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Full Length Research Paper

PCR technique to identify the transgenes Cry1Ac and Cry2Ab in clonned recombinant cells

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Cloning is the process of producing populations of genetically-identical individuals that occur in nature when organisms such as bacteria, insects or plants reproduce asexually. Modern cloning vectors include selectable markers (most frequently antibiotic resistance markers) that allow only cells in which the vector, but not necessarily the insert, has been transformed to grow. Additionally, the cloning vectors may contain color selection markers which provide blue/white screening on X-gal medium. Nevertheless, these selection steps do not absolutely guarantee that the DNA insert is present in the cells. Further investigation of the resulting colonies is required to confirm that cloning was successful. This may be accomplished by means of PCR. TOPOTA cloning provides one step cloning strategy for direct insertion of Taq polymerase amplified PCR products into a plasmid vector ligation (TOPO Cloning Reaction). In the presence of salt in TOPO Cloning reaction by mixing PCR product and TOPO vector, for transforming bacterial strain into competent cells, DH51α is supplied with the kit. Analysing transformants take 10 white colonies and culture them overnight in LB medium containing 50 µg/ml ampicillin or 50 µg/ml kanamycin. The plasmids were analyzed by PCR to confirm the presence of insert, after which PCR product of the Cry1A(c) and Cry2Ab transgene of Bt-cotton was produced to be cloned and ligated with vector.

Key words: TOPO-TA Cloning, PCR, BT-Cotton, Recombinant and Non-Recombinant colonies.

INTRODUCTION

The first transgenic plant was produced in 1983, when a tobacco line expressing Kanamycin resistance was produced. While 'Flavr Savr' tomato was the first transgenic variety which reached the market, the expansion in the cultivation of Genetically Modified (GM) crops has been very rapid in the recent past. Till 1994, there were no commercial farms producing GM crops in the world while by the end of 1998, about 29 million hectares of land were devoted to the production of GM crops (James, 2009). USA, Australia, Canada and Mexico took up the advancements in agri-business and lead the world in GM crop production (James, 2009). Even the Asian countries identified the need and usefulness of GM crops and adapted the GM crop production. In China, 650,000 farmers planted Monsanto's Bollgard cotton (GM cotton variety Bt-cotton) in 1998 which increased to 1 million in 1999 (Gandhi and Namboodiri, 2006). The all over increase in GM crop production extended to 102 million hectares from 1.7 million hectares in a short span of 1996 to 2006 (Gandhi and Namboodiri, 2006; James, 2009). In India, GM crops are regulated under the purview of 1986 Indian Environmental Protection Act. These rules cover all the areas of research as well as large scale application of Genetically Modified Organisms (GMOs) and products throughout the country involving manufacture, use, import, export, storage and research (GOI, 1998; Bhat and Chopra, 2005; Gandhi and Namboodiri, 2006). Till date no GM crop/products are allowed in India to be marketed. However, Bt-cotton is the only transgenic crop, approved for commercial cultivation in few states of India. *Bacillus thruingiensis* is a bacterium that naturally produces some proteins that are lethal to insect larvae (Cantwell et al., 1964; Cannon, 1996; Kamale et al., 2011). By transforming the genes that encode these proteins into cotton balls, scientists have created a type of cotton that produces its own pesticides, making it resistant to insects. This variety of cotton was given the

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name Bt-cotton event Bollgard II (Kranthi et al., 2005).

Bollgard II cotton event 15985 was developed by Monsanto Company to produce the Cry2Ab insect control protein, which provides effective season-long control of key lepidopteron insect pests (Kranthi et al., 2005). This product was produced by the re-transformation of Bollgard® cotton event 531, which produces the Cry1Ac insect-control genes and the nptII selectable marker gene. Therefore, Bollgard II cotton produces two transgenes, Cry1Ac and Cry2Ab, for effective control of the major lepidopteran insect pests of cotton, including the cotton bollworm, tobacco budworm, pink bollworm and armyworm (Kranthi et al., 2005; Shree et al., 2012). Along with the increase in the market of the transgenic crops, the demand for testing GMOs and for certifying non-GMO foodstuffs has increased dramatically (Amiri et al., 2013). Within the area of expanding techniques for identification and quantification of transgenic crops, two major approaches for detecting GMOs are still applicable on large scale (Yates, 1999; Shree et al., 2012). First method includes the identification of proteins produced by the introduced trait gene through the detection of its specific antibody, such as by Enzyme Linked Immunosorbent Assay (ELISA) (Brett, 1999; Ahmed, 2002; Chalam and Khetarpal, 2012), while the second method employs the identification of specific DNA sequence used for gene modification by Polymerase Chain Reaction (PCR) (Khetarpal and Kumar, 1996; Gachet et al., 1999).

In the present study, authors have adapted PCR technique to identify the transgenes Cry1Ac and Cry2Ab in recombinant cells after successfully cloning occurs. Molecular cloning refers to the procedure of isolating a defined DNA sequence and obtaining multiple copies of it *in vivo*. It collectively refers to processes used to create copies of DNA fragments (molecular cloning), cells (cell cloning), or organisms.

Modern cloning vectors include selectable antibiotic resistance markers, which allow only cells in which the vector has been transfected, to grow. Additionally, the cloning vectors may contain colour selection markers which provide blue/white screening (α factor complementation) on X-gal medium. Nevertheless, these selection steps do not absolutely guarantee that the DNA insert is present in the cells obtained. Further investigation of the resulting colonies is required to confirm that cloning was successful. This may be accomplished by means of PCR, restriction fragment analysis and/or DNA sequencing.

Although these steps are invariable among cloning procedures, a number of alternative routes can be selected, these are summarized as a 'cloning strategy'. Initially, the DNA of interest needs to be isolated to provide a DNA segment of suitable size. Subsequently, a ligation procedure is used where the amplified fragment is inserted into a vector (piece of DNA). The vector which is frequently circular is linearised using restriction enzymes, and incubated with the fragment of interest under appropriate conditions with an enzyme called DNA ligase. Following ligation, the vector with the insert of interest is transfected into cells. A number of alternative techniques are available, such as chemical sensitivation of cells and electroporation. Finally, the transfected cells are cultured. As the after mentioned procedures are of particularly low efficiency, there is a need to identify the cells that have been successfully transfected with the vector construct containing the desired insertion sequence in the required orientation.

MATERIALS AND METHODS

Materials required

The materials required are spreaders, Petri plates, S.O.C medium (supplied with the kit), L.B medium, Amplicillin (50 µg/ml) or Kanamycin (50 µg/ml), 40 mg/ml X-gal in dimethylformamide (DMF), 100 mM IPTG in water, and vials of competent cells (supplied with the kit).

Plant material

BT-Cotton (BG-II) event MON 15985 was given by Dr. V. Kumar, Main Cotton Research Station Navasari Agricultural University, Athwa Farm, Ghod Dod Road, Surat, Gujarat.

DNA extraction

GeneiPure Column kit was used for DNA isolation. GeneiPure Column kits provide a fast and simple way to isolate DNA from seeds. Up to 25 mg of tissue can be processed using GeneiPure Column kits. Purification was done as per the suggested protocols with slight modification in the incubation time from 15 min to 1 h at the first step to get better yield of DNA as experienced in our earlier attempts. Genomic DNA of Bt-Cotton was run on 0.8% agarose gel electrophoresis. The gel was run for 1 h in 1X TBE buffer at 100V. The gels were visualized by ethidium bromide staining under UV light (254–366 nm) and pictures were captured using UV Gel Documentation System.

Qualitative polymerase chain reaction (PCR)

All PCR reactions were performed in a final volume of 50 µ,l using 2.5 units proof reading Taq DNA polymerase (Sigma), 50 µM of each forward
mM of each dNTP and 3 µl of DNA. PCR was carried out in DNA Engine MJ Research, PTC-200 (Peltier Thermal Cycler) using Sigma PCR mixtures. Thermal cycling conditions were 94°C for 15 min followed by 30 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 10 min with a final extension step of 10 min at 72°C. As a result, the obtained PCR products were kept at 4°C till gel electrophoresis. An aliquot of the PCR product was analyzed on a 2% agarose gel. The gel was run for 1 h in 1X TBE buffer at 100V. The gels were visualized by ethidium bromide staining under UV light (254–366 nm) and pictures were captured using UV Gel Documentation System. 100 bp DNA Ladder Plus (MBI Fermentas)

consisting of DNA fragments ranging in size from 50 to 1000 bp, was used as DNA molecular weight marker. PCR involves two primers (one reverse and one forward) usually for detection of transgene in transgenic rice.

Experimental outline

1) Producing PCR product of the gene to be cloned.
2) Setting up TOPO Cloning reaction by mixing PCR Product and TOPO Vector (Ligation).
3) Incubation for 5 min at room temperature.
4) Transforming TOPO Cloning reaction into One Shot Chemically Competent cells (Transformation).
5) Selecting and analyzing white (recombinant clones) or light blue colonies for insert.
6) Store in Glycerol Stock.

About TOPOTA cloning

TOPOTA cloning provides a highly efficient one step cloning strategy for direct insertion of Taq polymerase amplified PCR products into a plasmid vector. The plasmid vector (pUC 19) is supplied in kit linearized form with single 3' thymidine overhangs for TA Cloning and Topoisomerase I covalently bound to vector (activated vector).

Ligation (TOPO cloning reaction)

In the presence of salt (200 mM NaCl; 10 mM $MgCl_2$) in TOPO cloning reaction by mixing PCR product and TOPO vector, inclusion of salt allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. Result is more intact molecules leading to higher transformation efficiencies.

Transforming chemically competent E. coli

For transforming bacterial strain into chemically competent cells DH5 α 1, adding 200 mM NaCl 10 mM $MgCl_2$ in TOPO Cloning reaction increases number of colonies over time. A salt solution (1.2 M NaCl; 0.06 M $MgCl_2$) is supplied with the kit.

Analysing transformants

1) Ten white colonies were taken and culture overnight in LB medium containing 50 µg/ml amplicillin.
2) The plasmids were analyzed by PCR to confirm the presence of insert.
3) Spreader was sterilized each time before use by dipping in 100% ethanol and then placing it over flame after which it was allowed to cool down before use. Selective LB plates were warmed at 37°C for 30 min, after which 40 µlX-gal and 5 µl IPTG were poured over the control and experimental Petri plates and then spread with the help of the spreader before pouring the transformation mixture over the Petri plates.

Producing PCR products for cloning reaction

Concentration of DNA sample = 250 ng

PCR for analyzing transformants

1 ml of inoculated LB broth containing recombinant white clones was taken in a fresh eppendrof and centrifuged at 5000 rpm for 10 min, after which the supernatant was discarded and the pellet was stored at -20°C overnight. The pellet was transfered into fresh eppendrof tube with the help of 200 µl pipette Elute with 30 µl TE buffer, after which it was mixed properly and boiled at 90°C for 3 min. Finally, it was centrifuged again and the supernatant was used as template DNA for PCR.

RESULTS AND DISCUSSION

Modern cloning vectors include selectable markers (most frequently antibiotic resistance markers) that allow only cells in which the vector, but not necessarily the insert, has been transformed to grow. Additionally, the cloning vectors may contain color selection markers which provide blue/white screening on X-gal medium. These selection steps do not guarantee that the DNA insert is present in the cells. Further investigation of the resulting colonies is required to confirm that cloning was successful. This may be accomplished by means of PCR, TA cloning vectors (Zhou and Gomez-Sanchez, 2000; Chen et al., 2009) which takes advantage of the well-known propensity of non-proofreading DNA polymerases to add a single 3´-A to PCR products that are also employed for cloning large PCR fragments. Proof-reading polymerases lack 5'-3' proofreading activity and are capable of adding adenosine triphosphate residues to the 3' ends of the double stranded PCR product. Such a PCR amplified product can then be cloned in any linearized vector with complementary 3' T overhangs. Figure 1 shows the PCR amplified product for Cry1Ac and Cry2Ab having 230 and 453 bp products used as a DNA. For cloning purposes, *E. coli* DH5 α expressing used. These cells are compatible with *lacZ* blue/white selection procedures, and are easily transformed with good quality plasmid DNA that can be recovered from transformants. DH5 α is one o for plasmid propagation. Figure 2 shows the duplicates of the control plates bacteria that are grown in LB Agar medium without antibiotics ampicillin for 12 h in incubator at 37°C. It shows numerous non-recombinant blue coloured bacterial colonies as well as fungal contamination. Figure 3 shows the duplicates of the masterplate recombinant white colonies that were seen in LB Agar medium with antibiotics ampicillin for 12 h in incubator at 37°C. It shows approximately 20 recombinant white colonies. The blue white screening is one of the most common molecular techniques that allow the successful ligation of the gene of interest to be detected in vector (Langley et al., 1975; Zamenhof and Ausubel, 1972; Ausubel et complementation plasmids are among the most commonly used vectors for cloning and sequencing

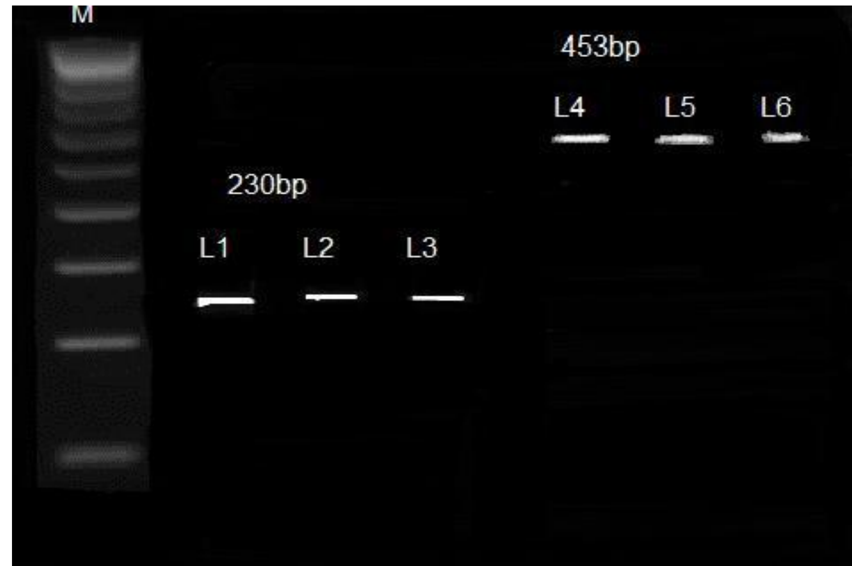

Figure 1. Transgene Cry1A(c) and Cry2Ab in Bt-Cotton before cloning.
M: shows 100 bp ladder; L1, L2 and L3 show 230 bp amplified product of Cry1A(c) gene; L4, L5 and L6 show 453 Cry2Ab gene Bt-cotton.

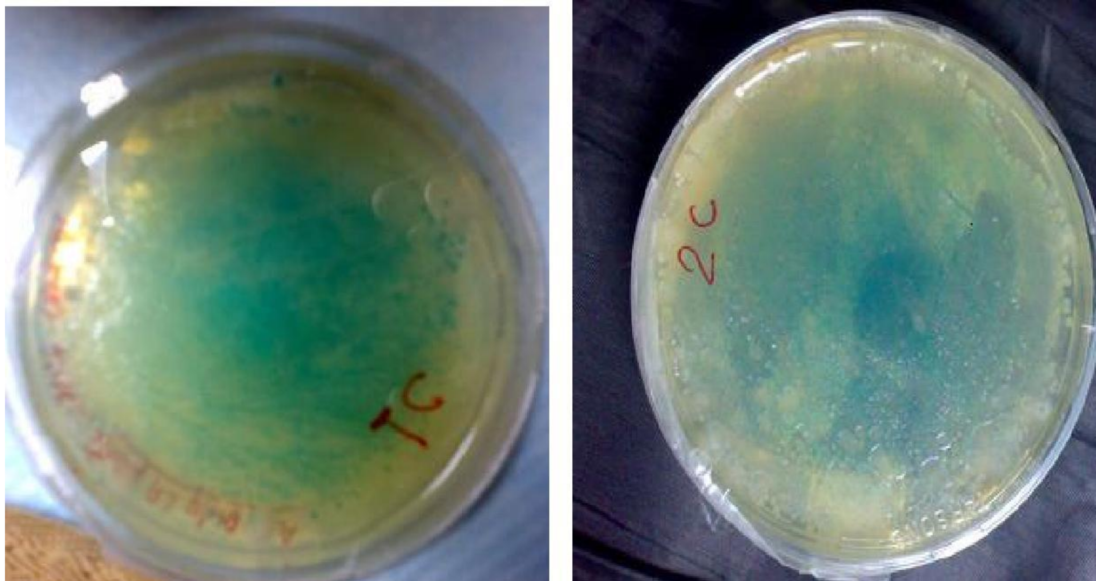

Figure 2 Control (1C) & (2C) Masterplate

Figure 2. Control (1C) and (2C) masterplate.
In controlIncontrol(1C)and(1C)&(2C),master(2C) platemasterbacteriaplatewerebacteriagrownin areLBAgargrownmediumin LBwithoutAgarantibioticsmediumampicillinwithoutfor12 antibioticsh in incubator at 37°C. It shows numerous non-recombinant blue coloured bacterial colonies as well as fungal contamination.

the DNA fragments, as they generally have a good multiple cloning site and an efficient blue-white screening system for identification of recombinants in presence of a histochemical dye, 5-bromo-4-chloro-3- indolyl- β -d-galactoside (X-gal) (Manjula, 2004). The molecular mechanism for blue/white screening is based on a genetic engineering of the *lac* operon in the *E. coli* as a host cell combined with a subunit complementation achieved with the cloning vector. The *lacZ* product gives rise to the functional enzyme after tetramerization (Jacobson et al., 1994) and is easily detected by c h r o m o g e n i c s u b s t -galactosidase f The c t i chemical required for this screen is X-gal, a colorless m o d i f i e d g a l a c t o s e s u g a r - t h a galactosidase to form 5-bromo-4-chloroindoxyl which is spontaneously oxidized to the bright blue insoluble pigment 5,5'-dibromo- 4,4'-dichloro-indigo and thus functions as a n i n d i c a t o r-D-1- I s o

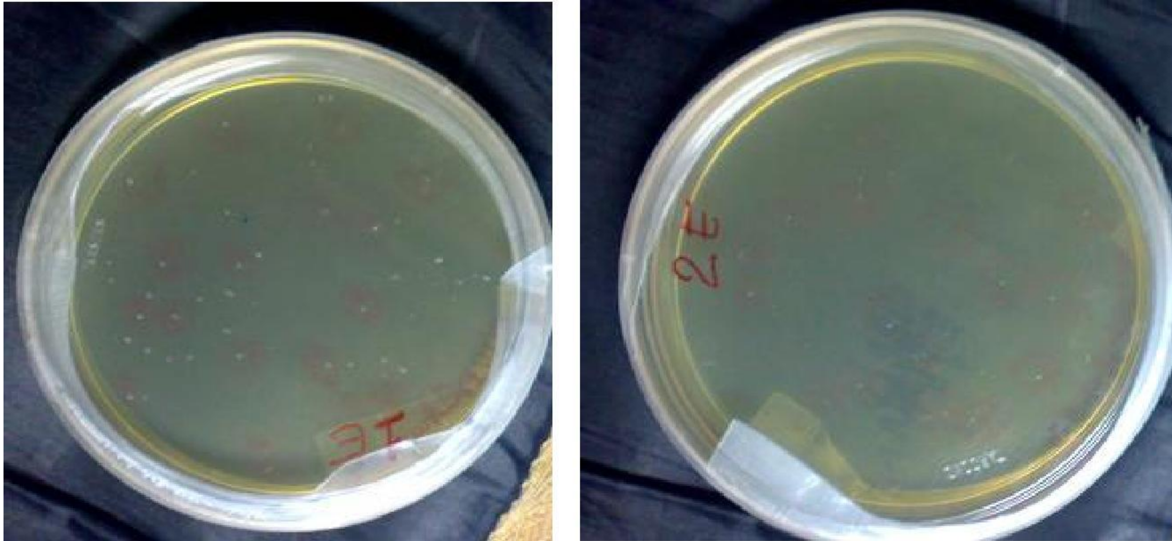

Figure 3. Experimental (1E) and (2E) masterplate
Figure 3 Experimental (1E)&(2E) Masterplate
In experimental (1E) and (2E), master plate recombinant white colonies were seen in LB Agar medium with antibiotics ampicillin for 12 h in incubator at 37°C. It shows approximate 20 recombinant white colonies.

In experimental (1E) &(2E) master plate recombinant white colonies were seen in LB Agar

Figure 4. 2% Agarose Gel Electrophoresis showing transgene Cry1Ac and Cry2Ab in event 15985 after cloning.
M shows 100 bp ladder; L1, L2 and L3 show 230 bp amplified product of Cry1A(c) in bt-cotton; L4, L5 and L6 show 453 bp amplified product of Cry2Ab in bt-cotton.

thiogalactopyranoside (IPTG) which functions as the inducer of the Lac operon can be used as an enhancer. The hydrolysis of colorless X-gal by β-galactosidase causes the characteristic blue colour in the colonies indicating that the colonies contain vector without insert. White colonies indicate insertion of foreign DNA and loss of the cells ability to hydrolyze the marker. Blue white screening is thus a quick and easy technique that allows for the screening of successful cloning reactions through the color of the bacterial colony. The correct type of vector and competent cells are important considerations when planning a blue white screen. After getting the recombinant clones we have to detect it by the PCR technique that we are getting the same product size after electrophoresis as shown in Figure 4. For PCR, 2 ml of LB broth was inoculated with recombinant white colonies. 1 ml inoculated LB broth was centrifuged at 5000 rpm for 10 min, after which the pellet obtained was then eluted in TE buffer and boiled at 90°C for 3 min. This mixture of pellet eluted in TE buffer was again centrifuged to rupture the membrane of bacteria so that plasmid DNA of recombinant colonies containing transgene Cry1A(c) and

Cry2Ab in Bt-Cotton get extracted into the supernatant which was used as template DNA for PCR. PCR followed by 2% Agarose Gel Electrophoresis shows 230 and 453 bp amplified product of transgene Cry1A(c) and Cry2Ab in Bt-Cotton (MON 15985). When 100 bp marker was used, samples L1, L2, L3 showed 230 bp, amplified product of Cry1A(c) and L4, L5, L6 showed amplified product of Cry2Ab having 453 bp (Kamale et al., 2011). We got the same size product from recombinant clones. Therefore we can say from the above results that successful cloning of transgene Cry1A(c) and Cry2Ab has taken place in samples L1, L2, L3, L4, L5 and L6, which can be used for quantitation purposes.

Conclusion

The protocol presented here is a streamlined procedure for fast and reliable cloning of genes of interest from PCR. This provides a highly efficient one step cloning strategy for direct insertion of Taq polymerase amplified PCR products into a plasmid vector protocol. In addition, the protocol could be adapted for high-throughput applications.

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