Modification of pEtc20 with a Modified *Cauliflower mosaic virus* 35S Promoter

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Abstract

The *Cauliflower Mosaic Virus* (CaMV) 35S promoter has been the go-to promoter for viral and mRNA work in plants. The promoter was originally mapped in turnip, a dicot. The promoter works well in dicots but does not have as much success in monocots, such as grasses. It is our aim to provide evidence that the CaMV 35S promoter is in need of modification to work in grasses. Prior to our experiment, K. Scheets constructed two *Maize chlorotic mottle virus* (MCMV) plasmids with CaMV 35S promoters. One plasmid, pCaMCMV, was constructed with a promoter: virus junction according to the published transcription start site of the 35S promoter. It was essentially noninfectious, infecting only 1% of plants inoculated. The second plasmid, pEtc20, was constructed with four additional bases at the beginning of the MCMV sequence. It was much more infectious, infecting 60% of the plants inoculated. Using this preliminary data, we attempted to assemble two MCMV plasmids with modified promoter/transcription start sites of CaMV 35S. Our initial attempt was unsuccessful, with either pEtc20 being recovered or no DNA recovered at all. New primers with revised melting temperatures were obtained. These were used for another attempt at joining the promoter and MCMV regions. If we are correct, researchers will be able to use the CaMV 35S promoter for better results in mRNA and viral work in monocots.

Keywords: MCMV, CaMV 35S promoter

Introduction

There are several methods of inducing infections of plants with plus sense single strand viruses via mechanical inoculation. One such method is to make the plasmid with an inserted eukaryotic promoter followed by the viral cDNA sequence. A method for accurate termination of the RNA transcript must also be included. Mechanical inoculation or the use of *Agrobacterium* is used to introduce the DNA into a plant and induce infection. After the viral RNA is synthesized, it is transported to the cytoplasm where it begins the viral replication cycle. The *Cauliflower mosaic virus* (CaMV) 35S promoter is the promoter of choice for this procedure. This promoter was originally mapped in a turnip.

The first infection of a plant completely originating from cDNAs was performed using *Brome mosaic virus* (BMV) cDNAs inserted between modified CaMV 35S promoter and terminator regions derived from CaMV DNA (Mori et al. 1991). Of the plants inoculated, only eudicots were able to be infected. Only *Barley mild mosaic virus* (BaMMV) with a modified CaMV 35S promoter has been able to successfully infect a cereal through rub inoculation, albeit at a low success rate (Meyer and Dessens 1997).

The Scheets lab assembled two similar versions of a CaMV 35S:*Maize chlorotic mottle virus* (MCMV) cDNA based on the turnip-mapped CaMV promoter. Both plasmids have the MCMV sequence identical to that of pMCM41, an infectious transcript cDNA (Scheets et al., 1993). The "correctly" assembled plasmid (pCaMCMV) is essentially noninfectious with only 1% of inoculated plants becoming infected. Another plasmid, pEtc20, was constructed and had 4 extra nucleotides between the promoter and the viral sequence. pEtc20 is readily infectious with

*=Mentor

pEtc20

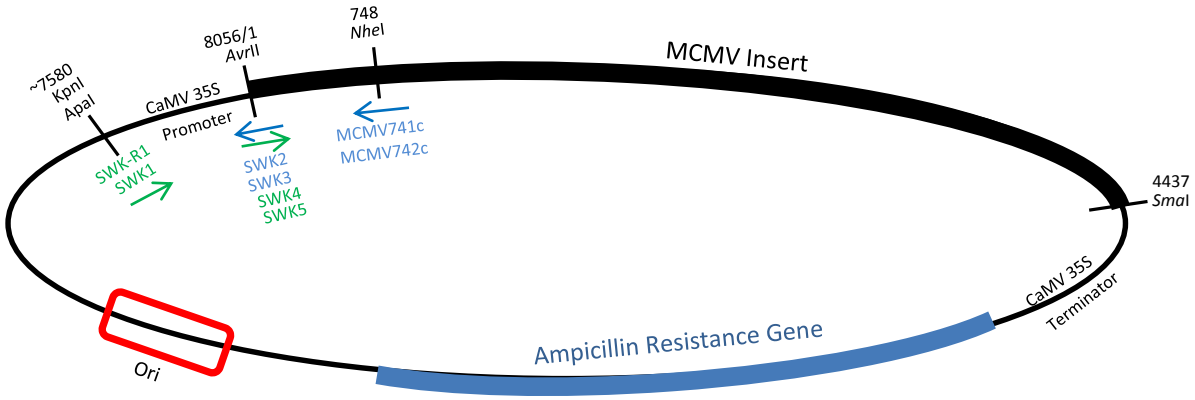

Figure 1 - Map of pEtc20. Relevant unique restriction sites are marked. Locations and orientations of forward (green) and reverse (blue) PCR primers are marked by arrows and are larger than actual annealing regions. MCMV cDNA is a heavy black line flanked by the CaMV 35S promoter and terminator sequnces. The ampicillin resistance gene is a heavy blue line. The origin of replication (ori) is marked by a red box.

60% of eighty-one plants becoming infected. More recent work in the Scheets lab included transcription mapping from these plasmids and transgenic maize using a CaMV 35S promoter.

The hypothesis is that the turnip-mapped transcription site of the CaMV 35S promoter is not the site that is most often used in maize and probably not used in other grasses. With the Scheets' lab preliminary results, new MCMV plasmids with a revised promoter:virus junction of CaMV 35S were designed (Fig.1). If we are correct, the new constructs will be more infectious at lower DNA inoculation concentrations than pEtc20. With this information, other researchers will be able to design DNA-inoculation plasmids for use in directly infecting grasses.

Methods

General Methods

General laboratory procedures for gel electrophoresis, restriction enzyme digests, phenol/$CHCl_3$ extraction, ethanol precipitation, bacterial plates, media preparations, and drop dialysis were used (Sambrook et al. 1998). A 0.7% agarose gel with a 0.8X TAE buffer was used for electrophoresis. Quantitation of purified DNA was done by using a Nanodrop 2000c (Thermo Scientific, Wilmington, DE).

Primers

Six primers were used to create two different versions of the CaMV 35S:MCMV junction. Both involved different pairs of primers and had different numbers of spacer sequences. All primers were from Integrated DNA Technologies (Coralville, IA). The primers were designed for use in overlap extension PCR to join the promoter and MCMV sequences. The outside primer sites, SWK1, SWK-R1, MCMV742c, and MCMV741c, were designed with two different unique restriction enzyme sites (*Kpn*I at 7583 bp or *Nhe*I at 748 bp) (Fig. 1) within the parent plasmid. Promoter primers designated SWK2 and SWK3 and MCMV primers designated SWK4 and SWK5 were designed to change the promoter:virus junction with a modified sequence.

PCR of replacement fragments

Polymerase chain reaction (PCR) was used to construct the desired fragments.

All PCR reactions were done following manufacturer's instructions by using either Q5 or Q5 Hot Start DNA polymerase (New England Biolabs, Ipswich, MA). Several different methods of PCR were used. These included overlap extension PCR, touchdown PCR, and temperature gradient PCR.

pEtc20 provided template DNA for synthesis of PCR2 through PCR5 and PCR2R through PCR5R. Primers for PCR2 were SWK1 and SWK2. Primers for PCR3 were SWK1 and SWK3. Primers for PCR4 for were MCMV742c and SWK4. Primers for PCR5 were MCMV742c and SWK5. Primers for PCR2R were SWK-R1 and SWK2. Primers for PCR3R were SWK-R1 and SWK3. Primers for PCR4R were MCMV741c and SWK4. Primers for PCR5R were MCMV741c and SWK5. For PCR24, low amounts of column purified SWK2 and SWK4 were used as templates with SWK1 and MCMV742c acting as primers. For PCR35, small amounts of column purified SWK3 and SWK5 were used as templates with primers SWK1 and MCMV742c.

DNA Fragment Purifications

Purification of fragments was done by using either the QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands) or NucleoSpin Gel and PCR Clean-up Kit (Machery-Nagel, Duren, Germany). Gel isolation of the 1.2 kb fragments was performed with the NucleoSpin kit.

Ligation of Inserts with Vector DNA

K. Scheets provided pEtc20 that was digested with *Kpn*I and *Nhe*I followed by phenol/$CHCL_3$ extraction and ethanol precipitation, and then dephosphorylated. The 1200 bp fragments were digested with *Kpn*I and *Nhe*I and K. Scheets used phenol/$CHCL_3$ extraction and ethanol precipitation to remove the enzymes. In separate reactions, PCR24 or PCR35 were ligated with digested, dephosphorylated pEtc20 using T4 DNA ligase (New England Biolabs) and ultrasonic ligation (Cooper et al. 1993).

Transformation and Selection

The ligation reactions were transformed into *Escherichia coli* DH5α using an ECM 600 Electroporator (BTX, Holliston, MA). Samples were then shaken at 37°C for 30 minutes. Samples were then plated on LB plates containing 75 µg/ml carbenicillin and incubated at 37°C overnight. Individual colonies were then picked from the plates and grown overnight in 2 ml of Terrific Broth. The culture was then transferred to 1.7 ml microfuge tubes and centrifuged. The DNA from the resulting pellet was using the EasyPrep method (Berghammer and Auer 1993).

Plasmid Analysis

Screening was done by performing a restriction digest with *Avr*II, as the original pEtc20 plasmid would have its restriction site while the new plasmid would not. The digests and uncut pEtc20 were then electrophoresed to check if the correct sized plasmids had been constructed.

Progress to Date

Amplification of PCR2, PCR3, PCR4, and PCR5 were successful, yielding

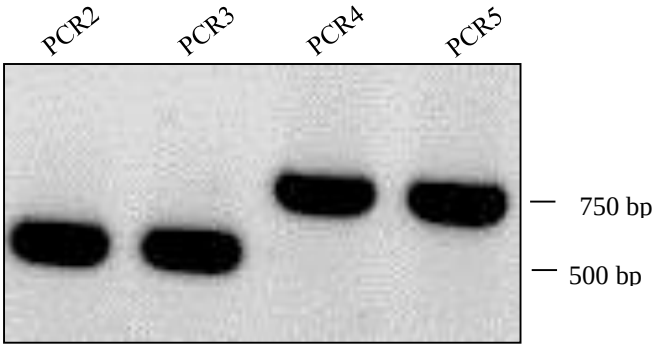

Figure 2 - Agarose gel of PCRs with original primers. The approximate sizes of the products are indicated on the right.

the desired size 500 bp or 750 bp fragments (Fig. 2). Our first attempt at an overlap extension PCR for the creation of the full inserts, PCR24 and PCR35, was also successful, producing the desired 1200 bp fragments. However, additional fragments varying from ~500 bp to just less than 2 kb in size were also present (Fig. 3A). Attempts at manipulating PCR conditions to produce only the desired 1200 bp fragments were unsuccessful. The 1200 bp fragments were gel purified. PCRs utilizing the outside primers , SWK1 and MCMV 742c, with gel purified, drop dialyzed 1200 bp fragments produced 1200 bp fragments as well as bands of additional sizes as seen with other PCRs (Fig. 3B). The overlap extension PCRs with the small fragments were digested with *Kpn*I and *Nhe*I in hopes of getting the desired transformation products. Carbenicillin plates yielded very few colonies. The *Avr*II digests were performed and found that either no DNA was present or what was present had the *Avr*II site (Fig. 4).

PCRs with the drop dialyzed fragments and the new outside primers with higher melting temperatures, SWK-R1 and MCMV741c were successful in amplifying the 1200 bp fragments, but unsuccessful in eliminating the small products (Fig. 5A). A new large fragment of pEtc20 was produced using *Apa*I and *Nhe*I since both of these enzymes can be readily heat-inactivated, eliminating multiple purification steps that can reduce yields of vector and insert DNAs (Fig. 5A). PCRs with the original SWK2, SWK3, SWK4, and SWK5 primers and either SWK-R1 or MCMV741c were performed. These PCRs were successful, producing the desired 500 bp and 750 bp fragments (Fig. 5B).

Discussion

The results of the restriction digests are evidence that our attempt to construct the new MCMV plasmid had failed. The desired plasmid should not have the *Avr*II site, while pEtc20 itself would have the restriction site. It is possible that a problem with the two outside primers caused this failure. The melting temperatures of the initially annealed portions of SWK1 and MCMV742c were 60°C and 63°C respectively. During the experiment, initial annealing temperatures of 62°C-63°C were used followed by higher annealing temperatures for most of the amplification cycles. The initial annealing temperatures apparently allowed MCMV742c to mis-prime to an internal region of the MCMV sequence, thus

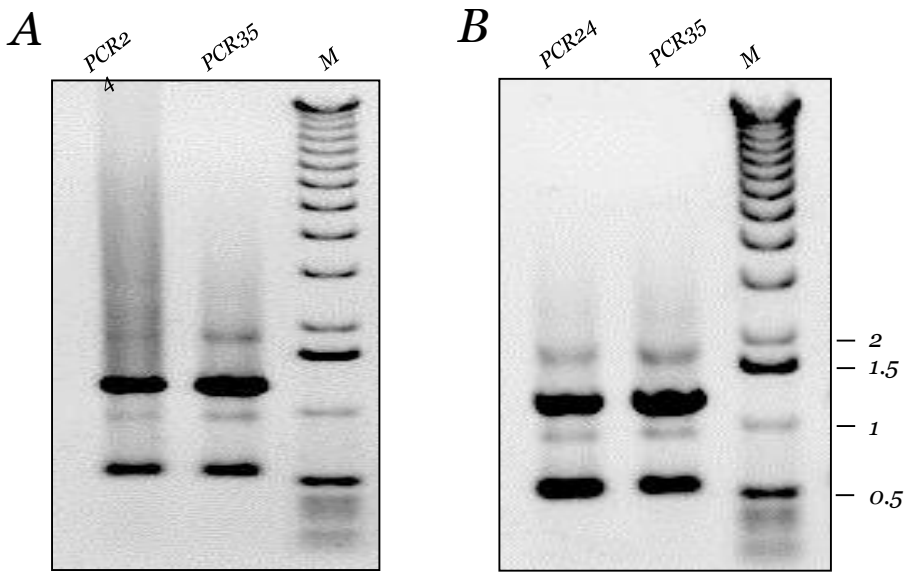

Figure 3 - Agarose gels of PCRs for amplification of 1200 bp DNAs. A) Templates were either PCR2 + PCR4 or PCR3 + PCR5 in overlap extension PCRs. B) PCRs used the gel purified, drop dialyzed 1200 bp DNAs from overlap extension PCRs as templates. Sizes in kb of some BRL 12 kb Ladder (M) bands are shown on the right.

producing the ~ 500 bp fragments alongside those of the expected size.

In an attempt to remedy this, two new primers SWK-R1 and MCMV741c were purchased. These primers were designed to be larger and have higher melting temperatures of ~70°C. The higher melting temperatures allow for a higher initial annealing temperatures. Attempts to use the original gel-purified, drop dialyzed fragments made previously were unsuccessful at amplifying only the 1200 bp fragment. Due to this, new constructs had to be made. Synthesis of PCR2R-PCR5R was successful, producing either 500 bp fragments or 750 bp fragments (Fig. 5B). Further work on pEtc20 improvements in the Scheets' lab will use the newly amplified PCR products with the higher melting temperature primers for synthesis of the entire 1.2 kb fragments. This should allow little or no production of the wrong insert fragments.

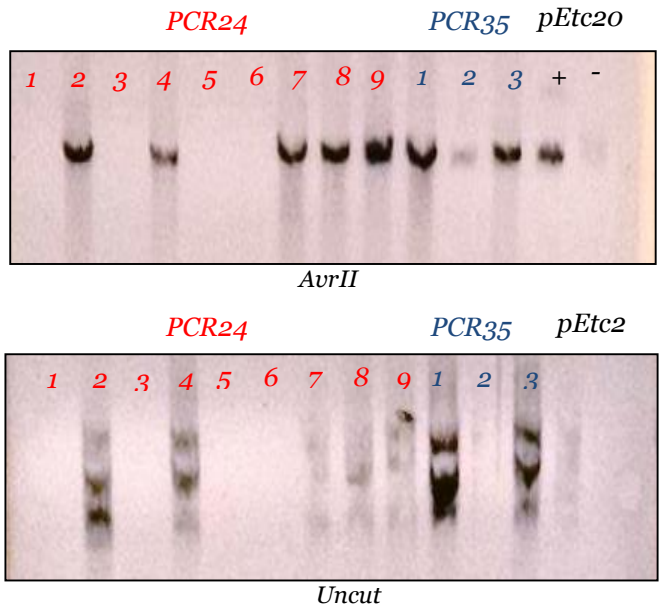

Figure 4 - Agarose gel analysis of plasmids recovered from bacterial transformations. Upper gel shows plasmids from ligations of dephosphorylated pEtc20 cut with KpnI and NheI with PCR24 (red) or PCR35 (blue) were digested with AvrII (upper panel). pEtc20 with (+) or without (-) AvrII digestion is included. Lower gel shows migration of uncut plasmids.

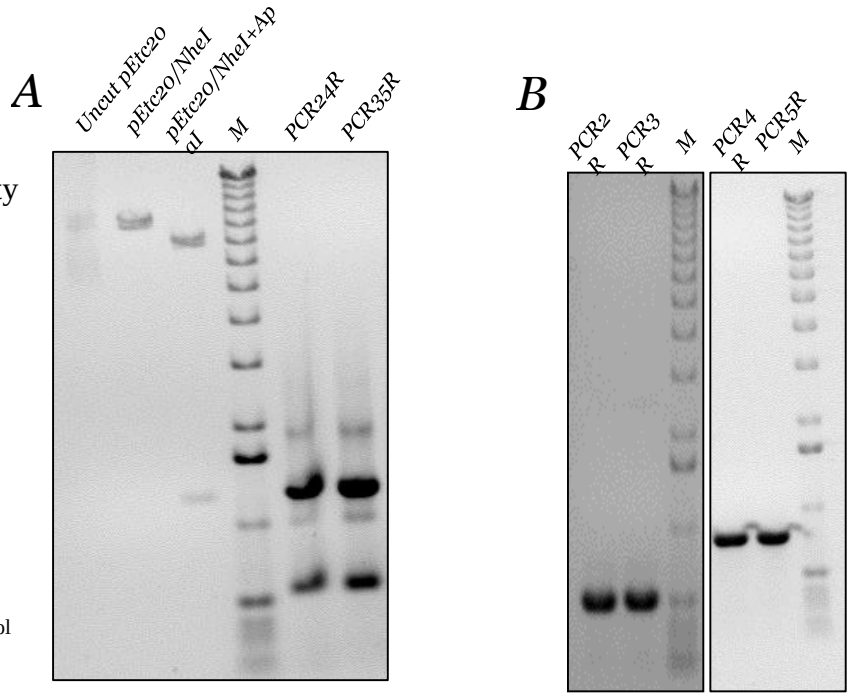

Figure 5 - Agarose gels of PCRs and pEtc20 digests. A) Single and double digests of pEtc20 with ApaI and NheI are on the left. PCRs using gel purified, drop dialyzed 1200 bp fragments with SWK-R1 and MCM741c (PCR24R and PCR35R) are on the right. B) Agarose gels of individual PCRs using primers SWK-R1 or MCM741c with pEtc20 template and relevant primers SWK2, SWK3, SWK4, and SWK5.

Acknowledgements

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