Approach Toward the Creation of Nonnarcotic Opium Poppy - Morphological and Genetical Analysis on "Thebaine Poppy" -

Noriaki KAWANO, Kayo YOSHIMATSU, and Fumiyuki KIUCHI
Division of Tsukuba, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation

Abstract. **Opium poppy (*Papaver somniferum* L.) is one of the most important medicinal plants, which is used as a sole commercial source of narcotic analgesic, morphine. The transformant of opium poppy we have established by infection of *Rhizobium rhizogenes* (formerly *Agrobacterium rhizogenes*) strain MAFF03-01724 showed aberrant morphology and altered opium alkaloid composition. The major alkaloid produced by this transformant was thebaine (16.3%, opium dry weight) instead of morphine. It is likely that this "thebaine poppy" phenotype was caused by the integration of T-DNA(s) into the poppy genome DNA, and their inserted loci are of great interest. To gain an insight into the mechanism of nonnarcotic thebaine accumulation for the further approach toward the creation of "codeine poppy" which produces codeine as a major alkaloid, the genetical and morphological analyses on the transformant was carried out. Here we report the results of the detailed analysis on the T-DNA inserted loci of T0 transfromant and the correlation between opium alkaloid composition and segregated T-DNA integration pattern in the self-pollinated T1 transformants.**

Introduction

Opium poppy (*Papaver somniferum* L.) is known to produce a vast variety of benzylisoquinoline alkaloids. We have focused on the transformant of this plant that demonstrates altered alkaloid composition compared with the wild type plant. It is an attractive experimental material because of its implication in the alkaloid biosynthesis, and of its potential to serve starting materials for semi-synthesis of novel pharmaceuticals with benzylisoquinoline backbone.

We have developed a *Rhizobium* transformant of *P. somniferum*, which shows an aberrant morphology and altered opium alkaloid composition. This transformant was established by infecting *Rhizobium rhizogenes* strain MAFF03-01724 to hypocotyls and a regenerated plantlet was maintained on MS medium. The acclimatized plantlet grown on soil flowered and developed capsule. The major opium alkaloid of this transformant was thebaine (16.3% dry weight), which was not accumulated in the non-transformant. The content of codeine also increased *ca.* three-fold in the transformant. These characteristics of the opium alkaloid

composition were similar to the results previously reported on the *in vitro* plantlet[1]. So, it is likely that "thebaine poppy" phenotype of the *Rhizobium*-transformant was caused by the stable insertion of T-DNA into the poppy genome DNA. To determine the inserted locus of T-DNA, inverse PCR (IPCR) and adaptor-ligation PCR (AI-PCR) were carried out. Then to gain an insight into the correlation between T-DNA integrated loci and thebaine accumulation, we have performed detailed analyses on opium alkaloid composition and T-DNA insertion pattern of self-pollinated T_1 transformants.

Materials and Methods

Plant materials. The *Rhizobium* transformant of *P. somniferum* var. Ikkanshu used in this study was established by infecting hypocotyl with *R. rhizogenes* strain MAFF03-01724 and regenerated as previously reported[1].

Self-pollinated seeds of T_1 transformant were obtained from T_0 transformant, which was transplanted onto soil. T_1 seeds were sowed in soil and then germinated plants were transplanted to a new pot one by one after five to six weeks. Finally sixty T_1 plants were flowered and developed capsules.

Extraction of opium alkaloids from the latexand HPLC analysis. Latex was collected from the unripe capsule then kept at 50°C, overnight to complete dryness. Five mL of methanol was added to *ca.* 5 mg of the latexthen sonicated for 30 min. After the extraction by sonication, 300 ml of methanol extract was transferred to Ultrafree-MC spin column (Millipore) and centrifuged shortly. The flow through of the column was subjected to the HPLC analysis. Samples were analyzed by the following HPLC system. The instrument was Waters Alliance PDA System (separation module: 2795, photodiode array detector: 2996) with TSK-GEL ODS100V column (4.6 x 250 mm, 5 mm, TOSOH, Japan). The alkaloids were separated at a flow rate of 0.7 mL"Emin-1 and using the gradient acetonitrile (A): 10 mM sodium 1-heptanesulphonate (pH 3.5) (B) (0-15 min 28-34 A%, 15-25 min 34-40 A%, 25-39 min 40 A%, 39-40 min 40-28 A%). Column temperature was 30°C. The alkaloids were detected by photodiode array detector in the range of 200 to 400 nm for qualitative analysis and at 284 nm for quantitative analysis.

Genomic DNA preparation and PCR analysis. Genomic DNA was extracted from leaves using DNeasy mini plant extraction kit (QIAGEN) with a slight modification. [Inverse PCR] Genomic DNA was digested with restriction enzymes such as *Kpn*I, *Hin*dIII, *Eco*RV, and *Pvu*II then self-ligated to produce circular genome DNA library. Inverse PCR (IPCR) was performed with *R. rhizogenes* MAFF03-01724 T-DNA specific sense and anti-sense

primers using circular DNA library as a template. Specifically amplified products by nested PCR were cloned into pT7Blue sequencing vector (Novagen, EMD Biosciences, USA). [Adaptor-ligation PCR] Genomic DNA was digested with blunt end cutters such as *Eco*RV, *Pvu*II, *Ssp*I, and *Stu*I. Digested DNA was ligated with adaptors then used as a template for adaptor ligation PCR (Al-PCR). PCR was performed with adaptor specific primers and T-DNA specific primers using adaptor-ligated genome library as a template. Following nested PCR and cloning of specific PCR product were performed as same as the IPCR.

DNA sequencing. DNA sequence of cloned PCR products was determined by ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems). Sequencing reaction was performed with BigDye 3.1 sequencing kit (Applied Biosystems) on both strands using Forward or Reverse primers specific to pT7Blue vector.

T-DNA integration pattern analysis (Multiplex PCR). Integration pattern for the four T-DNAs was analyzed by detecting the four junctions of T-DNA RB ends and adjacent genomic regions simultaneously. Primers specific for genome DNA were designed from the sequence information of T-DNA adjacent DNA fragments, namely RB1-460A, RB2-393A, RB3-588A, and RB4-261A. Multiplex PCR was performed by the mixture of these four anti-sense primers and T-DNA specific primer, then PCR products whose expected size for RB1, RB2. RB3, and RB4 junctions were 573 bp, 493 bp, 691 bp, and 375 bp respectively, were analyzed by agarose gel electrophoresis.

Results and Discussion

T-DNAs were Integrated into the Poppy Genome in Complicated Manner

Unknown genomic DNA sequence adjacent to the T-DNA RB or LB region was amplified by IPCRor Al-PCR. These PCR products were cloned into T-vector and their sequences were determined. Finally amplified products were classified into three classes (Fig.), the first one was the genome DNA connected with a T-DNA end, and the second was the T-DNA end connected with another T-DNA end, and the last was the T-DNA end connected with partial fragment of T-DNA orf13 region.

The first class, the genomic fragments connected with T-DNA borders was further classified in five types. Four of those, RB1, RB2, RB3, and RB4 were genomic DNA fragments connected with RB T-DNA border, and LB1 was the fragment connected with LB. PCR analysis using wild type genome DNA as a template revealed that the fragments RB2 and LB1 were the pair of the ends of single T-DNA. These results suggest that T-DNA insertion has occurred in four loci of the poppy genome, at least. The second class of PCR

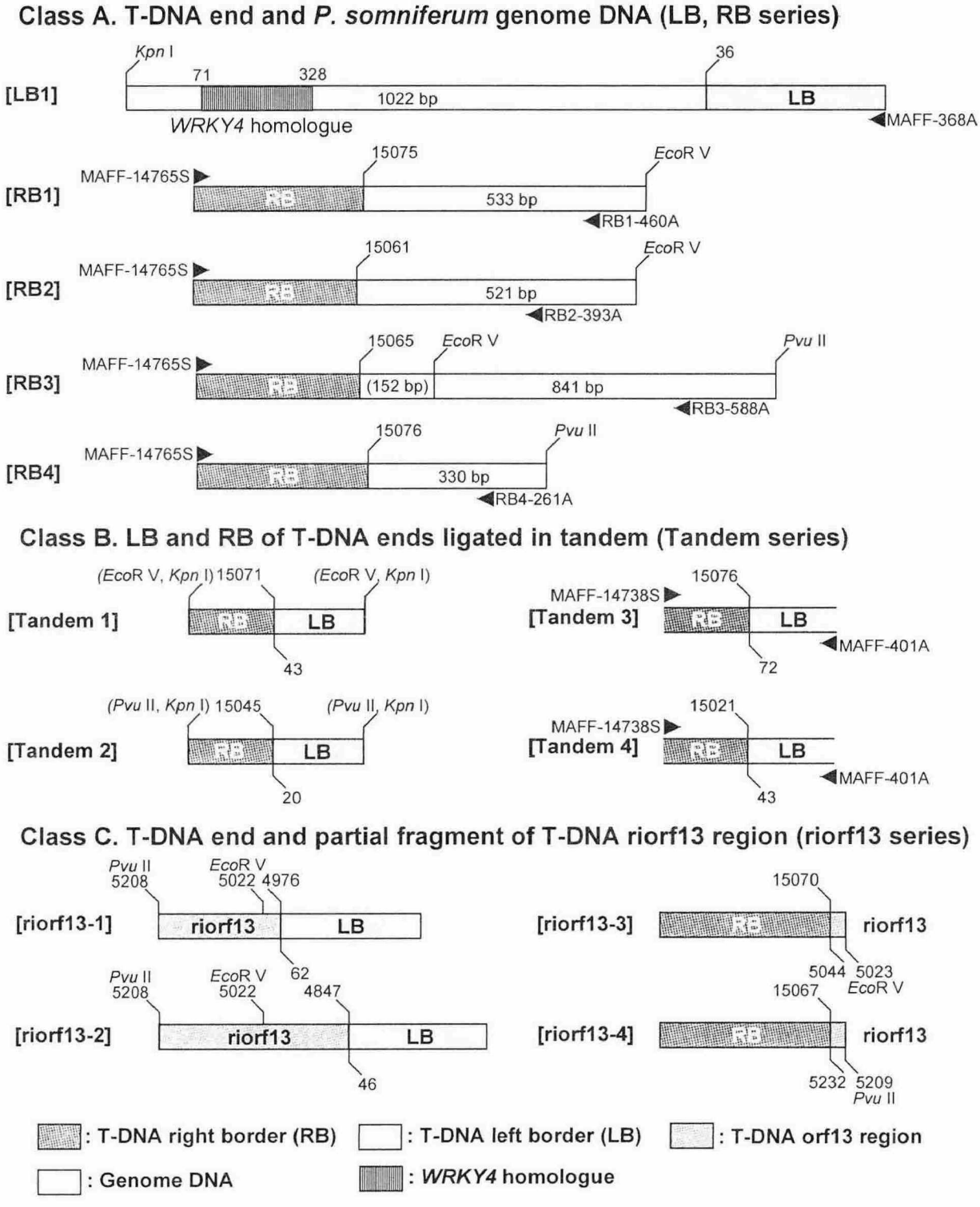

Fig. Schematic representation for the T-DNA ends and their adjacent sequences amplified by IPCR and Al-PCR

Genomic DNA was digested by restriction enzymes shown at the end of genomic DNA region. The position of primers were indicated by the arrow head. Primers used for the multiplex PCR are also shown.

Numbers above the junctions indicate the positions of border of T-DNA RB or LB. LB1 and RB2 are the pair of the ends of a single T-DNA. Partial ORF homologous to *A. thaliana WRKY4* gene was found at *ca.* 0.7 kbp upstream of LB border on LB1 (vertical stripe region).

Tandem 3 and 4 were obtained by direct amplification of T-DNA borders.

product, the border of T-DNAs connected in tandem, was further classified into four types and the third class was classified into four types.

Characteristic "Thebaine Poppy" was Well Conserved in T_1 Transformants

Opium alkaloids in the latex of T_1 transformants were analyzed by HPLC. The thebaine content ranged from 0.32% to 26.5% (%, opium dry weight). Most of the T_1 transformants showed high thebaine phenotype, and these high thebaine strains and wild type (low thebaine and high morphine) strains did not segregated in Mendelian manner.

T-DNA Integration Pattern was Not Correlated with Thebaine Content

By the multiplex PCR performed on T-DNA and RB adjacent genome junction, T-DNA integration pattern was analyzed on twenty-four transformant strains representing high-thabaine, moderate-thebaine, and low-thebaine strains. As a result, no correlation between integration pattern for these four T-DNAs and thebaine content was observed. For example, high thebaine strain (#2-17) and wild type train (#2-1) showed the same T-DNA insertion profile; RB1(+), RB2 (-), RB3(+), RB4(-) (+: positive, -: negative).

Results of IPCR and Al-PCR analysis revealed that at least four T-DNAs were integrated into the genome DNA of T_0 transformant. Furthermore, T-DNAs connected in tandem or the partial fragments derived from T-DNAs ligated with each other were found. The functional implication or the mechanism how these fragments have occurred remains unknown. These complicated data make it difficult to overview the whole structure of T-DNA integrated loci.

The opium alkaloid composition pattern of sixty T_1 transformants did not segregate in Mendelian manner. These phenomena implicate that the altered alkaloid composition, especially the high amount accumulation of thebaine, was not caused by the modification of single gene locus.

Further analysis on the genomic sequence around the T-DNA inserted loci is still in progress. The effects of expressed proteins transcribed from the components of T-DNA itself, such as *rol* gene cluster should also be taken into consideration.

For the genetic manipulation of morphine biosynthetic pathway at will, the complete understanding of the pathway, including the regulation mechanism of biosynthesis, is necessary. Our "thebaine poppy" is a milestone for the creation of nonnarcotic opium poppy. Genetical analysis on this plant may sure provide a clue for the further creation of "codeine poppy".

Literature Cited

1) Yoshimatsu K. and Shimomura K., *Plant Cell Rep.* 11, 132-136 (1992)

Noriaki KAWANO

Present Address: Division of Tsukuba, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, 1-2 Hachimandai, Tsukuba, Ibaraki, Japan 305-0843

E-mail: nkawano@nibio.go.jp

Education

Ph.D. 2004 The University of Tokyo (Pharmaceutical Sciences)
M.S. 2001 The University of Tokyo (Pharmaceutical Sciences)
B.S. 1999 The University of Tokyo (Pharmaceutical Sciences)

Professional Experience

2005-Present Researcher, Division of Tsukuba, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation
2004-2005 Researcher, Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences

Publications

1. Ichinose K., You S., Kawano N., Hayashi K., Yao X.-S., and Ebizuka Y. Heterologous expression of furostanol glycoside 26-*O*-β-glucosidase of *Costus speciosus* in *Nicotiana tabacum*. *Phytochem.* 51, 599-603 (1999)
2. You S., Kawano N., Ichinose K., Yao X.-S., and Ebizuka Y. Molecular cloning and sequencing of an *Allium macrostemon* cDNA probably encoding oxidosqualene cyclase. *Plant Biotech.* 16, 311-314 (1999)
3. Kawano N., Ichinose K., and Ebizuka Y. Molecular Cloning and Functional Expression of cDNAs Encoding Oxidosqualene Cyclases from *Costus speciosus*. *Biol. Pharm. Bull.* 25, 477-482 (2002).