Two mRNA Species Encoding Calcium-Dependent Protein Kinases Are Differentially Expressed in Sexual Organs of Marchantia polymorpha through Alternative Splicing

Rie Nishiyama, Hiroshi Mizuno, Sachiko Okada, Tomoya Yamaguchi, Mizuki Takenaka, Hideya Fukuzawa and Kanji Ohyama

Laboratory of Plant Molecular Biology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto, 606-8502 Japan

In plants, calcium-dependent calmodulin-independent protein kinases (CDPKs) are the predominant calcium-regulated protein kinases and their genes are encoded by a multigene family. A CDPK gene was cloned from a liverwort, Marchantia polymorpha, which showed a high level of sequence similarities to other higher plant CDPK genes. The liverwort CDPK gene consisted of 9 exons and 8 introns. The 6th and 7th exons (Exon 6A and Exon 6B) were almost identical except for 4-amino acid substitutions, both of which coded for EF-hands in the calcium-binding domain. RT-PCR analysis revealed that two species of mature mRNA containing either Exon 6A or Exon 6B were generated from a single CDPK gene by mutually exclusive alternative splicing. Both histidine-tagged fusion proteins derived from cDNAs containing either Exon 6A or Exon 6B exhibited calcium-dependent protein kinase activity in vitro. Preferential accumulation of the mature mRNA with Exon 6A detected in male sexual organ implies possible sexual control of the ratio between the two CDPK isozymes through alternative splicing. Functions and evolution of CDPKs are discussed based on the structure and expression of the liverwort CDPK gene.

Key words: Alternative splicing — Calcium-dependent protein kinase — Liverwort — Plant — Sexual organs.

Materials and Methods

Plant materials—The liverwort suspension-cultured cells were originally derived from a female thallus and maintained on
The male and female thalli were derived from a respective gemma grown in the field of Kyoto City. They were sterilized and maintained on M51C solid agar medium (Ono et al. 1979). The male and female sexual organs were collected from wild populations.

Preparation of genomic DNA and PCR—Genomic DNA was prepared from male and female thalli using cetyltrimethylammonium bromide (Ausbel et al. 1987). The PCR reaction mixture (20 μl) contained 20 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 μg/ml gelatin, 1.5 mM MgCl₂, 20 μM dNTPs, 0.1 μCi of [α²³P]dCTP, and 0.0125 units μl⁻¹ of Taq DNA polymerase (PE Applied Biosystems). The PCR products were separated by electrophoresis on 5% polyacrylamide gel, and visualized by autoradiography.

Rapid amplification of cDNA ends (RACE)-PCR was performed using 1 μg Poly(A)⁺ RNA and oligonucleotide primers, 5′ cap primer-1 (5′-CTGACACAACCACCTTTCCGTC-3′), or 5′ cap primer-2 (5′-GAGGTTGAATATGGATGC-3′). Cloning and sequence analysis of PCR fragments—The amplified PCR fragments which were unique to either male or female genomes were recovered from polyacrylamide gels and cloned into pBluescript II KS+. DNA sequencing was performed by the dideoxy chain-termination method using ABI PRISM Dye primer Cycle Sequencing Kit (PE Applied Biosystems) and a DNA sequencer (PE Applied Biosystems; Model 377). Sequence homology searches were performed with the Genbank, EMBL, SwissProt, and PIR Databases.

DNA and RNA blot hybridizations—DNA fragments were labelled with [α²³P]dCTP using the Megaprime DNA Labelling System (Amersham) and used as a probe. Two μg each of genomic DNA isolated from female suspension-cultured cells was digested with either HindIII or Smal. The DNA fragments were electrophoresed in a 0.7% agarose gel. After alkaline treatment and blotting onto nylon membrane, hybridization was performed in a solution containing 5 × Denhardt's reagent, 6 × SSPE, 0.5% SDS, 100 μg ml⁻¹ denatured salmon sperm DNA, and 50% formamide at 42°C (Ausbel et al. 1987). The filter was washed twice with a solution containing 5 x Denhardt's reagent, 6 x SSPE, 0.5% SDS, 10 mM dithiothreitol (DTT), 200 μg ml⁻¹ salmon sperm DNA, 500 μg ml⁻¹ denatured salmon sperm DNA, and 50% formamide at 42°C (Ausbel et al. 1987). The filter was washed twice with a solution containing 1 x SSPE and 0.1% SDS at room temperature followed by washing with that of 0.1 x SSPE and 0.1% SDS at 42°C for 10 min and then autoradiographed.

Poly(A)⁺ RNA was isolated from the female suspension-cultured cells using the PolyATtract System 1000 (Promega) according to the manufacturer's instruction. One μg of poly(A)⁺ RNA was electrophoresed in a 0.8% denaturing agarose gel containing formaldehyde (Ausbel et al. 1987) and transferred onto nylon membrane. Hybridization was carried out according to the method described above for the genomic Southern hybridization.

RT-PCR and analysis of alternatively spliced products—Using 0.5 μg of Poly(A)⁺ RNA from female suspension-cultured cells, the reverse transcription was carried out using the Ready To Go T-Primed First-Strand Kit (Pharmacia Biotech) according to the manufacturer's instruction. The first strand of the cDNA mixture was then amplified in a 100 μl solution containing 0.5 μM oligonucleotide primers, EX5-F (5′-AAAGCTTGCAGAGTCAGGATGC-3′) and EX7-R (5′-GAGTCCCATCCCATCGGTTA-3′), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP mixture, and 0.025 units μl⁻¹ of Taq DNA polymerase (TAKARA). Twenty five cycles of PCR (94°C for 1 min, 57°C for 1 min, 72°C for 1 min) were carried out. The PCR products were subjected to either a single or double digestions with EcoT22I and FokI, separated by electrophoresis on 5% polyacrylamide gels, and visualized by staining with ethidium bromide. For analysis of molar ratio of mRNA species, poly(A)⁺ RNA samples prepared from male, female sexual organs, and male, female thalli were subjected to RT-PCR (PCR in conjunction with reverse transcription) in the presence of 5 μCi of [α²³P]dCTP. The PCR products were then washed five times with TE-buffer using membrane filters SUPREC 02 (TAKARA). Approximately 1,000 cpm of PCR products were digested with EcoT22I and FokI, separated by electrophoresis using 5% polyacrylamide gel, and visualized using a image analyzer (BAS 2000, FUJIFILM).

In vitro protein kinase assay—Two cDNA fragments coding for CDPK-A (containing exon 6A) and CDPK-B (containing exon 6B) were amplified by PCR using the 5′-primer (5′-GGAGTACATATGGGCAACTGCGTG-3') and 3′-primer (5′-CAAGATCCACCAGGGTCGACATCA-3') (Ndel overlapping with translation initiation codon and BamHI site used for subcloning are indicated by underlines). The PCR products were digested with Ndel and BamHI, and the resulting fragments were inserted into the corresponding sites of the pET-15b vector (Novagen). Both of these cloned fragments were verified by DNA sequencing.

The fusion proteins with histidine-tags were expressed in E.coli BL21 (DE3) pLysS and purified according to the manufacturer's instruction. The protein kinase assays were performed at 25°C for 15 min in a 20 μl solution containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM CaCl₂, 4 μCi of [α²³P]ATP, 10 μg of a-casein (Sigma), and 500 ng of fusion protein. To measure the kinase activities in the absence of Ca²⁺ ions, 5 mM EGTA (ethylen glycol bis[β-aminoethylether]-N,N,N',N'-tetraacetic acid) was added as a chelating agent. Reaction mixtures were subjected to 12% SDS-polyacrylamide gel electrophoresis and to autoradiography.

Results

Isolation and characterization of the CDPK gene from M.polymorpha—Genomic DNAs of male and female thalli were amplified using five arbitrary 15-mer oligonucleotide primers for the purpose of detection of differences between male and female genomes. Ten PCR products which were detected to either male or female genomes were cloned and their sequences were determined. By chance, a deduced amino acid sequence from one of the clones, F3C containing 280-bp PCR fragment amplified using primer-2, showed significant similarities with a consensus sequence (called EF-hand) of CDPK genes. In order to obtain the full-length of the putative CDPK gene, a genomic library for the female suspension-cultured cells of liverwort (Akashi et al. 1996) was screened using the PCR fragment in the F3C as a probe. One lambda clone containing a 5.4-kb Ndel-SacI fragment was selected and the nucleotide sequence of the 5.4-kb fragment was determined. By comparing the nucleotide sequence with the databases,
Fig. 1 Structure of the liverwort CDPK gene (A) and comparison of partial amino acid sequences deduced from Exon 6A and Exon 6B (B). (A) Solid boxes represent exons and two nearly identical exons, Exon 6A and Exon 6B, are shown as shaded boxes with 6A and 6B, respectively. Arrow heads with F and R indicate the positions of RT-PCR primers, EX5-F and EX7-R, respectively. Restriction sites: B, BgIII; E, EcoRI; H, HindIII; N, NcoI; S, Sall; Sc, SacI; X, XbaI. (B) Amino acid sequences, Exon 6A and Exon 6B have a unique site for EcoT22I and FokI, respectively (Fig. 3B). In fact, the PCR product digested with EcoT22I produced three fragments; 224-bp fragment derived from either Exon 6A or Exon 6B, and 127-bp and 97-bp fragments from Exon 6A (Fig. 3A, lane 2). When digested with FokI, it gave three fragments; 224-bp fragment derived from Exon 6B, and 127-bp and 97-bp fragments from Exon 6A (Fig. 3A, lane 2). In addition, by double digestions with EcoT22I and FokI, it gave four fragments; 127-bp and 97-bp fragments from Exon 6A, and 160-bp and 64-bp fragments from Exon 6B (Fig. 3A, lane 3). Thus, these results indicate that two very similar mRNAs having either Exon 6A or Exon 6B are processed from a single CDPK gene by alternative splicing, and the post-transcriptional product derived from this gene is a mixture of two kinds of mRNA molecules having either Exon 6A or Exon 6B.

Differential expression of alternatively spliced products—We further surveyed the ratio of the accumulation of mRNAs having either Exon 6A or Exon 6B in different organs of the liverwort. Due to differences in the restriction sites of Exon 6A and Exon 6B, the ratio of alternatively spliced mRNAs can be estimated by comparing the levels isoforms which are translated from alternatively spliced mRNA molecules.

Accumulation of alternatively spliced mRNA molecules—To identify actual splicing products containing Exon 6A and/or Exon 6B, the region of the mRNA sequences ranging from Exon 5 to Exon 7 was amplified by RT-PCR using a set of primers, EX5-F and EX7-R (as shown in Fig. 1A). A PCR product of 224 bp was detected as a single band (Fig. 3A, lane 1), which indicates that the 224-bp fragment derived from either Exon 6A or Exon 6B (Fig. 3B), and a mature mRNA containing both Exon 6A and Exon 6B was not detected. Based on their nucleotide sequences, Exon 6A and Exon 6B have a unique site for EcoT22I and FokI, respectively (Fig. 3B). In fact, the PCR product digested with EcoT22I produced three fragments; 224-bp fragment derived from Exon 6B, and 127-bp and 97-bp fragments from Exon 6A (Fig. 3A, lane 2). When digested with FokI, it gave three fragments; 224-bp fragment derived from Exon 6A, and 160-bp and 64-bp fragments from Exon 6B (Fig. 3A, lane 3). In addition, by double digestions with EcoT22I and FokI, it gave four fragments; 127-bp and 97-bp fragments from Exon 6A, and 160-bp and 64-bp fragments from Exon 6B (Fig. 3A, lane 4). Thus, these results indicate that two very similar mRNAs having either Exon 6A or Exon 6B are processed from a single CDPK gene by alternative splicing, and the post-transcriptional product derived from this gene is a mixture of two kinds of mRNA molecules having either Exon 6A or Exon 6B.

Differential expression of alternatively spliced products—We further surveyed the ratio of the accumulation of mRNAs having either Exon 6A or Exon 6B in different organs of the liverwort. Due to differences in the restriction sites of Exon 6A and Exon 6B, the ratio of alternatively spliced mRNAs can be estimated by comparing the levels...
Alternative splicing of CDPK gene in *M. polymorpha*

Fig. 3 Restriction analysis of RT-PCR products. (A) First strands of cDNA mixture were generated from poly(A)⁺ RNA isolated from female liverwort suspension-cultured cells and amplified with EX5-F and EX7-R primers (lane 1). The RT-PCR products were digested with *EcoT22I* (lane 2), *FokI* (lane 3) and both enzymes (lane 4). (B) Schematic illustration of RT-PCR products shown in (A). Restriction endonuclease *EcoT22I* cleaved RT-PCR product derived from Exon 6A into two fragments with sizes of 97 bp and 127 bp, and *FokI* digested that containing Exon 6B into two fragments with the sizes of 64 bp and 160 bp.

of RT-PCR products followed by restriction digestions, if the PCR products are generated under non-saturating conditions. Since the two kinds of PCR products with Exon 6A or Exon 6B have the same length of 224 bp and they have almost identical nucleotide sequences which differ by 20 nucleotides in the exon regions, the molar ratio of the two PCR products should reflect that of the two kinds of mRNA molecules produced in respective male, female sexual organs, and male, female vegetative organs called thalli. Therefore, poly(A)⁺ RNA samples were isolated from each organs and subjected to RT-PCR for 23 cycles under non-saturating condition (data not shown) in the presence of 5% polyacrylamide gel, and then followed by autoradiography (Fig. 4A). Relative intensities of radioactivity in each band in the autoradiogram was estimated by image analyzer and shown in Fig. 4B. From the autoradiogram, the percentages of the accumulated PCR fragments with Exon 6A in the total PCR products prepared from female sex organ and thalli were from 38% to 44% (lanes 2-4). In contrast, that of the accumulated PCR fragments with

Fig. 4 Alternatively spliced products in different organs of the liverwort. (A) Poly(A)⁺ RNA was isolated from male sexual organs (lane 1), female sexual organs (lane 2), male thalli (lane 3) and female thalli (lane 4), and subjected to RT-PCR. The PCR products labelled with ³²P were completely digested with *EcoT22I* and *FokI*. (B) Schematic illustration of the relative intensities of radioactivity which were obtained by scanning of the autoradiogram as shown in panel (A). Total radioactivities of the four bands in each lane are shown as 100%.

Exon 6A prepared from male sex organ was approximately 60% (lane 1). These data suggest that a higher level of the mRNA transcripts with Exon 6A are accumulated in the male sexual organ compared with those of the mRNA with Exon 6B. This analysis was repeated ten times with two different preparations of poly(A)⁺ RNA samples and two different preparations of cDNA samples and the almost same accumulation pattern of RT-PCR products was observed in each autoradiograms (data not shown).

Purification of two fusion proteins of CDPK and protein kinase assay—The cDNA fragments coding for CDPK isoforms, CDPK-A derived from Exon 6A and CDPK-B from Exon 6B, were obtained by RT-PCR using a set of primers, 5'-primer and 3'-primer, and then inserted into the pET-15b expression vector. The two fusion proteins which were designed to have six histidine residues to the amino terminus (His-CDPK-A and His-CDPK-B) were expressed in *E. coli* and purified by Ni⁺⁺-affinity column chromatography (Fig. 5A). Each partially purified fraction contained 63-kDa protein corresponding to the expected molecular size of 62.6 kDa as a major component, although degraded bands were detected at 40 kDa and 30 kDa in SDS-PAGE (Fig. 5A, lanes 2 and 4). Both two fusion proteins, His-CDPK-A and His-CDPK-B, were used for an in vitro kinase assay using casein as a substrate (Fig. 5B). In the presence of Ca²⁺, phosphorylation of casein was observed in the presence of either fusion pro-
Alternative splicing of CDPK gene in *M. polymorpha*

Discussion

**Amino acid sequence conservation between liverwort and higher plant CDPKs**—The alternatively spliced liverwort CDPKs (CDPK-A from Exon 6A and CDPK-B from Exon 6B) each have 548 amino acid residues with an estimated molecular weights of 60418 and 60477, respectively. CDPK-A and CDPK-B shared significant amino acid sequence similarities (approximately 60% identity throughout their length) with previously characterized calcium-dependent and calmodulin-independent serine/threonine protein kinases of higher plants, *Arabidopsis* (64%) (Hong et al. 1996), rice (62%) (Breviario et al. 1995), maize (61%) ( Estruch et al. 1994), 44% identity with *Chlamydomonas* CDPK (Siderius et al. 1997), and 33% identity with *Drosophila* CaMK (Ohsako et al. 1993) (Fig. 6). Although the N- and C-terminal regions of the liverwort CDPKs did not show significant similarity to other plant CDPKs, liverwort CDPKs contain the following three conserved domains characteristic to CDPKs; First, the kinase domain is subdivided into twelve kinase subdomains (I-VIA, VIB-XI) as reported previously (Hanks and Hunter 1995) and shows a high level of local sequence similarity (193 amino acids identical in 259 amino acids) with the kinase domain of the maize CDPK which expresses specifically in pollen (Estruch et al. 1994). Twenty-three consensus amino acids predicted in the kinase domain (Stone and Walker 1995) were all conserved in the two liverwort CDPK isoforms (depicted as asterisks in Fig. 6). Second, the autoinhibitory domain, which functions as a pseudo-substrate for the kinase and inhibits kinase activity in the absence of Ca$^{2+}$ (Harmon et al. 1994), is highly conserved in the liverwort CDPK isoforms (30 amino acids identical in 34 amino acids with *Arabidopsis* CDPK6). Third, the calcium-binding domains forming a three-dimensional structure that consists of four helix-loop-helix domains (so called EF-hand motifs) are conserved in the liverwort CDPKs. Five amino acid residues, DxDx(D/S)/NxxxD(D/T/N)xExE (x, unconserved amino acid residues), in the loops of liverwort CDPK isoforms correspond to those which bind to Ca$^{2+}$ in other calcium-binding proteins as depicted previously (da Silva and Reinach 1991, Marsden et al. 1990). In addition, 6th Gly residue and 8th Ile residue in the EF-hands are conserved in the liverwort CDPK isoforms as shown previously (da Silva and Reinach 1991, Marsden et al. 1990). It has been reported that a single amino acid substitution in the EF-hands causes a dramatic reduction of calcium-binding affinity (Drake et al. 1996, Steinmetz et al. 1998), or drastic decrease in enzyme activity (Drayer et al. 1995). In the two liverwort CDPKs, there are three substitutions in the loops and one in a helix between the loops (shown in Fig. 1B and Fig. 6). In particular, the second calcium-binding loop contains an amino acid substitution (Asp in CDPK-A, and Asn in CDPK-B) involved in calcium-binding. A substitution between Asp and Glu, which is located in the helix (Fig. 1B) could reflect the calcium-binding affinity as reported that Glu residue in the helices modulate the calcium-binding affinity through the stability of three dimensional structure of the enzyme (Fujimori et al. 1990). These substitutions might reflect a different calcium-binding affinity or phosphorylation activity between the two liverwort isoforms, although both isozymes showed calcium-dependent protein kinase activities in vitro (Fig. 5B). Although higher levels of phosphorylation of casein and autophosphorylation were observed in one of the iso-zyme CDPK-B (Fig. 5B), it can not be concluded that CDPK-B has a higher specific activity than CDPK-A because of enzyme degradation or impurities in the two different preparations. Thus, further quantitative analy-
Fig. 6 Alignment of deduced amino acid sequences of the liverwort CDPK isoforms with those of other calcium-dependent protein kinases. Two liverwort CDPKs, CDPK-A (Liverwort-A) and CDPK-B (Liverwort-B), derived from either Exon 6A or Exon 6B, respectively. The compared sequence data are ATCDPK6 of Arabidopsis (Hong et al. 1996) (Genbank U20623), CDPK of rice (Breviarlo et al. 1995) (Genbank X81394), pollen specific CDPK of maize (Estruch et al. 1994) (Genbank L37484), CDPK of Arabidopsis (Sudieris et al. 1997) (Genbank D13330), and calcium/calmodulin-dependent protein kinase of Drosophila (Ohsako et al. 1993) (Genbank D13330). Amino acid residues are shown in single letter symbols. Colons indicate amino acids identical to those of the corresponding position in the kinase domain. Autoinhibitory domain is boxed by broken lines. Calcium-binding loops in EF-hands are boxed. Open arrowheads represent amino acid substitutions between liverwort CDPK-A and CDPK-B. Calcium-binding residues in EF-hands are shown as plus marks. Arrowheads represent positions corresponding to mRNA splicing sites. Numbers at the end of sequences indicate alternative splicing of CDPK gene in *M. polymorpha*.
ses of phosphorylation activities and calcium-binding affinities are required to determine the difference of these two isoforms in respect to amino acid sequence substitutions.

**Alternative splicing in the liverwort CDPK gene—**

Post-transcriptional alternative splicing has been discovered in eukaryotes as a mechanism to expand the limited genetic information for specific regulatory functions but the mechanism of alternative splicing in plants is not well documented. Interestingly, the liverwort CDPK gene has two almost identical and contiguous exons that are alternatively spliced into two mRNAs, and a mRNA containing both exons was hardly detectable. These results indicate that the intron between Exon 6A and Exon 6B (intron [6A–6B]) is not independently spliced out. A mature mRNA having Exon 6A results from elimination of the intron [5–6A] and the intron [6A–7] containing Exon 6B. On the other hand, a mature mRNA having Exon 6B is produced by eliminating the intron [5–6B] containing Exon 6A and the intron [6B–7]. Thus, the contiguous 5' and 3' splice sites of the intron [6A–6B] are not spliced at the same time. It has also been reported that an alternative splicing of rat alpha-tropomyosin gene involves mutually exclusive utilization of two tandem exons Exon 2 and Exon 3 (Smith and Nadal-Ginard 1989). Splicing of Exon 2 to Exon 3 is blocked. This block to splicing can be relieved by insertion of spacer elements that increase the 5' donor site—the lariat branch point separation. Thus, it has been suggested that the mutually exclusive alternative splicing of the two tandem exons is determined by the proximity of the 5' donor site to the branch point of the intron 2-3, which prevents the two exons from being spliced together. In the case of the liverwort CDPK gene, although the lariat branch point of the intron [6A–6B] is not clear, it is possible that the 63-nucleotide intron [6A–6B] in length is too short to be spliced out. Or it is possible that essential elements necessary to the independent splicing are missing in the intron [6A–6B], resulting in mutually exclusive alternative splicing.

**Functions and evolution of the liverwort CDPK gene—**

It is known that development and differentiation of organs or tissues are regulated by alternative splicing in plants (Görlich et al. 1995, Kopriva et al. 1995) as well as in mammals (Wieczorek et al. 1988, Libri et al. 1989). Our preliminary demonstration that a higher level of mRNA molecules encoding one type of kinase CDPK-A is accumulated in the male sexual organ than the other organs (Fig. 4), suggests that a differential expression of CDPKs in the sexual organs is controlled by alternative splicing. Alternative splicing events are actually reported in genes related with sex determination in *Drosophila* (Kelly and Kuroda 1995) and mating type locus in heterothallic ascomycete *Cochliobolus* (Leubner-Metzger et al. 1997). Considering these examples in other organisms, it might be possible that sex differentiation in the liverwort is regulated with differential signal transductions through two species of the liverwort CDPKs, although further experimental studies are required to prove it.

In plants, CDPKs are usually encoded by a set of genes namely gene family and those genes are expressed in a tissue-specific and stage-specific manners. To our knowledge, no example of alternative splicing has been reported for CDPK genes. Finding that CDPK isoforms are encoded by a single copy gene producing two almost identical species of mRNAs by alternative splicing in liverwort cells leads us to propose two possibilities on the evolution and diversification of CDPKs. One possibility is that the progenitor of land plants had only a single CDPK and that liverwort has acquired CDPK isoforms by the alternative splicing of a single gene after divergence of bryophytes and other plants. In contrast, higher plants have acquired isoforms encoded by multigene families through gene duplications. The other possibility is that the progenitor of land plants has already had CDPK isoforms through alternative splicing of a premature mRNA encoded by a single gene like liverwort, and that higher plants have lost alternative exons after gene duplication resulting multigene families. To discuss the evolution of CDPK, further informations about the insertional positions of introns in CDPK genes from other plants including ferns and higher plants are required.

The authors thank Dr. W. Gruissem, University of California, Berkeley, for his valuable suggestions. This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (No. 06454076 and No. 07281101), and by PROBRAIN.

**References**


Alternative splicing of CDPK gene in *M. polymorpha*


(Received October 28, 1998; Accepted December 2, 1998)