

Northern hybridization

Soybean poly(A)+ RNA (5 µg/lane) and RNA markers (4 µg/lane RNA Millennium Markers, Ambion, Austin, Texas) were electrophoresed on agarose gel (1% agarose in NorthernMax-Gly kit, Ambion). The poly(A)+ RNA was transferred to a nylon membrane (BrightStar-Plus Positive Charged Nylon membrane, Ambion). The RNA was downwardly transferred for one hours and thirty minutes with a stack assembly (TurboBlotter, Schleicher & Schuell, Keene, NH) and transfer buffer (Transfer Buffer in NorthernMax-Gly kit, Ambion). The blotted poly(A)+ RNA was crosslinked with UV light (120 mj/cm²). The crosslinked RNA was hybridized (63°C, overnight) with DIG labeled RNA probe (0.1 nM) in a hybridization solution (hybridization solution in NorthernMax-Gly kit, Ambion). The membrane with hybridized RNA and probe was washed twice in a low stringent solution (63°C, 5 minutes each time, #1 Washing Solution in NorthernMax-Gly kit, Ambion) and twice in high stringent solution (63°C, 15 minutes each time, #2 Washing Solution in NorthernMax-Gly kit, Ambion).

DIG labeled RNA probes were synthesized according to the manufacturers' protocol (Strip-EZ RNA T7 Strippable Probe Synthesis Kit, Ambion, Austin, Texas). The transcription mix (60 µl) included transcription buffer, ATP (500 µM), modified CTP (200 µM), GTP (500 µM), UTP (400 µM), DIG-11-UTP (100 µM), template (23 to 45 nM) and enzyme mixture (60 U). The transcription was carried out at 37°C for 2 hours. The template DNA was removed by RNase-free DNase I (6 U) at

37°C for 15 min. Heating at 75°C for 5 min destroyed the DNase.

The Large Subunit A probe (305-bp RNA) was synthesized with the template, the 3' untranslated region of the cDNA encoding Large Subunit A. Unlike the translated region, or the coding region, the 3' untranslated region usually does not have significant homology with other cDNAs encoding a homologous protein. Therefore, the Large Subunit A probe should hybridize only to Large Subunit A mRNA without cross hybridization to other RNR large subunit mRNAs. For the same reason, the small subunit RNA probe (285-bp RNA) was synthesized with the template, the 3' untranslated region of the cDNA encoding the small subunit.

Detection of hybridized nucleic acid

Hybridized to nucleic acid, DIG-labeled probe was recognized and combined by an antibody (Anti-DIG-AP, 37.5 mU/ml, Boehringer Mannheim, Indianapolis, IN) conjugated to alkaline phosphatase. The alkaline phosphatase hydrolyzed a chemiluminescent substrate (0.25 mM CDP-Star, Tropix, Bedford, MA). Indicating the location and quantity of targeted DNA or mRNA, the hydrolyzed substrate was detected by exposure to film (Biomax MR, Kodak, Rochester, NY) for 20 min to 1 hour according to the different amount of nucleic acid detected. The relative amount of nucleic acid detected were measured, calculated and displayed with a digital imaging system (AlphaImager, Alpha Innotech Corporation, San Leandro, CA).

Results

Cloning and sequence of the cDNA encoding the small subunit of ribonucleotide reductase in soybean

A middle fragment of the cDNA encoding the small subunit of RNR in soybean was first amplified, which had 770 bp, the expected size according to the primer positions (Figure 9). This fragment encoded a sequence of amino acids similar to the middle region of the small subunits of RNR in other organisms (Figure 14).

The 3' ends of the cDNA amplified by 3' RACE had sizes around 650 bp (Figure 10). They encoded a sequence of amino acids similar to the carboxyl-terminal of the small subunits of RNR in other organisms (Figure 14).

The 5' ends of the cDNA amplified by 5' RACE had sizes between 300 and 440 bp (Figure 11). The 5' ends varied in size, because the reverse transcriptase may have paused and the synthesis may have terminated prematurely. They encoded a sequence of amino acids similar to the amino-terminal of the small subunits of RNR from other organisms (Figure 14).

A cDNA fragment containing the complete coding region of the small subunit of RNR had 1227 bp, the expected size according to the primer positions (Figure 12). It was amplified with soybean cDNA template, the downstream-stop-codon primer and the upstream-start-codon primer (Table 3).

The cDNA encoding the small subunit of RNR in soybean included a complete open reading frame of 1020 bp, a 5' untranslated region of 78 bp, and a 3' untranslated region of 247 bp plus a poly(A) tail (Figure 13). The small subunit of RNR in soybean had 339 amino acids and a predicted molecular mass of 39 kDa.

Two characteristics of this sequence showed completion of the long open reading frame at its 5' end. First, two successive stop codons were found upstream from the start codon and in frame with the open reading frame (Figure 13). Second, the amino-terminal (N-terminal) residues between the small subunit of RNR in soybean and that in *Arabidopsis* were similar (Figure 14).

Two characteristics of the cDNA sequence indicated that this cDNA encoded a small subunit of RNR in soybean. First, the predicted amino acid sequence of this cDNA had significant overall similarities to the small subunits of RNR in diverse organisms, which represent five kingdoms (Table 7). Second, the amino acid residues essential for catalytic activity and for enzyme structure of the small subunits of RNR were conserved in the predicted amino acid sequence of this cDNA. The small subunit of RNR has 17 extremely conserved amino acid residues (Nordlund and Eklund 1993) (Chaboute et al. 1998). All these 17 amino acid residues were preserved in the predicted amino acid sequence of this cDNA as well as in the small subunit of RNR from 5 other organisms, which represent 5 kingdoms (Figure 14).

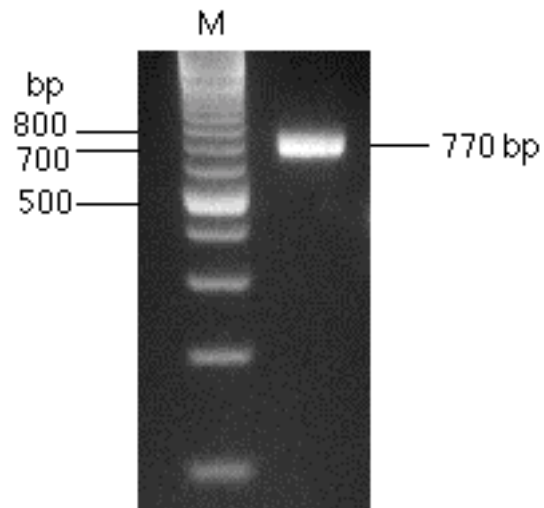


Figure 9. An amplified middle fragment of the cDNA encoding the small subunit of RNR in soybean. Right lane shows a middle fragment (770 bp) of cDNA encoding the small subunit of RNR in soybean. The fragment was amplified by PCR with soybean cDNA as template and with degenerate primers. 10 μ l PCR product was loaded. Left lane shows a DNA marker (100 bp ladder, Gibco BRL).

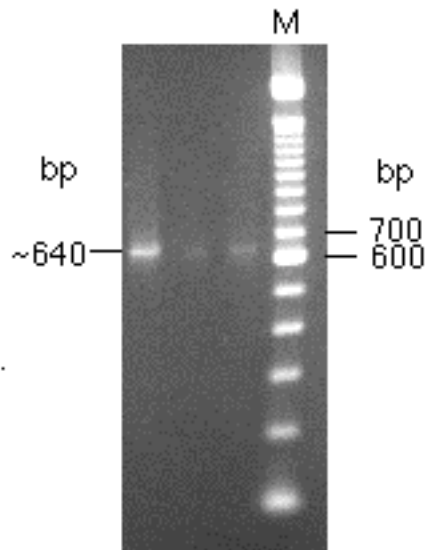


Figure 10. Amplified DNA fragments containing 3' ends of the cDNA encoding the small subunit of RNR in soybean

Left lane shows DNA fragments (around 640 bp) containing 3' ends of cDNA that encodes the small subunit of RNR in soybean. They are PCR products from 3'-rapid-amplification-of-cDNA-ends technique. Right lane shows a DNA marker (100 bp ladder, Gibco BRL).

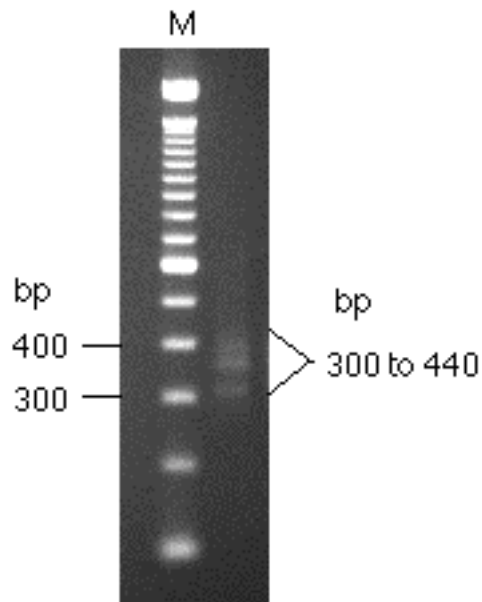


Figure 11. Amplified DNA fragments containing 5' ends of the cDNA encoding the small subunit of RNR in soybean
Right lane shows DNA fragments (between 300 and 440 bp) containing 5' ends of the cDNA encoding the small subunit of RNR in soybean. They are PCR products from 5'-rapid-amplification-of-cDNA-ends technique. Left lane shows a DNA marker (100 bp ladder, Gibco BRL).

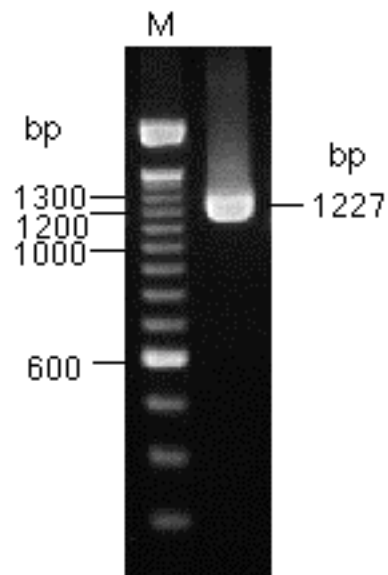


Figure 12. The amplified cDNA encoding the entire small subunit of RNR in soybean. Right lane shows a cDNA (1227 bp) encoding the entire small subunit of RNR in soybean. The cDNA was amplified with soybean cDNA, the downstream-stop-codon primer and the upstream-start-codon primer (Table 3). Left lane shows a DNA marker (100 bp ladder, Gibco BRL).

GTTTTGGGTGTCTTGGATCAGCGTGCTTTCTGATAGATAAGGGGTTTTTGTAGGGTTTAT 60
 TCTATTCATCCAAAAACAATGGGGTCTTTGGAAAATAATGGAGTAGACAAAGCAAGGGAT 120
 M G S L E N N G V D K A R D
 GAACAAGAACCAATTCTAATGGAGCAAAGCCAGAGGTTTTGCATGTTCCCATTCGCTAC 180
 E Q E P I L M E Q S Q R F C M F P I R Y
 AAGCAAATTTGGGAGATGTACAAGAAGGCTGAAGCCAGTTTTCTGGACTGCTGAAGAGTT 240
 K Q I W E M Y K K A E A S F W T A E E V
 GATCTCTTTATGATGTACAACATTGGGAGACCTTGTCTGTCTCTGAGAAGCACTTCATA 300
 D L S Y D V Q H W E T L S V S E K H F I
 ACACATGTCCTAGCTTTTTTGGCTGCTTCTGATGGAATTGTCTTGGAGAATTTGGCTGCA 360
 T H V L A F F A A S D G I V L E N L A A
 AGGTTTCTGAGTGATGTTCAAATTCCTGAGGCTCGGGCATTTCAGATAGCA 420
 R F L S D V Q I P E A R A F Y G F Q I A
 ATGGAGAATATTCATTCAGAGATGTACAGCTTGTCTGCTGGAGACGTATATTAAGATTCA 480
 M E N I H S E M Y S L L L E T Y I K D S
 AGAGAAAAACATAAATTATTCAATGCAATCGAAAATTTGCCTTGTGTTGCAAGGAAGGCA 540
 R E K H K L F N A I E N L P C V A R K A
 GAATGGGCATTGAGTTGGATTCATAGTTCCACTTCATTTGCAGAGAGACTTGTGCTTTT 600
 E W A L S W I H S S T S F A E R L V A F
 GCATGCGTTGAAGGGATATTTTCTCAGGAAGCTTCTGTGCCATATTCTGGCTTAAAAAG 660
 A C V E G I F F S G S F C A I F W L K K
 AGAGGATTGATGCCAGGTTTGACATTCTCAAATGAGCTAATCTCTAGAGATGAGGGCCTT 720
 R G L M P G L T F S N E L I S R D E G L
 CATTGTGATTTTGTCTTCTGTACAGTTTGTACGGAAGCCGCTAATTTCTGATCAG 780
 H C D F A C L L Y S L L R K P L I S D Q
 GTTCATAAGCTTGTACACGAAGCTGTTGAAATTGAACTGAGTTTGTGTGTGACGCCCTC 840
 V H K L V H E A V E I E T E F V C D A L

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CCCTGTCATTGATTGGCATGAACTCAGTGCTCATGAGCCAGTACATAAAATTTGTTGCT
----- 900
P C A L I G M N S V L M S Q Y I K F V A
GACAGGCTGTTGGTTGCCTTGGGGTACCAAAGAAAGTACAATGTGGAAAATCCCTTTGAT
----- 960
D R L L V A L G Y Q R K Y N V E N P F D
TGGATGGAGTTTATTTCTTTGCAAGGAAAGGCCAACTTTTTTCGAGAGAAGGGTGGGTGAT
----- 1020
W M E F I S L Q G K A N F F E R R V G D
TATCAAAAAGCGTCTGTGATGTCAAGCCTCCAAGATGCCGGGAAAAACTTTGTTTTCAAG
----- 1080
Y Q K A S V M S S L Q D A G K N F V F K
CTTGATGAGGACTTCTAATTATGATTTATCTGTTTCAGCTTCAATTATTCTGCGGATATA
----- 1140
L D E D F .
CTGAATTAGGCATCGGAAATTTCAATTTACTATTTTTTTTTTCAGTAACATGTTTAGTCTAC
----- 1200
ACACATAACTTAAGTGATTCCCTAGCCGCTGGTATCTATCTGCACTCAGGTCACCTTGATA
----- 1260
ATATGTTTCGTTATATTCTAATGGCATGTAATATTTGTAACGGTAGCCAAAACGGCCTA
----- 1320
GTCATAGTCATTAGTGATTAAGGAGAAAAAAAAAAAAAAAAAAAA
-----> 1361

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Figure 13. Sequence of the cDNA and predicted amino acid of the small subunit of ribonucleotide reductase in soybean

Two asterisks show two successive stop codons (TGA and TAG) upstream from the start codon and in frame with the long open reading frame, indicating completion of the open reading frame at its 5' end.

Table 7. Percentage identity between the amino acid sequence of the small subunit of ribonucleotide reductase in soybean and that in other organisms

Organism	Kingdom represented	Identity
Arabidopsis	Plantae	77.0 %
Human	Animalia	64.9 %
Baker's yeast	Fungi	60.2 %
Dictyostelium	Protista	64.8 %
<i>E.coli</i>	Monera	19.5 %

Soybean					
Arabidopsis					
Human			MLSLRVPLAPITDPQQQLSPLKGLSLVDKENTPPALSGTRVLA		
Baker's yeast			MPKETPSKAAADALSDLEIKDSSNLNKELETTLREENRVKSDMLKEKLSK		
Dictyostelium					
E.coli					
Soybean			MGSLENNGVDKARDE-QEPILMEQSQRFCMF-PIRYKQIWEM	40	
Arabidopsis			MGSLKEGQGRDMEEGESEPELLMAQNQRFTMF-PIRYKSIWEM		
Human			SKTARRIFQEPTEPKTKAAAPGVEDEPLLRENPRRFVIF-PIEYHDIWQM		
Baker's yeast			DAENHKAYLKSHQVHRHKLKEMEKEEPELLNEDKERTVLF-PIKYHEIWQA		
Dictyostelium			MEEINKKDTFIEPILKENKDRFVLF-PIKYPDIWRM		
E.coli			MAYTTFSTQTKNDQLKEPMFFGQPVNVARYDQQKYDIFEKL		
		49	59		
Soybean			YKKAEEASF ⁴⁹ WTAAEVDLSY ⁵⁹ DVQHWE-TLSVSEKHFITHVLAFFAASDGIVL	89	
Arabidopsis			YKKAEEASF ⁴⁹ WTAAEVDLST ⁵⁹ DVQQWE-ALTDSEKHFISHILAFFAASDGIVL		
Human			YKKAEEASF ⁴⁹ WTAAEVDLSK ⁵⁹ DIQHWE-SLKPEERYFISHVLAFFAASDGIVN		
Baker's yeast			YKRAEEASF ⁴⁹ WTAAEIDLK ⁵⁹ DIHDWNNRMNENERFFISRVLAFFAASDGIVN		
Dictyostelium			YKKALASHWVAEEIDLGN ⁵⁹ DNDWEYKLT ⁵⁹ DNERHFISHVLAFFAASDGIVN		
E.coli			IEKQLSFFWRPEEVDVSR ⁵⁹ DRIDYQ-ALPEHEKHIFISNLKYQTL ⁵⁹ LDSIQG		
			116 119 123		
Soybean			ENLAARFLSDVQIPEARAFYGFQIAMENI ¹¹⁶ HSEM ¹¹⁹ YSL ¹²³ LLETYIKDSREKHK	139	
Arabidopsis			ENLA-RFLNDVQVPEARAFYGFQIAMENI ¹¹⁶ HSEM ¹¹⁹ YSL ¹²³ LLETFIKDSKEKDR		
Human			ENLVERFSQEVQITEARCFYGFQIAMENI ¹¹⁶ HSEM ¹¹⁹ YSL ¹²³ LIDTYIKDPKERE		
Baker's yeast			ENLVENFSTEVQIPEAKSFYGFQIMENI ¹¹⁶ HSET ¹¹⁹ YSL ¹²³ LIDTYIKDPKESEF		
Dictyostelium			ENLATRFMSEVQIPEARCFYGFQIAIENI ¹¹⁶ HSET ¹¹⁹ YSL ¹²³ LIETYIKDKQTKDK		
E.coli			RSPNVALLPLISIPELETWVETWAFSE ¹¹⁶ TI ¹¹⁹ SR ¹²³ SYTHIIRNIVNDP---SV		
Soybean			LFNAIENLPCVARKAE-----WAL-----SWIHSSTS-FA	168	
Arabidopsis			LFNAIETIPCISKKAK-----WCL-----DWIQSPMS-FA		
Human			LFNAIETMPCVKKKAD-----WAL-----RWIGDKEATYG		
Baker's yeast			LFNAIHTIPEIGEKAE-----WAL-----RWIQDADALFG		
Dictyostelium			LFNAIETIPCISKKAE-----WAL-----RWINDSDS-FA		
E.coli			VFDDIVTNEQIQKRAEGISSYDELIEMTSYWHLLGEGTHTVNGKTVT ¹⁶⁸ VS		
		178 182 186		208	
Soybean			ER-----LVAFACVEGIF ¹⁷⁸ SGSF ¹⁸² CAIFWLKKRGLMPGLTFSNELIS	209	
Arabidopsis			VR-----LVAFACAE ¹⁷⁸ GIF ¹⁸² SGSF ¹⁸⁶ CAIFWLKKRGLMPGLTFSNELIS		
Human			ER-----VVAFAAVEGIF ¹⁷⁸ SGSF ¹⁸² ASIFWLKKRGLMPGLTFSNELIS		
Baker's yeast			ER-----LVAFASIEGV ¹⁷⁸ SGSF ¹⁸² ASIFWLKKRGMPGLTFSNELIC		
Dictyostelium			ER-----LVAFAAVEGIF ¹⁷⁸ SGSF ¹⁸² CSIFWLKKRGLMQGLTFSNELIS		
E.coli			LRELKKKLYLCLMSVNALEAIR ¹⁷⁸ FV ¹⁸² SFACSF ¹⁸⁶ AFAERELMEGNAKIIRLIA		
	210 212 215				
Soybean			RDEGLH ²¹⁰ CDFA ²¹² CLLYSL ²¹⁵ LRKPLISDQVHKL ²¹⁵ VHEAVEIETEFVCDALP----	255	
Arabidopsis			RDEGLH ²¹⁰ CDFA ²¹² CLLISL ²¹⁵ LQLHVPLEKVYQIVHEAVEIETEFVCKALP----		
Human			RDEGLH ²¹⁰ CDFA ²¹² CLMF ²¹⁵ KHLVHKPSEERVREIIINAVRIEQEFLTEALP----		
Baker's yeast			RDEGLHTDFA ²¹⁰ CLLF ²¹² FAHLKNKPDPAIVEKIVTEAVEIEQRYFLDALP----		
Dictyostelium			RDEGLH ²¹⁰ CDFA ²¹² CLLYTKLQRKLDPKVIEENDKSAVECEKEFICESLP----		
E.coli			RDEALH ²¹⁰ LTGTQHMLNLLRSGADDP ²¹² EMAETAE ²¹⁵ ECKQECYDLFVQAAQ ²¹⁵ QEKD		

			292
Soybean	-----CALIGMNSVLMSQYIKFVADRLLVALGYQRKYNV-ENPF	DWM	296
Arabidopsis	-----CDLIGMNSNLMSQYIQFVADRLLVTLGCERTYKA-ENPF	DWM	
Human	-----VKLIGMNCTLMKQYIEFVADRLLMELGFSKVFRV-ENPF	DFM	
Baker's yeast	-----VALLGMNADLMNQYVEFVADRLLVAFGNKKYYKV-ENPF	DFM	
Dictyostelium	-----VDLIGMNSRSMSQYIEFCADRLVVS LGYKKIFNS-SNPF	FEWM	
E.coli	WADYLF RDGSMIGLNKDILCQYVEYITNIRMQAVGLDLPFQTRS	NP	IPIWI
		309	315
Soybean	E--FISLQGANFFERRVGDYQKASVM---SSLQDAGKNFVFKLDEDF		339
Arabidopsis	E--FISLQGKTNFFEKRVG EYQKASVM---SNLQNGNQNYEFTTEEDF		340
Human	E--NISLEGKTNFFEKRVG EYQRMGVM-----SSPTENSFTLDADF		389
Baker's yeast	E--NISLAGKTNFFEKRVS DYQKAGVMSKSTKQEAGA----FTFNEDF		399
Dictyostelium	E--MISLQRKSNFFEKGVAEYAKTGVAIQGNNQKNNQSR TLVLDEDF		338
E.coli	NTWLVS DNVQVAPQEEV EYSSY----LVGQIDSEVD TDDLSNFQL		376

Figure 14. Alignment of the predicted amino acid sequence of the small subunit of ribonucleotide reductase in soybean with that in other organisms. The 17 most conserved amino acid residues essential for catalytic activity and for enzyme structure are highlighted: the iron ligand residues and their environment (Trp 49, Glu 116, His 119, Glu 178, Asp 211, Glu 212; His 215), the tyrosyl radical and its environment (Tyr 123; Phe 182, Ser 185, Phe 186; Ile 208), the residues for the binding of the large subunit (Asp 59, Arg 210, Glu 309, Tyr 315) and the residue for a helix turn (Pro 292). The numbering is based on the amino acid sequence of the small subunit of RNR in soybean. Soybean, accession number AAD32302; *Arabidopsis*, P50651; Human, PRRM2; Baker's Yeast, P09938; *Dictyostelium*, P42521; and *E.coli*, P00453.

Cloning and sequence of the cDNA encoding Large Subunit A of ribonucleotide reductase in soybean

The same strategy used to clone the small subunit of RNR in soybean was used to clone the large subunits (Figure 8). Three different large subunits of RNR in soybean were cloned; they are designated Large Subunit A, Large Subunit B and Large Subunit C.

A middle fragment of the cDNA encoding Large Subunit A of RNR in soybean was first amplified, which had 530 bp, the expected size according to the primer positions (Figure 15). This fragment encoded a sequence of amino acids similar to the middle region of the large subunits of RNR in other organisms (Figure 21).

The 3' ends of the cDNA amplified by 3' RACE had sizes between 540 and 800 bp (Figure 16). They encoded a sequence of amino acids similar to the carboxyl-terminal of the large subunits of RNR in other organisms (Figure 21).

The 5' ends of the cDNA amplified by 5' RACE had various sizes (Figure 17). The 5' ends varied in size, because the reverse transcriptase may have paused and the synthesis may have terminated prematurely. A cDNA of about 2.4 kb was cloned. It encoded a sequence of amino acids similar to the amino-terminal of the large subunits of RNR from other organisms (Figure 21).

A cDNA fragment containing the complete coding region of Large Subunit A of RNR had 2542 bp, the expected size

according to the primer positions (Figure 18). It was amplified with soybean cDNA template, the downstream-stop-codon primer and the upstream-start-codon primer (Table 4).

The cDNA encoding Large Subunit A of RNR in soybean included a complete open reading frame of 2430 bp, a 5' untranslated region of 287 bp, and a 3' untranslated region of 314 bp plus a poly(A) tail (Figure 19). Large Subunit A of RNR in soybean has 809 amino acids and a predicted molecular mass of 91 kDa.

Two characteristics of this sequence showed completion of the long open reading frame at its 5' end. First, a stop codon was found upstream from the start codon and in frame with the open reading frame (Figure 19). Second, the amino-terminal (N-terminal) residues between Large Subunit A of RNR in soybean and the large subunits of RNR in other eukaryotic organisms were similar (Figure 21).

Two characteristics of the cDNA sequence indicated that this cDNA encoded a large subunit of RNR in soybean. First, the predicted amino acid sequence of this cDNA had significant overall similarities to the large subunits of RNR in diverse organisms, which represent five kingdoms (Table 8). Second, the amino acid residues essential for catalytic activity of RNR were the same between Large Subunit A of RNR in soybean and the large subunits of RNR in six other organisms, which represent five kingdoms (Figure 21).

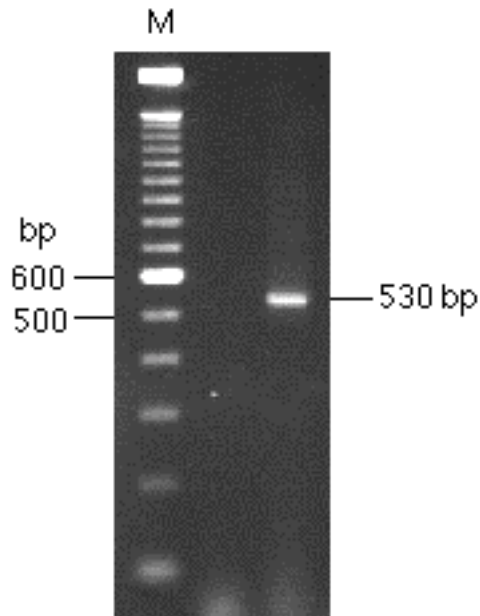


Figure 15. An amplified middle fragment of the cDNA encoding Large Subunit A of RNR in soybean. Right lane shows a middle fragment (530 bp) of cDNA encoding Large Subunit A of RNR in soybean. The fragment was amplified by PCR with degenerate primers and soybean cDNA as template. Left lane shows a DNA marker (100 bp ladder, Gibco BRL).

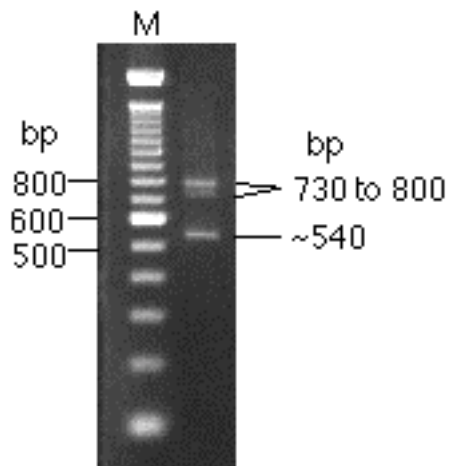


Figure 16. Amplified DNA fragments containing 3' ends of the cDNA encoding Large Subunit A of RNR in soybean

Right lane shows DNA fragments (between 540 and 800 bp) containing the 3' ends of cDNA encoding Large Subunit A of RNR in soybean. They are PCR products from 5'-rapid-amplification-of-cDNA-end technique. Left lane shows a DNA marker (100 bp ladder, Gibco BRL).

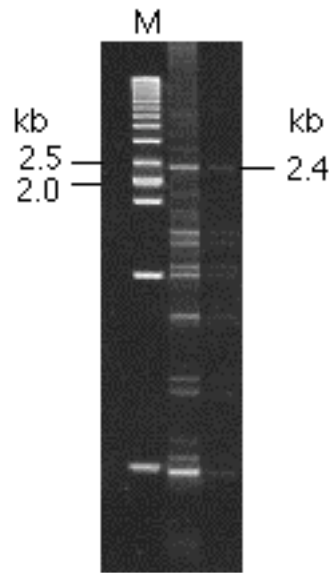


Figure 17. Amplified DNA fragments containing 5' ends of the cDNA encoding Large Subunit A of RNR in soybean
Right lane shows DNA fragments containing the 5' ends of cDNA encoding Large Subunit A of RNR in soybean. They are PCR products from 5'-rapid-amplification-of-cDNA-ends technique. The band of about 2.4 kb was cloned later. Left lane shows a DNA marker (500 bp ladder, Gibco BRL).

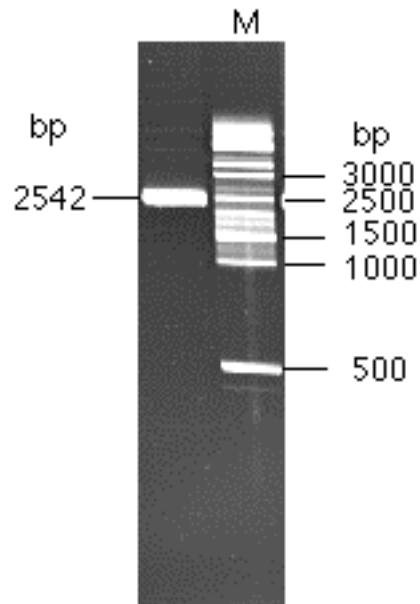


Figure 18. The amplified cDNA encoding entire Large Subunit A of RNR in soybean. Left lane shows a cDNA (2542 bp) encoding entire Large Subunit A of RNR in soybean. The cDNA was amplified with soybean cDNA as template, the downstream-stop-codon primer and the upstream-start-codon primer (Table 4). Right lane shows a DNA marker (500 bp ladder, Gibco BRL).

ACTCACTATAGGGCTCGAGCGGCCGCCGGGCGAGGTCTCCACAATTCATTTAATTCAAG 60
|-----*-----|
*
TTCTGAATTCCAAATGCAACGTTCTCGCGGGAAAATTTCGAAATTGCACACATTTGCGAG 120
|-----|
GGAACCCTCATTATTCAGCCGGCAAAAATAAAAAAAGAAATCTTTCACATATCCCAACCC 180
|-----|
CTCAGCACACAAACACTCTATTTATACCCTCGCGCCTGCGCCAAAAGTTCGACGAAAT 240
|-----|
TCTCCACTGAGAGACATAGAAAACCGAGAAAAGAAAAAAGACAATATGTATGTGGTGA 300
|-----|
M Y V V
AGAGAGATGGGAGGCAAGAGACGGTGCATTTTGATAAAAATCACCGCGCGTCTCAAGAAAC 360
|-----|
K R D G R Q E T V H F D K I T A R L K K
TTAGTTACGGTTTAAGCACCGAACACTGTGACCCTGTTCTGGTGTCTCAGAAAGTGTGG 420
|-----|
L S Y G L S T E H C D P V L V S Q K V C
CTGGCGTCTACAAAGGCGTCACCACTAGCCAGCTCGATGAGTTGGCGGCGGAAACCGCCG 480
|-----|
A G V Y K G V T T S Q L D E L A A E T A
CGGCTATGACCGCCAACCACTGACTATGCTTCTTTGGCTGCGAGGATTGCCGTTTCAA 540
|-----|
A A M T A N H P D Y A S L A A R I A V S
ATCTGCACAAGAATACGAAGAAGTCATTTTCTGAAACGATTAAGGTTATGTATTACCATT 600
|-----|
N L H K N T K K S F S E T I K V M Y Y H
TTAATGAGAGATCTGCGATGAAGGCTCCTCTGATTGCTGATGACGTCTATGAAATTATCA 660
|-----|
F N E R S A M K A P L I A D D V Y E I I
TCAAGAATGCTGCTCGATTGGACAGTGAGATAATCTATGATAGGGACTTTGACTATGATT 720
|-----|
I K N A A R L D S E I I Y D R D F D Y D
ACTTTGGTTTCAAACCTCTTGAGAGGTCATACCTCTGAAGGTTCAAGGAAAGGTTGTGG 780
|-----|
Y F G F K T L E R S Y L L K V Q G K V V
AAAGGCTCAGCACATGTTGATGAGGGTTGCTGTTGGAATTCATAAGGATGACATTGATT 840
|-----|
E R P Q H M L M R V A V G I H K D D I D

CTGCTGTCAGAACATACCACATGATGTCTCAGAGATGGTTCACTCATGCATCTCCAACAC 900
 S A V R T Y H M M S Q R W F T H A S P T
 TTTTCAATGCAGGAACACCTAGGCCTCAGTTGAGTAGTTGCTTCCTTGTGTGCATGAAAG 960
 L F N A G T P R P Q L S S C F L V C M K
 ATGATAGTATAGAGGGAATATATGACACTTTGAAGGAGTGTGCAGTCATTAGCAAATCAG 1020
 D D S I E G I Y D T L K E C A V I S K S
 CTGGAGGAATTGGTGTTAGTGTTCACAACATTCGTGCCACAGGAAGTTACATCCGTGGCA 1080
 A G G I G V S V H N I R A T G S Y I R G
 CAAATGGGACATCAAATGGTATTGTTCCAATGCTGCGTGTGTTCAACGATACTGCTCGCT 1140
 T N G T S N G I V P M L R V F N D T A R
 ATGTTGATCAAGGGGGAGGCAAGAGGAAAGGTGCATTTGCTGTGTACCTGGAGCCATGGC 1200
 Y V D Q G G G K R K G A F A V Y L E P W
 ATGCTGATATATTTGAATTCTTGATTTAAGGAAAAACCATGGAAAGGAAGAGCATCGAG 1260
 H A D I F E F L D L R K N H G K E E H R
 CTCGAGATTTGTTTTATGCTCTTTGGGTGTCTGATCTCTTTATGGAAAGAGTTCAGAGCA 1320
 A R D L F Y A L W V S D L F M E R V Q S
 ATGGGCAATGGTCTTTGTTTTGCCCAATGAAGCGCCAGGTTTGGCTGATTGCTGGGGTG 1380
 N G Q W S L F C P N E A P G L A D C W G
 AAGAATTTGAGAAATTGTACTCAATATGAAAGAGAAGGAAAAGCAAAGAAGGTTGTTC 1440
 E E F E K L Y T Q Y E R E G K A K K V V
 AGGCACAGAATCTCTGGTTTGAATTTTGAAGTCCCAGATAGAACTGGAACCTCCTTACA 1500
 Q A Q N L W F E I L K S Q I E T G T P Y
 TGCTTTTTAAGGACACTTGAATAAAAAAGCAACCAACAGAATTTGGGCACAATCAAGT 1560
 M L F K D T C N K K S N Q Q N L G T I K
 CATCAAACCTGTGCACTGAGATAATTGAGTATACAAGTCCAACAGAAACGGCTGTGTGCA 1620
 S S N L C T E I I E Y T S P T E T A V C

ACTTGGCATCGATTGCCCTGCCGCGATATGTAAGAGAGAAGGGTGTGCCCATGGAATCTC 1680
 N L A S I A L P R Y V R E K G V P M E S
 ATCCATCTAAGCTTGTTGGAAGCAGAGGCTCAAAAAACCGATATTTTGACTTTGACAAAC 1740
 H P S K L V G S R G S K N R Y F D F D K
 TGGGAGAGGTTACTGCAATAGTGGCAACAAACCTCAATAAAATAATTGATGTTAATTACT 1800
 L G E V T A I V A T N L N K I I D V N Y
 ACCCAGTTGATACTGCTAGAAAGATCAAACATGCGACACCGACCCATTGGTATTGGAGTTC 1860
 Y P V D T A R R S N M R H R P I G I G V
 AGGGTCTTGCCGATACCTTCATACTACTAGGTGTGGCATTGATTACCAGAGGCTCAGC 1920
 Q G L A D T F I L L G V A F D S P E A Q
 AGTTGAACAAGGATATATTTGAGACTATATACTATCATGCACTAAAACTTCATCTGAAT 1980
 Q L N K D I F E T I Y Y H A L K T S S E
 TGGCTGCAAAAAGAAGGTCCTATGAAACATATAGTGGTAGTCCTATAAGCAAGGGAATTC 2040
 L A A K E G P Y E T Y S G S P I S K G I
 TTCAGCCAGACATGTGGGGTGTGATGCCCTCAAGTCGTTGGGATTGGGATGCACTTCGGG 2100
 L Q P D M W G V M P S S R W D W D A L R
 AGATGATAGCAAAGACGGGTGTGAGGAACTCACTTCTTGTGGCCCTATGCCAACTGCAT 2160
 E M I A K T G V R N S L L V A P M P T A
 CAACTAGCCAGATTCTTGGCAATAATGAGTGTTTTGAGCCATATACTTCTAATATCTACA 2220
 S T S Q I L G N N E C F E P Y T S N I Y
 GCCGCAGAGTTCTAAGTGGTGAATTTGTTGTAGTGAACAAGCATTACTTCATGACTTGA 2280
 S R R V L S G E F V V V N K H L L H D L
 CTGAAATGGGACTTTGGTCTCCTACAATCAAGAATAATATTATCTATGAGGATGGTTTCAG 2340
 T E M G L W S P T I K N N I I Y E D G S
 TTCAGAAAATCCCAGAAATACCTGATGATCTGAAAATCATATAACAAGACTGTTTGGGAGA 2400
 V Q K I P E I P D D L K I I Y K T V W E

```

TTAAGCAAAGACATTGGTTGATATGGCTGTTGATAGAGGATGCTACATAGACCAGAGTC 2460
I K Q K T L V D M A V D R G C Y I D Q S
AGAGCTTGAATATTCACATGGATCAACCCAACTTTGGAAAGTAACTTCTTTGCATTTCT 2520
Q S L N I H M D Q P N F G K L T S L H F
ATGCATGGTCAAAGGGTTTGAAGACTGGGATGTATTATCTGCGATCACGTGCCGCAGCAG 2580
Y A W S K G L K T G M Y Y L R S R A A A
ATGCTATCAAGTTTACTGTTGATACCTCTATGCTCCATGAAAAACCTATGGCAGAGGAAG 2640
D A I K F T V D T S M L H E K P M A E E
AGGATGATAATACCAAGATGGCACAGATGGTGTGCTCTTTAACAAACCGAGAAGAGTGT 2700
E D D N T K M A Q M V C S L T N R E E C
TGGCTTGTGGAAGTTGAAAGCATTCCAAGTTCATTTTCCCGGATTGATTTTGGGCCTAA 2760
L A C G S .
AAACCCTTAATAAGTGCAAACCTCAAAAATTTGTTTTAGGCCTTAATGATATAAGGTTCA 2820
ACTCATACATGATAATCTTAAGCATTCTAAATTATCTTTAGAGCTTTGTATAGTACTTAT 2880
GGTGTTAATTTCAATTTGTACCTGTTTATGTATTGTATGTTAAATAGTTGAAAGATGTCAT 2940
TGTTGGTAGCATGTCAACTGTCCAGTGAGATTCTTTATTTCTACATTTAAATGTTACTA 3000
TTTGCTTATCTATTCCTTCTAAAATTTTAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 3060
A
> 3061

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Figure 19. Sequence of the cDNA and predicted amino acid of Large Subunit A of ribonucleotide reductase in soybean

An asterisk shows a stop codon (TAG) upstream from the start codon and in frame with the long open reading frame, indicating completion of the open reading frame at its 5' end.

Table 8. Percentage identity between the amino acid sequences of large subunits of ribonucleotide reductase in soybean and that in other organisms

Organism	Kingdom represented	Identity with Soybean Large Subunit A	Identity with Soybean Large Subunit B
Tobacco	Plantae	90.6 %	89.2 %
Human	Animalia	68.9 %	67.8 %
Fission yeast	Fungi	65.6 %	65.1 %
Plasmodium	Protista	64.7 %	63.2 %
<i>E.coli</i>	Monera	29.0 %	28.1 %

Cloning and sequence of the cDNA encoding Large Subunit B of ribonucleotide reductase in soybean

The cDNA encoding Large Subunit B of RNR in soybean included a complete open reading frame of 2427 bp , a 5' untranslated region of 39 bp, and a 3' untranslated region of 164 bp plus a poly(A) tail (Figure 20). Large Subunit B of RNR in soybean has 808 amino acids and a predicted molecular mass of 91 kDa. Large Subunits A and B of RNR in soybean share 92.3% identity of amino acid sequence.

The amino-terminal (N-terminal) residues between Large Subunit B of RNR in soybean and the large subunits of RNR in other eukaryotic organisms were similar (Figure 21), suggesting that the long open reading frame was complete at its 5' end.

Two characteristics of the cDNA sequence indicated that this cDNA encoded another large subunit of RNR in soybean. First, the predicted amino acid sequence of this cDNA had significant overall similarities to the large subunits of RNR in diverse organisms, which represent five kingdoms (Table 8). Second, the amino acid residues essential for catalytic activity of RNR were the same between Large Subunit B of RNR in soybean and the large subunits of RNR in six other organisms, which represent five kingdoms (Figure 21).

CCTGTTCTCACTTCACACTCCATCACCAGATATCACAAAATGTATGTCGTAAAGAGGAAC 60
 M Y V V K R N
 GGGCGGCAAGAGACGGTTCACTTCGACAAAATAACCGCGCGCTCAAGAAGCTTAGTTAT 120
 G R Q E T V H F D K I T A R L K K L S Y
 GGCCTCAGCTCCGATCACTGCGACGCCGTTTTGGTCGCTCAGAAAGTCTGCGCCGGCGTT 180
 G L S S D H C D A V L V A Q K V C A G V
 TACAAAGGCGTCACCACCAGCCAACCTTGACGAATTGGCCGCCGAAACTGCTGCCGCCATG 240
 Y K G V T T S Q L D E L A A E T A A A M
 ACCGCCAACCATCCCGACTACGCTTGCTTGGCTGCTAGGATTGCCGTTTTCGAATCTGCAC 300
 T A N H P D Y A C L A A R I A V S N L H
 AAGAACACCAAGAAGTCGTTCTCGGAGACGATCAAGATCATGTACTATCATTTTAATGAG 360
 K N T K K S F S E T I K I M Y Y H F N E
 AGATCTGGGCTGAAGGCTCCTCTGATCGCCGATGATGTTTATGAAATAATTATGAAGAAT 420
 R S G L K A P L I A D D V Y E I I M K N
 GCTGCTCGTCTAGACAGCGAGATAATCTATGACAGGGACTTTGACTATGATTATTTTGGA 480
 A A R L D S E I I Y D R D F D Y D Y F G
 TTCAAACCCCTTGAGAGGTCTTACCTCTTGAAGGTTCAAGGGCAGGTTTTGGAAAGGCCT 540
 F K T L E R S Y L L K V Q G Q V L E R P
 CAACACATGTTGATGAGGGTTGCTGTTGGGATTACAAGGATGACATAGATTCTGCTGTC 600
 Q H M L M R V A V G I H K D D I D S A V
 AAAACTTACCACATGATGTCTCAACGATGGTTACCCATGCATCTCCGACACTTTTCAAT 660
 K T Y H M M S Q R W F T H A S P T L F N
 GCTGGAACACCTAGGCCTCAACTGAGTAGTTGCTTCTTGTGTGCATGAAAGATGATAGT 720
 A G T P R P Q L S S C F L V C M K D D S
 ATAGAGGGGATATATGAAACTTTGAAGGAGTGTGCTATCATCAGCAAATCAGCTGGAGGA 780
 I E G I Y E T L K E C A I I S K S A G G

ATTGGTGTCTCTATTCATGACATTCGTGCTACAGGCAGTTATATTCGTGGGACAAATGGG 840
 I G V S I H D I R A T G S Y I R G T N G
 ACATCCAATGGCATTGTCCCGATGTTGCGTGTGTTCAATGATACTGCCCGCTATGTTGAT 900
 T S N G I V P M L R V F N D T A R Y V D
 CAGGGGGGAGGCAAAAGGAAAGGTGCATTTGCTGTGTACTTGGAGCCATGGCATGCTGAT 960
 Q G G G K R K G A F A V Y L E P W H A D
 ATGTTTGAATTCTTGGATTTAAGGAAAAATCATGGGAAGGAAGAGCATCGTGCTCGAGAT 1020
 M F E F L D L R K N H G K E E H R A R D
 CTGTTTTATGCTCTCTGGGTGCCTGATCTCTTTATGGAAAGAGTTCAGAGCAATGGCCAA 1080
 L F Y A L W V P D L F M E R V Q S N G Q
 TGGTCTTTGTTTTGTCCAGTGAAGCACCGGGTTTGACAGATTGTTGGGGTGAGAAATTT 1140
 W S L F C P S E A P G L T D C W G E K F
 GAGGAGCTTTATCTTCAATATGAAAGAGAAGGAAAAGCAATGAAGGTTGTCCAGGCACAG 1200
 E E L Y L Q Y E R E G K A M K V V Q A Q
 AGCCTCTGGTTCGAAATTTCTGAAGTCACAGATAGAAACCGGAACCCCTACATGCTTTTT 1260
 S L W F E I L K S Q I E T G T P Y M L F
 AAGGATACTTGCAATAGGAAAAGCAATCAACAGAATTTGGGTACAATTAATCGTCAAAC 1320
 K D T C N R K S N Q Q N L G T I K S S N
 TTGTGTAAGATAATTGAATATTCAAGTCCAAGTCAAAGTCTGTGTGCAATCTGGCA 1380
 L C T E I I E Y S S P T E T A V C N L A
 TCAATTGCACTACCACGATATGTAAGAGAAAAGGGTGTACCAATGGAGTCCCATCCATCC 1440
 S I A L P R Y V R E K G V P M E S H P S
 AAGCTTGTGGGAAGCACATGCTCTGGAATCGGTATTTTGACTTTGATAAACTAGCAGAG 1500
 K L V G S T C S G N R Y F D F D K L A E
 ATTACTGCACTGGTCACAACAAACCTGAACAAAGTAATTGATGTTAATTACTACCCAGTT 1560
 I T A L V T T N L N K V I D V N Y Y P V

GAAAATGCAAAACGGTCAAACCTTGCGGCACAGACCAATTGGTATTGGAGTACAGGGTCTT 1620
E N A K R S N L R H R P I G I G V Q G L
GCTGATACTTTCATACTCCTTGGCATGGCATTGATTACACCAGAGGCTCAGCAGTTAAAC 1680
A D T F I L L G M A F D S P E A Q Q L N
AAGGAGATATTTGAGACTATATATTATCATGCTCTAAAAACTTCATGTGGTTTGGCTGCA 1740
K E I F E T I Y Y H A L K T S C G L A A
AAGGAAGGTCCTATGAAACATACAGTGGTAGTCTATAAGCAAGGGAATTCTTCAGCCG 1800
K E G P Y E T Y S G S P I S K G I L Q P
GACATGTGGGGTGTGGCACCTCAAATCGTTGGGATTGGGATGCACTTCGGGAGATGATA 1860
D M W G V A P S N R W D W D A L R E M I
TCAAAGAATGGTGTGAGAACTCACTTCTCGTGGCCCCTATGCCTACTGCATCTACTAGC 1920
S K N G V R N S L L V A P M P T A S T S
CAGATTCTTGAAATAACGAGTGTGTTTGAACCATATACTTCAAACATATACAGTCGTAGA 1980
Q I L G N N E C F E P Y T S N I Y S R R
GTTTTAAGTGGTGAATTTGTTGTTGTGAACAAGCATCTTCTTCATGACTTGACTGAAATG 2040
V L S G E F V V V N K H L L H D L T E M
GGACTGTGGTCTCCTACATTAAGAATAAGATTATCTATGAAGATGGCTCAGTTCAGAAA 2100
G L W S P T L K N K I I Y E D G S V Q K
ATTCCAGAAATACCTGCTGACCTGAAGAATATATACAAAACCTGTTTGGGAGATTAACAA 2160
I P E I P A D L K N I Y K T V W E I K Q
AGGACATTGGTAGATATGGCTGCTGATCGAGGATGCTACATAGATCAGAGCCAAAGCTTA 2220
R T L V D M A A D R G C Y I D Q S Q S L
AATATTCACATGGATCAACCCAACCTTTGGAAAACCTGACTTCCTTGCAAGTTTTATGCATGG 2280
N I H M D Q P N F G K L T S L Q F Y A W
TCCAAGGGTCTAAAAACTGGAATGTATTATCTTCGATCACGTGCTGCAGCTGATGCTATT 2340
S K G L K T G M Y Y L R S R A A A D A I

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AAGTTCACCGTTGACACCTCTGCTCTCAGAGAAAAATCCAATGTGGAGGATGATGATAAT 2400
K F T V D T S A L R E K S N V E D D D N
ACAAAAATGGCACAGATGGTGTGCTCTTTAACAAACCGTGACGAGTGCTTAGCTTGTGGG 2460
T K M A Q M V C S L T N R D E C L A C G
AGTTGAAATGTTTCAAAAGTAATGCTTTCATTAGAGGCGACAAAGATAAATCACGTGTTA 2520
S .
ATCTATAGCTTTTTCAAAGCTTTGAACATAGTTGTCATCAATATCATTTGTATTCTTGCA 2580
TATCCTTGTATCAGATTTATTTACTCAGTAGTATAAATACCAAGTCAGTTAAAAAAAAA 2640
AAAAAAAAAAAAAAAAA
-----> 2656

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Figure 20. Sequence of the cDNA and predicted amino acid of Large Subunit B of ribonucleotide reductase in soybean

Soybean A	MY---- <td>DKITARLKKLSY---GLSTEHC</td> <td>DPVLVSQKV</td> <td>43</td>	DKITARLKKLSY---GLSTEHC	DPVLVSQKV	43		
Soybean B	MY---- <td>DKITARLKKLSY---GLSSDH</td> <td>CDAVLVAQKV</td> <td></td>	DKITARLKKLSY---GLSSDH	CDAVLVAQKV			
Tobacco	MY---- <td>DKITARLKKLSY---GLSPDH</td> <td>CDPVLVAQKV</td> <td></td>	DKITARLKKLSY---GLSPDH	CDPVLVAQKV			
Arabidopsis	MY---- <td>DKITARLKKLSY---GLSSDH</td> <td>CDPVLVAQKV</td> <td></td>	DKITARLKKLSY---GLSSDH	CDPVLVAQKV			
Human	MH---- <td>DKITSRIQKLCY---GLNMD</td> <td>FVDPAQITMKV</td> <td></td>	DKITSRIQKLCY---GLNMD	FVDPAQITMKV			
Fission yeast	MF---- <td>DKITARVSR</td> <td>LCY---GLDSDHVPVEITQKV</td> <td></td>	DKITARVSR	LCY---GLDSDHVPVEITQKV			
Plasmodium	MY---- <td>DQILKRIQRLSY---GLH-</td> <td>ELVDPARVTQGV</td> <td></td>	DQILKRIQRLSY---GLH-	ELVDPARVTQGV			
E.coli	MNQNL	LVTKRDGSTERINLDKI-HRV--LDWAAEGLHN-----VSISQ--				
Soybean A	CAGV-----YKGV	TTSQ	LDELAAETA	AAMTANH-PDYASLAARIAVS	84	
Soybean B	CAGV-----YKGV	TTSQ	LDELAAETA	AAMTANH-PDYACLAARIAVS		
Tobacco	CAGV-----YKGV	TTSQ	LDELAAETA	AAMTANH-PDYACLAARIAVS		
Arabidopsis	CAGV-----YKGV	TTSQ	LDELAAETA	AAMTCNH-PDYASLAARIAVS		
Human	IQGL-----YSGV	TVE	LDTLAAETA	AATLT	TKH-PDYAILAARIAVS	
Fission yeast	ISGV-----YPGV	TTE	LDNLAAETA	AATMT	TKH-PDYAILAARIAVS	
Plasmodium	INGM-----YSGIK	TCE	LDELAAQT	CAYMATTH-PDFSILAARITTD		
E.coli	--- <td>KTSDIHETIIKAAADLISR</td> <td>DAPDYQYLAARLAI</td> <td>F</td> <td></td>	KTSDIHETIIKAAADLISR	DAPDYQYLAARLAI	F		
Soybean A	NLHKNTKKS	FSFE-TIKVMYYHF	NERSAMKAPLI	ADDVYEII	IKNAARL--	131
Soybean B	NLHKNTKKS	FSFE-TIKIMYYHF	NERSGLKAPLI	ADDVYEII	IMKNAARL--	
Tobacco	NLHKTPKKS	FSFE-TIKDMYYHV	SERSGLKAPL	VSDEVYEII	IMKNAARL--	
Arabidopsis	NLHKNTKKS	FSFE-TIKDMFYHV	NDRSGLKSPLI	ADDVFEII	MQNAARL--	
Human	NLHKETK	KVFS	D-VMEDLYNY	INPHNGKHSP	MAKSTLDIVLANKDRL--	
Fission yeast	NLHKQTE	KVFS	T-VVQQ	LHDYVNP	KTDK	PAPMISDKIYDIVMKHKDEL--
Plasmodium	NLHKNTS	DDVAE-VAE	ALTYK	DVR-GRPASLI	SKEVYDFILLHKDRL--	
E.coli	HLRK--- <td>QFEP</td> <td>PALYDHV-----VKM</td> <td>VEMGKYD-----NH</td> <td>LLE</td> <td></td>	QFEP	PALYDHV-----VKM	VEMGKYD-----NH	LLE	
Soybean A	-----DSEII	YDRDF	YDYFG	FKTLERSYLL--KVQ	GKVVERPQH	169
Soybean B	-----DSEII	YDRDF	YDYFG	FKTLERSYLL--KVQ	GQVLERPQH	
Tobacco	-----DSEII	YDRDF	YDYFG	FKTLERSYLL--KIQ	GKVVERPQH	
Arabidopsis	-----DSEII	YDRDFEY	YDYFG	FKTLERSYLL--KVQ	GTVVERPQH	
Human	-----NSAI	YDRDFS	YNYFG	FKTLERSYLL--KING	KVAERPQH	
Fission yeast	-----DSAI	YDRDF	TYNFF	FGKTLERSYLL--RIDG	KVAERPQH	
Plasmodium	-----NKEI	DYTRDF	NYDYFG	FKTLERSYLL--RIN	KIIERPQH	
E.coli	DYTEEE	FKQMDTFIDH	DRDMTFSYAAV	KQLE	GKYL	VQNRVTGEIYESAQF
Soybean A	MLMRVAVGIHKD-----	DIDSAVRTYH	HMMSQRWF	THASPTL	205	
Soybean B	MLMRVAVGIHKD-----	DIDSAVKTYH	HMMSQRWF	THASPTL		
Tobacco	MLMRVAVGIHKD-----	DIESVIKTYH	LMMSQRWF	THASPTL		
Arabidopsis	MLMRVAVGIHKD-----	DIDSVIQTYH	LMMSQRWF	THASPTL		
Human	MLMRVAVGIHKE-----	DIDAAIETY	NLLSERWF	THASPTL		
Fission yeast	MIMRVAVGIHGE-----	DIEAAIETY	NLMSQRYF	THASPTL		
Plasmodium	LLMRVAVGIHID-----	DIDKALETYH	LMMSQKYF	THATPTL		
E.coli	LYILVAAC	LFSNYPRE	TRLQYVKRFYD---AVST	FKI-----SL	PTPIM	

217 218

Soybean A	FNAG--TPRPQLSSCFLV-CMKDDSI EGIYDTLKECAVI---SKSAGGIG	249
Soybean B	FNAG--TPRPQLSSCFLV-CMKDDSI EGIYETLKECAII---SKSAGGIG	
Tobacco	FNAG--TPRPQLSSCFLI-CMKEDSI EGIYDTLKECAVI---SKSAGGIG	
Arabidopsis	FNAG--TPRPQLSSCFLV-CMKDDSI EGIYETLKECAVI---SKSAGGIG	
Human	FNAG--TNRPQLSSCFLV-SMKDDSI EGIYDTLKQCALI---SKSAGGIG	
Fission yeast	FNAG--TPRPQLSSCFLV-TMKDDSI EGIYDTLKMCAVI---SKTAGGIG	
Plasmodium	FNSG--TPRPQMSSCFLV-SMKADSI EGIYFETLKQCALI---SKTAGGIG	
E.coli	--SGVRTPTRQFSSCVLIEC--GDSLDSINAT--SSAIVKYVSRAG-IG	
Soybean A	VSVHNIRATGSYIRGTNGTNSGIVPMLRVFNDTARYVDQGGGKRKGA---	296
Soybean B	VSIHDIRATGSYIRGTNGTNSGIVPMLRVFNDTARYVDQGGGKRKGA---	
Tobacco	VSVHNIRATGSYIRGTNGTNSGIVPMLRVFNDTARYVDQGGGKRKGA---	
Arabidopsis	VSVHNIRATGSYIRGTNGTNSGIVPMLRVFNDTARYVDQGGGKRKGA---	
Human	VAVSCIRATGSYIAGTNGNSNGLVPMIRVYNNNTARYVDQGGNKRPGA---	
Fission yeast	INIHNIRATGSYIAGTNGTNSGIVPMIRVYNNNTARYVDQGGNKRPGA---	
Plasmodium	VAVQDIRGQNSYIRGTNGISNGLVPMIRVYNNNTARYVDQGGGKRKGS---	
E.coli	INAGRIRALGSPIRGGEAFHTGCIPFYKHFQTAVKSCSQGGV-RGGAATL	
Soybean A	FAVYLEP-WHADIFEFLDLRKNHGKEEHRARDLFYALWVSDLFM-ERVQS	344
Soybean B	FAVYLEP-WHADMFEFDLRKNHGKEEHRARDLFYALWVPDLFM-ERVQS	
Tobacco	FAVYLDP-WHADIFEFLDLRKNHGKEEHRARDLFYALWVPDLFM-QRVQS	
Arabidopsis	FAVYLEP-WHADVYEFLELRKNHGKEEHRARDLFYALWLPDLFM-ERVQN	
Human	FAIYLEP-WHLDIFEFLDLKKTGKEEQRARDLFFALWIPDLFM-KRVET	
Fission yeast	FAAYLEP-WHADVMDFEFLRKTGHNEDFRAREMFYALWIPDLFM-QRVER	
Plasmodium	FAVYIEP-WHSDIFEFLDLRKNHGKEELRARDLFYAVWVPDLFM-KRVKE	
E.coli	F--Y--PMWHEVESLVLKNNRVEGVRVHMDYGVQINKL-MYTRLLK	
Soybean A	NGQWSLFCPNEAPGL-----ADCWGEEFEKLYTQYEREGKAKKV-VQAQN	388
Soybean B	NGQWSLFCPSEAPGL-----TDCWGEKFEELYLQYEREGKAMKV-VQAQS	
Tobacco	NGQWSLFCPNEAPGL-----ADCWGEDFEKLYINYEKEGKAKKV-VQAQN	
Arabidopsis	NGQWSLFCPNEAPGL-----ADCWGAEFETLYTKYEREGKAKKV-VQAQQ	
Human	NQDWSLMCPNECPGL-----DEVWGEFEKLYASYEKQGRVRKV-VKAQQ	
Fission yeast	NEQWTFFCPNEAPGL-----ADVWGEFVALYKEYEKENRGRRS-LPAQK	
Plasmodium	NKNWTL MCPNECPGL-----SETWGEFEKLYTKYEEENMGKKT-VLAQD	
E.coli	GEDITL FSPSDVPGLYDAFFAD--QEEFERLYTKYEKDDSI RQVRKAVE	
	427 429 431	
Soybean A	LWFEIL-KSQIETGTPYMLFKDTCNRKSN-QQNLGTIKSSNLCTEIIIEYT	436
Soybean B	LWFEIL-KSQIETGTPYMLFKDTCNRKSN-QQNLGTIKSSNLCTEIIIEYS	
Tobacco	LWFEIL-KSQIETGTPYMLYKDTNRKSN-QQNLGTIKSSNLCTEIIIEYT	
Arabidopsis	LWYEIL-TSQVETGTPYMLFKDSCNRKSN-QQNLGTIKSSNLCTEIIIEYT	
Human	LWYAIL-ESQTETGTPYMLYKDTNRKSN-QQNLGTIKCSNLCTEIVEYT	
Fission yeast	VWYAIL-QSQVETGNPFMYKDTNRKSN-QKNVGTIRCSNLCTEIVEYS	
Plasmodium	LWFAIL-QSQIETGTPYMLYKDTNRKSN-QKNLGTIKCSNLCTEIIIEYT	
E.coli	L-FSLMMQERASTGRIYIQNVDCNTHSPFDPAIAPVRQSNLCTEIIIEY	

		444	459	
Soybean A	SPT-----	ETAVCNLASIALPRYVR-	EKGVPMESHPSKLVGSRG	474
Soybean B	SPT-----	ETAVCNLASIALPRYVR-	EKGVPMESHPSKLVGSTC	
Tobacco	SPA-----	ETAVCNLASIALPRYVR-	EKGVPSSESQPSKLVGSRG	
Arabidopsis	SPT-----	ETAVCNLASIALPRFVR-	EKGVPLDSHPPKLAGSLD	
Human	SKD-----	EVAVCNLASLALNMYVT-	-----	
Fission yeast	SPD-----	EVAVCNLASVALPTFIK-	-----	
Plasmodium	SPD-----	EVAVCNLASIALCKFVDLEK-	---KE-----	
E.coli	LPTKPLNDVNDENGEIALC	TLSAFNL-	-----GAIN	

		478		
Soybean A	SKNR	YFDFDKLGEVTAIVATNLNKIIDVNY--	YPVDTARRSNMRHRPIGI	522
Soybean B	SGNR	YFDFDKLAEITLVTNLNKVIDVNY--	YPVENAKRSNLRHRPIGI	
Tobacco	SKNR	YFDFDKLAEVTALVTNLNKIIDVNY--	YPVETAKRSNLRHRPIGI	
Arabidopsis	SKNR	YDFEKLAEVTATVTVNLNKIIDVNY--	YPVETAKTSNMRHRPIGI	
Human	SEHTY-	DFKKLAEVTKVVRNLNKIIDINY--	YPVPEACLSNKRHRPIGI	
Fission yeast	-DGKY-	NFQKLHDVVKVTRNLNKIIDVNY--	YPVPEARSNMRHRPVGL	
Plasmodium	-----	FNFKKLYEITKIIITRNLDKIIERNY--	YPVKEAKTSNTRHRPIGI	
E.coli	-----	NLDELEELAILAVRALDALLD--	YQDYPIPAAKRGAMGRRTLGI	

Soybean A	GVQGLADTFILLGVAF-----	DSPEAQQLNKIDIFETI-YYHALKT	561
Soybean B	GVQGLADTFILLGMAF-----	DSPEAQQLNKEIFETI-YYHALKT	
Tobacco	GVQGLADTFMLLGMFAF-----	DSREAQQLNKIDIFETI-YYHALKA	
Arabidopsis	GVQGLADAFILLGMPF-----	DSPEAQQLNKIDIFETI-YYHALKA	
Human	GVQGLADAFILMRYPF-----	ESAEAQLLNKQIFETI-YYGALEA	
Fission yeast	GVQGLADAFFALRLPF-----	ESAGAKKLNQIFETI-YHAALEA	
Plasmodium	GVQGLADTFMLLRYPY-----	ESDAAKELNKRIFETM-YYAALEM	
E.coli	GV-----	INFAYYLAKHGKRYSDGS-ANLTHKTFEAIQYYL-LKA	

Soybean A	SSELAAKEGP---YE--	TYSGSPISKGILQPDMWGVMPSSR-----	597
Soybean B	SCGLAAKEGP---YE--	TYSGSPISKGILQPDMWGVAPSNR-----	
Tobacco	SSELAAKEGP---YE--	TYAGSPVSKGIIQPDMWGVTPSDK-----	
Arabidopsis	STELAARLGP---YE--	TYAGSPVSKGILQPDMWNVIPSDR-----	
Human	SCDLAKEQGP---YE--	TYEGSPVSKGILQYDMWNVTPDL-----	
Fission yeast	SCEIAQVEGT---YE--	SYEGSPASQGILQYDMWNVNPTDL-----	
Plasmodium	SVELASIHGP---YE--	SYQGSPASQGILQYDMWNAKVDNKY-----	
E.coli	SNELAKEQGACPFNETTYA-----	KGIL-----PIDTYKKDLDTIA	

Soybean A	-----	WDWDALREMIAKTGVNRSLLVAPMPTASTSQILGNNECFEP---Y	639
Soybean B	-----	WDWDALREMISKNGVRNLLVAPMPTASTSQILGNNECFEP---Y	
Tobacco	-----	WDWVALREMITKNGVRNLLVAPMPTASTSQILGNNECFEP---Y	
Arabidopsis	-----	WDWAVLRDMISKNGVRNLLVAPMPTASTSQILGNNECFEP---Y	
Human	-----	WDWKVLKEKIAKYGIRNLLIAPMPTASTAQILGNNESIEP---Y	
Fission yeast	-----	WDWAELEKKEIAKHGIRNLLVAPMPTASTSQILGFNECFEP---Y	
Plasmodium	-----	WDWDELKAKIRKHGLRNLLLAPMPTASTSQILGNNESFEP---Y	
E.coli	NEPLHYDWEALRESIKTHGLRNSTLSALMPSETSSQISNATNGIEPPRGY		

Soybean A	TSNIYSRRVLSGEFVVVNKHLHDLTEMGLWSPTIK-NNIIYEDGSVQKI	688
Soybean B	TSNIYSRRVLSGEFVVVNKHLHDLTEMGLWSPTLK-NKIIYEDGSVQKI	
Tobacco	TSNIYSRRVLSGEFVVVNKHLHDLTEMGLWSPALK-NRIIYEDGSVQKI	
Arabidopsis	TSNIYSRRVLSGEFVVVNKHLHDLTDMGLWTPTLK-NKLINENGSIYVNV	
Human	TSNIYTRRVLSGEFQIVNPHELLKDLTERGLWHEEMK-NQIIACNGSIQSI	
Fission yeast	TSNMYQRRVLSGEFQIVNPWLLKDLVERDLWNEDMK-NKLVMLDGSIQAI	
Plasmodium	TSNIYYRRVLSGEFFVVNPHELLKDLFDRGLWDEDMK-QQLIAHNGSIQYI	
E.coli	VS-----IKASK----DGILRQV	
Soybean A	PEIPD--DLKIIYKTVWE-----IKQKTLVDMAVDRGCYIDQS	724
Soybean B	PEIPA--DLKNIYKTVWE-----IKQRTLVDMAADRGCYIDQS	
Tobacco	PEIPD--ELKEIYKNVWE-----IKQRTLVDMAVDRGCYIDQS	
Arabidopsis	AEIPD--DLKAIYRTVWE-----IKQRTVVDMAADRGCYIDQS	
Human	PEIPD--DLKQLYKTVWE-----ISQKTVLKMMAAERGAFIDQS	
Fission yeast	PEIPQ--DLKDLYKTVWE-----ISQKTVIDYAADRGPFIDQS	
Plasmodium	SEIPD--DLKELYKTVWE-----IKQKNIIDMAADRGIFFIDQS	
E.coli	--VPDYEHLHDAYELLWEMPNGNDGYLQLVGMQK-----FIDQS	
		756 757
Soybean A	QSLNIHMDQPNF--GK-----LTSLFYAWSKGLKTGMYYLRSRA	762
Soybean B	QSLNIHMDQPNF--GK-----LTSLQFYAWSKGLKTGMYYLRSRA	
Tobacco	QSLNIHMDQPNF--GK-----LTSLFHTWSRGLKTGMYYLRSRA	
Arabidopsis	QSLNIHMDKPNF--AK-----LTSLFHYTWKGLKTGMYYLRSRA	
Human	QSLNIHIAEPNY--GK-----LTSMHFYGWKQGLKTGMYYLRTRP	
Fission yeast	QSLNIHLKDPSY--GK-----ITSMHFYGWKKGLKTGMYYLRTMA	
Plasmodium	QSLNIYIQKPTF--AK-----LSSMHFYGWEKGLKTGAYYLRTQA	
E.coli	ISANTNYDPSRFPSPGKVPMQQLLKDLLTAYKF-----GVKT-LYYQNTRD	
Soybean A	AADAIKFTVDT----SMLHE-----KPM-----EEE-----	785
Soybean B	AADAIKFTVDT----SALRE-----KSNV-----ED-----	
Tobacco	AADAIKFTVDT----AMLKE-----KPKT-----AVD-----	
Arabidopsis	AADAIKFTVDT----AMLKE-----KPSVAEGDKEVEEEE-----	
Human	AANPIQFTL-----NKEK----LKD-KEKVS----KEE-	
Fission yeast	ASAAIKFTVDP----VALRARNEESNEENKKPVIKNGKAEISAEPTKEEI	
Plasmodium	ATDAIKFTVDTHVAKNAVKLKNADG-----VQITREVSRETI	
E.coli	GAE-----DAQ-----	
Soybean A	----DDNTKMAQMVCSL--TNREECL--ACGS	809
Soybean B	----DDNTKMAQMVCSL--TNRDECL--ACGS	808
Tobacco	----DD-TKMAQMVCSL--TNREDCL--SCGS	808
Arabidopsis	----DNETKLAQMVCSL--TNPEECL--ACGS	816
Human	---EEKERNTAAMVCSL--ENRDECL--MCGS	792
Fission yeast	DIYNEK-----VLACSI--KNPEACE--MCSA	811
Plasmodium	ST----ESTVTQNVCLRRNNDQCL--MCSG	806
E.coli	---DD-----LVPSIQ---DDGCESGACKI	761

Figure 21. Alignment of the amino acid sequence of the large subunits of ribonucleotide reductase in soybean with that in other organisms. Soybean A = Soybean Large Subunit A. Soybean B = Soybean Large Subunit B. The amino acid residues essential for the catalytic activity of RNR are highlighted: the cysteines for the reduction of the ribose of the substrate (Cys 218, 429, 444), the tyrosines for the transfer of the free radical from the small subunit (Tyr 756, 757), and the amino acid residues for binding and transformation of the ribose moiety of the substrate (Ser 217, Asn 427, Glu 431) (Uhlin and Eklund 1994). A motif of 20 amino acids (from Gly 459 to Asn 478) unique to plants is also highlighted. The numbering is based on the amino acid sequence of Soybean A. Soybean A, accession number AF118784; Soybean B, AF118785; Tobacco, CAA71815; *Arabidopsis*, AAD20398; Human, P23921; Fission's Yeast, P36602; Plasmodium, P50648; and *E.coli*, P00452.

Cloning and sequence of the cDNA encoding partial Large Subunit C of ribonucleotide reductase in soybean

When the 3' end of the cDNA encoding Large Subunit B of RNR in soybean was amplified and cloned, a cDNA fragment encoding part of another large subunit (Large Subunit C) of RNR was unintentionally cloned. The cDNA fragment includes an open reading frame of 390 bp (Figure 22). The open reading frame is probably incomplete at its 5' end. The cDNA has a 3' untranslated region of 138 bp plus a poly(A) tail.

The open reading frame encodes a partial protein of 129 amino acids. It shares 88.4% and 94.6% identity, respectively, with the same region of Large Subunit A and B of RNR in soybean. This region of Large Subunit A and B share 88.4% identity. The amino acid residues essential for catalytic activity of RNR are conserved in this partial protein as in other large subunits of RNR (Figure 23). According to above evidences, this cDNA clone probably encodes the C-terminal part Large Subunit C of RNR in soybean.

```

TATGAAGATGGCTCTGTTTCAGAAAATTCAGAAATGCCTGCTGTAAGAGTATATAC 60
|-----|
Y E D G S V Q K I P E M P A V L K S I Y
AAACTGTTTGGGAGATTAACAAAGGACATTGGTAGATATGGCTGCTGATCGAGGATGC 120
|-----|
K T V W E I K Q R T L V D M A A D R G C
TACATAGATCAGAGCCAAAGTTTAAATATTCACATGGATCAACCCAACCTTGGAAAGCTG 180
|-----|
Y I D Q S Q S L N I H M D Q P N F G K L
ACTTCCTTGCAATTTCTATGCATGGTCCAAGGGTCTAAAGACTGGAATGTATTATCTTCGA 240
|-----|
T S L H F Y A W S K G L K T G M Y Y L R
TCGCGAGCTGCAGCCGATGCTATTAAGTTCACCGTTGACACCTCTGCTCTCAAAGAAAAA 300
|-----|
S R A A A D A I K F T V D T S A L K E K
TCCAATGTAGAGTATGATGATAATACAAAAATGGCACAGATGGTGTGCTCTTTAACAAC 360
|-----|
S N V E Y D D N T K M A Q M V C S L T N
CGTGAAGAGTGCTTAGCTTGTGGGAGTTGAACTGTTTCAAAGTAATGCTTCTTTAGAGA 420
|-----|
R E E C L A C G S .
TGGCAAAGATAAATGTTTCAAGATAAATCACGTGTTAATCTATAACTTTCTGGAACGATT 480
|-----|
TCAAAGCTTTGTTTCATATTTGTCATTAATATCATTGTATCTTTGCTAAAAAAAAAAAAA 540
|-----|
AAAAAAAAAAAAAAAAA
-----> 555

```

Figure 22. Sequence of the cDNA fragment and predicted amino acid of partial Large Subunit C of ribonucleotide reductase in soybean

```

Soybean A Part  YEDGSVQKIPEIPDDLKIIYKTVWEIKQKTLVDMAVDRGCIYIDQSQSLNI 729
Soybean B Part  YEDGSVQKIPEIPADLKNYKTVWEIKQRTLVDMAADRGCYIDQSQSLNI
Soybean C Part  YEDGSVQKIPEMPAVLKSIIYKTVWEIKQRTLVDMAADRGCYIDQSQSLNI

                               756 757
Soybean A Part  HMDQPNFGKLTSLH FYAWSKGLKTGMY Y LRSRAAADAIKFTVDTSMLHEK 779
Soybean B Part  HMDQPNFGKLTSLQ FYAWSKGLKTGMY Y LRSRAAADAIKFTVDTSALREK
Soybean C Part  HMDQPNFGKLTSLH FYAWSKGLKTGMY Y LRSRAAADAIKFTVDTSALKEK

                               785
Soybean A Part  PMAEEEDDNTKMAQMVCSLTNREECLACGS 809
Soybean B Part  SNVED- DDNTKMAQMVCSLTNRDECLACGS
Soybean C Part  SNVEY- DDNTKMAQMVCSLTNREECLACGS

```

Figure 23. Alignment of the amino acid sequence of C-terminal regions of Large Subunit A, B and C of ribonucleotide reductase in soybean.

The residues essential for catalytic activity of RNR are highlighted: the tyrosines for the transfer of free radical from the small subunit (Tyr 756,757). A missing amino acid residue (Glu 785) in Large Subunit B and C is also highlighted. The numbering is based on the amino acid sequence of Large Subunit A.

The gene or gene family encoding the subunits of ribonucleotide reductase in soybean

In order to determine whether a single gene or a gene family encodes the large and small subunit of RNR in soybean, Southern analysis was performed with different restriction digests. The results imply that a gene family of at least three members encodes different large subunits of RNR, and that a single gene encodes the small subunit of RNR in soybean.

Southern analysis of the genes encoding the large subunits of RNR showed three restriction fragments digested by either *Bbu* I or *Bgl* II (Figure 24). The three restriction fragments obtained by *Bbu* I digestion and detected by Southern analysis were 7.7, 9 and 14 kb. The 9-kb fragment probably represents the Large Subunit A gene, because it showed the strongest hybridization intensity among the three fragments. The probe was a 401-bp Dig-labeled DNA. It had the sequence from 1467th to 1867th nucleotide in the coding region of Large Subunit A cDNA. The probe should hybridize to Large Subunit A gene with the strongest intensity due to perfect matches of the sequences. The probe should hybridize to other RNR large subunit genes with weaker intensity than to Large Subunit A gene due to mismatches of the sequences. For example, between the probe and the cDNA of Large B Subunit, there were mismatches of 11.2% of the nucleotides (45 out of 401 nucleotides did not match).

The three restriction fragments obtained by *Bgl* II digestion and detected by Southern analysis were 4.9, 5.8 and 6.6 kb. The 5.8-kb fragment probably represents the Large Subunit A gene, because it showed the strongest hybridization intensity among the three fragments.

Southern blot analysis of the gene encoding the small subunit showed a single restriction fragment digested by *Bbu* I, *Bgl* II or *Eco*R I (Figure 25). This result implies that a single gene may encode the only small subunit of RNR in soybean. The probe was a 448-bp DIG-labeled DNA. It had the sequence from 84th to 531st nucleotide in the coding region of the small subunit cDNA.

The restriction enzymes used in this study were selected based on two reasons. First, these restriction enzymes did not have restriction site in the probed regions of the cDNA according to the sequence analysis. Therefore, these enzymes might lack restriction site in the probed regions of the genomic DNA. However, if there were unknown introns within the probed regions of the genomic DNA, extra restriction sites were still possible and would result in multiple hybridizing fragments. Second, based on preliminary results, these restriction enzymes completely or nearly completely digested soybean genomic DNA (Table 9). Some restriction enzymes can not completely digest soybean genomic DNA, because different restriction enzymes have different sensitivities to site-specified methylation of DNA, and because methylation of DNA is more extensive in higher plants than in animals (Zhu et al. 1994).

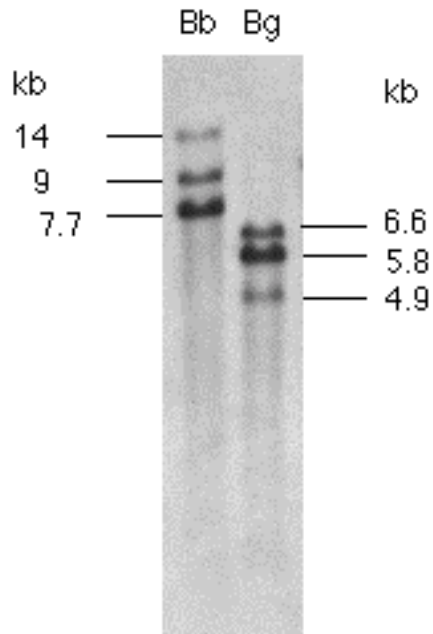


Figure 24. Southern blot analysis of genes encoding the large subunits of ribonucleotide reductase in soybean
 Soybean genomic DNA (5 µg/lane) was digested with restriction enzyme *Bbu* I (Bb) or *Bgl* II (Bg), electrophoresed through an agarose gel, and transferred to a membrane. A 401-bp DIG-labeled DNA probed the membrane. The probe DNA had the sequence of a part coding region in the cDNA that encodes Large Subunit A of RNR in soybean.

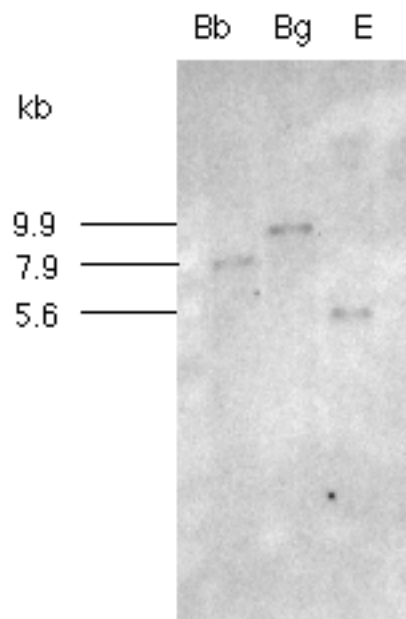


Figure 25. Southern blot analysis of genes encoding the small subunit of ribonucleotide reductase in soybean

Soybean genomic DNA (5 µg/lane) was digested with restriction enzyme *Bbu* I (Bb), *Bgl* II (Bg), or *EcoR* I (E). The digested DNA was electrophoresed through an agarose gel and transferred to a membrane. A 448-bp DIG-labeled DNA probed the membrane. The probe DNA had the sequence of part coding region in the cDNA that encodes the small subunit of RNR in soybean.

Table 9. Digestibility of Soybean Genomic DNA by Restriction Enzymes

Enzyme	Effect
<i>Bam</i> H I	C
<i>Bbu</i> I = <i>Sph</i> I	B
<i>Bgl</i> II	B
<i>Eco</i> R I	A
<i>Kpn</i> I	C
<i>Sal</i> I	D
<i>Xba</i> I	B
<i>Xho</i> I	D

- A. complete digestion
- B. small amount of undigested genomic DNA remained
- C. moderate amount of undigested genomic DNA remained
- D. large amount of undigested genomic DNA remained

Gene expression of ribonucleotide reductase in soybean

In order to determine if light influences RNR gene expression, relative levels of RNR mRNA in primordial shoots of young soybean seedlings were estimated with Northern hybridization.

RNR mRNA was detected from soybean seedlings grown in the dark and from those exposed to light. The relative level of Large Subunit A mRNA in primordial shoots in seedlings exposed to light was 23% less than that in seedlings grown in the dark (Figure 27). The relative level of the small subunit mRNA in seedlings exposed to light was 33% less than that in seedlings grown in the dark (Figure 28). The amount of the RNA loaded was the same for the two gel lanes that represent two growth conditions (Figure 26).

When the Large Subunit A probe was used, a transcript of approximately 2.9 kb was detected. From this result one can conclude that the Large Subunit A clone of the 3.06 kb-long cDNA is nearly a full-length cDNA clone.

When the small subunit probe was used, a transcript of around 1.4 kb was detected. From this result one can conclude that the small subunit clone of 1.36 kb-long cDNA is nearly a full-length cDNA clone.

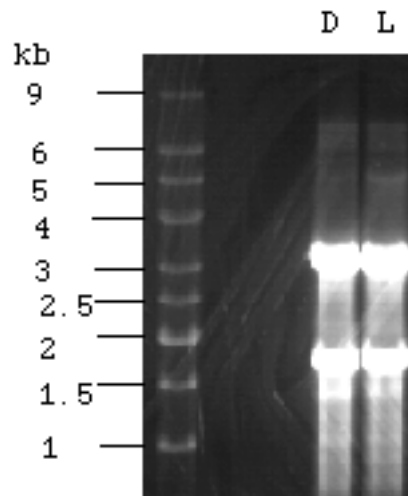


Figure 26. Electrophoresis of mRNA from soybean

Poly(A)⁺ RNA was isolated from the primordial shoots of 4-day old soybean seedlings. After soybean seeds germinated for four days in the dark and the seedlings emerged from soil, the seedlings were grown for additional three hours in the dark (D), or in the light (L). The same amount of poly(A) RNA (5 µg per lane including remnant ribosomal RNA) from the primordial shoots under these two growth conditions was electrophoresed through an agarose gel and stained with ethidium bromide. The RNA was subsequently transferred to a membrane for mRNA level analysis. Left lane shows a RNA marker.

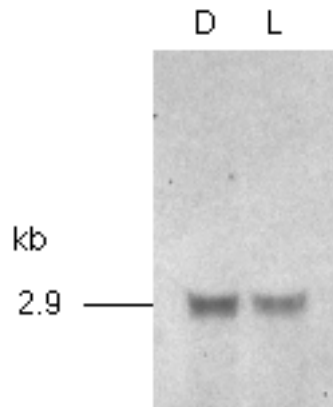


Figure 27. Northern blot analysis of the mRNA encoding Large Subunit A of ribonucleotide reductase in soybean.

Poly(A)⁺ RNA was isolated from the primordial shoots of 4-day old soybean seedlings. After soybean seeds germinated for four days in the dark and the seedlings emerged from soil, the seedlings were grown for three additional hours in the dark (D), or in the light (L). The poly(A) RNA (5 µg per lane including remnant ribosomal RNA) was separated on an agarose gel and transferred to a membrane. The membrane was probed by a 305-bp DIG-labeled RNA, which hybridized to the mRNA encoding Large Subunit A of RNR in soybean.

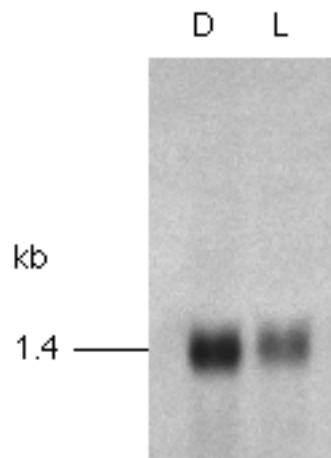


Figure 28. Northern blot analysis of mRNA encoding the small subunit of ribonucleotide reductase in soybean.

Poly(A)⁺ RNA was isolated from the primordial shoots of 4-day old soybean seedlings. After soybean seeds germinated for four days in the dark and the seedlings emerged from soil, the seedlings were grown for three additional hours in the dark (D), or in the light (L). The poly(A) RNA (5 µg per lane including remnant ribosomal RNA) was separated on an agarose gel and transferred to a membrane. The membrane was probed by a 285-bp DIG-labeled RNA, which hybridized to the mRNA encoding the small subunit of RNR in soybean.

Multiple poly(A) sites in mRNAs encoding ribonucleotide reductase in soybean

Among the six sequenced cDNA clones encoding Large Subunit A of RNR in soybean, five different poly(A) sites were found (Figure 29). These poly(A) sites resulted in five different lengths of the 3' untranslated regions of the cDNAs. The length of these 3' untranslated regions, or the distance from the stop codons to the poly(A) tails, varied from 289 to 314 nucleotides. The two far away poly(A) sites were 26 nucleotides apart. The two closest poly(A) sites were next to each other.

Similarly, three different poly(A) sites were found from three clones encoding the small subunit of RNR in soybean (Figure 30). The two far away poly(A) sites were 63 nucleotides apart. To the contrary, only a single poly(A) site was found from three clones encoding Large Subunit B of RNR in soybean (Figure 31). Polyadenylation signal AAUAAA sequence was not discerned upstream these poly(A) sites in the mRNA encoding Large Subunit A, Large Subunit B and the small subunit.

Clone A AAGCATTCCAAGTTGCATTTTCCCGGATTGATTTTGGGCCTAAAAACCCT 50
Clone B AACCCT 50
Clone C AAGCATTCCAAGTTGCATTTTCCCGGATTGATTTTGGGCCTAAAAACCCT 50
Clone D AAGCATTCCAAGTTGCATTTTCCCGGATTGATTTTGGGCCTAAAAACCCT 50
Clone E AAGCATTCCAAGTTGCATTTTCCCGGATTGATTTTGGGTCTAAAAACCCT 50
Clone F AAGCATTCCAAGTTGCATTTTCCCGGATTGATTTTGGGCCTAAAAACCCT 50

Clone A TAATAAGTGCAAACCTCAAAAATTTGTTTTAGGCCTTAATGATATAAGGT 100
Clone B TAATAAGTGCAAACCTCAAAAATTTGTTTTAGGCCTTAATGATATAAGGT 100
Clone C TAATAAGTGCAAACCTCAAAAATTTGTTTTAGGCCTTAATGATATAAGGT 100
Clone D TAATAAGTGCAAACCTCAAAAATTTGTTTTAGGCCTTAATGATATAAGGT 100
Clone E TAATAAGTGCAAACCTCAAAAATT-GTTTTAGGCCTTAATGATATAAGGT 99
Clone F TAATAAGTGCAAACCTCAAAAATTTGTTTTAGGCCTTAATGATATAAGGT 100

Clone A TCAACTCATACATGATAATCTTAAGCATTCTAAATTATCTTTAGAGCTTT 150
Clone B TCAACTCATACATGATAATCTTAAGCATTCTAAATTATCTTTAGAGCTTT 150
Clone C TCAACTCATACATGATAATCTTAAGCATTCTAAATTATCTTTAGAGCTTT 150
Clone D TCAACTCATACATGATAATCTTAAGCATTCTAAATTATCTTTAGAGCTTT 150
Clone E TCAACTCATACATGATAATCTTAAGCATTCTAAATTATCTTTAGAGCTTT 149
Clone F TCAACTCATACATGATAATCTTAAGCATTCTAAATTATCTTTAGAGCTTT 150

Clone A GTATAGTACTTATGGTGTTAATTTTCATTTGTACCTGTTTATGTATTGTAT 200
Clone B GTATAGTACTTATGGTGTTAATTTTCATTTGTACCTGTTTATGTATTGTAT 200
Clone C GTATAGTACTTATGGTGTTAATTTTCATTTGTACCTGTTTATGTATTGTAT 200
Clone D GTATAGTACTTATGGTGTTAATTTTCATTTGTACCTGTTTATGTATTGTAT 200
Clone E GTATAGTACTTATGGTGTTAATTTTCATTTGTACCTGTTTATGTATTGTAT 199
Clone F GTATAGTACTTATGGTGTTAATTTTCATTTGTACCTGTTTATGTATTGTAT 200

Clone A GTTAAATAGTTGAAAGATGCCATTGTTGGTAGCATGTCAACTGTCCAGTG 250
Clone B GTTAAATAGTTGAAAGATGTCATTGTTGGTAGCATGTCAACTGTCCAGTG 250
Clone C GTTAAATAGTTGAAAGATGTCATTGTTGGTAGCATGTCAACTGTCCAGTG 250
Clone D GTTAAATAGTTGAAAGATGTCATTGTTGGTAGCATGTCAACTGTCCAGTG 250
Clone E GTTAAATAGTTGAAAGATGTCATTGTTGGTAGCATGTCAACTGTCCAGTG 249
Clone F GTTAAATAGTTGAAAGATGTCATTGTTGGTAGCATGTCAACTGTCCAGTG 250

Clone A AGATTCTTTATTTCCCTACATTTAAATGTTA 280
Clone B AGATTCTTTATTTCCCTACATTTAAATGTTA 280
Clone C AGATTCTTTATTTCCCTACATTTAAATGTTA 280
Clone D AGATTCTTTATTTCCCTACATTTAAATGTTA 280
Clone E AGATTCTTTATTTCCCTACATTTAAATGTTA 279
Clone F AGATTCTTTATTTCCCTACATTTAAATGTTA 280

Clone A	CTATT-GCTC-----	289(290)
Clone B	CTATTTGCTC-----	290(290)
Clone C	CTATT-GCT-ATC-----	291(293)
Clone D	CTATTTGCTTATCTATTCCTTC-----	302(302)
Clone E	CTATTTGCTTATCTATTCCTTCT-----	302(303)
Clone F	CTATTTGCTTATCTATTCCTTCTAAAATTTTAGC	314(314)

Clone A	AAAAAAAAAAAAAAAAAAAAA
Clone B	AAAAAAAAAAAAAAAAAAAAA
Clone C	AAAAAAAAAAAAAAAAAAAAA
Clone D	AAAAAAAAAAAAAAAAAAAAA
Clone E	AAAAAAAAAAAAAAAAAAAAA
Clone F	AAAAAAAAAAAAAAAAAAAAA

Figure 29. Heterogeneity of 3' untranslated regions in mRNAs encoding Large Subunit A of ribonucleotide reductase in soybean. The complete 3' untranslated region of six clones encoding Large Subunit A of RNR in soybean are shown (part of Clone B is missing). The numbers without parentheses indicate the nucleotide position downstream from the stop codon. The numbers in parentheses indicate the nucleotide position referring to the consensus sequence. Missing nucleotides in the sequences caused the difference between the two numbers.

Clone A	TTATGATTTATCTGTTTCAGCTTCAATTATTCTGCGGATATACTGAATTA	50
Clone B	TTATGATTTATCTGTTTCAGCTTCAATTATTCTGCGGATATACTGAATTA	50
Clone C	TTATGATTTATCTGTTTCAGCTTCAATTATTCCGCGGATATACTGAATTA	50
Clone A	GGCATCGGAAATTTCACTTACTATTTTTTTTTT-CAGTAACATGTTTAGTC	99
Clone B	GGCATCGGAAGTTTCATTTACTATTTTTTTTTTTCAGTAACATGTTTAGTC	100
Clone C	GGCATCGGAAATTTCACTTACTATTTTTTTTTT-CAGTAACATGTTTAGTC	99
Clone A	TACACACATAACTTAAGTGATTCCTAGCCGCTGGTATCTATCTGCACTCA	149
Clone B	TACACACATAACTTAAGTGATTCCTAGCCGCTGGTATCTATCTGCACTCA	150
Clone C	TACACACATAACTTAAGTGATTCCTAGCCGCTGGTATCTATCTGCACTCA	149
Clone A	GGTCACCTTGATAAATATGTTTCGTTATATTCCCGT-----	184
Clone B	GGTCACCTTGATAAATATGTTTCGTTATATTCCCTAATGGCATGTAATATTT	200
Clone C	GGTCACCTTGATAAATATGTTTCGTTATATTCTTAATGGCATGTAATATTT	199
Clone A	-----	184
Clone B	GTAACGGTAGC-----	211
Clone C	GTAACGGTAGCCAAAACGGCCTAGTCATAGTCATTAGTGATTAAGGAG	247
Clone A	AAAAAAAAAAAAAAAAAAAAA	
Clone B	AAAAAAAAAAAAAAAAAAAAA	
Clone C	AAAAAAAAAAAAAAAAAAAAA	

Figure 30. Heterogeneity of 3' untranslated regions in mRNAs encoding the small subunit of ribonucleotide reductase in soybean. The complete 3' untranslated regions of three clones encoding the small subunit of RNR in soybean are shown. The numbers indicate the nucleotide position downstream from the stop codon.

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Clone A  AATGTTTCAAAAGTAATGCTTTCATTAGAGGCGACAAAGATAAAATCACGT  50
Clone B  AATGTTTCAAAAGTAATGCTTTCCTAGAGGCGACAAAGATAAAATCACGT  50
Clone C  AATGTTTCAAAAGTAATGCTTTCATTAGAGGCGACAAAGATAAAATCACGT  50

Clone A  GTTAATCTATAGCTTTTTCAAAGCTTTGAACATAGTTGTCATCAATATCA  100
Clone B  GTTAATCTATAGCTTTTTCAAAGCTTTGAACATAGTTGTCATCAATATCA  100
Clone C  GTTAATCTATAGCTTTTTCAAAGCTTTGAACATAGTTGTCATCAATATCA  100

Clone A  TTTGTATTCTTGCATATCCTTGTATCAGATTTATTTACTCAGTAGTATAA  150
Clone B  TTTGTATTCTTGCATATCCTTGTATCAGATTTATTTACTCAGTAGTATAA  150
Clone C  TTTGTATTCTTGCATATCCTTGTATCAGATTTATTTACTCAGTAGTATAA  150

Clone A  ATACCAAGTCAGTT  164
Clone B  ATACCAAGTCAGTT  164
Clone C  ATACCAAGTCAGTT  164

Clone A  AAAAAAAAAAAAAAAAAAAAAA
Clone B  AAAAAAAAAAAAAAAAAAAAAA
Clone C  AAAAAAAAAAAAAAAAAAAAAA

```

Figure 31. Uniformity of 3' untranslated regions in mRNAs encoding Large Subunit B of ribonucleotide reductase in soybean.

The complete 3' untranslated regions of three clones encoding Large Subunit B of RNR in soybean are shown. The numbers indicate the nucleotide position downstream from the stop codon.

Discussion

cDNA cloning of large and small subunits of ribonucleotide reductase in soybean

In this study, cDNAs encoding Large Subunit A, Large Subunit B, Large Subunit C (partial), and the small subunit of RNR in soybean were amplified, cloned and sequenced.

Two characteristics of these sequences show completion of the open reading frames in these cDNAs at their 5' ends. First, stop codons were found upstream from the start codons and in frame with the long open reading frames (Figure 13, 19). Therefore the start codons are the most upstream among the possible start codons in the open reading frames. Second, the amino-terminal (N-terminal) residues between these subunits of RNR in soybean and those in other eukaryotic organisms are similar (Figure 14, 21).

Two characteristics of these sequences indicate the identity of these cDNAs. First, the predicted amino acid sequence of these cDNAs have significant overall similarities to the subunits of RNR in diverse organisms, which represent five kingdoms (Table 7, 8). These sequences of RNR subunits in soybean show a high homology (77.0% to 90.6% identity) to those in other plant species, a high homology (60.2% to 68.9% identity) to those in the organisms representing other eukaryotic kingdoms, and a homology (19.5 % to 29.0 % identity) to those in *E. coli*. Second, the amino acid residues essential for catalytic

activity and for enzyme structure of these subunits of RNR are conserved in the predicted amino acid sequences of these cDNAs. All the 17 conserved amino acid residues featured the small subunit of RNR are conserved in the small subunit of RNR in soybean (Figure 14). Likewise, all the 8 conserved residues featured the large subunit are conserved in the large subunits in soybean (Figure 21).

A recognizable fragment, or a motif, of 20 amino acids was found in both Large Subunit A and B of RNR from soybean (Figure 21). All the known five RNR large subunits in plants (two from soybean, two from tobacco, and one from *Arabidopsis*) have this motif (Chaboute et al. 1998) (Lin et al. 1999). However, it is absent in the RNR large subunits in the organisms belonging to other kingdoms (Figure 21). Further searched with Basic Local Alignment Search Tool (BLAST) programs (Altschul et al. 1990), this motif could not be found from any known amino acid sequences of proteins from animals, fungi, protista or bacteria. Also, it could not be found from plants except the sequence of the RNR large subunits. Therefore it appears to be specific for the RNR large subunit in plants.

Proteins often use motifs as building blocks to organize secondary and tertiary structures and to perform functions (Unger and Sussman 1993). If a motif is well known it can be used to predict a protein function (Bork and Koonin 1996). Because this motif is little known, three possible functions are hypothesized as follows.

This motif may not be directly involved in catalytic functions of RNR, because it can not be found from RNR in organisms other than plants. All the class I RNR in different kingdoms have the same catalytic functions and cofactor, so they should have similar well-conserved motifs involved in the catalytic functions.

The first possible function of this motif is binding another subunit of plant RNR. Unlike animals and protista, plants have multiple large subunits of RNR. This motif may bind different large subunits and contribute to forming a RNR complex.

The second possible function of this motif is targeting to a particular subcellular location in plant cells. The subcellular compartments and internal membranes that are unique in plant cells may require this motif to locate RNR.

The third possible function of this motif is binding to a regulatory molecule, and the regulatory molecule could in turn influence the activation or life span of the enzyme. Plants have to stand harsh environments, such as ultraviolet light and extreme temperature, instead of avoiding them as animals do. Plant cells also need to regulate cell division according to light condition. Therefore, this plant-specific motif may be beneficial to plants for regulating RNR.

For understanding the function of this plant-RNR-specific motif, one approach could be X-ray crystallography; another approach could be site-specific mutagenesis. X-ray

crystallography would show the position of this motif on the three-dimensional structure of a plant RNR, and the position may reveal the motif's function. A site-specific-mutant gene encoding the large subunit without this motif could be constructed by deleting the relevant nucleotides. The mutant large subunit gene could be delivered in plant cells, or large amounts of the modified large subunit protein could be produced in *E. coli*, so the function of the large subunit protein without the motif could be studied.

The gene or gene family encoding ribonucleotide reductase in soybean

Based on the Southern hybridization results it appears that a small gene family encodes at least three different large subunits of RNR in soybean, whereas a single gene encodes the small subunit of RNR. These Southern hybridization results are consistent with the sequenced cDNAs in this study. These cDNAs encode three different large subunits and one small subunit of RNR in soybean. These results are also consistent with the report that a small gene family encodes different large subunits of RNR in tobacco (Chaboute et al. 1998), and a single gene encodes the only small subunit of RNR in tobacco (Chaboute et al. 1998) and in *Arabidopsis* (Philipps et al. 1995).

How could different large subunits of RNR compose the enzyme complex? One possibility is that the different large subunits could make up the same enzyme complex (i.e. $L_A L_B SS$,

L_AL_CSS or L_BL_CSS). This would mean that the RNR complex in plants has non-homodimer structures. Alternatively, the different large subunits could make up different enzyme complexes, but the same large subunits compose each enzyme complex (i.e. L_AL_ASS, L_BL_BSS and L_CL_CSS). This would mean plants have different RNR isozymes.

It is not clear why plants may have non-homodimer RNR or different RNR isozymes; in other words, it is not clear why some plants need different large subunits of RNR. Four hypotheses are suggested; they are spare-part rescue, non-homodimer, tissue-specific and environment-specific.

First, spare-part rescue of RNR could be advantageous in plants, because RNR is an indispensable enzyme for *de novo* DNA synthesis, and because plants are exposed to much more sunlight than animals and fungi due to requirement for photosynthesis. It is well known that ultraviolet radiation in sunlight causes various kinds of DNA damage including formation of thymine dimers. If a RNR gene in a plant cell is damaged by sunlight, another spare-part RNR gene would be crucial for the cell to survive. In fact, a cell under ultraviolet radiation needs not only at least one normal RNR gene to survive, but also more RNR gene expression to repair damage on its genomic DNA (Filatov et al. 1996).

Second, non-homodimer RNR may function better than homodimer RNR in plants. Evolution procession may have refined the large subunits of RNR in plants, from the homodimer to the non-homodimer, therefore improved the

subunit binding, active-site efficiency or allosteric regulation.

Third, different cell-types, tissues and organs in a plant may use different RNR to adapt their differentiation. The fourth hypothesis is that plants may use environment-specific large subunit of RNR. For example, under normal condition plant cells could use one kind of RNR to prepare for cell division. Under ultraviolet radiation, the cells could use another kind of RNR to repair damaged DNA.

Different approaches could be used to understand the reason for multiple large subunits. One approach could be evaluating the mRNR levels of the different large subunits by Northern hybridization or in situ hybridization.

Different large subunit mRNAs could be distinguished with gene specific probes. These mRNR levels in different cell-types, tissues and organs under different environments would reveal the relationship and change of the gene expression of the large subunits of RNR in plants. Another approach could be evaluating the protein levels of different large subunits by Western blotting or fluorescence microscopy. Different large subunits could be distinguished from each other with monoclonal antibodies.

Based on amino acid sequences, two evolutionary relationships among the three large subunits of RNR in soybean can be speculated. First, Large Subunit B and C are closer to each other than to Large Subunit A in terms of phylogeny. The counterparts of Large Subunit B and C share 94.6% sequence identity with each other, whereas both of

them share 88.4% identity with the counterparts of Large Subunit A. Both Large Subunit B and C lack the same amino acid (Glu 785) compared to Large Subunit A (Figure 19). Second, Large Subunit A may be the most basal lineage among the three large subunits. This lineage is supported by the fact that Large Subunit A has a higher homology to the large subunits of RNR in other organisms than Large Subunit B does (Table 7).

Gene expression of ribonucleotide reductase in soybean

In this study Northern hybridization results show that RNR large and small subunit genes in soybean are expressed both in the dark-grown and light-grown seedlings, and that light does not increase the mRNA levels. When the same amount of poly(A) RNA (5 µg) was analyzed, the Northern hybridization intensity of RNR mRNA in primordial shoots grown in the light was less than that grown in the dark (23% to 33% less). However, the total amount of poly(A) RNA from primordial shoots grown in the light was more than that grown in the dark (about 18% more, data not shown). Therefore, the RNR mRNA level in shoots grown in the light was just slightly less than that grown in the dark (10% to 20% less).

Contrary to expectation, light did not increase RNR mRNA levels in primordial soybean shoots. The expected increase of RNR mRNA levels was based on the fact that when young soybean seedlings are grown in the dark, the primordial shoots remain yellow and in bud form; after transfer to

light for a couple of hours, the shoots begin to green, grow and develop. Because RNR is a key enzyme for DNA synthesis, one could predict that light would turn on or increase RNR gene expression for cell division.

To explain why light does not increase RNR mRNA levels, one could speculate that light increases RNR enzyme activity by post-transcriptional regulation without increasing RNR mRNA levels. Evaluating the RNR protein levels with Western blotting could test this speculation.

Multiple poly(A) sites in mRNAs encoding ribonucleotide reductase in soybean

Multiple poly(A) sites and different lengths of the 3' untranslated regions were found in cDNAs encoding some subunits of RNR in soybean. The same cis-acting elements may imprecisely locate some poly(A) sites and cause different poly(A) sites in different mRNR.

Multiple poly(A) sites were found in mRNAs encoding Large Subunit A and the small subunit of RNR in soybean. Five different poly(A) sites were found in six Large Subunit A clones sequenced; three different poly(A) sites were found in three small subunit clones sequenced. The high frequency of clones with different poly(A) sites suggests that even more poly(A) sites will be found if more clones are sequenced.

Polyadenylation signal AAUAAA sequence was not found upstream these poly(A) sites. This finding is not surprising because the polyadenylation signals in plants differ between mRNAs and are hard to discern by sequence inspection alone (Wu et al. 1995).

The RNA poly(A) sites are close to each other. Among the five poly(A) sites in mRNAs encoding Large Subunit A of RNR, the farthest two poly(A) sites are separated by 25 nucleotides; the closest two poly(A) sites are separated by only one nucleotide.

Some researchers consider multiple cis-acting elements as the cause for multiple poly(A) sites in plants (Li and Hunt 1997). They suggest that in a pea gene, four near-upstream elements and four dinucleotide Y(= C or T)A elements result in four poly(A) sites, respectively. They also suggest that a far-upstream element results in three of the four poly(A) sites, and that a second far-upstream element results in the fourth poly(A) site.

Although Li's hypothesis can explain the basis for four poly(A) sites in a plant gene, it can not explain the basis for many more poly(A) sites in other plant genes; also it can not explain why some poly(A) sites are close to each other. For example, 14 different poly(A) sites were found from 22 clones encoding a chloroplast RNA-binding protein in tobacco (Klahre et al. 1995). Among these 14 poly(A) sites, the farthest two poly(A) sites are separated by 94 nucleotides; the closest two poly(A) sites are separated by only one nucleotide. Because these 14 poly(A) sites are so

close to each other, it is almost impossible that there are distinguishable 14 near-upstream and multiple far-upstream elements to locate these sites respectively.

One could speculate that the same cis-acting elements may locate a poly(A) site at an approximate position, instead of a fixed position. This imprecise location would cause different poly(A) sites in different mRNA molecules encoding the same RNR subunit, and cause close poly(A) sites.

Imprecise location of poly(A) sites by the same cis-acting elements can explain other researchers' results (Klahre et al. 1995), so imprecise location of poly(A) sites may also occur in plants other than soybean and in mRNA encoding genes other than RNR. In the past, most researchers have sequenced only a few cDNA clones encoding the same plant protein, because sequencing was time consuming; their focus was on the coding regions instead of 3' untranslated region of the cDNA. More and more sequence data of 3' untranslated region of plant cDNA may reveal that multiple poly(A) sites in plant mRNA are more common than previously thought.

Summary

In summary, cDNAs encoding Large Subunit A, Large Subunit B, Large Subunit C (partial), and the small subunit of RNR in soybean were amplified, cloned and sequenced. The RNR large subunits in soybean contain a motif with 20 amino acids, which appears to be specific for the RNR large subunits in plants.

Southern hybridization results imply that a small gene family encodes at least three different large subunits of RNR in soybean, and that a single gene encodes the small subunit. The presence of three different large subunits of RNR in soybean suggests that the RNR complex in plants may have a non-homodimer structure. Alternatively, plants may have different RNR isozymes.

Northern hybridization results show that RNR large and small subunit genes in soybean are expressed both in dark-grown and light-grown seedlings, and that light does not increase RNR mRNA levels.

Multiple poly(A) sites and different lengths of the 3' untranslated regions were found in cDNAs encoding some subunits of RNR in soybean. The same cis-acting elements may imprecisely locate some multiple poly(A) sites in plants.

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