



Analysis of tandem repeat units of the promoter of capsanthin/capsorubin synthase (*Ccs*) gene in pepper fruit

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Abstract Capsanthin/capsorubin synthase (*Ccs*) gene is a key gene that regulates the synthesis of capsanthin and the development of red coloration in pepper fruits. There are three tandem repeat units in the promoter region of *Ccs*, but the potential effects of the number of repetitive units on the transcriptional regulation of *Ccs* has been unclear. In the present study, expression vectors carrying different numbers of repeat units of the *Ccs* promoter were constructed, and the transient expression of the β -glucuronidase (*GUS*) gene was used to detect differences in expression levels associated with the promoter fragments. These repeat fragments and the plant expression vector PBI121 containing the 35s CaMV promoter were ligated to form recombinant vectors that were transfected into *Agrobacterium tumefaciens* GV3101. A fluorescence spectrophotometer was used to analyze the expression associated with the various repeat units. It was concluded that the constructs containing at least one repeat were associated with *GUS* expression, though they did not differ from one

another. This repeating unit likely plays a role in transcription and regulation of *Ccs* expression.

Keywords *Ccs* · *GUS* · Promoter · Tandem repeat units · Transient expression

Abbreviations

<i>Ccs</i>	Capsanthin/capsorubin synthase
<i>GUS</i>	β -Glucuronidase
PL0	Vector carrying full-length <i>Ccs</i> promoter fragments with W box and AACA motif
PL1	Vector carrying full-length <i>Ccs</i> promoter fragments without W box or AACA motif
PL2	Vector carrying two repeat units of the promoter fragment
PL2-1	Vector carrying one repeat unit of the promoter fragment
PL3	Vector carrying no repeat units of the promoter fragment

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Introduction

Transcriptional promoters are regions of DNA that initiate transcription of specific downstream genes; generally, they are located quite near the transcription start site of the genes they regulate (Smale and Kadonaga 2003). As an important *cis*-regulatory element, promoters are involved in the regulation of gene transcription and expression (Segal et al. 2002), and a promoter can activate RNA polymerase to interact with the DNA template and begin transcription (Smale and Kadonaga 2003).

Capsanthin/capsorubin synthase (*Ccs*) gene is a key gene that regulates the synthesis of capsanthin in pepper fruits, which are unable to develop red coloration if *Ccs*

has been deleted or contains a loss of function mutation. The promoter of *Ccs* gene is distinctive; it contains three tandem repeat units of 176 bp in *Ccs* promoter region. These repeat units start 165 bp upstream of the *Ccs* gene transcription start site, which is the main area in which promoters occur (Li et al. 2013). In plants, it is very rare that a tandem repeat appears within 165 bp upstream of a promoter. The existence of these tandem repeats may have important significance for capsanthin synthesis in pepper fruits.

In this study, ‘the ‘Yiduhong’ pepper cultivar was used as the experimental organism to examine the effect of the repeat units within the *Ccs* promoter; the main research objective was to elucidate the function of different numbers of repetitive units of the promoter. Three main experiments were conducted to fulfil this objective: (1) construction of expression vectors of different repeat unit lengths, (2) *GUS* reporter gene expression levels were assayed for transient expression, and (3) the expression activity associated with promoters of different repeat units was analyzed using a fluorescence spectrophotometer to detect differences among *Ccs* promoter repeat unit lengths in pepper fruits.

Materials and methods

Plant and expression vector materials

Seeds for the ‘Yiduhong’ pepper cultivar (*Capsicum annuum*) and the ‘Micro-Tom’ tomato (*Solanum lycopersicum*) cultivar were provided by the *Capsicum* Research Group in the College of Horticulture at Northwest A&F University, Shaanxi Province, People’s Republic of China. The PBI121 expression vector was also provided by the *Capsicum* Research Group at Northwest A&F University.

Construction of the cloning vector

PCR amplification bands (L1, L2, L2-1, and L3) were cut and purified with a Wizard PCR purification kit (TianGen Co., Ltd., Beijing, China). The purified PCR products (i.e., L1, L2, L2-1, and L3) were cloned into a pMD19-T vector and transformed into *E. coli* DH5 α . The positive clones were screened on LB media plates with ampicillin, and detected by colony PCR. The recombinant plasmids (pMD-L0, pMD-L1, pMD-L2, pMD-L2-1, and pMD-L3, respectively) were digested with both *Bam*HI and *Hind*III. The fragments were identified and sent to Sangon, Shanghai Co., Ltd. for sequencing.

Construction of the expression vector

Extraction of genomic DNA and detection in pepper fruits

Seeds were removed from pepper fruits, and five fruit pericarps from the same cultivar were mixed together and triturated in liquid nitrogen. A cetyltrimethylammonium bromide (CTAB) extraction solution was added to the resulting powder. DNA was extracted from samples following the procedure described by Tian et al. (2015). The DNA samples were dissolved in Tris–EDTA (TE) buffer solution and stored at $-20\text{ }^{\circ}\text{C}$ before gene identification. DNA primers were designed using Primer 5 software based on pepper gene sequences obtained from GenBank (*Ccs*: GenBank No. X77289.1).

Cloning Ccs promoters with various numbers of repeat units

A primer was designed based upon sequences (GenBank) from the *Ccs* gene promoter in *Capsicum* (Supplementary Table 1). The repeat units of the promoters of various lengths were amplified using PCR. The PCR products were detected using agarose gel electrophoresis, purified with a wizard PCR purification kit (Promega, USA) and then cloned into a pMD19-T vector using T4 DNA ligase. The cloned vector was transformed into *E. coli* DH5 α . The recombinant clones were screened and identified using PCR, restriction endonuclease digestion analysis, and sequencing. The different repeat promoter expression vectors were constructed from DNA fragments that were in accordance with the known promoter fragments (Fig. 1).

Construction of cloning vectors from Ccs gene promoters with various numbers of repeats

A combination of the PBI121 plasmid, the expression vector (PBI121), and the cloning vector carrying the target sequence were digested with double-strand exonucleases and then incubated for 10 h at $37\text{ }^{\circ}\text{C}$ in a water bath. The enzyme product was recovered using a DNA extraction kit [TaKaRa Biotechnology (Dalian) Co., Ltd]. The target gene fragments were ligated to the PBI121 expression vector with T4 DNA ligase (Supplementary Fig. 1–5) while incubated at $16\text{ }^{\circ}\text{C}$ overnight and then transformed into *E. coli* DH5 α . The target gene fragments were screened and identified by colony PCR.

Construction of the plant expression vector

The enzymatic reaction system is presented in Supplementary Table 2. The plant expression vectors (i.e., PL0, PL1, PL2, PL2-1, and PL3) were constructed by cloning

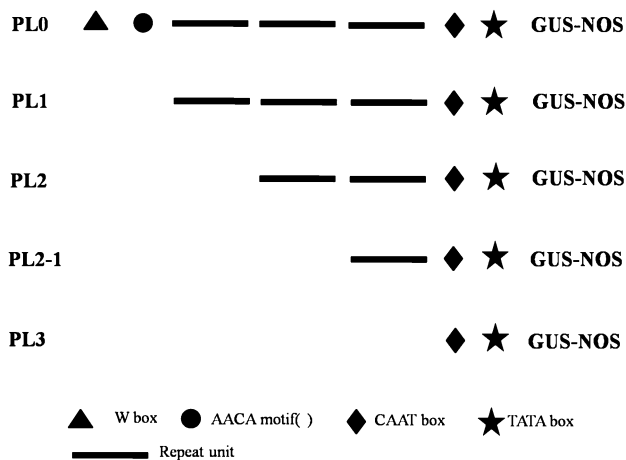


Fig. 1 The recombinant PBI121 vector containing tandem repeat sequences of the *Ccs* gene promoter. PL0, PBI121 vector carrying full-length *Ccs* promoter fragments with a W box and AACA motif; PL1, PBI121 vector carrying full-length *Ccs* promoter fragments without a W box and AACA motif; PL2, PBI121 vector carrying two repeat units of the promoter fragment; PL2-1, PBI121 vector carrying one repeat unit of the promoter fragment; PL3, PBI121 vector carrying zero repeat units of the promoter fragment

the fusion gene into the PBI121 plasmid. The enzymatic reaction system is presented in Supplementary Table 3. The expression vectors were constructed and confirmed by restriction enzymes digests followed by fragments sequencing. The correct target fragments were extracted and the plasmids were named PL3, PL2, PL1, PL2-1, and PL0. The recombinant promoter expression vectors were introduced into *Agrobacterium tumefaciens* GV3101 using the freeze–thaw method (Wang et al. 2013). The promoter regions from the target repetitive units were detected by PCR, and the bacterial cultures were preserved at $-80\text{ }^{\circ}\text{C}$ for further use.

The preparation of *Capsicum* and tomato materials

Capsicum and tomato seeds were germinated as previously described (Tian et al. 2014). The germinated seeds were sown into wells on a plate. When three to four true leaves were present, the seedlings were transplanted into plastic pots and maintained under field conditions until green fruits developed. These potted *Capsicum* and tomato plants were then moved into controlled climate conditions.

Transient expression of the *GUS* reporter gene in *Capsicum* and tomato fruits with various numbers of promoter repeats

A solution of *Agrobacterium* (20 μL) containing the *Ccs* gene promoter fragment was transferred into 20 mL of LB

liquid medium (50 mg/L Kan, 50 mg/L Gen, 10 mg/L Rif) for overnight incubation at $28\text{ }^{\circ}\text{C}$ while being centrifuged at $35\times g$ (Kapila et al. 1997). After overnight incubation, the *Agrobacterium* culture was centrifuged at $2504\times g$ at room temperature conditions for 10 min. The concentration of the bacterial suspension was diluted with 10 mL of penetration buffer solution (0.5% D-glucose, 50 mM MES, 2 mM $\text{Na}_3\text{PO}_4\cdot 12\text{H}_2\text{O}$, 0.1 mM AS) (Sparkes et al. 2006) and the resulting supernatant was discarded. These steps were repeated in order to remove as much excess LB liquid medium as possible. Then, the absorbency at 600 nm of 2.5 mL of the resuspended *Agrobacterium* solution containing the *Ccs* gene promoter fragments was measured. The solution was then diluted with penetration buffer to $\text{OD}_{600} = 0.5$ for subsequent genetic transformation.

Capsicum and tomato plants were placed in controlled climate conditions with high intensity irradiation for 1 h prior to injection in order to allow the plants to acclimatize to the new environment. Green fruits of same age were selected and tagged. Fruit stalks were selected as the injection location and the *Agrobacterium* solution (0.8 mL) was injected into *Capsicum* and tomato fruits with a 1 mL syringe. The bacterial culture was introduced slowly into *Capsicum* and tomato fruits. Five fruits were selected for each bacterial culture carrying a different *Ccs* gene promoter fragment, and each treatment was repeated five times. After inoculation with *Agrobacterium*, *Capsicum* and tomato plants were transferred into a controlled environment at $28\text{ }^{\circ}\text{C}$ with a 14 h day/10 h night photoperiod for two days. These processed materials were divided into two parts, one for *GUS* histochemical staining and the other for the determination of *GUS* activities.

GUS histochemical staining

Capsicum and tomato pericarp tissue was smooth and very dense. To facilitate *GUS* histochemical staining, the experimental tomato materials were divided into thin layers and placed into a sterilized Petri dish containing *GUS*-staining liquid (Supplementary Table 4). The Petri dishes were sealed with plastic film and promptly placed in a homoeothermic incubator at $37\text{ }^{\circ}\text{C}$ for 24 h. The *GUS*-staining solution was removed and replaced with 70% ethyl alcohol to remove excess pigments and was then incubated at $37\text{ }^{\circ}\text{C}$ for 6–8 h. After the initial incubation, the 70% ethyl alcohol was replaced with 90% ethyl alcohol and incubated at $37\text{ }^{\circ}\text{C}$ for 10 h. The 90% ethyl alcohol was removed, and changes in chlorophyll were observed. Then, the plates were flushed with 90% ethyl alcohol continuously for an additional 10–12 h until no chlorophyll remained. The material was then placed on a light box and photographs were taken (Subramanyam et al. 2013).

The determination of *GUS* activities

To prepare a *GUS* enzyme extract, 0.1 g of tomato fruit that had been previously treated with *GUS* histochemical stain was ground in liquid nitrogen using a mortar and pestle. *GUS* extraction buffer was added to the mortar, and grinding was continued until the material became a uniform powder. The powder was then poured into a microcentrifuge tube and shaken for 5 min. Then the powder was centrifuged at 12,000 rpm for 10 min, and the resulting supernatant was removed and stored at 4 °C.

To conduct the enzymatic reaction, 4-methylumbelliferone (4-MU) was used for fabrication of the fluorescence standard curve. Then 1 mM 4-methylumbelliferyl glucuronide (MUG) was added into the extraction buffer to form the reaction buffer. The reaction buffer was placed in a water bath at 37 °C for 10 min, and 20 µL of *GUS* extraction buffer was added into the 200 µL reaction buffer volume and mixed thoroughly. Then, 100 µL of mixture buffer was removed immediately and added to 900 µL of 0.2 M Na₂CO₃ to terminate *GUS* activity. The remaining reaction mixture volume was reacted at 37 °C for 30 min, and then 900 µL of 0.2 M Na₂CO₃ was added to terminate the reaction. The fluorescence intensity, 4-MU content, and *GUS* activity were then measured (Yang et al. 2000).

Statistical analysis

SAS 6.12 software (SAS Institute, Gary, NC, USA) was used for data analysis. Duncan's multiple-range test was used, and Least significant ranges (LSR) analysis was conducted at $P < 0.05$.

Results and discussion

DNA extraction of pepper fruits

The extracted DNA was not contaminated with RNA, protein, or phenol and the target bands were clearly visible (Supplementary Fig. 6).

PCR amplification of various promoter fragments

The PL0 bands (full-length promoter fragments) and PL3 bands (proximal promoter fragments) were clearly visible on 1% agarose gel electrophoresis. The PL0 fragment was about 1000 bp, which was consistent with the target fragment length (Fig. 2). Sequence analysis of different repeat units of the *Ccs* promoter had revealed that there were no frameshifts and/or base mutations. This verified that the promoters of different repeat unit lengths (i.e., PL0 and PL3) were obtained successfully (Fig. 2); however, the

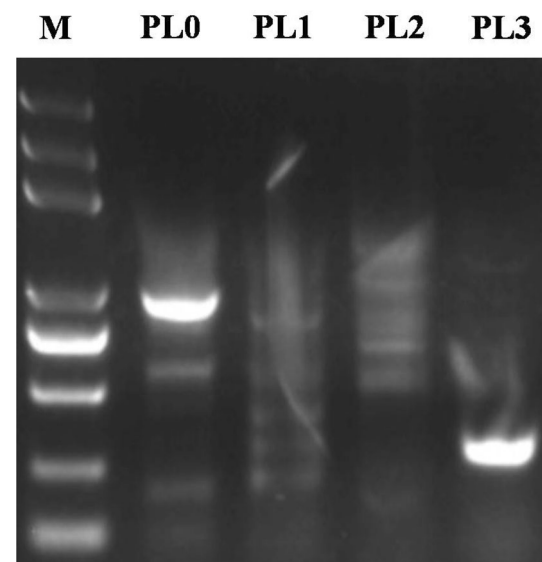


Fig. 2 Different specific tandem repeat regions for PCR amplification

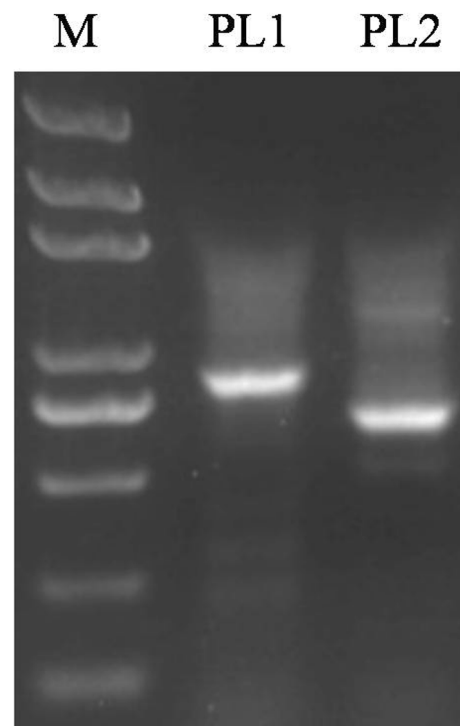


Fig. 3 PCR amplification of the PL1 and PL2 tandem repeat regions

PL1 and PL2 bands were faint (Fig. 2), and PCR amplification was therefore repeated for PL1 and PL2 (Fig. 3). PL0 was used as a template to re-amplify PL1 and PL2, and the target bands for PL1 and PL2 were thus obtained. Sequence analysis of PL1 and PL2 revealed that there were no frameshift and/or base mutations. This showed that promoters of intermediate repeat unit lengths (i.e., PL1 and PL2) were successfully obtained (Fig. 3).

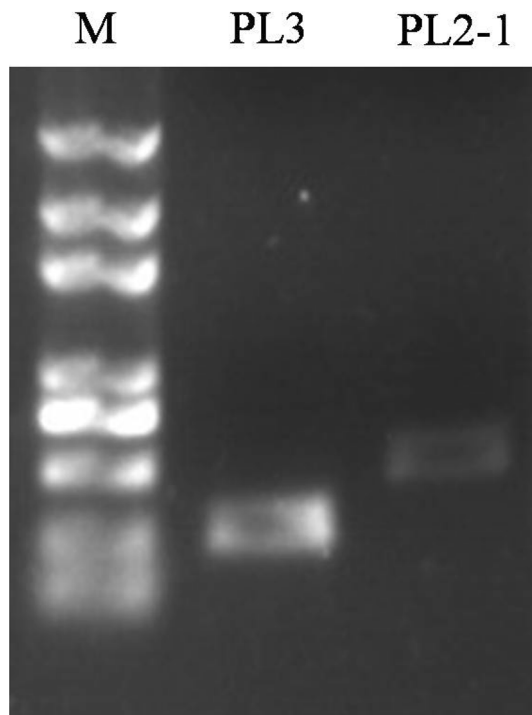


Fig. 4 PCR amplification of the PL2-1 and PL3 tandem repeat regions

The same method was followed, and PL2 PCR amplicons were used as the template to amplify PL2-1, thus obtaining the PL2-1 target band. PL2-1 was digested ligated to the pMD19-T vector, and transformed into *E. coli* DH5 α . Sequence analysis of PL2-1 revealed that there were no frameshift and/or nucleotide base mutations (Fig. 4).

Detection of expression vectors from promoter repeats

Five expression vectors carrying different repeat units were digested with *Bam*HI and *Hind*III, respectively, and their banding patterns were analyzed after 1% agarose gel electrophoresis. Their lengths were approximately 1000, 900, 700, 500, and 380 bp, respectively (Fig. 5). Sequencing results of the DNA bands were in accordance with the known different repeat units of the *Ccs* promoter. Sequence analysis of expression vectors carrying different repeat units revealed that there were no frameshift and/or nucleotide base mutations. This confirmed that the expression vectors were successfully constructed (Fig. 5).

Transient expression in mature green *Capsicum* fruits

Each partial and full-length *Ccs* promoter was expressed normally in mature green *Capsicum* fruit (Fig. 6).

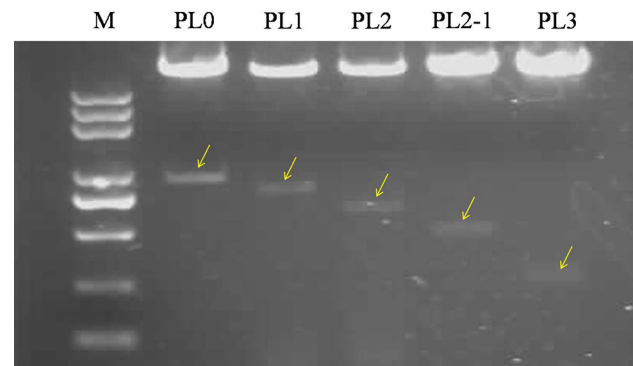


Fig. 5 Expression vectors digested with *Bam*HI and *Hind*III. Expression vectors carrying different repeat units (i.e., PL0, PL1, PL2, PL2-1, and PL3) were digested with *Bam*HI and *Hind*III, respectively, and were analyzed using 1% agarose gel electrophoresis. The lengths of these bands were approximately 1000, 900, 700, 500, and 380 bp, respectively

Transient expression various promoters mediated by *Agrobacterium* in mature green tomatoes

Each variable repeat *Ccs* promoter (i.e., PL0, PL1, PL2, and PL2-1) was expressed normally in mature green tomato fruits except for PL3; the fruit carrying PL3 did not properly stain blue because there was no repetition of the *Ccs* gene promoter in PL3. These highly similar repetitive sequences (i.e., PL1, PL2, PL2-1, and PL0) were able to promote *Ccs* expression in the fruits. No clear differences were observed among the various levels of blue staining in PL0, PL1, PL2, and PL2-1 (Fig. 7).

A comparison of Figs. 6 and 7, reveals that pepper and tomato yielded similar expression results. The ‘Micro-Tom’ tomato cultivar has a short life cycle as it is a dwarf plant, thus, the fruiting period is early and uniform, ‘Micro-Tom’ fruits are therefore an ideal material for studying transient expression.

Activity analysis of different repetitive units

To further confirm the differences among PL0, PL1, PL2, and PL2-1 constructs, an activity analysis of *GUS* expression for constructed with different numbers of promoter repeats was conducted. Promoters consisting of each of the various repetitive units were active, and there were no obvious differences among PL0, PL1, PL2, and PL2-1. However, the activity of PL3 was extremely low (Fig. 8).

Promoter function studies have primarily focused on the construction of deletions, mutations, and function verification of promoter regions. Usually, the expression level of the *GUS* reporter gene decreases as promoters are shortened (Li et al. 2012; Liang et al. 2012). Han et al. (2007) constructed a series of deletion fragments of the LRP16 promoter and conducted a promoter activity analysis that

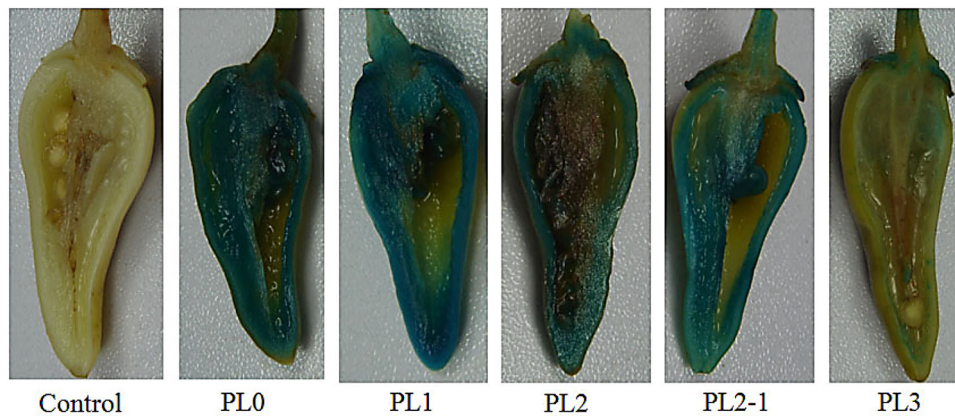


Fig. 6 Expression analysis of tandem repeat promoter regions in pepper fruits at the green ripening stage. Control, the control group (PBI121 vector without any *Ccs* promoter repeat units); PL0, PBI121 vector carrying full-length *Ccs* promoter fragments with a W box and AACA motif; PL1, PBI121 vector carrying full-length *Ccs* promoter fragments without a W box and AACA motif; PL2, PBI121 vector

carrying two repeat units of the promoter fragment; PL2-1, PBI121 vector carrying one repeat unit of the promoter fragment; and PL3, PBI121 vector carrying zero repeat units of the promoter fragment. The *GUS* reporter gene in mature, green pepper fruits was expressed by all containing *Ccs* promoter fragments containing repeat units (PL0, PL1, PL2, and PL2-1)

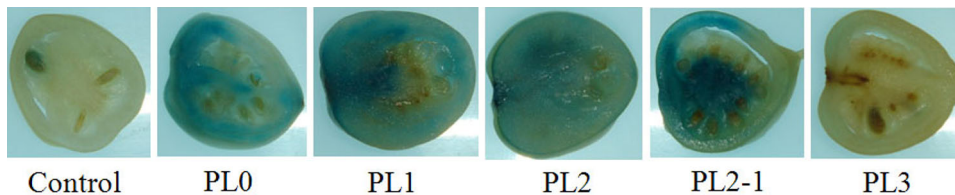


Fig. 7 Expression analysis of different specific tandem repeats in the promoter region in 'Micro-Tom' fruits. Control, the control group (PBI121 vector without any *Ccs* promoter repeat units); PL0, PBI121 vector carrying full-length *Ccs* promoter fragments with a W box and AACA motif; PL1, PBI121 vector carrying full-length *Ccs* promoter fragments without a W box and AACA motif; PL2, PBI121 vector

carrying two repeat units of the promoter fragment; PL2-1, PBI121 vector carrying one repeat unit of the promoter fragment; PL3: PBI121 vector carrying zero repeat units of the promoter fragment. The *GUS* reporter gene in mature, green tomato fruits was expressed by all plants containing *Ccs* repeat units (PL0, PL1, PL2, and PL2-1)

showed that the LRP16 promoter was a typical type II promoter with a maximally active region 200–600 bp upstream (Han et al. 2007). Liang et al. (2012) found that all deletion fragments of a promoter could be detected from the activity of the *GUS* reporter gene, but the activity of deletion fragments differed with construct lengths. Li et al. (2012) found that *GUS* expression activity increased as the 5' terminal deletions typically increased *GUS* activity. Similarly, Li et al. (2013) revealed that the expression of a gene became weaker with the decreases in promoter length. Our results indicated that the *GUS* activity associated with the *Ccs* promoter repeats were not related to promoter length. No apparent differences were observed in the activity of *GUS* between plants with the deletion of the full-length promoter, PL0 (which included three repeat units), and the promoter with the fewest repeats PL2-1 (with one repeat unit; Fig. 8).

In our experiment, the expression level of the *GUS* reporter gene did not decrease with the deletion promoter deletion size. The different repeat units of the *Ccs* gene promoter were not obviously associated with differences

in *GUS* activity. Previous studies focusing on the *Ccs* gene promoter did not report differences in effects associated with the number of repeat units in the promoter region. Any sequence deletion in a promoter changes the structure and function of the promoter (Han et al. 2007; Liang et al. 2012; Li et al. 2013); however, every repeat unit of the *Ccs* gene promoter in *Capsicum* was a relatively complete repetitive sequence, although the partial sequences did not compose the full-length promoter. This complicated our results as there were no obvious differences in *GUS* activity among the repetitive units of the *Ccs* gene promoter. Specific repetitions of the *Ccs* gene promoter appear to be redundant. Each deletion in the series of partial *Ccs* gene promoters contained at least one complete repeat unit. The expression level of the *GUS* reporter gene showed that a single repeat was sufficient to ensure *Ccs* gene expression, in other words, each repeat was an adequate substitute that ensured normal *Ccs* gene expression for color formation in pepper fruits. However, for other gene promoters, totally lacking repeated units, the partial deletions of promoter sequences rendered their

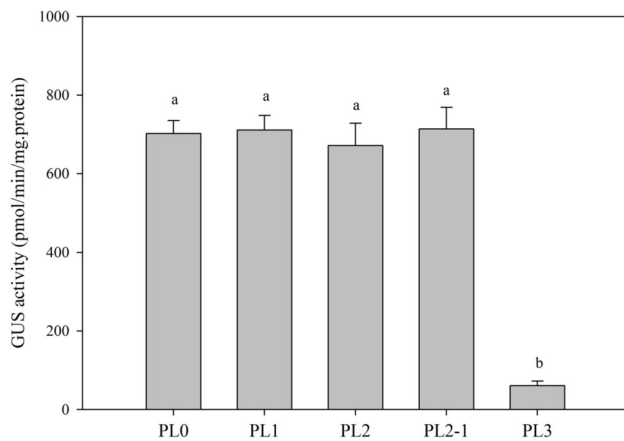


Fig. 8 Analysis of *GUS* activity in ‘Micro-Tom’ fruits for different specific repeat sequences of the *Ccs* gene promoter. PL0, PBI121 vector carrying full-length *Ccs* promoter fragments with a W box and AACA motif; PL1, PBI121 vector carrying full-length *Ccs* promoter fragments without a W box and AACA motif; PL2, PBI121 vector carrying two repeat units of the promoter fragment; PL2-1, PBI121 vector carrying one repeat unit of the promoter fragment; and PL3, PBI121 vector carrying zero repeat units of the promoter fragment. Activity of the *GUS* reporter gene was observed in plants with all numbers of *Ccs* promoter repeat units; however, there were no obvious differences among PL0, PL1, PL2, and PL2-1 plants in expression level, while *GUS* expression in PL3 plants was extremely low

structure incomplete in comparison with a single-repeat *Ccs* gene promoter, which thus impaired promoter function (Liang et al. 2012; Li et al. 2013). Our study provides the basis for further research on *cis*-regulatory elements of the *Ccs* promoter and the effect of *Ccs* expression on pepper fruit color.

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