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Unusual Orientation of cDNAs Found in a cDNA Library

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Abstract

Many cloning vectors in which cDNAs can be inserted to the sense orientation have been developed. Uni-ZAP XR vector (Stratagene) should contain clones that are oriented to sense direction with respect to T3 RNA polymerase primer. Unexpectedly, large portions of cDNAs in Chinese cabbage cDNA library showed unusual insertions, antisense orientation and a hybrid of two different clones. Using two clones, *4H03* and *53-B10*, derived from different cDNA libraries, we proposed and demonstrated the possibility of unusual-construct formation by *in vitro* translation and northern blot analysis. The *4H03* clone was inserted with inverse direction, and its transcript and translation product could be produced by T7 RNA polymerase, indicating that this clone is definitely inserted into inverse orientation. The *53-B10* that contains two independent genes was turned out to be a hybrid in which two genes are inserted to opposite direction each other. All unusual constructs might be due to the presence of small fragments of DNA, like adapter. However, the mechanism underlined the formation of unusual constructs is still remain to be solved.

Key words: λZAPII, directional cloning, *Eco*RI adapter, cDNA library, Chinese cabbage

Introduction

Complementary DNA (cDNA) library contains informations for mRNA population in particular tissue or at the developmental stage of an organism. Since reverse transcriptase was discovered (Baltimore 1970; Temin and Mizutani 1970), *in vitro* synthesis of DNA complementary (cDNA) to RNA was established by three different groups using an oligo (dT) primer in order to synthesize single-stranded DNA from mRNA templates (Bank et al. 1972; Ross et al. 1972; Verma et al. 1972). The cloning method of the cDNA into a vector has been improved after its establishment in mid 1970s (Rougeon et al. 1975; Rabbitts 1976). Most common vectors used in the cloning are λZAP (Short et al. 1988) and its derivatives (Stratagene). These are the most popular bacteriophage λ vector, which allows recovery of cDNA inserts as plasmid. λZAP carries a polycloning site embedded in the region of the *lac*Z gene coding for the acomplementation fragment of β-galactosidase. Relatively long cDNAs (up to 10 Kb) can be inserted to the downstream of *lac*Z promoter and expressed after *in vivo* excision.

The ZAP-cDNA synthesis kit (Stratagene) uses a hybrid oligo (dT) linker-primer that contains an *Xho*I restriction site and *Eco*RI adaptor for direction cloning (Krug and Berger 1987; Kretz et al. 1989). Therefore, one can obtain more information for cDNAs, such as 3'-noncoding sequence information and 5'-information for confirming presence of translation starting codon. The overall process of cDNA synthesis is well described in Manufacturer's instruction manual (Stratagene). Using mRNA, the first-strand cDNA synthesis is performed with a 50-base oligonucleotide (5'-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTT-3') that consists of "GAGA" sequence to protect the *Xho*I restriction enzyme recognition site, *Xho*I site (CTCGAG) and an 18-base poly(dT) sequence. The *Xho*I restriction site allows the finished cDNA to be inserted into the Uni-ZAP XR vector (Short et al. 1988) in a sense orientation (*Eco*RI-*Xho*I) with respect to the *lac*Z promoter. The poly (dT) region binds to the 3' poly (A) region of the mRNA template, and MMLV-RT begins to synthesize the first-strand cDNA. After synthesis of the second-strand DNA synthesis using RNaseH and DNA polymerase I, *Eco*RI adapters composed of 9- and 13-mer oligonucleotide are is ligated to the blunt ends of cDNA.

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Then, cDNAs are digested with *Xho*I and the residual linker-primer from the 3' end of the cDNAs can be eliminated by gel filteration on a drip column containing Sepharose CL-2B gel. The size-fractionated cDNAs are then precipitated and ligated to the Uni-ZAP XR vector. The size fractionation and phenol extraction could be enough to eliminate any small fragments of DNA before ligation proceeds, thereby all constructs should have sense orientation. However, large quantities (ca. 31%) of inserts obtained from Chinese cabbage cDNA library were inserted in antisense (reverse) orientation and some clones consist of two genes. In this study, we describe how unusual orientation can be occurred as well as how to prove it.

Materials and Methods

Plant material

Brassica rapa L. ssp. *pekinensis* inbred line, 'Chiifu', was grown either in field from September to November in South Korea or in growth chamber at 23±2℃ under light/ dark cycles of 16/8 h with the light intensity of 150 μmol $m^{-2}s^{-1}$.

Selection of cDNA clones and sequence analysis

Poly (A^+)-mRNA was isolated from total RNA using paramagnetic oligo (dT) beads (PolyAtract mRNA isolation II, Promega) according to the manufacturer's procedures. The cDNAs were cloned into λZAPII (Stratagene) following the recommended procedures. Two clones used in this study, *53-B10* and *4H03*, were selected from EST and microarray experiment, respectively. The former one was selected from EST experiment using cDNA libraries constructed in Chinese cabbage inbred line 'Chiifu' leaves after one week exposure to frost (daily low temperature is below 0℃) in field. The latter one was down-regulated upon light-chilling treatment. The cDNAs were sequenced using an ABI 3730x1 automated sequencer (Applied Biosystems) with four fluorescent dyes. The similarity of DNA sequences was determined with the known sequences available in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) with the BLAST search program. Open reading frames were determined by a program of http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html.

In vitro transcription and translation

To obtain riboprobes (sense and anitisense probes), *in vitro* transcription was carried out using T7 and T3 RNA polymerases (Promega), in the presence of α-^{32}P-rCTP (Amershamparmacia), for 1h at 37℃. Then, unincorporated nucleotides were removed by column chromatography using Sephadex G-50 column. To examine whether two cDNA clones can be translated *in vitro*, the clones were translated using T_NT® Coupled Wheat Germ Extract System (Promega), in the presence of ^{35}S-methionine (Amershamparmacia). The products were separated on 10% or 15% SDS-PAGE, dried and exposed to Personal FX (BIO-RAD) screen.

RNA isolation and northern hybridization

Northern hybridization with total RNAs of isolated from Chinese cabbage leaves using Trizol Reagent was performed according to the standard protocol (Sambrook and Russell 2001). Twenty micrograms of total RNA were subjected to electrophoresis and transferred to Nylon (Schleicher & Schuell) using VacuGene™ XL Vacuum Blotting System (Phamacia Biotech). Hybridization with ^{32}P-labelled probe and washing procedures were followed by the manufacture r's protocol. Autoradiography was carried out by exposing the membrane to Screen of Personal FX (BIO-RAD). Five micrograms of the poly (A^+)-mRNA isolated by paramagnetic oligo (dT) beads (PolyAtract mRNA isolation II, Promega) were also subjected to northern blot analysis with either sense- or antisense-riboprobes.

Results and Discussion

Possible construct

According to flow chart of cDNA synthesis supplied by manufacturer's manual (Stratagen), all clones in resulting cDNA library should have sense orientation with respect to T3 RNA polymerase primer. However, unexpected percent (5-35%) of clones examined showed antisense-oriented cDNAs at least in Chinese cabbage libraries. Particularly, some clones turned out to be a hybrid of two independent genes. Using two clones, *4H03* and *53-B10*, derived from different cDNA libraries, we postulated and examined the possible unexpected outcome.

As shown in Figure 1A, *Eco*RI adapter present in 3'-*Xho*I site should be cut out from the cDNA and eliminated from subsequent gel filteration with Sepharose CL-2B gel. These gel filteration and phenol extraction followed by ethanol precipitation will be enough to eliminate small fragments of DNA including linker or adapter molecules. If small fragments were not removed, however, several possible constructs might be resulted from rejoining of adapter and cDNAs (Figure 1B, C, D). If *Xho*I recognition sequences attached to

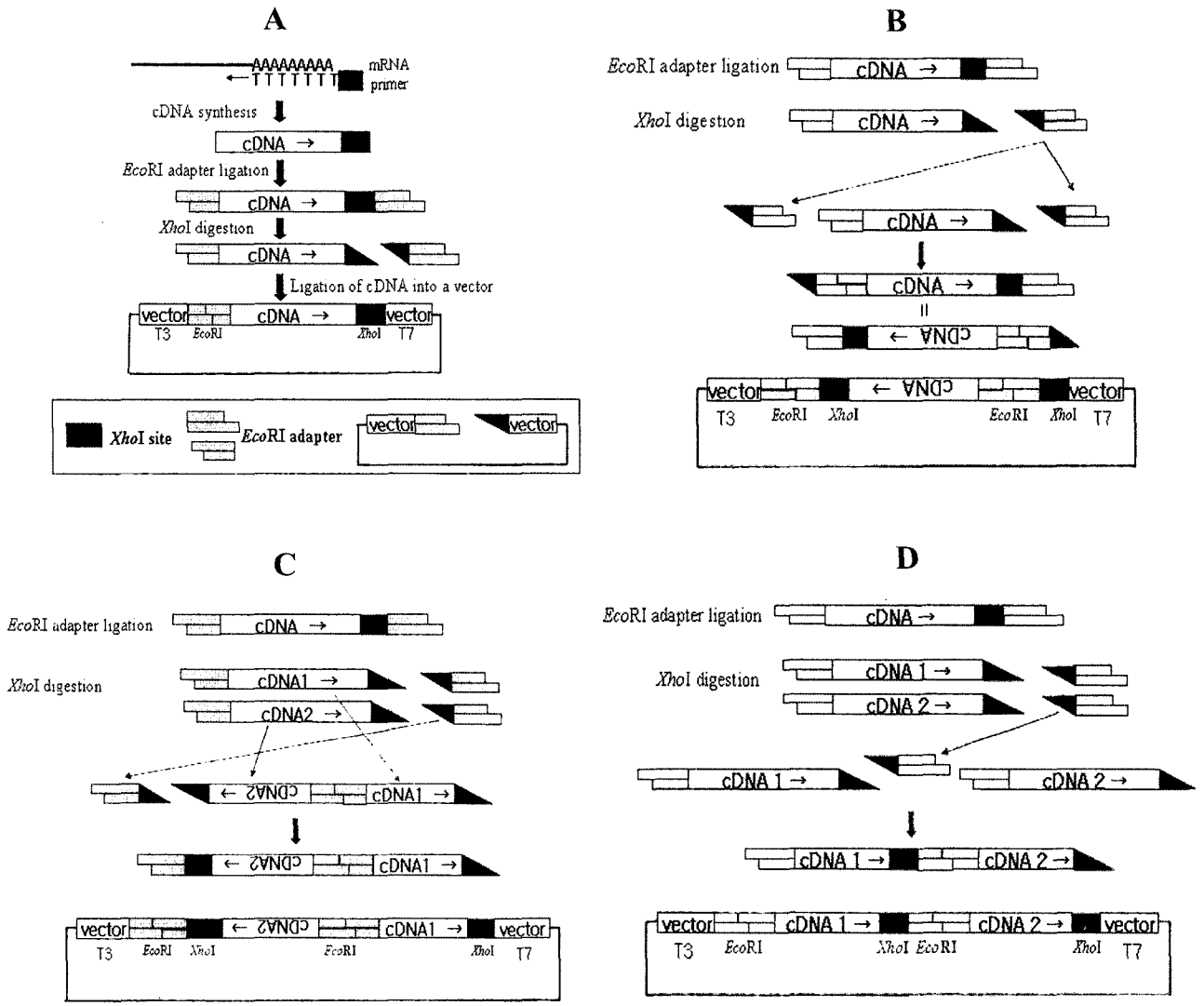

Figure 1. Schematic illustration of cDNA library generated in Lab. A, Simplified scheme of cDNA synthesis flow chart by Stratagene's manual. B, Possible insertion of cDNA with reverse orientation. C, Possibility of two clones that are inserted to either direction. D, Possibility of two clones that are inserted to direct orientation. Arrow indicates direction of clone, 5' →3', with respect to translation-start site.

*Eco*RI adapter are present and they are ligated at *Eco*RI site before cDNA insertion into the vector, antisense-oriented cDNA can be formed (Figure 1B). If two cDNAs join together at *Eco*RI site after *Xho*I cut and *Xho*I recognition sequences attached to *Eco*RI adapter is ligated to *Xho*I site of one of two cDNA, two cDNAs can be inserted into the vector (Figure 1C). However, two cDNAs should be either direction in this case. If *Xho*I recognition sequences attached to *Eco*RI adapter is ligated to one cDNA after *Xho*I digestion, another cDNA digested with *Xho*I can be joined to this cDNA, thereby two cDNAs are ligated with sense direction (Figure 1D).

Inversely oriented clone

We choose a clone, *4H03* clone of 2,016 bp, that is composed of 1, 140 bp-ORF, 564 bp-5' UTR and 325-3' UTR. Its predicted polypeptide has 380 amino acids containing serine/threonine protein kinase domain as well as tyrosine protein kinase domain. This deduced protein shows 93% identity to *Arabidopsis thaliana* putative protein kinase (At3g-22750)(NM_113174) at the amino acid sequence level. However, this clone is inserted in the antisense manner with respect to T3 RNA polymerase primer (Figure 2A).

To examine orientation of this clone, *in vitro* translation was performed with T3 and T7 RNA polymerases using

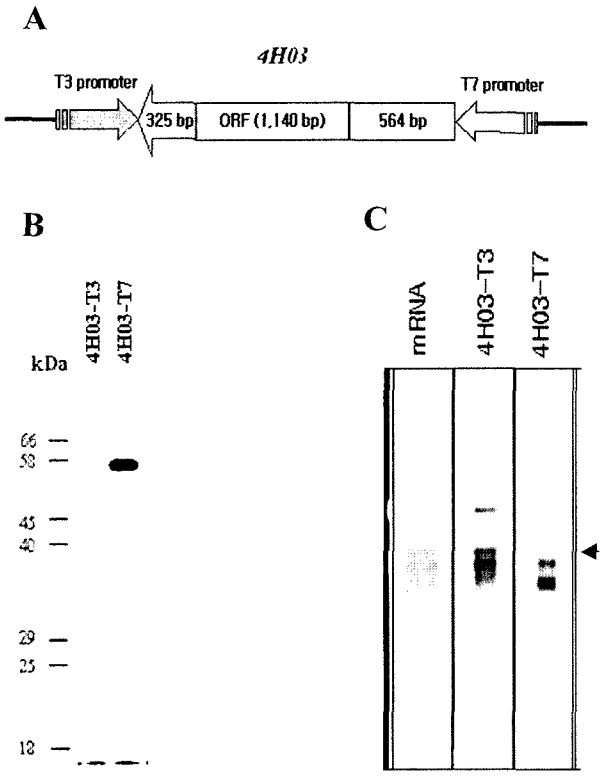

Figure 2. Analysis of *4H03* clone inserted with antisense orientation. A, *4H03* clone in the polylinker region of λZAP vector. B, *In vitro* translation products of *4H03* clone with T3 and T7 RNA polymerases. The product was separated on 10% SDS-PAGE, dried and exposed to Personal FX (BIO-RAD) screen to obtain image. Numbers at left indicate in the positions of molecular weight markers. C, Northern blot analysis of poly(A^+)-mRNA with sense and antisense riboprobes. Arrow indicates the predicted size of *4H03* transcript.

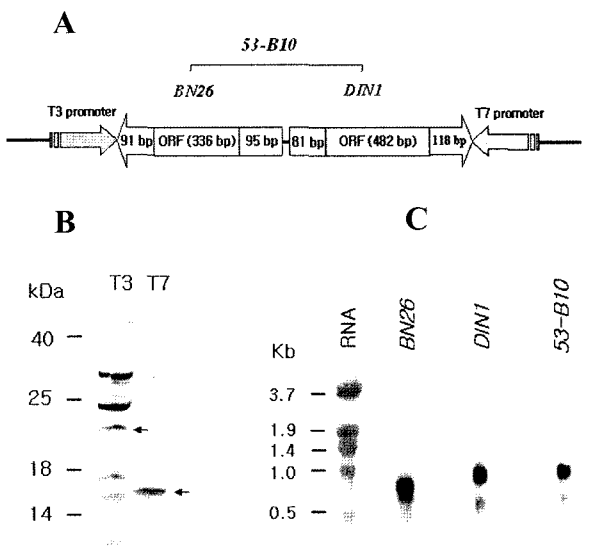

Figure 3. Analysis of *53-B10* clone, a hybrid. A, *53-B10* clone in the polylinker region of λZAP vector. B, *In vitro* translation products of *53-B10* clone with T3 and T7 RNA polymerases. The product was separated on 15% SDS-PAGE, dried and exposed to Personal FX (BIO-RAD) screen to obtain image. The positions of the molecular weight markers are shown at the left and expressed in kDa. C, Northern blot analysis of *53-B10* with three independent probes, *BN26*, DIN1 and *53-B10*. Total RNAs were extracted from leaf samples collected either before (for *DIN1* and *53-B10*) or after (for *BN26*) one week exposure to frost (daily low temperature is below 0℃) in field. RNA represents methylene blue-stained blot showing RNA to be transferred. Numbers at left indicate in the positions of molecular weight markers as kilobase.

Wheat Germ Extract (Promega). Figure 2B showed the *in vitro* translation product only from T7 RNA polymerase, an antisense orientation. The size of the product was about 58 kDa that is predicted molecular weight from deduced amino acid sequences. However, partial polypeptides were synthesized from T3 RNA polymerase, a sense direction. This result indicates that 4H03 clones might be inserted into an antisense orientation.

To confirm the *in vitro* translation data, northern blot analysis was carried out with poly (A^+)-mRNA and riboprobes, antisense probe derived from T3 and sense probe from T7 RNA polymerase (Figure 2C). As shown in Figure 2C, signal having the expected size was observed in the hybridization with the antisense riboprobe, indicating that a putative protein kinase gene could be expressed *in vivo* in Chinese cabbage and it would be inserted into antisense direction during cDNA library synthesis.

Hybrid clone

As a result of nucleotide sequence analysis of *53-B10* selected from EST analysis with mature leaf cDNA library, this clone showed very high redundancy (14/3,000 ESTs) and included two genes that are homologous to known genes. As shown in Figure 3A, the first one, *BN26*, has 98% identity to *Brassica napus* low temperature regulated transcript (S68727) and second one 93% identity to the *Raphanus sativus* mRNA for *DIN1* (AB004242)(a dark inducible, senescence-associated gene). *BN26* is oriented to antisense direction, while *DIN1* to sense direction with respect to T3 RNA polymerse primer. As expected as Figure 3A, two clones are linked with *Eco*RI enzyme site.

To analyze the *53-B10* clone, *in vitro* translation was first carried out with T3 and T7 RNA polymerases using Wheat Germ Extract (Promega). Deduced amino acid sequence of *BN26* will be 135 amino acid polypeptide with a theoretic molecular weight of 14.5 kDa, while that of *DIN1* 182 amino acid polypeptide with a theoretical molecular weight of 20

kDa. As expected, *in vitro* translation products of *BN26* and DIN1 were 15 and 20 kDa, respectively (arrows in Figure 3B). This result also support that *53-B10* clone is consisted of two independent cDNAs.

To confirm the *in vitro* translation data, northern blot analysis was carried out with three different probes, *BN26*, *DIN1* and both (*53-B10*)(Figure 3C). The hybridization signal with *BN26* probe was detected at about 700 nucleotide, while the signal with *DIN1* at around 1 kb. All these sizes are correspondence to expected sizes of two clones. In addition, similar size of signal as *DIN1* case was observed in the hybridization with full-*53-B10* probe. *BN26* signal was detected only on the membrane constructed with RNAs isolated from leaves that was collected after one week exposure to frost (daily low temperature is below 0℃) *in* field, whereas *DIN1* transcript was present in normal mature leaves. This is the reason why we could not detect both transcripts in the case of *53-B10* clone. We postulated that same sizes of hybridization signals were detected in all three cases if *53-B10* is not a hybrid. However, the result of northern blot analysis implied that 53-B10 is a hybrid of two cDNAs, resulting from unexpected linker ligation during cDNA library construction. Moreover, when we analyzed *Arabidopsis* genomic sequences, homologous clones to two Chinese cabbage ones were located in different chromosomes, *BN26* (At2g42530) and *DIN1* (At4g35770). All these informations can support that *53-B10* is a hybrid of two cDNAs.

In summary, many cloning vectors have been developed to clone cDNAs with desirable direction with respect to translation-start site. In many cases, however, the clones were inserted with unexpected manner, largely inverse direction and a hybrid, even though the library construction procedure includes steps necessary for elimination of possible contaminants by small fragments of DNA. We demonstrated formation of undesirable constructs from Chinese cabbage cDNA libraries by *in vitro* translation and northern blot analysis. However, the mechanism underlined the formation of unusual constructs is still remain to be solved.

Acknowledgments

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