CHAPTER 4:
Ligation independent cloning

ABSTRACT:

We describe a technique to clone kilobase size fragments of double-stranded DNA from any source into plasmid vectors, as long as the sequence of the insert termini are known. The thermostable DNA polymerase purified from *Pyrococcus furiosus* is used for this technique. Recombinant plasmids are generated and recovered in *E. coli* in one day. To demonstrate the utility of this method we cloned genomic fragments encoding chalcone synthase (CHS) and stilbene synthase (STS) from *Arabidopsis thaliana* and *Arachis hypogaea*, respectively, into the phagemid pBluescript KS+. This method has been termed ligation-independent cloning (LIC).

INTRODUCTION:

The shuttling of genes between organisms has been a major goal since the determination that DNA is the molecule of heredity (Hershey and Chase, 1952). Since then a flood of new techniques to clone genes from all cell types has revolutionized how scientists study protein function. Obstacles that prevent the study of scarce proteins are overcome by cloning the gene into, and purifying the protein from, bacteria.

Conventional methods of gene cloning into bacteria can be costly and time consuming. This process generally involves the isolation of a double-stranded DNA (dsDNA) fragment encoding the desired sequence. The fragment is then covalently joined with an autonomous self-replicating circular plasmid. Restriction enzymes are typically used to digest the DNA molecules to produce compatible ends and DNA ligase is required to join the fragments together. The DNA must be purified between each successive enzymatic manipulation and this can lead to loss of substrate and may involve many days of work.

Here we describe an alternative method to clone dsDNA fragments from most any source as long as the sequence of the insert termini is known. The origins of ligation independent cloning (LIC) stem from the development of the polymerase chain reaction (PCR) (Saiki et al., 1988) and difficulties in cloning PCR products during the stilbene synthase (STS) analysis (CHAPTER 3). Yon (1989) showed that PCR could be used to join two fragments of dsDNA together. This led to the idea that a DNA insert could be joined with a plasmid to create recombinant molecules *in vitro*. Two limitations to this approach are, however, the size of the products that can be made *in vitro* and the accuracy of the replicated sequence. These limitations can be overcome by using the thermostable DNA polymerase isolated from *Pyrococcus furiosus* (*Pfu*) (Lundberg et al., 1991). This polymerase is capable of amplifying products as large as 5 kb and, unlike *Taq* DNA polymerase, has 3’ to 5’ proofreading activity (Barnes, 1994; Cline et al., 1996). We demonstrate the utility of this approach by directly cloning fragments (Figure 1) of the genomic loci encoding CHS and STS from *Arabidopsis thaliana* and *Arachis hypogaea*, respectively.

MATERIALS and METHODS:

Plant Material and Growth Conditions.

A. thaliana ecotype Columbia and *A. hypogaea* cultivar NC7 were used in these studies. Approximately 1000 (25 mg) wild-type *A. thaliana* seeds and 100 *A. hypogaea* seeds were stratified on Murashige and Skoog-sucrose medium in the dark at 4°C for four days (Kubasek et al., 1992). Seeds were transferred to soil and germination was induced with constant light at 22°C. Mature plants were grown in soil at 22°C under a 16-hr-light / 8-hr-dark cycle. 3-week-old plant tissue was harvested and stored at -70°C.

DNA isolation.

A. thaliana genomic DNA was isolated according to Watson and Thompson (1986). These samples were further purified through a CsCl equilibrium centrifugation gradient (Maniatis et al., 1982) and stored in 10 mM tris pH 7.2, 1 mM EDTA at 4°C. *A. hypogaea* genomic DNA was isolated using a modified citrymethylammonium bromide (CTAB) technique. Ten grams of *A. hypogaea* tissue was homogenized with 35 ml of extraction buffer (0.1M Tris pH 7.5, 0.5 M EDTA, 20 mM Na-bisulfate, 6.4% sorbitol) and filtered through 2 layers of cheesecloth and 1 layer of miracloth. The filtrate was centrifuged at 1200 x g for 15 min at 4°C. The pellet was resuspended in 5 ml of extraction buffer then 5 ml of nuclei lysis buffer (0.2 M Tris pH 7.5, 0.05 EDTA, 2 M NaCl, 2% CTAB, and 1 ml of 10% sarcosyl were added. The sample was mixed gently and incubated at 60°C for 1 hour, then extracted once with 15 ml of chloroform/IAA (24:1). The aqueous phase was isolated and 1 volume of isopropyl alcohol was used to precipitate high molecular weight DNA. DNA was washed with 70% ethanol to remove salt and stored at 4°C.

Vector preparation.

One microgram of pBluescript KS(+) (Stratagene, La Jolla) was linearized by digestion with EcoRV (Promega, Madison) at 37°C for 4 hours. Linear dsDNA was purified away from proteins and buffer components using Promega Wizard PCR-preps columns (Promega, Madison) according to the manufacture's instructions and eluted in ddH_2O to a final concentration of 0.5 μg/ml.

PCR reactions.

Reactions containing 1 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 X buffering salts, and 2.5 units of *Pfu* DNA polymerase (Stratagene) were used to amplify the CHS or STS locus from 100 ng of *A. thaliana* or *A. hypogaea* genomic DNA, respectively. The two CHS primary PCRs contained the following primer combinations: reaction #1, pBSKas13 + a8; reaction #2, s13 + pBSKΩa8. The two STS primary PCRs contained the following primer combinations: reaction #1, G107aΩ + C98a; reaction #2, G107a + C98aΩ. Samples were initially denatured at 94°C for 90 s, then cycled at 94°C (90 s), 55°C (90 s), 72°C (180 s) for 35 rounds. Ten microliter aliquots of these reactions were examined by agarose gel electrophoresis and ethidium bromide staining. Remaining amounts of primary products were purified from unincorporated primers and nucleotides using the Wizard PCR preps columns and eluted in 50 μl distilled water.

One microliter of each purified primary product was mixed with 10 ng of EcoRV-digested pBluescript vector (Stratagene) in the presence of 1 μM of each appropriate secondary primers, 0.2 mM dNTPs, 1 X buffering salts, and 2.5 units of *Pfu* DNA polymerase. Template/primer combinations for the secondary CHS PCRs were: reaction A, template #1 and pBSKΩ + a8; reaction B, template #2 and s13 + pBSKa. Template/primer combinations for the secondary STS PCRs are: template #1 and pBSKΩ + C98a; reaction B, template #2 and pBSKa + G107a Samples were denatured at 94°C for 4 min, annealed at 60°C (for CHS reactions) or 52°C (for STS reactions) for 4 min, and extended at 72°C for 4 min. This step was repeated once to ensure the

creation of the chimeric fusion template. Samples were cycled at 94°C (75 s), 52°C (120 s), 72°C (5 min) for 33 rounds. Samples were extended with a final incubation at 72°C for 4 min.

Secondary products were purified from unincorporated primers and nucleotides using Wizard PCR preps columns and eluted in 50 µl distilled water. Twenty microliters of complementary purified chimeric fusions were mixed together and denatured at 94°C for 5 min., allowed to re-anneal at 60°C for 4 hours, then stored at 4°C. Three microliters of this product were used to transform *E. coli* JM109 by electroporation (Smith et al., 1990). Transformants were selected on 100 µg/ml ampicillin and recombinant plasmids were screened with isopropyl-B-D-thiogalactopyranoside (IPTG) and (X-gal) (Maniatis et al., 1982). Sequences of all primers are shown in table I.

PCR colony screen.

To confirm that transformed plasmids were recombinant, a single *E. coli* colony was suspended in 10 mM Tris pH 9.8 and 0.1% Triton X-100, boiled for 5 min, allowed to cool to room temperature, then centrifuged at 16,000 X g for 3 min. Three microliters of the supernatant of this sample was used as template in a PCR containing 1 μM T3 and T7 primer each, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 X buffering salts, and 2.5 units *Taq* DNA polymerase (Promega) in a total volume of 30 μl. Samples were denatured at 94°C for 45 s, then incubated at 94°C (70 s), 46°C (70 s), 72°C (180 s) for 40 cycles. Samples were stored at 4°C. Ten microliters of these samples were analyzed by agarose gel electrophoresis and ethidium bromide staining (Maniatis et al., 1982).

Plasmid analysis:

Plasmid DNA for restriction digest and sequence analysis was isolated from *E. coli* clones using a modification of the alkaline lysis miniprep (Birnboim and Doly, 1979) in which the initial precipitate was pelleted at 50,000 rpm in a TLA-100 ultracentrifuge (Beckman, Palo Alto, CA). One μl of plasmid DNA was digested with EcoRI and HindIII at 37°C for 2 hours and then restriction products were analyzed by agarose gel electrophoresis and ethidium bromide staining (Maniatis et al., 1982). Sequencing of recombinant plasmids using T3 and T7 primers was performed as described previously (Shirley et al., 1992).

RESULTS:

LIC works by using chimeric primers to fuse vector sequences to a desired DNA insert during an initial primary (1°) PCR reaction (Figure 1). This product then serves as a primer to fuse the insert DNA to the cloning vector in a secondary (2°) PCR reaction that amplifies the chimeric fusion of insert and plasmid species. These products are denatured and allowed to hybridize together. Combinatorial hybridization between chimeric fusions produces recombinant nicked circular plasmids that can be recovered by transformation of *E. coli*. The total process requires two PCR reactions, two DNA purifications, and transformation of *E. coli*. Recombinant plasmids can thus be obtained in one day.

Cloning the *A. thaliana* genomic CHS locus.

To demonstrate the feasibility of LIC we initially chose to clone a fragment of the CHS locus from *A. thaliana* genomic DNA (Figure 2). Amplification of the CHS insert was verified by agarose gel electrophoresis (Figure 3). This product was then used to synthesize the chimeric fusions with pBluescript KS(+) by PCR (Figure 3). The products of this reaction were denatured and allowed to anneal in order to create recombinant nicked circular plasmids capable of being replicated in *E. coli*. These samples were used to transform *E. coli* JM109. One thousand transformants were obtained per microliter of renatured chimeric DNA fusion. A transformation efficiency of 10^8 cfu/μg supercoiled pBluescript was obtained with JM109 under these conditions. Greater than 98% of these ampicillin-resistant colonies displayed a negative phenotype in a blue/white screen for b-galactosidase activity.

To determine if recombinant transformants actually contained the desired fragment of the *A. thaliana* genomic CHS locus, eleven random white clones were screened by PCR using plasmid-specific primers. One blue clone was included as a negative control. All eleven white clones produced the expected 874 bp product consistent with the presence of the CHS fragment (Figure 4). The one blue clone produced a 164 bp product consistent with a non-recombinant pBluescript plasmid. Clones containing multiple inserts were not observed. The clones were further characterized by sequencing plasmid DNA with T3 and T7 primers. No errors in the sequence of the CHS clone or the insert/vector junctions were observed. The insert was in the correct orientation as predicted by primer design.

Cloning the *A. hypogaea* STS genomic locus.

The same technique was used to clone a fragment of the STS genomic locus from *A. hypogaea*. Although the specific primers used were different from those used for *A. thaliana* CHS, the vector and strategy were identical (Figure 5). Five white transformants were screened by PCR with vector-specific primers (Figure 6). The products amplified from all five clones were consistent with the presence of a recombinant plasmid containing an insert the size of the published *A. hypogaea* STS fragment. To confirm these results plasmid DNA was isolated from three of these clones and digested with EcoRI and HindIII. Fragments consistent with the published *A. hypogaea* genomic STS sequence were obtained in these reactions as well (figure 6). Sequencing of the three purified plasmids confirmed the identity of the cloned fragment as the desired *A. hypogaea* STS fragment containing no detectable errors due to amplification. The orientation of these clones was also as predicted by primer design.

DISCUSSION:

The results of this study indicate that the LIC method can be used successfully to clone fragments of the *A. thaliana* CHS and *A. hypogaea* STS loci into the pBluescript KS(+) phagemid vector. Similar results for both cloning events were obtained, about 2000 total recombinant colonies were recovered of both CHS and STS clones. PCR screening for clones of CHS determined that all nine recombinant clones examined in this experiment contained the CHS insert. Similar results were obtained for five STS clones. This suggests that the LIC technique is extremely efficient. Sequence analysis verified the correct orientation of the clones as originally designed as well as the fidelity of sequence amplification. These results were consistent with previous studies that showed *Pfu* DNA polymerase amplifies template DNA with minimal misincorporation of nucleotides (Barnes, 1994). Because this method worked with two completely different sets of primers to clone two unrelated genes, this method should be useful for cloning any DNA using genomic DNA as the starting material.

Although LIC and conventional cloning methods recover recombinant plasmids in *E. coli*, the construction of recombinant vectors *in vitro* is quite different. Only one enzyme is used to manipulate the DNA during LIC. This reduces the opportunity for loss of material due to nuclease contamination. Moreover, minute quantities of DNA may be cloned directly because the method amplifies the target sequences. Importantly, LIC does not rely on the presence of compatible restriction sites in the insert and vector. Therefore, any fragment of DNA that can be amplified using PCR may, in theory, be cloned in this fashion.

Introduction of point mutations during the amplification steps could prevent obtaining useful clones. Proteins expressed from cloned genes containing mutations could have undesired and artifactual properties. In this case it is essential to amplify inserts using a polymerase with proofreading ability. The size of the 1° PCR products and the sequence of CHS locus clone were as predicted. These results indicate that *Pfu* is capable of amplifying templates under 5 kb with high fidelity. 98% of all the clones contained an insert and of those examined all were in the specified orientation. Moreover, no concatemers were detected which indicates that amplification of artifact PCR products by *Pfu* was minimal.

There appears to be a limit, however, to the size of constructs that can be made by *Pfu* DNA polymerase. When attempting to insert CHS and CHI cDNAs into two yeast two-hybrid vectors, pPC62 and pPC86 (Chevray and Nathans, 1992), the 2° products, which were no less than 7 kb in size, could not be amplified under conditions used to clone CHS and STS (data not shown). This may be simply due to the inherent limit on the ability of *Pfu* to amplify templates longer than 7 kb, stalling at misincorporated nucleotides (Barnes, 1992; Lawyer et al., 1993). A combination of *Pfu* and an N-terminally truncated variant of *Taq* polymerase, however, has been used to amplify products as large as 35 kb from l bacteriophage templates (Barnes, 1994). The more processive form presumably does most of the bulk synthesis while the 3’ to 5’ exonuclease activity of the native form repairs misincorporated bases.

Other methods to use PCR to construct recombinant vectors *in vitro* have been proposed. One technique exploits the 3’ to 5’ exonuclease activity of T4 DNA polymerase to create single-stranded termini on both the insert and vector (Charalampos and de Jong, 1990). In this method, insert and vector are annealed via complementary termini and then transformed into *E. coli*. Although, like LIC, this method does not require DNA ligase, it does require several enzymatic manipulations in order to generate complementary ends. Another technique described by Shuldiner (1990) used chimeric primers to synthesize fusions between vector and insert during PCR in a similar manner to the method outlined here.

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Table I: Primers used for LIC cloning of *A. thaliana* CHS and *A. hypogaea* STS. Sequences of primers complementary to pBluescript KS(+) are indicated in bold.

s13	ccaaatacacctaacttgtt
a8	tccgaccccacaatgag
pBSKas13	**ccgggctgcaggaattcgat**ccaaatacacctaacttgtt
pBSKΩa8	**cgacggtatcgataagcttgat**tccgaccccacaatgag
G107a	ggtggcactgtccttcg
C98a	gagaccagggccaaaac
G107aΩ	**ggctgcaggaattcgat**ggtggcactgtccttcg
C98aΩ	**cggtatcgataagcttgat**gagaccagggccaaaac
pBSKΩ	atcaagcttatcgataccg
pBSKa	atcgaattcctgcagcc
T7	taatacgactcactataggg
T3	taaccctcactaaaggga

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Figure 1. Schematic of the ligation independent cloning (LIC) procedure.

Two complementary PCRs are performed in parallel. Products that include sequences complementary to the vector are synthesized during a primary PCR amplification. These products serve as primers to form a chimeric fusion between amplified insert and vector. Fusions are amplified using secondary primers during another round of PCR. When chimeric fusions of parallel reactions are mixed, denatured, and annealed, hybridization forms nicked, circular, recombinant plasmids.

Figure 2. Diagram of the CHS and STS genomic loci.

In each case the fragment to be cloned is indicated by the bar. The entire box represents the transcribed region. The black box indicates the 5' untranslated region. The gray box represents intron sequences. The asterisk indicates the conserved active site cysteine in these enzymes. Arrows indicate the annealing sites for the 1° primers.

A. Diagram of the *A. thaliana* CHS locus.

B. Diagram of the *A. hypogaea* STS locus.

Figure 3. Cloning of the CHS genomic fragment.

A. Agarose gel analysis of primary CHS PCR products. Ten microliters of each parallel primary PCR (lanes 1 and 2) was fractionated on a 1% agarose gel and DNA visualized with ethidium bromide staining. 100 ng of *A. thaliana* genomic DNA was used as template with nonchimeric primers as a positive control (G). A negative control, containing no DNA template, is also shown (lane NT).

B. Agarose gel analysis of chimeric fusion secondary products. Ten microliters of each parallel secondary PCR (A and B) was fractionated on a 1% agarose gel and DNA visualized with ethidium bromide staining. Specific nonchimeric primers were used to amplify the pBluescript vector as a size control indicated in lane P (plasmid). Notice that the chimeric fusion products are larger in size than the linear plasmid.

Figure 4. Colony PCR screen for recombinant CHS clones.

Eleven white (lanes 1 - 11) and one blue (lane B) colony were screened for the presence of recombinant plasmids by PCR using T3 and T7 primers. Ten microliters of each PCR were fractionated in a 1% agarose gel and DNA was visualized by ethidium bromide staining.

Figure 5. Cloning of the STS genomic fragment.

A. Agarose gel analysis of primary STS PCR products. Ten microliters of each parallel primary PCR (lanes 1 and 2) was fractionated on a 1% agarose gel and DNA visualized with ethidium bromide staining. One hundred nanograms of *A. hypogaea* genomic DNA was used as template with nonchimeric primers as a positive control (G).

B. Agarose gel analysis of chimeric fusion secondary products. Ten microliters of each parallel secondary PCR (A and B) was fractionated on a 1% agarose gel and DNA visualized with ethidium bromide staining. Specific nonchimeric primers were used to amplify the pBluescript phagemid vector (P) as a size and amplification control.

Figure 6. Colony PCR and restriction analysis of STS clones from *A. hypogaea*.

A. Five white transformants were screened for the presence of recombinant plasmids by PCR using T7 and T3 primers. Ten microliters of each PCR (lanes 1 - 5)were fractionated on a 1% agarose gel and DNA was visualized by ethidium bromide staining.

B. Restriction digest analysis of recombinant plasmids. Ten microliters of restriction digest (lanes A, B, and C) were fractionated on a 1% agarose gel and DNA was visualized by ethidium bromide staining.