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# Analysis of AtCLV3 promoter and its utilization for expressing cre recombinase in Indian mustard

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## ABSTRACT

Constitutive expression of the transgene is not always desirable because of its pleiotropic effects and metabolic pay-off. Therefore, tissue-specific expression of the transgene can be more significant in reducing the unintended effects of the transgene. The strength as well as tissue and temporal specificity of the promoter activity is dependent on *cis*-acting elements and transcriptional factors. The objective of this study was to analyse the promoter elements in Arabidopsis CLV3 promoter and its utilization in driving tissue specific expression of the *cre recombinase* gene in Indian mustard. The study was carried out at ICAR-NIPB, New Delhi, during 2015–19. In this study, *in silico* analysis of Upstream Regulatory Modules (URMs) of CLV3 gene led to the identification of several sequence motifs related to its meristematic tissue specific expression. Further, the CLV3 promoter was used for driving the expression of a *cre recombinase* gene in transgenic lines of *B. juncea* plants for functional analysis. The Cre-transgenic lines are useful for Cre/*lox* mediated marker gene excision in transgenic *B. juncea*.

**Keywords**: *B. juncea*, CLAVATA3 gene, *Cre recombinase*, *Cis*-acting elements, Upstream Regulatory Modules

The shoot apical meristem (SAM), a source for totipotent stem cells, plays a pivotal role in all post-embryonic organ development. A balance between cell proliferation and differentiation maintains a functional SAM. Signals from the neighbouring cells decide the fate of the stem cells and their proliferation (Spradling *et al.* 2001, Weigel and Jürgens 2002). The organization and functioning of the SAM are maintained by a negative feedback loop of the *WUS* and *CLAVATA3* (*CLV3*) genes. The *WUSCHEL* (*WUS*) transcription factor gives a positive signal to the stem cells for maintaining their undifferentiated state, whereas CLV3 interact with *CLV1/CLV2* receptor complex to produce a negative signal to restrict the number of stem cells by limiting the WUS expression (Brand *et al.* 2000 and Schoof *et al.* 2000). In many transgenic studies constitutive promoters have been used despite the disadvantageous pleiotropic effects and metabolic pay-off by the constitutive expression of the transgene (Walters and Heil 2007, Liu *et al.* 2008, Kasuga *et al.* 1999, Brini *et al.* 2011). This is primarily due to the unavailability of well characterized specific promoters for diverse experimental needs. Tissue-specific promoters drive the expression of a gene in specific tissues, where their regulation is coordinated by transcription factors and cis-elements (Cornejo *et al.* 1993 and Park *et al.* 2010). Thus, analysis of promoters in terms of their cis-elements is important for understanding the regulation of the promoter activity.

CLV3, a member of the CLE family (CLAVATA3/ESR-related) of peptides, maintain stem cell population within SAM in Arabidopsis (Ito *et al.* 2006 and Kondo *et al.* 2006). During the reproductive stage, reproductive SAM gives rise to flowers which sets seeds after fertilization. Hence, upstream promoter elements of CLV3 are involved in determining floral meristematic tissue specific expression of the gene. In the present study, the upstream promoter elements of the Arabidopsis CLV3 were analysed for identifying the important *cis*-elements using the web-based tool PlantCARE. The ectopic promoter activity was validated in Indian mustard, *Brassica juncea* for expressing the *cre recombinase* gene of *E. coli*.

## MATERIALS AND METHODS

The *cis*-elements were analysed in both the strands (+ and -) of the CLV3 promoter sequence by using PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plant care/html/).

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Seeds of *B. juncea* cv. Varuna were grown in 8-inch pots filled with soil and soilrite. The plants were maintained at 24±2°C with 16/8 h light/dark cycle and relative humidity of 65-70% in the glass house of National Phytotron Facility of ICAR-Indian Agricultural Research Institute, New Delhi. All the experiments were carried out at ICAR-NIPB, New Delhi during 2015–19. CLV3 promoter (1.2 kb), *cre recombinase* gene (1.1 kb), and CLV3 terminator (1.5 kb) were PCR amplified from three recombinant plasmids pENTR899-CLV3 promoter, pPZP200-*cre* and pENTR898-CLV3 terminator, respectively using gene-specific primers. The primers were designed in Primer Quest tool IDT (https://eu.idtdna.com/PrimerQuest/Home/Index) (Table 1). For directional cloning of the CLV3 promoter, the forward primer contained the *Eco*RI and *Pac*I sites, and the reverse primer *Sma*I restriction site at the 5/ end of the primers. For cloning the *cre recombinase*, gene specific forward and reverse primers were hooked with *Xba*I restriction site and for CLV3 terminator primers were hooked with *Sal*I and *Pst*I restriction sites on the 5/ end of the primers.

PCR reaction was set up for 20 µl volume containing 2 µl of 10X *Pfu* DNA polymerase buffer,1 µl of dNTP mix (10 mM), 1 µl each of the forward and reverse primer (10 mM), 2 µl *Pfu* DNA polymerase (Thermo Scientific, USA) and 100 ng of the template plasmid DNA. The final reaction volume was made up with nuclease-free water. The PCR reaction was carried out in thermal cycler (Applied Biosystems, USA) which was programmed as: initial denaturation at 94ºC for 4 min followed by 35 repeated cycles of denaturation at 94ºC for 1 min, primer annealing at 55ºC for 35 s and primer extension at 72 ºC for 1 min followed by a final extension at 72ºC for 10 min. The amplicons were separated on 1.2% agarose gel and extracted from the agarose gel by QIAquick® Gel extraction kit (Qiagen, USA). The eluted amplicons were cloned in pGEM-T easy vector (Promega, USA) and validated through Sanger sequencing (SciGenom Labs Pvt Ltd, India). After confirmation through sequencing, the CLV3 promoter, *cre* gene, and CLV3 terminator were assembled in pUC19 vector for constituting the *Cre* expression cassette.

The *Cre*-expression cassette was further subcloned in a binary vector pPIPRA560 in *Pac*I site (Chi-Ham *et al*. 2012). The positive clones were confirmed by colony PCR using gene-specific primers and release of the insert by restriction digestion. The recombinant binary vector was named as pPIPRA:CLV3-*cre*. The confirmed recombinant binary vector was mobilized into the *Agrobacterium tumefaciens* strain GV3101. *A. tumefaciens* carrying pPIPRA:CLV3-*cre* construct was used for genetic transformation of *B. juncea* plants by floral spray method described in Aminedi *et al*. (2019). The harvested seeds from the treated *B. juncea* plants were germinated and grown in soilrite inside a growth chamber under controlled conditions. For molecular analysis, the genomic DNA was isolated from the true leaves of the putative *B. juncea* transgenic plants as well as from the control plants by CTAB method (Doyle 1991). Putative transgenic plants were screened by PCR using Emerald Amp GT PCR Master Mix (Takara Bio Inc., Japan) and by using promoter-specific and *cre* gene-specific primers (Table 1).

For RT-PCR analyses, total RNA was extracted from the PCR-positive plants using RNAiso Plus (Takara Bio Inc., Japan) according to the manufacturer's instructions and treated with DNase using TURBO DNA free (Ambion Inc., USA). cDNA was synthesized by reverse transcription of 4 µg of DNA free RNA using PrimeScript™ 1st strand cDNA synthesis kit (Takara Bio Inc., Japan). cDNA was diluted 10 times for qRT-PCR. The qRT-PCR was performed on a StepOnePlus Real-Time PCR system (Applied Biosystems, USA) using the SYBR Premix Ex Taq II (Tli RNaseH Plus) kit. Co-amplification of GAPDH cDNA was taken as the internal control (Chandna *et al.* 2012). A 10 µl qRT-PCR reaction mixture contained: 5 µl 2X SYBR Premix ExTaq II, 0.2 µl 50X ROX reference dye, 0.4 l each of the forward and reverse primers (10 µM), 1 µl of diluted cDNA, and 3.4 µl nuclease-free water. The qPCR was programmed as: initial denaturation at 95ºC for 30 s followed by 40 repeated cycles at 95ºC for 10 s, 60ºC for 30 s, and 72ºC for 30 s. The relative transcript level was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

## RESULTS AND DISCUSSION

*Sequence analysis and cis-elements in CLV3 promoter*: The diverse *cis*-elements in the promoter region regulate the promoter activity in terms of strength and specificity. Thus, identification of the promoter elements is important for gaining a better understanding of the function of

Table 1 List of primers used for PCR and qRT-PCR

| S. No. | Name | Primer sequences (5'-3') | Amplicon size |
|---|---|---|---|
| 1 | CLV3 promoter | F-GGAATTCCCTTAATTAAGGTGGTAAATCAGTAAATTAG<br>R-TCCCCCGGGAGAGAGAAAGTGACTGAGTGAGAGAG | 1.5 kb |
| 2 | *Cre recombinase* | F-GCTCTAGAAATGTCCAATTTACTG<br>R-GCTCTAGAGCTAATCGCCATCTTC | 1.1 kb |
| 3 | CLV3 terminator | F -GCGTCGACCCTTAATTAACCTAATCTCTTGTTGC<br>R- AACTGCAGTATGTGTGTTTTTTC | 1.2 kb |
| 4 | q*Cre* | F –CTGACGGTGGGAGAATGTTAAT<br>R-CATCGCTCGACCAGTTTAGTT | 150 bp |
| 5 | qGAPDH | F - TCAGTTGTTGACCTCACGGTT<br>R-CTGTCACCAACGAAGTCAGT | 100 bp |

the promoter. Therefore, we attempted to identify the major *cis*-acting elements present in the CLV3 promoter using the PlantCARE database. Total 110 *cis*- elements belonging to 26 different motif types were identified in the CLV3 promoter sequence. On the basis of function, the identified *cis*-elements were categorized into five groups: light-responsive, hormone-responsive, stress-responsive, tissue-specific, and other regulatory elements (Fig 1a). Among these, the stress-responsive elements were found to be maximum in number (8%) followed by light-responsive elements (7%), hormone responsive elements (4%), and tissue-responsive elements 1%. *In silico* analysis of CLV3 promoter sequence also identified the core promoter elements such as the TATA box (total number 70) and the CAAT box (total number 26). Additionally, numerous *cis*-acting elements like light-responsive motifs such as G-Box, GA motif, I-Box, BoxG and ATCT motif (Martínez-García *et al*. 2000), hormone-responsive elements like ABRE (ABA-responsive element), tissue responsive element CAT box, and stress-responsive element like MBS were also identified in the CLV3 promoter.

*PCR based cloning of CLV3 promoter and terminator of Arabidopsis*: CLV3 promoter from *A. thaliana* specifically shows higher activity in the stem cell reservoirs of shoot and floral meristematic cells (Fletcher 1999). The Arabidopsis CLV3 promoter cloned in pENTR899 was PCR amplified using sequence specific primers and ligated in pGEM-T Easy vector (Promega, USA). The recombinant colonies were selected on the basis of blue-white screening assay. The recombinant clones were validated by release of the cloned fragment by restriction digestion and sequencing. The promoter was further sub-cloned in pUC19 vector within the restriction sites *Eco*RI-*Sma*I. The CLV3 terminator sequence from *A. thaliana* was PCR amplified using sequence specific primers with *Sal*I (forward) and *Pac*I-*Pst*I (reverse) restriction sites from the recombinant pENTR898 plasmid and cloned in pGEM-T Easy vector. The cloning of insert was confirmed by restriction analysis and sequencing. The CLV3 terminator was further sub-cloned in *Sal*I and *Pst*I sites and next to the CLV3 promoter in recombinant pUC19 in which CLV3 was cloned earlier.

*Developing a plant transformation vector for expressing cre recombinase under CLV3 promoter*: The *cre* gene codes for recombinase which mediate specific cleavage at lox sequence and thus has been used frequently in Cre/*lox* mediated marker excision from the transgenic plants. The *cre* gene was PCR amplified from a recombinant plasmid pPZP200 using gene specific primers hooked with *Xba*I restriction sites in forward and reverse primers and cloned into pGEM-T Easy vector. The cloning was confirmed by restriction digestion and sequencing of the clones. The *cre* gene was further subcloned at the *Xba*I site in recombinant CLV3pro-CLV3ter-pUC19 vector in between the CLV3 promoter and the CLV3 terminator sequence. The desired orientation of the *cre* gene was checked by restriction digestion using *Bam*HI enzyme which released a fragment of 350 bp. The *cre* expression cassette, CLV3pro-*cre*-CLV3ter in pUC19 was released by *Pac*I restriction digestion and sub-cloned into a binary vector pPIPRA560 linearized with *Pac*I. The recombinant pPIPRA560-*cre* construct was mobilized into *Agrobacterium tumefaciens* strain GV3101 by freeze thaw method (Weigel and Glazebrook 2006).

*Development of transgenic Indian mustard with Cre recombinase under CLV3 promoter*: The pPIPRA560-*Cre* binary construct was used for *Agrobacterium*–mediated transformation of *B. juncea* cv. Varuna by floral spray transformation method. The putative transgenic plants were screened for presence of the *cre* recombinase gene using gene specific primers. The amplification of 1.1 kb amplicon by PCR from the genomic DNA of the putative transgenic plants confirmed the presence of the transgene. The amplified fragment was further validated through sequencing. No amplification was seen in case of untransformed *B. juncea* plants. The transgenic lines scored positive in PCR analysis were further analysed for the expression of *cre* gene by qRT-PCR.

*Analysis of cre gene expression by qRT-PCR in the transgenic lines*: To check the CLV3 promoter activity and expression of the *cre* gene in the transgenic *B. juncea* plants,

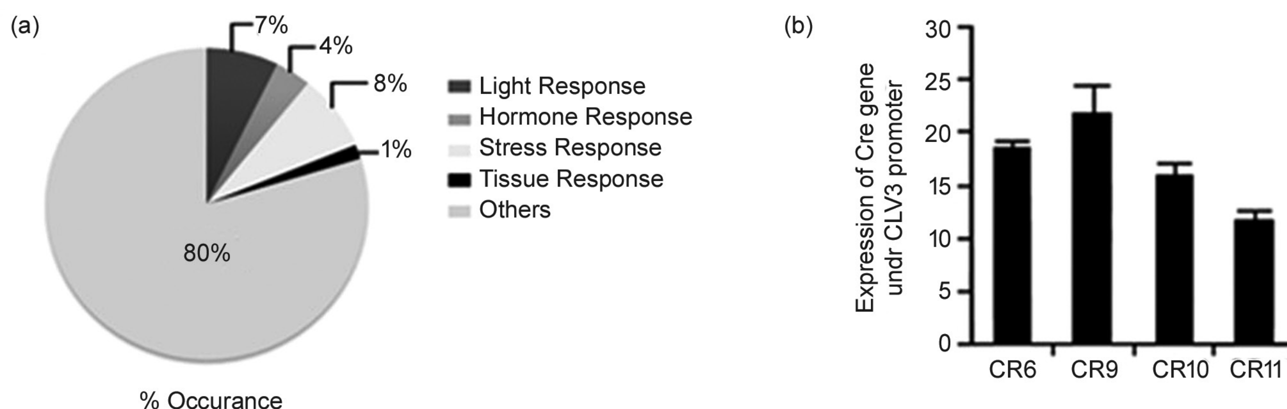


Fig 1 (a) Pie chart showing frequency of *cis*-elements present in CLV3 promoter (b) Gene expression analysis of *cre recombinase* by qRT-PCR - Relative transcript levels of *cre* recombinase gene under CLV3 promoter in different *B. juncea* transgenic lines.

qRT-PCR analysis was performed. For qRT-PCR analysis the total RNA was isolated from the leaves of different transgenic *B. juncea* lines. The results of qRT-PCR showed variable transcript levels of the *cre recombinase* across the different transgenic lines (Fig 1b). The maximum level of *cre* transcript was observed in CR9 followed by CR6 and CR10, whereas CR11 showed the least level of the transcripts. Thus, qRT-PCR analysis results validated ectopic promoter activity and expression of the *cre recombinase* in the transgenic lines of *B. juncea*.

The developed transgenic Cre lines are useful for deploying in hybridization with the other transgenic *B. juncea* lines for rescuing progeny plants free from selectable marker gene based on the Cre/*lox* mediated recombination. However, for marker excision it is important that the marker gene in the parent transgenic lines is flanked by *lox* sequences. The developed Cre lines of Indian mustard will be an important resource in transgenic research of Brassica biotechnology programme.

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