



Methodology

Simple and efficient method for isolating cDNA fragments of *lea3* genes with potential for wide application in the grasses (Poaceae)

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ABSTRACT. cDNA fragments of *lea3* genes with a high GC content (from 68 to 77%) were found in several Poaceae, including *Sorghum vulgare*, *Saccharum officinarum*, *Oryza officinalis*, *Oryza meyeriana*, *Ampelocalamus calcareus*, *Cynodon dactylon*, and *Zizania latifoli*. They were successfully isolated by means of optimal experimental parameters, which included dimethyl sulfoxide as additive and degenerate primers “AGETKAS” and “AGKDKTG”, and their sequences were analyzed. Compared to the method of isolating genes by screening of a cDNA library using abscisic acid- and other stress-responsive cDNA clones, which is time-consuming and costly, this method is relatively easy and inexpensive. Using this new method, many new homologue *lea3* genes were rapidly determined.

Key words: Gramineae plants; *lea3* gene; High GC content sequence; Degenerate primers

INTRODUCTION

Late embryogenesis abundant (LEA) proteins are mainly low-molecular weight (10-30 kDa) proteins, that are involved in protecting higher plants from damage caused by environmental stress especially drought. Based on their amino acid sequences, LEA proteins are basically divided into five groups (Dure, 1993; Zhang and Zhao, 2003). To date, there have been some reports about the *lea3* gene, while its mechanism of function in plants has not been clearly revealed. Therefore, it is necessary to isolate more *lea3* genes to provide a reference for their structural and functional research. Unlike most of eukaryotic genes, which contain GC-rich sequences mainly in the promoter region, the GC content of *lea3* gene is higher in all the coding region.

A variety of polymerase chain reaction (PCR) additives and enhancing reagents have been used to increase the yield, specificity and consistency of PCR. It has been reported that some reagents such as tetramethylammonium chloride, DMSO, betaine, non-ionic detergents, and formamide are capable of improving the efficiency and specificity of PCR (Weissensteiner and Lanchbury, 1996; Kovarova and Draber, 2000). Recently, it was found that PCR could be dramatically enhanced by Au nanoparticles (Li et al., 2005). In this research, DMSO, formamide, glycerol, NP-40 (Nonidet P 40) and Tween20 were investigated in order to determine which additive could effectively enhance the amplification of the *lea3* gene. By means of the degenerate primers designed, fragments of *lea3* genes from several species in the Gramineae were isolated by PCR with appropriate additives.

MATERIAL AND METHODS

Plant materials

Fresh seedlings from *Saccharum officinarum*, *Oryza officinalis*, *Ampelocalamus cal-careus*, *Cynodon dactylon*, and *Zizania latifoli* were obtained from the surrounding area of Kunming. After harvesting, the whole fresh seedlings or cuttings were kept at room temperature for 4 h for RNA extraction. *Hordeum vulgare* and *Sorghum vulgare* seeds were soaked in water for 12 h and transferred to three layers of filter paper with water for 3 days under light. Germinated seedlings were air-dried for 4 h at room temperature for RNA extraction.

Design of degenerate primers

The sequences of *lea3* genes of *Hordeum vulgare* (GenBank ID: X13498), *Oryza sativa* (GenBank ID: OSU57641, RICWSI18), *Triticum aestivum* (GenBank ID: AY148492, X56882), *Zea mays* (GenBank ID: BT023964, DQ244556, DQ246069, EU955058, NM-001111828), and *Bromus inermis* (GenBank ID: AB181485, AB181486) were analyzed by DNAMAN (Figure 1) for primer designing. Degenerate primers were designed as follows: forward, 5'-gccggcgagRc caaggSMc-3' (R: g/a, S: c/g, M: c/a); reverse, 5'-gScggWYttgtccttgccgg-3' (S: c/g, W: a/t, Y: c/t).

RNA extraction and synthesis of cDNA

RNA was extracted from plant tissues using the RNAsimple Total RNA kit (Tiangen, Beijing, China). First-strand cDNA synthesis was carried out using the First-Strand RT-PCR kit (TaKaRa, Dalian, China). PCR was carried out using the plant material cDNA samples described above.

RT-PCR and sequence analysis

Amplification reactions were carried out under the following conditions: 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.2 μM primers, 0.01 μg/μL template, 0.2 mM each dNTP, 8% DMSO, and 0.05 U/μL Taq polymerase (TransGen, Beijing, China). The cycling conditions in a Biometer[®] T Gradient thermal cycler consisted of an initial incubation for 5 min at 94°C followed by 35 cycles each of 30 s of denaturation at 94°C and 30 s of annealing at 53°C and for 50 s of extension at 72°C, along with a final elongation step at 72°C for 7 min. Amplified materials were subjected to electrophoresis on a 1% agarose gel stained with ethidium bromide, visualized on a UVP Imaging System and documented by VisionWorksLS. Recovery of the amplified fragment was conducted according to manufacturer instructions for the Gel Extraction Mini Kit (Huashun, Shanghai, China). The purified PCR products were cloned into plasmid vector pMD18-T (TaKaRa, Dalian, China). By DNA transformation techniques, transformed susceptibility cells were cultured on a 50-mg/L ampicillin plate for 9-16 h at 37°C. After single-clone amplification in LB, harvested product was used for sequence analysis by Shanghai Shengggong Biotechnology Company, Ltd. The deduced amino acid sequences were determined by DNAMAN.

RESULTS AND DISCUSSION

Optimization of experimental parameters

The amplification of the barley HVA1 gene was carried out in order to determine which additive is efficient for isolating the *lea3* gene. The results showed that it was very difficult to amplify the *lea3* gene using genomic DNA of barley as template without any additive. DMSO at a concentration of 3 to 15% improved the yields. This finding was similar to that of Bookstein et al. (1990), while the product could be tested even when the concentration of DMSO was increased up to 15%. It was different from other results of previous reports (Saiki et al., 1990; Kawasaki, 1990), showing that the product was less when formamide concentration was increased from 1 to 3% in PCR and that glycerol and non-ionic detergents such as NP-40 and Tween20 did not work effectively. Among the combinations of DMSO and glycerol at different concentrations, it was found that only the combination of 1% glycerol and 5% DMSO could increase the product yield slightly, and that others could not improve the amplification of the *lea3* gene (Figure 1).

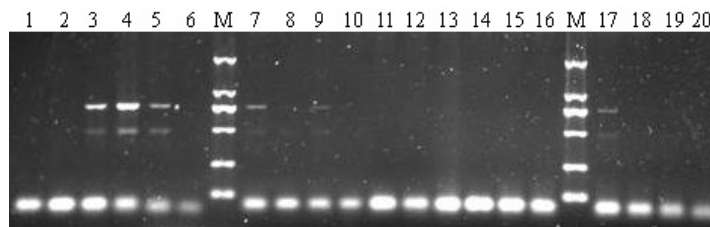


Figure 1. The effects of different additives on PCR. M = DL2000 marker; lane 1 = no additive as control; lane 2 = 2% DMSO; lane 3 = 5% DMSO; lane 4 = 10% DMSO; lane 5 = 15% DMSO; lane 6 = 20% DMSO; lane 7 = 1% formamide; lane 8 = 2% formamide; lane 9 = 3% formamide; lane 10 = 1% glycerol; lane 11 = 3% glycerol; lane 12 = 5% glycerol; lane 13 = 0.2% Tween20; lane 14 = 0.8% Tween20; lane 15 = 0.2% NP-40; lane 16 = 0.8% NP-40; lane 17 = combination of 1% glycerol and 5% DMSO; lane 18 = combination of 2% glycerol and 5% DMSO; lane 19 = combination of 1% glycerol and 10% DMSO; lane 20 = combination of 2% glycerol and 10% DMSO.

Structural analysis of *lea3* gene cDNA fragments

Under optimal conditions, fragments of *lea3* genes from some other species in the Gramineae such as *H. vulgare*, *A. calcareus*, *S. vulgare*, *S. officinarum*, *O. officinalis*, *C. dactylon* and *Z. latifoli* have been attained by means of the degenerate primers designed, and their sequences were analyzed. Each of their nucleotide sequences and their coding amino acid numbers were different, *S. vulgare* coded for 139 amino acids, *S. officinarum* for 128 amino acids, *O. officinalis* for 117 amino acids, *O. meyeriana* for 133 amino acids, *A. calcareus* for 145 amino acids, *C. dactylon* for 113 amino acids, and *Z. latifoli* for 100 amino acids. Their coding amino acid sequences were analyzed, and the alignment result (Figure 2) showed that they contained several LEA consensus sequences as reported previously (Dure, 1993). Furthermore, besides known 11-amino acid motifs in *lea3* proteins, it is interesting that the amino acid sequences deduced from the degenerate primer sequences, which are composed of “AGETKAS” and “AGKDKTG”, respectively, are also conserved in the Gramineae. Therefore, it could be suggested that partial cDNA fragments of *lea3* genes even in all Gramineae plants could be amplified simply by PCR using the degenerate primers. Actually, based on these fragments, full-length cDNA sequences of *lea3* genes from *S. vulgare*, *A. calcareus* and *Z. latifoli* were obtained successfully by 3' RACE and 5' RACE, for which Genbank accession numbers are GQ494000, GU433601 and GQ494017, respectively.

Sorghum vulgare	AGERAKRTTEKSGQAMGATKIDTAQHAKETTQKKA.SDSDTG.SYLG.....QKTEERBHK	53
Saccharum officinarum	RGETKRAAEAKHK.....ASHAADPRAMGMG.HDAADRSMGMG.HDAKDGAVRERDA	49
Oryza officinalis	RGETKRAHT.....EAKDRTSDAAGHAAGKGHDTKG..ATKEKAYEEROR	42
Oryza meyeriana	RGETKRAHTTEKAGQVMGAGKDKACEAKDRTSDAAGHAAGKGHDTKG..ATKEKAYEEROR	58
Ampelocalamus calcareus	RGETKRPCRAEDGVRDGRGABEGAGGQGHGVRRRG.PRDSPGPRQSEATKEKAYEEROK	59
Cynodon dactylon	RGETKRAEAEAKOK.....ASAAAEFAMGRG.HDAKEA.....TKDRAWOFKDA	42
Zizania latifoli	RGETKRAAEETG.....QMTGAT.....QEKARDKQOT	29
Sorghum vulgare	AGQITTESTKQKAGQTTAAKQKAAETTEETKQKAEETTE.....ATKQKTAETTEATK	106
Saccharum officinarum	ASDANGPAMD...C.....AABATRSGAEARBSASGAAGAGDHARDGACQTG	95
Oryza officinalis	ASETAQAAKDRTSG.....TAQAAKTAAEASKD.....QTGF LGKTEQAK	84
Oryza meyeriana	ASETAQAAKDRTSG.....TAQAAKTAAEASKD.....QTGF LGKTEQAK	100
Ampelocalamus calcareus	ASETAQAAKDRAGGGKDTGSGFLGQKTEAAKQKASETTE.....AAKQKASETTEATK	112
Cynodon dactylon	ASDANGRVADRGRG.....AABETKRAHEA.....GDGAREGACQTG	80
Zizania latifoli	VSDATGFAVDKGRG.....AABETKRAHDT.....AGSADRAGQTG	67
Sorghum vulgare	QKAAEAMETTKQKAAEAGQVAKETVVSQKDKTG	139
Saccharum officinarum	SYIACTABARQKRAASAAQVAKETVVSQKDKTG	128
Oryza officinalis	QKAAETACBARQKRAETAQVTKDSAVAGKDKTG	117
Oryza meyeriana	QKAAETACBARQKRAETAQVTKDSAVAGKDKTG	133
Ampelocalamus calcareus	QKAAETTEBARQKRAEDARQVARGSAVAGKDKTG	145
Cynodon dactylon	GYVACTABARQKRAAGAAQVAKETVVSQKDKTG	113
Zizania latifoli	SYIGQIABARQKRAETAQVTKDSAVAGKDKTG	100

Figure 2. Sequence alignments of Group 3 LEA protein fragments of some Gramineae plants.

Gene expression and regulation of LEA proteins

Expression of the *lea3* gene starts from the late period of maturation and initiation period of drying, reaches its peak in progressive dehydration, and sharply decreases after some hours of germination (Han and Kermode, 1996; Brands and David Ho, 2002). Many reports show that the expression of the *lea3* gene has no tissue-specificity, as the gene is expressed in cotyledons, panicles of seeds and also in stems, leaves and roots (vegetative tissues) (Federspiel, 2000). In this research, *lea3* genes could be expressed after seedlings or cuttings had been kept at room temperature for a few hours. Compared to the previous re-

search, which needed treatment with appropriate concentration of ABA (abscisic acid), NaCl and PEG6000 or desiccation for several days (Shih et al., 2004), the treatment of seedlings or cuttings in this research is simpler and more convenient.

In conclusion, partial cDNA fragments of *lea3* genes from several species of the Gramineae, in which GC content is high as 77%, were successfully isolated by using the simple and efficient method described in this paper. DMSO was suggested as an additive since it could remarkably increase specificity and yield of amplification. Compared to isolating genes by screening of an ABA- or other stress-responsive cDNA library, which is time-consuming and costly, this method is more convenient and economical.

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