A HYBRID CLONING PROCEDURE FOR CONSTRUCTION OF PLANT TRANSFORMATION VECTORS FOR ihpRNA-MEDIATED GENE SILENCING

G. Tonkovska, A. Atanassov, I. Atanassov
AgroBioInstitute, Sofia, Bulgaria

ABSTRACT
A hybrid cloning procedure for construction of gene silencing transformation vectors, involving intron-hairpin structures, was developed. The intron-hairpin structure was initially assembled through PCR-amplification and digestion/ligation of the target gene and intron regions. The obtained ihp-fragments were directly cloned into plant transformation vectors or Gateway™ entry vectors employing a BP Clonase® reaction of the Gateway™ cloning system.

Introduction
The induction of post-transcriptional gene silencing is one of the most effective and direct ways to study gene function and has increasing functional genomics applications. Both suppression (antisense RNA) and co-suppression (overexpression of sense RNA) approaches were applied to induce silencing of the target gene in transgenic plants (1, 2). It was demonstrated that, efficiency of gene silencing induction depends substantially on the target gene, parameters of the transformation vector, position and structure of T-DNA insertions etc. (3, 4, 5). The discovery and study of the RNA interference phenomenon, in which double-stranded RNAs (dsRNA) elicits degradation of a target mRNA containing homologous sequence, led to development of more effective dsRNA-mediated gene silencing methods (6, 7, 8). In plants, a particularly effective method for induction of dsRNA-silencing is transformation of the plant with chimeric gene construct producing hairpin RNA (hprRNA) (9). Such construct consists from inverted regions of the target gene sequence, separated by an appropriate spacer. The insertion of a functional plant intron region as a spacer fragment additionally increases the efficiency of the gene silencing induction, due to generation of an intron spliced hairpin RNA (ihpRNA) (10, 11).

Methods for construction of ihpRNA transformation vectors
The development of efficient and reliable procedure for construction of ihpRNA vectors is crucial for the successful application of this gene silencing technology in the transgenic plant research. Two general protocols for assembling of ihpRNA constructs were proposed by Helliwell and Waterhouse, 2003 (11): (i) consecutive cloning of PCR amplified gene sequences in inverted position on both sides of the intron region. This protocol includes repeated cloning steps, which make it labor- and time-consuming and restrict its high-throughput application. (ii) the second method involves assembling and cloning of the entire intron-hairpin structure using a Gateway™ Cloning System. The protocol is based on the PCR-amplification of a
gene sequence with simultaneously addition of attB1 and attB2 sites. Through site-specific recombination, the att-tailed PCR fragments are inserted unidirectional at the both sides of the intron fragment in specially constructed plant transformation vector. Although allowing high throughput application, the method has disadvantage to add to the generated dsRNA extra sequences derived from att-ends. This protocol also requires the use of a specially constructed vector possessing an intron region surrounded by att-sequences under specific arrangement.

In a preliminary experimental work we tried to develop an efficient cloning protocol using combinations of PCR-amplification and DNA digestion/ligation steps, followed by re-amplification and cloning of the assembled DNA fragments. Although the intron-hairpin structures were obtained, they could not be successfully re-amplified and cloned. In order to increase efficiency and specificity of the direct cloning of the assembled intron-hairpin structure we introduced single Gateway™ cloning step and developed an efficient hybrid cloning procedure.

Hybrid cloning of intron-hairpin vectors
The outlines of the developed cloning protocol are shown on Fig. 1. The procedure demonstrates the cloning of an intron-hairpin structure including reverse orientated fragments from a region of GUS gene, placed on the both sides of the second intron of ran gene from Nicotiana sylvestris. Initially, two PCR fragments corresponding to 820 bp region of GUS coding sequence were amplified through two step PCR reaction. During the first step, the regions were amplified within five PCR cycles with the primer pairs F1 x R and F2 x R. Both F1 and F2 primers contain the same 3'-end sequence matching to the one end of the target gene region and also include at the 5'-end: (i) sites for digestion with Bam HI (F1) and Sac I (F2) enzymes and (ii) sequences for tailing with B1 (F1) and B2 (F2) primers. Accordingly, two sites for digestion with Xho I and Xba I enzymes were inserted in the 5'-end of R1 primer, Table. During the second amplification step, attB1 / attB2 sequences were added to the F1 / F2 ends of the fragments after thirty cycles of PCR amplification from DNA fragments obtained within the first PCR step, with primers B1 x R and B2 x R, see Table for primer details. The amplified PCR fragments were digested with Xho I (B1,F1 x R1 fragment) and Xba I (B2,F2 x R1 fragment). In parallel to preparation of gene fragments, the region of second intron from ran gene of N. sylvestris was amplified using RF x RR primers. The sites for Xho I (RF) and Xba I (RR) enzymes were inserted at the 5'-end of the pointed primers respectively, Table. The amplified intron region included both exon / intron borders and thus provided the successful splicing of the sequences cloned at its both ends.

Fig. 1. Outlines of hybrid cloning procedure for construction of ihp- transformation vectors. PCR# I involves five cycles amplification of target gene region with the primer pairs F1 x R and F2 x R. One µl of PCR# I amplifications was subject of second PCR# II amplification of thirty cycles with primers B1 x R and B2 x R, see Table for primer details. The amplified PCR fragments are digested with Xho I (B1,F1 x R1 fragment) and Xba I (B2,F2 x R1 fragment). DNA fragment contained intron region from ran gene of N. sylvestris, with inserted Xho I and Xba I sites on the ends, was obtained through PCR# III amplification of nsran (AY563050) genomic DNA with RF x RR primers. Alternatively, the PCR tailed (FR x RR) intron region is cloned in plasmid vector and pDNA is isolated. The obtained PCR fragments are digested with Xho I and Xba I, and ligated. Assembled attB1-gus/intron/gus-attB2 fragments are cloned into: (i) att- sites of pDONOR entry vector or (ii) att-sites placed downstream of plant promoter region in plant transformation vector, through Clonase BP reaction. The intron-hairpin fragment inserted into entry plasmid vector is re-cloned with Bam HI and Sac I sites of suitable plant transformation vector.
**PCR# I**

- **target gene**
  - F1 / F2
  - R1

- **intron**
  - RF
  - RR

**PCR# II**

- **attB1**
  - B
  - XbXh

- **PCR# III**

**Digestion with XhoI and XbaI enzymes**

- **attB1**
  - B
  - Xh

- **attB2**
  - S
  - attB2

**Ligation**

- **attP1**
  - ccdB
  - attP2

**BP Clonase**

- **35S**
  - ihp
  - S
  - NOS

- **35S**
  - ihp
  - NOS

**Fig. 1.**
TABLE
Structure and sequence of the primers used in the hybrid cloning procedure for construction of \textit{ihp}-
transformation vectors. The gene-specific sequences are present with small italic letter; intron-specific
with small letter; sequences of restriction sites are marked with underlined capital letter; overlapping
sequences related to B1 and B2 primer tailing are present in bold / italic capital letter. (See Fig. 1, for
details concerning the position and orientation of the primers)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>GGTCTAGACTCGAGctatcaccgtttgtgtg</td>
<td>Gene specific primer</td>
</tr>
<tr>
<td></td>
<td>\textit{XbaI} \textit{XhoI}</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>CAAAAAACGAACGGCTGGATCCggttcggtgcaata</td>
<td>Gene specific primer with \textit{B1} tail</td>
</tr>
<tr>
<td></td>
<td>\textit{BamHI}</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>CAAGAAAAGCTGGGTAGCCTgggtcgtgcaata</td>
<td>Gene specific primer with \textit{B2} tail</td>
</tr>
<tr>
<td></td>
<td>\textit{SacI}</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>GGGGACAAAGTTTGTAACAAAAAAGCAGGCT</td>
<td>Primer for addition of \textit{attB1} region after F1 tailing</td>
</tr>
<tr>
<td>B2</td>
<td>GGGGACAACTTTGTAACAAAGAAGCCTG GGT</td>
<td>Primer for addition of \textit{attB2} region after F2 tailing</td>
</tr>
<tr>
<td>RF</td>
<td>AGATCTCGAGgtatgcatctagtttag</td>
<td>Intron specific primer</td>
</tr>
<tr>
<td></td>
<td>\textit{BglII} \textit{XhoI}</td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>GAATTCTAGAacttgaaaagattttag</td>
<td>Intron specific primer</td>
</tr>
<tr>
<td></td>
<td>\textit{EcoRI} \textit{XhoI}</td>
<td></td>
</tr>
</tbody>
</table>

sides. The obtained PCR fragments were
digested with \textit{XhoI} and \textit{XbaI}, thus gene-
rating compatible ends with the two GUS
fragments, Fig. 1. Alternatively to the PCR
amplification the \textit{FR} x \textit{RR}- tailed intron
region, preliminary cloned in plasmid vec-
tor, could be excised through enzyme di-
gestion. The performed ligation reaction
with all three fragments resulted in forma-
tion of intron-hairpin fragments, possessing
\textit{attB1} and \textit{attB2} sequences at their both
ends, Fig. 1.

Carrying out the BP Clonase® reaction
the obtained \textit{attB1-gus/intron/gus-attB2}
fragments were cloned into: (i) \textit{att}- sites of
pDONOR entry vector or (ii) directly
cloned downstream of the plant promoter
region in a plant transformation vector
containing \textit{att}-sites. Designing of the vec-
tors with lethal \textit{cdB} gene between the atte-
sites substantially facilitates the cloning
through elimination of the background
colonies carrying the functional \textit{cdB-}
plasmid (Gateway™ Technology, Invitro-
gen). When the intron-hairpin fragment is
cloned into the entry plasmid vector, e.g.
pDONOR, the re-cloning of the entire \textit{ihp}-
cassette in \textit{BamHI} and \textit{SacI} sites of a sui-
table plant transformation vector has to be
accomplished, Fig. 1.

Applications of the hybrid cloning
procedure
The described hybrid cloning procedure
could be directly applied using only the
available sequence data. A set of three
primers has to be designed prior starting of
the cloning. The sequences from the ends
of the target gene region are placed in the
3'-end of \textit{F1/F2} and \textit{R} primers, Table. The
sequences of the rest of the primers: \textit{RF};
\textit{RR}; \textit{B1}; and \textit{B2}, do not depend on the tar-
get gene and could be used in different
cloning experiments. If transformation
vector, containing \textit{attP1/ccdB/attP2} cas-
sette downstream of a plant promoter is
available, the assembled intron-hairpin
structure could be directly cloned in it.
Such transformation vector could be easily
constructed by PCR amplification of
\textit{attP1/ccdB} \textit{attP2} region from pDONOR
vectors and inserted downstream of a plant
promoter. [Gateway™ Technology, Invitrogen]. Alternatively, the intron-hairpin cassette could be readily re-cloned by using the restriction sites inserted in F1 and F2 primers. Since F1 and F2 primers are determined for each target gene, an efficient cloning strategy could be directly designed considering the cloning sites available in the transformation vector. The described cloning procedure is also very flexible in term of origin of the intron region and restriction sites used for assembling of the intron-hairpin structure. The using of different intron regions and restriction enzymes could be easily accomplished through designing of RF/RR and R primers, for details see Table. Introducing of two restriction sites in each intron primer gains more flexibility for selection of restriction enzymes for assembling of ihp-structure, for example BglII / XhoI in RF and EcoRI / XbaI in RR primers.

In conclusion, the application of the described hybrid cloning procedure allows reducing the time and the effort for construction of ihp-transformation vectors compared to the DNA digestion/ligation cloning, as well as does not require using of a specific transformation vector and lowers the cloning cost compared to Gateway™ cloning. The protocol could be applied in high-throughput manner, employing simultaneously ihp-cloning of more than ten target genes.

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REFERENCES