Appl Microbiol Biotechnol (2009) 84:323–333
DOI 10.1007/s00253-009-1967-y

Production of double-stranded RNA for interference with TMV infection utilizing a bacterial prokaryotic expression system

Guohua Yin · Zhaonan Sun · Nan Liu · Lin Zhang · Yunzhi Song · Changxiang Zhu · Fujiang Wen

Received: 15 October 2008 / Revised: 11 February 2009 / Accepted: 14 March 2009 / Published online: 28 March 2009
© Springer-Verlag 2009

Abstract In many species, the introduction of double-stranded RNA (dsRNA) induces potent and specific gene silencing, a phenomenon called RNA interference (RNAi). RNAi is the process of sequence-specific, posttranscriptional gene silencing (PTGS) in animals and plants, mediated by dsRNA homologous to the silenced genes. In plants, PTGS is part of a defense mechanism against virus infection, and dsRNA is the pivotal factor that induces gene silencing. Here, we report an efficient method that can produce dsRNA using a bacterial prokaryotic expression system. Using the bacteriophage λ-dependent Red recombination system, we knocked out the *rnc* genes of two different *Escherichia coli* strains and constructed three different vectors that could produce dsRNAs. This work explores the best vector/host combinations for high output of dsRNA. In the end, we found that strain M-JM109 or the M-JM109lacY mutant strain and the vector pGEM-CP480 are the best choices for producing great quantities of dsRNA. Resistance analyses and Northern blot showed that Tobacco mosaic virus infection could be inhibited by dsRNA, and the resistance was an RNA-mediated virus resistance. Our findings indicate that exogenous dsRNA could form the basis for an effective and environmentally friendly biotechnological tool that protects plants from virus infections.

G. Yin
College of Plant Protection, State Key Laboratory of Crop Biology, Shandong Agricultural University,
Tai'an, Shandong 271018, China

Z. Sun · N. Liu · L. Zhang · Y. Song · C. Zhu (✉) · F. Wen (✉)
College of Life Sciences, State Key Laboratory of Crop Biology, Shandong Agricultural University,
Tai'an, Shandong 271018, China
e-mail: zhchx@sdau.edu.cn
e-mail: fjwen@sdau.edu.cn

Keywords Double-stranded RNA · RNase III · TMV · RNA-mediated virus resistance

Introduction

The emergence of RNA interference (RNAi) as a central eukaryotic posttranscriptional regulatory mechanism has sparked continuous interest in the nucleases (ribonuclease III, RNase III) that both recognize and cleave double-stranded RNA (dsRNA). RNase III (transcribed and translated by the *rnc* gene in *Escherichia coli*) enzymes occur ubiquitously in different organisms. They have now been shown to occupy a central position in mediating dsRNA-dependent processes, including RNA maturation, RNA decay, gene silencing (responsible for generating microRNAs or short interfering RNA), and a range of other cellular activities (Apirion and Gitelman 1980; Dunn and Studier 1973; Elela et al. 1996; Fire et al. 1998; Krautz-Peterson and Skelly 2008, Zhang et al. 2004). The isolation of a point mutation in the chromosomal gene (*rnc105*) that abolished its enzyme activity (Kindler et al. 1973) revealed that the *rnc* gene is not an essential gene and can be knocked out.

With the development of molecular biology, an efficient bacteriophage λ-dependent Red recombination system has been developed for genetic engineering. Red recombination is used to substitute the exogenous linear polymerase chain reaction (PCR) fragments for the target gene in a chromosome by integrating a defective phage λ into the *E. coli* chromosome or a plasmid. The Red recombination system includes three genes: *bet*, *exo*, and *gam*, and their products are Bet, Exo, and Gam, respectively (Datsenko and Wanner 2000). These Red proteins enable the occurrence of recombination events between DNA species with as little as 40–60 bp homologous sequences (Baba et al.

2006; Yu et al. 2000; Datsenko and Wanner 2000; Poteete and Fenton 2000).

Studies show that complete cleavage of dsRNA by *E. coli* RNase III produces 12–15 bp duplex products on average (Amarasinghe et al. 2001), and these short dsRNAs could not induce gene silencing. Actually, in fungi, plants, and most invertebrates, longer dsRNA could be more effective in inducing RNA silencing and in the regulation of gene expression, in addition to its role as a tool in functional genomics analysis (Dykxhoorn et al. 2003; Meister and Tuschl 2004). Current approaches to producing long dsRNA rely on annealing sense and antisense single-stranded RNA strands. However, this may produce poor-quality dsRNA. The *E. coli* strain HT115 has a mutation in the *rnc* gene and could be used to produce long dsRNA for RNA-mediated virus resistance and studies in gene function (Tenllado and Díaz-Ruíz 2001; Tenllado et al. 2003, 2004; Timmons et al. 2001).

Numerous studies have reported protecting plants from virus infection by using dsRNA or hairpinRNA (hpRNA) derived from sequences of plant virus species (Liu et al. 2007; Smith et al. 2000; Waterhouse et al. 1998; Zhu et al. 2008). In 2001, Tenllado and his colleagues discovered that direct transformation of nontransgenic plant species with dsRNA produced by an in vitro expression system could efficiently interfere with virus infection. Thereafter, Tenllado et al. (2003) developed an in vivo expression system to produce large amounts of virus-derived dsRNAs in bacteria. The results showed that delivering dsRNA by a combination of lysing cells with the French Press onto plant surfaces could efficiently prevent plants from virus infections. In the antiviral response, the delivered dsRNA, transgenes, or endogenous genes are first processed by an RNase III-like nuclease, called Dicer in *Arabidopsis*, into 21–23 nt small interfering RNAs (siRNAs). Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). The siRNA strands are then unwound to form activated RISCs and subsequently guide the RISCs to complementary RNA molecules and destroy the homologous target mRNAs (Hamilton and Baulcombe 1999; Hammond et al. 2000; Zamore et al. 2000). DsRNAs or self-complementary hairpin RNAs produced during the intermediate steps of genome replication were shown to be key triggers of RNA silencing (Bass 2000; Elbashir et al. 2000; Hamilton and Baulcombe 1999; Molnár et al. 2005; Vogler et al. 2007). In this paper, we constructed different prokaryotic bacterial expression systems to produce dsRNA derived from the Tobacco mosaic virus (TMV) coat protein (CP) gene. This paper aims to discover the best vector/host combinations for the highest output of dsRNA and explore the resistance of exogenous dsRNA or hpRNA for protecting the plant from virus infections.

Materials and methods

Bacterial strains, plasmids, media, chemicals, and other reagents

Table 1 lists bacterial strains and plasmids used in this work. Glucose, L-arabinose, *Taq* DNA polymerase, agarose, and other chemicals came from TaKaRa (Dalian, China). The *Dpn*I was from New England Biolabs (Ipswich, MA, USA); KOD-plus DNA polymerase was from Toyobo (Tokyo, Japan). Luria–Bertani, Super Optimal Broth, and Super Optimal Broth with catabolite repression media were prepared as described elsewhere (Sambrook et al. 1989). *Taq* DNA polymerase was used in all PCR tests. KOD-plus DNA polymerase was used to generate DNA for cloning and mutagenesis. Tiangen products (Tiangen Biotech, Beijing, China) were used to isolate plasmid DNAs, gel-purify fragments, or purify PCR products. The concentration and purity of all PCR products, extracted dsRNA, and DNA were determined with a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Construction of different vectors and amplifying the targeting fragments

Table 2 lists all primers used in this study. The TMV CP gene was amplified by real-time (RT)-PCR from extracted TMV mRNA using primers TMVCPII-5 and TMVCPII-3 and subcloned into the *Pst*I and *Sal*I sites of vector pUC19 to give pUC19CP (Fig. 1). The subcloned fragment consisted of a 480-bp cDNA presenting the entire coding region of the TMV CP gene and a 120-bp spacer (often called "loop" in hpRNA) representing sequences of the bacterial glucoronidase gene (Chen et al. 2003). Plasmid pUC19CP was used as a template for the construction of three different vectors (LCP480, pGEM-CP480, and pET-CP480) for the production of dsRNA in vivo after transformation into different *E. coli* strains (Fig. 1). We constructed vector LCP480 by inserting a 480-bp TMV CP gene digested with *Pst*I and *Sal*I into the multicloning sites of plasmid L4440 digested with the same restriction endonuclease sites. We constructed vectors pGEM-CP480 and pET-CP480 with inverted repeat of the 480-bp TMV CP gene and separated the two DNA units by the spacer sequence (a 120-bp glucuronidase gene).

The primers RNaseIII50-5 and RNaseIII50-3 and LacY-5 and LacY-3 were used for amplifying the chloramphenicol resistance gene and kanamycin resistance gene with 50-bp homologous sequences with the *rnc* and *Lac*Y genes, respectively. The targeting PCR fragments with cat^R or kan^R were generated independently and were amplified in the Eppendorf Mastercycler (Eppendorf, Germany) accord-

Table 1 *E. coli* strains and plasmids used in this study

Strains or plasmids	Genotype/properties/specific use	Resistance genes	Source/reference
Strains			
HT115	F-mcrA mcrB IN (rrnD-rrnE) 1, rnc14::Tn10(DE$_3$ lysogen:lacUV5 promoter-T7 polymerase)	*tet*^{R}	Dasgupta et al. 1998; Takiff et al. 1989
JM109(DE3)	*end*A1, *rec*A1, *gyr*A96, *thi, hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, λ-, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15], lDE3	–	Promega, USA
HMS174 (DE3) PLysS	F$^-$ *recA1 hsdR*(r_{K12}^- m_{K12}^+) (DE3) PLysS	*rif*^{R}, *cat*^{R}	Novagen, Germany
M-JM109	the *rnc* mutant strain of JM109(DE3)	*cat*^{R}	This study
M-HMS174	the *rnc* mutant strain of HMS174 (DE3)PLysS	*rif*^{R}, *cat*^{R}, *kan*^{R}	This study
M-JM109LacY	the *rnc* and *Lac*Y mutant strain of JM109(DE3)	*kan*^{R}	This study
Plasmids/vectors			
pKD3	used to obtain the chloramphenicol resistance gene with 50-bp homologous sequences with the *rnc* gene	*amp*^{R}, *cat*^{R}	Datsenko and Wanner 2000, AY048742
pKD4	used to obtain the kanamycin resistance gene with 50-bp homologous sequences with the *rnc* or *Lac*Y gene	*amp*^{R}, *kan*^{R}	Datsenko and Wanner 2000, AY048743
pKD46	used for the expression of Red recombinases (Bet, Exo, and Gam)	*amp*^{R}	Datsenko and Wanner 2000, AY048746
pBI121	used as a template for amplifying a 120-bp glucuronidase gene	*amp*^{R}	Chen et al. 2003, AF485783
pUC19	used for the cloning of TMV CP gene	*amp*^{R}	Yanisch-Perron et al. 1985
pUC19CP	used as a template for the construction of LCP480, pET-CP480, and pGEM-CP480	*amp*^{R}	This study, DQ014551
pCP20	used for the elimination of antibiotic resistance-marker gene	*cat*^{R}, *amp*^{R}	Datsenko and Wanner 2000
L4440	used for the construction of LCP480	*amp*^{R}	Timmons and Fire 1998
pGEM®-T Easy	cyclized at the *Eco*RI site and used for the construction of pGEM-CP480	*amp*^{R}	Promega, USA
pET-22b (+)	used for the construction of pET-CP480	*amp*^{R}	Novagen, Germany
LCP480	used for dsRNA production	*amp*^{R}	This study
pGEM-CP480	used for dsRNA production	*amp*^{R}	This study
pET-CP480	used for dsRNA production	*amp*^{R}	This study
pEASY-T1	used to clone the recombined DNA fragments and sequence analysis	*amp*^{R}	TransGen Biotech, China

tet^R tetracycline resistance gene, *rif*^{R} rifampicin resitance gene, *cat*^{R} chloramphenicol resistance gene, *amp*^{R} ampicillin resistance gene, *kan*^{R} kanamycin resistance gene

ing to the protocols suggested by KOD-plus instruction (Toyobo). The PCR products were digested with *Dpn*I, ethanol precipitated, and resuspended in the appropriate TE buffer (10 mmol/L Tris–HCl, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), pH 7.5). Afterwards, the PCR products were size-fractionated on a 1% agarose gel in Tris–acetate–EDTA buffer. The corresponding band was excised and purified with TIANgel Midi Purification Kit (Tiangen Biotech) according to the manufacturer's protocol, except that in the last step, we suspended the amplified linear DNAs in sterile water.

Gene knock-out, mutant selection, elimination of pKD46, and the resistant marker gene

JM109 (DE3) and HMS174 (DE3) PLysS were transformed with plasmid pKD46 according to the method suggested by Sambrook et al. (1989). Knock-out of the *rnc* and *Lac*Y genes, mutant selection, and elimination of pKD46 and the antibiotic-resistant marker gene were performed according to the protocols suggested by Datsenko and Wanner (2000) with small modifications. The primers (RNaseIII50-5 and RNaseIII50-3; LacY-5 and LacY-3) and plasmids (pKD3 or pKD4) were used to knock out the *rnc* and *Lac*Y genes, respectively. Colony-direct PCR (CD-PCR) was used to show that all mutants had the correct structures.

Dot-blot hybridization and sequence analysis for knock-out of the *rnc* and *Lac*Y genes

Genomic DNA of *E. coli* was extracted using the Biospin Bacteria Genomic DNA Extraction Kit (Bioer Technology, Hangzhou, China) according to the instructions of the supplier. The *E. coli rnc* and *lac*Y mutation strains were

Table 2 The primers used for gene knock-out, mutantscreening, and vector construction

Primer	Primer sequence (5′→3′)[a]	Specific use
RNaseIII50-5	ATGAACCCCATCGTAATTAATCGGCTTCAACGGAAGCTGGGCTACACTTT*AGCGATTGTGTAGGCTGGAG*	Amplification of the *rnc* gene
RNaseIII50-3	CTGATCGTGCGCTTCGCCACGTACCTGGACTACCAGATAAGTCGGCAGCG*TTAACGGCTGACATGGGAATTAG*	Amplification of the *rnc* gene
LacY-5	GCCGCTATTTCTCTGTTCTCGCTATTATTCCAACCGCTGTTTGGTCTGCT*AGCGATTGTGTAGGCTGGAGCT*	Amplification of the *Lac*Y gene
LacY-3	ACCCAGCACCAGATAAGCGCCCTGGAAACCGATGCTTTCATACATATTGC*TTAACGGCTGACATGGGAATTAGC*	Amplification of the *Lac*Y gene
Cat-5	AGCGATTGTGTAGGCTGGAGCT	Mutant screening
Cat-3	TTAACGGCTGACATGGGAATTAGC	Mutant screening
JD-5	ACCGGTAAACTGAAACTGCA	Mutant screening
JD-3	TGGAGATTTTCTGCCCCAG	Mutant screening
LacY-JD5	GGGTAAACTGGCTCGGAT	Mutant screening
LacY-JD3	CGACTTCATTCACCTGACG	Mutant screening
RNC5	TAATCATCAGGAACTGTTGCAG	Preparation of the *rnc* gene probe
RNC3	GCAGATGGCGACCCTGCAAAT	Preparation of the *rnc* gene probe
LACY-5	TTCTGACAAACTCGGGCTGCG	Preparation of the *lac*Y gene probe
LACY-3	CCGCCAGTACAGACATAAAAATC	Preparation of the *lac*Y gene probe
CPI-5	GCGC**CTCGAG**ATGTCTTACAGTATCACTAC	Construction of pET-CP480
CPI-3	GCGC**AAGCTT**TCAAGTTGCAGGACCAGAG	Construction of pET-CP480
CPII-5	GCGC**GTCGAC**TCAAGTTGCAGGACCAGAG	Construction of pET-CP480
CPII-3	GCGC**GAATTC**ATGTCTTACAGTATCACTAC	Construction of pET-CP480
GUSI-5	GCGC**AAGCTT**GCAGATGACAGTGGCATCG	Construction of pET-CP480
GUSI-3	GCGC**GTCGAC**GACTGCCTCTTCGCTGTAC	Construction of pET-CP480
TMVCPI-5	GC**GGGCCC**ATGTCTTACAGTATCACTAC	Construction of pGEM-CP480
TMVCPI-3	GCGC**GAATTC**TCAAGTTGCAGGACCAGAG	Construction of pGEM-CP480
TMVCPII-5	GCGC **CTGCAG**TCAAGTTGCAGGACCAGAG	Construction of pGEM-CP480
TMVCPII-3	GCGC **GTCGAC**ATGTCTTACAGTATCACTAC	Construction of pGEM-CP480
GUSII-5	GCGC**GAATTC**GCAGATGACAGTGGCATCG	Construction of pGEM-CP480
GUSII-3	GCGC **CTGCAG**GACTGCCTCTTCGCTGTAC	Construction of pGEM-CP480
M13F-47	CGCCAGGGTTTTCCCAGTCACGAC	Identification of pGEM-CP480
M13R-48	AGCGGATAACAATTTCACACAGGA	Identification of pGEM-CP480
CP-F	ATTAGACCCGCTAGTCACAGCAC	Amplification TMV CP gene
CR-R	GTGGGGTTCGCCTGATTTT	Amplification TMV CP gene
16S-F	CATGCCGCGTGTATGAAGAA	Amplification *E. coli* 16 S rRNA
16S-R	CGGGTAACGTCAATGAGCAAA	Amplification *E. coli* 16 S rRNA

[a] The underlined sequences of primers were the homologous sequences to the target genes, the italic sequences were used for amplifying the resistance-marker genes. The bold-faced sequences are different restriction endonuclease sites. Primers GUSI-5 and GUSI-3, GUSII-5 and GUSII-3 were used to amplify the glucuronidase gene of plasmid pBI121.

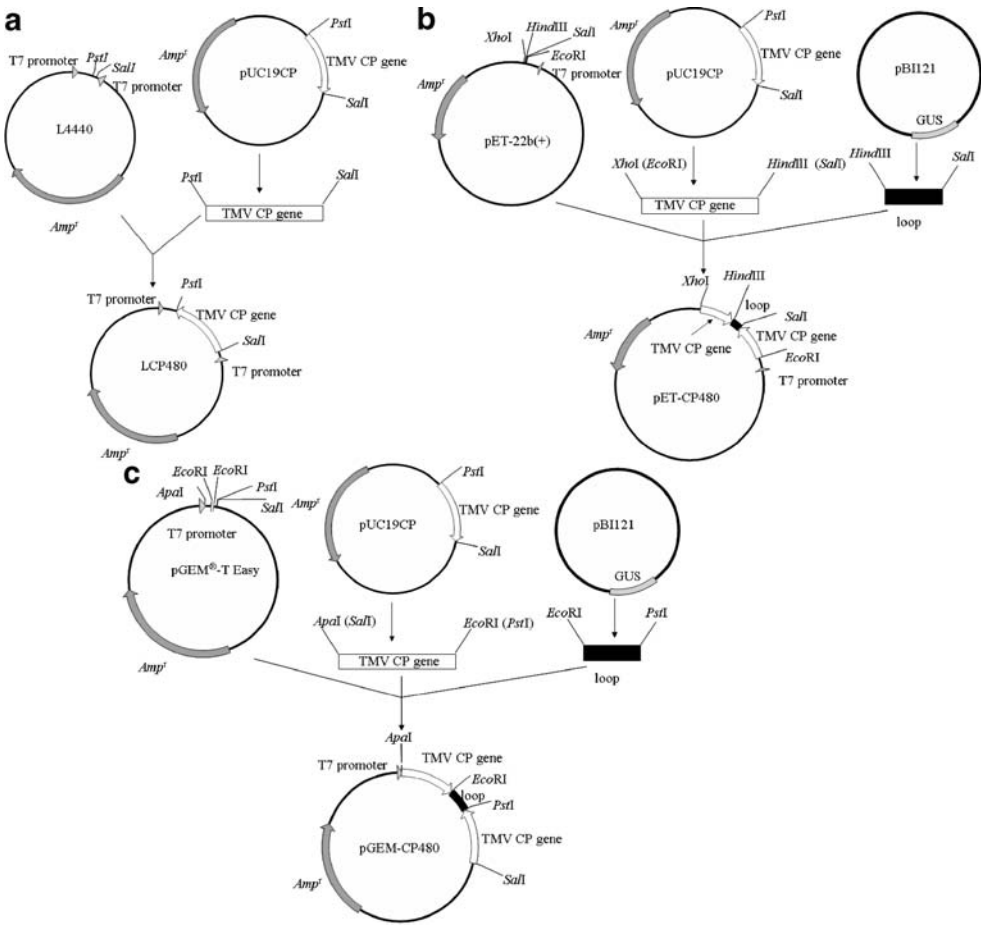

Fig. 1 Map of three different vectors. Schematic representation of three different vectors carrying the TMV CP cDNA sequence: **a** LCP480, two T7 promoters; **b** pET-CP480, one T7 promoter, inverted-repeat TMV cDNA sequence; and **c** pGEM-CP480, one T7 promoter, inverted-repeat TMV cDNA sequence

further verified by dot-blot hybridization using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany) according to the instructions of the supplier. Dot-blot hybridization conditions and detection were performed according to Sambrook et al. (1989). The recombination fragments of different mutations were cloned into the vector pEASY-T1 and sequenced by TaKaRa for further verification. The correct mutant strains have been deposited at China General Microbiological Culture Collection Center (CGMCC). The collection numbers for M-JM109, M-JM109lacY, and M-HMS174 are CGMCC 1.8744, CGMCC 1.8745, and CGMCC 1.8746, respectively.

Induction and extraction of dsRNA

The induction of dsRNA from different prokaryotic bacterial expression systems was performed according to Tenllado et al. (2003) with small modifications. TRI_{ZOL} Reagent (Invitrogen, USA) was used for the isolation of total RNA from bacteria as described in the instructions in the supplier's instructions. The nucleic acid pellet obtained from 1 mL culture of *E. coli* cells was resuspended in 20 μL TE buffer and used for 2% agarose gel electrophoresis. Accumulation of dsRNA in bacterial extracts and its purity were confirmed by treatment with RNaseA under high salt conditions (Tenllado et al. 2003).

Quantitative real-time PCR and resistance analysis of extracted dsRNA transcripts

One-step real-time RT-PCR of the dsRNA transcripts were carried out in a Bio-Rad iQ5 system using a Quant One Step RT-PCR (SYBR Green I) Kit (Tiangen Biotech) according to the supplier's instructions with small modifications. All samples were run in triplicate, and relative amounts of dsRNA were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). One-way analysis of variance (ANOVA) analysis and Duncan's multiple-range tests were performed with SAS 8.2 software (SAS Institute, Cary, NC, USA).

We performed resistance tests following methods proposed by Tenllado et al. (2003). The tested tobacco was kept in growth chambers with a 16-h light and an 8-h dark cycle at 25°C. We observed the development of TMV-infection symptoms (first, a light-green coloration between the veins of young leaves followed by a mosaic on the tobacco leaves) for as long as their life cycle (2 months post-inoculation). The content of the TMV virus in tested

plants was detected by indirect enzyme-linked immunosorbent assay (ELISA), according to Liu et al. (2007).

Small RNA fraction extraction and Northern blot

We extracted small RNA fractions of the tested tobacco using PureLink™ miRNA Isolation Kit (Invitrogen) according to the instructions of the supplier. Electrophoresis, blotting, and EDC cross-linking of small RNA fractions were carried out sequentially according to Pall et al. (2007). Probe synthesis, siRNA hybridization, and chemiluminescent detection (DIG Northern Starter Kit, Roche) were performed according to Goto et al. (2003).

Results

Knock-out of the *rnc* and *Lac*Y genes

Plasmid pKD46 was transformed into JM109 (DE3) and HMS174 (DE3) PLysS, respectively. Knock-out mutants of strains JM109 (DE3) and HMS174 (DE3) PLysS with a precise deletion of a 458-nt segment in the *rnc* gene were produced as described earlier in the "Materials and methods" section. Representative kanamycin-resistant and chloramphenicol-resistant transformants were characterized after transformation of PCR-fusion products of the cat^R gene of pKD3 and *rnc* sequences and of PCR-fusion products of the kan^R gene of pKD4 and *rnc* sequences into the different *E. coli* hosts JM109 (DE3) and HMS174 (DE3), respectively. Both carried the pKD46 plasmid for Red-mediated recombination. PCR tests using locus-specific primers and *cat*- or *kan*-specific primers (Table 2) revealed that all mutants from the strains had new junctions and locus-specific fragments of the expected size (Fig. 2). For convenience, we named the *rnc* gene mutants of these strains M-JM109 and M-HMS174, respectively.

The lac permease (*lac*Y) mutation (Tuner™ strains) allows uniform entry of isopropyl-β-D-thio-galactoside (IPTG) into all cells in the population, which produces a concentration-dependent, homogeneous level of induction (Minea et al. 2005). In response to this, we knocked out the *Lac*Y gene of strain M-JM109, which produces more dsRNA than other *E. coli* strains (Figs. 3 and 4). To avoid using more antibiotics, we eliminated the resistant-marker gene with plasmid pCP20. On elimination of the chloramphenicol resistance-marker gene, resulting sensitive clones in PCR tests using locus- and cat^R-specific primers (Table 2) produced new fragments, as expected from the loss of the cat^R gene. Subsequently, we used PCR-fusion-products of the kan^R gene of pKD4 and of *Lac*Y sequences (for details, see the "Materials and methods" section) in transformation of one of the chloramphenicol-sensitive descendants of mutant M-JM109 in order to knock out precisely a 911-nt segment in the *Lac*Y (Fig. 2) gene. For convenience, we gave the *Lac*Y gene mutant of M-JM109 the name M-JM109lacY. We further verified accuracy of all mutations by dot-blot hybridization analysis and sequence analysis (data not shown).

Production of dsRNA by different *E. coli* strains defective for RNase III

For comparison, different prokaryotic expression systems were designed to express dsRNA or hpRNA containing TMV CP cDNA sequence under the control of the T7 promoter. To simplify the descriptions of the bacterial strains used in this paper, we used the following terminology: HT115 is an RNase III-mutant bacterial host harboring a λDE3 lysogen (source of T7 polymerase), and HT115/LCP480, HT115/pGEM-CP480, and HT115/pET-CP480 are lysogenic strains harboring the plasmids LCP480, pGEM-CP480, and pET-CP480, respectively. M-JM109/LCP480, M-JM109/pGEM-CP480, M-JM109/pET-CP480, M-HMS174/LCP480, M-HMS174/pGEM-CP480, M-HMS174/pET-CP480, M-JM109lacY/LCP480, M-JM109lacY/pGEM-CP480, and M-JM109lacY/pET-CP480 are named analogously to the plasmid-harboring HT115 derivatives. Inducing the T7 promoter with IPTG, we found that the HT115, M-JM109, M-HMS174, and M-JM109lacY strains accumulated significantly higher levels of dsRNA, while the accumulation of dsRNA by the RNase III-expressing strains was not detectable by gel electrophoresis (Fig. 3).

As shown by the agarose gel in Fig. 3, there is a clear band about 660-bp extracted from strains HT115/LCP480, M-HMS174/LCP480, M-JM109/LCP480, and M-JM109lacY/LCP480, respectively. LCP480 has two convergent T7 polymerase promoters in opposite orientation, and both can be induced by IPTG. LCP480 therefore produces (+) and (−) ssRNA, which then leads to the formation of dsRNAs inside bacterial cells. Because the transcribed dsRNA contains (next to the TMV CP gene sequence) a 180-bp sequence with the multicloning region of plasmid L4440, the dsRNA produced by plasmid LCP480 is 660-bp dsRNA long. There are two obvious dsRNA bands extracted from strains containing either pGEM-CP480 or pET-CP480. The reason for this phenomenon is a 120-bp hairpin loop in the self-complementary RNAs of pGEM-CP480 and pET-CP480, respectively. RNAs with a hairpin loop move slowly in agarose gels compared to dsRNA without a loop region. Thus, the upper band in Fig. 3 localizes at a place corresponding to a sequence size of 550-bp dsRNA, while the other band was found below the regular position of 500-bp dsRNA likely because a 480-bp dsRNA without a loop was formed due to degradation of the loop by RNaseA.

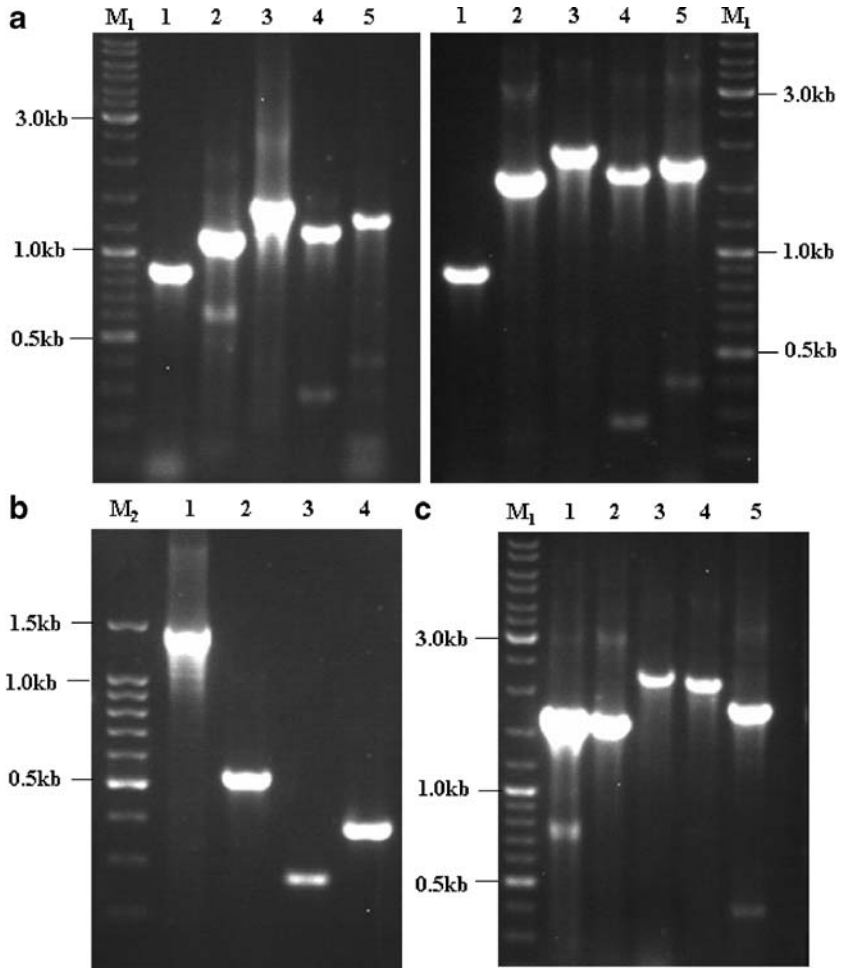

Fig. 2 PCR analysis to verify the successful construction of *E. coli* mutants. PCR analysis to verify mutants M-JM109 and M-HMS174 (**a**, *left* and *right*, respectively), the elimination of the chloramphenicol marker gene in M-JM109 (**b**) and in the M-JM109lacY mutant strain (**c**). M_1 Gene Ruler™ DNA Ladder Mix, M_2 100 bp DNA-Ladder Marker. **a** *Left*, *lane 1* PCR performed on wild-type JM109 (DE3) strain with the primers JD-5 and JD-3, expected fragment size 879 bp; *lane 2* the targeting linear DNA fragment performed on pKD3 with the primers RNaseIII50-5 and RNaseIII50-3, expected fragment size 1,152 bp; *lane 3* PCR performed on M-JM109 with the primers JD-5 and JD-3, expected fragment size 1,473 bp; *lane 4* PCR performed on M-JM109 with the primers JD-5 and Cat-3, expected fragment size 1,201 bp; *lane 5* PCR performed on M-JM109 with the primers Cat-5 and JD-3, expected fragment size 1,322 bp. **a** *Right*, *lane 1* PCR performed on wild-type HMS174 (DE3) PlysS strain with primers JD-5 and JD-3, expected fragment size 879 bp; *lane 2* the targeting linear DNA fragment performed on pKD4 with the primers RNaseIII50-5 and RNaseIII50-3, expected fragment size 1,615 bp; *lane 3* PCR performed on M-HMS174 with the primers JD-5 and JD-3, expected fragment size 1,936 bp; *lane 4* PCR performed on M-HMS174 with the primers JD-5 and Cat-3, expected fragment size 1,664 bp; *lane 5* PCR performed on M-HMS174 with the primers Cat-5 and JD-3, expected fragment size 1,787 bp. **b** *Lane 1* PCR performed on M-JM109 with the primers JD-5 and JD-3, expected fragment size 1,473 bp; *lane 2* PCR performed on chloramphenicol eliminated mutant with the primers JD-5 and JD-3, expected fragment size 543 bp; *lane 3* PCR performed on chloramphenicol eliminated mutant with the primers JD-5 and Cat-3, expected fragment size 271 bp; *lane 4* PCR performed on chloramphenicol eliminated mutant with the primers Cat-5 and JD-3, expected fragment size 394 bp. **c** *lane 1* PCR performed on M-JM109 with primers LacY-JD5 and LacY-JD3, expected fragment size 1,692 bp; *lane 2* the targeting linear DNA fragment PCR performed on pKD4 with the primers LacY-5 and LacY-3, expected fragment size 1,615 bp; *lane 3* PCR performed on M-JM109lacY with the primers LacY-JD5 and LacY-JD3, expected fragment size 2,296 bp; *lane 4* PCR performed on M-JM109lacY with the primers LacY-JD5 and Cat-3, expected fragment size 2,155 bp; *lane 5* PCR performed on M-JM109lacY with the primers Cat-5 and LacY-JD3, expected fragment size 1,656 bp

Quantitative real-time PCR of dsRNA

To compare dsRNA produced by different dsRNA prokaryotic expression systems, we performed quantitative real-time (qRT)-PCR and measured the absorbance of extracted dsRNA at 260 nm. To further corroborate differences in dsRNA production by the different bacterial expression systems, we used the qRT-PCR. ANOVA analysis and multi-comparison analysis were carried out from the average results of three independent experiments. According to a Duncan all-pairwise comparison test, the three vectors LCP480, pET-CP480, and pGEM-CP480

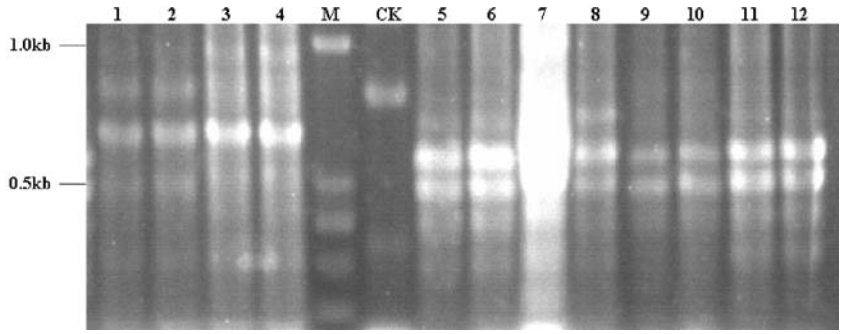

Fig. 3 Production of dsRNA by different *E. coli* strains defective for RNaseIII. Approximately 10 μg of total RNA extracted from different *E. coli* strains was loaded into each lane. *Lane M* dsRNA Marker; *CK* total RNA extracted from the wild-type strain JM109 (DE3) containing LCP480; *lanes1–4* dsRNA extracted from M-HMS174/ LCP480, HT115/LCP480, M-JM109/LCP480, and M-JM109lacY/ LCP480, respectively; *lanes 5–8* dsRNA extracted from HT115/ pGEM-CP480, M-JM109/pGEM-CP480, M-JM109lacY/pGEM-CP480 and M-HMS174/pGEM-CP480, respectively; *lanes 9–12* dsRNA extracted from M-HMS174/pET-CP480, HT115/pET-CP480, M-JM109/ pET-CP480 and M-JM109lacY/pET-CP480, respectively

show differences to each other in dsRNA production ($P<0.01$). The vector pGEM-CP480 is best, the pET-CP480 is second best, and the vector LCP480 is third. Two of the four mutations, M-JM109 and M-JM109lacY, show differences to M-HMS174 and HT115 ($P<0.01$). However, M-JM109 showed no difference to M-JM109lacY, and M-HMS174 showed no difference to HT115. The differences are as follows: M-HMS174/LCP480 (1.00±0.01)< HT115/LCP480 (1.34±0.08)<M-JM109/LCP480 (1.72± 0.40)<M-JM109lacY/LCP480 (2.14±0.10), M-HMS174/ pET-CP480 (1.87±0.13)<HT115/pET-CP480 (2.22±0.04)< M-JM109/ pET-CP480 (3.85±0.36)<M-JM109lacY/pET-CP480 (4.31±0.06), and M-HMS174/pGEM-CP480 (3.10± 0.13)<HT115/pGEM-CP480 (3.72±0.04)<M-JM109/ pGEM-CP480 (6.50±0.69)<M-JM109lacY/ pGEM-CP480 (7.28±0.56). In total, strain M-JM109 or the M-JM109lacY strain and the vector pGEM-CP480 are the best choices for dsRNA production (Fig. 4).

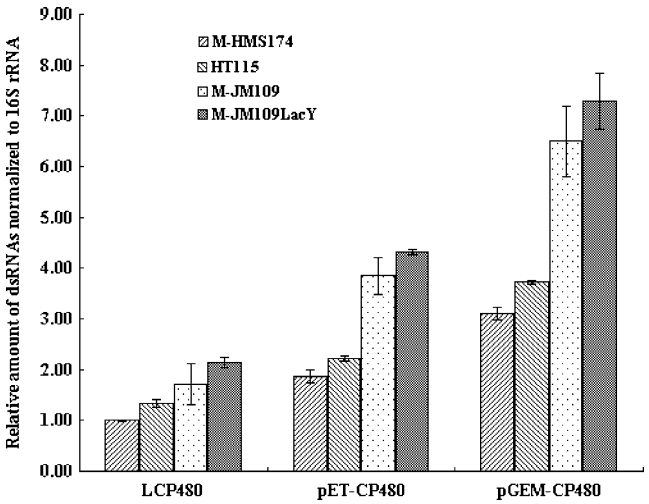

Fig. 4 Quantitative analysis of dsRNA production in different *E. coli* expression systems. Averages and standard deviations were calculated from $n=3$

Resistance analysis of extracted dsRNA transcripts

To prove whether the bacterial-produced dsRNA could interfere with TMV infection, we tested 20 tobacco plants for each vector. The tobacco plants were inoculated with mixtures of TMV plus bacterial-dsRNA extracts obtained from *E. coli* strain M-JM109lacY. Per tested plant, 300 μg dsRNA was applied regardless of the vector used for dsRNA production. The titer of TMV was 2 μg/mL of purified virus. The plants were inoculated with equal volume mixtures (100 μL per tobacco leaf) of TMV plus dsRNA produced by three different vectors (LCP480, pET-CP480, and pGEM-CP480). At 10 days post-inoculation (dpi), all wild-type tobacco showed the characteristic TMV mosaic on their leaves. The tested tobacco was assessed approximately every 10 days. The final classification refers to the state of the plants after the last assessment date. We discerned two major categories of plants after simultaneous treatment of TMV and bacterial dsRNA, resistant and susceptible. The susceptible tobacco showed varying degrees of symptoms; some had very mild symptoms (very mild green mosaics compared with the TMV-infected control tobacco). In all three cases, 20% of the total tested plants showed very mild symptoms, and 30% of the total tested plants showed the characteristic TMV mosaic on their leaves. The resistant plants remained symptom-free, even beyond 2 months post-inoculation. The dsRNA produced by the three vectors LCP480, pGEM-CP480, and pET-CP480 could all protect tobacco from TMV infection. They showed no great difference in resistance to TMV infection (regardless of whether the produced dsRNA contained a hairpin loop or not). In all three cases, 50% of the tested plants were resistant. The TMV-ELISA values of resistant tobacco show

no great difference with those of the negative control tobacco, and the virus content in the susceptible tobacco was obviously lower than in the positive control tobacco but higher than in the negative control tobacco (Table 3).

Northern blot of siRNA extracted from the tested tobacco

Virus resistance in transgenic plants containing a virus-derived transgene is frequently caused by posttranscriptional gene silencing of the transgene (Smith et al. 2000; Vargas et al. 2008; Waterhouse et al. 1998). To prove whether the employment of exogenous dsRNA could induce virus resistance, we investigated the presence or absence of siRNA in tested tobacco plants at the end of the experiment. We also analyzed susceptible tobacco and wild-type tobacco nontreated with TMV of the same age as positive and negative controls, respectively. TMV RNA signals were detected in the susceptible tobacco but not in the resistant tobacco and TE buffer-treated wild-type tobacco (data not shown). We detected siRNA signals in the resistant and susceptible tobacco but not in the wild-type tobacco (Fig. 5). Northern blot results, showing a decreased accumulation of TMV RNA in tested resistant and susceptible tobacco (data not shown) and the presence of siRNAs (Fig. 5) in tested resistant and susceptible tobacco, strongly support the conclusion that resistance to TMV is an RNA-mediated virus resistance.

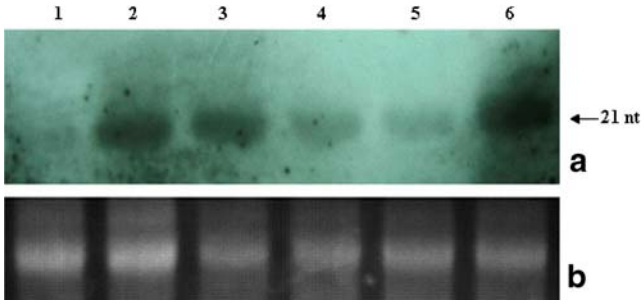

Fig. 5 Northern blot analysis of siRNA from tobacco plants. Two fully expanded tobacco leaves were mechanically inoculated per assay using carborundum as an abrasive. Northern blot analysis of small RNA fractions extracted from the tested plants at about 15 days postinoculation (about six-leaf stage). Approximately 15 μg of small RNA fractions were loaded into each lane. **a** *Lane 1* small RNA fractions extracted from wild-type tobacco; *lanes 2–5* small RNA fractions extracted from resistant tobacco; *lane 6* small RNA fractions extracted from susceptible tobacco. **b** 5 S rRNA was used to show that an equal amount of small RNA fraction was loaded in *lanes 1–6*

Discussion

In this work, using Red-mediated recombination, we generated the RNase III-defective *E. coli* mutant strains M-JM109, M-JM09lacY, and M-HMS174 for producing great quantities of dsRNA. These mutants were proved to be efficient in producing dsRNA by lack of dsRNA-specific RNases, just as the previously described strain HT115 does (Tenllado et al. 2003).

In this study, we discovered that the strains M-JM109 and M-JM109lacY are the best choices for producing great quantities of specific dsRNA. The main reasons might be as follows. The genotype of strain JM109 (DE3) is *end*A1and *rec*A1 (Table 1). The *end*A1 gene encodes endonuclease I, which has an important role in genomic DNA and plasmid replication and recombination, whereas the *rec*A1 gene expresses an adenosine triphosphate-dependent DNA recombinase. The *end*A and *rec*A1mutation will make the

Table 3 ELISA detection of resistance mediated by dsRNA transcripts

DsRNA preparation[a]	Phenotype[b] (R/S)	Virus titer (OD490nm)[c]		
		10dpi	20dpi	30dpi
Positive control	S 10/10	0.411±0.008	0.521±0.003	0.551±0.005
Negative control	R 10/10	0.020±0.003	0.022±0.004	0.021±0.002
M-JM109lacY/LCP480	R 11/20	0.022±0.003	0.021±0.004	0.023±0.003
	S 9/20	0.227±0.012	0.323±0.014	0.425±0.009
M-JM109lacY/pGEM-CP480	R 10/20	0.023±0.004	0.022±0.007	0.021±0.005
	S 10/20	0.216±0.011	0.314±0.016	0.405±0.017
M-JM109lacY/pET-CP480	R 10/20	0.021±0.005	0.023±0.004	0.024±0.006
	S 10/20	0.225±0.012	0.317±0.13	0.414±0.015

[a] The plants were inoculated with equal volume mixtures (100 μl per tobacco leaf) of TMV sap (2 μg/ml) plus dsRNA (3 μg/μl in TE buffer) produced by different vectors (LCP480, pGEM-CP480 and pET-CP480). The positive control only inoculated with TMV sap; the positive control inoculum was 1:10 dilution (*w*/*v*) of TMV-infected leaves ground in 10 mmol/L sodium phosphate buffer (pH 7.0). The negative control was inoculated with TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 7.5)

[b] The number of plants showing resistant (R) or susceptible (S) penotypes to TMV infection is indicated

[c] The virus titer is the average±standard derivation (n=3)

exogenous plasmids or the transcribed dsRNA more stable, and then the degradation of dsRNA may be lower (Kowalczykowski et al. 1994; Sambrook et al. 1989; Yanisch-Perron et al. 1985).

The output of dsRNA produced by different bacterial-expression systems was greatly different due to the different vectors in our study. In the end, we discovered that the output of dsRNA produced by pGEM-CP480 was the highest of the three vectors. Compared to pET-CP480 and LCP480, pGEM-CP480 has a high copy number plasmid, which can reach several hundred copies per cell (Bao and Cagan 2006; Sambrook et al. 1989). Secondly, although pGEM-CP480 as pET-CP480 has only one T7 promoter and from which the (+) and (−) ssRNA are transcribed in a single unit linked together by a loop, they might easily and quickly form dsRNA after transcription. The two T7 promoters of LCP480 separately transcribe (+) and (−) ssRNA; the (+) and (−) ssRNA are not linked by a loop after transcription. Accordingly, they might be vulnerable and easily degraded by the RNA endonucleases (such as RNaseA, RNaseB) in *E. coli* before forming the dsRNA (Kowalczykowski et al. 1994; Sambrook et al. 1989).

TMV causes serious disease in tobacco plants, with systemic mosaic symptoms and great losses in yield and quality of tobacco leaves (Lucas 1975; Zhu et al. 2008). The RNAi technique has been proved to be a powerful tool for pathogen-derived resistance. In this paper, the TMV CP gene was used for bacterial RNAi vectors, and the exogenous dsRNA produced by *E. coli* has been shown to be very effective in inhibiting TMV infection.

At present, researches need a safe and efficient strategy for antiviral gene engineering. Based on RNAi technology, delivery of dsRNA or hpRNA derived from plant virus sequences may be an important new strategy for virus resistance in transgenic or nontransgenic plants (Duan et al. 2008; Liu et al. 2007; Tenllado and Díaz-Ruíz 2001; Tenllado et al. 2003; Vargas et al. 2008). Compared to acquiring transgenic plants, using dsRNA transcripts provided by this strategy for RNAi has apparent advantages. First, this method avoids time- and labor-consuming, cost-intensive production of transgenic plants. Transgenic plants have increasingly raised concern for having potential negative ecological effects, such as heterologous encapsidation, complementation, synergy, and genetic flow between organisms (Tenllado et al. 2004). Second, the method offered here can deliver multiple virus dsRNAs to disrupt several virus species at once and may achieve multiple virus resistances at one time. Third, this method is safer than pesticides, which potentially cause chemical residue accumulation in the environment. Finally, dsRNA produced by this method remains are stable in vitro for several days after inoculation, even after exposure of the plants to a rain storm (Tenllado and Díaz-Ruíz 2001). This method will make RNAi technology widely available and applicable for protecting crops from virus infection. In this study, we have established a simple, fast, safe, and inexpensive strategy to produce large amounts of dsRNA derived from viral sequences using a bacterial-expression system. We hope this strategy will widely be used in the near future.

Acknowledgments We acknowledged Li Wei for the kind gift of the bacterial strain HT115. We also thank Zhang Zhao-shan, Zhang Wei-cai, Zheng Cheng-chao, Yang Zheng-you, Gu Hong-cang, Francisco Tenllado, and Barry L. Wanner for guidance on this paper. We thank Lv Shi-en and An Hai-long for revising the paper. This work was supported by the Excellent Youth and Middle Age Scientists Fund of Shan Dong Province [no. 2007BS06007] and the National Natural Science Foundation of China [no. 30771408].

References

Amarasinghe AK, Calin-Jageman I, Harmouch A, Sun W, Nicholson AW (2001) *Escherichia coli* ribonuclease III: affinity purification of hexahistidine-tagged enzyme and assays for substrate binding and cleavage. Methods Enzymol 342:143–158

Apirion D, Gitelman DR (1980) Decay of RNA in RNA processing mutants of *Escherichia coli*. Mol Gen Genet 177:339–343

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol . doi:10.1038/msb4100050

Bao S, Cagan R (2006) Fast cloning inverted repeats for RNA interference. RNA 12:2020–2024

Bass BL (2000) Double-stranded RNA as a template for gene silencing. Cell 101:235–238

Chen PY, Wang CK, Soong SC, To KY (2003) Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol Breed 11:287–293

Dasgupta S, Fernandez L, Kameyama L, Inada T, Nakamura Y, Pappas A, Court DL (1998) Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the *Escherichia coli* endoribonuclease RNase III - the effect of dsRNA binding on gene expression. Mol Microbiol 28:629–640

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645

Duan CG, Wang CH, Fang RX, Guo HS (2008) Artificial microRNAs highly accessible to targets confer efficient virus resistance in plants. J Virol 82:11084–11095

Dunn JJ, Studier FW (1973) T7 early RNAs and *Escherichia coli* ribosomal RNAs are cut from large precursor RNAs *in vivo* by ribonuclease III. Proc Natl Acad Sci USA 70:3296–3300

Dykxhoorn DM, Novina CD, Sharp PA (2003) Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol 4:457–467

Elbashir SM, Lendeckel W, Tuschl T (2000) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15:188–200

Elela SA, Igel H, Ares MJ (1996) RNase III cleaves eukaryotic preribosomal RNA at a U3 snoRNP-dependent site. Cell 85:115–124

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811

Goto K, Kanazawa A, Kusaba M, Masuta C (2003) A simple and rapid method to detect plant siRNAs using nonradioactive probes. Plant Mol Biol Rep 21:51–58

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950–952

Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404:293–296

Kindler P, Keil TU, Hofschneider PH (1973) Isolation and characterization of all RNase III deficient mutant of *Escherichia coli*. Mol Gen Genet 126:53–69

Kowalczykowski SC, Dixon DA, Eggleston AK, Lauder SD, Rehrauer WM (1994) Biochemistry of homologous recombination in *Escherichia coli*. Microbiol Rev 58:401–465

Krautz-Peterson G, Skelly PJ (2008) *Schistosoma mansoni*: the dicer gene and its expression. Exp Parasitol 118:122–128

Liu HM, Zhu CX, Zhu XP, Guo XQ, Song YZ, Wen FJ (2007) A link between PVY^N CP gene-mediated virus resistance and transgene arrangement. J Phytopathol 155:676–682

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

Lucas GB (1975) Diseases of tobacco, 3rd edn. Biological Consulting Associates, Raleigh, NC

Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431:343–349

Minea R, Swenson S, Costa F, Chen TC, Markland FS (2005) Development of a novel recombinant disintegrin, contortrostatin, as an effective anti-tumor and anti-angiogenic agent. Pathophysiol Haemost Thromb 34:177–188

Molnár A, Csorba T, Lakatos L, Várallyay É, Lacomme C, Burgyán J (2005) Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. J Virol 79:7812–7818

Pall GS, Codony-Servat C, Byrne J, Ritchie L, Hamilton AJ (2007) Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. Nucleic Acids Res 35:e60

Poteete AR, Fenton AC (2000) Genetic requirements of phage lambda red-mediated gene replacement in *Escherichia coli* K-12. J Bacteriol 182:2336–2340

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, NY

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. Nature 407:319–320

Takiff HE, Chen SM, Court DL (1989) Genetic analysis of the *rnc* operon of *Escherichia coli*. J Bacteriol 171:2581–2590

Tenllado F, Díaz-Ruíz JR (2001) Double-stranded RNA-mediated interference with plant virus infection. J Virol 75:12288–12297

Tenllado F, Martínez-García B, Vargas M, Díaz-Ruíz JR (2003) Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. BMC Biotechnol 3:3

Tenllado F, Llave C, Díaz-Ruíz JR (2004) RNA interference as a new biotechnological tool for the control of virus diseases in plants. Virus Res 102:85–96

Timmons L, Fire A (1998) Specific interference by ingested dsRNA. Nature 395:854

Timmons L, Court DL, Fire A (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene 263:103–112

Vargas M, Martínez-García B, Díaz-Ruíz JR, Tenllado F (2008) Transient expression of homologous hairpin RNA interferes with PVY transmission by aphids. Virol J 5:42

Vogler H, Akbergenov R, Shivaprasad PV, Dang V, Fasler M, Kwon MO, Zhanybekova S, Hohn T, Heinlein M (2007) Modification of small RNAs associated with suppression of RNA silencing by tobamovirus replicase protein. J Virol 81:10379–10388

Waterhouse PM, Graham MW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci U S A 95:13959–13964

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc Natl Acad Sci USA 97:5978–5983

Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101:25–33

Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W (2004) Single processing center models for human Dicer and bacterial RNase III. Cell 118:57–68

Zhu CX, Song YZ, Yin GH, Wen FJ (2008) Induction of RNA-mediated multiple virus resistance to Potato virus Y, Tobacco mosaic virus and Cucumber mosaic virus. J Phytopathol 157:101–107