Symmetrical directional cloning: An efficient method to prepare hairpin RNA interference constructs*

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Abstract RNA interference (RNAi) is a loss-of-function approach by which double-stranded RNA (dsRNA) initiates degradation of homologous mRNAs in a sequence specific manner. The dsRNA molecules can be produced *in vitro* or *in vivo*, and can be introduced to cells in a number of ways. Here we report a more efficient method for the cloning of inverted repeat DNA fragments into expression vectors that can be transcribed into effective dsRNA molecules *in vivo* or *in vitro*. This method, named Symmetrical Directional Cloning (SDC), takes the advantage of compatible non-palindromic restriction enezyme sites, which allow one to directionally clone a single PCR product in both the sense and antisense orientations together into a vector. SDC allows for the directional cloning of inverted repeats using a single PCR product; it requires only one cut site on each side of the loop. Hence this method is more cost effective and less time-consuming. At least 21 commercially available restriction endonucleases can be used as cloning sites for the SDC method. The efficacy of dsRNA expression vectors prepared by SDC has been demonstrated by targeting a negative regulator of the signaling pathway mediating the response of cells to phytohormone, gibberellins (GA), in the aleurone cells.

Keywords: **RNA interference, hormone, hpRNA, symmetrical directional cloning.**

Loss-of-function experiments are of central importance for the elucidation of gene function. Knockout techniques that dominated in the past focused on disrupting a gene at the DNA level by chemical mutagenesis or insertional mutations by transposable elements. While these methods are still very useful and economical, RNAi has become the preferred method for loss-of-function experiments in many eukaryotic systems including plants[1~3], fungi[3,4], protozoans[5,6] and animals[7,8].

The phenomenon is a process by which dsRNA triggers the silencing or reduction of expression of an endogenous homologue (s)[9]. RNAi is genetically and mechanistically related in all eukaryotes examined with dsRNA[10], Dicer[11] and RISC[12] being the centerpieces to silencing. First, dsRNA is cut by Dicer into 21~26 nucleotide (nt) fragments and then separated into single-stranded small interfering RNAs (siRNAs). The siRNAs are used as a guide molecule for the ribonuclease complex, RISC, which degrades any RNAs sharing homology with the bound siRNA[13,14]. The mechanism is more elaborate in many organisms but these steps are common to all eukaryotes in which RNAi is applicable[9]. The RNAi technique is broadly applicable for both stable and transient knockouts across the eukaryotic spectrum, from plant development and hormone signal transduction research[2,15] to showing promising potential for the treatment of modern plagues like HIV[16] and cancer[17]. In the past, RNAi methods employed the introduction of either longer dsRNA (200~1000 nt) or shorter siRNA (21~23 nt). These have been chemically synthesized or obtained by *in vitro* transcription with T7 RNA polymerase[7,18]. siRNAs can also be generated by the enzymatic digestion of longer dsRNA by purified *E. coli* exonuclease III[19]. An advantage of this method is that a great variety of siRNAs are produced with different sequence specificities that target different regions of an mRNA. Several methods are in use for the introduction of dsRNA or siRNA and are generally specific to certain types of organisms. dsRNA may be injected[7], soaked into[20] or fed to[21] *C. elegans*. *Drosophila* cultures are incubated in media containing dsRNA[22]. Introduction of siRNA into mammalian cells requires the use of a transfection reagent[23]. In mammalian systems, the long dsRNA induces a generally cytotoxic interferon response causing transcription to be shut

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down in a sequence-nonspecific manner[24]. This problem is circumvented by the application of short runs of dsRNA; hence microinjection of short siRNA or dsRNA is commonly used in mammalian systems[18,25].

Recently, *in vivo* expression from DNA-based expression vectors has been adopted by many as a more desirable approach. There are mainly three strategies for the expression of dsRNA. First, dsRNA is produced by the simultaneous transcription of sense and antisense strands by separate promoters[10,26]. The second approach is expression of inverted repeats separated by a spacer region, which is transcribed into hairpin RNA (hpRNA). These long hairpins of 200～1000 bp of dsRNA are separated by a long loop region (>100 bp), which may contain an intron to enhance efficacy[27,28]. Third, short hairpin RNA (shRNA) is the predominant choice for mammalian systems. shRNAs usually take the form of ～19 nt of perfectly matched base-pairing separated by ～8 nt of spacer and ends in 2 nt 3′ overhangs[18,24,25]. In vertebrates, shRNAs are driven by type III class RNA polymerase III promoters[29] such as HI[30] and U6[8], which possess a well-defined transcription start site and a termination signal of five consecutive thymidines. For longer hpRNAs, expression is driven by RNA polymerase II promoters; the transcription start and termination sites are not critical[31]. In plants, DNA constructs for RNAi can be introduced into *Arabidopsis* and other plant species that can be easily transformed via the *Agrobacterium*-mediated method[2,32,33]. Recently, RNAi constructs have been introduced by gene gun biolistics to achieve transient interference in barley aleurone cells[15].

The most commonly used method of preparing hpRNA constructs is to amplify a target DNA fragment twice by PCR, each with a pair of primers containing different restriction enzyme sites. These PCR fragments are inserted into either side of the loop respectively. If a single site is used on each side, each insertion requires that the orientation to be confirmed. Directional cloning could be employed in this case by putting two cut sites on either side of the loop. Here we introduce a more efficient method, named SDC, for the production of hpRNA vector constructs. SDC allows for the directional cloning of inverted repeats using a single PCR product; it requires only one cut site on each side of the loop. Hence this method is more cost effective and less time-consuming.

1 Materials and methods

1.1 Chemicals and enzymes

T4 DNA ligase was obtained from Promega (Madison, WI). Restriction enzymes were acquired from both Promega and New England Biolabs (Beverly, MA). KlenTaq LA DNA polymerase, 4-methylumbelliferyl-β-D-glucuronide trihydrate and 4-methylumbelliferone were obtained from Sigma Chemical Co. (St. Louis, MO). Luciferin was from BD Pharmingen (San Diego, CA).

1.2 Plant materials and genomic DNA isolation

Rice (*Oryza sativa* L. ssp *japonica*) seeds were kindly provided by Dr. Kent McKenzie at California Rice Experiment Station. Barley (*Hordeum vulgare* cv Himalaya) seeds (1998 harvest) were purchased from Washington State University (Pullman, WA).

Rice and barley seeds were germinated on wet Whatman paper saturated with imbibing solution (20 mmol/L $CaCl_2$ and 20 mmol/L sodium succinate) in the dark at 26 ℃. Genomic DNA was isolated from the shoot tips of 10-day-old seedlings. Sterile rice and barley shoots were frozen in liquid nitrogen and ground into a powder. The frozen powder tissue was suspended in CTAB extraction buffer (55 mmol/L cetyl-trimethyl-ammonium bromide, 1.4 mol/L NaCl, 100 mmol/L Tris-HCl pH 8.0, 20 mmol/L EDTA) plus 2% β-mercaptoethanol. The homogenate was incubated at 55 ℃ for 30～45 min, cooled to room temperature and extracted twice with an equal volume of chloroform. The DNA was precipitated with isopropanol and then redissolved in TE buffer plus RNase A (20 μg/mL). After incubation at 37 ℃ for 1 h, the DNA was precipitated with ammonium acetate and ethanol, and then dissolved in TE buffer.

1.3 Preparation of DNA constructs

Plasmid pMBL022 (*Amy32b-GUS*) was used as the reporter construct, which was made by linking the promoter (up to −331), the entire 5′ untranslated sequence, and the first intron of the low pI α-amylase gene, *Amy32b*, to the GUS coding sequence and the 3′ untranslated region of the same α-amylase gene[34]. Plasmid pAHC18 (*UBI-Luciferase*), which contains the luciferase reporter gene driven by

the constitutive maize ubiquitin promoter[35], was used as an internal control construct to normalize GUS activities of the reporter construct[36,37].

The different RNAi effector constructs were made in a vector derived from pAHC18[35], which contains the maize *UBI* promoter and 3′ *NOS* terminator. *UBI-SLN1* (*RNAi1*) was made by the SDC method. A synthetic polylinker containing the *Asc*I site (underlined) is obtained from the annealing of two oligonucleotides 5′-GATCCGCGGC GGCCGCGATA TCTTAATTAA GGCGCGCCG-3′ and 5′-GATCCGGCGC GCCTTAATTA AGATATCGCG GCCGCCGCG-3′. The linker was inserted into the *Bam*HI site of a pAHC18 derivative that lacks the *Luciferase* ORF. A 1273-bp fragment covering the second intron of the *OsGAMyb* gene[38] was amplified by PCR using 5′-AATGGCGCGC C*GCTTAGC*CC ACCAGGTAAT AGTTTTTCTT GCC-3′ (sense) and 5′-AATGGCGCGC C**CCTTAGG**CT GCGTCGACTT GCAACAGG-3′ (antisense) primers. The sense primer contains *Asc*I (underlined) and *Blp*I sites (italicized) and the antisense primer includes *Asc*I (underlined) and *Bsu*36I sites (bold). After digestion with *Asc*I, the PCR product was inserted into the *Asc*I site of the intermediary plasmid, giving rise to the pre-RNAi construct. A 621-bp *HvSLN1* fragment was obtained by PCR with the genomic barley DNA template and the following primers: 5′-TATT*GCTTAG C*CGTGAACTC AGTCTTCGAG ATG-3′ (sense primer) and 5′-CGAA*GCTAAG C*TCTCACGAT GTCTACTTAC AGC-3′ (antisense primer). The 5′ ends of both primers contain a *Blp*I site (italicized). This fragment was inserted into the *Blp*I site of the pre-RNAi construct in one orientation, and then into the *Bsu*36I site of resulting plasmid construct in the opposite orientation, generating *UBI-SLN1* (*RNAi1*). *UBI-SLN1* (*RNAi2*) was prepared by inserting a 672-bp cDNA fragment of *HvSLN1* into either side of a 227-bp loop fragment as an inverted repeat[15].

1.4 Particle bombardment assay

The detailed descriptions of the transient expression procedure with the barley (*Hordeum vulgare*) aleurone system and the particle bombardment technique have been published before[36]. De-embryonated half-seeds of Himalaya were imbibed for 2.5 ~ 3 days, and then the pericarp and testa were removed. The DNA mixture (in 1∶1 molar ratio) of *Amy32b-GUS* and *UBI-Luciferase*, along with or without an effector construct, was bombarded into barley embryoless half-seeds (four replicates per test construct). For each bombardment, 8 prepared half seeds were arranged in a small circle (about 1.8 cm in diameter) to maximize the bombarded surface area. After bombardment, 4 half-seeds were incubated in 4 mL of the imbibing solution containing 50 μg/mL of chloramphenicol with or without 1 μmol/L GA_3 in small (6 cm in diameter) Petri dishes. After 24 h of incubation at 24 ℃ with gentle shaking, the bombarded seeds in sets of four were homogenized using a mortar and pestle in 800 μL of grinding buffer (100 mmol/L sodium phosphate pH 7.0 ~ 7.2, 5 mmol/L DTT, 10 μg/mL Leupeptin, 20% glycerol)[36]. The homogenates were centrifuged at 10000 g for 5 min, and the supernatants were retained for enzyme assays. For luciferase assays, 100 μL of the supernatant was mixed with 150 μL of 2X luciferase assay buffer (60 mmol/L Tris·SO_4 pH 7.7, 20 mmol/L $MgCl_2$, 20 mmol/L DTT, 2 mmol/L EDTA), 2 μL of 100 mmol/L γATP, and 8 μL of 25 mmol/L luciferin immediately before placing into the Sirius luminometer (Berthold Detection System GmbH, D75173 Pforzheim, Germany). For GUS assays, 50 μL of the supernatant was diluted into 200 μL of GUS assay buffer (2.5 mmol/L 4-methylumbelliferyl-β-D-glucuronide, 50 mmol/L sodium phosphate, pH 7.0 ~ 7.2, 10 mmol/L EDTA, 10 mmol/L DTT, 10 μg/mL Leupeptin, 20% methanol, 0.02% NaN_3)[36] and incubated at 37 ℃ for 20 h. Then the reaction mixture was centrifuged at 10000 g for 5 min, and 50 μL of the supernatant was diluted into 2 mL of 0.2 mol/L Na_2CO_3 and the resulting fluorescence was measured in a Sequoia-Turner model 450 fluorometer (Mountain View, CA) where 1 μmol/L 4-methylumbelliferone gives a reading of 1000 units. The normalized GUS activity represents the total number of fluorescent units in 20 h from an aliquot of extract that contained 200000 relative light units per second (RLU/s) of luciferase activity.

2 Results and discussion

2.1 Strategy of SDC

The SDC method takes advantage of restriction endonucleases that recognize non-palindromic variable sequences, which allows for orientation-specific ligation. If two compatible enzymes are used, a single PCR fragment can be inserted twice into a vector as inverted repeats. In this report, *Blp*I and *Bsu*36I

sites were used as an example to illustrate the strategy of SDC. The recognition sequences of *Blp*I and *Bsu*36I are GC′TNA GC and CC′TNA GG respectively. After digestion, both enzymes produce a 5′ overhang of 3 nucleotides "TNA". The central nucleotide in the cut sites is an "N", which is variable (N=A, C, G or T). This allows one to design experiments that make the compatibility of the two cut sites orientationally specific. The method we report here starts with the PCR amplification of a target fragment flanked by a *Blp*I site, in which the central nucleotides were chosen to be a "T" in the top strand and an "A" in the bottom strand (Fig. 1(a)). This is followed by the construction of a pre-RNAi vector that contains the ubiquitin promoter[35], a loop region (the 2nd intron of OsGAMyb, 39) and Nos terminator[35]. The loop is flanked by the *Blp*I and *Bsu*36I sites. The requirement of these sites is that they give compatible non-palindromic overhangs and they do not exist in the target region. In this case, the central nucleotides were chosen to be a "T" and an "A" in the top strand of the *Blp*I and *Bsu*36I sites respectively (Fig. 1(b)). This method forces the insert (Fig. 1(a)) to be ligated into the *Blp*I site in one orientation (Fig. 1(c)) and subsequently into the *Bsu*36I site in the opposite orientation (Fig. 1(d)). As a result, a sense-loop-antisense construct for hpRNA is produced.

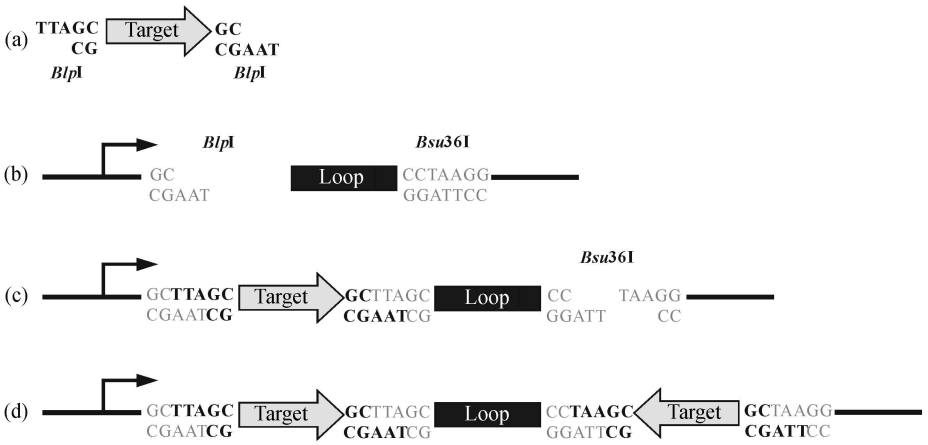

Fig. 1. SDC strategy for construction of RNAi expression vectors. (a) PCR product of the target fragment flanked by non-palindromic sticky ends that are generated by digestion with *Blp*I; (b) pre-RNAi expression vector containing a loop region flanked by non-palindromic cut sites, *Blp*I and *Bsu*36I (shown cut with *Blp*I); (c) construct containing the target fragment directionally cloned in the sense orientation into the *Blp*I site (shown cut with *Bsu*36I); (d) the final construct with the target fragment and loop region cloned in the sense-loop-antisense configuration.

2.2 A highly effective RNAi construct prepared by SDC

To demonstrate the efficacy of SDC, two hpRNA constructs targeting *HvSLN1* that were made by two different methods were compared. HvSLN1 protein is a negative regulator of the GA signaling pathway in barley aleurone cells[15,40,41]. It blocks the transcription of hydrolase genes such as those encoding α-amylases[15,40,41]. The loss-of-function mutation of *HvSLN1* results in the constitutive expression of α-amylase genes such as *Amy32b*[42,43].

UBI-HvSLN1(RNAi1) was constructed by the SDC method. A 1273-bp fragment that contains the second intron of the *OsGAMyb* gene was used as a "loop" in this RNAi construct. A 621-bp *HvSLN1* gene-specific DNA fragment was subcloned into both sides of the loop (Fig. 2(a)). UBI-HvSLN1 (RNAi2) was made by a conventional method. It was produced by subcloning a 672-bp fragment of SLN1 cDNA into both sides of a 227-bp loop region[15]. Both UBI-HvSLN1 (RNAi1) and UBI-HvSLN1 (RNAi2) effector constructs were then tested using the particle bombardment transient expression system.

As shown in Fig. 2(b), the expression level of the *Amy32b* promoter was low at all time points in the absence of GA. Exogenous GA treatment led to a high-level induction of the *Amy32b* expression. When the UBI-HvSLN1(RNAi1) effector construct was co-expressed, a gradual derepression of *Amy32b* occurred over time (Fig. 2(b)). After incubation for 72 h in the absence of GA, the level of *Amy32b* ex-

pression was comparable to those of the GA-treated samples. Consistently, the construct prepared by the conventional method, UBI-HvSLN1(RNAi2), had the same effect as that prepared by SDC, UBI-HvSLN1(RNAi1)(Fig. 2(b)), suggesting that the SDC strategy results in a highly effective hpRNA construct.

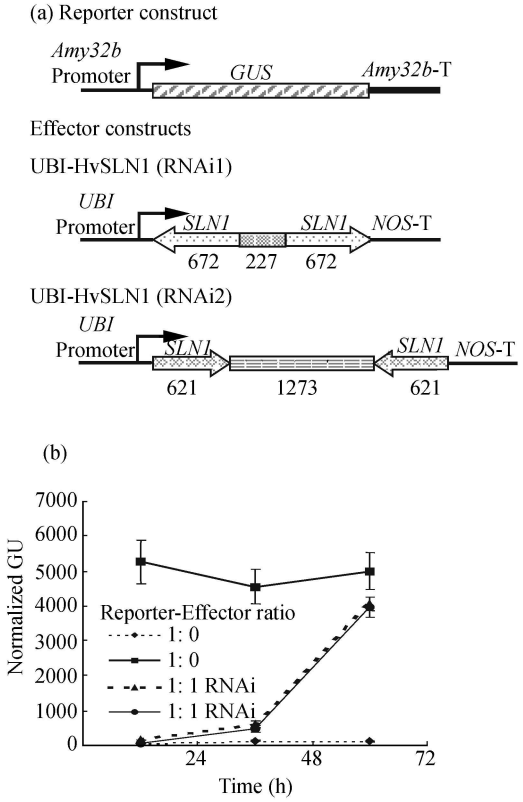

Fig. 2. The RNAi effector construct made by SDC is almost equally as effective as the one made by a conventional method. (a) Schemes of gene constructs. The target fragments in the effector constructs share 100% sequence identity with part of the endogenous *SLN1* transcripts. Arrowheads indicate the orientation of the gene fragments. Numbers below the effector constructs represent the size (in bp) of every segments (not drawn to scale). *UBI* promoter, maize ubiquitin promoter and its first intron; *NOS*-T, terminator of nopaline synthetase gene. (b) The reporter construct, *Amy32b-GUS*, and the internal construct, *UBI-Luciferase*, were cobombarded into barley half-seeds with or without the effector constructs [UBI-HvSLN1(RNAi1) or UBI-HvSLN1(RNAi2)] by using the same molar ratio (1:1) of effector and reporter constructs. GUS activity was normalized in every independent transformation relative to the luciferase activity. Bars indicate GUS activities ± SE after 24, 48 or 72 h of incubation of the bombarded half-seeds with or without 1 μmol/L GA_3. Data are means ± SE of four replicates.

2.3 Enzymes that can be used in SDC

Endonucleases used in this method must be able to recognize sequences that are nonpalindromic. This is the key to the orientation specific nature of the method. Usable enzyme combinations may produce 5′ or 3′ overhangs of 1, 3 and 4 nucleotides. Table 1 outlines the compatible restriction enzymes that can be used together for the cloning of inverted repeats. There are at least 21 commercially available restriction endonucleases that are usable for this approach. This makes the technique flexible enough to use with virtually any target gene because it is highly likely that a DNA target fragment lacks at least one of restriction enzyme sites listed in Table 1.

Table 1. Sets of restriction endonucleases that can be used for SDC

First endonuclease[a)]	Second endonuclease
*Acc*B7I (CCAN NNN′NTGG)	*Bgl* I, *Bst*API, *Mwo*I or *Sfi*I
*Ahd*I (GACNN N′NNGTC)	*Xcm*I
*Alw*NI (CAG NNN′CTG)	*Bgl*I, *Dra*III, *Mwo*I or *Sfi*I
*Ban*II (G RGCY′C)	*Bst*XI
*Bgl*I (GCCN NNN′NGGC)	*Acc*B7I, *Alw*NI, *Bst*API or *Dra*III
*Blp*I (GC′TNA GC)	*Bpu*10I or *Bsu*36I
*Bme*1580I (G KGCM′C)	*Bsp*1286I or *Bst*XI
*Bpu*10I (CC′TNA GC)	*Blp*I or *Bsu*36I
*Bsi*HKAI (G WGCW′C)	*Bst*XI
*Bsp*1286I (GDGCH′C)	*Bme*1580I or *Bst*XI
*Bst*API (GCAN NNN′NTGC)	*Acc*B7I, *Bgl*I or *Sfi*I
*Bst*XI (CCAN NNNN′NTGG)	*Ban*II, *Bme*1580I, *Bsi*HKAI or *Bsp*1286I
*Bsu*36I (CC′TNA GG)	*Blp*I or *Bpu*10I
*Dra*III (CAC NNN′GTG)	*Alw*NI, *Bgl*I, *Mwo*I or *Sfi*I
*Eco*109I (RG′GNC CY)	*Rsr*II
*Eco*NI (CCTNN′N NNAGG)	*Pfl*FI
*Mwo*I (GCNN NNN′NNGC)	*Acc*B7I, *Alw*NI or *Dra*III
*Pfl*FI or *Tth*111I (GACN′N NGTC)	*Eco*NI
*Rsr*II (CG′GWC CG)	*Eco*O109I
*Sfi*I (GGCCN NNN′NGGCC)	*Acc*B7I, *Alw*NI, *Bst*API or *Dra*III
*Xcm*I (CCANNNN N′NNNNTGG)	*Ahd*I

Each applicable enzyme is listed alphabetically in column 1 along with the sequence it recognizes. In column 2 are the enzymes that give compatible overhangs. Overhangs can be 5′ or 3′ of 1, 3 or 4 nucleotides. No usable 2 nucleotide overhangs were found from New England Biolabs or Promega at this time. Note that first endonucleases in column 1 are compatible with all of their corresponding second endonucleases in column 2. However, enzymes within a group of second endonucleases may not be compatible with each other. All usable enzymes are listed in both columns. a) Single letter codes: D=A/G/T, H=A/C/T, K=G/T, M=A/C, N=A/C/G/T, R=A/G, W=A/T, Y=C/T.

SDC requires the construction of an intermediate vector. However, once made, this intermediate can be used for the preparation of countless specific hpRNA constructs. To go from pre-RNAi vector (Fig. 1(b)) to final hpRNA construct (Fig. 1(d)) requires one PCR reaction, three digestions and two

ligations. The orientations of the ligations are controlled so the efficiency of the process is greatly increased. To reverse the orientation, a different pre-RNAi vector is not necessary. For the example illustrated in Fig. 1, to produce an antisense-loop-sense construct requires a different set of primers that switch the central nucleotide in the *Blp*I site from T to A in the sense primer and the central nucleotide in the *Blp*I site from A to T in the antisense primer, which forces the flip of insertion orientations of the PCR fragment. SDC increases the efficiency of cloning and is cost-effective.

The constructs created in this report were used for transient interference of hpRNA in barley aluerone cells by particle bombardment. SDC, however, is not limited to this system. In *Arabidopsis*, *Agrobacterium*-mediated transient expression system has been successfully used to target the *HvSLN1* orthologue[44,45]. SDC should help improve the efficiency of producing this type of RNAi constructs. In other systems, several different classes of hpRNA product molecules have been defined[28]. All classes are compatible with the SDC method.

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