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ARTICLE

Detection of self-complementary inverted repeats by single forward primer driven PCR

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ABSTRACT Inverted repeat gene structures designed for silencing functional genes have been widely used both in academic and applied research. The correct orientations of such structures are usually validated with restriction analysis and/or sequencing. We speculated that the inverted repeat nature of such constructs can be shown by a simple PCR reaction with a single forward primer. To test this hypothesis five different constructs were established from grapevine sequences in a hairpin-intron style silencing system. We were able to amplify the appropriate products in each case. Thus a forward-primed PCR alone may be sufficient to prove the inverted repeat nature of the desired constructs. **Acta Biol Szeged 58(1):65-68 (2014)**

KEY WORDS

Agrobacterium tumefaciens
crown gall
Phire Taq polymerase
plant diseases
RNA interference

Gene silencing plays an increasing role both in plant and animal biology to study physiological processes of living cells. It has also become a popular strategy to establish disease resistance in plants (Doran and Helliwell 2009). RNA-silencing or RNA interference (RNAi) is based on the sequence-specific recognition and subsequent degradation of target mRNA through RNA-induced silencing complexes (RISCs). RISCs contain short 21 bp RNA sequences that recognize the complementary RNA sequences resulting in its complete degradation by the RISC complex (Rana 2007; Pratt and MacRae 2009). RNAi-based strategies have been extensively studied in agriculture, e.g., to elaborate new strategies for controlling viral diseases of plants (Bonfim et al. 2007; Nahid et al. 2011; Wang et al. 2012; Shimizu et al. 2013), for silencing *Agrobacterium tumefaciens* oncogenes to prevent crown gall disease (Alburquerque et al. 2012) as well as for nematode (Gheysen and Vanholme 2007; Huang et al. 2014) and insect pest control (Gu and Knipple 2013).

From the technical aspect, gene constructs producing hairpin-structures of self complementary RNA in which the inverted repeats are separated by an intron sequence proved to be a highly efficient tool for gene silencing. To establish such structures several improved applications of the Gateway technology based on site specific DNA-recombination and related pHellsgate vectors have been constructed and widely used (Wesley et al. 2001; Helliwell et al. 2002; Helliwell and Waterhouse 2003; Earley et al. 2006; Traore and Zhao 2011). An improved technology, 'Golden Gate' (Engler et al. 2008) uses the type II restriction enzyme *BsaI*. A special characteristic of this enzyme is that it cleaves DNA adjacent to the recognition site. This feature of the enzyme has two important impacts on cloning strategy. On one hand, after cleavage and subsequent ligation the recognition site is not included in the DNA fragment anymore, such cleavage and ligation of both vector and insert can be carried out in one tube and one step, making the cloning process very simple. The second important feature of the enzyme is that different sticky ends can be created by the digestion with the same enzyme, which means that directional cloning of the PCR product in two copies and opposite orientation can be also achieved in the same digestion/ligation step. Thus cloning of genes of interest in opposite orientation becomes possible in a single restriction digestion-ligation step into the pRNAi-GG binary vector (Yan et al. 2012). The vector is specifically designed for the fast and effective creation of inverted repeat constructs. pRNAi-GG is introducing a complex selection system for clones including both copies of the insert and a copy of a pyruvate dehydrogenase kinase (Pdk) intron between the two copies (Fig. 1.). The opposite oriented insert PCR products are replacing two copies of the bacterial toxin CcdB, thus selecting for clones carrying both arms of the hairpin structure. The inclusion of the Pdk intron is ensured by a chloramphenicol resistance gene included in the intron sequence (for more detailed description of the cloning system see Yan et al. 2012).

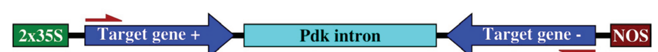

Figure 1. The schematic representation of the self-complementary inverted repeat clones established during this study.

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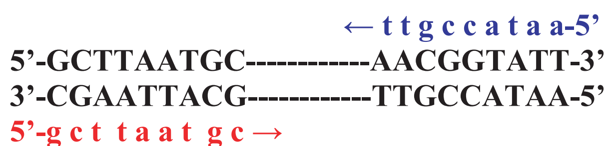
Although the use of the 'Golden Gate' cloning system highly simplified cloning of genes coding for self-complementary hairpin RNAs, the identification of the correct orientation of clones needs detailed PCR analysis, restriction analysis and/or sequencing (Nahid et al. 2011; Alburquerque et al. 2012; Yan et al. 2012). Here we show that a single forward primer designed for the gene of interest (cloned in inverted orientation surrounding an intron sequence) reliably detects self-complementary RNAi structures in one PCR reaction.

Materials and Methods

Homologous genes were selected from the published grapevine genome sequence (Jaillon et al. 2007). Five genes of interest, each contributing to *Agrobacterium*-transformation in tobacco or *Arabidopsis* were chosen for this study: RTNL2 and Rab8a (Hwang and Gelvin 2004), Hta2 and Hta10 (Zhu et al. 2003) and Vip1a (Li et al. 2005). Partial sequences of these genes were amplified by PCR with specific primers (Table 1.) using the cDNA from the grapevine variety *Vitis berlandieri* x *Vitis rupestris* cv. 'Richter 110' as template. RNA was extracted from *in vitro* plants using PureLink Plant RNA Reagent (Life Technologies) following the manufacturer's instructions. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Life Technologies) in 20 µl reaction volume from 600 ng total RNA. PCR products were cloned into pJet vector using the CloneJET PCR Cloning Kit (Thermo Scientific). The sequence accuracy of the cloned PCR products was confirmed by sequence analysis.

Chosen sequences were cloned into pRNAi-GG in inverted orientation into the left and right side of a Pdk intron containing a chloramphenicol resistance gene using a single *Bsa*I digestion-ligation step as described by Yan et al. (2012).

Conventional PCR



Single-primed PCR for inverted repeats

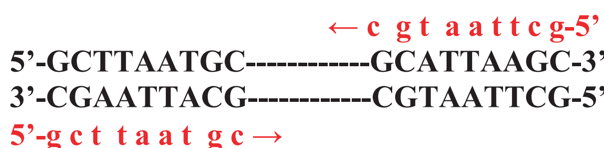


Figure 2. Schematic presentation of amplification of inverted repeats with a single forward primer. In conventional PCR (above) two different primers, called forward (red) and reverse (blue) primers are used that are designed to the two DNA strands in opposite orientation. In the case of inverted repats (below) the forward primer alone directs the synthesis of DNA in both strands in opposite orientation yielding well-defined amplification products.

The schematic structure of these clones is shown on Figure 1. The cloned genes were transformed into *Escherichia coli* DH5α for further work using kanamycin/chloramphenicol selection.

Restriction analysis

The pRNAi-GG plasmids containing the silencing constructs were isolated from *E. coli* DH5α cell suspensions using the

Table 1. Primers used for cloning (forward and reverse for each sequence, see Fig. 1) and PCR analysis of inverted repeat constructs (forward only) designed on the basis of grapevine sequence data (Jaillon et al. 2007). The 5' end of the primers includes 15 base pair long (*underlined italics*) flanking sequences used for the cloning procedure but they are not essential for the amplification. Further details on the grapevine genes involved in this study have been described earlier (Deák et al. 2013).

Primer name (specificity)		Primer sequence (5'→3')	Size of the cloned fragment (bp)	Size of the product amplified by the forward primer (bp)
VvHta2	Forward	*ACCAGGTCTCAGGAG*ACGGGATCTCAGTGGCTTCT	165	1,995
	Reverse	*ACCAGGTCTCATCGT*TTCCACAATTGGTCTGTTAGGA		
VvHta10	Forward	*ACCAGGTCTCAGGAG*CGATTCTCATGAAGTACAAAACG	268	2,201
	Reverse	*ACCAGGTCTCATCGT*GGAAACCAAACATCCCAGTC		
VvVip1a	Forward	*ACCAGGTCTCAGGAG*CTAATTGGCAGGAAGGCAGA	214	2,093
	Reverse	*ACCAGGTCTCATCGT*TCCATCCATTAAATGCTCCA		
VvRab8a	Forward	*ACCAGGTCTCAGGAG*CCAGGGGAAGGTTCTTCAAG	124	1,913
	Reverse	*ACCAGGTCTCATCGT*TGCCAAAGCCAGTAGTTGAA		
VvRTNL2	Forward	*ACCAGGTCTCAGGAG*TTCCAAGTGGTAGGGCAATC	289	2,243
	Reverse	*ACCAGGTCTCATCGT*CTGGGCAGAAGGAACAATGC		

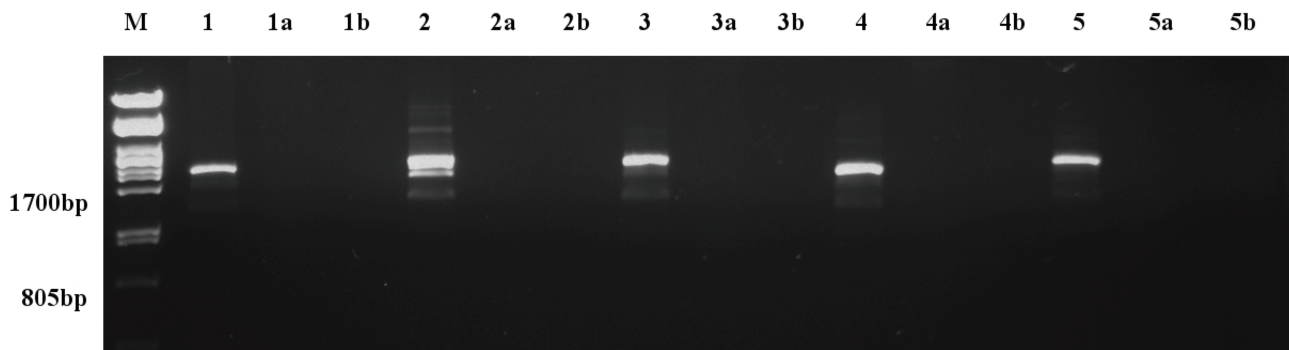


Figure 3. Detection of inverted repeats with single forward primed PCR. M: size marker (*PstI*-digested λ-Phage DNA), followed by reactions with forward primers specific for VvHta2 (1-1b), VvHta10 (2-2b), VvVip1a (3-3b), VvRab8a (4-4b) and VvRTNL2 (5-5b). Single numbers indicate the inverted repeat constructs, „a" lanes contain DNA samples of the empty pRNAi-GG vector, and „b" samples are DNA-free controls.

alkaline lysis method (Sambrook et al. 2001). The extracted plasmid DNA was digested with *Bgl*II and *Pst*I Fast Digest restriction enzymes (Fermentas) at 37 °C for 20 min. The digested DNA fragments were separated on agarose gels and stained with ethydium bromide dye.

PCR conditions

PCR reactions were performed using an Applied Biosystems 9700 thermal cycler in 20 μl final volume with 0.4 μl of Phire Hot Start II Polymerase (Thermo Scientific) in 1x buffer supplemented with 0.5 μM of forward primer (Table 1) and 0.2 mM of each dNTP. One single colony of *E. coli* DH5α cells containing the pRNAi-GG vector with the corresponding silencing construction was used as template of the PCRs. Bacterial colonies containing blank pRNAiGG vectors were used as negative control samples with all of the applied primers. Template DNA from *E. coli* was isolated by lysing the cells in hot Triton X-100/sodium-azide buffer (Abolmaaty et al. 2000). The cycling parameters were: 98 °C for 3 min, 98 °C for 10 s, 66 °C for 5 s, 72 °C for 20 s, for 35 cycles, and then 72 °C for 3 min.

Results and Discussion

In this work we used the Golden Gate cloning system according to Yan et al. (2012) to set up silencing constructions harbouring self complementary hairpin structures in pRNAi-GG vectors. Short (124-289 bp) sequences of five different grapevine genes (Table 1.) were cloned into the pRNAi-GG vector on both sides of the 1599 bp long Pdk intron in opposite orientation, resulting in five constructions: pRNAiGG-VvVip1si, pRNAiGG-VvRtnl2si, pRNAiGG-VvRab8si, pRNAiGG-VvHta2si and pRNAiGG-VvHta10si. The results of the one step digestion-ligation process were checked by restriction analysis using *Bgl*II and *Pst*I enzymes. Digestion by the *Pst*I enzyme proves the incorporation of the inverted repeats surrounding the Pdk intron, while the *Bgl*II digestion provides information about the direction and incorporation of the Pds intron.

Due to the structure of inverted gene repeats yielding self-complementary RNAs we speculated that a single forward primer should amplify the full length of „gene of interest→intron←gene of interest" sequence since the forward primer designed corresponding to the 5'-3' strand will behave as a reverse primer and direct an 5'-3' DNA synthesis at the 3' of this strand (Fig. 2). To prove if this works, we tested various conventional PCR with *Taq* polymerases, but the expected relatively long DNA sequences were not properly amplified.

When PCRs were challenged by Phire Hot Start II polymerase (Thermo Scientific) specifically designed for the synthesis of long or difficult DNA templates, we obtained the expected DNA fragments. Figure 3. shows the products of colony PCR synthesized by using only the forward primers of the five constructs. The templates were *E. coli* DH5α colonies containing the pRNAi-GG vectors with the inserted silencing constructions. Colonies with pRNAi-GG vectors did not yield any PCR products (Fig. 3).

The identification of the inverted repeat constructions requires multiple PCR analysis, or restriction analysis of purified plasmid DNA, and/or DNA sequencing (Nahid et al. 2011; Alburquerque et al. 2012; Yan et al. 2012). The strategy described here offers a fast and cost-effective method to directly screen inverted repeat constructions from bacterial colonies after cell lysis by a simple PCR reaction using only the forward primer. Moreover, the same PCR primers which were applied during the cloning process can also be used for the detection. Thus these results show that the structure of hairpin constructs containing self complementary inverted repeats can be confirmed by a single primer driven PCR.

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