Expressing stem-loop RNAs in plants, fungi, and animals efficiently silences homologous target gene expression. We devised a novel PCR strategy, called inverted repeat PCR (IR-PCR), which allows rapid assembly and cloning of stem-loop-containing constructs in any vector. IR-PCR relies on differentially tagging antisense and sense copies of the target in one round of PCR and assembling them in a second. We used IR-PCR to assemble constructs targeting profilin, actin, and actin-related protein (ARP) transcripts from Arabidopsis. Immunoblotting of lines expressing a profilin PRF1 3’ untranslated region (UTR)-specific construct demonstrated a 77 to 97% reduction in PRF1 protein, but not other profilin isovariants.

Keywords: Post-transcriptional gene silencing — RNAi — Stem-loop RNA — Transcriptional gene silencing.

Abbreviations: ARP, actin-related protein; GUS, β-glucuronidase; IR-PCR, inverted repeat PCR; NOS, nopaline synthase; PEPC, phosphoenolpyruvate carboxylase; PTGS, post-transcriptional gene silencing; RDRP, RNA-dependent RNA polymerase; RNAi, RNA interference; UTR, untranslated region; WT, wild type.

RNA interference (RNAi) is now commonly used to knock down gene expression in plants, animals, and fungi (Baulcombe 2004, Meister and Tuschl 2004). RNAi can target specific RNA sequences for degradation or translational inhibition by a process called ‘post-transcriptional gene silencing’ (PTGS), or it can block gene expression in a related process termed ‘transcriptional gene silencing’ that affects promoter activity (Meister and Tuschl 2004). In plants, the expression of stem-loop RNAs in which the double stranded RNA stem is homologous to the target gene or its RNA is more efficient at producing knockdown phenotypes than is antisense RNA (Chuang and Meyerowitz 2000, Mette et al. 2000). Furthermore, dissecting gene function using RNAi mediated gene silencing is often more efficient than isolating T-DNA insertion mutations of a target gene.

The demand of genomics for high-throughput analysis of gene function by RNAi has led to the development of several RNAi cloning vectors that express RNAs containing inverted repeats with stems derived from the target gene of interest. These vectors require PCR of the target gene to introduce novel end sequences and multiple restriction enzyme-based or recombination-based DNA cloning steps that produce inefficiencies in assembling some sequences as inverted repeats (Wesley et al. 2001, Helliwell and Waterhouse 2003). We have devised a novel alternative strategy for making RNAi gene constructs, called inverted repeat PCR (IR-PCR), which requires only two rounds of PCR and one cloning step. IR-PCR allows assembly and cloning of RNAi constructs in any vector in a single day, as outlined in Fig. 1. As case studies, we show the assembly of inverted repeat constructs for profilin PRF1, actin ACT8 and ACT3, and actin-related protein ARP4 genes from Arabidopsis. The molecular phenotypes resulting from expressing the construct prf1Ri targeting PRF1 transcripts were examined in Arabidopsis.

The profilins, actins, and ARPs are all encoded by gene families in Arabidopsis (Kandasamy et al. 2002, Meagher and Fechheimer 2003, Kandasamy et al. 2004). In order to dissect all the functions of one gene family member without disrupting the expression of others, we utilized the distinct 3’ untranslated region (UTR) of each gene in the inverted repeat (stem) region of our RNAi constructs. For example, in plants, profilins are encoded by a family of diverse gene sequences predating the split between monocots and dicots 200 million years ago (Huang et al. 1996). The family of five Arabidopsis profilin genes encodes at least five protein isovariants. Arabidopsis PRF1, PRF2, and PRF3 proteins are constitutively expressed throughout the plant, whereas PRF4 and PRF5 proteins are relatively pollen-specific (Christensen et al. 1996, Huang et al. 1996, Kandasamy et al. 2002). By targeting the distinct 3’ UTR of PRF1 we had the potential to silence the expression of one profilin isovariant without impacting the others.

The strategy for performing an IR-PCR assembly is outlined in Fig. 1. The construction began in the first round of PCR with creation of the three individual DNA pieces: the target gene sequence in the antisense orientation, the same target
gene sequence in the sense orientation, and an 1,060 bp β-glucuronidase (GUS) linker (Fig. 1A, B). IR-PCR relies on differentially tagging the sense and antisense oriented target gene sequences with novel sequences in this first amplification. When the three fragments were mixed together to begin the second round of PCR, their complementary 3′ ends annealed...
and extended using each other as templates by overlap extension (Ho et al. 1989) (OE-PCR, Fig. 1C). The full-length assembled product was then amplified from the mix with terminal primers, and the RNAi assembly was cloned into vector (Fig. 1D), which expressed the stem-loop RNA product (Fig. 1E).

The reaction products from IR-PCR assemblies targeting four example genes (PRF1, ACT8, ACT3, and ARP4) from the model plant Arabidopsis thaliana are shown in Fig. 2. Starting with the first nucleotide after the stop codon and extending downstream into the 3' UTR, a sequence of 300 bp was amplified from PRF1, ACT8, and ACT3 and 200 bp from ARP4 (left side of each frame in Fig. 2). Again, the key to IR-PCR is that the sense and antisense fragments were differentially tagged at both ends to assemble the stem region in each construct. The four pairs of 3' UTR sequences were combined by OE-PCR with the 1,060 bp GUS linker region to assemble the final products of 1.6, 1.6, 1.6 and 1.4 kb for prf1Ri, act8Ri, act3Ri, and arp4Ri, respectively (right side of each frame in Fig. 2). The sequences of each set of four DNA primers that differentially tagged the sense and antisense target sequences for each of the four RNAi constructs and the GUS linker primers are shown in Table 1.

The 1.6 kb prf1Ri fragment was subcloned under the control of constitutive promoters actin ACT2 and CaMV 35S to make constructs A2p:prf1Ri and 35Sp:prf1Ri. Both constructs were designed to express an identical 1.6 kb RNA product with a stem-loop structure (Fig. 1E, 2A). These constructs were transformed into Arabidopsis. PRF1 protein levels in lines expressing prf1Ri from both promoters were determined for several dozen T2 generation, 6-day-old seedlings on quantitative immuno-blots using a suite of antibodies (McKinney et al. 2001, Kandasamy et al. 2002). The monoclonal antibody MAbPRF1, which is specific for the PRF1 isovariant, revealed a 77% to 97% reduction in protein levels relative to wild type (WT), as shown for several randomly chosen A2p:prf1Ri lines in Fig. 3 (lines 2, 6, 8, 9, 10). The seedling protein levels ranged between 3% and 23% of WT levels for these five typical prf1Ri lines expressing A2p:prf1Ri (Fig. 3B). Similar results were achieved with the majority of lines expressing 35Sp:prf1Ri (not shown).

In order to compare the PRF1 expression of the various prf1Ri plant lines with a bona fide knockout mutant, we searched various databases for mutants in the PRF1 gene, and identified a new T-DNA insertion allele prf1-3. This mutant line was backcrossed twice to WT. We screened for the prf1-3 allele by PCR using the primers described in Table 1. DNA sequencing determined that the prf1-3 insertion was located between codons 17 and 18 in exon 1 of the PRF1 gene, as

![Fig. 2](https://example.com/fig2)

Fig. 2  IR-PCR assembly of constructs targeting four genes. The PCR amplifications of component fragments and the IR-PCR assembly of RNAi constructs targeting four Arabidopsis genes are shown: (A) PRF1, (B) ACT8, (C) ACT3, and (D) ARP4. The PCR products were examined by agarose gel electrophoresis. Each product fragment of interest is indicated by an arrowhead (a grey dot indicates a truncated fragment) along with its size estimated in kb (kilo base pairs). Molecular weight standards are shown (Std).
Inverted repeat PCR

shown in Fig. 3G. Thus, this should be a null mutation, knocking out all expression of the PRF1 protein. No PRF1 protein was detected by the Western analysis of homozygous prf1-3 mutant seedlings (Fig. 3B, left lane). Hence, MAbPRF1 is indeed isovariant specific for PRF1 as reported previously in assays on recombinant protein expressed in *Escherichia coli* (McKinney et al. 2001, Kandasamy et al. 2002).

Using a second monoclonal antibody, MAbPRF1,2, that reacts specifically with the constitutive profilins PRF1, PRF2, and PRF3, we can measure the remaining profilin protein levels in the PRF1 knockout and knockdown lines (McKinney et al. 2001). MAbPRF1,2 detected only 40% of the WT levels of profilin in the prf1-3 mutant seedlings, the residual PRF2 and PRF3 (Fig. 3C). The five prf1Ri knockdown lines showed a drop in residual profilin approximately proportional to their drop in PRF1 levels. Thus, this monoclonal detected PRF2, PRF3, and the residual PRF1 in the RNAi lines. The levels of remaining PRF2 and PRF3 in the RNAi lines compared favorably with the value in prf1-3. Using the polyclonal PAbPRF antibody that recognizes all five *Arabidopsis* profilins, Western analysis revealed a similar decrease in overall seedling profilin protein levels in the prf1Ri lines and prf1-3 line compared to WT (Fig. 3D).

Expression of the pollen-specific profilin isovariants PRF4 and PRF5 was unaffected in the flowers of prf1Ri and prf1-3 lines, as determined by Western analysis with a pollen profilin isovariant-specific monoclonal antibody (mAbPRF4,5 Fig. 3F) (Kandasamy et al. 2002). Similar specific drops in the levels of PRF1 protein were obtained with the 35S constitutively expressed RNAi lines (not shown). These data with four distinct profilin antibodies confirmed that severely knocking down PRF1 protein levels by targeting the 3′ UTR of the PRF1 transcript had little effect on expression levels of the other four profilin isovariants, PRF2, 3, 4 or 5. Phosphoenolpyruvate

### Table 1  PCR primers used in IR-PCR assembly and segregation analysis

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5′-3′</th>
<th>Primer type</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRF1CL-S</td>
<td>TGATAGTGATAGTGATAGTG/CCATGG/GGATCC/AACCTCTCTTTGTTT-GCAAAACCA</td>
<td>A1</td>
</tr>
<tr>
<td>PRF1CL-A</td>
<td>AGCGTTAGCGTTAGCGTTAG/AGATCT/GGTACC/AACCTCTCTTTGTTT-GCAAAACCA</td>
<td>A2</td>
</tr>
<tr>
<td>PRF1-GUS1</td>
<td>TTTTCTTCGCTTTTCCTCG/AAACCAAGGTTCATTTGCGGTTTCT</td>
<td>S1</td>
</tr>
<tr>
<td>PRF1-GUS2</td>
<td>ACTGAAAGAAGACTTCTCG/AAACCAAGGTTCATTTGCGGTTTCT</td>
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</tr>
<tr>
<td>ACT3-3′BAMA</td>
<td>TTAGACTAGTAGTAGTAG/CCATGG/TTAAAAATTAGAGGTTAGACTAAT</td>
<td>A1</td>
</tr>
<tr>
<td>ACT3-3′KNP</td>
<td>AGCGTTAGCGTTAGAGGTTACC/AGATCT/TTAAAAATTAGAGGTTAGACTAAT</td>
<td>A2</td>
</tr>
<tr>
<td>ACT3-GUS3′S</td>
<td>ACTGAAAGAAGACTTCTCG/AAACCAAGGTTCATTTGCGGTTTCT</td>
<td>S1</td>
</tr>
<tr>
<td>ACT3-GUS3′S</td>
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<td>S2</td>
</tr>
<tr>
<td>ACT5CL-S</td>
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</tr>
<tr>
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<td>LBa1</td>
<td>TGGTCTACGAGTAGGGGCCGAC</td>
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* Restriction enzyme sites are underlined, linker sequences are italicized, and target gene sequences are written in bold. All DNA sequences are written in a 5′ to 3′ orientation.

* For primer types refer to Fig. 1 and 3.
carboxylase (PEPC) protein levels were also analyzed and demonstrated equal loading and efficient membrane transfer among samples (PEPC, Fig. 3A, E). PEPC is a high molecular-weight protein (110 kDa) that was analyzed independently from profilin (14 kDa) on a strip cut from the top of each Western imprint.

We have had very few difficulties using IR-PCR to assemble RNAi constructs with the desired gene targeting sequence. Perhaps the greatest advantage of IR-PCR is the ability to make constructs destined for any vector and with any linker sequence. We have assembled much shorter (70 bp) and much longer (500 bp) target sequences into RNAi constructs using IR-PCR by decreasing or increasing the extension time of the second round PCR, respectively, relative to the conditions described in Materials and Methods (Kandasamy et al. 2005a, Kandasamy et al. 2005b, Zimeri et al. 2005, Deal et al. 2005). We have made novel two-piece assemblies with short linkers by altering the S1 and S2 primers (Table 1) to overlap with each other and replacing the GUS linker sequences, but we have not tested the efficacy of such two-piece constructs in silencing gene expression (R.B. Deal and R.B. Meagher, unpublished ). One artifact of the IR-PCR protocol should be mentioned. Occasionally, in the second round of PCR a smaller 1.3 kb product was amplified along with the expected 1.6 kb fragment (Fig. 2A, B, C). Note that the arp4Ri assembly did not produce an analogous 1.2 kb truncated product (Fig. 2D). The truncated products were assemblies of only two of the three fragments (Fig. 1C) and were due to contaminating L1

**Fig. 3** Profilin protein expression compared among A2p:prf1Ri lines and the T-DNA mutant prf1-3. Western blots examined profilin protein levels among various plant lines. (A–D) Analysis of constitutive profilins in 6-day-old seedling samples. (A) PEPC (phosphoenolpyruvate carboxylase) levels were examined from the high molecular weight region of B to confirm equal loading and membrane transfer among protein samples. This blot was probed with rabbit polyclonal anti-PEPC antibody that reacts with most plant PEPCs. A similar control PEPC blot was run for C and D (not shown). (B) The low molecular weight region of the same blot shown in (A) was probed with monoclonal antibody MAbPRF1 that is specific for the Arabidopsis PRF1 isovariant. The fraction of the PRF1 level found for each line is indicated below the Western blot. The five prf1Ri RNAi lines exhibited a 77 to 97% reduction in PRF1 protein levels compared to WT. No PRF1 expression was detected in the prf1-3 mutant. (C) The combined expression levels of PRF1, PRF2 and PRF3 were examined with monoclonal antibody MAbPRF1,2. A drop in profilin expression was observed that was proportional to the drop in PRF1 levels. Prf1Ri lines 8 and 10 were slightly under and over loaded relative to WT based on the PEPC control. (D) Total profilin levels were assayed by probing a Western blot parallel to that in (B) and (C) with the polyclonal antibody, PAbPRF, which reacts equally with all five Arabidopsis profilin isovariants. (E, F) Analysis of reproductive profilins PRF4 and PRF5 in flowers. The molecular weights of profilin and PEPC in kDa are indicated. (G) Map of the PRF1 gene and location of T-DNA insertion generating the prf1-3 mutant allele. PRF1 is made up of three exons and two introns (ivs). The T-DNA insertion in the mutant line prf1-3 is located after codon 17 in the first exon disrupting PRF1 expression. A left border sequence (LB) from this T-DNA insertion faces toward the 3′ UTR of the gene. Also indicated are locations of the various PCR primers (arrows) used to examine the structure and segregation of the prf1-3 allele and WT PRF1 allele (Table 1). The location of the 3′ UTR target by RNAi is indicated with a black line. The prf1-3 mutant was obtained from The Arabidopsis Information Resource (TAIR) at www.arabidopsis.org and was originally part of the Salk Institute Arabidopsis T-DNA Insertion collection.
and L2 primers carried into the second round of PCR. However, these products did not pose a problem for the IR-PCR protocol, because they contained only one of the two restriction enzyme sites required in the subsequent cloning step (Fig. 1D). This is evidenced by the fact that in the cloning of RNAi constructs described herein and those targeting more than a dozen other genes, we have never cloned these shorter truncated fragments.

Targeting the 3′ UTR of mRNAs encoding individual members of a gene family gives greater target-gene specificity to the RNAi approach of activating PTGS, because of the lack of sequence conservation and hence diversity of 3′ UTR sequences. Either degrading the 3′ UTR or blocking access of the translational machinery to the 3′ UTR of the target mRNA should knock down protein expression significantly given the evidence that most translation occurs on circular polysomes in which the 3′ polyadenylated end of the mRNA is bound via poly(A) binding protein to the ribosomal complex (Sachs et al. 1997, Wells et al. 1998, Palanivelu et al. 2000). Thus, even when the RNAi approach is used to target 3′ UTR mRNA sequences it does not depend upon the predicted transitive properties of the RNAi machinery to move upstream of the targeted sequence, but only upon direct effect on the targeted 3′ UTR sequence itself. While we have demonstrated that RNAi specifically silenced PRF1 protein expression, we have not determined if silencing occurred via a mechanism of RNA degradation or RNA translational inhibition or transcriptional silencing, which are all possible (Hannon 2002, Baulcombe 2004, Schmitz et al. 2004). Because plants have gene sequences expressing RNA-dependent RNA polymerase (RDRP) that have been shown to play roles in plant gene silencing (Dalmay et al. 2000), it might be expected that transitivity caused by RDRP would spread RNA silencing to conserved regions upstream of the 3′ UTR in the PRF1 mRNA (Himber et al. 2003). However, in spite of the high degree of sequence homology among the three constitutive profilin mRNAs (McKinney et al. 2001, Kandasamy et al. 2002), any transitivity that does occur in plants was not strong enough to transmit RNAi from the 3′ UTR of the PRF1 mRNA through its upstream coding sequence to the conserved coding regions of the other profilin mRNAs, such as PRF2 and 3. Our data from Western blotting with four profilin antibodies with distinct specificities suggest that any negative effects on the expression of the other four profilin proteins that may have occurred were insignificant. Other recent papers also suggest the benefits of targeting the 3′ UTR of distinct gene family members (Iifuju et al. 2003, Fukusaki et al. 2004, Ogita et al. 2004).

In a separate study, the arp4Ri assembly (shown in Fig. 2D) containing inverted repeats from the 3′ UTR of the nuclear chromatin remodeling protein ARP4 was expressed constitutively in Arabidopsis (Kandasamy et al. 2005b). A vast majority of independent arp4Ri plant lines were silenced for ARP4 protein expression and showed strong developmental defects. Those plants with ARP4 knocked down to less than 20% of WT levels were dwarfed, flowered early, showed altered flower morphology and flower senescence, and both male and female sterility. Again, targeting the 3′ UTR was quite effective at silencing gene expression.

IR-PCR joins inverted repeat sequences without special cloning vectors or a site-specific recombination system and allows RNAi constructs expressing stem-loop RNAs to be assembled and cloned in a single day. Besides speed and simplicity, a significant advantage of IR-PCR over currently used methods is that the assembled product can be cloned into any expression vector with any linker. We have used constructs assembled by IR-PCR to successfully knock down the expression of profilin PRF1 and several other plant genes including actins, actin-related proteins, and metallothioneins (Kandasamy et al. 2005a, Kandasamy et al. 2005b, Zimeri et al. 2005, Deal et al. 2005).

Materials and Methods

IR-PCR protocol

All first-round PCR amplifications to create the three parts of the assembly were carried out as follows: 94°C for 2 min, then 40 PCR cycles (94°C for 1 min, 52°C for 1 min, and 72°C for 1 min 30 s), then one cycle of 72°C for 7 min. The common linker fragments used in all IR-PCR constructs and other products were stored frozen at –20°C. PCR products were purified with a Qiagen PCR purification kit, digested with appropriate restriction enzymes, and cloned into pBluescriptII SK (Stratagene, La Jolla, CA, USA). DNA sequencing did not reveal base mutations introduced by PCR for 90% of the constructs we made. The assembly was subcloned as a NotI–BglII fragment under control of the actin ACT2 gene promoter and terminator (A2p:prf1Ri) (Fig. 1D) (Zimeri et al. 2005) and as a KpnI–SacI fragment under control of the constitutive CaMV 35S promoter and nopaline synthase (NOS) terminator (35Sp:prf1Ri, not shown) into a modified pBIN19 plant binary vector (Dhankher et al. 2005). The A2p:prf1Ri and 35Sp:prf1Ri containing vectors were transformed into Agrobacterium tumefaciens. After infiltration of the two Agrobacterium strains into Arabidopsis, populations of T1 seedlings were selected on agar plates containing 35 µg ml⁻¹ Kanamycin.

Plant growth conditions

Seeds were sterilized by soaking in 70% ethanol for 2 min, followed by 20 to 30 min in 30% Clorox bleach containing 0.02% Triton X-100 (BioRad, Hercules, CA, USA). Seeds were then washed 3 to 5
times in sterile distilled water. Seeds were placed on 0.8% phytagar plates (Life Technologies, Inc., Rockville, MD, USA) containing half-strength Murashige and Skoog salts (Murashige and Skoog 1962) (Life Technologies, Inc.) and 1% sucrose. To stratify seeds, plates were stored at 4°C overnight then transferred to 22°C growth chambers with cycles comprised of 16 h of light and 8 h of darkness. Plants were later transferred to soil and placed in larger growth rooms that were at 21°C and had a 16 h photoperiod.

Western analysis of protein expression

Protein samples were prepared, resolved by SDS-PAGE on 12 or 13% gels, and analyzed by protein gel blotting (Kandasamy et al. 1999) using the multiple profilin antibodies and PEPC (Rockland, Gilbertsville, PA, USA) antibody as described previously (McKinney et al., 2001, Kandasamy et al., 2002).

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References


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