

利用长引物嵌套式反向 PCR 方法克隆雅致枝霉 Δ^6 -脂肪酸脱氢酶基因上游序列

A Method Using Long Primers for Cloning the Upstream Sequence of Delta-6 Fatty Acid Desaturases Gene of *Thamnidium elegans* by Nested Inverse PCR

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摘 要 用限制酶 *EcoR* I、*Kpn* I 分别对雅致枝霉 As3.2806 基因组 DNA 进行消化,然后在低浓度条件下利用 T4DNA 连接酶使 DNA 自身环化。根据已知基因序列,设计一对长度为 35nt 的长反向引物和两对较短的引物,以基因组连接产物为模板,通过三轮嵌套式 PCR 反应,获得一长度约为 4kb 的扩增片段。经序列测定表明得到了 Δ^6 -脂肪酸脱氢酶基因上游序列约为 1.3kb,初步序列分析显示该序列为一潜在的启动子序列。

关键词 雅致枝霉,长引物,巢式反向 PCR, Δ^6 -脂肪酸脱氢酶

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Abstract *Thamnidium elegans* is a kind of phycomycete that produces essential unsaturated fatty acids, particularly γ -linolenic acid. In this process, Δ^6 -Fatty acid desaturase (D6D) plays a key role due to its enzymatic properties that catalyze the Δ^6 site dehydrogenation of precursor linoleic acid (18 $2\Delta^9,12$ -n-6) and α -linolenic acid (18 $3\Delta^9,12,15$ -n-3). This reaction is the first and rate-limiting step of highly unsaturated fatty acid (HUFA) synthesis pathways. After we have isolated and cloned the gene coding Δ^6 -fatty acid desaturase from *Thamnidium elegans* As3.2806 (GenBank accession number DQ099380), our interest focuses on the promotion and regulation of the gene transcription. To achieve this aim, we designed long primers and used nested inverse PCR to amplify DNA flanking sequences. First, genome of *Thamnidium elegans* was extracted and digested with restriction enzymes *EcoR* I and *Kpn* I, respectively. Then we ligated the digested DNA with T4 ligase at low concentration which is propitious for linear DNA to joint intramolecule. According to the sequence of Δ^6 -fatty acid desaturase gene of *Thamnidium elegans*, we designed a couple of 35nt long inverse primers and two couples of shorter inverse primers for inverse PCR. Three rounds of PCR reactions were performed. In the primary reaction, the ligated DNA was used as a template, and the product was used as the template of the secondary reaction, the tertiary reaction was achieved in the same way. After all the three rounds of reactions, we got a nice product about 4kb from the *EcoR* I digested sample, in which a 1.3kb 5' upstream sequence (GenBank accession number DQ309425) of Δ^6 -fatty acid desaturase gene containing several putative regulatory elements including TATA

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box, FSE-2, AP-1 sites, CCAAT *cis*-element site and STRE-binding site was derived after sequencing. All of these implied intensely that this 1.3kb fragment is a condition-regulated promoter. It is the first report about *Thamnidium elegans* Δ^6 -fatty acid desaturase gene promoter. The procedure described here is a rapid and simple method and particularly useful to isolate flanking sequences from fungal genome.

Key words *Thamnidium elegans*, long primers, nested inverse PCR, Δ^6 -fatty acid desaturases

高不饱和脂肪酸(Highly unsaturated fatty acids, HUFA)如花生四烯酸(20:4 n-6)和二十二碳六烯酸(DHA)不仅具有多种生理功能包括促进脑发育,心血管功能,炎症应答反应等^[1-4],而且是构成生物膜磷脂的重要组成,起到细胞内信号传导和基因表达,细胞分化,调节脂代谢的功能^[5-8]。 Δ^6 -脂肪酸脱氢酶作用于生物体合成高不饱和脂肪酸的第一步催化反应将亚油酸转化为亚麻酸,是高不饱和脂肪酸合成过程的限速酶,因此受到多个水平的调节,虽然目前已从多种生物体内克隆到 Δ^6 -脂肪酸脱氢酶基因序列,但对丝状真菌来源的启动子序列的克隆分析尚未见报道,所以其转录水平的调节有待于深入研究。在这一背景下,本研究室以雅致枝霉(*Thamnidium elegans*)As3.2806为研究对象,在克隆了其 Δ^6 -脂肪酸脱氢酶基因序列(GenBank注册号: DQ099380)的基础上,利用长引物嵌套式反向PCR方法克隆该基因上游1.3kb的序列,通过分析具有多个转录调节元件(GenBank注册号: DQ309425),是首次报道 Δ^6 -脂肪酸脱氢酶基因上游序列,通过实验证明该方法是克隆丝状真菌已知基因上游序列简单有效的方法。

1 材料与方法

1.1 材料

1.1.1 实验菌株:雅致枝霉(*Thamnidium elegans*)As3.2806,购自中国科学院微生物研究所。大肠杆菌(*Escherichia coli*)DH5 α ,本研究室保存。

1.1.2 质粒和基因序列:pGEM-T vector,购自Promega公司;雅致枝霉As3.2806 Δ^6 -脂肪酸脱氢酶基因,本研究室克隆,GenBank号:DQ099380。

1.1.3 培养基:

(1)土豆培养基(1L):土豆200g,葡萄糖20g, KH_2PO_4 3g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.5g,琼脂12g, pH自然。(2)LB培养基(1L):蛋白胨10g,酵母粉5g, NaCl 5g,用NaOH调pH至7.4,固体培养基补加15g琼脂。

1.1.4 生化试剂:各种限制酶及T4DNA连接酶购自大连宝生物工程公司;PCR用2 \times Master Mix、植物基因组提取试剂盒、PCR产物回收试剂盒、核酸分子量标准(Marker)购自北京天为时代有限公司;T-载体连接试剂盒购自Promega公司;寡核苷酸引物由北京奥科生物公司合成;其余有机试剂和药品均为国产分析纯。

1.2 方法

1.2.1 基因组的提取:按植物基因组提取试剂盒说明书进行。

1.2.2 限制性内切酶消化:分别用EcoRI、KpnI对基因组DNA进行消化,反应体系如下:基因组DNA 15 μL (30ng/ μL), 10 \times Buffer 5 μL ,相应的限制性内切酶 1 μL (10~15u/ μL),加水补至50 μL ,在37 $^\circ\text{C}$ 反应2h,65 $^\circ\text{C}$ 水浴15min灭活限制性内切酶。

1.2.3 连接反应:取酶切产物6 μL (9ng/ μL),加10 \times Ligase Buffer 2 μL , T4连接酶 1 μL (350u/ μL),加水补至20 μL ,4 $^\circ\text{C}$ 反应过夜。PCR反应体系:2 \times Master Mix 12.5 μL ,上下游引物(20 $\mu\text{mol/L}$)各1 μL ,模板DNA(连接产物或上一轮PCR产物)1 μL ,加水补至25 μL 。PCR产物回收连接至pGEM-T载体,E. coli DH5 α 感受态细胞的制备、转化及转化子的筛选、质粒提取等见参考文献[9]。引物合成于北京奥科公司(见表1),测序由北京三博公司完成。

表1 实验所用引物

Table 1 Primers used

No.	Sequence	Vsage
P1	5'-CCTFGGGTGATTGGTTCATGGGTGGTTGAAGTAT-3'	First round PCR reaction
P2	5'-GATGCCACGACAGCCAAAGTGAAGTACACCATAA-3'	First round PCR reaction
P3	5'-TGCCTCGTCACAACCTGCCTAC-3'	Second round PCR reaction
P4	5'-ACGCATGGCATGAAATACGTC-3'	Second round PCR reaction
P5	5'-CACTGGATATTACATCCAAGCTCTC-3'	Third round PCR reaction
P6	5'-TGAACCTCATAGCCTCTCATCTCC-3'	Third round PCR reaction
P7	5'-CGTCTAGAGAATTCCTTCAAACAACAATTT-3'(Xba I)	PCR detection and subcloning
P8	5'-CGAAGCTTCATCTTACTGGTAATTAATTATGTC-3'(Hind III)	PCR detection and subcloning

表 2 各 PCR 反应条件

Table 2 Parameters for every PCR

PCR reaction	Primers	Thermo cycle parameters	Cycles
First round PCR (two-step PCR)	P1、P2	94°C 3min	1
		94°C 30s 68°C 10min	35
		72°C 10min	1
Second round PCR (touch-down PCR)	P3、P4	94°C 3min	1
		94°C 30s 60°C 1min 72°C 9min	2
		94°C 30s 58°C 1min 72°C 9min	2
		94°C 30s 56°C 1min 72°C 9min	2
		94°C 30s 54°C 1min 72°C 9min	25
		72°C 10min	1
Third round PCR (touch-down PCR)	P5、P6	94°C 3min	1
		94°C 30s 60°C 1min 72°C 9min	2
		94°C 30s 58°C 1min 72°C 9min	2
		94°C 30s 56°C 1min 72°C 9min	2
		94°C 30s 54°C 1min 72°C 9min	25
		72°C 10min	1

2 结果

2.1 雅致枝霉 Δ^6 -脂肪酸脱氢酶基因旁侧序列的扩增

利用第一轮长引物两步法反向 PCR, 及随后两轮嵌套式落降 PCR(见图 1) 扩增出 4.0kb 左右的片段(图 2) 将其回收连接于 pGEM-T, 转化 DH5 α , 测序结果表明克隆得到长 3.8kb 序列, 与已克隆雅致枝霉 Δ^6 -脂肪酸脱氢酶基因序列(GenBank 号: DQ099380) 对比, 分别在雅致枝霉 Δ^6 -脂肪酸脱氢酶基因 5' 端上游延伸 1.3kb 与该基因 5' 端有 132bp 重叠(见图 3), 在 3' 端下游延伸 2.5kb, 与基因 3' 端有 122bp 重叠(见图 4), 并具有 *Sac* I 酶切位点, 与质粒双酶切鉴定结果一致。

2.2 对扩增产物的 PCR 检测

为验证克隆的正确性从得到的 5' 上游序列两端设计引物 P7 和 P8, 分别以带有 3.8kb 扩增片段的

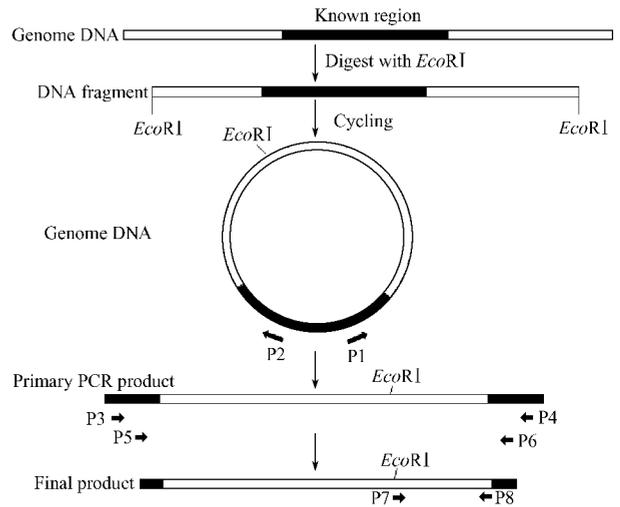


图 1 反向 PCR 及 PCR 示意图

Fig.1 The process of inverse PCR

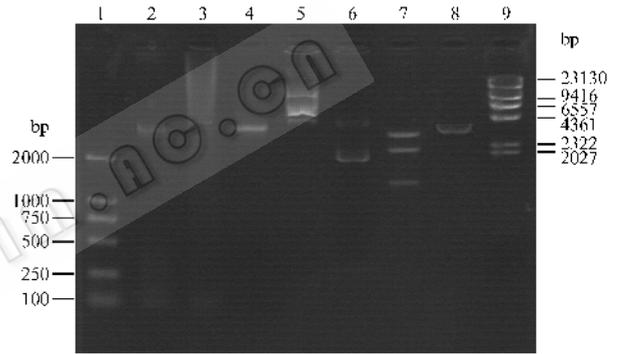


图 2 PCR 产物电泳分析

Fig.2 Analysis of the result of inverse PCR

1: DL2000 marker; 2: secondary PCR product; 3: primary PCR product; 4: tertiary PCR product; 5: T-vector with insertion fragment; 6: T-vector plasmid; 7: insertion plasmid digested by *Sac* I and *Nco* I; 8: T-vector plasmid digested by *Sac* I and *Nco* I; 9: λ DNA digested by *Hind* III.

质粒、基因组 DNA 和 *Eco*RI 消化后的基因组 DNA 为模板进行扩增, 反应条件: 94°C 3min; 94°C 30s, 45°C 1min, 72°C 2min, 5 cycles; 94°C 30s, 58°C 1min, 72°C 2min, 25 cycles; 72°C 10min. 结果如图 5 所示,

5'-PCR	CTTCCTCTCTCTTTCAATTTCCCAACTCTCCTACTTCACTAATTAATTAC	1300
genome DNAAACTCTCCTACTTCACTAATTAATTAC	27
5'-PCR	CAGTAAAAGAATGAGTACATTAGATCGTCAATCTATTTTTCACAAATAAAGGA	1350
genome DNA	CAGTAAAAGAATGAGTACATTAGATCGTCAATCTATTTTTCACAAATAAAGGA	77
5'-PCR	ATTAGAGTCGATCAGTCAACCGTATTCATGATGGAGATGAGGAGGCTATGA	1400
genome DNA	ATTAGAGTCGATCAGTCAACCGTATTCATGATGGAGATGAGGAGGCTATGA	127
5'-PCR	AGTT	1405
genome DNA	AGTT	132

图 3 反向 PCR 结果与雅致枝霉 Δ^6 -脂肪酸脱氢酶基因 5' 端重叠区

genomeDNA	CACTGGATATTACATCCAAAGCTCTCTTTGCAATTAAGCAAAAAGTCGTTT	50
3'-PCR	CACTGGATATTACATCCAAAGCTCTCTTTGCAATTAAGCAAAAAGTCGTTT	50
genomeDNA	TAATTCATGATACCTTTTAGAAAAGCATAAAAATAAAATCTTTCAAAAAAAA	100
3'-PCR	TAATTCATGATACCTTTTAGAAAAGCATAAAAATAAAATCTTTCAAAAAAAA	100
genomeDNA	AAAAAAAAAAAACTTTTTACGAC.....	122
3'-PCR	AAAAAAAAAAAACTTTTTACGACAAAGATTTTCTGTTTCATCCGCCAAAAAT	150

图4 反向 PCR 结果与雅致枝霉 Δ^6 -脂肪酸脱氢酶基因 3' 端重叠区

Fig.4 Sequences overlaps between the result of inverse PCR and *Thamnidium elegans* delta-6 fatty acid desaturases 3' end

在三个模板中均扩增得到 1.3kb 的特异性片段,与预期片段大小相符。

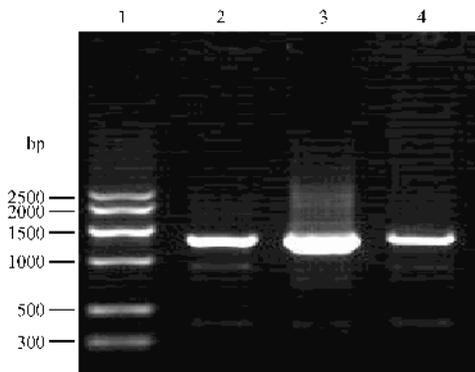


图5 克隆片段的 PCR 鉴定

Fig.5 Identification of the cloning fragment by PCR

1: marker VII ; 2: product from genome DNA ; 3: product from plasmid ;
4: product from *EcoR* I digested DNA.

2.3 克隆得到的 Δ^6 -脂肪酸脱氢酶基因 5' 上游序列的分析

对雅致枝霉 Δ^6 -脂肪酸脱氢酶基因上游 1318bp 序列分析显示该序列中含有 5 个 TATA box, 和多个脂肪酸脱氢酶转录因子结合位点(见图 6)。其中 CCAAT *cis*-element 和 AP-1 是存在于硬脂酰脱氢酶 *Scd1* 等多个脂肪酸基因转录表达调控元件^[10], 而且发现存在于在丝状真菌夹膜组织胞浆菌 (*Histoplasma capsulatum* Downs ATCC38904) Δ^9 -脂肪酸脱氢酶基因启动子中 AP-1 元件(保守序列 TGACTAA)对温度敏感型菌株受温度影响发生两型性生长具有重要的转录调节作用^[11]。FSE-2(fat-specific element-2)是存在于人类 *SCD* 和小鼠 *Scd1*、*Scd2* 启动子中的保守序列^[12]。STRE(Stress-responsive element)同样存在于鲁氏毛霉(*Mucor rouxii*) Δ^9 -脂肪酸脱氢酶基因启动子序列中^[13]。CCAAT *cis*-element 在真核生物启动子中普遍存在,是真核生物转录因子的结合位点^[14], 并已发现的在人类肝细胞硬脂酰脱氢酶基因上有保守的顺式调控元件^[13]。这些结合位点的存在意味着该序列是一个潜在的真核生物启动子,受到各种转录激活因子、阻遏蛋白、增强因子

的调控。据此推测该序列为一在脂肪酸代谢中具有重要作用的启动子调控序列。

3 讨论

目前扩增邻近未知序列的方法有文库筛选法、热不对称交错 PCR(TAIL PCR)法、接头连接 PCR(Adaptor-ligation PCR)法、反向 PCR(inverse PCR)法等。反向 PCR(inverse PCR)最早由 Ochman 开发^[15], 是将复杂的模板 DNA(如基因组 DNA、YAC 克隆 DNA 等)用单一限制性内切酶或产生可互补粘性末端的两种限制性内切酶进行消化,然后在低浓度条件下进行连接反应,使其自身环化并以此为模板,从已知序列向未知序列方向设计引物,扩增已知序列的相邻序列的方法。

3.1 基因组的提取

完整的基因组 DNA 是反向 PCR 成功的关键,选用植物基因组提取试剂盒利用硅质材料对 DNA 进行特异性吸附,最大限度的减小液体对 DNA 的剪切,保证基因组的完整,得到高质量的丝状真菌基因组 DNA(图 7)。

3.2 限制酶的选择

限制酶必需选用在已知序列中不存在酶切位点的酶,并应尽量选择酶切效率高、特异性好、能产生粘性末端且常用的酶,选择产生平末端的酶则给连接反应带来额外的困难。识别 6bp 序列的限制酶,在基因组平均中每隔 $4^6 = 4096$ bp,与预期所克隆的序列长度匹配,此外考虑到基因组的甲基化等问题,在本实验中选用 *EcoR* I、*Kpn* I 对基因组 DNA 进行消化,结果 *EcoR* I 消化基因组 DNA 得到了很好的结果。另外,可以对样品 DNA 同时选用几种限制性内切酶分别进行消化,分别进行 PCR 扩增,择优选用,提高了实验的效率,同时提供平行对照,增加实验的准确性。

3.3 连接反应的体系

长的线状 DNA 在低浓度下有利于自身环化连接,但具体浓度随 DNA 复杂程度和所选用的内切酶

述不同的 PCR 技术的有机组合,成功扩增出已知基因的旁侧序列,其中 5'上游 1.3kb,3'下游 2.3kb,为进行上游启动子功能分析奠定了基础。

通过实验证明利用反向 PCR 技术,先使用长引物两步法 PCR 获得初步扩增产物,然后再用嵌套引物和降落 PCR 增加产物特异性,是扩增已知序列旁侧序列的有效而简便的方法。在 PCR 技术日益发展的背景下,应用不同技术的组合,往往能够在实验中取得事半功倍的效果。同时我们在实验中优化的条件和方法,也为今后类似的真菌分子生物学的研究工作提供了一种途径。

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