

Full Length Research Paper

Building a bi-directional promoter binary vector from the intergenic region of *Arabidopsis thaliana cab1* and *cab2* divergent genes useful for plant transformation

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The ability to express genes in a controlled and limited domain is essential to succeed in targeted genetic modification. Having tools by which to rapidly and conveniently generate constructs which can be assayed in a diverse array of plant species expedites research and end-product development. Targeting specifically green plant tissues offers an opportunity to effect changes to diverse processes such as water use efficiency, photosynthesis, predation and nutrition. To facilitate the generation of transgenes to be expressed in this domain, we created a series of plasmids called p2CABA based on the *Arabidopsis thaliana* chlorophyll *a/b* gene promoter, a single natural bidirectional promoter that can drive and express two different genes at the same time. Studies we carried out showed reporter gene, *GUS* expressed in leaves and stems but not in the roots, as expected since this endogenous promoter controls the expression of two photosynthetic genes in *A. thaliana*. We, therefore, utilized the intergenic region between the *A. thaliana cab1* and *cab2* divergent genes to design and construct a bidirectional promoter vector containing two multiple cloning sites and a gateway recombination cassette. This in turn will help minimize gene silencing and achieve desirable expression pattern of transgenes, a critical issue in plant genetic engineering and in this report we show their use in Medicago and tomato.

Key words: *Arabidopsis cab* genes, bidirectional promoters, gene expression, plant genetic engineering, reporter genes, photosynthetic genes.

INTRODUCTION

Genetic engineering is the deliberate manipulation of an organism's genome using modern DNA recombinant technology and this involves the introduction of foreign DNA or synthetic genes into the organism of interest. Genetic engineering of plants offers a wide range of advantages achieved through the introduction of a number of different desirable genes at a single event and reducing the time needed to integrate introduced traits into an elite genetic background. This therefore, widens

the potential pool of useful genes such as herbicide resistance, increased virus tolerance, or decreased sensitivity to insect or pathogen attack and drought tolerance. The generation of transgenic plants is increasingly a routine activity for important agricultural plants and this is achieved mainly by using *Agrobacterium tumefaciens* and particle bombardment methods.

Genetic engineering in plants is highly dependent on the transformation efficiency and the transgene expression levels in specific tissue (Koyama et al., 2005). The problem arising from transgene expression levels can be addressed by the promoter chosen in the gene construct. These promoters can be constitutive or tissue

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specific. The cauliflower mosaic virus (CaMV) 35S promoter and its derivatives are commonly used in dicots. Modern recombinant DNA technologies have vastly expanded what is possible with the study of molecular biology and in crop improvement.

For many applications, the use of a single transgene is insufficient and therefore use, of multiple transgenes is required (Halpin, 2005) as would be the case in pathway engineering. Also, regulatory requirements are increasingly requesting multiple genes for insert control to prolong development of resistance (Kumar et al., 2008). When multiple genes are required to act in concert to produce a given function, two or more promoters are generally required to drive expression (Koyama et al., 2005).

Using multiple copies of identical promoters can lead to transgene silencing (Meyer, 1996; Mitra et al., 2009) and promoters with different sequences may not have completely overlapping expression domains or similar levels of expression, which may be exacerbated when used in multiple species. There are still a very limited number of well characterized promoters, and fewer still characterized in divergent plant species (Carre and Kay, 1995). Therefore, the practice of gene stacking causes more frequent transgene silencing often caused by repeated DNA sequences and a greater chance of rearrangement among the repeatedly used promoter sequences (Mitra et al., 2009).

Plastids are becoming important targets for biotechnological improvement. These organelles are responsible for intercepting light energy, chemically fixing inorganic carbon dioxide and executing a host of other biochemical functions. However, plastid transformation is a cumbersome procedure and is still a routine in only a very small number of species (Maliga, 2004). Therefore, the natural plastid targeting sequences present in the 5' coding sequences of *A. thaliana* chlorophyll a/b, that is, *Arabidopsis cab1* and *cab2* (AtCAB1 and AtCAB2) can be harnessed to create a bidirectional promoter transformation vector which retain either of the two targeting sequences so that the relative ease of nuclear transformation could be taken advantage of to direct high levels of recombinant protein to the chloroplast.

Therefore, we used the *Arabidopsis cab* region to construct three general purpose cloning and or transformation vectors, p2CABA, p2CABB and p2CABC. p2CABA contains only the promoter and 3'UTR regions of AtCAB1 and AtCAB2. p2CABB and p2CABC each contain one of the plastid targeting sequences from AtCAB1 and AtCAB2 respectively. A version containing both targeting peptides was not created in order to avoid repetitive sequences within the t-DNA.

The GateWay vector system provided the backbone of these binary vector series with single natural bidirectional promoter. The high expression and bidirectionality of the AtCAB intergenic region has been previously verified in the widely divergent species *Nicotiana tabacum*. There

are two multiple cloning sites on opposite sides of the *bidirectional* promoter to make it more user friendly, in addition to a Gateway option for cloning.

MATERIALS AND METHODS

Bacteria strains

Escherichia coli strains One Shot® *ccdB* Survival™ 2 T1R competent cells (Invitrogen Corporation, Grand Island, NY, USA), DB3.1™ competent cells (Invitrogen Corporation, Grand Island, NY, USA) and *Agrobacterium tumefaciens* GV3101 were used.

Construction of the destination binary p2CABA vector

Gateway™ vector pKGW, intergenic region from divergent *Arabidopsis cab1* and *cab2* genes, *E. coli* strains *ccdB* Survival™ 2 T1R and DB3.1, 35S and octopine synthase (OCS) terminators were used in this study. We followed these steps; (i) vector backbone from pKGW, modified with a t35S terminator to the right side of multiple cloning site (MCS2), (ii) inserted the tOCS terminator to the left multiple cloning site (MCS1), and (iii) finally addition of the *cab1/2* gene promoter to complete the p2CABA vector (Figure 1). Polymerase chain reaction (PCR) cloning of the promoter of the OCS terminator was carried out while the CaMV 35S terminator was added through traditional restriction and ligation. Traditional restriction digestion and ligation was used for cloning t35S into linearised pKGW while tOCS was cloned using PCR and In Fusion™ PCR Cloning system was used for insertion into the pKGW+t35S vector backbone.

We incorporated the *SpeI* sites into promoter near the *attR* sites to remove the recombination cassette in the vector pKGW, placed *AvrII* at Kan end of promoter and *HindIII* and the t35S terminator inserted. Ligate were later transformed into DB3.1 *E. coli* cells and selection was done on Luria-Bertani (LB) spectinomycin plates. Colonies grown on LB spectinomycin plate were checked for insert presence by restriction digestions using the reconstituted restriction sites of each insert. The resultant construct, pKGW+t35S was digested with *XbaI* and *AvrII* to which PCR cloned tOCS modified with *XbaI* and *AvrII* sites (Figure 2) was inserted through In Fusion system (Clontech Laboratories, Inc. CA, USA) and transformation of *ccdB* Survival™ 2 T1R competent cells (Invitrogen Corporation, USA) by heat shock method. Resultant colonies were checked for tOCS insert presence using colony PCR with primers OCSA: (5'-ACCCGGGGATCCTCTAGACTGCTGAGCCTCGACATGTTGTGC) and OCSB: (5'-CGTCTCGCATATCTCATTAAGCAGCCTAGGCTTAATTAATCGC GAAGGCCTAAAGAGCTCCATTTAAATACTAGTGACGTCGCATG CTCCCGGCCGCA). The vector pKGW+t35S+tOCS were linearised with enzymes *StuI* and *SacI* and the bidirectional promoter modified with *StuI* and *SacI* was PCR cloned and inserted using In Fusion system. To circumvent the *ccdB* effects, we performed the LR recombination reaction (recombination reaction between an entry clone containing *attL* and a destination vector containing *attR* sites).

Gateway cloning was used to place the GUS coding sequence downstream of the CAB2 transcriptional start site. For use in other experiments, we inserted a cassette containing an inducible promoter driving CRE recombinase flanked by two LOX-P sites into MCS2. For GUS assays, we stained plants that had undergone excision of the LOX-P Cre-cassette leaving only a single LOX-P site in MCS2 which is part of the 5' UTR of the GUS transcript. The sequence data of this bidirectional promoter transformation vector, p2CABA have been submitted to the GenBank databases under accession number JQ965697 (Figure 1).

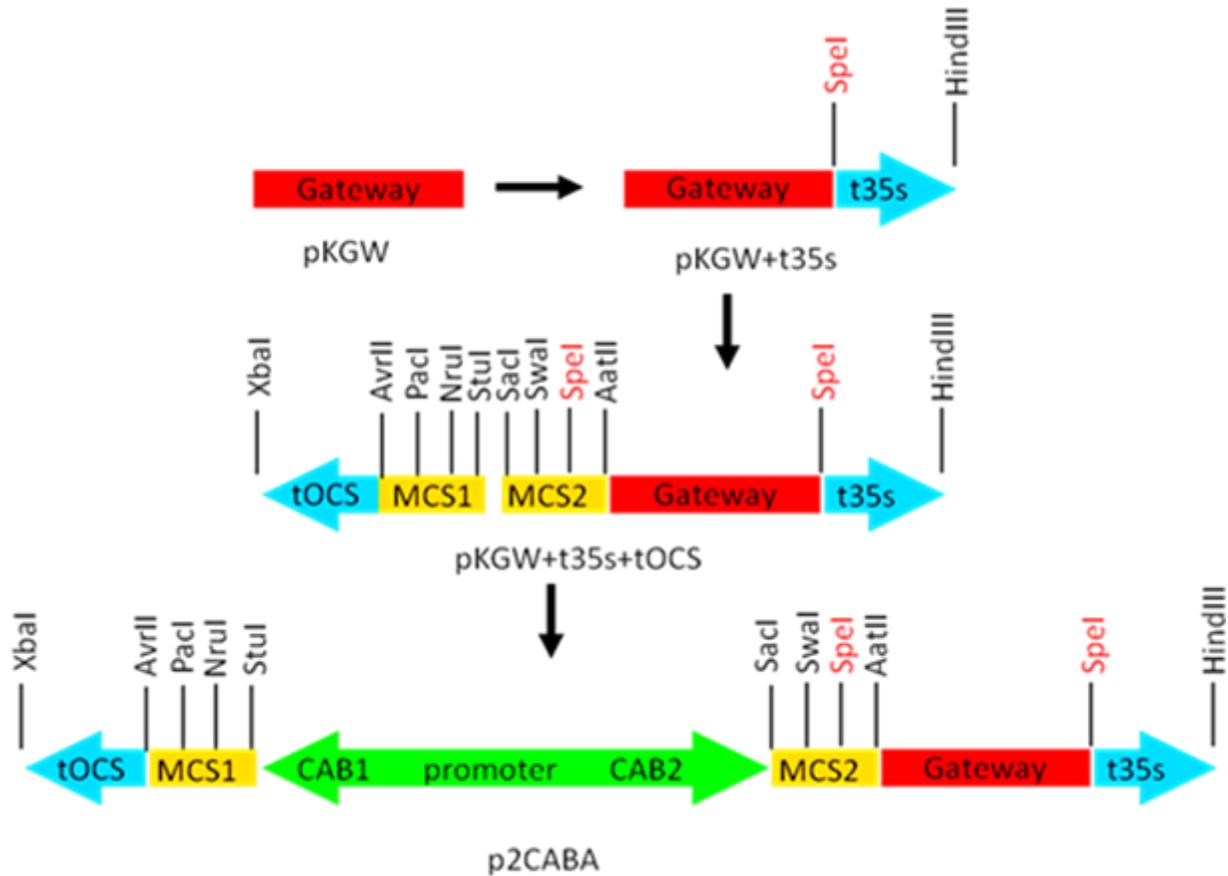


Figure 1. Schematic diagram of steps followed in building the bidirectional binary vector p2CABA from the backbone of Gateway vector pKGW.

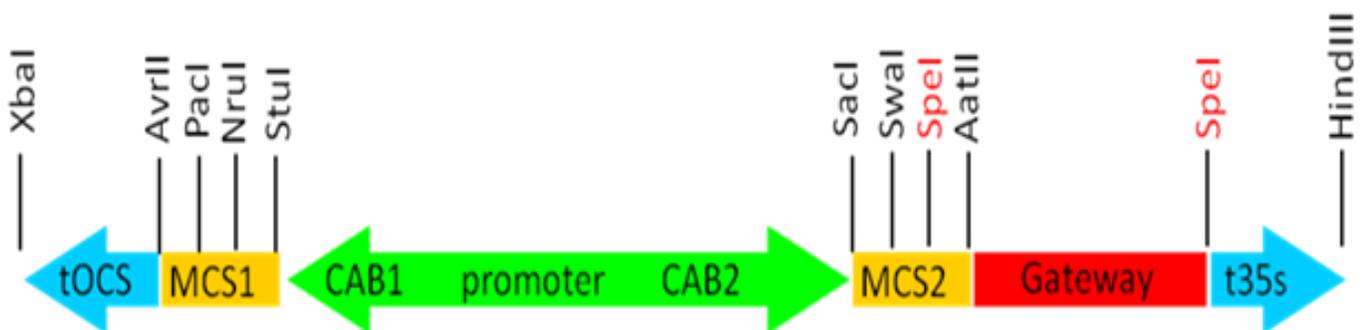


Figure 2. Vector cassette map. tOCS, the polyadenylation signal from the T-DNA-encoded octopine synthase gene; MCS1, the first Multiple cloning site; CAB1/2 prom, the bidirectional promoter of the intergenic region from divergent *Arabidopsis cab1* and *cab2* genes; MSC2, the second multiple cloning site; Gateway, part of the Gateway LR recombination sites *attR1* and *attR2*; t35S, polyadenylation signal of the CaMV 35S terminator.

Plasmid DNA isolation

All plasmid DNA was isolated using QIAprep® Spin Miniprep Kit. QIAGEN GmbH, D-40724 Hilden.

Transformation of *Agrobacterium* by electroporation

Agrobacterium strain GV3101 was transformed via electroporation (2.5 Kv/uM/mSec) and incubated in 0.5 mL liquid LB for 3 h at 28°C

with shaking. 50 μ L of transformation culture was plated onto LB medium plates with the following antibiotics: rifampicin (Sigma-Aldrich, St. Louis Missouri, USA) 100 μ g/ml; gentamycin (PhytoTechnology Laboratories, Shawnee Mission, Kansas, USA) 100 μ g/ml and spectinomycin (PhytoTechnology Laboratories, Shawnee Mission, Kansas, USA) 200 μ g/ml.

A single colony was selected after 48 h growth at 28°C and placed into 10 mL LB with the above antibiotics and grown overnight with agitation at 28°C. *Agrobacterium* transformation was confirmed by restriction digestion on plasmid extracted with Qiagen plasmid mini kit (QIAprep® Spin Miniprep Kit. QIAGEN GmbH, D-40724 Hilden). 8 mL were used for plasmid extraction in four 2 mL Eppendorf tubes. At the neutralization step after centrifugation, each tube was inverted gently a few times and recentrifuged until the membranous layer could no longer be reduced. Each of the four neutralized lysate was passed successively through a single Qiagen column to accumulate plasmid and the rest of the protocol was followed as per product manual. Restriction digestions were carried out on 17 μ L of eluted plasmid with 2 μ L digestion buffer and 1 μ L restriction enzyme and product visualized using standard gel electrophoresis. Glycerol stocks were made after confirmation and freshly streaked plates made.

Plant transformation

The Tomato Transformation Protocol (Ralph M. Parsons Foundation, 2011) was used in plant transformation and this was done in The Ralph M. Parsons Foundation Plant Transformation Facility, University of California Davis. California. USA.

GUS staining

All plant tissue materials from leaf, shoot and flower sample were collected and placed directly into vials containing hexane (Sigma-Aldrich, St. Louis Missouri, USA) for 5 min at room temperature to remove cuticle and facilitate staining. Ice-cold 90% acetone (Fisher Scientific, Hampton, New Hampshire, USA) was then poured into the vials and the vials placed on ice for 30 min with 1 change of fresh 90% acetone (Fisher Scientific, Hampton, New Hampshire, USA) at 15 min to remove remaining hexane. 90% acetone was poured off and replaced with cold GUS staining buffer (Without X-GLUC) and allowed to stand for 2 min to remove most of the remaining acetone. Wash GUS buffer was poured off and replaced with room temperature GUS staining buffer containing X-GLUC and vials were placed into a vacuum incubator at 37°C and exposed to soft vacuum overnight. After GUS staining reaction, used GUS buffer was poured off and replaced with 75% ethanol (Sigma-Aldrich, St. Louis Missouri, USA) and allowed to stand for 30 min with periodic gentle agitation. 75% ethanol was poured off and replaced with 100% methanol (Sigma-Aldrich, St. Louis Missouri, USA) to facilitate chlorophyll removal. Changes were done hourly until no chlorophyll was visible in the tissues or the methanol. Tissues were stored indefinitely in 100% methanol with no noticeable solubilization of GUS after three months.

Solutions

Phosphate buffers A and B were made in 1 L stock solutions. Buffers K3 and K2 were made in 50 ml stock solutions. All stock solutions were stored at room temperature. GUS staining buffer was made prior to each use; buffer A: 27.59 g/L Na₂HPO₄ (Sigma-Aldrich, St. Louis Missouri, USA) and buffer B: 28.39 g/L NaH₂PO₄ (Sigma-Aldrich, St. Louis Missouri, USA); 0.1 N working phosphate buffer: 39 ml buffer A, 61 ml buffer B. H₂O to 200 ml, K3 ferricyanide

(Sigma-Aldrich, St. Louis Missouri, USA): 0.0167 g/ml and K2 ferrocyanide (Sigma-Aldrich, St. Louis Missouri, USA): 0.0211 g/ml; GUS staining buffer for 10: 5 ml working phosphate buffer, 100 μ L K3, 100 μ L K2, 100 μ L Triton X-100 (Sigma-Aldrich, St. Louis Missouri, USA), 100 μ L dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis Missouri, USA), 200 μ L 0.5 M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis Missouri, USA), 0.01 g X-GLUC (Rose Scientific Ltd, Edmonton Alberta, Canada) dissolved in 200 μ L dimethylformamide (DMF) (Sigma-Aldrich, St. Louis Missouri, USA). H₂O to 10 ml.

RESULTS

Resultant vector, p2CABA (Figure 3) has kanamycin plant selection with spectinomycin selection for *E. coli* and *Agrobacterium*. The p2CABA has a *ccdB* gene which limits its use in transformation of *E. coli* with the *E. coli* strain with resistance to *ccdB* effects and can support the propagation of plasmids containing the *ccdB* gene.

Consistent with the reports of this promoter expression in tobacco (Mitra et al., 2009), we found strong even expression in leaves and stems of tomato and alfalfa. GUS expression was detectable in sepals but not petals. GUS was only visible in the green portions of the tomato leaves (Figure 4i) while flower, petals and anthers were free of GUS staining (Figure 4ii).

Interestingly, in developing leaves of both species, GUS activity was only detectable initially at the distal portion of the leaflets (Figures 4A and B). This moved basipally as young leaves developed, corresponding to the wave of differentiation as the cells stop dividing and growth as a consequence of cell expansion begins. Consequently, expression in tissues undergoing maturation and in mature tissues but not in developing organs will reduce the risk of developmental abnormalities due to high levels of expression of heterogonous transgene products in young organ primordia.

DISCUSSION

To facilitate applications of plant research and biotechnology, we constructed a series of convenient vectors using the well characterized *Arabidopsis* chlorophyll *a/b*-binding (*cab1/2*) protein gene family (*Cab*) promoter. In the study, we made a bidirectional promoter – GUS construct of p2CAB was transformed into tomato and later performed a GUS staining experiment which showed all but only the green tissues of the plants stained blue colouration which clearly showed and confirmed that this promoter only expresses genes in chlorophyll containing tissues (Figure 4i, ii). Our studies using the heterologous species *N. tabacum* have shown that expression of the reporter gene GUS is high in leaves, stems and other green tissues but not in roots. In Figure 4ii, there is a clear distinction between the yellow

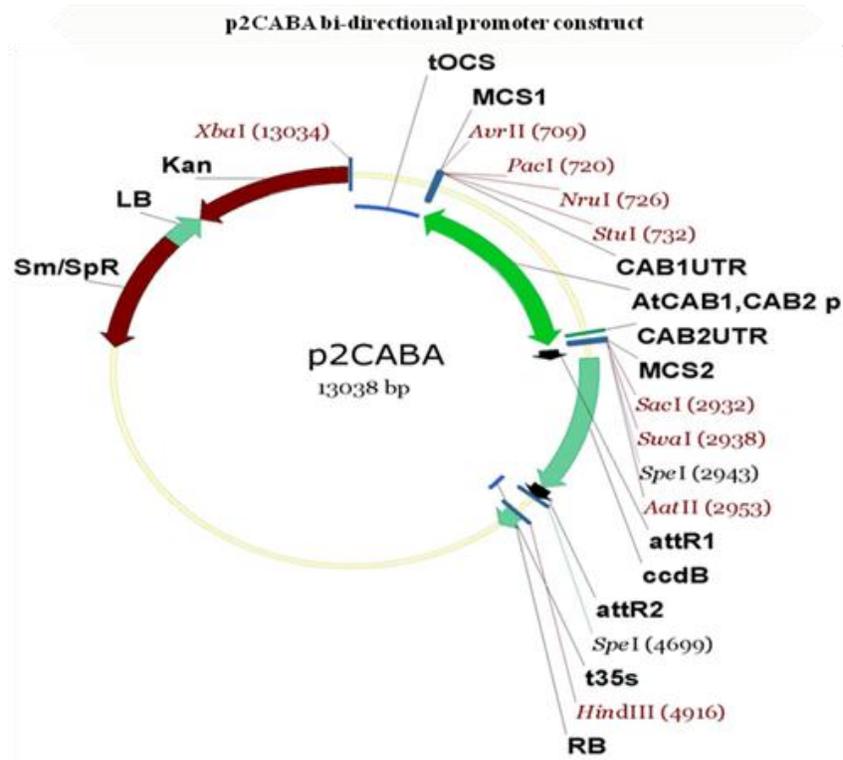


Figure 3. Vector map of p2CABA generated by Vector NTI Advance 9, Invitrogen.

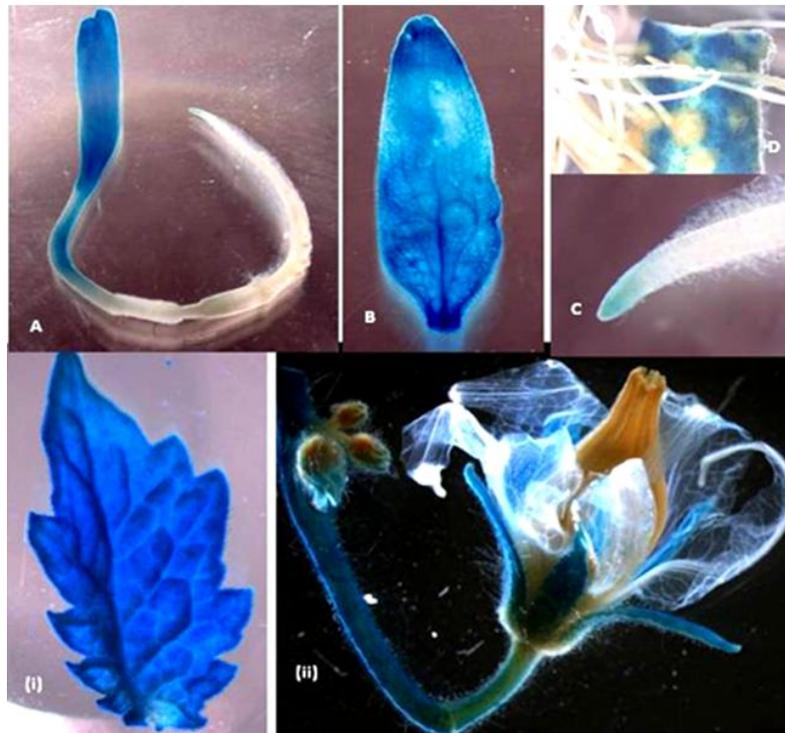


Figure 4. Expression of p2CABA-GUS in alfalfa and tomato tissues. **A)** Alfalfa shoot and root. **B)** Alfalfa leaf. **C)** Alfalfa root. **D)** Alfalfa seedlings. **(i)** Tomato leaf. **(ii)** Tomato flower.

therefore, shows a clear confirmation that this promoter is tissue specific and its expression is only in chlorophyll containing plant tissue. This clearly relates to the available literature where this endogenous promoter controls the expression of two photosynthetic chlorophyll *a/b* genes in *A. thaliana*. The ability of this small intergenic sequence to drive two coding sequences at high levels in green tissues and at high expression levels made this an ideal system on which to build a general use construct which can be used in a broad variety of plant species.

Conclusion

We sought to verify that the expression was restricted to green tissues in two additional species, *Medicago sativa* and *Solanum lycopersicum*, which belong to rosids and asteroids, respectively. The p2CABA vector will help minimize gene silencing and achieve desirable expression pattern of transgenes, a critical issue in plant genetic engineering. Therefore, the use of this vector in heterologous background will provide a means to express multiple genes in transgenic plants enhancing genetic engineering-based crop improvement especially in green tissue expressed genes against biotic and abiotic stresses like foliage diseases and pests.

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