Rolling circle amplification-mediated hairpin RNA (RMHR) library construction in plants

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ABSTRACT

Long hairpin RNA (lhRNA) construct-induced gene silencing facilitates the study of gene function in plants and animals, but constructing multiple lhRNA vectors using traditional approaches is both time-consuming and costly. Also, most of the existing approaches are based on sequence-specific cloning of individual sequences, and are therefore not suitable for preparing hpRNA libraries from a pool of mixed target sequences. Here we describe a rolling-circle amplification (RCA)-mediated hpRNA (RMHR) construction system suitable for generating libraries of lhRNA constructs from any gene of interest or pool of genes. Using RMHR we successfully generated a lhRNA library from a *Arabidopsis* cDNA population containing known and unknown genes, with an average size of 500–800 bp for the inverted-repeat inserts. To validate the RMHR system, lhRNA constructs targeting the β-glucuronidase (*GUS*) gene were tested using *Agrobacterium* infiltration and shown to be effective at inducing *GUS* silencing in tobacco leaves. Our results indicate that the RMHR technique permits rapid, efficient and low-cost preparation of genome-wide lhRNA expression libraries.

INTRODUCTION

RNA interference (RNAi)-mediated gene silencing has been used as an effective tool in gene function studies in a variety of organisms. This RNAi-based technology has also been successfully used in genetic engineering for virus resistance and improvements of food quality in crop plants (1). In a range of plant species, gene silencing efficiency with long hairpin RNA (lhRNA) constructs generally reaches over 70%, much higher than that with antisense constructs (2). Therefore, lhRNA constructs have been so far the most widely used technology for silencing genes in plants.

A typical lhRNA construct consists of a sense and an antisense sequence of a target gene separated by a spacer. This spacer is necessary for maintaining the stability of the construct in bacterial cells, as long perfect inverted repeats are known to be unstable in bacteria. Several cloning vectors have been developed to aid lhRNA construct preparation, including Phannibal (2), pFGC, pGSA and pGFPm-T (3) vectors involving two-step cloning, and the pHELLSGATE series and pANDA vector based on the GATEWAY cloning system (4). An inverted-repeat PCR (IR-PCR) strategy has recently been reported to allow rapid assembly and cloning of stem–loop-containing constructs in any vector (5). However, these approaches are all based on sequence-specific amplification of individual sequences, and therefore not suitable for construction of lhRNA libraries from a population of mixed target sequences, which requires simultaneous cloning of multiple sequences.

Since short hairpin RNA (shRNA) constructs are particularly effective at inducing silencing in mammalian cells, much effort has been made recently to construct shRNA libraries targeting animal genes, and several restriction enzyme-based methods have been developed. In the siRNA production by enzymatic engineering of DNA (SPEED) and enzymatic production of RNAi library (EPRIL) systems, small DNA fragments from restriction enzyme or DNase digestion are first ligated to a hairpin loop adaptor containing the recognition sequence for MmeI, and the ligation product is then digested with MmeI, releasing fragments containing 20-nt target sequences. The digestion product is then ligated to a linear DNA adaptor to allow for strand-replacement

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or primer-extension polymerase reaction to synthesize double-stranded DNA containing single-unit inverted repeat encoding shRNA (6,7). The restriction enzyme-generated siRNA (REGS) method also involves ligation to a hairpin-loop adaptor containing the MmeI site which cleaves exactly 20-nt target sequences from the ligated DNA. The MmeI-digested fragments are then made into palindromic structures using hairpin loops and isothermal rolling-circle amplification (RCA) (8). These techniques have proven successful but are designed to generate hpRNA with short stem lengths that are dependent on the features of a restriction enzyme used (i.e. MmeI). Another recently reported method, also designed to generate shRNA libraries, allows for flexible length of the short hairpin stems (9). It is based on ligation of de-phosphorylated short DNA fragments with phosphorylated hairpin-loop adaptors and subsequent strand-replacement polymerase reactions from the nicks of the ligation product. However, this method does not allow for amplification of the ligation and polymerase extension product.

Unlike animals, sRNA constructs have not proven very effective at inducing gene silencing in plants. Here, we described a novel technique based on RCA of DNA for generating multiple lhRNA constructs that target an individual gene or pool of cDNAs. Phi29 DNA polymerase was chosen for this RCA-mediated hpRNA (RMHR) construction system because of its capacity to perform strand displacement DNA synthesis for more than 70 000 nt, and its stability that allows efficient DNA synthesis to continue for many hours (10). Using the β-glucuronidase (*GUS*) gene as a target, we show that RMHR-generated lhRNA constructs are effective at inducing gene silencing in plants. The RMHR system is expected to provide a simple and low-cost approach for preparing lhRNA constructs for silencing genes in plants as well as animals.

MATERIALS AND METHODS

Construction of pBsa vector and subcloning of *GUS* gene fragments

Two complementary oligonucleotides were synthesized, including the sense strand Bsa(+) (5′ GGTCTCTGG GATATCGGTCTCTGAAG 3′) and the antisense strand Bsa(−) (5′ CTTCAGAGACCGATATCCCAG AGACC 3′). The two oligonucleotides were annealed and subsequently cloned into the Ecl136II site of the vector pQE30 (Stratagen, Qiagen, Hilden, Germany) to form the intermediate vector pBsa.

The *GUS* coding sequence was isolated from the pBI121 vector, and then fragmented with MspI digestion followed by treatment with T4 DNA polymerase. The samples were separated in 1.5% agarose gel, and the DNA fragments of 200–400 bp in size were excised and purified, and inserted into the EcoRV site of pBsa. The ligation products were transformed into *Escherichia coli* JM109, and 10 of resulting colonies were randomly picked and sequenced, including pBsa-GUSF1.

Generation of closed circular DNA and amplification with Phi29

Two oligonucleotides, miniHP1 and miniHP2, were synthesized, 5′-GAAGGCGATCGCTCTAGAGAGCT GGTTTCCTCTTTAGGTGAGCTCACTAGAGCGAT CGC (miniHP1) and 5′-TCCCAGGGCCCACTGCAG GGATCGGTTTCTCTTTTAGGTGGATCCATGCAG TGGGCCCT (miniHP2). The oligonucleotides were annealed and phosphorylated as previously described (11).

For ligation with the two hairpin-loop oligos, the ∼300 bp insert was excised from pBsa-GUSF1 with BsaI and gel-purified, and 20 ng of the purified fragment was mixed with 10 ng each of miniHP1and miniHP2, 0.5 μl of T4 DNA ligase (MBI) and 2 μl of ligation buffer in 20 μl. After overnight incubation at 16°C, the ligation reaction was precipitated with 0.3 M NaOAc and 2.5 volumes of ethanol, and dissolved in 50 μl sterile water.

For RCA, 14 μl of the ligation products was mixed with 2 μl of Phi29 reaction buffer and 2 μl of 100 μM 6-nt random primers. The mixture was heated at 95°C for 3 min and chilled in ice for 15 min, to which 0.5 μl of 10 mM dNTPs, 0.5 μl of 10 mg/ml BSA and 0.5 μl of Phi29 polymerase (MBI) were then added. After 6–8-h incubation at 30°C, the DNA was purified by precipitation with 0.3 M NaOAc and 2.5 volumes of ethanol and dissolved in 30 μl of sterile water.

Construction of hairpin RNA vector and transient expression analysis in tobacco leaves

Phi29-amplified products were digested with XbaI and SacI, and separated in 1% agarose gel. DNA fragments of ∼600 bp in size were excised and purified using a Qiagen agarose gel purification kit. The sample was ligated to pBI121 vector digested with XbaI and SacI, generating the pBI121HPGUS vector. pBI121 was digested with SmaI and Ecl136II and self-ligated to form pBI121-35S for use as control. pBI121, pBI121-35S and pBI121HPGUS were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation.

For *Agrobacterium* infiltration experiments, a 3-ml *Agrobacterium* culture was grown overnight at 28°C in the presence of 50mg/l rifamycin and 50 mg/l kanamycin. The culture was then inoculated into 15 ml of YEB containing the same antibiotics and incubated for about 6 h in a 28°C shaker. Subsequently 10 mM MES and 20 mM acetosyringone were added and the culture was shaken for further 2 h. *Agrobacterium* cells were harvested and resuspended in infiltration buffer (10 mM MgCl2, 10 mM MES and 200 mM acetosyringone) to an final OD_{600} value of 2.0, and left at room temperature for 2 to 3 h. *Agrobacterium* containing pBI121 was mixed with *Agrobacterium* containing pBI121HPGUS in a 1:4 ratio (0.5 ml versus 2 ml) and infiltrated into leaves of *Nicotiana benthamiana* that had been grown to 8–10 leaves in pots at ∼24°C under a photoperiod of 16 h light/8 h dark. Similarly, 0.5 ml of *Agrobacterium* containing pBI121 was mixed with 2 ml of *Agrobacterium* containing pBI121-35S and infiltrated into leaves of *N. benthamiana* for use as a control. After infiltration, plants were kept under the constant conditions and

grown for 48 h. The infiltrated leaves (50–70 mg) were then excised from the plants and used for *GUS* expression and small RNA analysis.

GUS enzyme assays

Agrobacterium-infiltrated leaf tissues were homogenized in protein extraction buffer (50 mM Na2H2PO4, 10 mMEDTA, 0.1% Triton X-100 and 1.0 g l^{-1} sarcosyl) and protein concentrations measured as described previously (12). For GUS activity determination, a fluorimetric assay was conducted essentially as described using Hoefer DyNA Quant 200 (Amersham Biosciences, Amersham Pharmacia Biotech, Uppsala, Sweden), with 4-methylumbelliferyl β-D-glucuronide as the substrate (13) and 4-methylumbelliferone (4-MU) as standard.

Northern blot analysis of small RNAs in infiltrated *N. benthamiana* leaves

Total RNA and small RNAs were isolated from the infiltrated leaves (100 mg) of *N. benthamiana* following the same procedure as described previously (14). The small RNAs were separated in 15% urea-polyacrylamide gel and transferred to Hybond-N+ filter. DNA fragment of ~250 bp in size from pBsa-GUSF1[#] were labeled with ^{32}P-dCTP using the Prime-a-Gene Labelling System (Promega, Madison, WI, USA), and used as probe to hybridize the Hybonnd-N+ filter. The hybridization was carried out as previously described (14) and hybridization signals were visualized by autoradiography.

Construction of *Arabidopsis* long hairpin RNA library

An *Arabidopsis* cDNA library was ordered from Arabidopsis Biological Resource Center (ABRC, Stock Number CD4-7). Plasmid DNAs were prepared following the recommended protocol. Construction of the *Arabidopsis* hpRNA library followed the same procedure for *GUS* hpRNA construction described above. Basically, plasmid DNA from the cDNA library was digested with SalI and NotI, and the cDNA inserts of 400–2000 bp in size were separated and purified in agarose gel. The purified samples were fragmented by AluI digestion and DNA fragments of 250–350 bp in size were purified from agarose gel for construction of *Arabidopsis* hpRNA library.

RESULTS

RMHR construction system

In nature, the replication of circular DNA molecules such as plasmids or viruses frequently occurs via a rolling circle mechanism. Linear RCA is prolonged extension of an oligonucleotide primer annealed to a circular template DNA *in vitro*, generating a continuous linear sequence consisting of tandem copies of the circle. Two primers are used to perform exponential RCA, one for each strand (15). Using random primers and Phi29 DNA polymerase, circular DNA templates can be amplified up to 10 000-fold in a few hours (16). Reaction products can be used directly for DNA sequencing, *in vitro* cloning, library construction, and other molecular biology applications.

The procedure for generating lhRNA vectors from double-stranded cDNAs was outlined in Figure 1. Basically, two different synthetic hairpin-loop DNA oligos (miniHP1 and miniHP2) were ligated to double-stranded DNA at the two termini to form closed circular DNA, which was then amplified by Phi29 polymerase. One of the loops, miniHP2, serves as spacer in the final hpRNA constructs. To introduce two different sticky ends to the DNA fragments for ligation with the two different hairpin-loop oligos, an intermediate vector pBsa was constructed by inserting a synthetic DNA linker into pQE30 vector, creating two BsaI sites flanking an EcoRV site into which DNA fragments were cloned. As BsaI recognizes the sequence GGTCTC(N)$_{1-5}$, the two introduced BsaI sites were designed as 'GGTCTC TGGGA' and 'GGTCTCTGAAG', so DNA fragments excised with BsaI contained unsymmetrical 4-nt 5′ overhangs 'GGGA' and 'CTTC', respectively at the two termini. This prevented the DNA fragments from both self-ligation and inter-fragment tandem ligation (Step 1; Figure 1).

Genes to be silenced were digested with the 4-base frequent cutter MspI or AluI to generate DNA fragments of 200–400 bp in size. The DNA fragments were first introduced to the EcoRV site in pBsa (Step 2; Figure 1), excised with BsaI, and then ligated with the two hairpin-loop oligos miniHP1 and miniHP2. miniHP1 contained an 5′ overhang 'GAAG' that is compatible with the 'CTTC' end of BsaI-digested fragments, whereas miniHP2 contained a 5′ overhang 'TCCC' compatible with the 'GGGA' end of the digested fragments. The two mini hairpins contained a sufficiently long self-complementary stretches to allow efficient self-annealing and ligation by T4 DNA ligase. Two restriction sites, XbaI and SacI, were introduced into miniHP1, for use in subsequent cloning of the inverted-repeat sequences into the expression vector (see step 5 in Figure 1). The restriction site PstI or BamHI was introduced into miniHP2 for subsequent characterization of the inverted-repeat fragments.

The ligation products had a single-stranded closed circular dumbbell structure (Step 3; Figure 1). RCA was then employed to amplify the circular DNA to create linear double-stranded DNA; Phi29 DNA polymerase and 6-nt random primers were used in RCA, yielding a concatemer of inverted repeats encoding lhRNA (Step 4; Figure 1). The amplification products were digested with XbaI and SacI, and the purified fragments were inserted into the pBI121 vector in place of the *GUS* coding sequence, forming the final lhRNA expression constructs (Step 5; Figure 1). This step-wise process was further detailed in the construction of *GUS* lhRNA constructs described below.

### Construction of *GUS* lhRNA constructs using RMHR

To test if lhRNA constructs prepared using RMHR were functional, the *GUS* coding sequence from pBI121 was fragmented by MspI and DNA fragments of 200–400 bp in size was purified and inserted into the EcoRV site of pBsa. Five clones were obtained (pBsa-GUSF1[#]-5[#]; Figure 2A), which contained 200–400-bp sequences
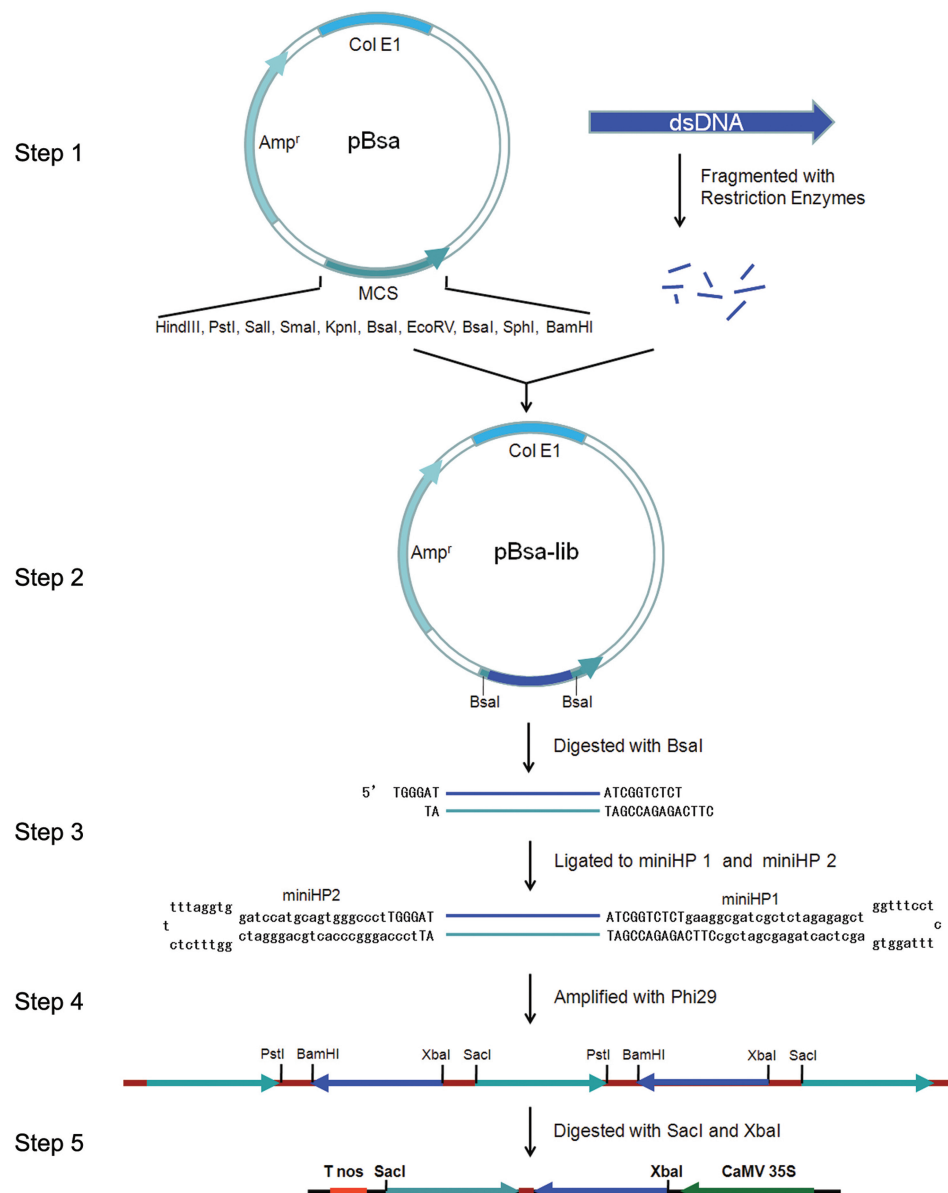
Figure 1. A schematic summary of the rolling circle replication-mediated hpRNA (RMHR) construction procedure. (Step 1) pBsa vector was constructed by inserting the synthesized DNA linker into pQE30 vector, in which two BsaI sites were introduced. As BsaI recognizes the sequence of GGTCTC(N)$_{1-5}$, the 4-nt overhangs (NNNN) in the two BsaI sites were designed to have different nucleotides for preventing self-ligation. (Step 2) Target sequences are fragmented using restriction enzymes (MspI or AluI) and inserted into the pBsa vector between the two BsaI sites. The purpose of this step is to generate sufficient amounts of BsaI-ended fragments for subsequent adaptor ligation. (Step 3) The fragments digested with BsaI were ligated to two hairpin loops, miniHP1 and miniHP2, which were formed from two oligonucleotides by self- annealing. The two mini hairpins contained a sufficiently long self-complementary stretches to allow efficient self-annealing and ligation by T4 DNA ligase. Two restriction sites, XbaI and SacI, were engineered into miniHP1, which were essential to subsequent cloning of inverted-repeat DNA into the expression vector (see step 5 below). Ligation products form a single-stranded closed circular dumbbell structure. (Step 4) RCA is used to amplify the circular DNA to create linear double-stranded DNA. The Phi29 DNA polymerase used in RCA displaces the newly synthesized strand in the presence of 6-nt random primers, yielding a concatemer of inverted-repeat double-stranded DNA encoding hairpin RNA. (Step 5) Amplification products were digested with XbaI and SacI, and inserted into vectors for plant transformation and expression of hairpin RNAs.

corresponding to different regions in the *GUS* sequence (Figure 2B). pBsa-GUSF1[#] with around 250-bp insert was chosen to construct a lhRNA vector. The BsaI-digested fragment of about 300 bp from pBsa-GUSF1[#] was ligated with miniHP1 and miniHP2 using T4 DNA ligase, generating closed circular DNAs. Agarose gel electrophoresis of the ligation mixture indicated that almost

all of the BsaI-digested DNA fragments were ligated to at least one adaptor, with over 65% ligated to both miniHP adaptors (Figure 2C). Assuming that some of the double-adaptor products contain nicks and that each of the four possible minHP1::DNA::miniHP2 ligations occurs at 65% efficiency, at least 18% (0.65^4) of the ligation products should be closed-circular molecules.
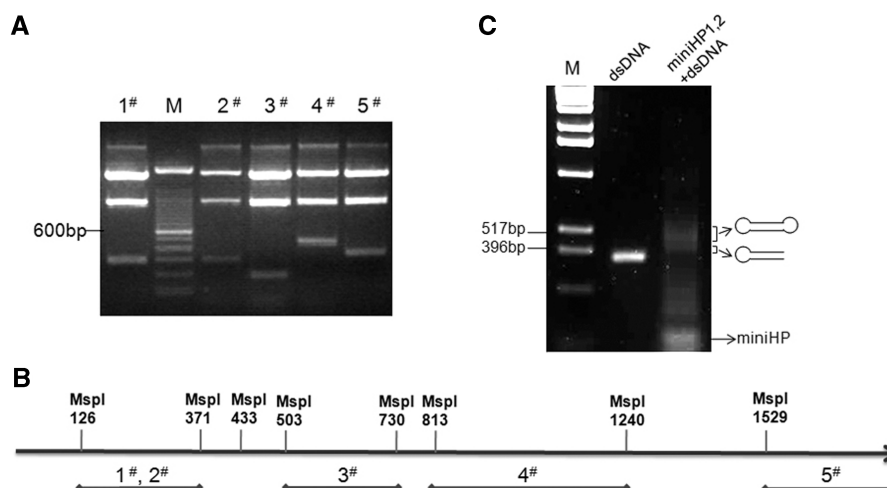
Figure 2. *GUS* coding sequence was fragmented with MspI and inserted into the pBsa vector. (**A**) BsaI digestion indicates that the 5 pBsa-GUSF clones contain inserts of 200–400 bp in size. M: DNA marker. (**B**) Distribution of the 5 *GUS* insert sequences along the coding region of the *GUS* gene. (**C**) Analysis of ligation efficiency between BsaI-digested DNA and the two hairpin adaptors miniHP1 and miniHP2. Note that the major band of BsaI-digested fragments (the middle lane) disappeared after ligation (the right lane), indicating near-complete ligation with at least one adaptor. The two predominant bands in the ligation reaction presumably represent ligation products with one adaptor and two adaptors, respectively. The smearing of the upper bands was likely due to presence of both close circular and nicked ligation products.
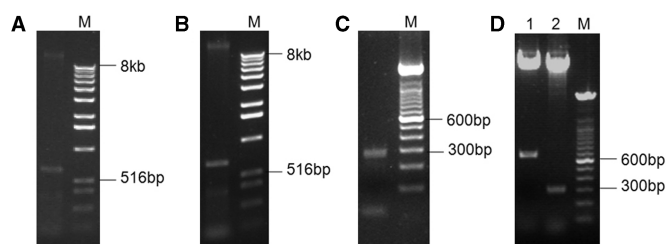
Figure 3. Amplification of circular DNA with Phi29 DNA polymerase and construction of *GUS* lhRNA vector. (**A** and **B**) Amplification products were digested with XbaI and SacI, respectively, which generated a ~600-bp fragment. (**C**) Digestion of the ~600-bp DNA with PstI generated a 300-bp fragment, indicating the formation of inverted repeat encoding lhRNA. (**D**) pBI121HPGUS was constructed by inserting the 600-bp XbaI/SacI fragment into pBI121. The construct was verified by digestion with XbaI and SacI (lane 1) and with XbaI, SacI and BamHI (lane 2).

The ligation products were amplified with Phi29 for over 6 h in the presence of 6-nt random primers. Large DNA fragments were observed in agarose gel, demonstrating that the circular DNAs served as templates for Phi29 to yield concatemers of double-stranded inverted-repeat DNA encoding hairpin RNA. This was confirmed by XbaI or SacI digestion of the amplification products which gave a linear DNA fragments of 600 bp (Figures 1, 3A and B) and by a further digestion of the 600-bp fragment with PstI that generated a 300-bp fragment (Figures 1 and 3C). To make the lhRNA constructs, the amplification products were double-digested with XbaI and SacI, and the resulting 600-bp fragment was ligated into pBI121, yielding pBI121HPGUS encoding *GUS* hairpin RNA. The construct was verified by restriction digestion and sequencing (Figure 3D).

Testing of *GUS* lhRNA construct

Agrobacterium infiltration-mediated transient assays have been widely used to study transgene expression and transgene-induced silencing in plants (17). We therefore chose *Agrobacterium* infiltration to investigate the silencing effect of pBI121HPGUS on the target *GUS* gene.

We mixed *Agrobacterium* carrying the 35S-GUS construct pBI121 with *Agrobacterium* harboring the lhRNA construct pBI121HPGUS and infiltrated the mixture into leaves of *N. benthamiana*. As a control, a mixture of pBI121-containing *Agrobacterium* with *Agrobacterium* carrying the empty vector pBI121-35S was infiltrated into different leaves of *N. benthamiana*. After the infiltrated plants were kept under constant conditions for 48 h, proteins were extracted for GUS activity assays. The result showed that leaves infiltrated with pBI121 and pBI121HPGUS had significantly reduced GUS activity than those infiltrated with pBI121 plus pBI121-35S; GUS activity was reduced by around 60% (Figure 4A). Northern blot hybridization of RNA isolated from infiltrated leaves showed that GUS-specific siRNAs accumulated in pBI121HPGUS-infiltrated tissues but not in pBI121-35S-infiltrated ones (Figure 4B). These results suggested that hpRNA was expressed from pBI121HPGUS and processed by Dicer into siRNAs, resulting in *GUS* silencing in *N. benthamiana* cells.

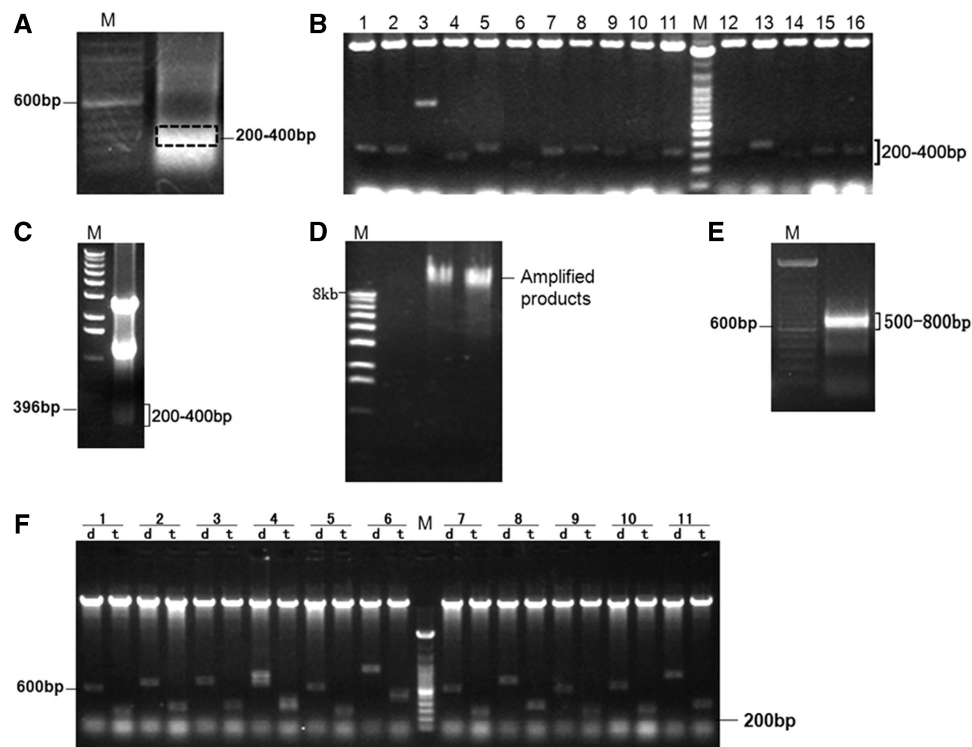## Construction of *Arabidopsis* long hairpin RNA library

An *Arabidopsis* cDNA library was obtained from ABRC, from which cDNA inserts of 400–2000 bp in size were separated. The samples were subsequently fragmented with AluI, and DNA fragments of 200–400 bp in size were separated (Figure 5A) and ligated into the pBsa vector, yielding a library named pBsa-At300 containing about 10^5 clones. Approximately 80% of clones contained inserts of 200–400 bp in size (Figure 5B). Plasmid DNA
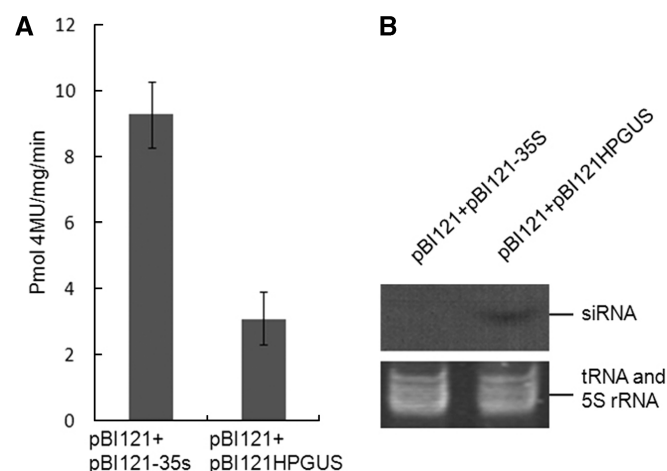
Figure 4. Analysis of GUS activity and small RNA accumulation in *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves. (**A**) GUS activity in *N. benthamiana* leaves infiltrated with pBI121 plus pBI121-35S (the left column) or infiltrated with pBI121 plus pBI121HPGUS (the right column). Note that *GUS* expression was clearly suppressed by the pBI121HPGUS construct. The data are an average of GUS activity in 10 *Agrobacterium*-infiltrated leaves. (**B**) Northern blot analysis of RNA isolated from a pool of *Agrobacterium*-infiltrated *N. benthamiana* leaves. siRNAs were detected in leaves infiltrated with pBI121 plus pBI121HPGUS (the right lane) but not in those infiltrated with pBI121 plus pBI12135S (the left lane).

was prepared from the pBsa-At300 library and digested with BsaI, and the DNA fragments were purified (Figure 5C) and ligated with miniHP1 and miniHP2, generating closed circular DNAs. The circular DNAs were amplified by RCA, yielding linear concatemers of inverted-repeat DNAs (Figure 5D). Following digestion with XbaI and SacI, fragments of 500–800 bp (Figure 5E) were inserted into the pBI121 vector, generating $\sim 10^4$ clones. Eleven clones were randomly selected and analyzed by restriction digestion. As shown in Figure 5F, double digestion with XbaI and SacI gave the expected 500–800-bp DNA fragments, whereas triple digestion with XbaI, SacI and BamHI generated the 250–400-bp half-sized fragments.

To examine if constructs in the lhRNA library contain diverse target sequences, ten random clones were sequenced and analyzed against *Arabidopsis* sequence databases. As shown in Table 1, nine of the 10 sequences match *Arabidopsis* cDNAs derived from chromosomes 1–5; the remaining sequence matches the vector of the library. Clones 5 and 7 had the same sequence while clone 4 contained a tubulin alpha-3 sequence and was likely to also target tubulin alpha-5 and tubulin alpha-1 because of the high sequence homology among tubulin genes. Thus, the 10 randomly selected clones contained eight different lhRNA constructs. This indicates that the

Figure 5. Construction of *Arabidopsis* lhRNA library. (**A**) *Arabidopsis* cDNAs were digested with AluI and DNA fragments of 200–400 bp were gel-purified and cloned into the pBsa vector, yielding a pBsa-300 cDNA fragment library. (**B**) Majority of the inserts in the pBsa-300 library had size ranging from 200 to 400 bp. (**C**) The pBsa library was digested with BsaI and DNA fragments of 200–400 bp in size were purified for ligation with the hairpin-loop daptors. (**D**) Circular DNAs were amplified with phi29 polymerase, generating DNA fragments of over 8 kb in average size (the two right lanes). (**E**) Amplification products were digested with XbaI and SacI to release single-unit inverted repeats. DNA fragments of 500–800 bp, representing the single unit inverted repeats, were clearly seen on the gel. (**F**) The 500–800 bp fragments were cloned into pBI121, forming the final lhRNA constructs. Eleven clones were verified by double (d) digestion with XbaI and SacI generating DNA fragments of 500–800 bp in size, and triple (t) digestion with XbaI, SacI and BamHI generating the half-sized 250–400 bp fragments.

Table 1. Sequence distribution of randomly selected clones in the *Arabidopsis* lhRNA library

Sequenced clones	Size of target sequence	Matched *Arabidopsis* locus	Sequence identity (%)	Target genes
1	173	AT1G03580	100	Pseudogene, meprin and TRAF homology (MATH) domain protein-related
2	331	AT1G76050	99	Pseudouridine synthase family protein
3	241	AT3G61830	100	ARF18 (AUXIN RESPONSE FACTOR 18)
4	224	AT5G19770	100	TUA3 (tubulin alpha-3)
		AT5G19780	98	TUA5 (tubulin alpha-5)
		AT1G64740	81	TUA1 (ALPHA-1 TUBULIN)
5	254	AT2G31140	100	Serine-type peptidase/signal peptidase
6	292	AT1G75750	100	GASA1 (GAST1 PROTEIN HOMOLOG 1)
7	253	AT2G31140	100	Serine-type peptidase/signal peptidase
8	270	AT4G28520	100	CRU3 (CRUCIFERIN 3); nutrient reservoir
9	227	AT3G15450	100	Unknown protein
		AT4G27450	80	Unknown protein
10	256	—	—	Vector sequence of cDNA library

lhRNA library constructed using the RMHR system is comprised of constructs that target a diverse population of *Arabidopsis* genes.

DISCUSSION

Long hpRNA constructs have been widely used for silencing genes in plants, but constructing multiple hpRNA vectors has proven a challenging task. A number of vector systems have been developed to facilitate the preparation of hpRNA constructs, including Gateway-based high-throughput vectors such as the pHELLSGATE series. However, these vector systems all require gene-specific amplification of target sequences, which is both time-consuming and expensive. The RMHR system developed in this work involves simultaneous formation of inverted repeats of multiple DNA sequences, allowing for preparation of multiple hpRNA constructs in the same reactions. Significantly, we demonstrate that the RMHR system can be used to make hpRNA constructs from both individual target genes and from a pool of target genes, with the latter allowing for construction of lhRNA libraries; the construction of a complex long stem hpRNA library targeting both known and unknown genes has not been reported before. Such lhRNA libraries would provide a cost-effective way for silencing genes in plants; lhRNA constructs targeting any particular gene or set of genes could be easily selected from a library using hybridization or PCR-based methods.

In this work we selected DNA fragments of 200–400 bp for hpRNA construction. Previous studies have indicated that fragments of about 120 bp should allow for efficient gene silencing in plants (2). We did not select longer fragments as we thought that inverted-repeat DNA with very long stems and a relatively short spacer (17 bp in this work) might not be stable in bacteria. However, the strategy can be modified to include a longer spacer to accommodate longer target gene sequences. It can also be modified to include an intron as the spacer, as intron-spliced hpRNA constructs were said to be more effective at inducing silencing in plants (18). The key for introducing a long spacer is to generate long single-stranded

hairpin-loop adaptors for ligation with the target DNA fragments. Several ways can be used to prepare long hairpin-loop adaptors. Long single-stranded DNA can be prepared using plasmids containing the F1 phage origin. Unidirectional PCR using a single primer with abundant DNA template is another possible strategy to generate long single-stranded DNA for use as hairpin-loop adaptors.

Efficient cloning of DNA fragments into the intermediate pBsa vector is an important step in the RMHR system, which is currently based on conventional blunt-end ligation. A possible improvement to allow more efficient cloning is to replace the EcoRV cloning site in pBsa with a sequence for recombination-based ligation of the GATEWAY system. Also, the RMHR system can be combined with various promoters including tissue-specific promoters to construct lhRNA libraries targeting both constitutively and differentially expressed genes.

In conclusion, the RMHR system provides a cost-effective method for rapid generation of multiple long stem hpRNA constructs of individual genes or sets of genes, and should be broadly useful in gene function studies in plants as well as in other organisms.

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