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A modified pod specific promoter for high level heterologous expression of genes in legumes

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ABSTRACT

Promoters are *cis*-acting regulatory elements that are usually present upstream to the coding sequences and determine the gene expression. Deployment of tissue specific and inducible promoters are constantly increasing for development of successful and stable multiple transgenic plants. To this end, as a strategy for enhanced expression of *cis* or transgenes, promoter engineering of the native *msg* promoter from soya bean has been carried out for executing pod specific expression of genes. *Cis* regulatory elements such as 5'UTR and poly (A) tract have been incorporated for imparting mRNA stability and translational enhancement to generate the modified 1.285 Kb pod specific promoter. Further to attain transcriptional enhancement the modified promoter has been cloned to generate Bi-directional Duplex Promoters (BDDP). The engineered *msg* promoter gene constructs can be deployed for high level tissue specific gene expression of cis/trans genes along with chosen terminator in chickpea. soybean and other legumes as well.

Key words: Bi-Directional Duplex Promoter (BDDP), Chickpea, Cis-engineering, Pod specific gene expression, Translational enhancement.

INTRODUCTION

Promoters are *cis*-acting DNA elements that govern the spatio-temporal and magnitude of expression of a gene. Promoters are generally situated upstream, although presence of internal promoters also has been well established (Horowitz *et al.,* 1983, Huang *et al.,* 2007). Core promoter region essentially governs whether transcription can take place or not. Whereas, the distal promoter region regulates the level of gene expression by harboring tissue specific sites and/or enhancer sequences that are bound by transcription factor (s), at times specifically produced in particular tissues (Lee *et al.,* 2000). Activator proteins when bound to DNA facilitate RNA polymerase binding and thereby increase transcriptional efficiency (Kolovos *et al.,* 2012). Promoters and enhancers coordinately act *via* multifaceted spatio-temporal interactions to regulate when, where the gene expression takes place and also determines the magnitude of expression (Pennacchio *et al.,* 2013). Therefore, the level and tissue/cell type/organ specific expression in heterologous systems is primarily dictated by the promoter and more specifically by the distal promoter. Emphasizing the importance on gene expression which is ultimately a culmination of the multifaceted regulation, the key role of promoter engineering has been a central focus of transgenic plant development for the past decade.

Heterologous expression of genes in plants is not only influenced by promoters but also several other factors that affect transcriptional and translational efficiencies (Streatfield *et al.,* 2007, Makhzoum *et al.,* 2014). High level heterologous expression of genes can be achieved by adopting various strategies like incorporation of untranslated regions (UTR) at the 5′ and 3′ end of mRNA that enhance its stability, elimination of mRNA destabilizing sequences, if any, found in the gene, optimization of sequences around the translational start codon and incorporation or replacement of the 4th nucleotide as Guanine (+4 rule) (Sugio *et al.,* 2010). The primary focus of such strategies relies heavily on stabilization of the mRNA level so that the probability of mRNA access by the translational machinery is increased. In this context, the ribosomal access to the mRNA can be augmented by manipulating the sequence context of the ribosomal binding site (RBS). Also the distance between the transcriptional start site and translational start codon has also been reported to influence the expression levels (Fessele *et al.,* 2002). Development of gene constructs considering such strategies can boost the expression of the transgenes in heterologous systems.

Traditionally deployment of strong constitutive promoters of CaMV35S, Ubiquitin, Actin, Tubulin and EIF (eukaryotic initiation factor) genes has been done on a large scale for developing the transgenic plants (Hernandez-Garcia *et al.,* 2014). However, constitutive expression of transgenes may often cause appearance of undesired phenotypes, including altered plant growth and development with fitness costs (Gurr and Rushton *et al.,* 2005).

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Alternatively the constitutive promoter deployed in a particular crop species may either completely fail or demonstrate low levels of expression in the desired tissue/ organ leading to ineffective levels of intended phenotype. For instance, deployment of CaMV35S promoter for expression of Cry protein in chickpea revealed low expression levels in post-flowering stage (116 DAS) compared to that of pre-flowering stage (88 DAS) (Das *et al.,* 2017). To this end, usage of tissue specific or inducible promoters that function in post-flowering stages and more specifically during pod development can overcome such a bottleneck. In this direction, *msg* promoter from soya bean has been reported to express in different parts of flowers, nectarines and young pods in both soybean and *Arabidopsis* (Stromvik *et al.,* 1999). Further the *msg* promoter has also been used for pod specific expression of fused *cry1Ab/Ac* in chickpea revealing strong expression in developing pods. However, the accumulation of the Cry proteins was reported to be in the range of 9-19 ng mg^{-1} TSP in contrast to the maximum expression up to 40 ng mg^{-1} TSP in chickpea (Mehrotra *et al.,* 2011). Gene regulation at the transcriptional level is one of the potential molecular mechanisms that can explicate and regulate variation in protein levels and introduction of such precise control is now feasible using engineered cis-regulatory elements specifically designed to bind transcriptional regulators. Therefore, in order to achieve high level expression of transgenes within specific tissues/ organs of chickpea and other legumes, *cis*-engineering of the promoters is pivotal to generate crops with enhanced agronomic traits.

In the present study the primary objective is to develop gene constructs for high level tissue specific expression of genes in pods of chickpea. Contextually, use of chimeric promoters or Bidirectional Duplex promoters (BDDP) in combination with strong terminators has been shown to drastically enhance transcriptional efficiency of genes (Chen *et al.,* 2018, Chennareddy *et al.,* 2017, Li *et al.,* 2004). However, use of tissue specific promoters as BDDP in combination with transcriptional and translational enhancers has not been reported yet. Therefore, this report describes the cis-engineering of the *msg* promoter and development of single and BDDP gene constructs for tuning genetic control as a prelude for expression of cis or transgenes in chickpea and other legumes.

MATERIALS AND METHODS

Plant materials and Plasmid vectors

Soya bean seeds were procured from local market and also seed of JS cultivar series (9305, 3305, 9560, 7105 and 9752) were germinated and genomic DNA was isolated. Two plasmid vectors: pUC57-Kan vector (Genewiz, United States) and pTZ57R vector (Thermo Scientific, India) were used. All restriction enzymes were procured from Thermo Scientific, India.

Promoter design for post-transcriptional transgene control

The nucleotide sequence of the native *msg* promoter was retrieved from the GenBank: AJ239127.1 and *in silico* restriction analysis of the sequence was done using NEB cutter tool. Further restriction enzyme sites of KpnI followed by HindIII were included in the 5′ end and another KpnI site was included on the 3′ end of the promoter sequence for facilitating cloning. A 36 nucleotides 5′untranslated leader sequences of the coat protein mRNA of alfalfa mosaic virus (AMV) (Jobling and Gehrke, 1987) followed by inclusion of poly A-tract of ten Adenine residues (Wang and Roossinck, 2006) were incorporated downstream to the promoter.

Site-directed mutagenesis and PCR based synthesis

An overlap extension PCR was performed using the soya bean genomic DNA template in combination with 60 nucleotide long primers. The modified sequence was used as the template sequence for designing overlapping primers of 60 nucleotides in length (Table 1). Two short external primers with KpnI and HindIII sites in the forward primer and KpnI site in the reverse primer were also designed. A master mix of all the primers was made with final concentration of each primer being 2 pM /µL. 0.5 µL of the reaction mixture was taken to which 1.5 µL of two short external primers were added at a concentration of 20 pM/µL. PCR was performed with long PCR Enzyme Mix (Thermo Scientific, K0182) comprised of unique blend of *Taq* DNA Polymerase and a thermostable DNA polymerase with proofreading activity was conducted for 30 cycles with denaturation at 90°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 20 seconds and a final extension at 72°C for 5 minutes. The PCR product was further subjected to blunting using Quick Blunting Kit (New

Table 1: List of Primers (5->3).

PRO F1	gaactgctttaaggaatattgtgctgaaggatgtcgggacacaatgctctgataggatgt
PRO R1	aatagattaacatgataaaattaatataaaaacttattatccaatgtcacatcctatcag
PRO F2	aatctattttattttacctcactgatttaacaaaatatttttataaatttgtgacggact
PRO R2	ctgcactattatcaattaaaacttattaaaaacatattcggctcaaaataagtccgtcac
PRO F3	atccaaagtcaggattcaaattctatatcgcttgcttattaaacacaaacttatttttta
PRO R3	aattattattaagtgtcatttttatgtttattttcagtgaaatatatatgtaaaaaataa
PRO F4	gtgatagttgctttcagataactgatagttgtgtgcaattatttggattcaagagttttt
PRO R4	ttttttttttggtggaagtatttgaaagaaaattaaaaataaaaactcttgaatccaaa
msgfwKandH	ggtaccaagcttctaagatgaactgctttaagga
msgrevKpnI	ggtacctttttttttttggtggaagtatttg

England Biolabs, E1201S) and processed for ligation. Bioserve Biotechnologies (India) Pvt. Ltd. synthesized and supplied all the primers.

Cloning strategy

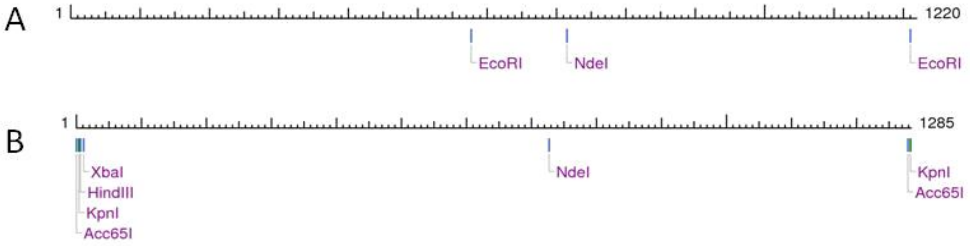
The PCR product was cloned into EcoRV site of pUC57-Kan vector by means of blunt end ligation and the recombinant clones were confirmed by PCR using M13 primers, restriction digestion by XhoI-HindIII enzymes and by sequencing. Further the cloned promoter was isolated by KpnI digestion and sub-cloned into KpnI site of the pTZ57R vector and confirmation of the recombinant clones and their orientation was done by restriction digestion with HindIII enzymes. All the recombinant clones, wherever required, were further confirmed by restriction digestion by other enzymes and sequencing.

RESULTS AND DISCUSSION

Cis-engineering of the native *msg* promoter

Transcriptional regulation mediated by the promoters in association with the *cis*-acting elements is pivotal for induction, activation and suppression of gene expression. Promoter engineering has become an essential criteria for trait development for attainment of desired magnitude and timing of expression of transgenes in heterologous systems. To this end, *cis*-engineering of the native *msg* promoter from soya bean has been carried out. The 1220 bp native sequence of the *msg* promoter has been analyzed for the presence of restriction enzyme recognition sites revealing the presence of two EcoRI sites located at 579 and 1211th nucleotide positions and NdeI site at 716th nucleotide position (Fig 1A). Presence of such commonly used sites located internal to the fragment to be cloned may complicate the cloning strategy; as such sites are often an integral part of the multiple cloning sites of commonly used vectors. Therefore, such sites are considered undesirable and the two EcoRI sites were eliminated by introducing A®G transition causing minimum change in the DNA sequence to facilitate cloning. However, the NdeI site was left unaltered for verification of the promoter in downstream processes like southern blotting of the transgenics. Additionally, restriction enzyme sites of KpnI followed by HindIII were included in the 5′ end of the promoter sequence and another KpnI site was included on the 3′ end of the promoter sequence for facilitating cloning (Fig 1B).

The process of translation initiation predominantly occurs in the 5′ untranslated leader sequences and therefore the UTR sequences have a considerable impact on the translational efficiency based on their length and the degree of the secondary structure adopted. The 67 nucleotides 5′ UTR of the TMV has been shown to boost the transgene expression by four to five fold in transgenic plants (Gallie *et al.,* 1987). Similarly, 36 nucleotides UTR of the Alfalfa Mosaic Virus (AMV) RNA4 encoding the coat protein, has also been shown to dramatically increase the transgene production by 35 fold **(**Jobling and Gehrke, 1987). UTR sequences are reported not only to be involved in enhancement of translational initiation but also enhancing the translational efficiency by adopting cap like secondary structures. Considering its relatively small size, fold increase in heterologous systems and legume based origin, we have chosen to incorporate the AMV 5′ UTR in the present gene construct. The ideal length that determines the functional efficiency of the UTR lies in the range of 40-80 nucleotides and shorter UTRs can impair the fidelity of translation. Therefore a poly-A tract of ten Adenine residues was introduced downstream to the 36 nucleotides 5′ UTR, followed by incorporation of six nucleotide enzyme KpnI site creating a total distance of 53 nucleotides. Appropriate measures have been taken not only to maintain the ideal distance range of 40- 80 but also to keep the DNA in the same phase (10.5 bp/turn × 5 turns = 52.5). Moreover, the poly-A tract generates secondary structure enhancing




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Optimized       ggtaccaagcttctagatgaactgctttaaggaatattgtgctgaaggatgtcgggacac
Native          ctagatgaactgctttaaggaatattgtgctgaaggatgtcgggacac
                **********************************************

Optimized       aatgctctgataggatgtgacattggataataagttttatattaattttatcatgttaa
Native          aatgctctgataggatgtgacattggataataagttttatattaattttatcatgttaa
                ************************************************************

Optimized       tctattttattttacctcactgatttaacaaaatatttttataaatttgtgacggactta
Native          tctattttattttacctcactgatttaacaaaatatttttataaatttgtgacggactta
                ************************************************************
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Fig 1: Continue...

Fig 1: Continue...

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Optimized       ttttgagccgaatatgtttttaataagttttaattgataatagtgcagtggatgtgaacc
Native          ttttgagccgaatatgtttttaataagttttaattgataatagtgcagtggatgtgaacc
                ************************************************************

Optimized       ttttagccatgtgaatttgttttccaatatttttatatatttttatttatatatacatata
Native          ttttagccatgtgaatttgttttccaatatttttatatatttttatttatatatacatata
                ************************************************************

Optimized       tgtcggggtagaggatgtcacatcttcactccaaacatctcattaatatcaatcatattc
Native          tgtcggggtagaggatgtcacatcttcactccaaacatctcattaatatcaatcatattc
                ************************************************************

Optimized       ttcttgattatcatatctagccacccttttatctcatttctttcactaggtgtccaataa
Native          ttcttgattatcatatctagccacccttttatctcatttctttcactaggtgtccaataa
                ************************************************************

Optimized       ttttttagtgtctaccaatccatttttatatatagatatatattgttgattatggtattg
Native          ttttttagtgtctaccaatccatttttatatatagatatatattgttgattatggtattg
                ************************************************************

Optimized       atataaaataattactattgaaagtaacgattaatattcattagaaatcgtgagcacact
Native          atataaaataattactattgaaagtaacgattaatattcattagaaatcgtgagcacact
                ************************************************************

Optimized       taaggtaagtactctcctttcattatgatttatttatatccaaagtcaggattcaaatt
Native          taaggtaagtactctcctttcattatgatttatttatatccaaagtcagaattcaaatt
                ********************************************** *********

Optimized       ctatatcgcttgcttattaaacacaaacttatttttacatatatatttcactgaaaata
Native          ctatatcgcttgcttattaaacacaaacttatttttacatatatatttcactgaaaata
                ************************************************************

Optimized       aacataaaaatgacacttaataataattgttttctttctatttttgaaataccaaaact
Native          aacataaaaatgacacttaataataattgttttctttctatttttgaaataccaaaact
                ************************************************************

Optimized       ttgtttcatatgtaaagggaaaaggaataatactattaagctacaccatgaaatcaaaca
Native          ttgtttcatatgtaaagggaaaaggaataatactattaagctacaccatgaaatcaaaca
                ************************************************************
Optimized       aaaattatcattgggaatgatataaaaaaatcaataaatttatccacatattataattta
Native          aaaattatcattgggaatgatataaaaaaatcaataaatttatccacatattataattta
                ************************************************************

Optimized       tgatttcatacacaataggataatagttttacactttcattgcaacagtatataatatga
Native          tgatttcatacacaataggataatagttttacactttcattgcaacagtatataatatga
                ************************************************************

Optimized       catatttatttctacgttgtgtgcccacttattctttaataatagtagaagaggaaaggg
Native          catatttatttctacgttgtgtgcccacttattctttaataatagtagaagaggaaaggg
                ************************************************************

Optimized       aaattcaatatttacttcttcattttccctttagccgttcatcatttttcaactttga
Native          aaattcaatatttacttcttcattttccctttagccgttcatcatttttcaactttga
                ************************************************************

Optimized       aagtttttttttgtctttttcaaaagaaattctcattgtattaatacattgagaaaaga
Native          aagtttttttttgtctttttcaaaagaaattctcattgtattaatacattgagaaaaga
                ************************************************************
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Fig 1: Continue...

Fig 1: Continue...

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Optimized       agacaaagataaaacaatgttaagtttaagatgtgaaaaaccacgaccatttgagcctct
Native          agacaaagataaaacaatgttaagtttaagatgtgaaaaaccacgaccatttgagcctct
                ************************************************************

Optimized       atatataggtgtctcgtatgctcaaacatgacaagccagtgatagttgctttcagataac
Native          atatataggtgtctcgtatgctcaaacatgacaagccagtgatagttgctttcagataac
                ************************************************************

Optimized       tgatagttgtgtgcaattatttggattcaagagttttattttaattttctttcaaata
Native          tgatagttgtgtgcaattatttgaattcaaga
                ************************  ********

Optimized       cttccaccaaaaaaaaaaaggtacc
Native          ___________________________
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Fig 1: Restriction map of the (A) native and (B) modified *msg* promoter restriction map. (C) Comparison of optimized and native *msg* promoter sequence. Additional restriction enzyme sites: KpnI-HindIII at the 5′ end and the KpnI at the 3′ end, introduced in the optimized are marked in blue. Two EcoRI sites present in the native sequence (highlighted in magenta), eliminated in the optimized sequence (highlighted in grey). AMV 5′ UTR followed by poly-Adenosine tract are highlighted in light green.

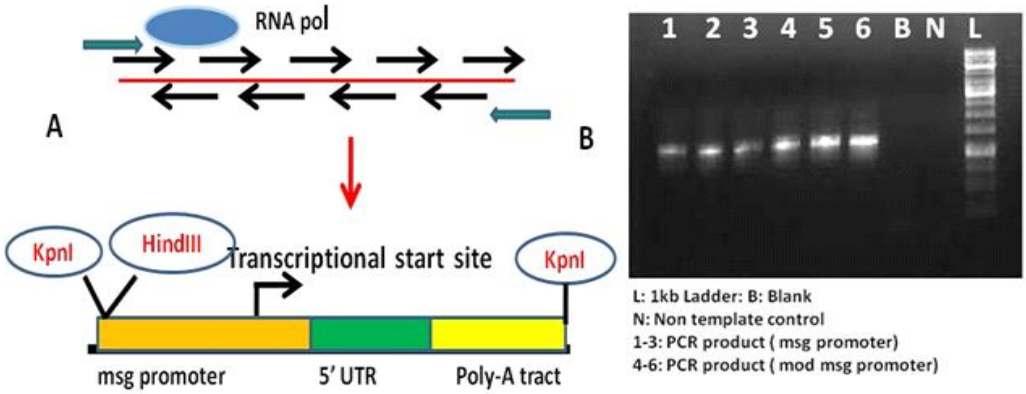


Fig 2: (A) Schematic representation of the overlap extension PCR and the resultant PCR product. (B) Electrophoretogram displaying the PCR based synthesis of the mod-*msg* promoter.

ribosomal binding efficiency, altogether a *cis*-engineered modified *msg* (mod-*msg*) promoter sequence of 1285 bp has been designed (Fig 1C).

PCR based promoter synthesis

Accurate synthesis of long DNA sequences using PCR based protocols have been well standardized (Xiong *et al.,* 2006) and previously employed for synthesis of codon optimized *Rs*AFP2 (*Raphanus sativus* Anti-fungal protein) and *ThEn*42 (*Trichoderma harzianum* endochitinase 42 (Konda *et al.*, 2009; 2010). However in the present study we have adopted a modified methodology (Fig 2A) employing a combination of template DNA and 60 oligonucleotides primers not only to introduce site specific mutations but also incorporation of UTR and poly-A tract simultaneously to yield the modified promoter (Fig 2B). This is the first report of *cis*-engineered pod specific promoter with potential applications for developing transgenic plants.

Cloning and confirmation of the pUC57-*msg* vector

The PCR product of 1.285 Kb length was cloned into pUC57-Kan vector and confirmed by double digestion with XhoI and HindIII (Fig 3A). The release of the 1.2 Kb band confirmed the cloning of the modified *msg* promoter (mod-*msg*). The pUC57-mod-*msg* vector (Fig 3C) was further confirmed by PCR amplification with M13 primers showing amplification in six transformants (Fig 3B). The sequence confirmation of the PCR products revealed 100% accuracy with the modified promoter sequence.

Development of single and Bi-Directional Duplex Promoter gene constructs

In previous studies, DNA elements pertaining to the tissue-specific expression of the *msg* promoter were shown to be located in the distal portion (Stromvik *et al.,* 1999). Taking advantage of the fact, cloning of two copies of *msg* promoters in bi-directional mode would result in development of BDDP. For this purpose, the mod-*msg* promoter from the pUC57-mod-*msg* construct was sub-cloned into pTZ57R vector at the KpnI site. The expected result in one particular orientation as depicted in the Fig 4C which would release the 1.2 Kb fragment when digested with HindIII enzyme. The pTZ57R-

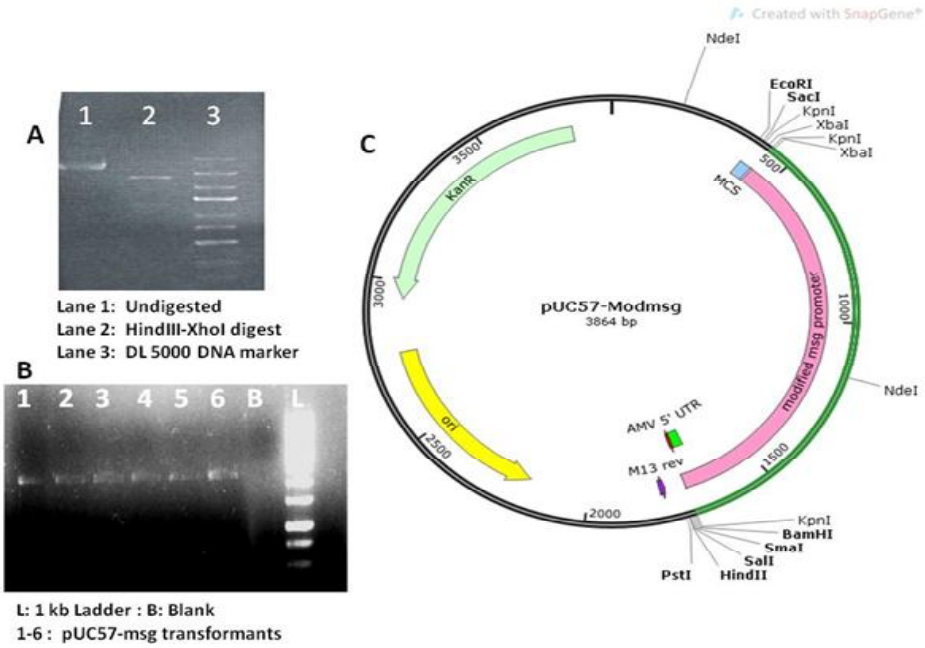


Fig 3: (A) Electrophoretogram displaying the confirmation of cloning of mod-*msg* promoter into pUC57 vector. (B) PCR confirmation of the pUC57-mod-*msg* clones. (C) DNA map of the pUC57-mod-*msg* vector.

mod-*msg* clones were confirmed by restriction digestion with HindIII enzyme and electrophoretogram is shown in Fig 4B. Interestingly, although 1.2 Kb band pertaining to mod-*msg* promoter can be observed in four lanes (1-4), simultaneously a variation in the band size of the pTZ57R vector backbone can also be observed. This increase in band size of the vector backbone in lanes 1 and 3 can be attributed to the presence of second copy of the mod-*msg* promoter.

The second copy of the mod-*msg* promoter can be cloned either in tandem manner or inverted direction or in Bi-directional mode (Fig 4A) and the expected outcomes of each possible cloning, when digested with HindIII are schematically explained in the Fig 4A. Presence of tandem promoter repeats will yield two fragments of approximately 1.2 Kb resulting in one band and the remaining vector backbone of 2.8 Kb. This possible outcome can be further differentiated by linearizing with any other single cut restriction enzyme or by sequencing. In the second possibility, wherein the promoters are expected to be cloned in inverted manner, two types of bands are expected with the size of 2.4 Kb insert size and 2.8 Kb band of vector backbone. This is primarily because the HindIII site is situated on the 5' side of the promoters, releasing both copies of the cloned promoters as a single fragment of 2.4 Kb. In the third possible outcome, wherein the promoters are expected to be in bi-directional manner, two bands of size 1.2 Kb insert and 4 Kb vector backbone combined with promoter are anticipated. This result can be observed in the lanes 1 and 3 of the electrophoretogram in figure 4B confirming the development of vectors with bi-directional duplex mod-*msg* promoters. Further, the recombinant vectors in the lanes numbered 2 and 4 were confirmed to contain single mod-*msg* promoters in desired direction, whereas the recombinant vector in lane 6 exhibiting single band of 4 Kb was confirmed to contain single mod-*msg* promoters in opposite direction.

Advantages and potential applications

Bi-directional duplex promoters comprising of CaMV35S core promoters with duplicated enhancers have been shown to significantly increase transgene expression in grape and tobacco (Li *et al.,* 2004). Alternatively native bidirectional promoters with tissue specific expression pattern in seed/ embryo have been reported in maize (Liu *et al.,* 2016), however deployment of such promoters in developing transgenics has not been done. The novelty of our study lies in exploitation of the modified *msg* promoter in bi-directional mode integrating tissue specificity with high level expression of transgenes. Apart from enhancing the expression, divergent orientation of mod-*msg* BDDPs prevent transcriptional read-through of the genes when cloned downstream of each of the units of the promoters. Moreover, such gene constructs are co-regulated and co-expressed subject to favorable histone modifications (Fang *et al.,* 2016) causing not only enhanced but also balanced expression of both the genes. The gene constructs developed may offer potential applications in developing transgenics for purposes such as

- Developing insect resistant transgenics by pod specific expression of two insecticidal genes like *cry* and *vip*.
- Developing nutritionally enhanced and improved quality pulses/legumes by pod specific down-regulation of endo--genous light signal transduction pathway genes.

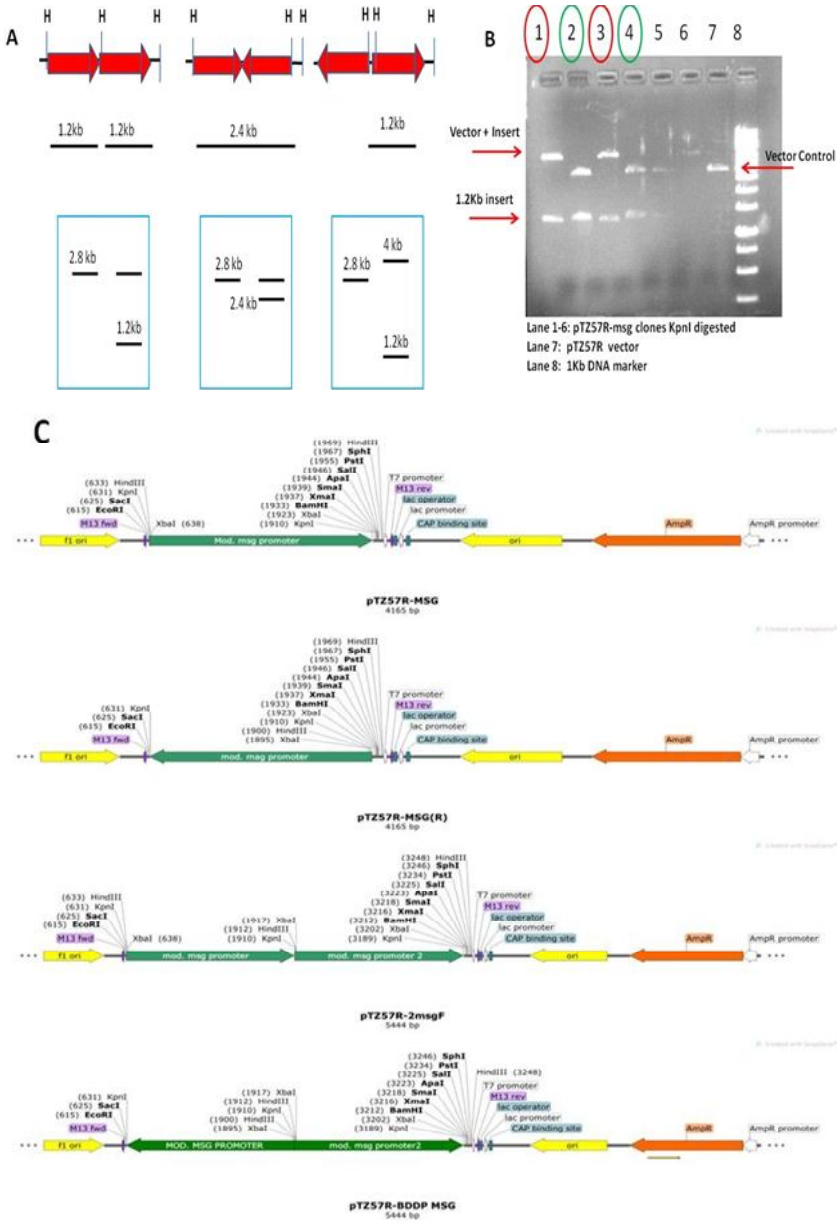


Fig 4: (A) Schematic representation of the possible orientations and outcomes of the mod-*msg* duplex promoters when digested with Hind III enzyme. (B) Electrophoretogram displaying the confirmation of cloning of mod-*msg* promoter into pTZ57R vector. (C) Design of the single and BDDPs vectors.

- Functional characterization of genes/miRNAs involved in pod development.

CONCLUSION

The present study primarily describes the site-directed mutagenesis of the native soya bean *msg* promoter eliminating undesired restriction enzymes and addition of desried sites at the 5′ and 3′ end. Furthermore *cis*-engineering of the promoter for translational enahancemnet has been carried out to accomodate 5′ AMV UTR sequence and polyadenosine tract. The modified promoter was used to develop constructs bearing single and bi-directional duplex promoters for transcriptionally enhanced tissue specific expression of candidate genes. These gene constructs can be used for pod specific expression of genes in chickpea and soya bean. Further characterization of the promoter in other legumes may offer potential solutions to the problem of pod borer and pod suckers in cowpea, pod fly, pod bug and pod wasp in pigeonpea and pod bug in *Vigna* species.

Future prospects

Development of an ideal expression vector is the key for generating effective transgenics. Design, development and

deployment of synthetic promoters in developing trasngenics with orchestered expression may be beneficial. Further measures for increasing transcriptional and translational efficiency by deployment of both 5′ and 3′ UTR regions needs to be focussed. Generation of BDDP gene constructs not only with single enhancers but with multiple enhancers may be given emphasis (Patro *et al.,* 2013). Additionally, measures for preventing transgene silencing upon integration into the heterochromatin genomic region by sequestration of matrix attachment regions on either side of the gene cassette needs to be employed (Van der Geest *et al.,* 2004). Furthermore approaches for directing the transgene product to specific cellular compartments reducing the interference in the cellular metabolism needs to be adopted for developing trasngenics plants with improved agronomic traits.

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Authors contributions

NPS and AKK conceived the concept for pod specific expression ; AKK designed and performed research; PS and KRS performed soya bean genomic DNA isolation, AKK, KRS analysed data and prepared figures; AKK with input from all authors wrote the paper.

Availablity of gene constructs

The gene constructs developed in the study are available at the Division of Plant Biotechnology, ICAR-Indian Institute of Pulses Research.

Conflicts of interest

The authors declare that they have no conflict of interest.

REFERENCES

Chen, Y.M., Dong, Y.H., Liang, Z.B., Zhang, L.H. and Deng, Y.Z. (2018). Enhanced vascular activity of a new chimeric promoter containing the full CaMV 35S promoter and the plant XYLOGEN PROTEIN 1 promoter. 3 Biotech. 8(9): 380. DOI:10.1007/s13205-018-1379-8.

Chennareddy, S., Cicak, T., Clark, L., Russell, S., Skokut, M., Beringer, J. *et al.* (2017). Expression of a novel bi-directional *Brassica napus* promoter in soybean. Transgenic Research. 26(6): 727-738. DOI:10.1007/s11248-017-0042-1.

Das, A., Datta, S., Thakur, S., Shukla, A., Ansari, J., Sujayanand, G.K., *et al.* (2017). Expression of a chimeric gene encoding insecticidal crystal protein Cry1Aabc of *Bacillus thuringiensis* in Chickpea (*Cicer arietinum* L.) confers resistance to gram pod borer (*Helicoverpa armigera* Hubner). Frontiers in Plant Science. 8: 1423. DOI:10.3389/fpls.2017.01423.

Fang, Y., Wang, L., Wang, X., You, Q., Pan, X., Xiao, J., *et al.* (2016). Histone modifications facilitate the coexpression of bidirectional promoters in rice. BMC genomics. 17(1): 768. DOI: 10.1186/s12864-016-3125-0.

Fessele, S., Maier, H., Zischek, C., Nelson, P.J. *et al.* (2002). Regulatory context is a crucial part of gene function. Trends in Genetics. 18(2): 60-63. DOI:10.1016/S0168-9525(02)02591-X.

Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C. and Wilson, T. M.A. (1987). The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. Nucleic Acids Research. 15(8): 3257-3273.

Gurr, S.J. and Rushton, P.J. (2005). Engineering plants with increased disease resistance: what are we going to express?. Trends in Biotechnology. 23(6): 275-282. DOI:10.1016/j.tibtech.2005.04.007.

Hernandez-Garcia, C. M., and Finer, J. J. (2014). Identification and validation of promoters and *cis*-acting regulatory elements. Plant Science. 217: 109-119. DOI:10.1016/j.plantsci. 2013.12.007.

Horowitz, H. and Platt, T. (1983). Initiation in vivo at the internal trp p2 promoter of *Escherichia coli*. Journal of Biological Chemistry. 258(13): 7890-7893.

Huang, P., Pleasance, E.D., Maydan, J.S., Hunt-Newbury, R., O'Neil, N.J., Mah, A. *et al.* (2007). Identification and analysis of internal promoters in Caenorhabditis elegans operons. Genome Research. 17(10): 1478-1485. DOI:10.1101/ gr.6824707.

Jobling, S.A. and Gehrke, L. (1987). Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence. Nature. 325(6105): 622. DOI:10.1038/ 325622a0.

Kolovos, P., Knoch, T.A., Grosveld, F.G., Cook, P.R. and Papantonis, A. (2012). Enhancers and silencers: an integrated and simple model for their function. Epigenetics and Chromatin. 5(1): 1. DOI: 10.1186/1756-8935-5-1.

Konda, A.K., Narasimha, R., Kompelli, S.K. and Dinesh, V.K. (2009). Codon optimization and PCR based synthesis of an antifungal plant defensin *Rs*AFP2 for expressing in Castor. Journal of Oilseeds Research. 26: 210-213.

Konda A.K., Narasimha, R., Kompelli, S.K. and Dinesh, V.K. (2010). Modification of *ThEn*42 gene for improved tolerance against Botrytis in Castor. Journal of Oilseeds Research. 27: 15-17.

Lee, T.I. and Young, R.A. (2000). Transcription of eukaryotic protein-coding genes. Annual Review of Genetics. 34(1): 77-137. DOI: 10.1146/annurev.genet.34.1.77

Li, Z.T., Jayasankar, S. and Gray, D.J. (2004). Bi-directional duplex promoters with duplicated enhancers significantly increase transgene expression in grape and tobacco. Transgenic Research. 13(2): 143-154. DOI: 10.1023B:TRAG.00000 26074.11859.77.

Liu, X., Yang, W., Li, Y., Li, S., Zhou, X., Zhao, Q. *et al.* (2016). The intergenic region of the maize defensin-like protein genes Def1and Def2 functions as an embryo-specific asymmetric bidirectional promoter. Journal of Experimental Botany. 67(14): 4403-4413. DOI: 10.1093/jxb/erw226.

Mehrotra, M., Sanyal, I. and Amla, D.V. (2011). RETRACTED ARTICLE: High-efficiency agrobacterium-mediated transformation of chickpea (*Cicer arietinum* L.) and regeneration of insect-resistant transgenic plants. Plant

Cell Reports. 30(9): 1603-1616. DOI:10.1007/s00299-011-1071-5.

Makhzoum, A., Benyammi, R., Moustafa, K. and Trémouillaux-Guiller, J. (2014). Recent advances on host plants and expression cassettes' structure and function in plant molecular pharming. BioDrugs. 28(2): 145-159. DOI: 10.1007/s40259-013-0062-1.

Patro, S., Maiti, I.B. and Dey, N. (2013). Development of an efficient bi-directional promoter with tripartite enhancer employing three viral promoters. Journal of Biotechnology. 163(3): 311-317. DOI:10.1016/j.jbiotec.2012.11.009.

Pennacchio, L.A., Bickmore, W., Dean, A., Nobrega, M.A. and Bejerano, G. (2013). Enhancers: five essential questions. Nature Reviews Genetics. 14(4): 288. DOI:10.1038/nrg3458.

Streatfield, S.J. (2007). Approaches to achieve high level heterologous protein production in plants. Plant Biotechnology Journal. 5(1): 2-15. DOI:10.1111/j.1467-7652.2006.00216.x.

Strömvik, M.V., Sundararaman, V.P. and Vodkin, L.O. (1999). A novel promoter from soybean that is active in a complex developmental pattern with and without its proximal 650 base pairs. Plant Molecular Biology. 41(2): 217-231. DOI:10.1023A:1006312228617.

Sugio, T., Matsuura, H., Matsui, T., Matsunaga, M., Nosho, T., Kanaya, S. *et al*. (2010). Effect of the sequence context of the AUG initiation codon on the rate of translation in dicotyledonous and monocotyledonous plant cells. Journal of Bioscience and Bioengineering. 109(2): 170-173. DOI: 10.1016/j.jbiosc.2009.07.009.

Van der Geest, A.H., Welter, M.E., Woosley, A.T., Pareddy, D.R., Pavelko, S.E. *et al*. (2004). A short synthetic MAR positively affects transgene expression in rice and Arabidopsis. Plant Biotechnology Journal. 2(1): 13-26. DOI:10.1046/j.1467-7652.2003.00044.x.

Wang, L. and Roossinck, M.J. (2006). Comparative analysis of expressed sequences reveals a conserved pattern of optimal codon usage in plants. Plant Molecular Biology. 61(4-5): 699-710. DOI:10.1007/s11103-006-0041-8.

Xiong, A.S., Yao, Q.H., Peng, R.H., Duan, H., Li, X., Fan, H.Q., *et al*. (2006). PCR-based accurate synthesis of long DNA sequences. Nature Protocols. 1(2): 791. DOI:10.1038/nprot.2006.103.