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Establishment of a Conditional Transgenic System Using the 2A Peptide in the Female Mouse Germline

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Abstract. Transgenic mice are essential research tools in developmental biology studies. The 2A peptide allows multiple genes to be expressed simultaneously at comparable levels in somatic cells, but there are no reports of it being used successfully in germ cells. We constructed a Cre/loxP-based conditional vector containing the 2A peptide to significantly enhance the expression of a reporter and target gene from a constitutive promoter in oocytes. Mice with a transgene insertion containing the chicken β -actin promoter, floxed EGFP-polyA cassette, mCherry reporter, 2A peptide and target gene DNA methyltransferase 3A2 (*Dnmt3a2*) were crossed with TNAP- or Vasa-Cre mice to produce offspring, in which mCherry and DNMT3A2 proteins were highly expressed in oocytes upon Cre-mediated removal of EGFP-polyA. This novel transgenic mouse line based on the 2A expression system can serve as a useful tool for examining gene function during oogenesis.

Key words: Oocytes, Transgene, 2A peptide

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Transgenic (Tg) mice have been employed as a useful experimental tool for investigating gene function during development and pathogenesis of disease [1, 2]. In these mice, a gene of interest, dominant-negative form of a gene or gain-of-function of a gene, is expressed using an exogenous or intrinsic promoter. Cytomegalovirus (CMV) or chicken β -actin gene (CAG) promoters are universally used to induce ubiquitous and robust expression of genes. However, constitutive overexpression can cause early embryonic lethality in some instances. Intrinsic promoters are used to induce tissue- and stage-specific expression of a target gene. Since they are generally weaker than constitutive promoters, the expression levels of the gene of interest may not be sufficiently high to produce an observable phenotype.

In the Cre/loxP system, the introduction of Cre recombinase leads to excision of loxP-flanked DNA sequences inserted into the host genome. Mice carrying a transgene in which a floxed polyA cassette is inserted between a constitutive promoter and the gene of interest (i.e., floxed mice) can be crossed with mice expressing Cre from a tissue- and/or developmental stage-specific promoter, thereby generating conditional Tg offspring that highly express the gene of interest in a spatially and temporally restricted manner by Cre-mediated removal of the floxed polyA sequence. By combining transgene insertion with Cre/loxP technology, this conditional expression system can circumvent lethality and ensure reliable expression of the target gene at higher levels.

The endogenous expression patterns of a gene of interest can be visualized by adding a reporter gene coding sequence in frame,

resulting in the production of a target-reporter fusion protein. However, fusion proteins can be misfolded and thereby compromise protein function. Internal ribosome entry sites (IRES) are used for polycistronic expression of target and reporter genes. The IRES comprises a regulatory sequence in the 5' UTR of mRNA, from which translation can be independently initiated. Hence, two or more genes separated by IRES sequences can be translated and expressed as individual proteins [3]. However, the presence of IRES may attenuate the expression of downstream open reading frames (ORFs). As an alternative, 2A peptide can be inserted between different protein-coding sequences within a single ORF. The 2A peptide sequence consists of a consensus motif (Asp-Val/Ile-Glu-X-Asn-Pro-Gly-Pro), in which normal peptide bond formation between the Gly and Pro residues is impaired, causing ribosomal skipping during translation and subsequent cleavage of the translated polypeptide at this site (Fig. 1A). This generates distinct polypeptides that are then independently folded into their native structures [4, 5]. Therefore, unlike the IRES, the 2A peptide allows multiple genes to be expressed simultaneously at comparable levels in mammalian cells, thus providing greater flexibility for gain-of-function studies [6].

To date, several Tg or knock-in mice using the 2A peptide expression system have been reported [6–8]. However, these studies demonstrated the function of the peptide in somatic cells, and there have been no reports of this strategy being used successfully in germ cells. The aim of the present study was to establish advanced methods for transgene expression during oogenesis. To this end, conditional Tg mice expressing DNA methyltransferase 3A2 (*Dnmt3a2*) and fluorescent reporter mCherry separated by a 2A peptide in oocytes were generated and characterized.

To establish the Tg system in the female germline and to examine the effects of DNMT3A2 gain-of-function in oocytes, we used a combined Cre/loxP and 2A system. Although DNMT3A is required for establishing genomic imprinting in germ cells [9, 10], it is unclear

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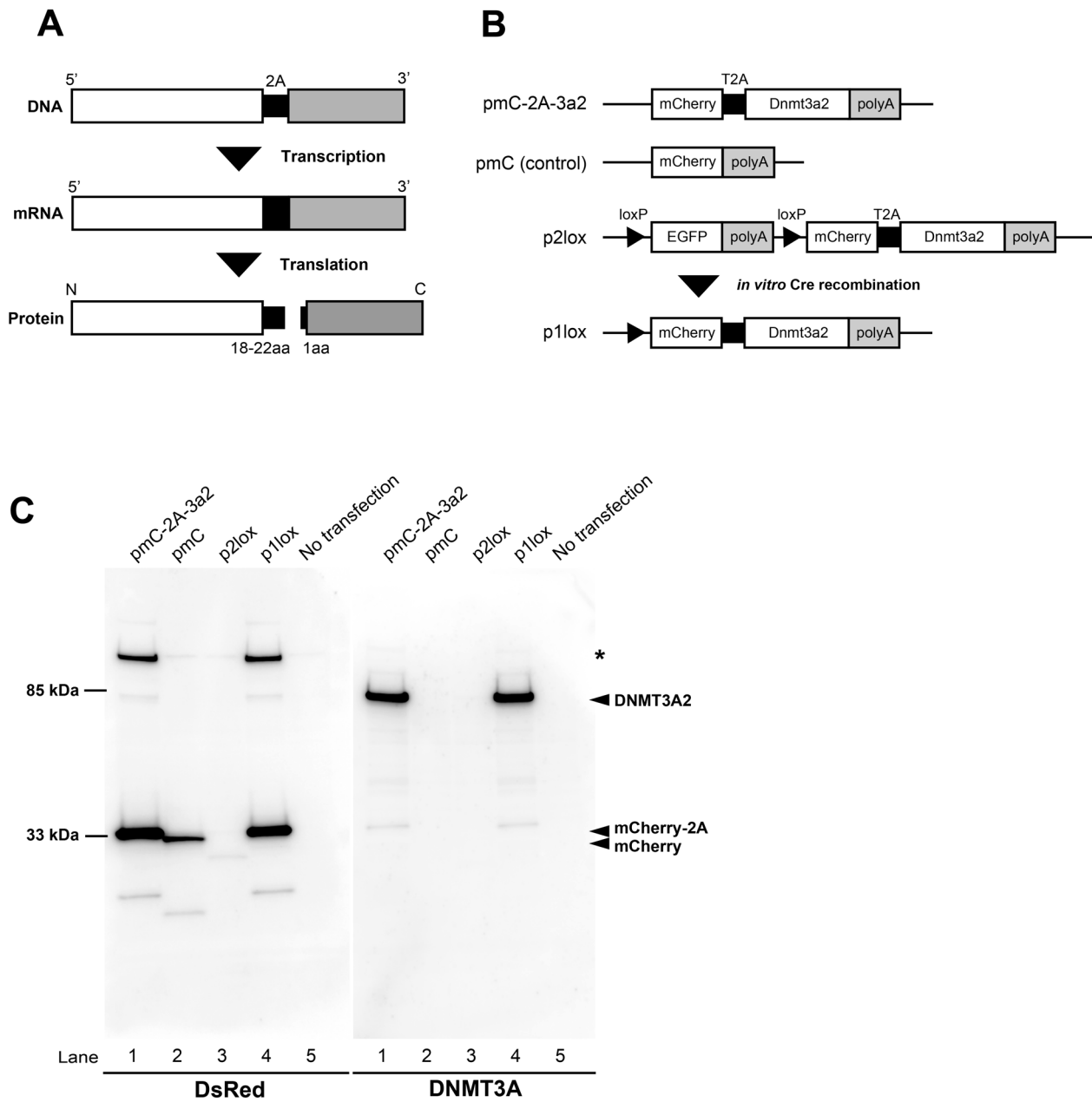


Fig. 1. Function of the 2A-based bicistronic vector in NIH3T3 cells. (A) Schematic representation of 2A peptide cleavage. Coding sequences for two genes of interest with an intervening 2A peptide sequence were transcribed as a single mRNA. During translation, the 2A peptide was cleaved into 20-aa and 1-aa portions, each of which was associated with the translated sequence of the genes. The translated polypeptides were independently folded into proteins. (B) Construction of vectors used in this study. The 2lox vector (p2lox) expressed EGFP from the CAG promoter but not downstream coding sequences. Like pmC-2A-3a2, the 1lox vector (p1lox), in which the EGFP cassette has been removed by *in vitro* Cre-mediated recombination, expresses mCherry and DNMT3A2. The control vector pmC expresses mCherry only. (C) Western blot of NIH3T3 cells transfected with pmC-2A-3a2 vector. The expression of mCherry (30 kDa), mCherry-2A (~33 kDa) and DNMT3A2 (~85 kDa) was detected (arrowheads). Signals that were detected with the anti-DsRed but not with anti-DNMT3A antibody (*) were likely to be nonspecific.

whether the presence of DNMT3A is sufficient for DNA methylation imprints. Two DNMT3A variants, DNMT3A1 and DNMT3A2, have been reported so far [11]. In this study, the shorter variant, DNMT3A2 was used. First, mCherry and DNMT3A2 coding sequences with an

intervening 2A sequence derived from the insect *Thosea asigna virus* (T2A) were inserted downstream of the CAG promoter (pmC-2A-3a2). A control vector was generated by inserting mCherry by itself into the same backbone (pmC) (Fig. 1B). The expression of these vectors

was verified by transfection into NIH3T3 cells. The mCherry (~33 kDa) and DNMT3A2 (~85 kDa) proteins were detected by Western blotting 48 h later. Since mCherry is a derivative of the fluorescent reporter DsRed [12], an anti-DsRed antibody was used to detect mCherry. As predicted, in cells transfected with pmC-2A-3a2, the molecular weight of mCherry was slightly higher than that in cells transfected with pmC owing to the presence of the N-terminal of T2A (20 amino acids). In addition, non-cleaved chimeric proteins double-labeled by antibodies against both DsRed and DNMT3A were not detected, suggesting that the gene products of pmC-2A-3a2 were efficiently cleaved in NIH3T3 cells (Fig. 1C).

Next, a floxed-EGFP-polyA cassette was inserted into pmC-2A-3a2 downstream of the CAG promoter (p2lox; Fig. 1B). The 1lox vector (p1lox) was produced from p2lox by *in vitro* Cre-mediated recombination. In Western blots of NIH3T3 cells transfected with p2lox or p1lox, mCherry and DNMT3A2 expression was detected in cells transfected with p1lox or pmC-2A-Dnmt3a2 but not in cells transfected with p2lox. Thus, the target protein DNMT3A2 was reliably expressed after recombination, which could be discerned based on the coexpression of mCherry instead of EGFP expressed in NIH3T3 cells (Fig. 1C).

To test these vectors *in vivo*, heterozygous Tg mice harboring linear p2lox DNA were crossed with heterozygous tissue-nonspecific alkaline phosphatase (TNAP)- or Vasa-Cre mice in order to produce double Tg (dTg) offspring carrying both 2lox and Cre alleles. The yield of both lines of dTg mice was at a normal Mendelian ratio (TNAP-Cre dTg, 17/63; Vasa-Cre dTg, 20/75). The TNAP promoter is not active until the early blastocyst stage but subsequently is activated exclusively in germ cells. Therefore, it is expected that TNAP drives Cre expression at an earlier stage of germ cell development from 9.5 days post coitum (dpc) [13, 14]. The Vasa, also known as Ddx4 or Mvh, promoter is active from 15.5 dpc until the meiotic stage, so Vasa-driven Cre is accumulated in ooplasm as well as maternal factors [15, 16]. It was predicted that oocytes derived from dTg mice would harbor a 1lox allele because of germ cell-specific expression of Cre and would therefore express only the mCherry reporter and not the EGFP reporter (Fig. 2A and B). To test this, nongrowing (ng) oocytes from the primordial ovarian follicles and fully grown (fg) oocytes from mature ovaries were obtained from dTg mice. Oocytes expressing mCherry only were observed, consistent with successful Cre-mediated EGFP-polyA removal (Fig. 2C). This mCherry expression was used as a measure of Cre efficiency, which

was defined as the number of mCherry-positive oocytes per total oocyte number. The Cre efficiencies in ng and fg oocytes from TNAP-Cre dTg (TNAP-dTg) mice were $72.5 \pm 19.7\%$ and $60.4 \pm 23.4\%$, respectively, compared with $88.3 \pm 3.8\%$ and $99.1 \pm 1.4\%$, respectively, in Vasa-Cre dTg (Vasa-dTg) mice (Table 1). These results indicated that Cre efficiency in 1lox oocytes was higher and less variable in Vasa-dTg mice than in TNAP-dTg mice. Unexpectedly, a subset of mCherry-positive oocytes was also EGFP-positive (Fig. 2C). This was probably due to the insertion of multiple copies of 2lox into the genome. Such double-positive oocytes were observed in 5% and 62% of ng oocytes derived from TNAP-dTg and Vasa-dTg mice, respectively (Fig. 2D). The observed differences in Cre efficiency using the two driver lines may be due to earlier activation of Cre recombinase in TNAP-dTg mice than in Vasa-dTg mice during embryogenesis. The specificity and the duration of Cre expression were associated with Cre efficiency. On the other hand, nearly all oocytes exclusively expressed mCherry in Vasa-dTg oocytes at the fg stage, with few EGFP/mCherry double-positive oocytes being observed (Fig. 2D). This finding suggests that sufficient accumulation of Cre in Vasa-dTg oocytes can ensure the recombination of nearly all inserted 2lox alleles.

To assess the function of the T2A sequence in oocytes, 1lox ovaries from 1 day postpartum mice (dpp) and 1lox oocytes from 20 dpp mice were analyzed and compared with ovaries or oocytes from 2lox littermates being used as controls. In agreement with the observations in NIH3T3 cells, Western blotting analysis showed a 14-fold higher expression of DNMT3A2 and mCherry protein in the ovary containing 1lox ng oocytes (Fig. 2E). Upregulation of DNMT3A1 was observed in ovaries and NIH3T3 cells harboring the 1lox allele; however, the cause for this was unclear in the present study. DNMT3A2 expression in 1lox oocytes was 1.3-fold greater than that in 2lox oocytes, suggesting that the upregulated DNMT3A2 was gradually masked by endogenous DNMT3A2 expression during oocyte growth [17, 18]. The protein size was comparable to endogenous DNMT3A2, indicating that non-cleaved proteins were not present (Fig. 2F). Based on these results, it was concluded that the T2A peptide is functional in female germ cells.

DNMT3A is a key enzyme responsible for the methylation of imprinted genes [9]. The effect of DNMT3A2 overexpression on genomic imprinting during early stage of oogenesis was assessed by analyzing the methylation status at imprinted loci in TNAP-dTg-derived 1lox ng oocytes. Maternally imprinted *Lit1* and paternally

Fig. 2. Establishment of germline-specific conditional Tg lines containing the 2A peptide. (A) Schematic representation of the conditional Tg allele. The 2lox vector (upper) expresses EGFP but not downstream sequences. In the 1lox allele, the floxed EGFP-polyA cassette is removed in the presence of Cre recombinase, with concomitant expression of mCherry and Dnmt3a2 (lower). Tg mice were generated by microinjection of the 2lox vector (7385 bp) linearized by double digestion with *Psp1406I* (P) and *BamHI* (B). (B) Schematic representation of the production of dTg mice. Female 2lox mice were crossed with TNAP- or Vasa-Cre males to generate dTg offspring carrying 2lox and Cre alleles. The Tg alleles in oocytes from dTg mice and 2lox littermates were 1lox (mCherry⁺/EGFP⁻) and 2lox (mCherry⁺/EGFP⁺), respectively. (C) Expression of the mCherry and EGFP reporters in ng oocytes from TNAP- and Vasa-dTg mice. (D) Percentage of 1lox ng and fg oocytes expressing mCherry and/or EGFP in dTg mice. The numbers above each graph indicate the total number of oocytes counted. (E) Western blot analysis of ovaries from 1lox and 2lox newborn pups. Protein samples were extracted from one ovary. NIH3T3 cells transfected with 1lox or 2lox vectors were loaded as positive and negative controls, respectively. Proteins of interest are indicated by black arrowheads. The ~135 kDa band (white arrowhead) is presumed to be DNMT3A1 expressed in ovarian somatic cells. Signals detected with anti-DsRed but not with anti-DNMT3A antibody (*) were considered as nonspecific. The ~140 kDa band (†) is also considered as nonspecific, since the molecular weight of non-cleaved mCherry/Dnmt3a2 protein is predicted to be 110–120 kDa. (F) Western blot of 1lox and 2lox growing oocytes; 150 oocytes were loaded in each lane. Signals detected with anti-DsRed but not with anti-DNMT3A antibody (*) were considered nonspecific. (G) DNA methylation status at *Lit1* and *H19* loci in 1lox and 2lox ng oocytes. Methylated and unmethylated CpG sites are represented by filled and open circles, respectively. Each line of the circles indicates one DNA strand.

Table 1. Cre efficiency in ng and fg oocytes from dTg mice

Oocytes	Promoter of Cre	Number of oocytes	
		Observed	mCherry-positive (average \pm standard deviation %) [†]
Nongrowing	TNAP	313	227 (72.5 \pm 19.7)
	Vasa	172	152 (88.3 \pm 3.8)
Fully grown	TNAP	43	26 (60.4 \pm 23.4)
	Vasa	109	108 (99.1 \pm 1.4)

[†]mCherry⁺/EGFP⁺ and mCherry⁺/EGFP⁻ oocytes were regarded as mCherry-positive oocytes.

imprinted *H19* loci remained hypomethylated in these oocytes, as was the case in control 2lox ng oocytes (Fig. 2G). This suggests that overexpression of DNMT3A2 is not sufficient to confer methylation marks at the imprinted loci. It is possible that other factors are required to recruit DNMT3A to the target region or to activate DNMT3A through association and/or modification [19–21].

In summary, these results demonstrate that a gene of interest (DNMT3A2) and reporter gene (mCherry) can be simultaneously expressed in female germ cells from a single transgene insertion using the 2A peptide. Such 2A peptide-based polycistronic Tg mice can be useful for live cell imaging of oocytes using multiple reporter genes and for other gain-of-function experiments. Additional studies are required to establish whether the overexpression of other epigenetic factors, or expression at earlier stages of oocyte development, would be sufficient to induce changes in the status of imprinted genes.

Methods

Vector construction and transfection in NIH3T3 cells

To construct the pmC-2A-3a2, mCherry and *Dnmt3a2* fragments were amplified from separate cDNA clones and ligated by recombinant PCR using the specific primers by adding the T2A sequence (listed in the Supplementary Table 1: on-line only) and KOD-FX DNA polymerase (Toyobo, Osaka, Japan; Supplementary Fig. 1: on-line only). In this fragment, a Kozak sequence was added to the 5' end of the sequence, and stop and start codons were removed from the mCherry and *Dnmt3a2* sequences, respectively. The 2lox vector was generated by inserting a floxed-EGFP-polyA cassette into the *EcoRI* site of the pCX vector [22]. The mCherry-T2A-*Dnmt3a2* fragment was inserted into this vector at the *EcoRV* site, downstream of the CAG promoter. To induce recombination *in vitro*, the 2lox vector was incubated with Cre recombinase (Takara Bio, Shiga, Japan) at 37 C for 20 min, which removed the floxed EGFP-polyA cassette and produced the 1lox vector.

NIH3T3 cells were cultured at 37 C and under 5% CO₂/95% air in Dulbecco's Modified Eagle's Medium (Invitrogen Life Technologies Japan, Tokyo, Japan) containing 10% fetal bovine serum. Vectors were transfected using Lipofectamine LTX (Invitrogen; Life Technologies Japan) in 1×10^5 cells. Cells were treated with 0.05% trypsin-0.53 mM EDTA and collected at 48 h after transfection.

Generation of conditional Tg mice

The 2lox vector was linearized by double digestion with *BamHI* and *Psp14061* (Takara Bio) and co-incubated with C57BL/6N freeze-thawed sperm in HTF medium. Tg mice carrying the 2lox

Tg allele (2lox mice) were generated by injection of the sperm into oocytes from C57BL/6N mice. The 2lox female mice were crossed with TNAP- or Vasa-Cre males to obtain Tg mice harboring 2lox and either of the Cre Tg alleles (dTg mice). The genotype of dTg mice was determined by PCR using primers specific to EGFP, *Dnmt3a2* and Cre. Primer sequences are listed in the Supplementary Table 1 (on-line only).

Oocyte collection and imaging

Ng and growing oocytes were collected from newborn (0–2 dpp) and 20 dpp female mice, respectively. Ovaries were treated with 0.1% collagenase in L-15 medium for 40 min and 0.05% trypsin/0.53 mM EDTA in PBS for 15 min at 37 C. Ng oocytes and ovarian somatic cells were suspended in M2 medium containing 5 μ g/ml cytochalasin B (Sigma-Aldrich Japan, Tokyo, Japan), and ng oocytes were isolated with a micromanipulator. Growing oocytes were further treated with 0.5% Pronase (Sigma-Aldrich Japan) in L-15 medium for 15 min at 37 C to remove the zona, and somatic cells were completely removed by pipetting.

Fg oocytes were obtained from the ovaries of 8- to 12-week-old adult mice at 44–46 h after injection of 5 IU equine chorionic gonadotropin (serotropin; ASKA Pharmaceutical, Tokyo, Japan). Cumulus cell-oocyte complexes were isolated, and cumulus cells were completely removed by pipetting. Fluorescence images of ng oocytes from dTg mice were obtained using a Zeiss LSM 710 laser confocal microscope (Carl Zeiss Japan, Tokyo, Japan).

Western blotting

Female 2lox mice were crossed with Vasa-Cre males, and their offspring were used as ovarian and oocyte donors. All samples were washed in PBS and lysed in sample buffer (0.25 M Tris-HCl, pH 6.8; 40% glycerol; 0.8% SDS; and 1% β -mercaptoethanol). SDS-PAGE was performed using 5–20% gradient gels (ATTO, Tokyo, Japan), and samples were transferred to PVDF membranes. Blocking and immunoreactions were performed using a Can Get Signal Immunoreaction Kit (Toyobo) with the following antibodies: anti-DsRed (1:2,000; Takara Bio, Otsu, Shiga, Japan), anti-DNMT3a (IMG-268A, 1:2,000; Imgenex, San Diego, CA, USA), anti-GAPDH (1:2,000; Trevigen, Gaithersburg, MD, USA) and anti- α Tubulin (1:2,000, Sigma-Aldrich Japan). The expression level of DNMT3A2 was normalized using GAPDH or α Tubulin expression.

DNA methylation analysis

For DNA methylation analysis, 400–500 ng oocytes were pooled and incubated in 18 μ l lysis buffer containing 1% SDS, 5 μ g proteinase

K and 2 μg *Escherichia coli* tRNA for 1 h at 37 C and treated with an EpiTect Bisulfite Kit (Qiagen, Tokyo, Japan) for sodium bisulfite conversion. Bisulfite-treated DNA was subjected to nested PCR using published primer sequences [23]. PCR products were cloned into the pGEM-T Easy vector (Promega, Tokyo, Japan), and sequencing was performed on an ABI Prism 3100 sequencing system (Invitrogen, Life Technologies Japan).

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