Generation and Selection of Promoter Trap Lines for the Investigation of Shoot Development in Arabidopsis

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T-DNA-mediated transformation is a common method for generating transgenic plants with insertional mutagenesis. In order to identify important genes involved in shoot development, a system of promoter trap insertional mutagenesis was employed in *Arabidopsis thaliana*. For this system, an efficient promoter trap vector, pFGL561 was developed. The pFGL561 includes a *basta-resistant* gene, an intron with multiple splicing donor and acceptor sites, and a promoter-less *GFP* reporter gene. Using floral-dipping method, we made total 300 T_1 promoter-trap lines which were screened for GFP expression. GFP signals in the T_1 plants were detected with high frequency, 26.7%, and the signals were reconfirmed in T_2 plants. To isolate the genes that are involved in shoot development, phenotypes were analyzed in T_2 plants of the 19 T_1 lines that had GFP signals in shoot apex, and 6 T_1 lines were selected that had abnormal shoot development. These lines will be very useful for the investigation of shoot development.

Key words – Arabidopsis, promoter trap lines, pFGL561, shoot development

Introduction

Arabidopsis is a member of the mustard (Brassicaceae) family, and offers important advantages for basic research in genetics and molecular biology as follows: (a) its a small genome (114.5 Mb/125 Mb) was sequenced in 2000 (b) extensive genetic and physical maps of all five chromosomes have been made (c) it has a rapid life cycle (about 6 weeks from germination to mature seed) (d) it has prolific seed production and is easily cultivated in restricted space (e) it allows for efficient transformation methods utilizing *Agrobacterium tumefaciens* (f) a large number of mutant lines and genomic resources are known, and (g) there is a multinational research community of academic, government and industry laboratories concerning this plant. Such advantages have made Arabidopsis a model organism for studies of the cellular and molecular biology of flowering plants[8].

T-DNA-mediated transformation is a common way for generating transgenic plants with loss-of-function. Using T-DNA allows for a large collection of independent insertional mutations to be used. Large T-DNA collections have been generated in Arabidopsis[1,2,3,9,11,22]. Systematic efforts are now underway to use these collections for "reverse genetic" screens to identify insertions in any cloned gene[11,15,23].

In order to identify important genes expressed in plant development, a system of promoter trap insertional mutagenesis was employed in Arabidopsis[4,12,13,19,20]. This system provides a way of identifying novel genes based on their expression patterns. Inserting the promoterless reporter also destroys normal gene function, resulting in a loss-of-function mutation. Such an approach can potentially allow the identification of functionally redundant genes that would not be detectable in conventional mutational screens[21].

To increase the expression of reporter proteins in the promoter trap system, an intron with multiple splicing acceptor and donor sites was introduced in front of the promoterless coding region of the reporter genes[17,18]. The multiple splicing acceptor and donor sites allow the reporter proteins to be expressed when the insertion occurs in either an exon or intron. Consequently, the expression of the reporter is more efficient than in constructs lacking the intron[18].

Even though promoter trap lines of Arabidopsis have been generated in many laboratories, some of them express reporter proteins less efficiently, are also not publicly available, or the number of the lines is insufficient for studying the genes involved in interesting plant development.

In this report, we describe the generation and selection of

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useful Arabidopsis promoter trap lines for the investigation of shoot development. An efficient promoter trap vector, pFGL561, was developed, and then the promoter trap lines were generated. The promoter trap lines that expressed reporter protein in shoot apex and showed abnormal phenotypes in shoot development were selected and analyzed for the study of shoot development in Arabidopsis.

Materials and Methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana Columbia* were surface -sterilized in 70% EtOH for 1 min and in 1/10-diluted commercial clorox (final 0.4% NaOCl) for 10 min, and then were washed four times with distilled water. Surface-sterilized seeds were plated on agar plates containing half-strength of Murashige and Skoog medium salts[16], vitamins, 1.5% sucrose, and 0.7% agar. The plates were placed for 3 days at 4℃ under dark condition, and then were placed under short-day (SD) conditions (8 h of light/16 h of dark) at 22±2℃ and at a photon flux density of 100 μ mol m^{-2} sec^{-1}. Ten- to twelve-day-old seedlings were transferred onto soil and grown under long-day (LD) conditions (16 h of light/8 h of dark) at 22±2℃

Plasmid constructions

To construct a binary vector for the promoter trap system, the *bar* gene fragment including the *mannopine synthetase* promoter and *ocs* terminator fragment was amplified by PCR using primers (5'-AAAGGATCCGCCAACAGAGC CTGGCGTTC-3' and 5'-CCCAAGCTTGAGATCTAGATAT CGATAAGC-3'), and the fragment was cloned into *Hin*dIII /*Bam*HI sites of pBSIIKS (pBSIIKS-BAR). The *Hin*dIII/*Bam*HI fragment of the pBSIIKS-BAR was transferred into *Hin*dIII /*Bam*HI sites of pPZP201 (pPZP201-BAR). And then, a fragment of pGA2713 including an intron with multiple donor/acceptor sites, sGFP, and NOS terminator was cloned into the *Bam*HI/*Sal*I sites of the pPZP201-BAR. The final construct was named as pFGL561.

Plant transformation and selection of transgenic plants

The pFGL561 were transformed into *Agrobacterium tumefaciens* strain, GV3101 (pMP90) by freeze-thaw method[5], and then into Arabidopsis plants by floral-dipping method[7]. Transformed plants were selected on soil by spraying 1/500-diluted commercial BASTA (Bayer Crop Science Ltd, Korea) or on agar plates containing 10 mg/L DL-phosthinothricin (BASTA) (Duchefa, Netherlands).

Image analysis and fluorescence microscopy

Images were acquired using a cooled charge-coupled device(CCD) camera (CoolSNAP, Roper Scientific Inc, USA) and a fluorescence phase microscope (Zeiss Axioskop, Carl Zeiss co., Jena, Germany). CCD camera was used to observe the GFP signals of T_1 plants. The GFP signals in whole seedlings and plant organs were observed by fluorescence phase microscope with the appropriate filters (Carl Zeiss 38 filter set; FT 495, BP 525.50 or BP 470.40). All images were processed with Adobe Photoshop version 7.0.

Results and Discussion

Construction of an efficient promoter trap vector

To gain high efficiency of promoter trapping in Arabidopsis, a vector, pFGL561 was constructed which includes a *basta-resistant* gene (*bar* gene) under a *mannopine synthetase* (*mas*) promoter, an intron with multiple splicing donor and acceptor sites, and a promoter-less *GFP* reporter gene (Fig. 1). This vector suits our purpose. First, the *bar* gene is a good selective marker, because it makes the isolation of transgenic plants very easy and economical. Second, the vector has an intron with multiple splicing donor and acceptor sites that is known to greatly increase the expression of a reporter protein[18]. Reporter gene can be

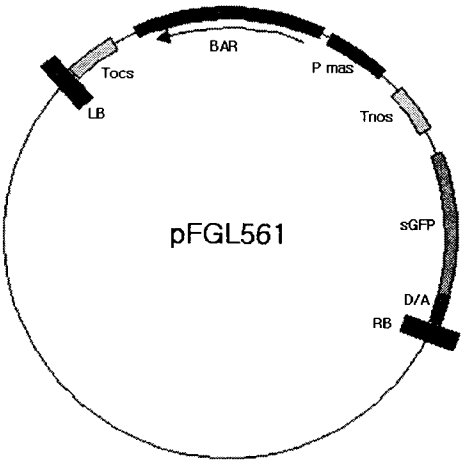

Fig. 1. Schematic map of a promoter trap vector, pFGL561. RB, right border; LB, left border; D/A, donor and acceptor splicing sites; *bar*, basta-resistant gene; Pmas, promoter of *mannopine synthase* gene; Tocs, terminator of *octopine synthase* gene; Tnos, terminator of *nopaline synthase* gene.

expressed very frequently due to an intron with the multiple splicing acceptor and donor sites in each of the three reading frames in front of the *GFP*-coding region. These sequences allow the GFP to be expressed when the insertion occurs in either an exon or intron. Third, the construct contains the promoter-less *GFP* reporter gene, which can be directly detected by illumination. Since no substrate is required, detection is relatively inexpensive, provided that the appropriate light source is available. Although GUS-based histochemical analysis is more sensitive than GFP analysis, the end product of the β-glucuronidase histochemical reaction is toxic for plant cells[14]. In contrast, detecting GFP activity can be done without the destruction, performed in live cells, and monitored during all of the developmental stages. Therefore, the GFP reporter gene is suitable for promoter trapping and *in vivo* expression studies.

Generation and screening of promoter trap lines

Several sets of transgenic plants with pFGL561 were generated, and were screened for GFP expression using a CCD imaging system, which visualizes the GFP image at a whole plant level. The CCD imaging system has a sensitive CCD camera and a intense light source with suitable band-pass filters.

GFP analysis from the promoter trap lines has revealed various expression patterns. A total of 300 T_1 seedlings at 10 days after germination (DAG) were analyzed. Eighty lines (26.7%) showed GFP activity (Table 1). Among the 80 T_1 lines, 19 lines showed GFP activity in shoot apex of the seedlings, 19 lines in hypocotyls, 9 lines in roots, 12 lines in shoot apex and hypocotyls, 2 lines in shoot apex and roots, 4 lines in hypocotyls and roots, and 15 lines in whole plant except cotyledons (Table 1 and Fig. 2). GFP

Table 1. Frequencies of GFP expression in T_1 seedlings

Organs showing GFP activity	No. of lines[a]
Shoot apex	19
Cotyledons	0
Hypocotyl	19
Root	9
Shoot apex + hypocotyl	12
Shoot apex + root	2
Hypocotyl + root	4
Shoot apex + hypocotyl + root	15
Total	80

[a]A total of 300 T_1 plants were examined using 10-day-old seedlings.

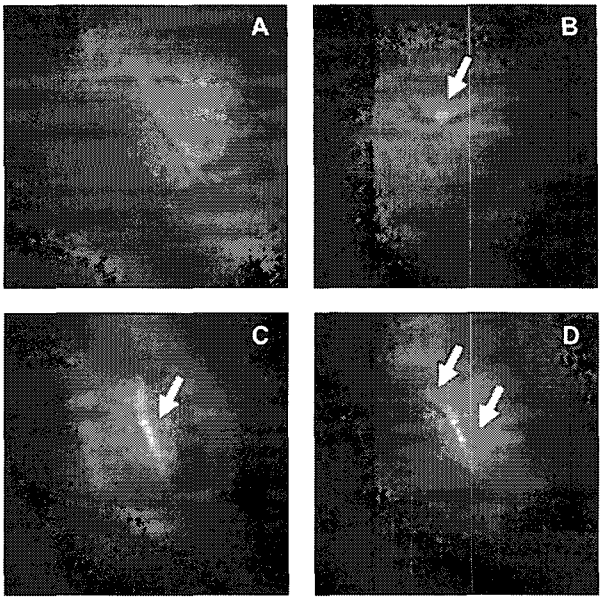

Fig. 2. Analysis of GFP signals in T_1 plants using CCD imaging system. GFP signals were observed in 10-day-old seedlings on the agar plates. Regions showing GFP signals are marked with arrows. GFP activity was not observed in WT (A). GFP activity was observed in shoot apex (B), in hypocotyl (C), and in shoot apex and hypocotyl (D) of T_1 plants.

signals in the T_1 plants occurred with high frequency (26.7%, 80/300), which is probably due to an intron with multiple splicing donor and acceptor sites. It is well reported that highly efficient gene detection by the gene trap is due not only to the installment of an intron in front of a reporter gene, but also to the presence of multiple splicing sites[6,10,18]. So far, the GFP signals in 7 T_1 plants were reconfirmed in T_2 plants under a fluorescence microscope (Fig. 3). We also screened for GFP signals in rosette leaves, floral buds, open flowers, and siliques of the mature T_1 plants, but the GFP signal was not detected in all of the organs (data not shown). The anthers could not be analyzed because they showed auto-fluorescence under both the CCD imaging system and the fluorescence microscopy (data not shown).

Analysis of the phenotypes of T2 transgenic plants

Promoter trapping is not only a means of identifying patterns of gene expression but also a tool for generating mutations by disrupting gene function. In addition to investigating the reporter expression patterns, we analyzed the promoter trap lines for visible mutant phenotypes.

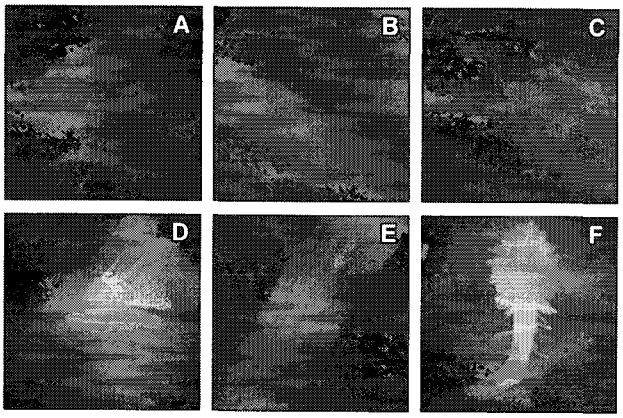

Fig. 3. Analysis of GFP signals in T_2 plants using fluorescence microscopy. GFP signals were investigated in 10-day-old seedlings on the agar plates. GFP activity was not observed in shoot apex (A), hypocotyl (B), and root (C) of WT. GFP activity was detected in shoot apex of T2 plant of T_1 line 64 (D), in hypocotyl (E) and root (F) of T_2 plants of T_1 line 147.

In order to isolate the genes involved in shoot development, phenotypic screens were done using the T_2 plants of the 19 T_1 lines showing GFP signals in shoot apexes (Table 1). The plants were screened mainly for conspicuous morphological changes in shoot architecture.

T_2 plants of 6 out of the 19 T_1 lines showed abnormal shoot development such as dwarfism, semi-dwarfism, and retarded shoot growth (Table 2 and Fig. 4). The abnormal shoot phenotypes were observed in 2 to 6 plants out of 25 or 30 T_2 plants, suggesting that probably one copy T-DNA is inserted into genomic DNA in T_1 plants of the 6 lines (Table 2). For the 6 T_1 lines, GFP signals in the T_1 plants were reconfirmed in the T_2 plants (Fig 3D). These results suggest that in the 6 T_1 lines, T-DNA is inserted in genes to play an important role in shoot development.

Table 2. T_1 lines showing abnormal shoot phenotypes in T_2 plants

T_1 Line No.	No. of investigated T_2 plants[a]	No. of plants showing abnormal shoot development
55	30	4
64	30	5
80	30	2
98	25	2
109	30	6
131	25	2

[a]T_2 plants of the 19 T_1 lines showing GFP signals at shoot apex were examined at 45 DAG.

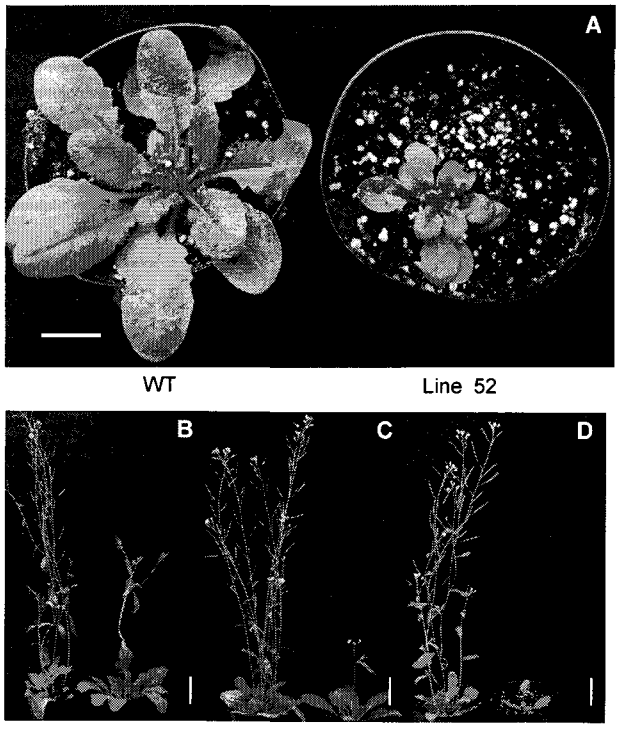

Fig. 4. *Phenotypes of* T_2 plants showing abnormal shoot development. Line 52 showed dwarfism at 35 days after germination (DAG) (A). Lines 109 (B), 80 (C), and 64 (D) also showed dwarfism or semi-dwarfism at 45 DAG. Bars=2 cm.

Taken together, in this study, a new useful vector for promoter trap, pFGL561, was developed, and the promoter trap lines having various *GFP* expression patterns were isolated. These promoter trap lines had highly efficient GFP signals. The selected promoter trap lines that had GFP signals at the shoot apex and abnormal shoot development will be very useful for investigating the regulatory mechanism of shoot development.

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초록 : 애기장대에 있어서 shoot 발달 연구를 위한 프로모터 trap 라인들의 제조 및 선별

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T-DNA 매개 형질전환은 삽입 돌연변이를 가지는 형질전환 식물체를 만들기 위한 유용한 방법이다. 애기장대의 shoot 발달 과정에서 중요한 역할을 하는 유전자를 확인하기 위해, 프로모터 trap 식물체 라인을 제작하고 분석하였다. 이를 위해 효율적인 프로모터 trap 벡터인 pFGL561을 제작하였다. pFGL561은 basta 저항성 유전자, multiple splicing donor acceptor 서열들, 그리고 프로모터가 없는 GFP 리포터 유전자를 포함하고 있다. Agrobacterium 균주인 GV3101에 pFGL561을 도입하고, 이를 매개로 하여 300개의 T_1 프로모터 trap 식물체를 제작하였고, 이들 식물체에서 GFP 발현을 조사하였다. T_1 식물체에서 GFP 발현 비율은 26.7%로 매우 높았고, 이러한 발현은 T_2 식물체에서도 재확인되었다. 한편, 식물의 shoot 발달에 관여하는 주요 유전자를 동정하기 위해서, shoot에서 GFP 발현을 보인 19개 T_1 식물체에서 유래한 T_2 식물체를 대상으로 표현형을 조사한 결과, 비정상적인 shoot 발달을 보이는 6개 T_1 라인을 확인하였다. 이들 식물체는 shoot 발달의 조절 기작 분석에 매우 유용하게 사용될 수 있을 것이다.