Original Article

Cloning of RNAi BBE Gene Fragments into the pGSA1285 Silencing Vector in Poppy Plant

Ali Ghorbani[1], Zabihollah Mohammad Ali Pur[2*], Ahmad Esmaili[3]

[1]MSc. in Biotechnology, University of Lorestan, Lorestan, Iran. [2]MSc. in Biotechnology, Payame Noor University of Isfahan, Isfahan, Iran. [3]PhD in Molecular Genetics, Tarbiat Modares University, Tehran, Iran.

Abstract

Poppy is one of the most important herbs in the pharmaceutical industry. It produces a wide range of isoquinoline alkaloids. Genetic manipulation of important enzymes involved in metabolic pathways could change poppy alkaloids. One of the techniques used for plant manipulation is gene silencing using RNA interference. In this study, RNAi technique was used to investigate the effect of gene silencing of Berberine Bridge Enzyme (BBE) gene on the major profile of Morphinan alkaloids network. After identification and isolation of BBE gene using specific primers, specific region of gene was selected for silencing. The target gene was cloned in T / A vector and sequenced. The fragment was then separated from the T / A vector by digestion. The silencing fragment was cloned in pGSA1285 vector in the sense strand. After confirmation of the presence of silencing fragment in the sense strand, pGSA1285 vector was digested again with digestive enzymes and the silencing fragment was cloned in antisense strand. Then, the whole silencing construct containing sense intron and antisense was confirmed by digestion.

Keywords: Gene silencing, RNA interference, BBE gene, Sense, Antisense

INTRODUCTION

Gene silencing is referred to as the phenomenon of complete or reduced expression of a previously expressed gene. RNA silencing was first observed in the experiments on the color of the petunia flower [1]. In recent years, various techniques have been used for manipulation of different plants, one of which is the gene silencing technique. BBE gene is one of the most important alkaloid pathway genes. The BBE enzyme is one of the most important enzymes of the network in the upstream of the three major branches of this alkaloid pathway [2]. RNAi gene silencing generally occurs at both transcriptional and post-transcriptional levels (PTGS). Silencing at the transcription stage is done by promoter methylation. Silencing after transcription is done by creating a double-stranded RNA and thus the desired product will not be produced. Antisense, RNAi, and virus induced gene silencing (VIGS) could be mentioned as after transcription methods. The RNAi mechanism is maintained in a wide range of eukaryotic organisms except *Saccharomyces cerevisiae*. In the antisense technique, the sense and the antisense fragments are linked together. The basis is a non-coding fragment that binds to the coding mRNA and prevents its expression. In RNAi technique, inhibition is accomplished by double-stranded small interfering RNA known as siRNA.

RNAi can be produced directly from dsRNAs, but in plants it is usually produced by changes in the construct and creation of hairpin RNAs. After transcription, the reverse sequence of a dsRNA structure and the intron fragment take the shape of a hairpin. Dicer, or Dicer-like protein (DCL), which is a part of the IIRNase I enzymes, then converts double-stranded RNA into 21-25 nucleotide fragments called siRNAs. The resulting siRNAs will be able to propagate through the phlegm throughout the plant. These siRNAs are then bound by the RISC complex, which is composed of several proteins, including argonate 5 protein. This complex will be able to identify and bind to its complementary sequence [3]. In the RNAi silencing mechanism, the enzyme responsible for mRNA cleavage is called a slicer. AGO1 is one of the most important slicers involved in RNAi [4].

Address for correspondence: Ali Ghorbani, MSc. in Biotechnology, University of Lorestan, Lorestan, Iran.
Email: zabihalipoor@yahoo.com

How to cite this article: Ghorbani, A., Mohammad Ali Pur, Z., Esmaili, A. Cloning of RNAi BBE Gene Fragments into the pGSA1285 Silencing Vector in Poppy Plant. Arch Pharma Pract 2020;11(S1):94-8.

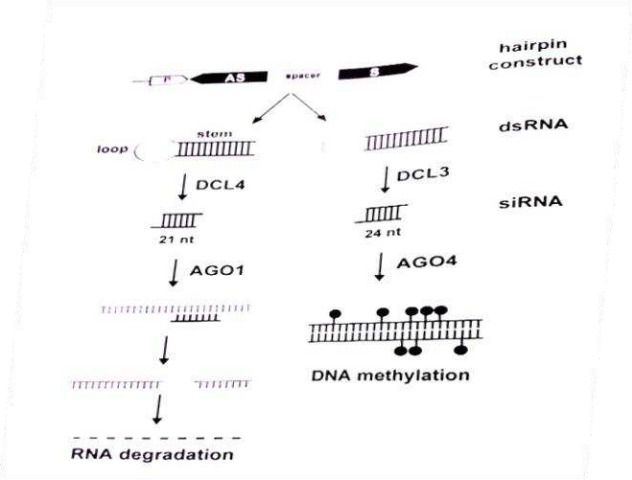


Figure 1- RNAi-Induced Silencing Pathways Post RNA transcription

The RNAi construct produces a double-stranded RNA product that is processed by both DCL3 and DCL4 enzymes separately. The DCL3 enzyme produces 24 nucleotide fragments of siRNA that bind to AGO4 and eventually methylate genomic DNA at the site of cytosines. But DCL4 produces 24 nucleotide fragments that bind to AGO1 to be directed to its complementary sequence in the mRNA.

In biotechnology and functional genomics applications, the 300-300 bp fragments of the target gene encoding are cloned into specific viral vectors such as PTRV2 (Tobacco Rattle Virus) through the VIGS technique and introduced into the plant by Agrobacterium along with the PTRV2 helper vector. After replication of viral fragments in vectors in the plant and production of dsRNA from the target gene, a large portion of transcripts related to that gene are [5]

In a study, the increased sybp80b3 gene expression was leaded to a 450% increase in the total amount of alkaloids as well as morphine and codeine. On the other hand, silencing of this gene by antisense method resulted in a decrease in the total profile of alkaloids. Temporal silencing by virus induced genes in both CODM and T6ODM genes blocked the alkaloids pathway at morphine and codeine levels [6, 7].

Materials and Methods

E.coli DH5a was used for building silencing constructs and its maintenance, as well as Agrobacter rhizogenes strains LBA9402, ATCC15834, A4 and MSU were used to induce and produce capillary roots. Antibiotics were used as selective markers in LB medium. Cramphenicol antibiotics were used to select pGSA1285. The primers used in this study were designed by vevtor nti software, which are listed below (Table 1):

Table 1. Primers sequences

Aim	Primer Sequence	Primer name
Silencing	5´ACTAGTGGCGCGCCCCATCTCT GAACTAAACACA3´	BBEQ-F
BBE gene	5´GGATCCATTTAAATTCTCCAAT CTATCCCTCCAATA3´	BBEQ-R
Transgenic Confirmation	5´CTCCTGACATCAAACTCGTC3´	RolC-F
Capillary roots	3´TGCTTCGAGTTATGGGTACA5´	RolC-R

The pTZ57R / T carrier was used for cloning and maintenance of pGSA1285 for silencing.

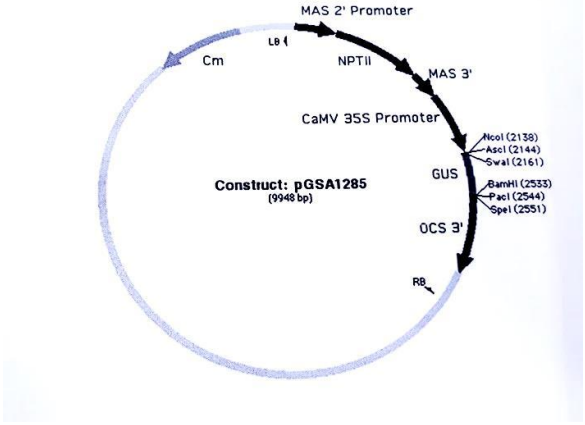


Figure 2. PGSA1285 Silencing Vector.

This vector has chloramphenicol and kanamycin bacterial resistance genes, hygromycin gene for plant, OCS3´ and MAS3 sequences which are signal for *Agrobacterium tumefaciens*, GAS gene, 360 bp long, ChsA intron, 1353 bp long, viral promoter caM34 and a plant promoter MAS1´.

BBEQ-F and BBEQ-R primers were designed to construct BBE gene silencing construct using vector NTI software. A region of 418 bp length was selected which was from nucleotide 1030 to nucleotide 1448 (Table 2). The cut site of this sequence was investigated using the NTI vector software,

Table 2- The sequence of the silencing primers and their cut site

Primer name	**Primer sequence**
	SpeI **AscI**
BBEQ-F	**5´ACTAGTGGCGCGCCCCATCTCTGAACTAAACA CA3´**

	BamHI SwaI
BBEQ-R	5´GGATCCATTTAAATTCTCCAATCTATCCCTCC AATA3´

To identify and amplify the BBE1 gene it is located at the center of the alkaloid network of the poppy plant and plays an important role in regulating several pathways, including sanuinarine morhinane. The full sequence of BBE1 gene with accession number AFO25430 is available at NCBI database. The sequences of this gene and the region selected for silencing are shown in Figure 3.

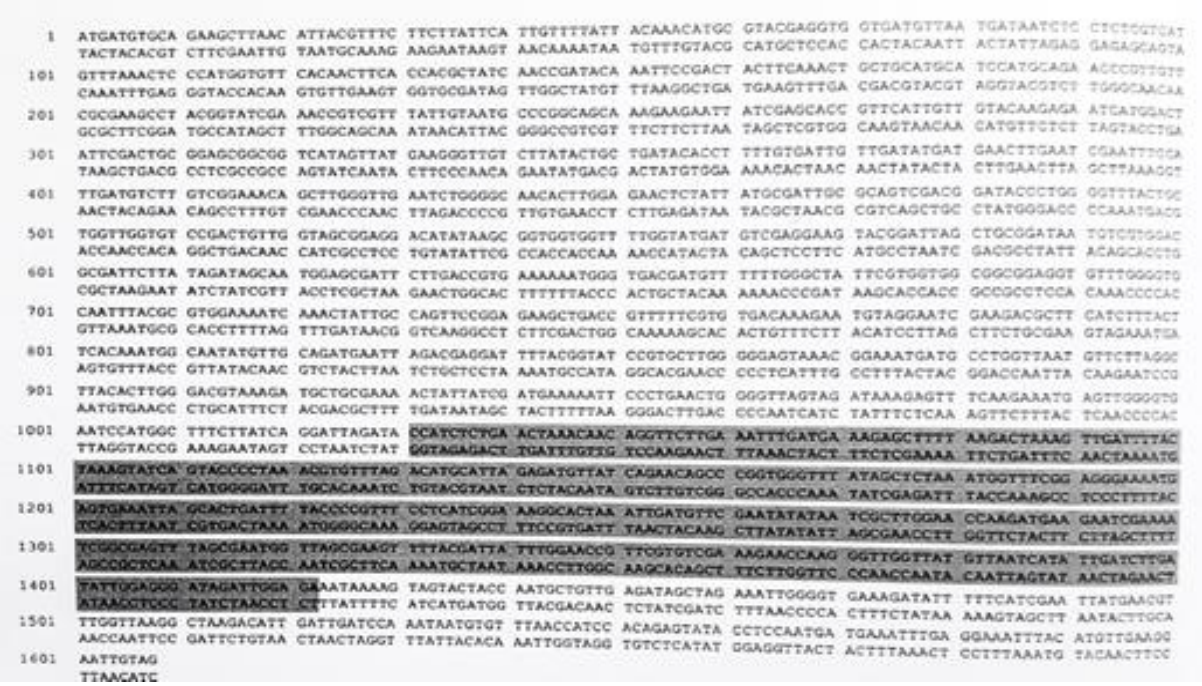


Figure 3- The sequence of the BBE1 gene and the region selected for silencing

To clone BBERNAI fragments into the pGSA1285 silencing vector two-step cloning was necessary that two fragments of senes and antisense should be transferred to this vector. In the first step, pGSA1285 and T / A vectors were double digested by Spel and Swal enzymes to put the clone of the sense fragment into the pGSA1285 vector. According to Fermentas company site information, the best reaction conditions were provided. The reactions were carried out for 16 hours at 37 ° C. The digested product was transferred to 1% gel electrophoresis. It was observed that a 418-bp fragment was ejected from the T / A vector, which was the desired silencing fragment, and pGSA1285 vector was linear. The desired fragments were separated from the gel. Immediately after extraction, the reaction of binding of the sense silencing fragment to the linearized vector pGSA1285 was done. The binding reaction components are as shown in Table 3.

Table 3. The components of the binding reaction of the sense fragment to the pGSA1285 vector

Amount (µl)	Reaction component	Amount (µl)	Reaction component
1	T4 ligase (10x)	6/5	Water nucleas free
13	Insert	2/5	Buffer (10x)
		7	pGSA1285 (55ng/ µl)

After confirmation of the presence of the sense fragment in pGSA1285, both PTZ57R / T and pGSA1285 (containing sense fragment) vectors were cut with BamHI and Spel digestive enzymes. The digestion reaction was carried out overnight at 37 ° C. The enzymatic digestion reaction products were purified by gel extraction kit. The connection steps were performed in the antisense direction, the same way as the sense binding stage. Transfection of the susceptible *E.coli* DH5a strain was performed by binding mix. The bacteria were cultured on solid LB medium containing chloramphenicol. Positive colonies were identified by PCR reaction and one of these colonies was cultured overnight for extraction of plasmid. The extracted plasmid was digested with the two BamHI and Spel enzymes to confirm once again the binding of the fragment to the antisense strand.

Discussion and Conclusion

After cloning of the silencing fragment in T / A vector to confirm the presence of silencing fragment, PCR was done

on the white colonies obtained by transgenesis. The expected band (418 bp) was amplified. The results can be seen in Figure 4.

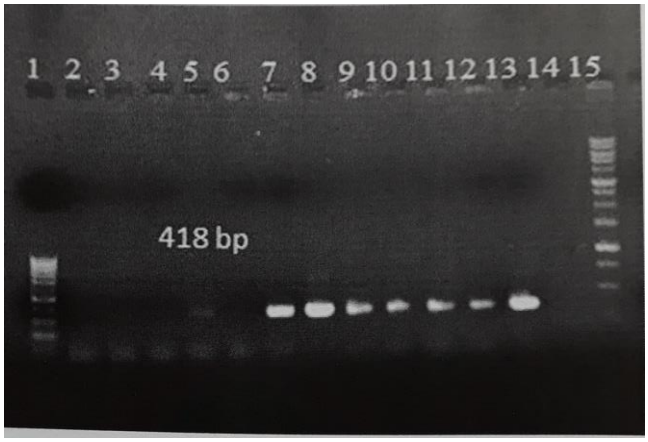


Figure 4- PCR colony to confirm the presence of the silencing fragment in the T / R vector. Wells 1 and 15: molecular weight markers of 100 pb and kb1, 14: negative control wells, 13: positive control, wells 2 to 12: silencing fragment amplification.

For further confirmation, plasmid positive colonies were extracted and digested with AscI and SwaI enzymes. The desired fragment of 418 bp was obtained. The PCR product was sequenced. The sequencing results were analyzed by BLAST software and the accuracy of the cloned gene sequences was determined. To clone the BBE silencing fragment into pGSA1285 vector, two empty pGSA1285 and T / A containing BBE silencing fragment vectors were digested simultaneously with both AscI and SwaI. Due to the proximity of the cut sites on the pGSA1285 vector, as expected, no bands were observed after digestion. Figure 5 shows the enzymatic digestion.

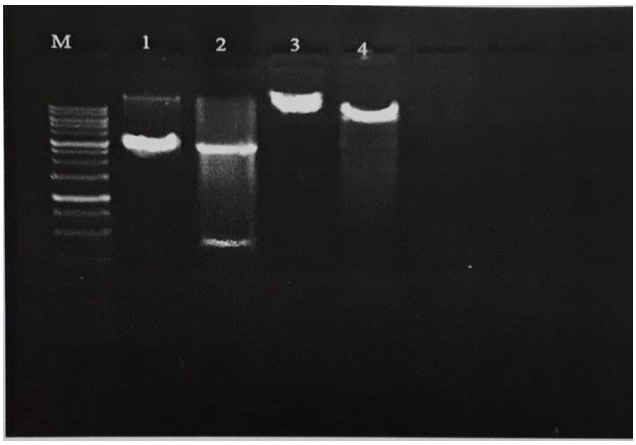


Figure 5- Digestion of two empty pGSA1285 and T / A containing M.BBE silencing fragment vectors, 1 kb molecular weight marker, well 1: digested undigested T / A vector, well 2: digested T / A vector containing BBE silencing fragment, well 3: non-digested pGSA1285 vector, well 4: digested pGSA1285 vector.

The confirmation of cloning BBE silencing fragment in pGSAA1285 vector was done by PCR of the clony and digestion. PCR with specific primers were used to confirm the presence of the constructs in the sense strand. Running of the PCR product on 1% agarose gel showed the amplification of 418 bp fragment.

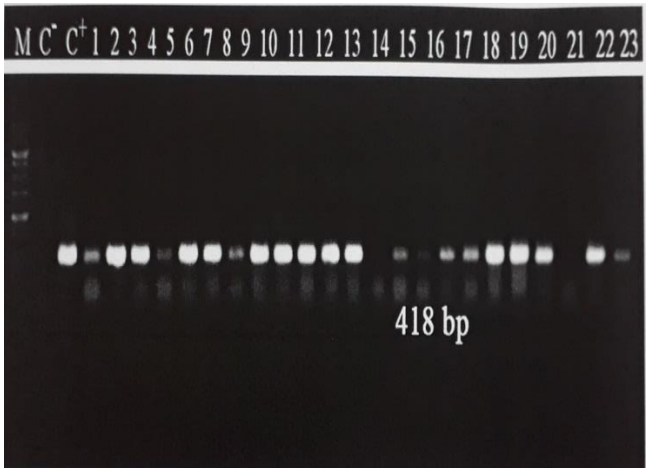


Figure 6- PCR cloning to confirm the insertion of the sense fragment in pGSAA1285 vector, well M: molecular weight marker, C-: negative control, C+:positive control, reaction, well 3: positive control of the reaction, well 1 to 14: amplification of the silencing fragment.

For further confirmation, plasmid positive colonies were extracted and digested with AscI and SwaI enzyme. The desired fragment with 418 bp length was obtained (Fig. 7).

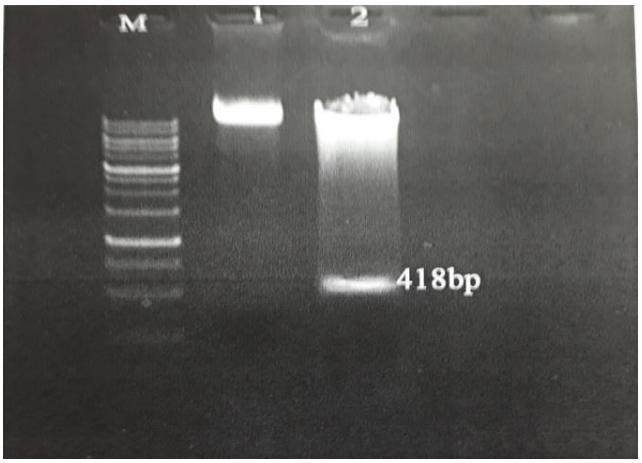


Figure 7- Digestion to confirm the insertion of the snse fragment in the pGSAA1285 vector. Well M: 1 kb molecular weight marker, well 2: non digested plasmid, well 3: digested plasmid.

The confirmation of cloning BBE silencing fragment in pGSAA1285 vector was done by PCR of the clony and digestion. PCR with specific primers were used to confirm the presence of the constructs in the sense strand. Running

of the PCR product on 1% agarose gel showed the amplification of 570 bp fragment.

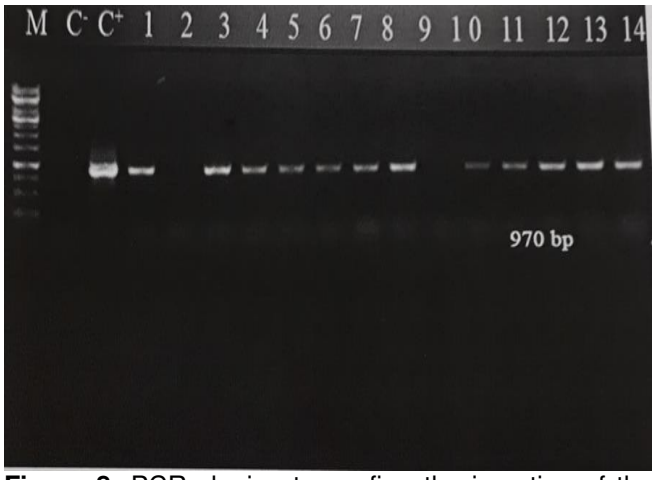
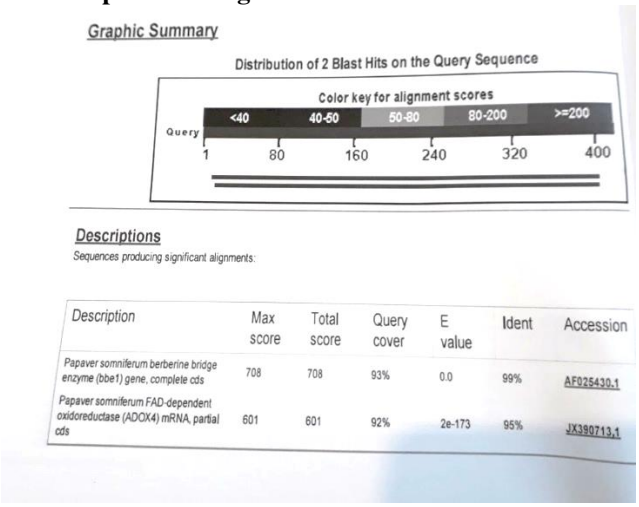


Figure 8- PCR cloning to confirm the insertion of the anti-sense fragment in pGSAA1285 vector, well M: molecular weight marker, C-: negative control, C+:positive control, well 1 to 14: amplification of the fragment.

Attachment 1:

Alignment and blast of the BBE gene in NCBI website

Appendix 7
Companion and gene BLAST BBE in NCBI site



REFERENCES

1. Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of achimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *The Plant Cell Online* 2, 279-289.
2. Dittrich, H., and Kutchan, T. M. (1991). Molecular cloning, expression, and induction of berberine bridge enzyme, an enzyme essential to the formation of benzophenanthridine alkaloids in the response of plants to pathogenic attack. *Proceedings of the National Academy of Science* 88, 9969-9973.
3. Gherbi H, Nambiar-Veetil M, Zhong C, Félix J, Autran D, Girardin R, Vaissayre V, Auguy F, Bogusz D, Franche C. Post-transcriptional gene silencing in the root system of the actinorhizal tree Allocasuarina verticillata. Molecular Plant-Microbe Interactions. 2008 May;21(5):518-24.
4. Hirai, S., and Kodama, H. (2008). RNAi vectors for manipulation of gene expression in higher plants. *Open Plant Sci* J 2,21-30.
5. Senthil-Kumar M, Mysore KS. New dimensions for VIGS in plant functional genomics. Trends in plant science. 2011 Dec 1;16(12):656-65.
6. Hagel, J. M., and Facchini, P .J. (2010) Dioxygenases catalyze the O-demethylation steps of morphine biosynthesis in opium poppy. *Nature Chemical Biology* 6, 273-275.
7. Hagel, J. M., and Facchini, P .J. (2013) Benzylisoquinoline alkaloid metabolism-acenttury of discovery and a brave new world. *Plant and Cell Physiology*, pct020.