Universidade Federal de Pelotas
Centro de Desenvolvimento Tecnológico
Bacharelado em Biotecnologia

Long-PCR/ QC-PCR/ Tail-PCR

GUILHERME INDA
KENNIA GALDINO

Sumário

1. Long-PCR

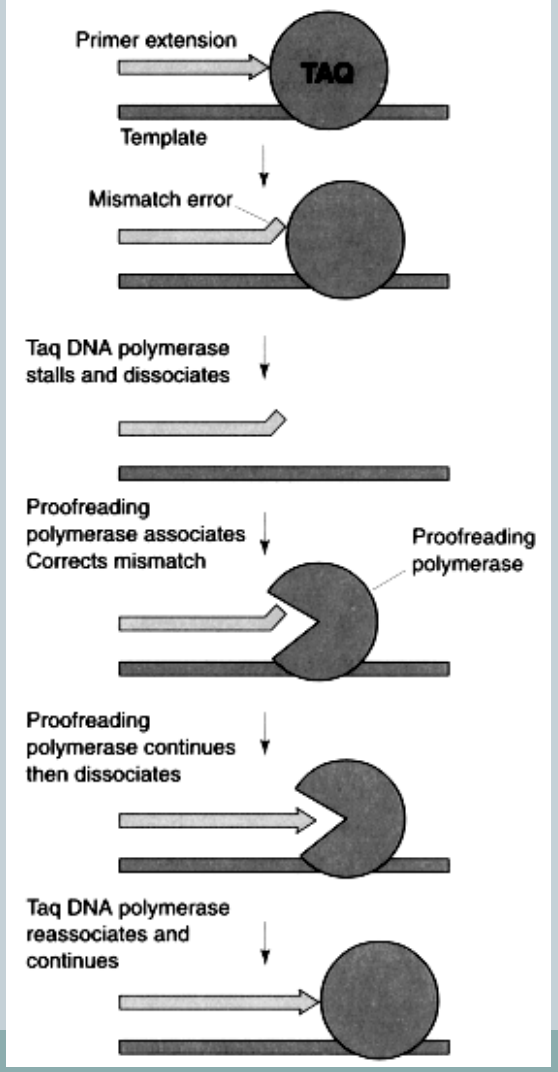
1.1. Apresentação da técnica de Long-PCR

- Amplificação de fragmentos longos de DNA (5-25kb)

- Amplificação de:
  Plasmídeos
  Cromossomas bacterianos
  Fragmentos maiores de DNA de eucariotos
  Regiões de DNA ricas em GC

- Eficiência do método depende do uso de duas polimerases:
  (1) non-proof reading polimerase (↑ cc)
  (2) proof reading polimerase (↓ cc/atividade exonucleotídica 3'-5')

1. Long-PCR


Primer extension
TAQ
Template
Mismatch error
Taq DNA polymerase
stalls and dissociates
Proofreading
polymerase associates
Corrects mismatch
Proofreading
polymerase
Proofreading
polymerase continues
then dissociates
Taq DNA polymerase
reassociates and
continues

1. Long-PCR

Long-PCR Protocol - Long PCR Reagents and Guidelines (Modified from Cheng et. al., 1994.

Generic Long PCR Program for the Perkin Elmer 9600

- Initial melting 94 degrees C 10-15 sec
- Cycles 1-15 94 degrees C 10 sec, 68 degrees C for n min (15 times)
- Cycles 16-30 94 degrees C 10 sec, 68 degrees C for n min +15 sec/cycle (15 times)

The 15 sec cycle extension for cycles 16-30 may be necessary for only the longest PCR (20 kb), please experiment.

* Uma Redução da Tm a 68°C deve ser feita para se evitar a perda da enzima durante as prolongadas extensões.

1. Long-PCR

1.2. Discussão dos artigos

Research

Optimization of Long-distance PCR Using a Transposon-based Model System

Lynne D. Ohler and Elise A. Rose[1]

Human Genome Laboratory, Perkin-Elmer Cetus Instruments, Emeryville, California 94608

1. Long-PCR

1.2. Discussão dos artigos

→ Objetivos do trabalho:

[...] explorar uma variedade de DNA polimerases termoestáveis sob diferentes condições de reações que pode permitir (1) a amplificação de produtos de PCR mais longos (5-25 kb) com elevado especificidade e fidelidade, e (2) a amplificação por PCR a partir de regiões que são atualmente difícil de amplificar (devido a estrutura secundária complexa e/ou elevado conteúdo de G + C).

1. Long-PCR

1.2. Discussão dos artigos

→ Metodologia:

- Moldes de DNA

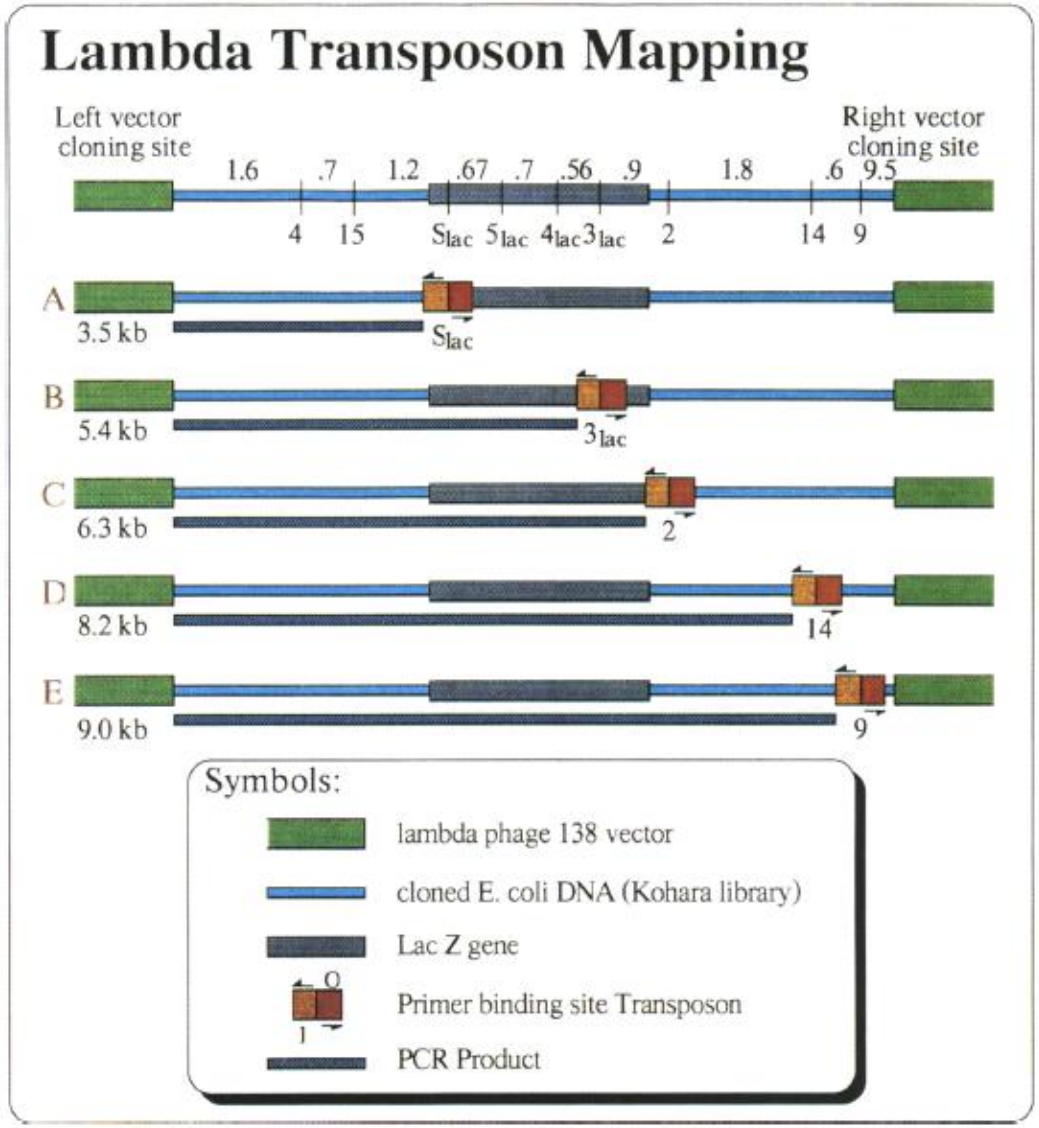
  → Clones de fagos Lambda 138 portadores do gene lacZ de E. coli com uma pequena quantidade de DNA circundante da bactéria.

  → 01 Transposon TN5supF aleatoriamente inserido.

- Primers

  → PCR realizada com primers complementares a sequências únicas perto de cada extremidade do transposon e com primers específicos para as duas extremidades do vetor.

1. Long-PCR


Lambda Transposon Mapping
Left vector cloning site
Right vector cloning site
1.6
.7
1.2
.67
.7
.56
.9
1.8
.6
9.5
4
15
Slac
5lac
4lac
3lac
2
14
9
A
3.5 kb
Slac
B
5.4 kb
3lac
C
6.3 kb
2
D
8.2 kb
14
E
9.0 kb
9
Symbols:
lambda phage 138 vector
cloned E. coli DNA (Kohara library)
Lac Z gene
Primer binding site Transposon
PCR Product

1. Long-PCR

1.2. Discussão dos artigos

→ Metodologia:

- Componentes da reação de amplificação

→Testar a atividade e estabilidade de diferentes DNA polimerases:

TABLE 1 Exonuclease Activities of DNA Polymerases

DNA polymerase	5′ → 3′ Exonuclease	3′ → 5′ Exonuclease	References
Tma	+	+	27
AmpliTaq	+	−	28
r*Tth*	+	−	20
Hot Tub	+	−	29
VENT	−	+	30
AmpliTaq Exo⁻ Mutein	−	−	26

+ indicates that enzyme possesses activity. − indicates that enzyme lacks activity.

1. Long-PCR

1.2. Discussão dos artigos

→ Metodologia:

- Componentes da reação de amplificação

  → Testar diferentes cc de reagentes, eficiência de aditivos (gelatina e Tween 20) e diferentes condições do ciclo

- Hot Start

  → Ampli-Wax PCR Gems

  → Diminuir a amplificação inespecífica do DNA

1. Long-PCR

1.2. Discussão dos artigos

→ Metodologia:

- Condições do ciclo de amplificação

  →Os melhores resultados foram obtidos quando as amostras foram submetidas a um período inicial de 10 ciclos cada um consistindo em: 95°C durante 10 seg, 58°C durante 30 segundos, e 72°C durante 3 min mais um incremento automático de 30 seg em cada ciclo.

- Eletroforese em gel e mapeamento dos Transposons

1. Long-PCR

1.2. Discussão dos artigos

→ Principais resultados:

1. Tthr DNA polimerase - produtos de PCR >3,5kb
2. gelatina a 0,01%, - efeito significativo para a amplificação de produtos de 9,0kb;
3. Hot start reduziu a formação de produtos não-específicos
4. Transposons

2. QC-PCR

2.1. Apresentação da Técnica

→ Quantitative competitive PCR

- Foi primeiramente descrita em 1990 por Gilliland e colaboradores.
- Quantificação precisa da amostra é difícil de ser obtida por PCR normal (qualitativa)

  → quantidade de produto amplificado não reflete necessariamente a quantidade de DNA inicial presente na reação.
- Este fato é devido a fase de Platô da PCR

2. QC-PCR

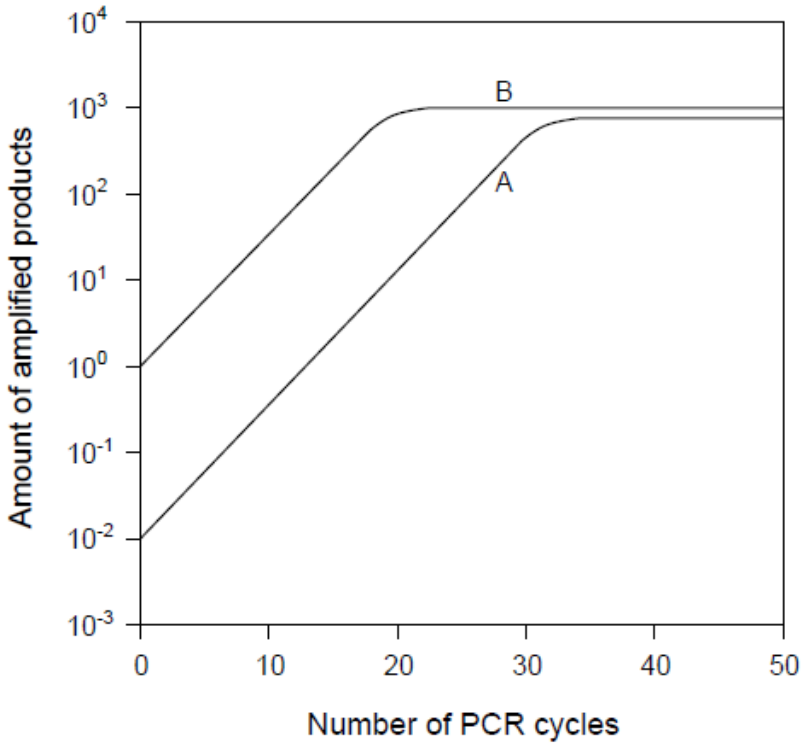


Figure 1. The plateau phase of PCR.
After approximately 30 cycles of PCR, almost the same amount of product will be obtained for samples A and B, even though they initially contain different amounts of template.

2. QC-PCR

→ Este efeito é causado por:

- A desativação da Taq DNA-polimerase
- A escassez de substratos de nucleotídeos
- Escassez de primer*
- A inibição pelo pirofosfato
- (Re)anelamento do DNA amplificado

→ Neste sentido a competitive-PCR foi desenvolvida para superar as dificuldade de quantificação.

2. QC-PCR

→ Princípios:

- Adição de quantidades conhecidas de um competidor

  → Fragmento de DNA que deve conter sequencias para os mesmos iniciadores da sequencia-alvo. Competindo ambos pelo mesmo conjunto de iniciadores.

- Uma vez que a quantidade inicial do competidor é conhecido, a quantidade de DNA alvo pode então ser estimada de acordo com a razão T:C:

  → T: quantidade de produto amplificado a partir do DNA/RNA alvo
  → C: quantidade de produto amplificado a partir de concorrente

2. QC-PCR

→ Um competidor ideal para um PCR quantitativo deve ser:

- Amplificado pelos mesmos iniciadores como o DNA alvo
- Distinguíveis do DNA alvo (tamanho diferente, o padrão dos fragmentos de restrição diferente, etc)
- Purificado e obtido numa quantidade ou concentração conhecida

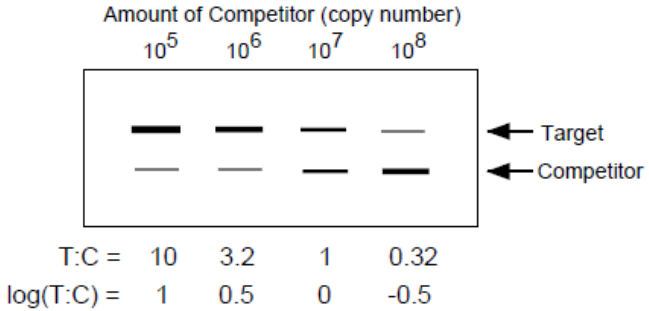


Figure 2. Principle of competitive PCR.
Samples are analyzed by agarose gel electrophoresis and the amount of competitor required to give a T:C ratio = 1 is determined. In this example, the amount of target DNA corresponds to 10^7 copies of competitor.

2. QC-PCR

→ Diferentes tipos de competidores:

- Competidor Homólogo - mesma sequência de nucleotídeos da sequencia alvo, mas contém uma deleção ou inserção, ou tem um sítio de restrição diferente, introduzida por mutagênese específica (possível formação de heteroduplex);
- Competidor Heterólogo - sequências de nucleotídeos diferentes do DNA/RNA alvo, exceto para as sequências de anelamento dos primers.

2. QC-PCR

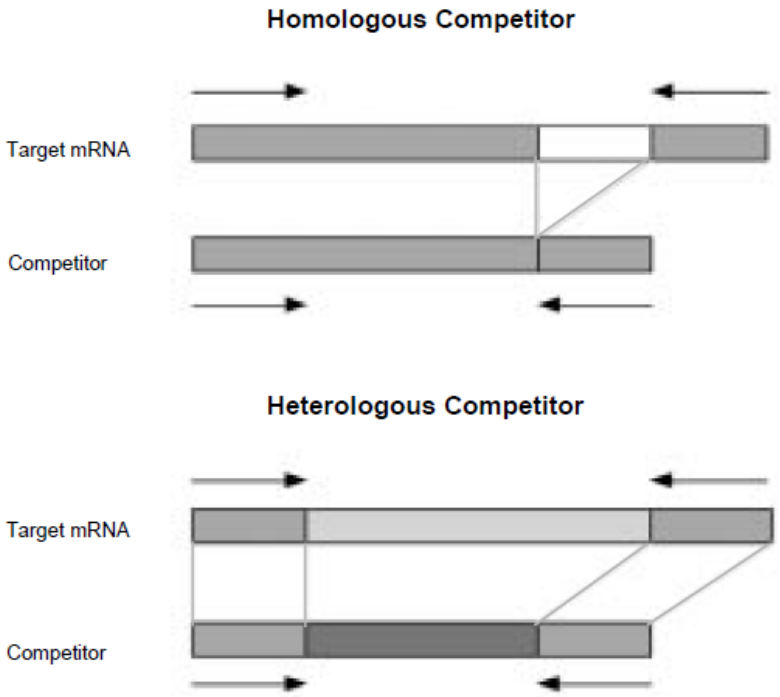


Figure 4. Types of competitors.

2. QC-PCR

2.2. Discussão do Artigo

ELSEVIER

Meat Science 57 (2001) 161–168

MEAT SCIENCE

www.elsevier.com/locate/meatsci

Quantitative competitive (QC) PCR for quantification of porcine DNA

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2. QC-PCR

→ Apresentação do trabalho:

- Várias abordagens analíticas têm sido feitos para identificar espécies de animais em produtos de carnes frescos ou processados, a fim de proteger os consumidores de fraudes e adulterações.
- "No entanto, até à data nenhum sistema QC-PCR foi relatado para a detecção de espécies de animais, em produtos alimentares."

→ Objetivo:

- Desenvolvimento e avaliação de uma Reação em Cadeia da Polimerase Quantitativa e Competitiva (QC-PCR) para detecção e quantificação de DNA suíno utilizando um novo sistema de PCR suíno-específica baseado no GH de *Sus scrofa*.

2. QC-PCR

→ Metodologia:

- Extração de DNA de diferentes amostras de alimentos a base de carne bovina e suína;
- PCR
- Construção do Competidor de DNA suíno

Table 1
Primer sequences[a], *mutagenic tail sequence* (30 bp) is written italic and *Pvu*II restriction site is underlined

Name	Sequence 5′–3′
L14735	aaaaaccacc gttgttattc aacta
H15149	gcccctcaga atgatatttg tcctca
GHP1	catccttggg ggtctc
GHP2	tttcatctct ttccattctt g
GHP1mut	*tactcaggat gtgctcgtca gctgcgtcca* ggtgcagtgg gtgtgta
GHP2mut	*tggacgcagc tgacgagcac atcctgagta* cactgctcag gtctgcagtc
LacZ f	*gccagggttt tcccagtcac ga*
LacZ r	*gagcggataa caatttcaca cagg*

[a] Primers L14735 and H15149 are used for cytochrome *b* amplification (Wolf, Rentsch & Hubner, 1999).

2. QC-PCR

→ Metodologia:

Clonagem vetor pGEM-T→

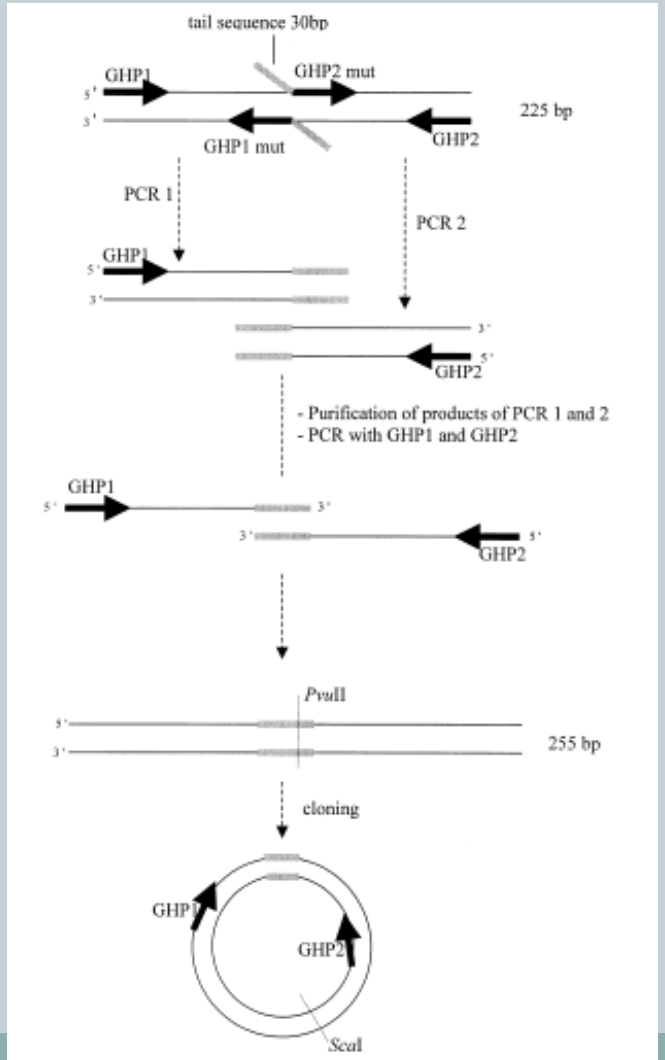


Fig. 1. Construction scheme of DNA competitor pGHP for QC-PCR.

2. QC-PCR

→ Metodologia:

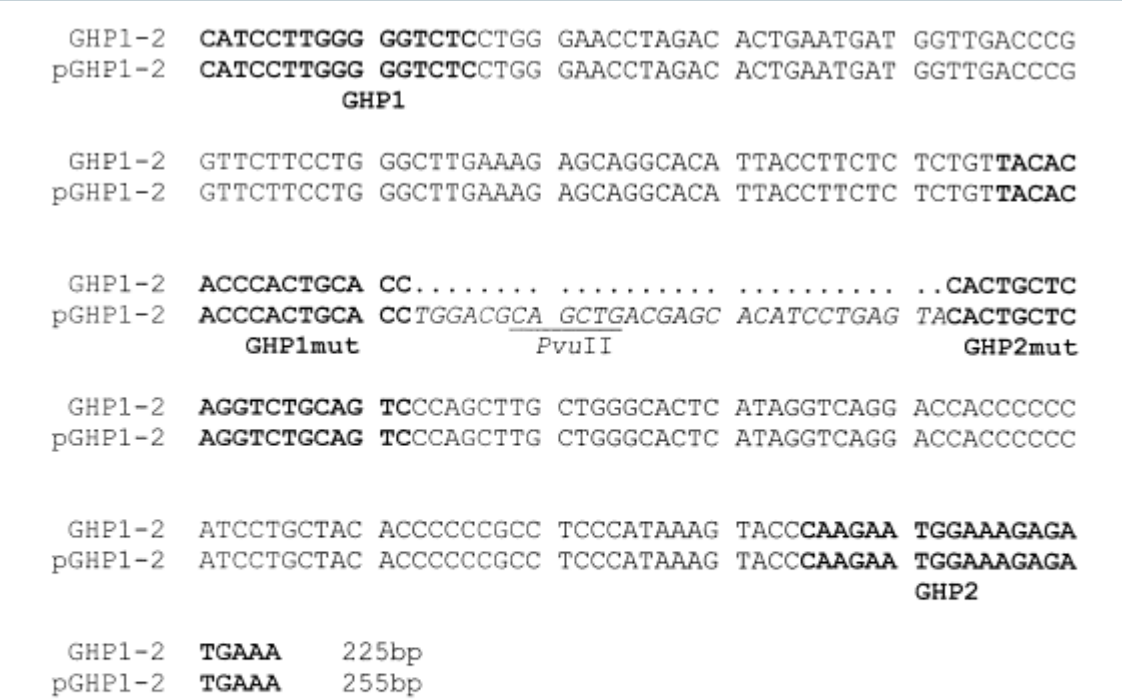


Fig. 2. Comparison of pGHP porcine DNA competitor sequence (255 bp) versus porcine growth hormone gene (225 bp GHP1-2 PCR product). Primers are written bold, the insertion is written italic and the *Pvu*II restriction site is underlined.

2. QC-PCR

→ Metodologia:

- RFLP

  → 20 µl mistura de PCR foram digeridos com RE AluI a 37°C durante 1 h. Os fragmentos de restrição foram separados num gel de agarose a 2,5%.

2. QC-PCR

→ Principais resultados:

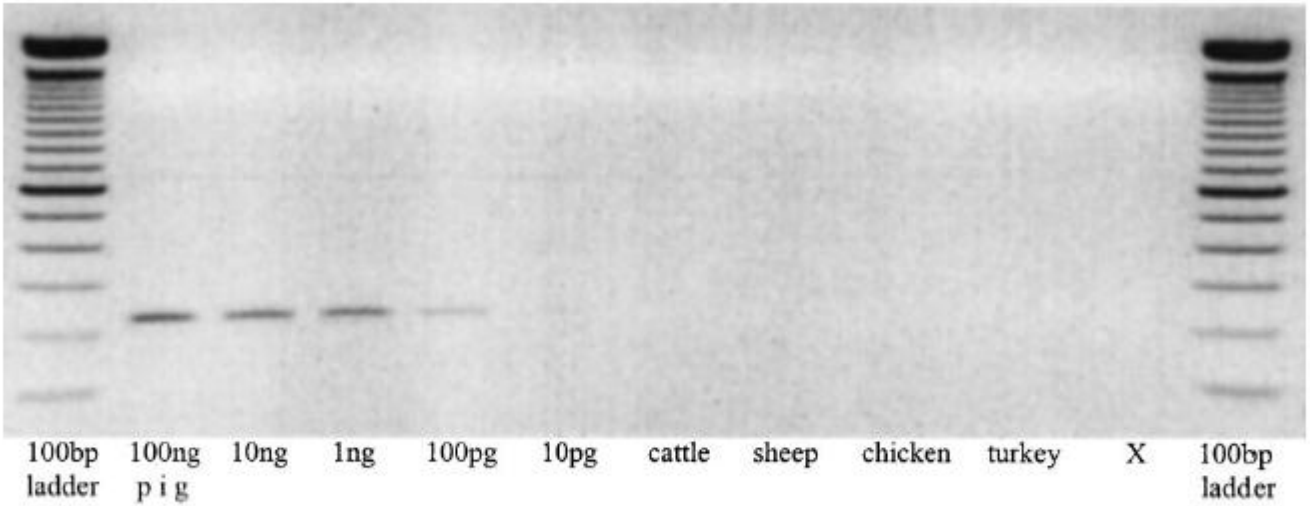


Fig. 3. Porcine growth hormone specific PCR with primer GHP1-2. Determination of sensitivity (100 pg) and selectivity of porcine DNA compared to DNA extracted from cattle, sheep, chicken and turkey. X = PCR negative control/mastermix without DNA.

2. QC-PCR

→ Principais resultados:

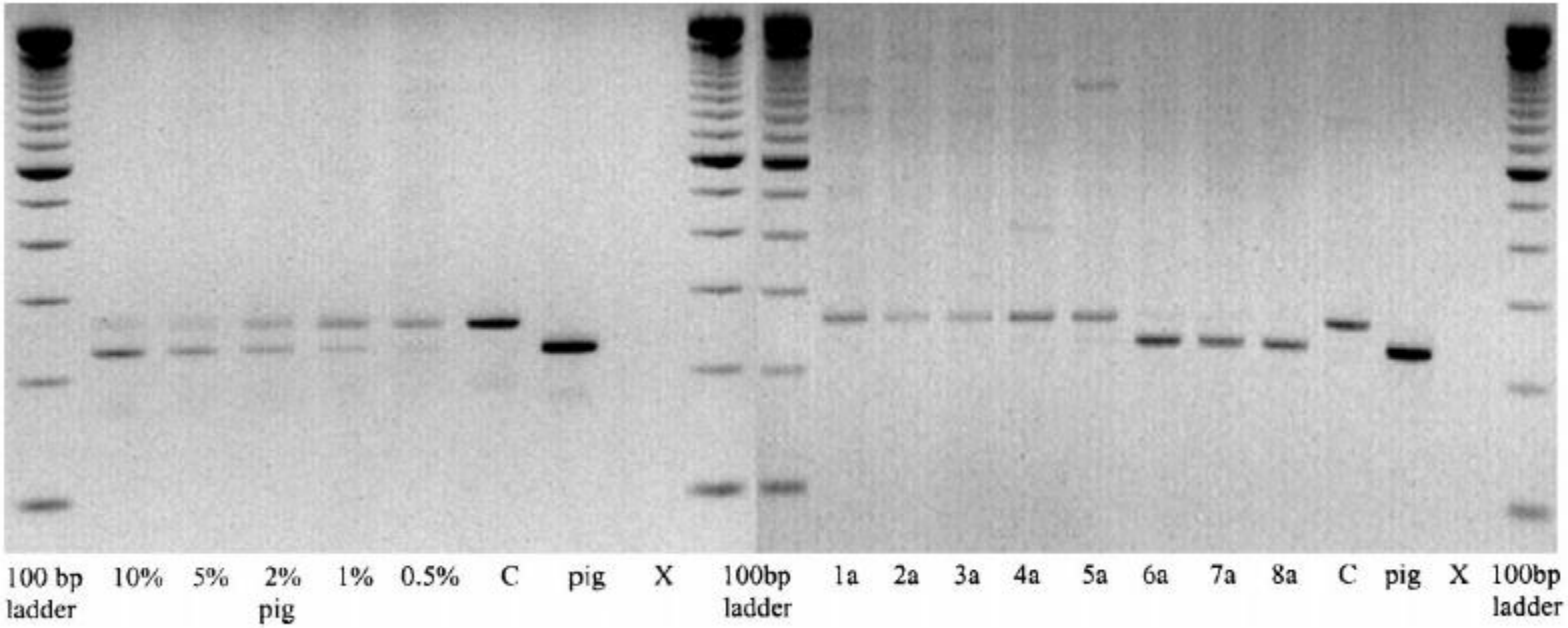


Fig. 4. 2% MS agarose gel: QC-PCR to show equivalence of 2% porcine DNA and 11 fg competitor (C) and its application to food samples (1–8). X = PCR negative control/mastermix without DNA.

3. Tail-PCR

→ Thermal Asymmetric Interlaced PCR

- Criado por Liu e Whittier em 1995;
- Técnica utilizada para recuperar fragmentos adjacentes de sequências conhecidas do DNA e para detectar sequências flanqueadoras do DNA;
- Utiliza um conjunto de nested primers junto com um arbitrary degenarated (AD) primer menor;
- As eficiências de amplificação de produtos específicos e nãoespecíficos podem ser termicamente controlados.

3. Tail-PCR

→Vantagens:

- Alta especificidade
- A proporção de produtos coamplificados não específicos é muito baixa
- Alta Eficiência
- Alto rendimento de produtos específicos com qualquer AD primer
- Simplicidade
- Análise em gel de agarose simples pode confirmar a especificidade do produto

3. Tail-PCR

→Vantagens:

- Velocidade:

  → As reações de amplificação sucessivas podem ser concluídas em 1 dia

- Alta sensitividade:

  → Sequência de cópia única do genoma pode ser amplificado

3. Tail-PCR

→ Detalhes e Passos:

- Modelos de primers
- Temperaturas de anelamento
- Ordem dos ciclos

3. Tail-PCR

→ Modelos de primers:

- Primers Específicos (SP):

  → Sequência específica de nested primer complementar a sequência vetor

  → Alta temperatura de fusão, Tm=58-63°C

- Arbitrary degenerate (AD) primer:

  → Relativamente menor

  → Temperatura de fusão menor, Tm =47-48°C

3. Tail-PCR

→ Temperatura de Anelamento:

- Ciclo de alto rigor:

Temperatura de anelamento = 63ºC

- Ciclo de rigor reduzido:

Temperatura de anelamento = 44ºC

- Ciclo de baixo rigor:

Temperatura de anelamento = 30ºC

3. Tail-PCR

→ Protocolo:

- 1º) Reação de PCR primária com SP1 e AD:

5 ciclos de alto rigor à 1 ciclo de baixo rigor à 10 ciclos de rigor reduzido à 12 super ciclos (TAIL)

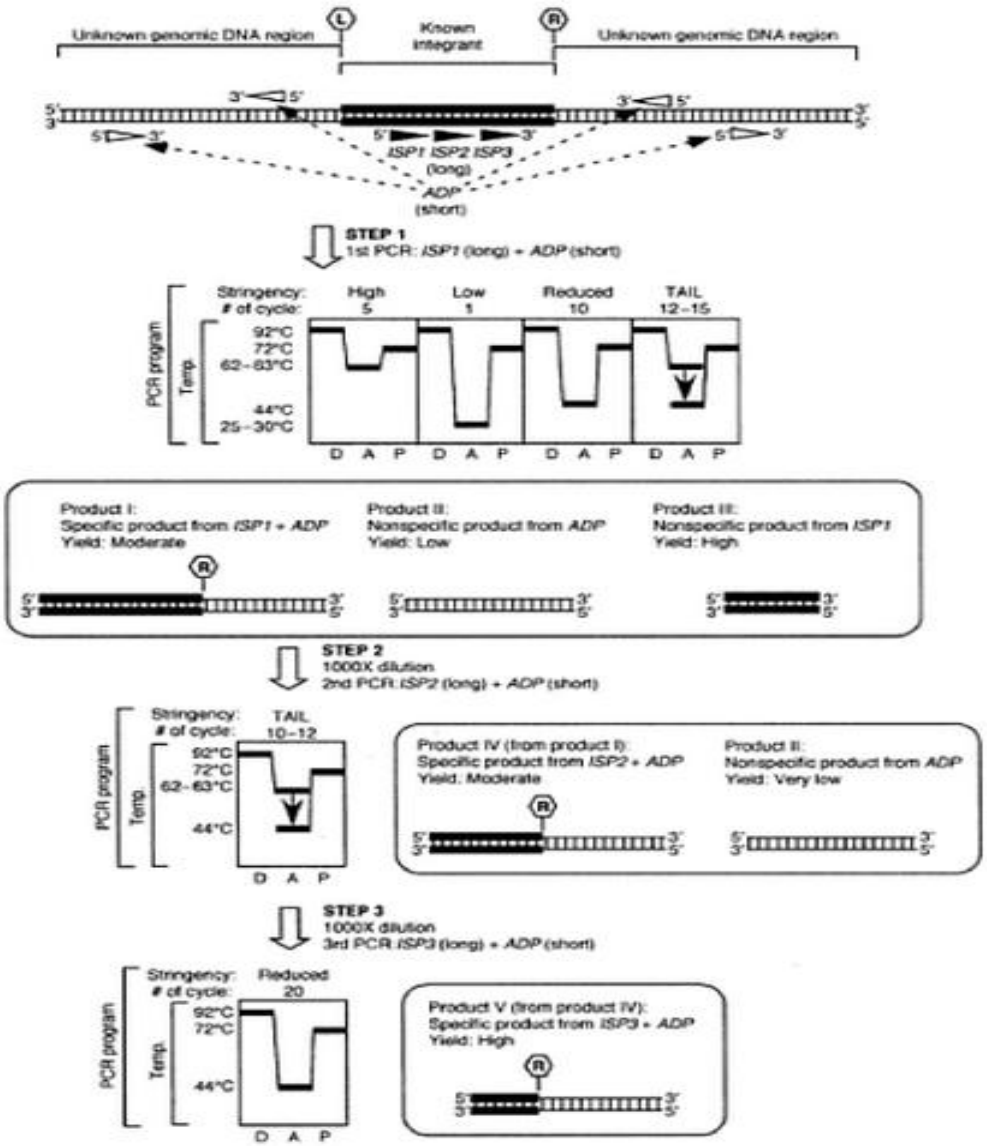
- 2º) Reação de PCR secundária com SP2 e AD:

10 super ciclos

- 3º) Reação de PCR terciário com SP3 e AD:

20 ciclos normais

3. Tail-PCR


Unknown genomic DNA region
L
Known integrant
R
Unknown genomic DNA region
ISP1 ISP2 ISP3 (long)
ADP (short)
STEP 1
1st PCR: ISP1 (long) + ADP (short)
Stringency:
# of cycle:
High 5
Low 1
Reduced 10
TAIL 12–15
PCR program
Temp.
92°C
72°C
62–63°C
44°C
25–30°C
D A P D A P D A P D A P
Product I:
Specific product from ISP1 + ADP
Yield: Moderate
Product II:
Nonspecific product from ADP
Yield: Low
Product III:
Nonspecific product from ISP1
Yield: High
STEP 2
1000X dilution
2nd PCR: ISP2 (long) + ADP (short)
TAIL 10–12
D A P
Product IV (from product I):
Specific product from ISP2 + ADP
Yield: Moderate
Product II:
Nonspecific product from ADP
Yield: Very low
STEP 3
1000X dilution
3rd PCR: ISP3 (long) + ADP (short)
Reduced 20
Product V (from product IV):
Specific product from ISP3 + ADP
Yield: High

3. Tail-PCR

Cycling Conditions Used for TAIL-PCR on the GeneAmp System 9600

Reaction	File no.	Cycle no.	Thermal condition
Primary	1	1	92°C (2 min), 95°C (1 min)
	2	5	94°C (15 s), 63°C (1 min), 72°C (2 min)
	3	1	94°C (15 s), 30 °C (3 min), ramping to 72°C over 3 min, 72°C (2 min)
	4	10	94°C (5 s), 44°C (1 min), 72°C (2 min)
	5	12[a]	94°C (5 s), 63°C (1 min), 72°C (2 min)
			94°C (5 s), 63°C (1 min), 72°C (2 min)
			94°C (5 s), 44°C (1 min), 72°C (2 min)
	6	1	72°C (5 min)
Secondary	7	10[a]	94°C (5 s), 63°C (1 min), 72°C (2 min)
			94°C (5 s), 63°C (1 min), 72°C (2 min)
			94°C (5 s), 44°C (1 min), 72°C (2 min)
	6	1	72°C (5 min)
Tertiary	8	20	94°C (10 s), 44°C (1 min), 72°C (2 min)
	6	1	72°C (5 min)

Note. The program files in each reaction were linked automatically.
[a] These are nine-segment super cycles each consisting of two high-stringency and one reduced-stringency cycle (see Fig. 1).

3. Tail-PCR

TAIL PCR (THERMAL ASYMMETRIC INTERLACED PCR)

TAIL-PCR is a powerful tool for the recovery of DNA fragments adjacent to known sequences. Basically, TAIL-PCR utilises three nested primers in consecutive reactions together with an arbitrary degenerate (AD) primer having a lower Tm (melting temperature) so that the relative amplification frequencies of specific and non-specific products can be thermally controlled.

Primary reaction.

In the primary reaction, one low stringency PCR cycle is conducted to create one or more annealing sites for the AD primer in the targeted sequence. Specific products are then amplified over non-specific ones by interspersion of two high-stringency PCR cycles with one reduced-stringency PCR cycle.

1. Set up 4 reactions as follows (one with each AD primer):

2 ml	10 X PCR buffer
1.2 ml	25 mM $MgCl_2$
0.2 ml	10 mM dNTP's
0.2 ml	100 $ngml^{-1}$ specific primer 1 (furthest away from AD) (0.15mM final)
2 ml	20 mM AD primer (2 mM final)
0.2 ml	Taq DNA polymerase
0.4 ml	DMSO
1 ml	DNA (1-20$ngml^{-1}$)
12.8 ml	H_2O

2. Cycle as follows: (if doesn't work, try dropping annealing step to 60°C)

92°C (3'), 95°C (1')	X 1
94°C (30s), 65°C (1'), 72°C (2')	X 5
94°C (30s), 25°C (2'), ramping to 72°C over 2', 72°C (2')	X 1
94°C (30s), 65°C (1'), 72°C (2')	
94°C (30s), 65°C (1'), 72°C (2')	
94°C (30s), 44°C (1'), 72°C (2')	X 15
72°C (5')	X1
cool to 4°C	

There is no need to run out this primary reaction. It should contain a medium yield of specific products, a high yield of non-targeted products, and a low yield of non-specific products. The nested primers used in the secondary and tertiary reactions result in very low yields of non-specific products, very high yields of specific products and no amplification of non-targeted products.

Secondary reaction

For the secondary reaction, a 1/40 dilution of the primary PCR product is used as template, and the specific primer is the middle one of the three specific primers.

1. Set up reaction as follows:

2.5 ml	10 X PCR buffer
1.5 ml	25 mM $MgCl_2$

3. Tail-PCR

0.25 ml	10 mM dNTP's
0.3 ml	100 $ngml^{-1}$ specific primer 2 (middle nested) (0.2 mM final)
2.5 ml	20 mM AD primer (2 mM final)
0.2 ml	Taq DNA polymerase
0.5 ml	DMSO
1 ml	DNA (1/40 dilution of primary PCR products)
16.25 ml	H_2O

2. Cycle as follows: (if doesn't work, try dropping annealing step to 60°C)

94°C (30s), 65°C (1'), 72°C (2')	
94°C (30s), 65°C (1'), 72°C (2')	
94°C (30s), 45°C (1'), 72°C (2')	X 12
72°C (5')	X 1
cool to 4°C	

Tertiary reaction

For the tertiary reaction, the SAME template (i.e primary PCR product) is used but this time in a 1/10 dilution. I usually simply add 4 X of the 1/40 dilution used for the secondary reaction. This removes the possibility of getting false positives. The specific primer used is the primer nearest the unknown sequence.

1. Set up reaction as follows:

5 ml	10 X PCR buffer
3 ml	25 mM $MgCl_2$
0.5 ml	10 mM dNTP's
0.6 ml	100$ngml^{-1}$ specific primer 3 (closest to AD) (0.2 mM final)
5 ml	20 mM any one AD primer (2 mM final)
0.4 ml	Taq DNA polymerase
1 ml	DMSO
4 ml	DNA (1/40 dilution of primary PCR products)
31 ml	H_2O

2. Cycle as follows:

94°C (30s), 45°C (1'), 72°C (2')	X 20
72°C (5')	X 1
cool to 4°C	

Agarose gel analysis

The secondary and tertiary products are run in adjacant lanes on a 1.2% agarose gel. The specificity of the products is confirmed by the expected size change between the secondary and tertiary products.

3. Tail-PCR

3.2. Discussão dos artigos

Mol Gen Genet (2000) 263: 554–560

ORIGINAL PAPER

R. Terauchi · G. Kahl

Rapid isolation of promoter sequences by TAIL-PCR: the 5′-flanking regions of *Pal* and *Pgi* genes from yams (*Dioscorea*)

Received: 10 October 1999 / Accepted: 31 January 2000

Abstract Using a modified TAIL-PCR technique, the 5′-flanking regions of the phenylalanine ammonia lyase (*Pal*) genes of a yam species, *Dioscorea bulbifera*, and the phosphoglucose isomerase (*Pgi*) gene of *D. tokoro* were successfully isolated. Two novel modifications of the TAIL-PCR procedure introduced here, namely (1) the use of a battery of random 10-mers (RAPD primers) as short arbitrary primers, and (2) the use of a total of five nested, gene-specific primers, allow the rapid isolation of the 5′-flanking region of any gene from organisms with large genomes. Isolated 5′-flanking regions were fused to the *gus* gene, and tested for transient expression in tobacco BY2 cells. All the isolated 5′-flanking regions were shown to drive reporter gene expression. Three *Pal* promoters responded to salicylic acid, presumably as a result of the binding of a MYB transcriptional activator to the multiple MREs (Myb Recognition Elements) present in these regions.

Key words TAIL-PCR · Promoter · *Dioscorea* · *PAL* gene · *PGI* gene

Introduction

The isolation of promoter and enhancer sequences is a crucial step in the study of the regulation of gene expression. Flanking regions of genes, containing these elements, have been conventionally isolated by screening genomic libraries using cDNAs as probes. However, the construction and screening of genomic libraries involves time-consuming procedures. As alternatives, PCR-based methods have increasingly been applied for this purpose. Inverse PCR (Ochman et al. 1988), and ligation-mediated PCR (Rosenthal and Jones 1990; Devon et al. 1995; Siebert et al. 1995; Balavoine 1996; Zhang and Chiang 1996) are the techniques most frequently used for the isolation of flanking regions of genes. These methods rely on the presence of restriction sites in the region to be isolated, so that the fragments can be self-ligated to form circular molecules (inverse PCR) or ligated to a DNA cassette (ligation-mediated PCR), prior to PCR. As information on restriction sites is usually not available in advance, there is no guarantee that digestion with a particular restriction enzyme will be successful, thereby necessitating trials with several different enzymes. Furthermore, in the case of ligation-mediated PCR, there exists the inherent problem of undesirable amplification of PCR products that are flanked by the DNA cassette sequence at both ends, at the expense of target sequence amplification. Several commercially available kits for the isolation of regions flanking a known DNA sequence try to minimize this problem by modifying the cassette structure (e.g. Siebert et al. 1995), but complete prevention of PCR amplification of non-target sequences is difficult. PCR products of the target sequence may be separated from non-target sequences by using biotinylated gene-specific primers and streptavidin-coated magnetic beads (Rosenthal and Jones 1990). However, this procedure requires biotin-labeling and capture/separation of the primers, which entails further costs, takes more time and reduces yields of target sequences. Therefore, simpler and more reliable techniques for

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primers of arbitrary sequence. An elaborate thermal cycling program composed of ``supercycles'', each consisting of one low-stringency cycle and two high-stringency cycles, allows only sequence-speci®c fragments to be exponentially ampli®ed. This method has been successfully used to isolate insert-end segments of P1 and YAC clones (Liu and Whittier 1995) and ¯anking regions of T-DNA inserts in Arabidopsis (Liu et al. 1995). But to the best of our knowledge, this method has never been employed for the isolation of ¯anking regions of any resident genes. Here we report the successful isolation of the 5¢ ¯anking regions of Pal and Pgi genes of yams using a modi®ed TAIL-PCR method. Two novel and essential modi®cations of the standard method were introduced for the systematic isolation of ¯anking regions of resident genes of organisms with large genome sizes: (1) use of a battery of random 10 mers originally developed for RAPD analysis (Williams et al. 1990) as the short arbitrary primers instead of three degenerate 16-mer primers as described in the original TAIL procedure (Liu and Whittier 1995), and (2) use of a total of ®ve nested gene-speci®c primers instead of three. By using modi®cation (1), we were able to exploit a whole battery of 10 mer primers from commercially available, low-cost primer sets designed for RAPD analysis (Williams et al. 1990). The use of a large number of 10 mers increases the probability of amplifying long target sequences. Modi®cation (2) was necessary to amplify target sequences from the complex genomes of the genus Dioscorea, which are about ®ve times larger (550 Mb; Arumuganathan and Earle 1991) than the Arabidopsis genome, for which the original TAIL-PCR technique was developed (Liu et al. 1995).

The Pal gene codes for phenylalanine ammonia lyase, the enzyme that catalyzes the conversion of phenylalanine to transcinnamic acid in the initial step of phenylpropanoid biosynthesis (Hahlbrock et al. 1976; Hahlbrock and Scheel 1989; Wanner et al. 1995). The Pal gene was one of the ®rst plant defense genes to be identi®ed, and was found to be induced by pathogens and environmental stresses (Kuhn et al. 1984; Edwards et al. 1985; Hahlbrock et al. 1995; Logeman et al. 1995). To obtain an elicitor-inducible promoter for the genetic engineering of yam crops, we have been trying to isolate the 5¢ ¯anking region of this gene. The Pgi gene codes for phosphoglucose isomerase, a key enzyme in glycolysis, which is known to be constitutively expressed. Molecular population genetic studies of the coding region of the Pgi from D. tokoro (Terauchi et al. 1997) prompted us to isolate and characterize the 5¢ ¯anking region of the gene.

Materials and methods

Plant materials, genomic DNA and cDNA

Total genomic DNA was extracted from individual Dioscorea bulbifera plants (cultivar sativa) and from D. tokoro using the standard CTAB (cetlytrimethylammonium bromide) method (Murray and Thompson 1980). DNAs were further puri®ed by CsCl ultracentrifugation. Total RNA was isolated from D. bulbifera leaf tissue by a phenol/SDS method (Palmiter 1974), and cDNA was obtained from it by reverse transcription using Superscript II (Gibco-BRL).

Isolation of Pal coding region from D. bulbifera

Alignment of the amino acid sequences derived from phenylalanine ammonia lyase (Pal) genes from 11 plant species (Arabidopsis thaliana, alfalfa, avocado, Camellia, Ipomoea, parsley, Pisum, Populus, rice, Trifolium, tobacco and tomato, obtained from the Genbank database) identi®ed highly conserved sequence regions. Based on the sequences of these regions, two degenerate primers were synthesized (Fig. 1): D0 (5¢CAYYTIGAAYGARGTIAARMRIATGGT-3¢ the forward primer) and U3 (5¢GMRCTICCRTC-

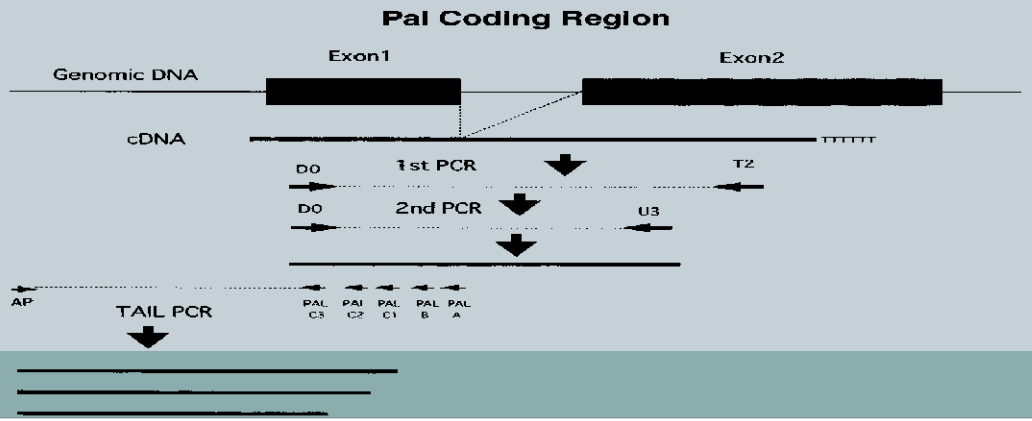

Fig. 1 Schematic outline of the procedures used to isolate Pal 5¢ ¯anking regions from Dioscorea bulbifera. First, a partial coding region of Pal was ampli®ed by two consecutive PCRs using the exon-speci®c primer pairs D0 and T2 (®rst PCR), and D0 and U3 (second PCR). On the basis of the sequence ampli®ed, ®ve gene-speci®c primers were synthesized and used in combination with an arbitrary primer (AP) for TAIL-PCR to obtain the 5¢ ¯anking region

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IARDATRTGYTCCAT-3¢ the reverse primer). In addition, the primer T2 (5¢ACRTCYTGRTTRTGYTGYTC-3¢ reverse primer) was synthesized according to Howles et al. (1994). PCR performed with the primer pair D0/T2 using D. bulbifera cDNA as template resulted in non-speci®c ampli®cation. An aliquot of this PCR product was then used as the template for the secondary PCR with primer pair D0/U3. The resulting discrete PCR product of the expected size was cloned into pBluescript, and sequenced.

The TAIL-PCR procedure

In order to allow chromosome walking beyond the known Pal and Pgi sequences into the unknown 5¢ ¯anking region, TAIL-PCR (Liu and Whittier 1995; Liu et al. 1995) was employed with two essential modi®cations: (1) the use of 10 mer random primers instead of degenerate 16 mers as the short primer, and (2) the use of a total of ®ve rather than three gene-speci®c primers in nested positions to ensure selection of the correct target fragments.

On the basis of the cDNA sequence of a Pal gene from D. bulbifera and a genomic Pgi DNA sequence from D. tokoro (Terauchi et al. 1997), a total of ®ve gene-speci®c primers in nested positions close to the 5¢ end of the coding regions were designed and synthesized. The primers for D. bulbifera Pal were PAL-A (5¢CCCTGCTTGGTYCTCCKATGAG-3¢), PAL-B (5¢CGCCAAAACCAGTAGTGACACC-3¢), PAL-C1 (5¢TCTTCATCACCCAATCACTGCT-3¢), PAL-C2 (5¢TGGTCTGGCCTCCTCTGAGAGC-3¢) and PAL-C3 (5¢CTAAGCTGACAACAGGATTCCT-3¢). The primers used for D. tokoro Pgi were PGI-A (5¢ACAGTGGCAGGAAGCACTCGTTGCCGGGAATAGTC-3¢), PGI-B (5¢GCATGAGATCGCGGAGATGCGTCTTCTTGATTTCT-3¢), PGI-C1 (5¢TTTGAGGGACGAATGGAGGAAGAG-3¢), PGI-C2 (5¢GATACATTGAGGTCCTTCCACTGC-3¢) and PGI-C3 (5¢TTCGCAGATAAGCGTGGACGTAGC-3¢) (Fig. 2). These primers were designed such that the Tm calculated according to Mazers et al. (1991) would be higher than 62 °C for the primers used in the primary PCR (PAL-A and PGI-A) and the secondary PCR (PAL-B and PGI-B), and higher than 57 °C for the tertiary PCR (PAL-C1±C3 and PGI-C1±C3). Sixteen arbitrary 10 mer primers each for Pal and Pgi were chosen from the 10 mer primer sets available (Roth), and care was taken to ensure that they were not predicted by the Oligo program (National BioSciences) to form stable duplexes with either of the two gene-speci®c primers (A and B).

Three rounds of PCR (Table 1) were carried out on a Perkin Elmer 9600 thermal cycler using the product of the previous PCR as template for the next, and employing a common arbitrary primer and nested gene-speci®c primers in a consecutive manner. The annealing temperature for the low-stringency cycle was set to 29 °C, instead of 44 °C as in the original protocol (Liu and

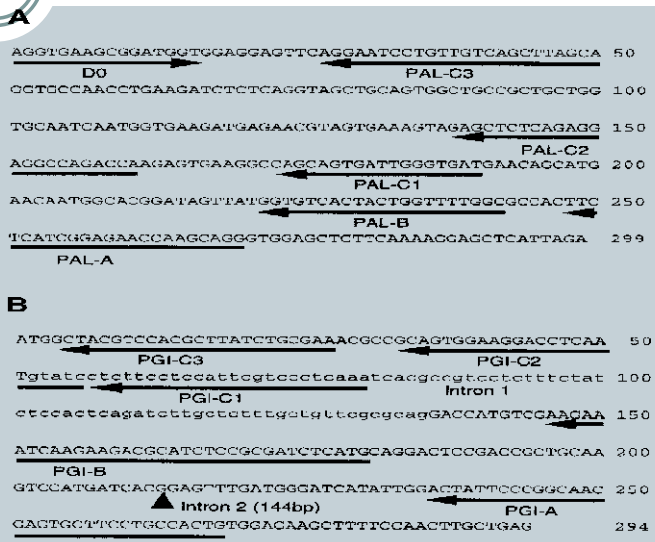

Fig. 2A, B Localization of the ®ve gene-speci®c primers for the Pal gene of D. bulbifera (A) and the Pgi gene of D. tokoro (B)

Whittier 1995). The primary PCR was carried out in a 20-l l volume containing 100 ng of genomic DNA, 0.2 l M gene-speci®c primer (Primer A), 2.0 l M 10 mer primer, 200 l M of each dNTP, 0.2 U Taq polymerase (Gibco-BRL) and 1´ Taq polymerase bu€er supplied with the enzyme. The secondary PCR was carried out with Primer B in combination with the same arbitrary primer as used in the primary PCR. The reaction solution was the same as for the primary PCR, except that 1 l l of a 1/50 dilution of the primary PCR product was used as template. For the tertiary PCR, three

Table 1 Reaction parameters for the TAIL-PCR used to amplify the 5¢ ¯anking regions of the Pal genes of Dioscorea bulbifera and the Pgi gene of D. tokoro

Reaction (primer combination)	Program no.	Number of cycles	Cycle (supercycle) parameters
Primary PCR			
(AP/Primer A)	1	1	93 °C, 1 min; 95 °C, 1 min
	2	5	94 °C, 30 s; 62 °C, 1 min; 72 °C, 2.5 min
	3	1	94 °C, 30 s; 25 °C, 3 min; ramping to 72 °C over 3 min; then 72 °C, 2.5 min
	4	15	94 °C, 10 s; 68 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 68 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 29 °C, 1 min; 72 °C, 2.5 min
	5	1	72 °C, 5 min
Secondary PCR			
(AP/Primer B)	6	12	94 °C, 10 s; 64 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 64 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 29 °C, 1 min; 72 °C, 2.5 min
	5	1	72 °C, 5 min
Tertiary PCR			
(AP/Primers C1±C3)	7	20	94 °C, 15 s; 29 °C, 30 s; 72 °C, 2 min
	5	1	72 °C, 5 min

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gene-specific primers (C1, C2 and C3) were separately used with the common arbitrary primer. The reaction solution for the tertiary PCR was the same as for the primary PCR except that 1 µl of a 1/10 dilution of the secondary PCR product was used as template, and the concentration of the arbitrary primer was 0.2 µM instead of 2.0 µM.

The products of the tertiary PCR (three PCRs for each arbitrary primer, corresponding to the three gene-specific primers C1 to C3) were separated in adjacent lanes on agarose gels to determine whether discrete PCR products from the three gene-specific primers show size differences corresponding to the relative positions of the nested primers. In the original protocol developed for *Arabidopsis* (Liu et al. 1995), PCR products of the secondary PCR (obtained with primer B) and tertiary PCR (obtained with primer C1) were separated in two adjacent lanes by agarose gel electrophoresis to detect the expected size difference. However, this procedure was not applicable for *Dioscorea*, as electrophoresis of the secondary amplicons usually resulted in smeared patterns. Use of three (C1, C2 and C3) instead of two primers for the tertiary PCR was also important to allow us to discriminate the true step-wise size differences corresponding to the different primer locations from spurious amplifications.

Cloning and sequencing of the tertiary PCR products

PCR products were excised from the agarose gel and reamplified. After polishing the ends with the Klenow fragment (NEB) and T4 polynucleotide kinase (NEB) in the presence of dNTPs and ATP, they were cloned into the *Sma*I site of pBluescript. DNA sequencing was performed on an ABI 373A automated sequencer.

Transient expression assay using cultured tobacco BY2 cells

Cloned 5′-flanking regions of *Pal* (PAL-AP140, PAL-AP122 and PAL-AP103) and *Pgi* (PGI-AP125) genes were PCR-amplified with primers carrying *Bam*HI (the distal end of the 5′-flanking regions) and *Xho*I (the proximal end) extensions at the 5′-ends. The products were ligated to a 2.6-kb *Xho*I-*Hin*dIII fragment containing the *gus* (*β*-glucuronidase) gene and the CaMV 35S polyadenylation signal derived from pRT101-gus (Töpfer et al. 1993), and cloned into the *Bam*HI-*Hin*dIII sites of pBluescript SK− (Stratagene), resulting in the plasmids pRG108 (PAL-AP140), pRG109 (PAL-AP122), pRG110 (PAL-AP103) and pRG103 (PGI2; see Fig. 5). Each of these GUS constructs was mixed in a 1:1 ratio with plasmid pRT101-LUC (a kind gift of Dr. C. Kirchner; Töpfer et al. 1993), harboring a firefly luciferase gene under control of the CaMV-35S promoter and 35S polyadenylation signal, and introduced into BY2 cells (*Nicotiana tabacum* cv. Bright Yellow; Ikeda et al. 1976) by particle bombardment (Biorad) following the manufacturer's instructions. Tobacco BY2 cells maintained in the BY-2 medium containing 2,4-D (Matsuoka and Nakamura 1991) were collected on a filter paper 4 days after subculturing, placed on a solid medium containing 0.2% gelan gum, and bombarded with the plasmids. After 24 h the cells were transferred to a solid BY-2 medium containing either (1) no added ingredients, (2) a culture filtrate of *Botrytis cinerea*, (3) N-acetylchitohexaose (0.02 mg/ml; Yamada et al. 1993), or (4) salicylic acid (SA; 20 µM), and cultured for another 24 h. Then cells were harvested and lysed in LC-*β* PicaGene cell lysis buffer (Wako Chemicals) by sonication. The lysate was centrifuged, and the supernatant immediately assayed for luciferase and *β*-glucuronidase activity using the PicaGene

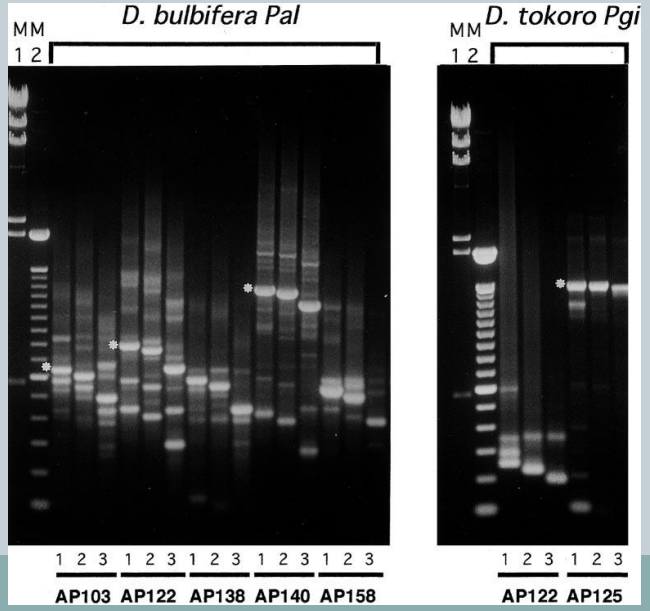

Fig. 3 Agarose gel electrophoresis of the tertiary PCR products of *D. bulbifera Pal* (left) and *D. tokoro Pgi* (right). M1 and M2 are molecular weight markers [*Hin*dIII digest of *λ*-DNA, and 100-bp ladder (Gibco-BRL), respectively]. DNA fragments marked with *asterisks* were excised from the gel and sequenced. The sequences of the arbitrary 10 mer primers were: 5′-GGTGCTCCGT-3′ (AP103), 5′-CGATGAGCCC-3′ (AP122), 5′-CTATCGCCGC-3′ (AP138), 5′-CGCAGACCTC-3′ (AG140), 5′-GTGTGCCCCA-3′ (AP158) and 5′-ACGGTGC-CTG-3′ (AP125)

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Luciferase detection kit (Wako Chemicals) and the Gus-Light GUS detection kit (Tropix), respectively, and an ATTO luminometer. Promoter activity was expressed as the GUS value divided by the LUC value, the latter serving as a normalizing factor to reduce the interexperimental ¯uctuation caused by di€erences in cell viability and/or e• ciency of plasmid delivery by particle bombardment.

Results and discussion

Isolation of the 5ϕ¯anking regions of Pal genes from D. bulbifera

Among the 16 arbitrary primers tested in combination with a set of Pal gene-speci®c primers (PAL-C1, PAL-C2 and PAI-C3), ten primers resulted in ampli®cation of discrete PCR products. Electrophoretic patterns obtained for ®ve arbitrary primers are shown in Fig. 3. Successful walking can easily be con®rmed by the stepwise change in the sizes of PCR products that correspond to the relative positions of the three nested, Pal-speci®c, primers (PAL-C1, PAL-C2 and PAL-C3). Further con®rmation came from the observation that DNA sequences of PCR products obtained by PAL-C1 primer overlapped perfectly with the 5ϕend sequence of a cDNA (data not shown). The sizes of PCR products ranged from 400 to 1300 bp (when PAL-C1 was used for walking).

Aligned DNA sequences of three PCR products (generated by the arbitrary 10 mers AP140, AP122 and AP103) are given in Fig. 4. DNA sequences were deposited in DDBJ, EMBL and Genbank under Accession Nos. AB016713±AB016715. The longest walk into the 5ϕ¯anking region was obtained with AP140 (1014 bp upstream of the start codon), followed by AP122 (510 bp) and AP103 (352 bp). Although the region close to the coding sequences is highly conserved, there are extensive di€erences between the products obtained with

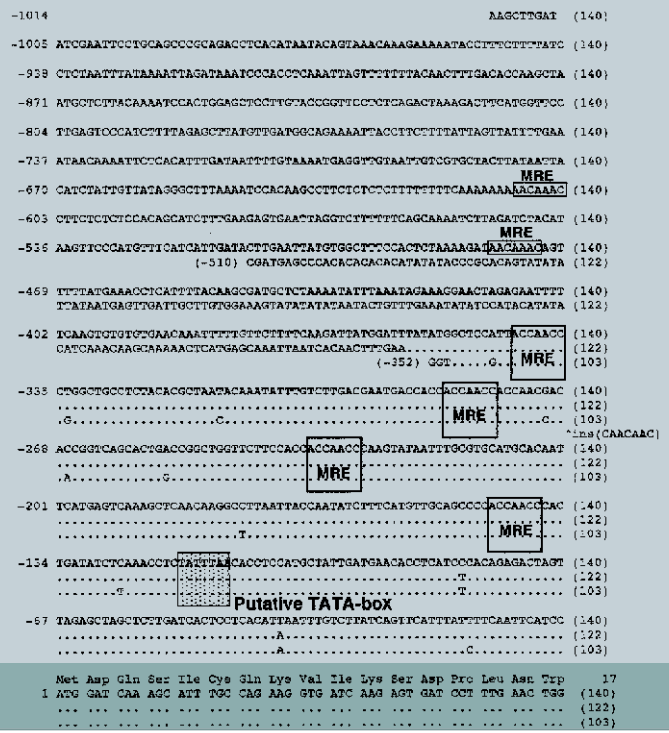

Fig. 4 Aligned DNA sequences of three TAIL-PCR products (ampli®ed with the arbitrary primers AP140, AP122 and AP103) obtained from the 5ϕ¯anking regions of Pal genes of D. bulbifera. The putative TATA box (sequence 5ϕTATTTAA-3ϕ) and MYB recognition elements (MRE consensus sequence: 5ϕACCAACC-3ϕ) are boxed

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regions of three *Pal* genes from *D. bulbifera* and the *Pgi* gene from *D. tokoro*. Transient expression studies showed that all the isolated sequences are transcriptionally functional. In the case of the *Pal* gene, 5′-flanking regions of multiple loci could be recovered. This demonstrates that we can isolate the promoter regions of most of the members of a particular gene family by systematically testing a large number of 10 mers of arbitrary sequence, in combination with consensus degenerate gene-specific primers. This versatile method recommends itself for the isolation of regulatory elements of genes from any organism.

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