on next page

Table 2. Continued.

cis-acting element	Position	Sequence	Description
Stress response element			
Dehydration response element	(+)782, (-)782	ACGT	ACGT sequence required for dehydration response
	(-)9, (-)47	CATGTG	MYC recognition site; dehydration response element
	(+)434, (+)1355, (+)1406	WAACCA*	MYB recognition site in dehydration-responsive gene rd22; W = A/T
	(+)1064	YAACKG*	MYB recognition site in dehydration-responsive gene rd22; Y = C/T; K = G/T
ARE	(+)1129, (-)582/(-)434, (-)1355	AAACAAA/TGGTTT	Anaerobic response element (ARE)
W-box	(+)261, (+)767, (-)7	TGAC*	W-box binding of the protein within the pathogenesis-related Class 10 (PR-10) genes
Pathogen- and salt-induced element	(+)889, (-)295, (-)450	GAAAAA	GT-1 motif involved in pathogen- and salt-induced expression
HSE	(+)147, (-)319	AAAAAATTTC	Cis-acting element involved in heat stress responsiveness
TC-rich repeats	(+)93, (-)928	ATTTTCTCCA	Cis-acting element involved in defense and stress responsiveness
WUN-motif	(+)261, (-)6	TGACY*	W box; May be involved in activation of ERF3 gene by wounding
	(-)1120	TCATTACGAA	Wound-responsive element
Hormone-induced element			
ARF-motif	(-)1002, (-)1283	TGTCTC	ARF (auxin response factor) binding site
Cytokinin-inducted element	(+)136, (-)1040	TATTAG	DNA sequence critical for cytokinin-enhanced protein
ERE	(-)326	AWTTCAAA	Ethylene-responsive element
ABA-inducted element	(+)9, (+)47	CACATG	MYC recognition site; involved in ABA-response element
	(-)268, (-)1278	CATGCA	RY repeat; ABA-response element
GARE-motif	(-)888	TTTTTTCC	Pyrimidine box; Gibberellin-response (GA) element
	(+)807, (-)755	CCTTTT	Pyrimidine box; GA-response element; sugar response element
	(+)509/(+)749, (+)1366	TCTGTTG/AAACAGA	Gibberellin-responsive element
TCA-element	(+)752	CAGAAAAGGA	Cis-acting element involved in salicylic acid responsiveness
Beta-amylase promoter region	(+)594, (-)550	TACTATT	DNA element in beta-amylase promoter
	(+)1073	TATCCA	DNA element in alpha-amylase promoters of rice
SURE	(+)678	AATAGAAAA	Sucrose responsive element (SURE)
Circadian	(+)300, (-)576	CAANNNNATC	Cis-acting element involved in circadian control
ARR1-binding site	Many	NGATT*	DNA motif binding of ARR1, a kind of response regulator; N = G/A/C/T
Dof binding site	Many	AAAG*	Core site required for binding of Dof proteins including PBF, an endosperm specific Dof protein in maize
GT-1 motif	(+)884	KWGTGRWAAWRW**	GT-1 motif; consensus sequence of rbcS BOX; K = G/T; W = A/T; R = A/G
GTGA motif	Many	GTGA*	DNA motif in the promoter of the tobacco (N.t.) late pollen gene g10
RAV1 binding site	(+)32, (-)511	CAACA*	Binding consensus sequence of *Arabidopsis* (A.t.) transcription factor, RAV1

*Conserved CREs among higher plants tested. **Specific CREs only found in potato.

T>C mutation at the 573rd position caused the loss of NGATT in Favorita; the T>C mutation at the 1007th position caused the loss of the light response element GT-1 in Zhongshu 7; the A>G mutation at the 989th position caused the loss of the root-specific motif ATATT in Désirée; and the T>A mutation at the 1211st position caused the loss of the GATA box, GRWAAW, and GATAA, connected with light and tissue specificity, in Désirée. In contrast, the G>A mutation at the 162nd position resulted in the Désirée, Favorita, and Zhongshu 7 cultivars gaining the CAAT box and the ATATT motifs (Figure 2). These results show that cultivars with higher levels of anthocyanins have more CREs related to tissue-specificity, light response, and anthocyanin biosynthesis.

Figure 2. Sequence alignment of the chalcone isomerase gene (*CHI*) promoter, StCHIP1, in different potato cultivars: Heijingang (purple skin and flesh), Désirée (red skin and yellow flesh), Zhongshu 7 (yellow skin and white flesh), and Favorita (yellow skin and flesh). The 665 bp sequence (StCHIP2) is italicized. Capital and small letters in bold type indicate single nucleotide differences in their respective positions. Corresponding motifs isolated from the PLACE database are shown in rectangular boxes. Compared with the Heijingang cultivars, cultivars with non-colored flesh have lost motifs (boxes with thick borders) and gained motifs (boxes with thin borders). Letters underlined denote putative transcription start sites (TSS).

Analysis of PCP activity

Whether nucleotide variations in PCP influence promoter activity was investigated further. The two PCP sequences, StCHIP1 and StCHIP2, were inserted into the binary vector pCMBIA1304 in place of the CaMV35S fragment upstream of *GFP* and *GUS*, respectively. The constructs, p1304-StCHIP1 and p1304-StCHIP2, were then introduced into onion epidermal cells and potato tuber flesh using an *Agrobacterium*-mediated method (Figure 4). Cytological observation showed that the green fluorescence signal was present in onion cells (Figure 5). X-gluc staining revealed that the GUS gene was expressed under the direction of StCHIP1 and StCHIP1 in the cultivars Heijingang, Désirée, HQ, Favorita, and Hui-2 (Figure 5). These results suggest that the single nucleotide variations in PCP were not the primary cause of the undetected expression levels of the CHI gene in potato cultivars with non-colored flesh.

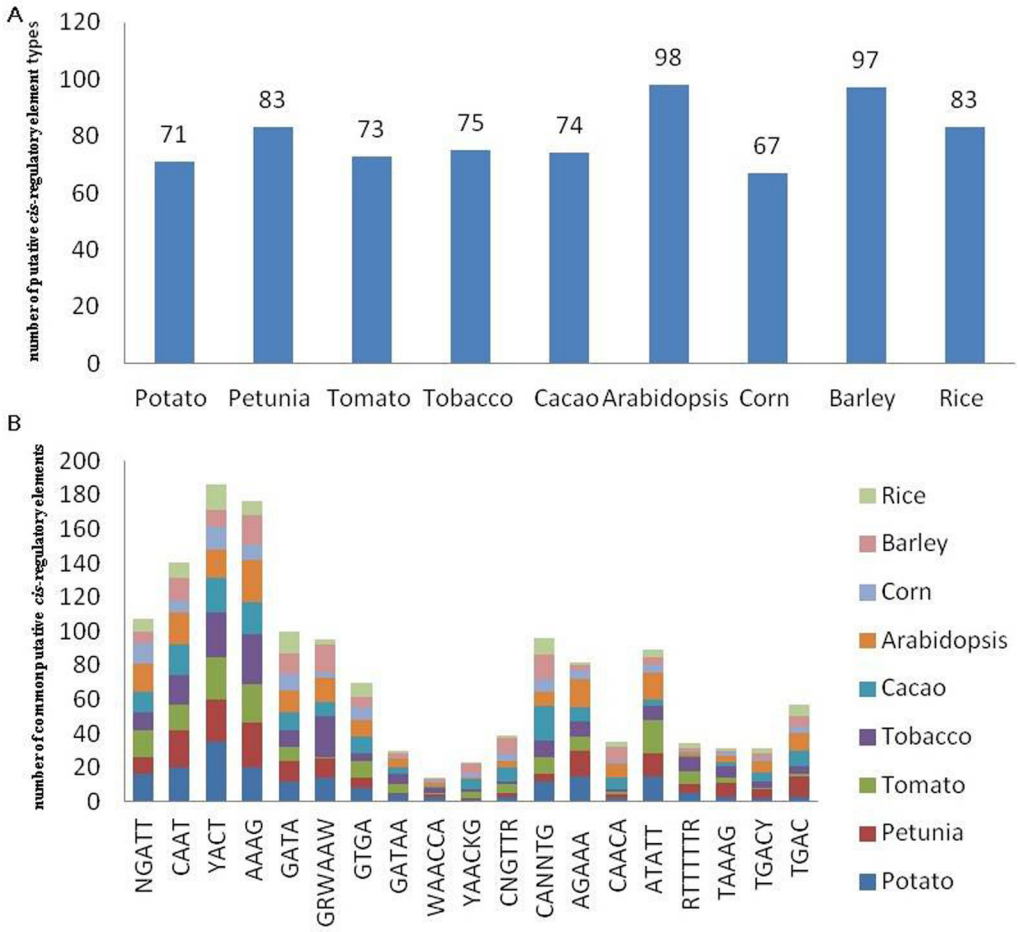

Figure 3. Putative *cis*-regulatory elements found in chalcone isomerase gene (*CHI*) promoters in 9 higher plants. (**A**) total number of putative *cis*-regulatory element types, (**B**) Occurrence of 19 common *cis*-regulatory elements.

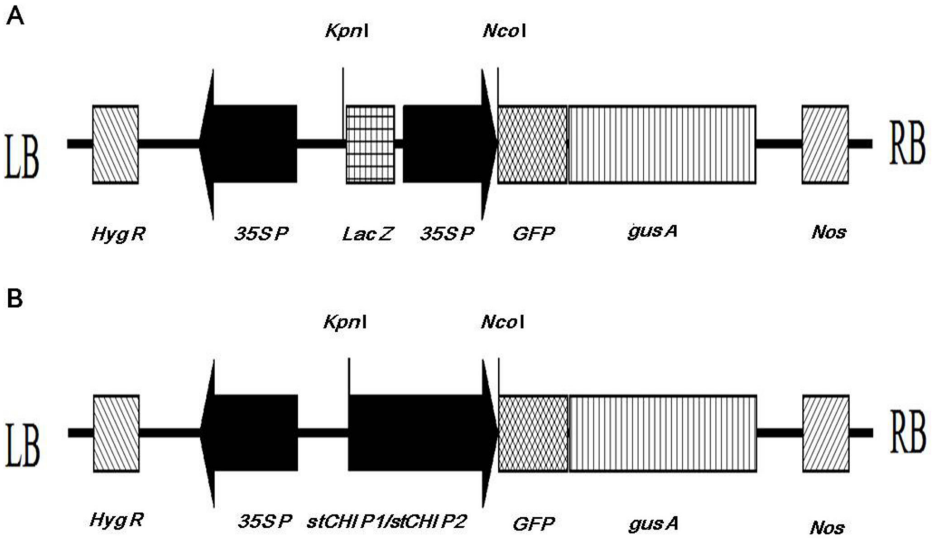

Figure 4. Schematic representation of the T-DNA region of the binary vector. The 1425 and 665 bp chalcone isomerase gene (*CHI*) promoter fragments amplified from the Heijingang cultivar were used to replace the 35S promoter upstream of the GFP gene in pCAMBIA1304 (**A**), and the recombinant vectors were named p1304-StCHIP1 and p1304-StCHIP2, respectively (**B**). LB, T-DNA left border; Hyg R, hygromycin (R); 35S P, CaMV 35S promoter; lacZ, lacZ lapha; GFP, green fluorescent protein gene; gusA, β-Glucuronidase gene; nos, Nos poly-A; RB, T-DNA right border.

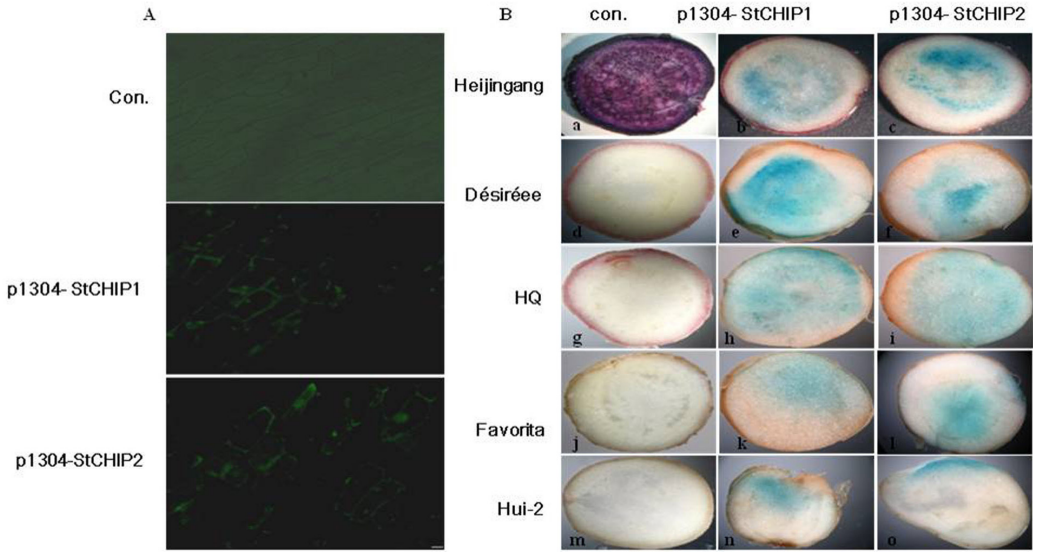

Figure 5. Transient expression of a green fluorescent protein gene (*GFP*) and a β-glucuronidase gene (*GUS*) driven by potato chalcone isomerase gene (*CHI*) promoters. (**A**) GFP expression in epidermal onion cells is driven by the vectors p1304-StCHIP1 and p1304-SCHIP2. Con, denotes non-tested onion epidermis control. (**B**) GUS expression in pigmented and non-pigmented potato tuber flesh. Panels a, d, g, j, and m corresponded to non-tested (control) potato flesh; panels b, e, h, k, and n corresponded to GUS expression driven by p1304-StCHIP1; and panels c, f, i, l, and o corresponded to GUS expression driven by p1304-StCHIP2.

DISCUSSION

The promoter is an essential regulatory element of gene expression. In the past, the CHI gene has been isolated from many plants, but its promoter has only been reported in *P. hybrida*, where *CHI* promoter sequences were isolated and the *cis*-element 'anther box' was discovered, revealing high homology among flavonoid gene promoters (van Tunen et al., 1989). In this study, PCP was cloned from 4 different potato cultivars (Heijingang, Zhongshu 7, Désirée, and Favorita; Figure 2). Sequence analysis showed that PCP exhibits high identity (~100%) among potato cultivars but low identity (~39 to 60%) among higher plants tested (data not shown), indicating that PCP is conserved in potato cultivars but variant among higher plants. To the best of our knowledge, similar studies investigating PCP have not been conducted; related research was about CHI gene observed to have low similarity in different dicot groups (~42 to 65%), except for the closely related legumes *Medicago sativa* and *Phaseolus vulgaris* (~80%; Druka et al., 2003).

In general, plant promoters consist of two types of elements: core elements and regulatory elements. The former influence promoter activity and the latter determine gene function. CHI is an important enzyme in anthocyanin biosynthesis and is often induced by environmental conditions (McKhann and Hirsch, 1994; Fofana et al., 2002; Pandey et al., 2014), thus, the *CHI* promoter inevitably possesses corresponding functional elements in its sequence structure. In this study, we observed that, aside from the core elements TATA-box and CAAT-box, there are several functional CREs in potato, which are related to tissue specificity, light response, stress, anthocyanin structural genes, etc.

Anthocyanin structural genes are uniformly controlled by special transcription factors in the plant metabolic pathway (Bovy et al., 2002; Jung et al., 2009), among which highly conserved tissue-specific motifs are isolated (van Tunen et al., 1989; Hartmann et al., 2005). Here, we isolated four important CREs connected with anthocyanin synthesis genes, including the GT-1 consensus found in the bean CHS15 promoter, the phenylalanine ammonia-lyase (PAL) promoter sequence found in the promoter of the PAL gene, which is upstream of *CHI* in the flavonoid biosynthetic pathway, the MYC consensus (E-box), which is the MYC recognition motif known in the anthocyanin gene promoter, and the MYB core found in the promoter of the *Petunia* CHSJ gene and bond of MYB.Ph3 involved in regulation of flavonoid biosynthesis (Solano et al., 1995). These results suggest that *CHI* and other anthocyanin structural genes may be regulated by common transcription factors, such as MYB and MYC proteins, in potato (Table 2).

MYB- and MYC-type proteins are important regulatory factors in regulating anthocyanin structural genes during tissue-specific accumulation of anthocyanins (Bovy et al., 2002; Shimizu et al., 2011; Pandey et al., 2014). The MYB transcription factor encoded by AN2 from potato influences pigment accumulation in the tubers (Jung et al., 2009). Anthocyanin accumulation is affected by many factors, including stress and light, and by controlling regulatory factors, such as MYB proteins (Procissi et al., 1997; Fofana et al., 2002). In a previous study, the *CHS* promoter showed activity under control of the MYB factor in *Arabidopsis thaliana* under light (Hartmann et al., 2005). Here, several putative motifs for defense were isolated in PCP, including two tissue-specific anthocyanin gene elements, MYB core for water stress and MYC consensus (E-box) for dehydration. Many other important light-responsive motifs were also found such as GATA box and the GT1 consensus in high occurrence (Figure 3). The environmental motifs required for light signal activation and stress may influence *CHI* expression by binding to anthocyanin regulatory factors.

Comparison of CREs among higher plants tested revealed 19 conserved CREs, consisting

of W-box, E-box, I-box, MYB, and MYC motifs involved in flavonoid synthesis, tissue specificity, light response, hormone response, and defense response (Figure 3). Interestingly, several CREs were mainly found in the Solanaceae, including potato, and were related to the promoter of the PAL gene, L1 layer-specific expression, seed-specific motif RY repeats, MYC recognition sites, dehydration response elements, ABA elements, GA elements, etc. (data not shown). In PCP, two light response motifs, KWGTGRWAAWRW and TGTATATAT, were found to only exist in the potato *CHI* promoter (Table 2). Therefore, PCP has retained several conserved sequence motifs of the CHI gene and produced its own specific motifs during its evolutionary history.

In biological evolution, nucleotide evolution caused by natural mutation can occur at any position on the DNA, and this mutation may be significant in biology (Druka et al., 2003). In previous studies, the eukaryotic promoter was changed by modifying 1 to 2 bases in the binding site, which affects gene expression levels, and proanthocyanidin-free barley mutants showed drastically reduced flavonoid levels compared to the wide-type control following single base changes in *CHI*, demonstrating that single nucleotide variations can alter gene expression (Druka et al., 2003; Rajkumar et al., 2013). In potato, tubers with different colored flesh result from anthocyanin accumulation. In order to determine the role of PCP in anthocyanin accumulation, we analyzed simple nucleotide variation in PCP in different potato cultivars. Nucleotide variation was evident at 6 sites in PCP, resulting in changes in CREs (Figure 2), similar to the variability in the 5'-UTR region of *CHI* represented by single nucleotide polymorphisms in the common bean (McClean and Lee, 2007). However, these single nucleotide variations did not appear to influence promoter activity, based on our PCP transient expression results (Figure 5). From a promoter perspective, this result revealed that PCP was not a decisive factor in anthocyanin metabolism in potato cultivars with non-colored tubers and silence of CHI gene expression may be attributed to special regulatory factors.

In conclusion, we isolated the potato *CHI* promoter from potato cultivars and analyzed its structural characteristics and differences in CREs between potato and other higher plants. We also assessed variation in PCP among different potato cultivars, revealing that PCP may be involved in growth and development, anthocyanin biosynthesis, and stress response, etc. However, we determined that single nucleotide variations in PCP were not responsible for silencing the CHI gene in the tested potato cultivars with non-colored flesh. These results may be helpful in furthering our understanding of PCP evolution and transcriptional regulation.

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