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***Original Paper***

# Expression of active enzymes from an inducible tomato-mosaic-virus-based vector in cultured transgenic tobacco BY-2 cells

Koji Dohi[1,2], Masashi Mori[1,2,*]

[1] Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nonoichi-machi, Ishikawa 921–8836, Japan; [2] CREST, Japan Science and Technology Agency, Kawaguchi, Saitama 332–0012 Japan
* E-mail: mori@ishikawa-pu.ac.jp Tel: +81-76-227-7527 Fax: +81-76-227-7557

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**Abstract** Previously, we reported on an inducible viral vector system for foreign protein production in cultured plant cells, in which the transcription of the recombinant viral vector RNA encoding a foreign protein is controlled by an estradiol-inducible promoter. In this study, we used the inducible virus vector system to test the efficiency of a modified tomato mosaic virus (ToMV) encoding the movement protein. The virus inducibly produced a foreign jellyfish green fluorescent protein encoded in the virus in transgenic tobacco BY-2 suspension-cultured cells as efficiently as modified ToMV without the movement protein. We then produced transgenic BY-2 cell lines ER–ToMV–MP–DHFR and ER–ToMV–MP–GUS, which encoded modified ToMV with *Escherichia coli* dihydrofolate reductase (DHFR) and $\beta$-glucuronidase (GUS), respectively. After estradiol was added to the cell culture, DHFR and GUS activity was detected in the ER–ToMV–MP–DHFR and ER–ToMV–MP–GUS cells, respectively. In contrast, no DHFR or GUS activity was detected in untreated transgenic lines. Three days after induction, DHFR accumulation accounted for up to 15% of the total soluble proteins extracted from the cells, indicating that an inducible viral vector is an effective option for efficiently producing active enzymes in cultured plant cells.

**Key words:** BY-2, tobacco, tobamovirus, virus vector.

Cultured plant cells have excellent potential for producing useful proteins such as biopharmaceuticals (Hellwig et al. 2004). Because plant cells are cultured in relatively inexpensive medium, large-scale industrial cultures are possible at moderate cost. The proteins produced can then undergo posttranslational modification, which is essential to activate some eukaryotic proteins. Another advantage of cultured plant cell proteins is the ease of extraction and purification, as the cells are uniform and contain no mycotoxins or mammalian pathogens. Some heterotrophic cell lines, such as tobacco BY-2 cells, do not contain large amounts of RuBisCO proteins, which may compete with the production of desired proteins. Thus, these cultured plant cells should be a good vehicle for economically and safely producing useful proteins.

Industrial protein production requires an efficient system for expressing the foreign gene. Recently, we reported a novel inducible virus infection system for producing a foreign protein in cultured plant cells (Dohi et al. 2006). In this system, the genomic RNA of a modified plant virus that encodes a foreign protein is expressed from stably transformed cDNA under the control of an inducible promoter (Figure 1). The inducibly expressed viral vector RNA replicates autonomously and transcribes subgenomic mRNA to produce a foreign protein (Figure 1). Using this system, we demonstrated the highly efficient production of green fluorescent protein (GFP) in transgenic BY-2 cells from an inducible modified tomato mosaic tobamovirus (ToMV; Dohi et al. 2006). However, whether the inducible ToMV vector can efficiently produce proteins other than GFP is unknown.

*Escherichia coli* dihydrofolate reductase (DHFR; 18 kDa) and $\beta$-glucuronidase (GUS; 68 kDa) have been used as representative prokaryotic enzymes to evaluate the efficiency of protein production in various expression systems (Madin et al. 2000; Marillonnet et al. 2005). In this study, we investigated E. coli DHFR and GUS expression from inducible ToMV vectors to evaluate whether active enzymes can be produced from inducible virus infections in cultured plant cells. Using the inducible virus infection system, we also tested the infection efficiency of ToMV derivatives encoding the viral movement protein.

Abbreviations: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; GFP, green fluorescent protein; ToMV, tomato mosaic virus; DHFR, dihydrofolate reductase; GUS, $\beta$-glucuronidase; NADPH, nicotinamide adenine dinucleotide phosphate
This article can be found at http://www.jspcmb.jp/

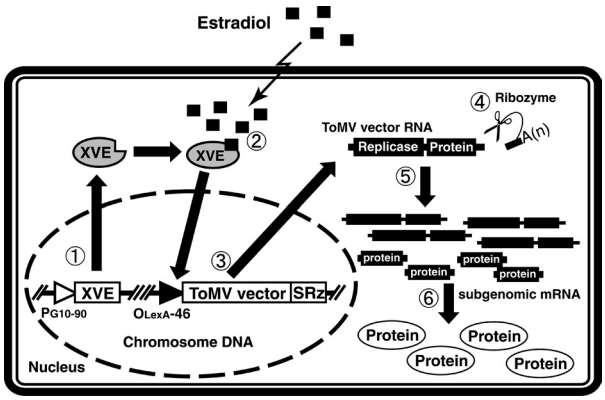


Figure 1. Schematic overview of the inducible ToMV vector system in cultured plant cells. XVE, a transcriptional activator that responds to estrogen. $O_{LexA}$-46, a promoter regulated by XVE; $P_{G10-90}$, a synthetic promoter for constitutive expression; SRz, a ribozyme sequence from tobacco ringspot virus satellite RNA. Step 1: XVE is expressed constitutively from the transgene. Step 2: Estradiol added to the culture activates XVE. Step 3: Activated XVE stimulates the transcription of the ToMV vector from the transgene controlled by XVE-regulated promoter $O_{LexA}$-46. Step 4: Nonviral sequences that interfere with the replication of the viral vector are removed from the 3′ terminus of the transcript by ribozymes. Step 5: The resulting viral vector replicates vigorously and transcribes subgenomic mRNA for a foreign protein. Step 6: The desired protein is translated from the subgenomic mRNA.

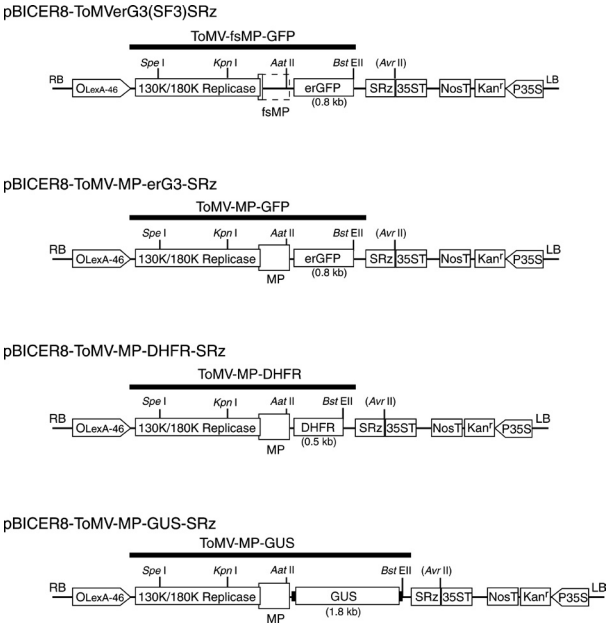


Figure 2. Schematic representation of the Ti plasmid vectors used in this study (not to scale). Bold lines above the constructs indicate the regions of modified ToMV cDNA. RB, right border; LB, left border; $O_{LexA}$-46, fusion promoter controlled by XVE; MP, 30 K viral movement protein gene; DHFR, *dhfr* gene; SRz, ribozyme sequence from tobacco ringspot virus satellite RNA; 35ST, cauliflower mosaic virus 35S terminator; NosT, nopaline synthase terminator; Kan$^r$, kanamycin-resistance gene; P35S, cauliflower mosaic virus 35S promoter; GUS, *uidA* gene encoding GUS. Bold regions adjacent to *uidA* (GUS) have sequences that differ from the corresponding regions of other constructs.

## Materials and methods

### *Construction of Ti plasmids*

The Ti plasmid pBICER8–ToMVerG3(SF3)SRz (Figure 2) has been described previously (Dohi et al. 2006). This plasmid encodes ToMV–fsMP–GFP, the ToMV derivative containing a frameshift mutation movement protein gene and a gene encoding an ER-localized GFP variant, G3GFP (Tamai and Meshi, 2001). The Ti plasmid pBICER8–ToMV–MP–erG3–SRz (Figure 2), which encodes a modified ToMV (ToMV–MP–GFP), was constructed as follows. The plasmid piL.erG3SRz(Avr) was constructed in the same manner as piL.erG3(SF3)SRz(Avr) (Dohi et al. 2006), except that the modified ToMV sequence was derived from piL.erG3 (Tamai et al. 2003), which encodes the wild-type 30 K movement protein, instead of from piL.erG3(SF3), which has a frameshift mutation in the 30 K movement protein gene. Then piL.erG3SRz(Avr) was digested with *Spe*I and *Avr*II, and a 5.6-kbp fragment was ligated into the *Spe*I site of pBICER8–ToMV5′–Spe (Dohi et al. 2006) to create pBICER8–ToMV–MP–erG3–SRz (Figure 2).

A Ti plasmid encoding the modified virus ToMV–MP–DHFR was constructed as follows. The *dhfr* gene from *E. coli* (Madin et al. 2000) was amplified by PCR from pEU–DHFR (Wakenyaku Co. Ltd., Kyoto, Japan) using the primers 5′-GGATCCAAGGAGATATAACAATGATCAGTCT-GATTGCGGC-3′ and 5′-GCCCCGGTTACCTTACCGCCG-CTCCAGAATCTC-3′. The PCR product was digested with *Aat*II and *Bst*EII and inserted between the *Aat*II and *Bst*EII sites of piL.erG3SRz(Avr). The resulting plasmid was digested with *Spe*I and *Avr*II, and the 5.3-kbp fragment was ligated into the *Spe*I site of pBICER8–ToMV5′–Spe to create Ti plasmid pBICER8–ToMV–MP–DHFR–SRz (Figure 2).

A Ti plasmid encoding the modified virus ToMV–MP–GUS was constructed as follows. The blunted *Sma*I–*Sac*I fragment of pBI121 (Jefferson et al. 1987) containing the *uidA* gene was inserted between the blunted *Sac*I and *Bst*EII sites of pLDE2 (Saito et al. 1987). The resulting plasmid was digested with *Kpn*I and *Bst*EII, and a DNA fragment containing the *uidA* gene was inserted between the *Kpn*I and *Bst*EII sites of piL.erG3SRz(Avr). The resulting plasmid was digested with *Spe*I and *Avr*II, and the 6.6-kbp fragment was ligated into the *Spe*I site of pBICER8–ToMV5′–Spe to create Ti plasmid pBICER8–ToMV–MP–GUS–SRz (Figure 2).

### *Culture, transformation, and estradiol treatment of BY-2 cells*

The culture and transformation of the cultured tobacco BY-2 cell line have been described previously (Dohi et al. 2006). To induce transcription from an estradiol-specific promoter, $\beta$-estradiol (Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to the medium at a final concentration of 10 $\mu$M (Dohi et al. 2006).

### *Detection of GFP fluorescence*

A BY-2 cell callus was photographed using the LAS-3000 imaging system with a 510DF10 filter (Fujifilm, Tokyo, Japan) to detect GFP fluorescence. GFP fluorescence in calli and cells was observed under a fluorescence stereoscopic microscope (SZX-12; Olympus, Tokyo, Japan) with an SZX-FGFP filter set

(excitation 460–490 nm, emission 510 nm; Olympus).

### *RNA and protein analysis*

Total RNA was prepared from the cultured cells and analyzed by Northern blotting to detect ToMV-related sequences, as described previously (Mori et al. 2001; Dohi et al. 2006).

Total soluble proteins were prepared from the cultured cells and analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), as described previously (Dohi et al. 2006). Western blot analysis to detect the movement and GUS proteins was conducted using anti-movement protein antibody (Meshi et al. 1992) with Immun-Star Goat Anti-Rabbit-AP Detection Kit (Bio-Rad, Hercules, CA, USA) and the anti-GUS IgG (Molecular Probes A5790; Invitrogen, Carlsbad, CA, USA ) with a Lumi-Light$^{PLUS}$ Western Blotting kit (Roche, Mannheim, Germany), respectively, according to the manufacturers' instructions.

### *Detection of enzyme activities*

DHFR activity in the BY-2 cell crude extract was measured essentially according to the method of Widemann et al. (1999). The cells were collected from the medium by centrifugation, resuspended with an equal volume of buffer A (0.05 M Tris–HCl, pH 7.5), and disrupted by sonication. An aliquot (10 $\mu$l) of the extract was mixed with 20 $\mu$l NADPH solution (0.5 mg ml$^{-1}$ NADPH in buffer A), added to 82.5 $\mu$l substrate solution [3.3 mg ml$^{-1}$ dihydrofolate ($FH_2$) and 20% (v/v) 2-mercaptoethanol in buffer A] in a well of a 96-well microplate, and incubated at room temperature. The absorbance of the mixture at 340 nm was measured to monitor NADPH oxidation using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA), at 40-s intervals. The protein concentrations of the extracts were measured using a protein assay (Bio-Rad), with bovine serum albumin (Toyobo, Tokyo, Japan) as the standard.

The BY-2 cells were stained to detect GUS activity, as described previously (Jefferson et al. 1987).

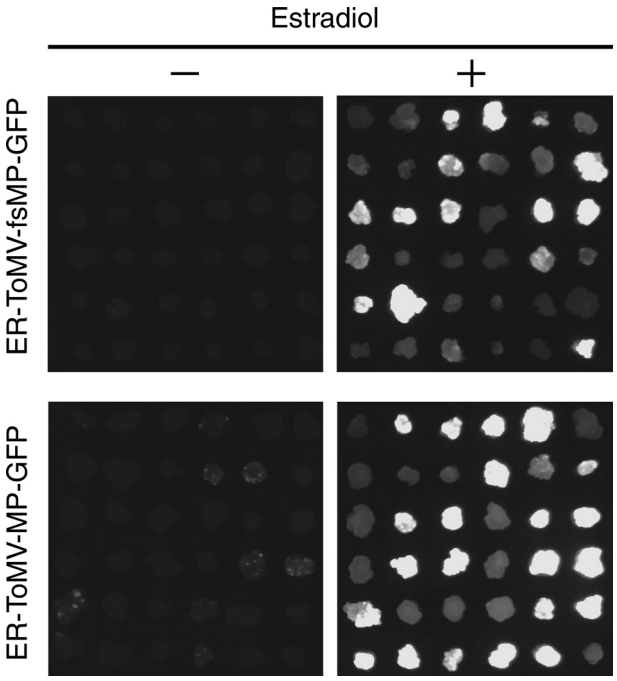


Figure 3. GFP fluorescence in calli of ER–ToMV–MP–GFP and ER–ToMV–fsMP–GFP cell lines. Thirty-six lines each of kanamycin-resistant calli were randomly selected from the transformants, placed on agar medium containing estradiol, and incubated for 4 days. White calli are those ones showing GFP fluorescence.

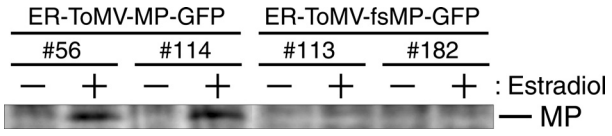


Figure 4. Western blot analysis of total soluble proteins (10 $\mu$g) to detect the viral movement protein. ER–ToMV–MP–GFP and ER–ToMV–fsMP–GFP cell lines were treated with estradiol 2 days after subculturing and then incubated for 24 h.

## Results

### *Inducible infection with ToMV encoding the viral movement protein*

In our previous study, ToMV–fsMP–GFP (Figure 2), a modified virus that encodes the movement protein gene with a frameshift mutation, was induced to efficiently express the foreign protein GFP in transgenic BY-2 cells (Dohi et al. 2006). Conversely, many tobamovirus-based vectors encode the movement protein, which facilitates cell-to-cell movement of the modified virus in inoculated leaves. In this study, we first investigated the replication efficiency of a modified ToMV containing the movement protein in an inducible viral vector infection system in cultured plant cells.

The cDNA of the modified ToMV genome, designated ToMV–MP–GFP, was constructed in the same way as ToMV–fsMP–GFP, but encoding the movement protein instead (Figure 2). ToMV–MP–GFP cDNA was inserted downstream from the estradiol-inducible promoter to create Ti plasmid pBICER8–ToMV–MP–erG3–SRz. The plasmid was introduced into the ER transgenic BY-2 cell line (Dohi et al. 2006), which constitutively expresses XVE, an estrogen-receptor-based transcriptional activator (Zuo et al. 2000), to produce ER–ToMV–MP–GFP cell lines. Drug-resistant calli were randomly selected from the resulting transformants, placed on agar medium containing estradiol, and incubated to investigate inducible GFP expression. As shown in Figure 3, GFP fluorescence was detected in some estradiol-treated ER–ToMV–MP–GFP lines. Without estradiol treatment, GFP fluorescence was not observed or observed only in a few cells. These results indicate that ToMV–MP–GFP is inducibly replicated and GFP is produced in response to estradiol treatment. Western blotting analysis revealed infection-specific accumulation of the movement protein in ER–ToMV–MP–GFP lines (Figure 4).

Viral RNA and GFP accumulations were compared

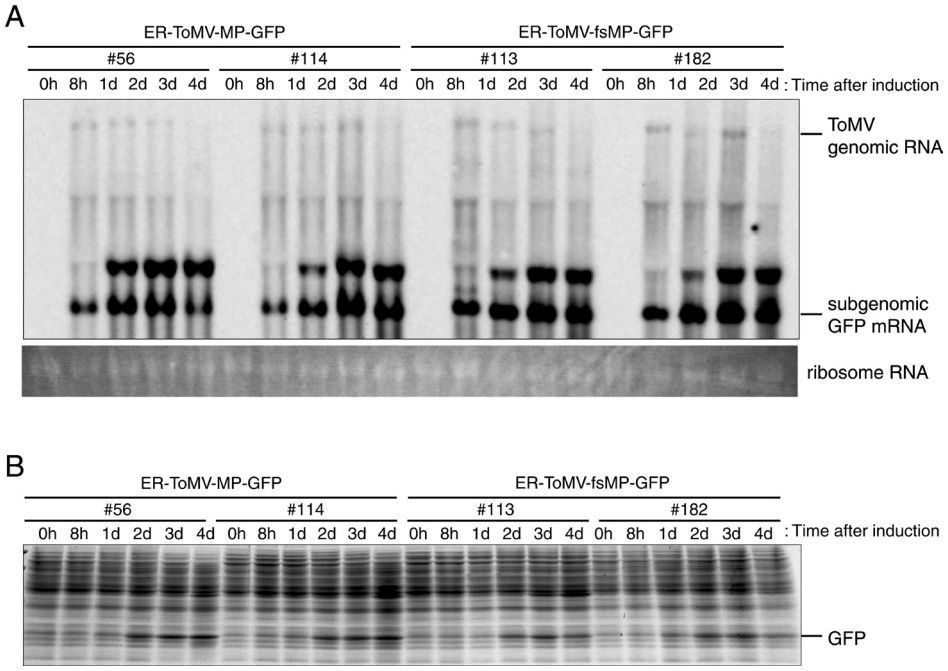


Figure 5. Accumulation of viral RNA and GFP in transgenic BY-2 cell lines. ER–ToMV–MP–GFP and ER–ToMV–fsMP–GFP cell lines were treated with estradiol 2 days after subculturing and then incubated for the indicated periods. (A) Northern blot analysis of ToMV-specific sequences in total RNA (2 $\mu$g). (B) SDS–PAGE (15% gel) of total soluble protein (7.5 $\mu$g) stained with Coomassie blue.

between ER–ToMV–MP–GFP lines #56 and #114 and ER–ToMV–fsMP–GFP lines #113 and #182, in which efficient GFP expression was induced in more than 80% of cells. Northern blotting revealed that viral RNA accumulation induced in ER–ToMV–MP–GFP cells was similar to that induced in ER–ToMV–fsMP–GFP cells (Figure 5A). GFP accumulation in ER–ToMV–MP–GFP cells was also similar to that in the ER–ToMV–fsMP–GFP cells (Figure 5B). The ratio of GFP-expressing cells in the ER–ToMV–MP–GFP lines decreased at lower concentrations of estradiol in the culture, as previously observed in ER–ToMV–fsMP–GFP lines (Dohi et al. 2006). These results suggest that, at least in efficient cell lines, the movement protein does not significantly affect ToMV RNA accumulation or GFP production.

### *Expression of DHFR from the inducible ToMV vector*

The production of functional enzymes is one of the main targets of transgenic biotechnology. To evaluate whether an active enzyme can be produced from the inducible viral vector, we constructed a ToMV vector encoding the *E. coli dhfr* gene, designated ToMV–MP–DHFR. Ti plasmid pBICER8–ToMVDHFRSRz, which encodes ToMV–MP–DHFR cDNA downstream from the estradiol-inducible promoter (Figure 2), was introduced into ER43, an XVE-expressing transgenic BY-2 cell line (Dohi et al. 2006; Nishikiori et al. 2006) using *Agrobacterium*-mediated transformation. A Northern blot of total RNA extracted from randomly selected kanamycin-resistant calli 4 days after estradiol treatment revealed specific signals corresponding to ToMV genomic RNA and subgenomic DHFR mRNA in several transgenic lines (Figure 6A). This indicates that ToMV–MP–DHFR genomic RNA was replicated and that subgenomic DHFR mRNA, which is a product of viral RNA replication, was successfully synthesized in these calli. The intensity of the bands varied significantly among the lines (Figure 6A), similar to the variation in inducible GFP expression in the ER–ToMV–MP–GFP and ER–ToMV–fsMP–GFP lines.

Three lines of ER–ToMV–MP–DHFR, in which strong signals for viral RNA were detected by Northern blotting, were suspension-cultured to investigate the production of DHFR protein. The cultures were treated with estradiol 2 days after subculture and incubated for a further 3 days before harvesting. SDS–PAGE analysis followed by Coomassie blue staining showed induction-specific bands corresponding to DHFR in the total soluble proteins extracted (Figure 6B). A comparison of the intensity of these bands with that for bovine serum albumin bands indicated that the amount of DHFR produced was approximately 15% (w/w) of the total soluble proteins. An enzyme assay revealed that DHFR was specifically detectable in the total soluble fractions extracted from calli treated with estradiol (Figure 6B). These results demonstrate that active DHFR protein was successfully produced in cultured plant cells using an inducible viral vector.

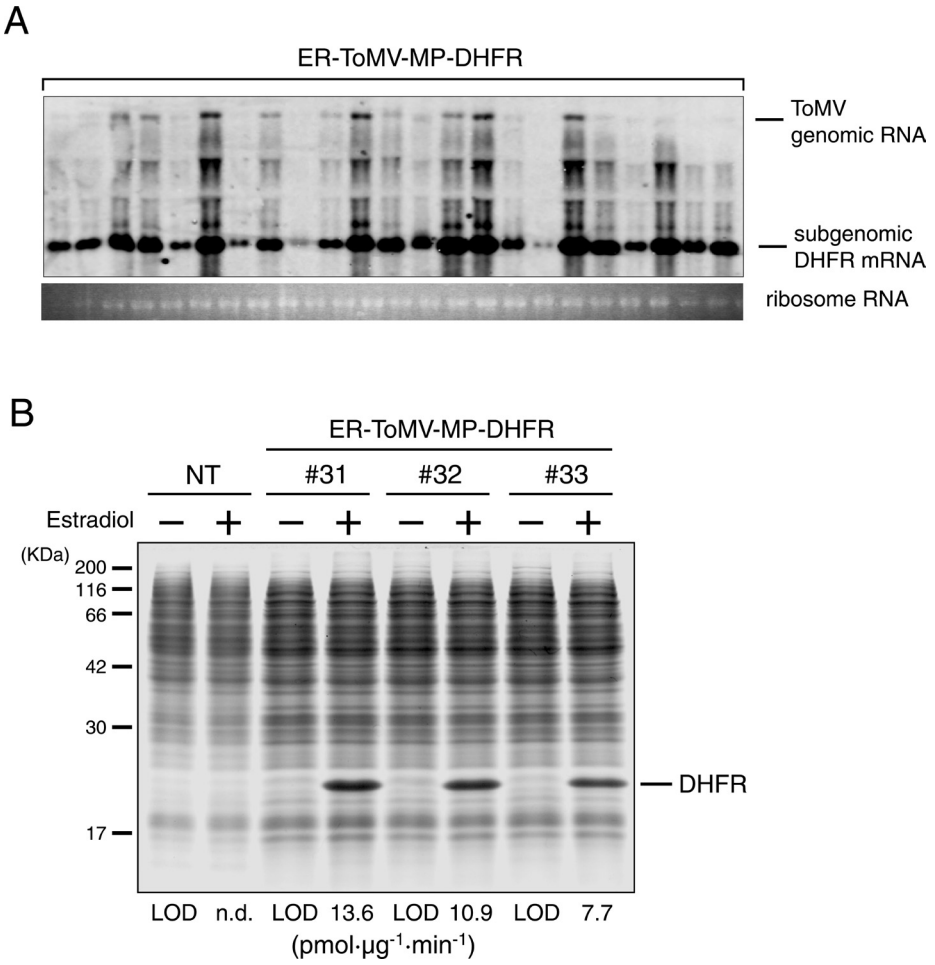


Figure 6. Accumulation of viral RNA and recombinant DHFR in transgenic BY-2 cell lines. (A) Northern blot analysis of ToMV-specific sequences in total RNA (2 $\mu$g). Twenty-three lines of kanamycin-resistant ER–ToMV–MP–DHFR calli were randomly selected, placed on agar medium containing estradiol, and incubated for 4 days before the extraction of total RNA. (B) SDS–PAGE (15% gel) of total soluble protein (10 $\mu$g) stained with Coomassie blue. Fractions of total soluble proteins were extracted from suspension-cultured ER–ToMV–MP–DHFR cells 3 days after estradiol treatment. The specific activity of DHFR in each extract is shown below the panel. LOD, less than the level of detection; NT, non-transgenic.

### *Expression of GUS from the inducible ToMV vector*

To further evaluate the performance of the inducible ToMV vector system, we constructed a modified ToMV-encoding GUS protein. Despite extensive efforts, we could not create Ti plasmids by replacing the *GFP* open reading frame with that of the *uidA* gene in pBICER8–ToMV–MP–erG3SRz and pBICER8–ToMVerG3(SF3)SRz. Therefore, we used the Ti plasmid encoding viral vector ToMV–MP–GUS, in which the 5′ and 3′ noncoding sequences adjacent to the *GUS* open reading frame differed from those of the corresponding regions in the other viral vectors used in this study, and was designated as pBICER8–ToMV–MP–GUS–SRz (Figure 2).

XVE-expressing ER cell lines (Dohi et al. 2006; Nishikiori et al. 2006) were transformed with pBICER8–ToMV–MP–GUS–SRz to produce ER–ToMV–MP–GUS lines. Kanamycin-resistant calli were placed on medium containing estradiol for induction and incubated for 3 days before GUS staining. Of the 376 lines tested, 224 lines were stained for GUS activity. No GUS activity was detected in lines not treated with estradiol, as shown in Figure 6A. Western blot analysis detected an induction-specific band corresponding to GUS (Figure 7B), indicating that an active GUS enzyme was inducibly produced in these cells. A comparison of purified GUS protein bands with a standard showed that the proportion of GUS protein in the total soluble protein of the induced cells was 0.01–0.02% (w/w; Figure 7B). Northern blot analysis of total RNA prepared from ER–ToMV–MP–GUS cell suspension cultures confirmed the estradiol-specific accumulation of ToMV–MP–GUS–RNA and subgenomic GUS mRNA (data not shown). The band intensities were significantly lower than those observed for ToMV–MP–GFP- and ToMV–MP–DHFR-expressing cell lines (data not shown), suggesting that the lower GUS production efficiency was due to lower ToMV–MP–GUS RNA accumulation.

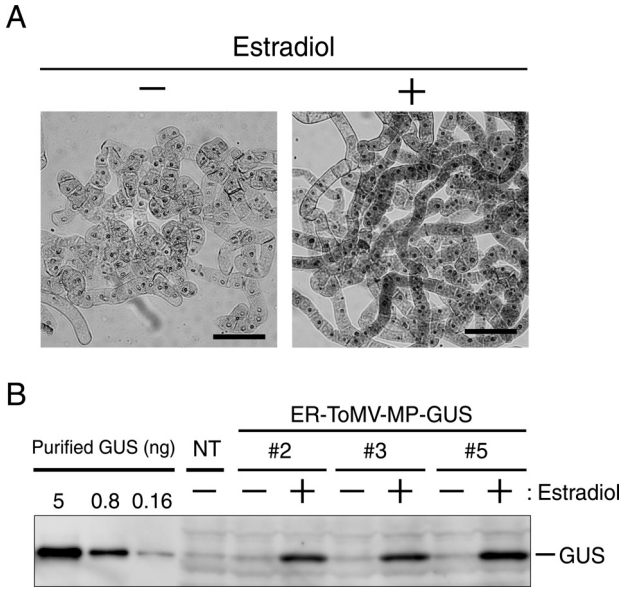


Figure 7. Inducible expression of recombinant GUS in ToMV–MP–GUS cells (3 days after estradiol treatment). (A) GUS staining in ToMV–MP–GUS cells with and without estradiol treatment (bars = 100 $\mu$m). (B) Western blot analysis of total soluble proteins (10 $\mu$g) to detect GUS protein. NT, non-transgenic.

## Discussion

DHFR and GUS activity was detected in the ER–ToMV–MP–DHFR and ER–ToMV–MP–GUS cell lines, respectively, in which viral vectors ToMV–MP–DHFR and ToMV–MP–GUS were inducibly expressed. In these cells, foreign protein expression was tightly suppressed in the absence of estradiol, demonstrating that the viral vector can inducibly produce these active enzymes.

DHFR accumulation in the ER–ToMV–MP–DHFR lines reached 15% of the total soluble intracellular proteins, which is comparable to GFP accumulation in our previous study (Dohi et al. 2006). This implies that the inducible virus vector system can be used to efficiently produce several different proteins in cultured plant cells. The efficiency and rapidity of foreign protein production reported here are among the highest recorded for foreign protein expression system in cultured plant cells (Hellwig et al. 2004). Although protein yield obviously depends on the protein in question, our results indicate that the inducible viral vector system is among the most promising methods for producing a foreign protein in cultured plant cells.

The rapid accumulation and high yields of DHFR confirm the merits of the inducible viral vector system for foreign protein production. Large-scale transgenic cell cultures are possible, as viral infection induction simply involves adding an inducer to the culture. It does not require inoculation or *Agrobacterium* treatment to achieve the transient infection of the viral vector. The DHFR yields that we achieved imply that several hundred grams of protein can be produced in $2\times10^4$ L cultures within several days of induction.

Although GUS activity was detected in the induced ER–ToMV–MP–GUS cells, the absolute concentration of GUS protein was significantly lower than that of DHFR in the ER–ToMV–MP–DHFR cell line or GFP in the ER–ToMV–MP–GFP cell line. The lower production of GUS was probably due to a lower accumulation of ToMV–MP–GUS RNA. Indeed, in BY-2 protoplasts, the accumulation of ToMV–MP–GUS genomic RNA, which was transcribed *in vitro* and inoculated into the protoplasts, was significantly lower than that of ToMV–MP–GFP RNA (Dohi and Mori, unpublished data). ToMV derivatives encoding large foreign proteins, such as GUS, may not be efficiently transcribed or replicated in the induced cells. Differences in the coding and flanking noncoding sequences of foreign protein genes could affect the infection efficiency of the virus.

Recently, various proteins, including bacterial antigens, have been produced efficiently in whole plants by using modified tobamovirus-based vectors that were transiently expressed from cDNA transfected by *Agrobacterium* infiltration (Marillonnet et al. 2005; Santi et al. 2006). These proteins also may be able to be efficiently produced via the inducible viral vector system in sterile-cultured transgenic plant cells.

BY-2 protoplasts have been used to reveal the intracellular localization of tobamovirus movement protein and intracellular trafficking mechanisms of the virus components (Más and Beachy, 2000). In ER–ToMV–MP–GFP lines, estradiol treatment specifically induced ToMV infection and expression of the movement protein. Large amounts of BY-2 cells expressing the movement protein, which could be obtained by simply adding estradiol to the culture, would be useful for basic research in elucidating the intracellular function of the movement protein, especially using biochemical approaches.

In conclusion, an inducible viral vector is one of the most effective options for efficiently producing foreign proteins, including active enzymes, in cultured plant cells.

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