Construction of plant high-frequency RNAi vector containing inverse-repeat terminator

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Abstract

Here we reported a vector, pMOG-DNOS, which contains a 35S promoter, a targeting gene *GUS* and an inverse-repeat sequence of *NOS* terminator in 3′ untranslated region. The idea of constructing this kind of vector is based on the recent progress in RNAi research. Transformation tests was carried out with oilseed rape (*Brassica napus* cv Westar). According to the X-Gluc staining result, for 360 transgenic plants of pMOG-DNOS, *GUS* was expressed strongly in 6 plants, weakly in 3 plants and negatively in 351 plants. Furthermore, ten of X-Gluc staining negative plants were random selected for transgene *NPTII* and *GUS* analysis by PCR. The result suggested that T-DNA was integrated in the genomes in all of selected plants. All of the analysis explained that the expression of *GUS* gene was suppressed in transgenic plants and vector pMOG-DNOS may use to gene function research by RNAi.

Key words: RNAi, Vector, inverted repeat sequence, *NOS* terminator, Oilseed rape, *Brassica napus*,

Introduction

RNA interference (**RNAi**) is a phenomenon in which double-stranded RNA (dsRNA) silences endogenous gene expression (Fire *et al*., 1998), and it can work in a remarkable variety of organisms, including animals, plants and fungi (Tuschl *et al*., 1999; Van der Krol *et al.*, 1990; Cogoni *et al.,* 1996http://www.pnas.org/cgi/content/full/99/7/4191?maxtoshow=&HITS=&hits=&RESULTFORMAT=&titleabstract=rnai &searchid=1049249846512_9687&stored_search=&FIRSTINDEX=0 - B1). By injecting pools of multiple double-stranded RNAs (dsRNAs) into animals or introducing transgenes which produce dsRNAs into plants and fungi, the sequence-specific degradation of RNA homologous to the triggers will be established. So, RNAi can be used as a tool to phenocopy the loss of function of one specific gene at a time, and it has been exploited in screens designed to identify developmental genes (Caplen, 2002).

Although RNAi is a powerful tool for functional genomics, but for plants, the construction of an RNAi vector(s) and the establishment of stable transformants are time-consuming and laborious. The constructs that give rise to dsRNA in plants are first designed by sense and anti-sense transgene expression in same T-DNA or transgenic plants (Waterhouse *et al.* 1998). However, as inverted-repeat DNA constructs containing an intron encoding RNA with a hairpin structure are included in an open reading frame, it can induce RNAi with high frequency up to 100% (Liu *et al.* 2000). A developed vector used in the Gateway™ cloning system, 'PHELLSGATE', is reported, which contains two pairs of the recombination sites attP1 and attP2 arranged in inverse orientation and interrupted by an intron (Wesley *et al*. 2001). By one simple step, a target sequence PCR product flanked by the attB1 and attB2sites can be directionally recombined into PHELLSGATE. This vector may facilitate high-throughput construction of hpRNA transgenes (Wang and Waterhouse, 2001).

Here we reported a vector, pMOG-DNOS, which contains a 35S promoter, a targeting gene *GUS* and an inverse-repeat sequence of *NOS* terminator in 3′ untranslated region that is unrelated to the target gene. The idea of constructing this kind of vector is based on the recent progress in RNAi research. RNAi is generally recognized that a silencing-inducing locus can efficiently reduce the expression of genes that give rise to transcripts partially homologous to those produced by the silencing-inducing locus. But the expression of gene that produces transcripts without homology to the silencing-inducing locus can also be decreased dramatically via transitive RNA silencing (Van Houdt H *et al*., 2003). Transitivity of RNA silencing is considered as increasing the initial pool of the short interfering (si)RNA raised from cleavage of long dsRNA by producing new siRNAs corresponding to sequences located outside the primary targeted regions of a transcript (Vaistij *et al*. 2002). In plants this transitivity is not only from 3′→5′ parts , but also from 5′→3′ parts of target transcripts (Voinnet et al., 1998; Vaistij et al., 2002). We believe that the expression of *GUS* gene in transgenic plants regenerated by plasmid pMOG-DNOS transformation would be suppressed by the 3 →5′ transitivity of RNA silencing originated inverse-repeat sequence of *NOS* terminator to the *GUS* transcripts. We investigated the expression of *GUS* gene in transgenic callus and plants by X-Gluc staining, and investigated the T-DNA integration of transgenic plants in the genome by PCR. The results suggest that the expression of *GUS* gene can be suppressed in most of transgenic plants. Furthermore, if a multiple cloning site is placed between promoter and inverse-repeat sequence of *NOS* terminator, vector pMOG-DNOS could be as a potential high-throughput construction used for the function analysis of any target sequence by transformation.

Material and Methods

Construction of transformation vector

To construct transformation vector pMOG-DNOS, two 256bp fragment containing intact NOS terminator and corresponding cut sites were isolated and respectively inserted into *Sal* I / *Hin*d III site and *Eco*R I / *Sca* I of plasmid pCAMBIA 1301 with bi-direction orientation, resulting in an intermediate plasmid p512M1. Another intermediate plasmid p520A3 was prepared by cloning de35S promoter-*GUS*-terminator *NOS* fragment into *Sal* I site of plasmid pMOG 800C, in which contains *Eco*R I and *Hin*d III sites in two sides of *NOS* terminator. Plasmid p523A3 was digested by *Eco*R I / *Hin*d III and filled with the bi-direction orientation sequence of *NOS* terminator in plasmid p512M1, resulting in plasmid pMOG-DNOS that contained a de 35S promoter, a GUS expression unit and two identical, divergently oriented *NOS* terminators separated by 30bp multiple cloning site (Fig 1).

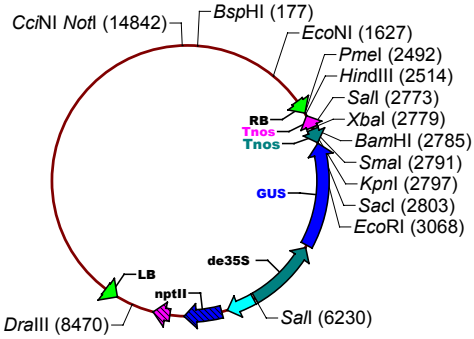

Fig.1 Construct map of plasmid pMOG-DNOS (*RB* right border sequence of a T-DNA, *LB* left border sequence of a T-DNA). The GUS is driven by the double enhanced CaMV 35S (de35S) promoter and terminated by the nopaline synthase terminator (*NOS*).

Plant material and culture media

Sterilized seeds of *Brassica napus* cv Westar were placed on M0 germination medium and grown in a dark chamber for seven days at 25±1°C. The seedlings were then used for explants.

M0 medium: only containing 10g / l agar and 50 mM $CaCl_2$. M1 medium: MS medium with 0.5 mg / l 2,4-D, 0.5 mg / l NAA, 0.5 mg / l 6-BA.. M2 medium: M1 medium with 300 mg / l Carboxyl and 25 mg/ l Kanamycin, 5.0 mg / l $Ag_2S_2O_3$. M3 medium: MS medium with 300 mg / l Carboxyl, 25 mg/ l Kanamycin, 1.0 mg / l Zt, 0.1 mg / l Kt and 6.0 g / l argrose. M4 medium: 1 / 2 MS medium with 0.1 mg / l IBA and 8.0 g / l agar.

Co-cultivation

Agrobacterium tumefaciens (LBA4404) with plasmid pMOG-DNOS was prepared overnight. Bacteria were harvested at time periods of 16-20 hours. Dilutions of bacteria to concentration of 10^4-10^5 bacteria per ml were prepared in M1 medium. The hypocotyls of seedlings were harvested and cut into pieces 0.3-0.5 cm in length and were soaked into the *Agrobacterium* suspension for about one hour. Then, the explants were dredged up from the infection medium. The co-cultivation of bacteria and explants was carried out for 60-72 hours without culture medium.

Callus induction and plant regeneration

After 3 days of co-incubation, hypocotyls were cultured in M2 medium for 15-20 days. The cultures then were transferred to M3 medium until shoots were regenerated. The regenerated shoots were transferred to M4 medium for root growth. The regenerated plants were transferred to soil and grown to maturity.

GUS assay

Harvested calli and leaves of regeneration plants were tested for histochemical *GUS* expression in an X-Gluc solution (Jefferson 1987).

PCR analysis

Genomic DNA was isolated from leaves of regeneration plants by AxyPrep Multisource Genomic DNA Miniprep Kit and used for PCR.

PCRs were carried out under standard conditions with 40s denaturation, 40s annealing, 2 min extension at 94°C, 57°C, and 72°C, respectively, for 30 cycles. Reaction products were resolved by electrophoresis in a 1.2% (wt / vol) agarose gel. The sequences of the PCR primers were as follows:

For GUS: 5′-GTGGAATTGATCAGCGTTGG-3′ and 5′-GCACCGAAGTTCATGCCAGT-3′.

For NPTII: 5′-GGCTATGACTGGGCACAACA and 5′-GAGCGGCGATACCGTAAA.

For Fad2: 5′-GCGAAACACCGCCCTTCACTG and 5′-CACCTTGCCTGTCCGGTTCCA.

Results

Normal expression of *GUS* gene was found in transgenic calli.

By the culture of 18d in M2 medium and 10d in M3 medium, some of transformed explants were used for analysis of *GUS* gene expression by X-Gluc staining. Almost all of transformed explants were stained by X-Gluc, explaining normal

expression of *GUS* gene, and RNAi did not happened in transgenic calli (Fig. 2A).

High suppression of GUS *gene expression is induced in transgenic plants*

Totally 360 transgenic plants was used for histochemical *GUS* expression. *GUS* was expressed strongly in 6 plants, weakly in 3 plants and negatively in 351 plants (Fig. 2B). The result suggested that RNAi might take place in transgenic plants obtained by transformation of vector pBI121-DN

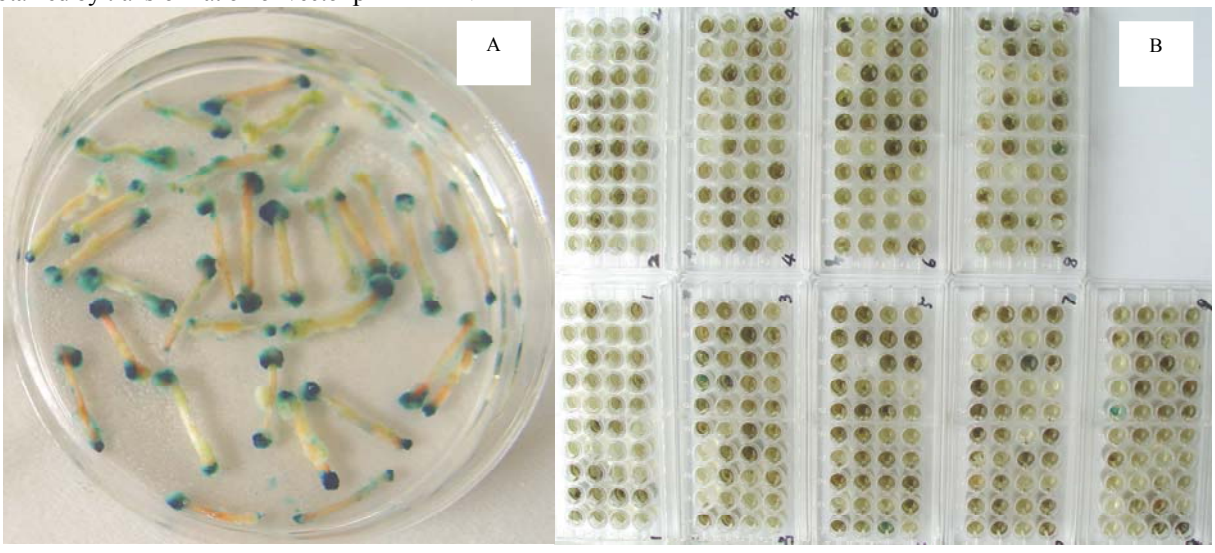

Fig.2 X-Gluc staining of transgenic calli (A) and plants (B).

T-DNA was integrated in the genomes

Ten of X-Gluc staining negative plants were random selected for transgene *NPTII* and *GUS* analysis by PCR and *Fad2* gene was used as internal marker. The result suggested that T-DNA was integrated in the genomes in all of selected plants (Fig. 3).

Discussion

It had been puzzling for more than a decade that introduction into plants of a transgene containing sequences homologous to an endogenous gene could result in silencing of the transgene and of all homologous host gene copies. Various hypotheses had been formulated to explain the phenomena (Meins and Kunz 1994). It is not until to the foundation of RNAi (Fire *et al*., 1998), the inverse-repeat construct which can produce dsRNA in transgenic plants is paid attention. The analysis of transgene locus structure explained that there was more than one inverse-repeat sequence in most of transgenic silencing plants (Marielle *et al*. 2000). The role of inverse-repeat sequence in gene silencing was directly proved by constructing a vector which contains various inverse-repeat sequence in ORF (Singh 2000, Lu 2004). However, we did not see any formal report about the vector which contains an inverse-repeat sequence outside the target gene

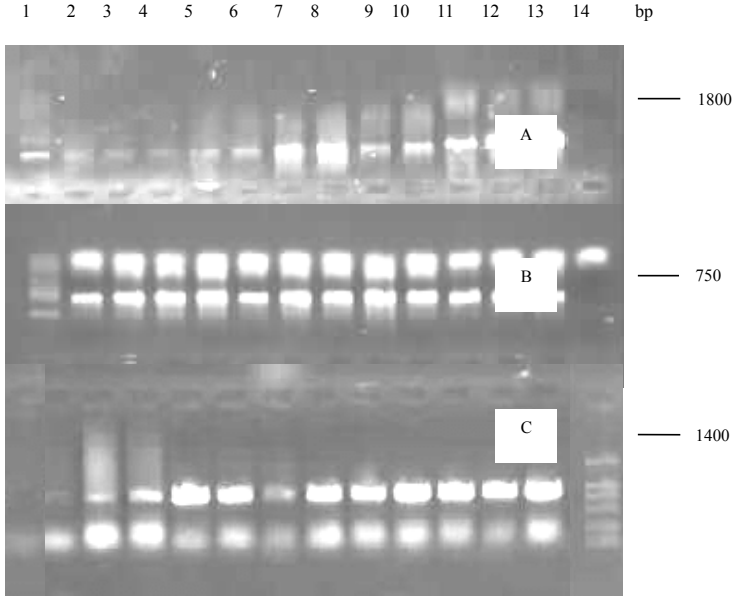

Fig.3 PCR of transgenic plants. A: GUS; B: NPTII; C: Fad2. 1. Negative control; 2-3. Positive control; 4-13, Transgenic plants, 14. PCR Marker 2000

In this experiment, vector pMOG-DNOS which contains an inverse-repeat sequence outside the target gene *GUS* was able to suppress the expression of *GUS* gene. The gene silencing appeared in this kind of transgenic plants may relate to transitivity of RNA silencing (Van Houdt H 2003).

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