Plant Science xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Construction of efficient, tuber-specific, and cold-inducible promoters in potato

Meng Li[a,b], Conghua Xie[a], Botao Song[a], Yongbin Ou[a], Yuan Lin[a], Xun Liu[a], Huiling Zhang[a], Jun Liu[a,*]

[a] National Center for Vegetable Improvement (Central China)/Key Laboratory of Horticulture Plant Biology (Huazhong Agricultural University), Ministry of Education/Huazhong Agricultural University, Wuhan 430070, People's Republic of China
[b] Central South University of Forestry and Technology, Changsha, People's Republic of China

ARTICLE INFO

Article history:
Received 11 December 2014
Received in revised form 21 February 2015
Accepted 21 February 2015
Available online xxx

Keywords:
Potato (*Solanum tuberosum* L.)
Promoter
Cold-inducible
C-repeat/dehydration-responsive element
Flanking sequence

ABSTRACT

Promoter activity is crucial for precise gene expression. Previously, a synthetic tuber-specific and cold-inducible promoter, pCL, containing a C-repeat/dehydration-responsive element (CRT/DRE) cassette and a tuber-specific fragment, was constructed in order to regulate cold-induced sweetening (CIS) in potatoes. However, the utility of pCL is limited due to its low activity. To improve its inducibility in response to low temperatures, we modified the CRT/DRE and flanking sequences. In particular, promoter activity was significantly improved by site-specific mutation of flanking sequences next to the core element (CCGAC) of CRT/DRE. We also inserted a modified CRT/DRE cassette into pCL; although this enhanced activity, it was not more effective than mutation of the flanking sequences. Indeed, up to 20-fold enhanced pCL activity could be achieved by replacing the CRT/DRE cassette in pCL with tandem repeats of two mutated CRT/DRE cassettes. This improvement was due to an enhanced affinity between the CRT/DRE cassette(s) and the StCBF1 transcription factor. Together, these data suggest that altering the structure of CRT/DRE can enhance CBF-related transcription complex formation and thus improve the activity of this cold-inducible promoter.

1. Introduction

Genetic engineering offers intriguing advantages for an in-depth understanding of gene function and the networks they are involved in [1]. Traditional biotechnology approaches, *e.g.*, gene overexpression or knockout, are described as 'all or nothing' and may thus fail to reveal the actual gene function under certain conditions [2]. In order to minimise undesirable changes in gene expression, a more precise tuning of promoter activity is required [3]. Various naturally inducible and/or tissue-specific promoters have been identified, but so far, none of them provides a wide dynamic range [2]. Therefore, synthetic promoters have been developed to provide more precise spatio-temporal regulation of gene expression [4,5]. Directed by the theory of synthetic biology, synthetic promoters can be constructed by using defined *cis*-elements as building blocks from diverse sources; thus, promoter engineering enables *cis*-elements with different properties to be combined [6,7]. Furthermore, diverse approaches, such as error-prone PCR, DNA shuffling, domain swapping, *cis*-element repetition and flanking sequence modification, can alter the properties of synthetic promoters in order to change their strengths, assign inducible or tissue-specific features, or combinations thereof [8–12].

Cold-induced sweetening (CIS) seriously impedes development of the potato processing industry. This process occurs in potato tubers during storage at low temperature [13], and results in the accumulation of reducing sugars (RS). RS can react with α-amino acids at high temperature, which causes unacceptable fried products with distorted colour and toxic acrylamide composition [14]. The majority of identified CIS-related genes participate in basic metabolic processes [15]; therefore, their disordered expression might impact homeostasis and cause negative or detrimental effects. For example, ectopic overexpression

Abbreviations: CBF, C-repeat binding factor; CIPP, *class I patatin* promoter; CIS, cold-induced sweetening; CRT/DRE, C-repeat/dehydration-responsive element; E3, *Solanum tuberosum* cv. E-potato 3; RS, reducing sugar; SOE PCR, gene splicing by overlap extension PCR.

* Corresponding author. Tel.: +86 27 87287381; fax: +86 27 87286939.
E-mail addresses: limeng0422@foxmail.com (M. Li), liujun@mail.hzau.edu.cn (J. Liu).

http://dx.doi.org/10.1016/j.plantsci.2015.02.014
0168-9452/

of the *AtCBF* gene inhibits tuber formation in potato [16]. For controlling CIS in potato, precise regulation of gene(s) at low temperature must be established specifically in the tubers. However, no natural promoters with such properties have yet been described.

A synthetic tuber-specific and cold-inducible promoter, pCL (GenBank accession number DQ494557), was previously constructed in order to improve the resistance to CIS in potato tubers [17]. The pCL promoter consists of two parts: a fragment from the potato *class I patatin* promoter (CIPP) region containing a tuber-specific cassette, TSSR [18,19] and a fragment derived from the promoter region of *Arabidopsis cor15a* containing a CRT/DRE cassette. The pCL promoter has been used for CIS regulation by driving an antisense expression of *StvacINV1*, the gene that encodes potato acid vacuolar invertase [20]. However, the weakness of this promoter limited its application. The CRT/DRE is responsive to low temperature, drought, high salt, and other types of abiotic stress [21,22], and the CCGAC core of the CRT/DRE element is critical for the cold inducibility of the promoter [23]. CRT/DRE is bound by CBF/DREB transcription factors [24–26]. The CBF pathway, independent of the ABA (abscisic acid) pathway, is an important signalling axis that mediates the response to low temperature in multiple plant species [27,28]. This has been exploited in biotechnology applications where the adoption of specific cold-inducible promoters has relieved the negative effects of endogenous promoters. For example, the *RD29A* promoter has been applied in genetic engineering of potato [16,29], tobacco [30], wheat [31], and mulberry [32].

Promoter strength is a prerequisite for successful construction of synthetic promoters [33] and it is affected by several variables such as *trans*-acting factors, *cis*-element composition, enhancers, space sequences, flanking sequences, and position effects. For synthetic promoter construction, repeats of characterised elements have been frequently used to enhance promoter strength [34–37]; other studies indicate that the length and base composition of flanking sequences can determine promoter properties [37–40]. The importance of sequences that flank CRT/DRE was proposed by Gilmour et al. [24]. This group fused three 27-bp fragments derived from the promoters of *Arabidopsis cor15a*, *cor15b*, or *cor78* to a minimal promoter, and investigated whether this affected their interactions with AtCBFs (including AtCBF1, AtCBF2, and AtCBF3). Although there is only a 4-bp disparity in the flanking sequences of CRT/DRE, the promoter fused with the *cor15b* fragment showed approximately 3-fold higher activity than those fused with the *cor15a* fragment. In contrast, although there is a 10-bp difference in the flanking sequences of *cor15a* and *cor78*, the level of expression elicited by these fragments was similar. The *cor15b* gene is highly responsive to cold [41], and its promoter activity was significantly higher than the *cor15a* promoter in potato and tobacco. Swapping experiments revealed that flanking sequences of CRT/DRE could affect the cold inducibility of the promoter [42]. However, the precise flanking sequences that determine the magnitude of the response have remained unclear.

For the cold-inducible promoters, their activity have a wide range from low to high, while the decision mechanism is unclear. Therefore, the key point is to clarify the factors that influence cold-inducible promoter strength in order to provide a theoretical basis for the design of synthetic plant cold-inducible promoters. The main objective of the present research was to construct efficient tuber-specific and cold-inducible promoter for manipulating CIS in potato tubers. Through the introduction of CRT/DRE repeats and flanking sequence mutations into the pCL promoter, we report the construction of an efficient synthetic cold-inducible and tuber-specific promoter and provide potential mechanistic explanation of this increased cold-inducible promoter strength.

2. Materials and methods

2.1. Plant materials and treatments

Potato (*Solanum tuberosum* L.) plants of cultivar E-potato 3 (E3) were used for transformation tests of the synthetic promoters. E3 is a hybrid variety bred by Southern Potato Research Centre (China) and widely cultivated in South China. It is a high yield variety, resistant to late blight but sensitive to CIS. A transgenic line, p35-7-E3, previously constructed by our group, containing a single copy of *GUS* gene driven by CaMV 35S promoter was used in present research. The plantlets and microtubers of both transgenic lines and nontransgenic E3 were produced *in vitro* with the protocols of Liu et al. [43]. The greenhouse plants were grown in 24-cm plastic pots at 20–25 °C and managed according to the general method of potato cultivation. The tissue culture plants were grown under the cycle of 16 h light (continuous illumination of approximately 40 $\mu mol\,m^{-2}\,s^{-1}$) and 8 h dark at 20 °C. The 8-week-old greenhouse plants and 6-week-old *in vitro* plantlets were subjected to the temperature treatments and the GUS assay of the leaves, stems and roots. Differently, the microtubers were obtained from the 8-week-old *in vitro* plantlets grown under 8 h light (continuous illumination of approximately 40 $\mu mol\,m^{-2}\,s^{-1}$) and 16 h dark at 16 °C according to the method of Liu et al. [43]. Before treatment, these materials were placed in chambers under 20 °C, continuous illumination of approximately 40 $\mu mol\,m^{-2}\,s^{-1}$ for 1 week to eliminate the effects caused by other factors such as diurnal, circadian, and photoperiodic regulation. Then, transgenic and control lines were sub-grouped into 4 °C or 20 °C chambers under continuous illumination of approximately 40 $\mu mol\,m^{-2}\,s^{-1}$ for 2 days before the promoter strength test. The normal tubers and stolons were harvested from the 16-week-old greenhouse plants and maintained at room temperature for 1 week, and then divided into two groups, which were separately kept in a 4 °C or 20 °C chamber under darkness for 2 days. After the treatment, different tissues were respectively sampled and rapidly frozen in liquid nitrogen.

2.2. SOE PCR for mutagenesis of pCL

The pCL promoter was previously constructed by Zhu et al. [17]. The modified pCL promoters were constructed by SOE (gene splicing by overlap extension) PCR [44]. The mutations or additional sequences were introduced to the original sequence through the use of primers designed to contain overlapping regions.

We designed the primer pair M1bF/M1bR with a 22 bp overlapping region including mutated sequences. Using the primer pairs ZL01L/M1bR and M1bF/ZC01R (ZL01L/ZC01R is the primer pair for complete sequence amplification of pCL which was constructed by Zhu et al. [17]), the mutated sequences were respectively introduced to the 3′-end of b1L fragment and 5′-end of b1R fragment. The b1L and b1R fragments were respectively amplified by PCR using LA Taq (Takara) from the plasmid pCL-121. Therefore, the b1L and b1R contain parts of the pCL sequence and mutated sequences in their cohesive ends, respectively. The two fragments were combined by SOE PCR through the overlapping region, then a fragment, pCL1b, with a 5′-*Hind*III and a 3′-*Bam*HI restriction site was constructed.

Subsequently, the primer pair M2bF/M2bR that has a 23 bp overlapping region sequence including an additional CRT/DRE cassette was designed. The primer pairs ZL01L/M2bR and M2bF/ZC01R were respectively used to amplify from pCL-121 using LA Taq, and the additional sequences were respectively introduced to the 3′-end of baL and 5′-end of baR by PCR. The two fragments with additional sequences were combined by SOE PCR to construct pCLab fragment. Similarly, using the primer pairs ZL01L/M2bR and M2bF/ZC01R, and pCL1b fragment as template, the additional sequences were respectively introduced to the 3′-end of b2L and 5′-end of b2R by PCR and

Please cite this article in press as: M. Li, et al., Construction of efficient, tuber-specific, and cold-inducible promoters in potato, Plant Sci. (2015), http://dx.doi.org/10.1016/j.plantsci.2015.02.014