

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

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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 argonaute 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].

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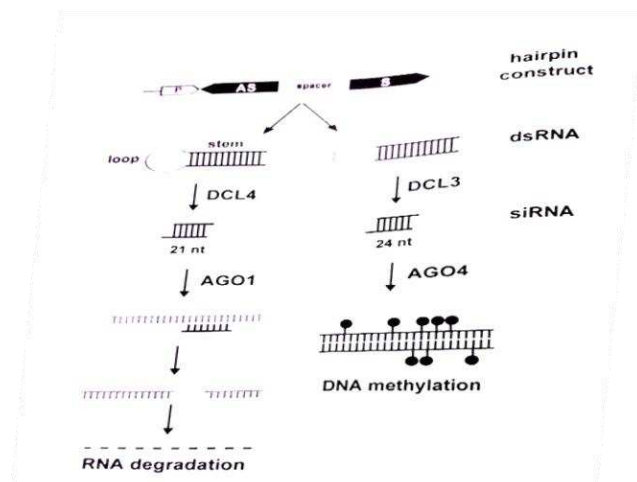


Figure 1- RNAi-Induced Silencing Pathways Post RNA transcription

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 led 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'ACTAGTGGCGCGCCCCATCTCTGA AACTAAACACA3'	BBEQ-F
BBE gene	5'GGATCCATTAAATTCTCCAAT CTATCCCTCCAATA3'	BBEQ-R
Transgenic Confirmation	5'CTCCTGACATCAAACCTCGTC3'	RoIC-F
Capillary roots	3'TGCTTCGAGTTATGGGTACA5'	RoIC-R

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

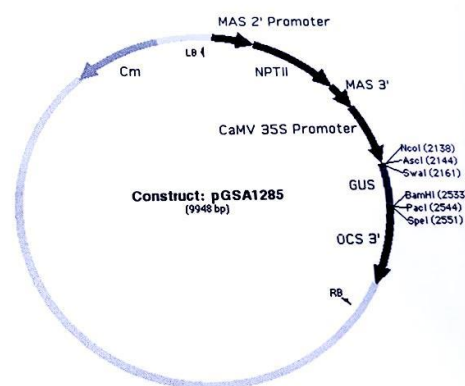


Figure 2. PGSA1285 Silencing Vector.

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	Ascl
BBEQ-F	5'ACTAGTGGCGCGCCCCATCTCTGA AACTAAACACA3'	

	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 morphinane. 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.

1	ATGATGTGCA GAAGCTTAAC ATTACGTTTC TCTTATTCA TTGTTTTATT ACAAACATGC GTACGAGGTG GTGATGTAA TGATAATCTC CTCCTGTTAT	TACTACACGT CTTCGAATTT TAATGCAANG AAGAATAAGT AACAAAAATAA TGTTTGTAGC CATGCTCCAC CACTACAATT ACTATTAGAG GAGAGCAATA
101	GTTTAAACTC CCATGGGTGT CACAACCTCA CCACGCTATC AACCGATACA AATTCGGACT ACTTGAAACT GCTGCATGCA ACCCTGTGTT	CAAAATTTGAG GGTACCACAA GTGTTGAAGT GGTGGGATAG TTGGCTATGT TTAAGGCTGA TGAAGTTTGA CGACGTACGT AGGTACGCTC TGGGCAACAA
201	CCGCAAGCCT ACGGTATCGA AACCGTGCTT TATTGTAATG CCGCGCAGCA AAGAAGAATT ATCGAGCACC GTTCATTGTT GTACAAGAGA ATCATGCACT	CGCGTTTCGA TCCCATAGCT TTGGCAGCAA ATAACATTAC GGGCGGTGCT TTCTCTTAA TAGCTCGTGG CAAGTAAGCA CATGTTCTCT TAGTACCTGA
301	ATTGCACTGC GGAGCGGCGG TCATAGTTAT GAAGGGTTGT CTTACTACTGC TGATACACCT TTTGTGATTG TTGATATGAT GAACCTTGAAT CGAATTTCGA	TAACTGTACG CTCGCGCGCC AGTATCAATA CTTCCCAACA GAATATGACG ACTATGTGGA AACTATACTA CTTGAACTTA CTTTAAAGCT
401	TTGATGCTCT GTCGGAAMCA GCTTGGGTGG AATCTGGGGC AACACTTGA GAACCTCTATT ATCGGATTGC GCAGTCGACG GATACCCCTG GGTTTACTGC	AACATACAGA CAGCGTTTGT CGAACCCCAAC TTAGACCCCG TTGTGAACCT CTTGAGATAA TACGCTAACG CGTCAGCTGC CTATGGGACC CCAATGACG
501	TGGTTGGTGT CCGACTGTTG GTAGCGGAGT ACATATAAGC GGTGGTGGTT TTGGTATGAT GTCGAGGAAG TACGGATTAG CTCGCGATAA TGTCTGGAC	ACCAACCCAA GGCTGACAAC CATCGCCTCC TGTATATTGG CCAACACCAA AACCTACTA CAGCTCCTTC ATGCTTAATC GACGCTATT ACAGCACTG
601	CGGATTCTTA TAGATAGCAA TGGAGCGGATT CTGACCGGTG AAAAAATGGG TGACGATGTT TTTTGGGCTA TFCGTTGGTG CGCGCGGAGT GTTTGGGGTG	CGCTAAGAAAT ATCTATCGTT ACCTCGCTAA GAACCTGGAC TTTTATACCC ACTGCTACAA AAAACCCGAT AAGCACCACC CCGCGCTGCA CAACCCCCAC
701	CAATTTACGC GTGGAAAATC AAATATTGCG CAGTTCGGGA GAAGCTGACC GTTTTTCGTG TGACAAAGAA TGTAGGAATC GAAGACGCTT CATCTTTACT	GTTAAATGCG CACCTTTTAG TTTGATAACG GTCAAGGCTC CTTGCACTG CAAAAGCAC ACTGTTTCTT ACATCCTTAG CTTCTCGGAA GTAGAAATGA
801	TCACAAATGG CAATATGTTG CAGATGAATT AGACGAGGAT TTTACGGTAT CCGTCTGGTG GGGAGTAAAC GGAAATGATG CCTGTTAAT GTTCTTAGG	AGTGTTTACC GTTATACAAAG GTCTACTTAA TCTGCTCTTA AAATGCCATA GCGCAGAAC CCCTCATTTG CTTTACTACT GGACCAATTA CAAGATCTCG
901	TTACACTTGG GACGTAAACG TGCTGCGAAA ACTATTATCG ATGAAAAAAT CCGTGAACCT GGGTTAGTAG ATAAAGAGTT TCAAGAAATG AGTTGGGGTG	AATGTGAACC CTGCATTTCT ACGACGCTTT TGATAATAGC TACTTTTAA GGGACTTGAC CCAATCATC TATTTCTCAA AGTTCTTTAC TCAACCCGAC
1001	AATCCATGCG TTTCTTATCA GGAATGATA CCACTCTGCA ACTAATCAAC AGGTTCTTGA AATTGTATGA AAGAGCTTTT AAGACTAAG TTGATTTTAC	TTAGGTACCG AAAGAATAGT CCTAATCTAT GGTAGAGACT TGATTTGGTG TCCAGAACT TTAACCTACT TCTCGAAA TCTGATTTC AACAAAAAG
1101	TAAAGTATCA GTACCCCTAA ACGTGTTTAG ACATGCATTA GAGATGYAT CAGAACAGCC CCGTGGGTTT ATAGCTCYAA ATGTTTTCGG AGGGAAAATG	ATTTCATAGT CATGGGGATT TGACAAAATC TGTACGTAAT CTCTACAATA GGTGTTGCGG GGCACCCAAA TATCGAGATT TACCAGAGCC TCCCTTTTAC
1201	AGTGAATTA GCAGTGATT TACCCGTTTT CTTATGCGA AAGGCACAA ATTGATGTTT GAATATATAA TCGCTTGGAA CCAAGATGAA GAATCGAAAA	CCGACTTAAT CCGTATGAAA ATGCGGCAAA GGAATGAGCT TTCCGTTGTT TAATACAAAG CTTATATATT AGCGAAGCTT GGTCTTACTT CTGAGCTTTT
1301	TCGCGAGTGT TAGCGAATGG TTAGCGAAGT TTTACGATTA TTTGGAACCG CCGTGTGCGA AAGAACCAGG GGTGGTTTAT GTTAATCATA TTGATCTTGA	AGCGGCTCAA ATCGCTTACC AATCGCTTCA AAATGCTAAT AAACCTTGCG AAGCAGACTC TCTGTGTTTC CCAACCAATA CAATTAGTAT AACTAGAATC
1401	TATTGGAGCG ATAGATTGGA GAAATAAAG TAGTACTACC AATGCTGTTG AGATAGCTAG AAATGGGGT GAAAGATATT TTTCTCGGAA TTATGAGCTG	ATAAGCTGCG TATCTAACCT CTTTATTTTC ATCATGATGG TTACGACAA CTTATCGATC TTTTACCCCA CTTTCTATAA AAGTAGCTTT AATACCTGCA
1501	TTGGTAAAG CTAGACATT GATTGATCCA AATAATGTGT TTAACCATCC ACAGAGTATA CTTCCAATGA TGAATTTTGA GGAATTTTAC ATGTTGAAAG	AACCAATTCG GATCTGTAA CTAACTAGGT TTTTACACA AATTGGTAGG TGTCTCATAT GGAGTTTACT ACTTTAAACT CTTTAAATG TACAACCTTC
1601	AATTGTAG TTAACATC	

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 senses and antisense should be transferred to this vector. In the first step, pGSA1285 and T / A vectors were double digested by SpeI and SwaI 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 nucleus 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 SpeI 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 SpeI 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.

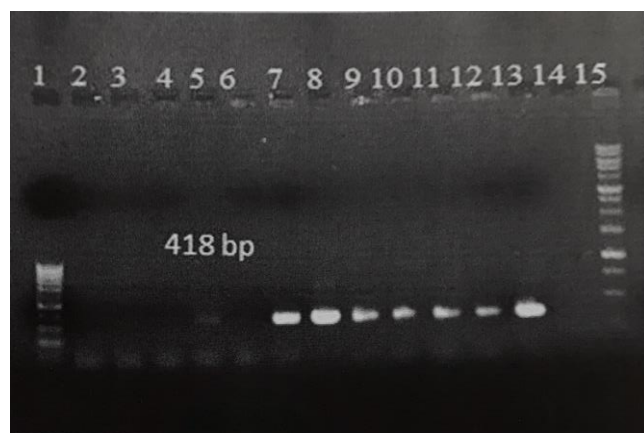


Figure 4- PCR colony to confirm the presence of the silencing fragment in the T / R vector. Wells 1 and 15: molecular weight markers of 100 pb and kb1, 14: negative control wells, 13: positive control, wells 2 to 12: silencing fragment amplification.

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.

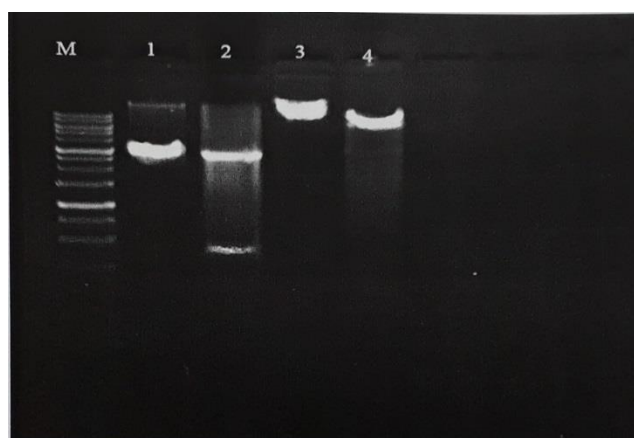


Figure 5- Digestion of two empty pGSA1285 and T / A containing M.BBE silencing fragment vectors, 1 kb molecular weight marker, well 1: digested undigested T / A vector, well 2: digested T / A vector containing BBE silencing fragment, well 3: non-digested pGSA1285 vector, well 4: digested pGSA1285 vector.

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.

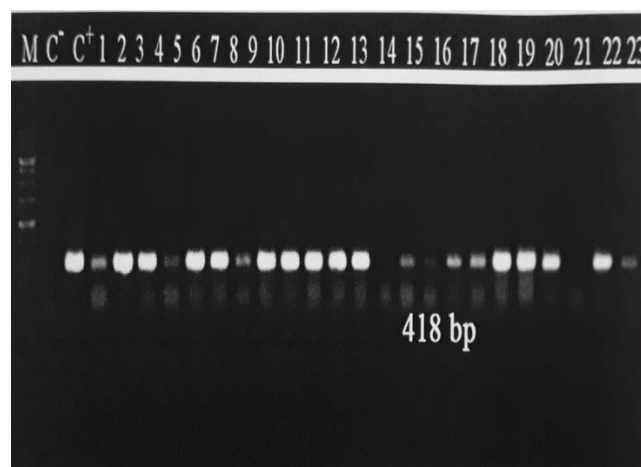


Figure 6- PCR cloning to confirm the insertion of the sense fragment in pGSAA1285 vector, well M: molecular weight marker, C-: negative control, C+:positive control, reaction, well 3: positive control of the reaction, well 1 to 14: amplification of the silencing fragment.

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).

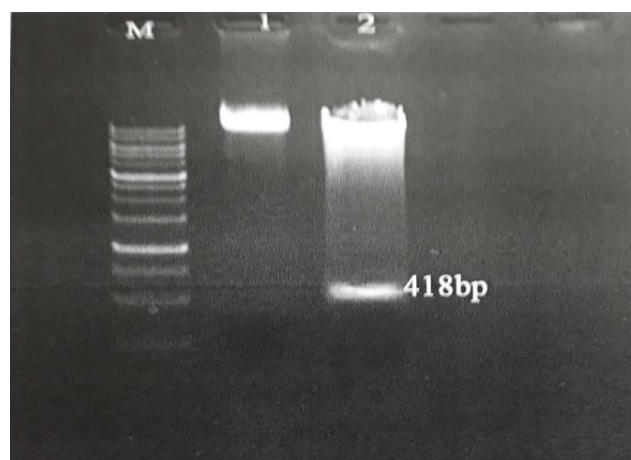


Figure 7- Digestion to confirm the insertion of the snse fragment in the pGSAA1285 vector. Well M: 1 kb molecular weight marker, well 2: non digested plasmid, well 3: digested plasmid.

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.

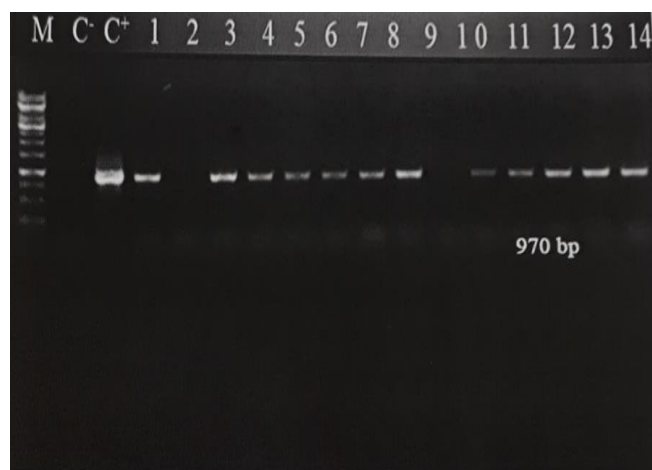


Figure 8- PCR cloning to confirm the insertion of the anti-sense fragment in pGSAA1285 vector, well M: molecular weight marker, C-: negative control, C+:positive control, well 1 to 14: amplification of the fragment.

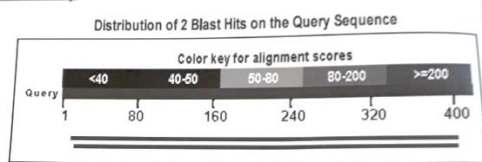
Attachment 1:

Alignment and blast of the BBE gene in NCBI website

Appendix 7

Companion and gene BLAST BBE in NCBI site

Graphic Summary



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
<i>Papaver somniferum</i> berberine bridge enzyme (bbe1) gene, complete cds	708	708	93%	0.0	99%	AF025430.1
<i>Papaver somniferum</i> FAD-dependent oxidoreductase (ADOK4) mRNA, partial cds	601	601	92%	2e-173	95%	JX390713.1

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