Structural organization and differential expression of rice α -amylase genes

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ABSTRACT

Rice α -amylases are encoded by a multigene family that has previously been classified into 5 hybridization groups. DNA sequence and Southern blot analysis identified three genes (*RAmy1A*, *RAmy1B* and *RAmy1C*) in Group 1 with DNA sequence identity of at least 90%. Hybridization Group 2 is represented by only one gene, *RAmy3D*, which is identical to a previously characterized cDNA, pOS137. *RAmy3D* is physically linked to the sole representative of Group 5, *RAmy3E*. The identity between these two genes is 81.4% in the coding region but less than 50% in the 5' and 3' flanking regions. Northern blot analysis and RNA-PCR were used to detect the expression of α -amylase genes in various tissues. Alpha-amylase mRNA was abundant in germinating seeds and callus. Some genes were also expressed at much lower levels in roots, young leaves and immature seeds. *RAmy1A* and *RAmy3E* were expressed in all tissues while *RAmy3D* was expressed in all tissues except the immature seeds. *RAmy1B* was weakly expressed only in callus. *RAmy1A* transcript was most abundant in the germinating seeds, while *RAmy3D* and *RAmy3E* transcripts were most abundant in callus and immature seeds, respectively.

INTRODUCTION

The process of cereal seed germination provides a number of opportunities to study the physiology and development of monocot plants. Among the enzymes expressed in the germinating seed, α -amylase is one of the most abundant and plays a crucial role in the hydrolysis of the starchy endosperm (see (1, 2, 3) for reviews). The cereal α -amylase gene/enzyme system is one of the best documented examples of hormonally regulated gene expression in plants. It has long been known that gibberellic acid (GA) can have a profound effect on the expression of α -amylase genes in germinating seeds (4, 5, 6). The α -amylase mRNA levels in isolated aleurone cells can increase 50 to 100 fold upon GA treatment (6, 7). For this reason, the cereal α -amylase genes have emerged as an ideal system for studying the molecular aspects of plant gene expression.

To facilitate more detailed studies of gene structure and GA regulation, α -amylase genes from rice, barley and wheat have been cloned and characterized (5, 6, 8, 9, 10). These studies reveal that the cereal α -amylases are encoded by multigene families. In barley and wheat, the α -amylase genes expressed in germinating seeds are classified as high pI and low pI classes (11, 12). In wheat, a novel class of α -amylase genes, (*Amy3*), is expressed only in the immature seed (8). It has been difficult to integrate the rice α -amylase genes into the high and low pI classification system used for barley and wheat (1). Based on DNA/DNA hybridization data, however, ten rice α -amylase genes can be classified into six distinct groups (9) (Ranjhan *et al.* manuscript in prep; Sutliff *et al.* manuscript in prep). These genes have been recently mapped to five of the twelve chromosomes of rice (Ranjhan *et al.* manuscript in prep) and given the gene designations, *RAmy1*–3 (Table 1). In this paper, we continue our analysis of genes in Groups 1, 2 and 5 with the eventual goal of elucidating the structure, organization and expression of the entire rice α -amylase multigene family.

MATERIALS AND METHODS

Gene cloning and DNA sequencing

Alpha-amylase gene cloning has been previously described (9). Appropriate restriction fragments containing α -amylase genes were subcloned into either pBluescript KS+/- (Stratagene) or M13mp19 (13) for restriction mapping and DNA sequencing. The sequencing procedure was the same as previously described (9). DNA sequence data were analyzed using the Sequence Analysis Software Package (14).

DNA, RNA isolation and Northern blotting

The procedure for the preparation of total rice (M202) DNA from 10 day old etiolated young leaves has been described (9). Total RNA was isolated from 6 day old germinating rice seeds, roots, etiolated young leaves, two month old callus and panicles 10 days after flowering following a procedure described in (15) and modified by Karrer *et al.* (manuscript in prep). One and 15 μ g of total RNA were separated on a 1% formaldehyde agarose gel and transferred onto nylon membrane. To ensure that

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Table 1. Nomenclature for rice α -amylase genes^a

Gene Name	Clones	Groups
<i>RAmy1A</i>	λ OSg2	1
<i>RAmy1B</i>	λ OSg3A	1
<i>RAmy1C</i>	(λ OSgX)	1
<i>RAmy2A</i>	λ OSg9C	4
<i>RAmy3A</i>	λ OSg7D	3
<i>RAmy3B</i>	λ OSg1	3
<i>RAmy3C</i>	λ OSg1	3
<i>RAmy3D</i>	λ OSg1A	2
<i>RAmy3E</i>	λ OSg7F	5
<i>RAmy3F</i>	(λ OSgY)	6

^aThe relationship among gene names, clone designations and hybridization groups is summarized in this table. Gene names are based on protein sequence comparisons among rice, wheat and barley α -amylase genes determined here (Fig. 5B) and elsewhere (7, 9) (Ranjhan *et al.*, manuscript in prep; Sutliff *et al.* manuscript in prep and Huang and Rodriguez, unpublished data). Parentheses around clones λ OSgX and λ OSgY indicate that the genes, *RAmy1C* and *RAmy3F* have been identified but corresponding clones have not been isolated from rice genomic libraries.

approximately the same amount of RNA was loaded into each lane, RNA samples were measured spectrophotometrically and then visualized by ethidium bromide staining of the agarose gel. Finally, the blot was probed with a wheat rDNA clone, pTA250.2 (16), to verify that equal amounts of RNA had been bound to the membrane in each lane. The blot was probed with the α -³²P labeled α -amylase cDNA clone, pOS103, (7), washed, and then exposed to X-ray film with intensifying screen (17).

Primer synthesis and polymerase chain reaction (PCR)

Primers for the various α -amylase genes were synthesized on a Cyclone Plus DNA Synthesizer (MilliGen/Biosearch). A common primer (3'-TTGAC CCTCA GCACC TCCTT CGTTC C-5') specific for a region in the second exon of *RAmy3E* (Fig. 4) was used for all amplifications. This 26-mer differs from corresponding regions in *RAmy3D* and *RAmy1* genes by only 3 or 4 bases at the 5' end of the oligonucleotide (Fig. 1 and 3). Gene-specific primers for the rice α -amylase genes *RAmy1A*, *RAmy3D* and *RAmy3E* were chosen from different locations in the 5' untranslated regions of these genes (Fig. 1, 3 and 4). The primers were designed so that the predicted RNA-PCR products for each gene could be distinguished by their size on polyacrylamide gels. Because the distance between the common 26-mer and the *RAmy1A* primer was 7 bp greater in *RAmy1B*, the *RAmy1A* primer could be used to detect PCR products specific to both genes. The expected molecular weights for PCR products specific to *RAmy1A*, *RAmy1B*, *RAmy3D* and *RAmy3E* mRNA are 160, 167, 193 and 148 bp, respectively.

PCR amplification with total RNA isolated from various rice tissues followed the procedure described in (18) with some modification to obtain a higher yield of expected PCR products. The primer annealing reaction was carried out in 10 μ l vol containing 15 μ g of total RNA and 1 μ M common primer in annealing buffer (0.04 M PIPES pH 6.5, 0.4 M KCl, 0.4 mM EDTA). The mixture was heated to 70°C for 3 min and then transferred to 42°C for 5 min. The cDNA synthesis step was performed in 100 μ l vol consisting of 10 μ l above annealing reaction, 1.6 u/ μ l RNasin, 1.5 mM each dNTP and 0.3 u/ μ l reverse transcriptase (RTase) in RTase buffer (0.05 M Tris pH 8.8, 10 mM MgCl₂, 1 mM DTT, 0.05 mg/ml BSA). After 60 min cDNA synthesis at 42°C the reaction was transferred to a 50°C heating block and incubated for 30 min. This reaction

served as cDNA stock for PCR and the remaining cDNA was stored at -20°C.

PCR amplification was performed in a TwinBlock (Ericomp) thermal cycler. The common primer was used in all amplifications. The amplification reaction of 50 μ l consisted of 5.0 μ l cDNA prepared above, 0.5 μ M common primer, 0.5 μ M gene specific primer, 0.2 μ g/ μ l BSA, 1 mM each dNTP and 0.025 u/ μ l *Taq* polymerase (US Biochemicals) in *Taq* polymerase buffer (20 mM Tris pH 8.8, 5 mM MgCl₂, 2.5 mM KCl, 15 mM (NH₄)₂SO₄). The DNA denaturation was set at 94°C for 1 min, the primer annealing at 60°C for 1 min and the primer extension at 72°C for 2 min. The reaction was allowed to run for 30 cycles before a final 8 min primer extension at 72°C.

The PCR products were first analyzed with a 2% agarose minigel. For better resolution, a 7.5% polyacrylamide gel was run in 1 X TAE buffer. The PCR products were visualized by ethidium bromide staining and by Southern blot analysis in which the separated PCR products were electro-blotted onto nylon membrane and probed with various α -amylase gene probes as described in the text and in the legend for Fig. 7.

RESULTS AND DISCUSSION

Three *RAmy1* genes are highly homologous

Previous work on the rice α -amylase multigene family (7, 9), described the isolation and classification of 30 α -amylase clones into 5 hybridization groups. Restriction mapping of the three Group 1 clones indicates that there are two different genes, represented by the clones λ OSg2 and λ OSg3A (9). The genes contained in these two clones have been given the names *RAmy1A* and *RAmy1B*, respectively (Table 1). The nucleotide sequence of *RAmy1A* was determined and found to be 85.5% identical to the high pI barley gene, *Amy6-4* (9). In order to compare the structure of *RAmy1A* to *RAmy1B*, a 3.8 kb *EcoRI* fragment from λ OSg2 and a 3.4 kb *EcoRI/HindIII* fragment from λ OSg3A were subcloned into pBluescript KS- (Stratagene). The restriction maps for these two genes were very similar in the coding region (Fig. 1C). DNA sequence information from the 5' flanking and part of the coding region of *RAmy1B* was determined and compared to the corresponding regions of *RAmy1A*. After establishing the best sequence alignment, the two genes were found to diverge by only 5% (Fig. 1A). It was previously shown that the cereal α -amylase genes contain either two or three introns, with the intron at the second position being variable (9). Since further DNA sequence analysis of *RAmy1B* revealed introns at the first and second positions in the gene (data not shown), we infer that *RAmy1B* contains three introns like its close relative, *RAmy1A*. Unlike *RAmy1A* which has two in-frame ATG translational start codons at the amino-terminus of exon I, *RAmy1B* has only one such codon (Fig. 1A).

Southern blot analyses were performed to determine the number of genes in the *RAmy1* subfamily and their relative homologies. One such blot containing genomic DNA digested with the restriction enzyme *KpnI* and probed with pOS103 (7), the cDNA analog of *RAmy1*, is shown in Fig. 1B. Three strong hybridization bands (3.7 kb, 4.1 kb and 5.7 kb) and some minor bands were observed when the blot was washed at 65°C. The additional bands, corresponding to other α -amylase genes, disappeared when the blot was washed at higher temperatures. Since there are no *KpnI* sites within the coding regions of *RAmy1A* and *RAmy1B* (Fig. 1C), the 5.7 kb band most likely represents a new *RAmy1* gene. Based on the intensity of the hybridization

Figure 1. Structural organization of *RAmyl* genes. **A.** Partial sequence of *RAmylB* aligned with a previously determined sequence of *RAmylA* (9). Gaps were inserted to maximize the homology. Vertical lines indicate identical nucleotides in both sequences. The oligonucleotides used in RNA-PCR (see Fig. 7) are indicated below the sequences. The intron/exon junction is indicated by a slash (/). Highly conserved sequences in the 5' flanking region of α -amylase genes (9) are underlined. **B.** Rice genomic Southern blot. Total leaf DNA was digested with restriction enzyme *KpnI*. Ten micrograms of digested DNA were separated on a 0.9% agarose gel and blotted onto a nylon membrane which was then probed with ^{32}P α -dCTP labeled rice α -amylase cDNA, pOS103 (7). The blot was washed at $0.2\times\text{SSPE}$ and 0.5% SDS at 65°C for an hour (lane 65°C). Two exposures were obtained after more stringent washes were performed at 71°C ($0.1\times\text{SSPE}$ and 0.5% SDS) (lane 71°C) and 77°C (lane 77°C) (17). The numbers adjacent to the DNA fragment refer to molecular weights in kb. **C.** Restriction maps of three rice *RAmyl* genes. The maps for *RAmylA* (1A) and *RAmylB* (1B) were derived from subclones (thick line) and their extension to *KpnI* site in the genomic clones (thin line) while the map for *RAmylC* (1C) was derived from genomic Southern blots. H, *HindIII*; S, *SalI*; B, *BamHI*; K, *KpnI*; X, *XhoI*; T, *PstI*; A, *ApaI*; R, *EcoRI*; P, *SphI*.

Figure 2. Restriction map of two linked rice α -amylase genes; *RAmy3D* and *RAmy3E*. A composite map was derived from three independent, overlapping clones indicated by lines beneath the map with clone designations to the right. The short bars labeled 'a' and 'b' were probes used to identify overlapping clones. p1ABK5 contains an insert from λOSg1A . A portion of vector DNA, indicated by a dashed line, was included to facilitate subcloning. p7FXba4.3 contained a 4.3 kb *XbaI* fragment from λOSg7F . Exons and introns are indicated by solid boxes and open boxes, respectively. Transcriptional orientations are indicated by arrows. Sa, *Sau3AI*; N, *NruI*; Xb, *XbaI*; H, *HindIII*; S, *SalI*; B, *BamHI*; K, *KpnI*; X, *XhoI*; P, *PstI*; A, *ApaI*; RV, *EcoRV*; Sm, *SmaI*.

signal observed at the higher wash temperature, we estimated this gene, designated *RAmylC*, to be greater than 90% identical to *RAmylA* and *RAmylB* (19). By comparing the sizes of the hybridization bands shown in Fig. 1B to the restriction maps of *RAmylA* and *RAmylB*, these bands can be related to the *KpnI* restriction fragments shown in Fig. 1C. Repeated attempts to isolate *RAmylC* from rice genomic libraries have yielded an additional 50 α -amylase clones. None of these clones, however, contained the *RAmylC* gene.

Group 2 and Group 5 genes are linked

Using the same experimental approach described above, it was determined that Group 2 and Group 5 each contained one α -amylase gene, *RAmy3D* and *RAmy3E*, respectively (Table 1). The suffixes 3D and 3E were used to name these genes because they showed the greatest degree of similarity to the Amy3 class of genes in wheat (see Fig. 5B). Since the other members of the *RAmy3* subfamily, (*RAmy3A*, *3B* and *3C*) are linked (9), we

looked for linkage between *RAmy3D* and *RAmy3E*, using the two-step chromosome walking experiment shown in Fig. 2. As can be seen in this figure, probe 'a' was used to identify genomic clone λ OSg17B which in turn provided probe 'b'. The latter probe hybridized to both λ OSg7F and λ OSg17B. Further subcloning, restriction mapping and hybridization revealed that the genes in Groups 2 and 5 (i.e., *RAmy3D* and *RAmy3E*, respectively) are separated by only about 4 kb of DNA. This conclusion is supported by Southern blot analysis and trisomic mapping experiments which indicate that *RAmy3D* and *RAmy3E* are present on the same restriction fragment and map to the same chromosome (Ranjhan *et al.*, manuscript in prep).

Nucleotide sequences of *RAmy3D* and *RAmy3E*

Linked genes of the same gene family are often highly homologous. In barley and wheat, α -amylase genes located on the same chromosomes are homologous in both the coding and 5' flanking regions (5, 6, 20, 21). In spite of their linkage, however, *RAmy3D* and *RAmy3E* have been classified into different gene groups based on DNA/DNA hybridization studies (9). This result implies DNA and amino acid sequence differences significant enough to warrant further investigation.

A total of 2938 bp of sequence was determined from both orientations of p1ABK5 (Fig. 2 and 3). This sequence included

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10      30      50      70      90
GATCTTCAACACCTGTGCTAGCTACTCCACTGCTCCATAGGCAATCATCAATCAGTAATCCGTTCTGAAAAGAGATAGGTGTGCGCAATCAGGAAC
110     130     150     170     190
GTTCTAGTTCGTGCTAGAAATCAGCAGCTCTCAAGTTAGCATCTCGATGAACCTAAATGCTCGCTGCGGCGTCCGCGGAGATGAAGTTTGTGATAAAC
210     230     250     270     290
TTGGTCAATGACATTCATATATGTGCTGTACGGAGTAGTTTCATCAGCAACATACACCTACTTCTACCTTATCCATTGGATTGCTATGCGCGGCTT
310     330     350     370     390
TGATATGGAATTTGTAATGAACCTGTTTATGACTTATGACATACTGATCTCGTAACATTCATAGATAGTACATAAATTCATCAACTACAAATAGATGAG
410     430     450     470     490
ATGGCTAGTCTTAGTAGAACAGTAGTCTCTCTTCCGGCTTGCTCCATTGGCTGATGACGATGAACAACCTCGGACTCATTGATTCAGCATTATCTGATT
510     530     550     570     590
CTCGCATTTCCAGGTCCGGATTAGGGTCTCACCAGATGTGATAGAATTGCCATGTCCAGAAATGAAGGAGSACGAGCCATATGTCATATACATGACG
610     630     650     670     690
GGAGATCAAGCGGCCAGTCAAGAGGCTAAGTCAACCCCTATTATATACGATCAGCCTGTAGAACACGTAGCACTGTCTTTTGTCTGAACCTCTGAAGA
710     730     750     770     790
TGAAAGGTTTCAGAGAAATGGCTCGCCTTATCCAAGCCGGCGATGGATGGAGGAGGAGTACCGCGCGCCGCTCAGGCAGTCTGCGCATCACGCCGCC
810     830     850     870     890
GCATCCCGTCCGCTTGAGACCGCGCCCGACCGCGCGCGCTACGTGGCCATGCTTTATGCTTATCCATATCCAGCCATTTATTGTGGT
910     930     950     970     990
CGTCTCTCTGATCATTCTCATTCCTCCGTCACCGGTGACCGTGCCCCGGTGTCTATATATGCCCCCGACGTGAGGTCATTCGCCACGAAACACATCG
1010    1030    1050    1070    1090
ATCATCATCATCTACAAGATCGATCAGTAGTGGTTAGCAGCAACTCACTATCGAACACGGTTTCAGTTTACACAGATATGAAGAACACCGACGCTT
1110    1130    1150    1170    1190
5'CATCTACAAGATCGATCAGTAGTGG 3' M K N T S S L
GTGTTTGTGCTCTCGTGGTCTCTGCAGCTTGACCTGTAACCTCGGGTCAAGCACAGTCTCTTCCAGGTACGTAGTACTTACTACCCATCATCTTC
1210    1230    1250    1270    1290
C L L L V V L C S L T C N S G Q A Q V L F Q
TGTAAGACTTTTGTGTAAGAAACATTAGAATTTTGTGATATTATGTGTGATGATGATCCTTACTTATAACATGATCAGGTTTC
1310    1330    1350    1370    1390
3'TTGACCTTCAGCACTCCTTCTGTTCC 5'
AACTGGGAGTCTGTAAGACGAGCAGGGTGGCTGGTACAACATGTTGAAAGGCCAAGTCGACGACATCGCCAAAGCCGGGGTCAACCCAGCTCTGGCTGCCG
1410    1430    1450    1470    1490
N W E S W K Q Q G G W Y N M L K G Q V D E I A K A G V T H V W L P P
CGCGCTCGCACTCCGTGGCGCGCAGGGTACATGCGCGGGCTCTTACGACCTGGACGCGTCAAGTACGCGCAGCGCGGCGGATCAAGTCGCTGAT
1510    1530    1550    1570    1590
P S H S V A P Q G Y M P G R L Y D L D A S K Y G T A A E L K S L I
CGCGCGCTTCCACGGAAGGGCTCCAGTGGCTCGCGACGCTCGTGATCAACCCCGTGGCGCGAGAAGGACGCGCGGGCTGTACTGCGTGTTC
1610    1630    1650    1670    1690
A A F H G K G V Q C V A D V V I N H R C A E K K D A R G V Y C V F
GAGGCGGGACGCCGACCGCTCGATGGGCCCCGCGATGATTCGACGACGACACGAGTACTCCGACGCGACGGGCCACCGGACCCGCGAGG
1710    1730    1750    1770    1790
E G G T P D R L D W G P G M I C S D D T Q Y S D G T G H R D T G E G
GGTTCGGCGCGCGCGACATCGACCACTCAACCCCGCGCTCCAGCGGGAGCTCACCAGTGGCTCAACTGGCTCAAGTCCGACGCTCGGCTTCGACGG
1810    1830    1850    1870    1890
F G A A P D I D H L N P R V Q R E L T D W L N W L K S D V G F D G
CTGGCGCTCGACTTCGCAAGGGATCTCCACGACATCGCTAAGATCTACGTGAGAGCTGCAAGCCGGCTTCGTCTGCGCGAGATATGGAATCG
1910    1930    1950    1970    1990
W R L D F A K G Y S T D I A K M Y V E S C K F G F V V A E I W N S
CTGAGCTACACCGCGACCGCAAGCCGGCGGCAACAGGACCGGCGGAGGCTGGTGAAGTGGTGAACCGCGTGGCGGGCGCGGATGACGT
2010    2030    2050    2070    2090
L S Y N G D G K P A A N Q D Q G R Q E L V N W V N A V G P A M T F
TCGACTTCACCAAGGCGCTCTGACGGCGGCTCCAGGCGGAGCTGTGGCGGTGCGGACGCGCAAGCGCGCGGCGATGATCGGTTGGCT
2110    2130    2150    2170    2190
D F T T K G L L Q A G V Q G E L W R L R D G N G K A P G M I G W L
GCCAGAGAGGCGGTACGTTCTGTCGACAAACGACACCGGCTCGACGCAAGCTTTGGCGTCCCTCCGACAGGTCATGACGGGTACGCTAC
2210    2230    2250    2270    2290
P E K A V T F V D N H D T G S T Q K L W P F P S D K V M Q G Y A Y
ATCTCACCCACCCCGAGTCCCTGATCGTAAGCAAAACATGCATTATAGTATTATATACCATGTCCTGATTAACTCCACCGTACAGTGTCTGAT
2310    2330    2350    2370    2390
I L T H P G V P C I
GAACGCTTCTTGTGGCAGTTCTACGACCATGTTCTGACTGGAACCTGAAGCAGGAGATAACCGCGCTGGCGGCGATCAGGAGAGGACGGCATCAACG
2410    2430    2450    2470    2490
F Y D H M F D W N L K Q E I T A L A A I R E R N G I N A
CCGGAGCAAGCTCCGATCGTCTCGCGACGCCAGCATACGTCGCGCTCGTCGACGAGAAGGTCTAGTGAAGATCGGACGAGGTACGACGTGGG
2510    2530    2550    2570    2590
G S K L R I V V A D A D A V A V V D E K V M V K I G T R Y D V G
CAACGCGAGTCCGCGATTTCATCAGAGGTGACGCGCAAGGACTACAGCGTCTGGGAGAAGGGTCCCTCCGCTCCCGCGGGCGGACATATAG
2610    2630    2650    2670    2690
N A V P S D F H Q T V H G K D Y S V W E K G S L R V P A G R H L *
CGGGCTCAAGCCCTAACTGAACGGGATAGTCATGCTCAACAGTTTCTACACGGAAGAATTTACTGATCTTATCTTTTTCAGTCAATTAATTAT
2710    2730    2750    2770    2790
GGTTTTATATATGTAATTTTGTATCCGATTGTAGCGTTCGAAATAGTAGGAGGCTCTTACGCTCTAGTTAATTTGCGGGCATATGTAGCTTGCCAG
2810    2830    2850    2870    2890
TTAATTGTGTTTGTATCAGCAGTTTGTAAACCGTTGGTGAATATATATGTGAGTTTCAAGTAAATAAATCATACTGCACCGATCAGTGAGTTT
2910    2930
TTATATACTGGTTTAAAGTGAGCACAGTACTAGTT

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Figure 3. Nucleotide sequence of the rice α -amylase gene *RAmy3D*. The coding region of *RAmy3D* was determined by sequence alignment to its cDNA analogue, pOS137 (7). The amino acid sequence in single letter code is shown beneath the DNA sequence. Putative transcriptional initiation and polyadenylation sites are shown in bold. The TATA box, 'CATC' box and polyadenylation signal sequences are underlined. Two sequence elements conserved between *RAmy3D* and *RAmy3E* are underlined with double- and wavy-lines. The oligonucleotides above the sequence served as primers for RNA-PCR (Fig. 7)

1080 bp of 5' flanking region and 338 bp of 3' flanking region. The α -amylase coding region contained two introns of 124 bp (intron I) and 88 bp (intron II). Both introns were in the same position as those found in other α -amylase genes (9). A comparison of the DNA sequence for *RAmy3D* (excluding introns) revealed that it was identical to the full length cDNA clone, pOS137 (7). Therefore, the transcription start site and polyadenylation site (indicated in bold) for *RAmy3D* were inferred from the pOS137 sequence (Fig. 3).

A total of 2683 bp of DNA sequence containing the complete *RAmy3E* gene on p7FXba.4.3 (Fig. 2) was determined from both orientations (Fig. 4). The 5' and 3' flanking regions are 520 bp and 621 bp, respectively. By comparing the *RAmy3E* sequence with the two rice α -amylase cDNA clones, pOS103 and pOS137 (7), two introns in the first and third positions were found. The length for introns I and II are 143 bp and 85 bp, respectively.

The three exons with lengths of 90 bp, 939 bp, and 285 bp encode a protein of 437 amino acids.

Comparison of the *RAmy3D* and *RAmy3E* gene sequences

A DNA sequence homology matrix comparing *RAmy3D* and *RAmy3E* showed extensive homology between the coding regions which diminished precipitously in the 5' and 3' flanking regions (data not shown). Direct sequence comparison of the coding regions for the two genes revealed that they were 81.4% identical at the DNA level and 72.7% identical at the protein level. Upon closer inspection of the 5' flanking regions, two conserved sequence elements of 10 bp and 31 bp were observed (Fig. 5A). These elements are underlined in Fig. 3 and Fig 4. Whether or not these elements are involved in the regulation of gene expression is not known. It should be noted, however, that *RAmy3D* and *RAmy3E* are expressed abundantly in rice callus;

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      10          30          50          70          90
GGTACCTGTTGGAACCACTGATCTGCGGCTCTCCGCTTGCCTTTATCAACGTATCGCGCTTCCGTCATCGGTGATCGAT
110          130          150          170          190
CACCAGAGAGACCGGACGACGAGTCCGAGAGCTCGCGCGCTCGATCGCGCGCGGTGACTCGAGCAGGGCTGAAGTAGTGCAGGGCTCAAGGC
210          230          250          270          290
GGCACTCCATCACCAGACACGGGGTCCAGACTACTCGTTTCGGTTGGAGAAATAACCACTTTATCCATGTGCTTATCCGTGAATTGCAACAGCATTG
310          330          350          370          390
ATTGTTCCGGTTAATTCGCTCGGCCATGTAACTCCGACCTGATCCTTTGGACACTATTAATAGAGCCAGTTCAGGCAATGCAAGAGCAGAGAAC
410          430          450          470          490
AGAGTACAGCAGGCGCTCTTCTCTCTTTCGGAAGTTGGCTACTTTGGCCAGCCATTAGGAAACAAGTTAGTTTGGAGAAGAAGCAGATTGAGACTGC
ATTTCATTGCTCTGTAGCC 3' 530          550          570          590
ATTTGCATTGCTCTGTAGCCATGGGCAAGCACCATTGTCACCTGTGTTTGTGCTGCTGCTGCGCTGCGCTCCAGCTTAGCACAAGCCCAAGT
      M G K H H V T L C C V V F A V L C L A S S L A Q A Q V
610          630          650          670          690
TCTCTCCAGGTAGTTAATTACTGACGCTTGGTGAAGTTTGTAACTTGATAATAATCTTGCACGGCAATATAATGACGCGCCGAGTCA
L F Q
710          730          750          770          790
GGAAGCTTGATTTGACCATGGGTTCGGTTTGGTGTTCGCTGACGTGACGGGTTTAACTGGGAGTCTGGAGGAAGCAAGGGCGGTGATCAACTT
      G F N W E S W R K Q G G W Y N F
810          830          850          870          890
TCTGCACGAGAAGGTGGAGGAGATCGCCAGCAGGGCGCCACCCAGCTTGGCTCCCGCCCGCTCGCACTCTGTCTCGCCGAGGGTTACATCGCCGGG
L H E K V E E I A S T G A T H V W L P P P S H S V S P Q G Y M P G
910          930          950          970          990
CGGCTCTACGACCTGGACGCTCCAGTACGGCAGGAGCGGAGCTCAAGTCGCTGATCGAGGCAATCCACGACAAGACGTCAGTGCCTCGCCGACA
R L Y D L D A S K Y G T E A E L K S L I E A F H D K N V E C L A D I
1010          1030          1050          1070          1090
TCGTCATCAACACCGCTGCGCCGACTACAAGGACAGCGCGCGCTGTACTGCTGTTTCGAGGGCGGACGCCGCGCGCTCGACTGGGCCCCGA
V I N H R C A D Y K D S R G V Y C V F E G G T P D G R L D W G P D
1110          1130          1150          1170          1190
CATGATCTGCAGCAGACACGAGTCTCAACGCCGCGCCACCCGACACCGCGCGCGGTTTCGGCGCCGCGCCGACATCGACACCTCAACCCG
M I C S D D T Q Y S N G R G H R D T G A G F G A A P D I D H L N P
1210          1230          1250          1270          1290
CGTGTCAGCGGGAGCTCACCAGTGGCTCAACTGGCTCAGGACCGACTCGGCTTCGACGGCTGGCGCTTCGACTTCGCAAGGGCTACTCCGCGCCG
R V Q R E L T D W L N W L R T D L G F D G W R L D F A K G Y S A P L
1310          1330          1350          1370          1390
TGGCAGGATCTACGTGCAACACCAACCGAGTTCGTCGTCGCGGAGATCTGGAGCTCGCTCATCAACGGCGAGCGCAAGCGCTCGACCAACCA
A R I Y V D N T N P T F V V G E I W S S L I Y N G D G K P S T N Q
1410          1430          1450          1470          1490
GGACCGGAGCAGGAGGAGTGGTGAATGGGTGGAGGCGCTCGGAAGCGCGGCGAGCGGCTTCGACTTCACCAAGGGCATCTCCAGCCCGCGGTG
D A D R Q E L V N W V E G V G K P A T A F D F T T K G I L Q A A V
1510          1530          1550          1570          1590
CAGGGCGAGCTGTGAGGCTCCACGACGGAACGGCAAGCGCGCGCTCATGGGTGGATGCCGATCAGGCCGTAACCTTCGTCGACAAACACGACA
Q G E L W R L H D G N G K A P G L M G W M P D Q A V T F V D N H D T
1610          1630          1650          1670          1690
CCGGCTCGACCCAGTCGCTCTGGCGTTCCCTTCCGACAAGGTATGACAGGGCTACGCTACATCTCACTCACCTGGCATCCCATGCTGATAGTAT
G S T Q S L W P F P S D K V M Q G Y A Y I L T H P G I P C I
1710          1730          1750          1770          1790
CACCACGAAATCTTCTCATCAATTCGTTTATATGTTGAGCTCATTTGTTGATGTGTACGTGTGTATGACGTTCTACGACCATGTGTTTCGACTG
      F Y D H V F D W
1810          1830          1850          1870          1890
GAACCTGCAGCAGAGATCGCGACGCTGGCTGAAATCCGGTCAAGGAACGGATCCATCGGAGAGCAGCTGGACATCTCAAGGCCGAGGGGACATC
N L Q H E I A T L A E I R S R N G I H A E S T L D I L K A E G D I
1910          1930          1950          1970          1990
TACGTCCGATGATCGACGGCAAGGTGATCAACAGCTCGGGCGAGGTACGACCGCGCGGATCATCCCTCCGACTTCCATGTCTGTCGCGCAGCGCA
Y V A M I D G K V I T K L G P R Y D A G G I I P S D F H V V A H G N
2010          2030          2050          2070          2090
ACGACTACTGCGTCTGGGAGAAGGAAGGCTCAGGGTTCTGCGGTTAGAAAGCACTATTAGCTTTAGCTATAGCGATCGAGTTGATGTTGCTTTCGAA
D Y C V W E K E G L R V P A G R K H Y *
2110          2130          2150          2170          2190
CCCTAGATAATATATACGTACGTGCTTATGATGAATCATGCAATTTTGTGCGAGATGTGTACGAGCGAGCTTCGATCGATGTACGCTTCTGTTAT
2210          2230          2250          2270          2290
AACTAGCGTTCTTCGAAATAAGTAATCGAATGTACCTGTAACTCTCGAGAAATGTAGGATGAATGAATAACTAGCTACTGTTCTGTTTCGATCCT
2310          2330          2350          2370          2390
CAAGAAAGACTTGCAAGATCTTGTCAGTTGACTTCACTTTTTTACTCCGCTTTTAGCGTCTGGATACCGTGGTGAATTGAAAGCTCAACTTATCCCG
2410          2430          2450          2470          2490
TTTGCCACGCAATATTAGCCGTAAGTAAACGAATGACACCTGCATATTCCGGCCCAAGCGCACGCTCGTGTCTCTCATTTAGCGTCCAAAGATA
2510          2530          2550          2570          2590
ATGGGACGAATGTTCTTCACAGCAACGATTTAGCCTAATATAATGGGCACTTTCCCTTTATAACCAAGGAATAAGTTCACTGGTCCCTTAATTTATC
2610          2630          2650          2670
AGCGAGTCTGAAATTTATCCCTAAACCGAAATACTGTATATAATTTGTCCTCCCAATTTTCAAAACGGTTCACTTAGAGGACCC

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Figure 4. Nucleotide sequence of the rice α -amylase gene *RAmy3E*. The coding region of *RAmy3E* was determined by sequence alignment to two cDNA clones, pOS137 and pOS103 (7). Important structural features in this gene are indicated as in Fig. 3.

Figure 5. Comparison of rice α -amylase genes *RAmy3D* and *RAmy3E*. **A.** Two conserved elements found in the 5' regions of *RAmy3D* and *RAmy3E*. Numbers refer to sequence coordinates for each element. Mismatches are indicated with lower case letter in *RAmy3E* elements. **B.** Protein sequence similarity between genes *RAmy3D* and *RAmy3E* and other α -amylase genes. Percent similarity was calculated pairwise for 16 α -amylase protein sequences from barley, rice and wheat using Sequence Analysis Software Package (14) with a gap weight of 1 and a gap length weight of 0.1. R = rice, B = barley and W = wheat. The sources of the sequences used in this analysis are as follows: *RAmy3D* and *RAmy3E* (this study), Amy1/13, Amy2/54, and Amy3/33 (8), *RAmy3A* and *RAmy3B* (Sutcliffe *et al.*, manuscript in prep), *RAmy1A* (9), Amy6-4 and Amy46 (6), gKAmY141 and gKAmY155 (10), gRAmy56 (21), Clone E (28), Amy32b (29) and *RAmy2A* (Huang and Rodriguez, unpublished data).

a feature not found in the other rice α -amylase genes (see Fig. 7). Other features shared by *RAmy3D* and *RAmy3E* include: (a) the same orientation relative to transcription; (b) the same number of introns; (c) similar sizes for introns I and II (124 bp, 88 bp of 3D and 143 bp, 85 bp of 3E, respectively) and (d) similar numbers of amino acid in the preprotein (435 and 437, respectively).

The degree of similarity between both *RAmy3D* and *RAmy3E* and other α -amylase genes from rice, barley and wheat was calculated and the values shown in Fig. 5B. As mentioned earlier, *RAmy3D* and *RAmy3E* have been tentatively assigned to the Amy3 class of α -amylase genes based on their similarity to Amy3/33 (8). As shown in Fig. 5B, the similarity between *RAmy3D*, *RAmy3E* and the Amy3 gene class was greater than 86% while the degree of similarity did not exceed 85% when these genes were compared to the Amy1 and Amy2 gene classes.

Expression of rice α -amylase genes in various tissues

O'Neill *et al.* (7) demonstrated that α -amylase mRNA in embryo-less half seeds can be induced at least 50-fold by treatment with GA₃. In this study, we examined the steady state levels of α -amylase mRNA in germinating seeds, roots, leaves, immature seeds (i.e., panicles) and callus tissues to better understand the developmental and tissue-specific expression of the various rice α -amylase genes. Total RNA from the aforementioned tissues was isolated and examined by Northern blot analysis. By probing the blot with an α -amylase cDNA clone at low stringency, it was possible to detect hybridization to transcripts from all five rice α -amylase gene groups (7, 9, 19). Although α -amylase mRNA was detected in all five tissues, it was most abundant in germinating seeds (Fig. 6, lanes 6 and 7). Based on the quantities

Figure 6. Rice α -amylase gene expression in various rice tissues. Lanes 1 and 2 contain 15 and 1 μ g total callus RNA, respectively; lanes 3, 4 and 5 contain 15 μ g total root, leaf and immature seed RNA, respectively; lanes 6 and 7 contain 1 and 15 μ g total RNA from germinated seeds, respectively.

of RNA loaded in lanes 6 and 7 (1 μ g and 15 μ g, respectively), we estimated that α -amylase mRNA level in germinating seeds was at least 20 fold higher than that in leaves (lane 4) and immature seeds (lane 5), which, in turn, were several fold higher than in root tissue (lane 3). RNA from embryo-derived callus also contained a high level of α -amylase transcript (lanes 1 and 2). This is consistent with a recent report by Simmons and Rodriguez (22) that describes the high level synthesis and secretion of α -amylase from rice callus. We conclude, therefore, that the increased α -amylase synthesis in callus tissue is due, at least in part, to increased levels of α -amylase mRNA. A more detailed study of α -amylase mRNA expression in callus tissue has recently been completed (Simmons *et al.*, manuscript in prep).

Gene expression detected by PCR

Although low stringency Northern blot analysis has proved useful for detecting the relative amounts of total α -amylase mRNA in different tissues, it is less effective at discriminating between transcripts from different α -amylase genes. In those tissues expressing low levels of α -amylase mRNA, high stringency wash conditions either remove all low-abundance hybrids from the membrane or fail to distinguish between hybridization to a low-abundance mRNA and cross hybridization to abundant transcripts from another α -amylase gene (unpublished observations). This problem was resolved by using RNA polymerase chain reaction, a recently-developed technique for detecting low level gene expression (23). RNA-PCR increases the sensitivity of detecting specific gene expression by several orders of magnitude (18, 24). Furthermore, the molecular weights of co-amplified PCR products for each gene under study can be predicted and resolved by gel electrophoresis.

To test the ability of α -amylase oligonucleotides to specifically prime an RNA-PCR reaction, control experiments were performed using the cDNA clones, pOS103 and pOS137 (Fig. 7A lanes 1 to 3). The PCR products in lanes 1 and 3 were consistent with the predicted molecular weights for both genes. Fig. 7A, lane 2 shows PCR products co-amplified with the primers for both genes. The identities of the PCR products were confirmed by Southern blot analysis (Fig. 7B lanes 1 to 3) of the same gel shown in Fig. 7A. No amplification of pOS137 in lane 1 and pOS103 in lane 3 was detected, although lanes 1 to 3 contained the same amounts of each cDNA template. Also, no amplification was observed when PCR was carried out in the same buffer and under the same conditions when the cDNA templates were omitted (data not shown).

Total RNA isolated from germinating rice seeds was amplified with the *RAmy1A* primer (lane 4) and the *RAmy3D* primer (lane

Table 2. Summary of α -amylase gene expression in various rice tissues

Germinated Seeds	Root	Leaves	Immature Seeds	Callus	
All Genes	100 ^a	1	3	4	65
<i>RAmy1A</i>	++++ ^b	++++	++++	+++	++
<i>RAmy1B</i>	—	—	—	—	+
<i>RAmy3D</i>	++	++	++	—	++++
<i>RAmy3E</i>	+++	+++	+++	++++	+++

^aRelative mRNA levels for all α -amylase genes normalized to the highest level of expression shown in lane 7 of Fig. 6.

^bRelative levels of mRNA for each gene as estimated from the PCR products shown in Fig. 7. The amount of PCR product is indicated by plus signs ranging from the highest (++++) to the lowest (+). Minus signs (—) indicate that no product was observed in the RNA-PCR reaction. Because of slight variations in the parameters and kinetics of each PCR reaction, only comparisons within each tissue can be made.

6) and co-amplified with both primers (lane 5). The banding patterns in lanes 4 to 6 were identical to those seen in lanes 1 to 3, indicating that *RAmy1A* and *RAmy3D* are expressed in germinating seeds. Lanes 7 to 9 show PCR products co-amplified with the same RNA as in lanes 4 to 6. The *RAmy3E* primer alone produced a single PCR product (lane 9) with the molecular weight expected for a correctly spliced transcript (Fig. 4). When the *RAmy3E* primer was co-amplified with either the *RAmy1A* primer (lane 8) or *RAmy3D* primer (lane 7), an extra band corresponding to the additional primer was detected. Lane 13 contains PCR products co-amplified from the same RNA with all three primers. These PCR products can not be due to the amplification of small amounts of contaminating DNA since the presence of introns in the DNA would generate PCR products much larger than those produced by mRNA (see Fig. 1, 3 and 4). In addition to their predicted molecular weights, the identities of the various PCR products were further confirmed by Southern blot analysis using probes specific for *RAmy1A*, *RAmy3D* and *RAmy3E* in either sequential (data not shown) or mixed hybridization experiments (Fig. 7B).

Assuming that the synthesis of cDNA in the reaction is equally efficient and that each type of the cDNA is proportionally co-amplified, then the relative levels of PCR product can be taken as an approximate measure of the relative abundance of the original transcript. The validity of these assumptions has been tested and confirmed in several laboratories and used to quantify relative levels of mRNA and gene copy number (24, 25, 26, 27). We have obtained similar results by co-amplifying a mixture of two α -amylase cDNAs in which the concentration of one was held constant and the other serially diluted (data not shown). Based on the results shown in Fig. 7A, we estimate the relative levels of α -amylase gene transcript in 6 day germinating rice seeds to be as follows: *RAmy1A* > *RAmy3E* > *RAmy3D*. These results are in agreement with the work of Karrer *et al.* (manuscript in prep.) who have used group-specific hybridization conditions to examine α -amylase mRNA accumulation in germinating rice seeds.

RNA isolated from germinated rice seeds gave no PCR products for *RAmy1B* and *RAmy1C* when the *RAmy1A* oligonucleotide was used to prime the reaction. Since the predicted PCR product made by the *RAmy1B* primer could be distinguished from that made by the *RAmy1A* primer, we infer that *RAmy1B* is inactive in this tissue. Since the nucleotide sequence for *RAmy1C* has not been determined, we can not rule out the possibility that PCR products for the *RAmy1A* and *RAmy1C* are similar in size and thus indistinguishable using this technique.

Figure 7. Detection of gene expression by RNA-PCR of α -amylase genes in various rice tissues. **A.** PCR products were separated on 7.5% polyacrylamide gel and visualized by staining with ethidium bromide. Control cDNAs, pOS103 and pOS137, were amplified with specific primers for *RAmy1A* (lane 1), *RAmy3D* (lane 3) and both (lane 2). Total RNA from germinating seeds was amplified with primers for *RAmy1A* (lane 4), *RAmy1A* and *RAmy3D* (lane 5), *RAmy3D* (lane 6), *RAmy3D* and *RAmy3E* (lane 7), *RAmy1A* and *RAmy3E* (lane 8), and *RAmy3E* (lane 9). Lanes 10 to 14 contained total RNA from roots, leaves, immature seeds, germinating seeds and callus, respectively, co-amplified with all three specific primers. **B.** Southern blot of the gel in 7A probed with mixed probes for *RAmy1A*, *RAmy3D* and *RAmy3E*. **C.** Lanes 15 and 18 are the same as the lanes 1 and 14 in 7A. PCR products of callus RNA amplified with *RAmy1A* primer (lane 17) were digested with *KpnI* (lane 16). **D.** Southern blot of gel in 7C probed with *RAmy1A* and *RAmy1B*.

Total RNA isolated from roots (lane 10), young leaves (lane 11), immature seeds (lane 12) and callus (lane 14) was also co-amplified with all three primers (Fig. 7A). Consistent with the Northern blot results shown in Fig. 6, α -amylase transcripts were detected in all tissues. No attempt was made to compare the relative abundance of α -amylase mRNAs between different tissues in the PCR reactions. We did, however, compare the relative abundance of different α -amylase mRNAs within each tissue based on the above assumptions. For example, the expression of *RAmy1A* and *RAmy3E* was observed in all five tissues. In immature seeds, *RAmy3E* transcripts were more abundant than those of *RAmy1A*, while *RAmy3D* transcripts were most abundant in callus tissue. The level of *RAmy1A* mRNA was found to be highest in the germinating seeds, roots and young leaves. These results suggest that all three α -amylase genes are expressed, albeit weakly, in young roots and leaves (Fig. 6 and 7). Whether or not the expression of these three genes persists in mature roots and leaves is not yet known. We are currently examining α -amylase gene expression in mature tissues under different growth conditions, and results of these experiments will be reported elsewhere. One PCR product observed in lane 11 was due to nonspecific amplification since Southern blot analysis showed that it did not hybridize to any α -amylase gene probes (Fig. 7B, lane 11).

No expression of *RAmy1B* was detected in rice roots, young leaves, immature seeds or germinating seeds. In callus (lane 14), however, a low-abundance PCR product of 167 bp (Fig. 7A, lane 14) was found to hybridize to the *RAmy1A* probe (Fig. 7B, lane 14). To better resolve this band and to provide further evidence that it was a true amplification product of *RAmy1B* mRNA, the following experiment was performed. *RAmy1A* primer was used to amplify callus RNA and the PCR products were digested with the restriction enzyme, *KpnI*. From the sequence in Fig. 1A, it was known that *KpnI* would cleave the PCR product of *RAmy1B*, but not *RAmy1A*. As expected, the 167 bp band seen in Fig. 7C, lane 17, was reduced in size to a 139 bp band seen in Fig. 7C, lane 16. When the blot shown in Fig. 7C was hybridized with a mixture of *RAmy1A* and *RAmy1B* probes, the identity of these bands was confirmed. This confirmation was further supported by sequentially probing the blot with *RAmy1A* and *RAmy1B*. We conclude that *RAmy1B* is weakly expressed in callus tissue only. For the sake of comparison, the results shown in Figs. 6 and 7 are summarized in Table 2.

Finally, although the *Amy3* genes of wheat are expressed only in immature seeds (8), *RAmy3D* was found to be expressed in all tissues examined except immature seeds. This raises the interesting question as to whether α -amylase genes in rice, wheat and barley should be classified according to their structure, characteristics of expression or both. Further analysis of the cereal α -amylase genes should provide the information needed to establish a consistent classification system and provide a better understanding of the function of these genes in the plant.

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