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# Temporal and Spatial Patterns of Accumulation of the Transcript of *Myo*-Inositol-1-Phosphate Synthase and Phytin-Containing Particles during Seed Development in Rice[1]

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***Myo*-inositol-1-phosphate (I[1]P) synthase (EC 5.5.1.4) catalyzes the reaction from glucose 6-phosphate to I(1)P, the first step of *myo*-inositol biosynthesis. Among the metabolites of I(1)P is inositol hexa*kis*phosphate, which forms a mixed salt called phytin or phytate, a storage form of phosphate and cations in seeds. We have isolated a rice (*Oryza sativa* L.) cDNA clone, pRINO1, that is highly homologous to the I(1)P synthase from yeast and plants. Northern analysis of total RNA showed that the transcript accumulated to high levels in embryos but was undetectable in shoots, roots, and flowers. In situ hybridization of developing seeds showed that the transcript first appeared in the apical region of globular-stage embryos 2 d after anthesis (DAA). Strong signals were detected in the scutellum and aleurone layer after 4 DAA. The level of the transcript in these cells increased until 7 DAA, after which time it gradually decreased. Phytin-containing particles called globoids appeared 4 DAA in the scutellum and aleurone layer, coinciding with the localization of the RINO1 transcript. The temporal and spatial patterns of accumulation of the RINO1 transcript and globoids suggest that I(1)P synthase directs phytin biosynthesis in rice seeds.**

*Myo*-inositol and I(1)P are essential for the survival of eukaryotic cells, because various metabolic routes diverge from these two compounds. A number of possible roles for *myo*-inositol and I(1)P metabolites in plant development have been demonstrated (for review, see Bohnert et al., 1995). I(1)P is utilized for the synthesis of plasma membrane phosphoinositides, which are involved in signal transduction as sources of second messengers (Gross and Boss, 1993). *Myo*-inositol combined with Gal is incorporated into the raffinose family of the vegetative storage form of carbohydrates (Kandler and Hopf, 1982). Oxidized inositols serve as noncellulosic cell wall components (Loewus and Loewus, 1983), and methylated inositols were shown to be involved in osmoprotection in a halophytic plant (Ishitani et al., 1996). In addition, I(1)P is further phosphorylated to inositol hexa*kis*phosphoric acid, or phytic acid, which strongly binds metallic cations such as K, Mg, Mn, Ca, Fe, and Zn via ionic associations with negatively charged phosphates (Maga, 1982; Lott et al., 1995) to form a mixed salt called phytin or phytate (Ashton, 1976). The synthesis of phytin as a storage form of phosphate and cations is a metabolic pathway unique to plants (Loewus and Dickinson, 1982).

Phytin is mainly stored in protein bodies in seeds as spherical inclusions called globoids (Lott et al., 1995). Cereal grains and oil seeds are particularly rich sources of phytin, and the phosphorus of phytin represents 80% to 90% of total phosphorus in these seeds (Maga, 1982). In rice (*Oryza sativa*) seeds phytin accumulates in the aleurone and scutellum cells but not in the starchy endosperm cells (Tanaka et al., 1973; Ogawa et al., 1977). During germination phytin is digested by an acid phosphatase called phytase (Laboure et al., 1993; Barrientos et al., 1994; Hubel and Beck, 1996), and the released phosphate, cations, and inositol are utilized by the seedlings. However, despite its biological importance, the regulation of phytin biosynthesis remains to be elucidated. Moreover, although its function in phosphorus storage is important for the growth of seedlings, monogastric animals are unable to digest phytin (Simons et al., 1990); therefore, the development of cultivars with a low seed phytin content is one of the major breeding objectives in several crop species.

I(1)P synthase (EC 5.5.1.4) catalyzes the reaction from Glc 6-P to I(1)P, the first step of inositol metabolism. Recently, cDNA clones with high homology to the I(1)P synthase gene of *Saccharomyces cerevisiae* (Johnson and Henry, 1989) have been reported from several plant species, including *Spirodela polyrrhiza* (Smart and Fleming, 1993), Arabidopsis (Johnson, 1994; Johnson and Sussex, 1995), *Citrus paradisi* (Abu-Abied and Holland, 1994), *Phaseolus vulgaris* (Wang and Johnson, 1996), and *Mesembryanthemum crystallinum* (Ishitani et al., 1996). In these studies transcripts were

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Abbreviations: DAA, days after anthesis; DIG, digoxygenin; I(1)P, *myo*-inositol-1-phosphate; 5′-RACE, rapid amplification of 5′-cDNA ends.

induced in various cellular processes, such as ABA-induced dormant bud formation in *S. polyrrhiza* (Smart and Fleming, 1993), salt and cold stresses in *M. crystallinum* (Ishitani et al., 1996), and photoperiodic response in *C. paradisi* (Abu-Abied and Holland, 1994). However, to our knowledge, there has been no report on the relationship between expression of the I(1)P synthase gene and phytin synthesis in developing seeds.

During the course of our research to isolate genes that may play a role in embryogenesis in rice (Yoshida et al., 1994a, 1994b; Mizobuchi-Fukuoka et al., 1996), we isolated cDNA clones that were preferentially expressed in calli in which somatic embryogenesis was induced (Mizobuchi-Fukuoka et al., 1996). In the present study we analyzed one of the clones, pRINO1, that showed a high homology to I(1)P synthase from yeast and plants. We report the accumulation pattern of the RINO1 transcript in planta during seed development of rice in relation to the accumulation of phytin-containing particles.

## MATERIALS AND METHODS

### Plant Material, Cell Cultures, and cDNA Clones

Rice (*Oryza sativa* L. var *japonica* cv Kamenoo) plants were grown in pots in a greenhouse under natural light conditions (for flowers and seeds), or in a growth chamber (LH-100, Nippon Medical and Chemical Instruments, Osaka, Japan) at 28°C with 14 h of illumination per day at an intensity of approximately 200 $\mu$E $m^{-2}$ $s^{-1}$ (for 7-d-old seedlings).

The initiation of callus suspension cultures from the scutellum of mature seeds and the induction of somatic embryogenesis and organogenesis (regeneration) have been described previously (Yoshida et al., 1994a).

The isolation of the pRINO1 (rice *myo*-inositol-1-phosphate synthase, see below) cDNA clone was described previously (Mizobuchi-Fukuoka et al., 1996). A cDNA library prepared from calli 7 d after induction of embryogenesis (embryogenic calli) was differentially screened using first-strand cDNA probes prepared from unorganized calli, calli 7 d after induction of organogenesis (organogenetic calli), and embryogenic calli. The pRINO1 clone showed markedly stronger signals with the embryogenic calli probe than with the other probes (referred to as pRSEM2 by Mizobuchi-Fukuoka et al., 1996).

### Extraction of RNA and Northern Analysis

Total RNA from shoots and roots of 7-d-old seedlings, from flowers just before heading, from immature embryos 7 DAA, and from the unorganized, organogenetic, and embryogenic calli was prepared using Isogen (Nippon Gene, Tokyo, Japan). Total RNA was fractionated on 1.2% agarose-formaldehyde gels and transferred to Hybond-$N^+$ membranes (Amersham). The *Eco*RI- and *Xho*I-excised insert of pRINO1 cDNA was labeled with [$\alpha$-$^{32}$P]dCTP by the random-priming method (Feinberg and Vogelstein, 1984) and used as a probe. Hybridization was carried out at 42°C according to the membrane manufacturer's instructions (Amersham). The final wash was in 0.1× SSPE/0.1% SDS at 42°C.

### Genomic Southern Hybridization

Rice genomic DNA was prepared from leaves as described by Murray and Thompson (1980). DNA was digested with restriction endonucleases, electrophoresed on a 0.8% agarose gel, and blotted onto Hybond-$N^+$ membrane. Restriction enzymes that do not digest the pRINO1 insert were used to digest the genomic DNA. The blot was probed with the pRINO1 insert labeled with [$\alpha$-$^{32}$P]dCTP by the random-priming method (Feinberg and Vogelstein, 1984). Hybridization was performed at 65°C according to the membrane manufacturer's instructions (Amersham). The final wash was in 0.1× SSPE/0.1% SDS at 65°C. For low-stringency hybridization the membrane was hybridized and washed at 58°C.

### DNA Sequencing and 5′-RACE

Both strands of the cDNA insert were sequenced using a dye-primer cycle-sequencing kit (Prism, Applied Biosystems) and an automatic DNA sequencer (model 377A, Applied Biosystems).

5′-RACE was carried out with the poly($A^+$) RNA from the 7-DAA zygotic embryos using a 5′-RACE system (Life Technologies). The primers used for 5′-RACE were 5′-dCCATTGTGGCATTGAACC-3′ for the 5′ extension reaction and 5′-dCCACAACACCACCACCTT-3′ for the nested amplification. The PCR fragment obtained was cloned into a pCRII vector (Invitrogen, San Diego, CA). The integrity of the junction between the 5′-RACE clone and the original clone was verified by sequencing the reverse-transcription PCR product.

Searches of nonredundant protein and nucleotide databases for sequence similarity were carried out using the Basic Local Alignment Search Tool (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD; http://www.ncbi.nlm.nih.gov/). Codon analysis and alignment of the amino acid sequences were carried out using the DNASIS program (Hitachi, Tokyo, Japan).

### In Situ Hybridization

In situ hybridization was carried out as described by Kouchi and Hata (1993) with some modifications. Developing seeds were fixed in 4% (w/v) *p*-formaldehyde/0.25% (w/v) glutaraldehyde and embedded in Paraplast Plus (Oxford, St. Louis, MO), and 8-$\mu$m sections were mounted on slides coated with Vectabond (Vector Laboratories, Burlingame, CA). The sections were hybridized with DIG-labeled RNA probes (Tautz and Pfeifle, 1989) synthesized from the pRINO1 cDNA using a labeling kit (Boehringer

Mannheim). Washing and detection procedures were as described by Kouchi and Hata (1993).

### Visualization of Globoids

Developing seeds were fixed for 3 to 4 h in a mixed aldehyde solution containing 1.4% glutaraldehyde and 2.0% *p*-formaldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, at 4°C. The fixed tissues were dehydrated through a graded ethanol series according to the method of Feder and O'Brien (1968), and then embedded in 2-hydroxyethyl methacrylate (Wako Pure Chemical, Osaka, Japan). Thin sections (2 $\mu$m) were stained with 0.5% toluidine blue O (Chroma-Gesellshaft Schmid GmbH, Köngen, Germany) in 5% ethanol. Phytin-containing particles (globoids) showed a purple metachromatic color (Jacobsen et al., 1971), whereas other cellular components appeared light blue to blue.

## RESULTS

### Accumulation of the RINO1 Transcript in Callus and in Planta

The cDNA clone pRINO1 showed a markedly stronger signal with the probe prepared from embryogenic calli than those from the organogenetic calli or unorganized calli (Mizobuchi-Fukuoka et al., 1996). Accumulation of the RINO1 transcript in calli was analyzed by northern analysis of total RNA isolated from the embryogenic calli, organogenetic calli, and unorganized calli. As shown in Figure 1A, the RINO1 transcript accumulated preferentially in the embryogenic calli. The RINO1 probe hybridized to a single band about 1.9 kb in length. A weak signal of the same size was detected in the organogenetic calli after a long exposure (data not shown).

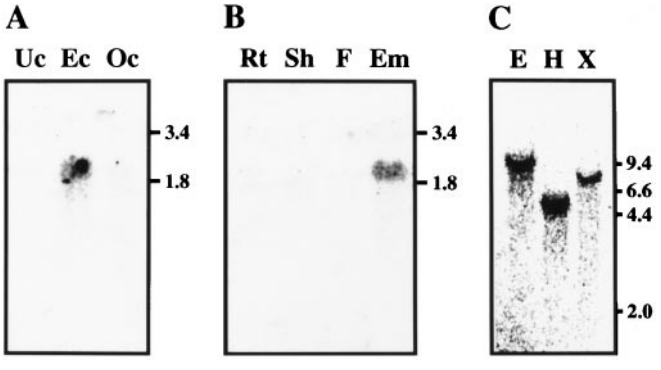


**Figure 1.** Northern and Southern analyses of RINO1. A, Total RNA (10 $\mu$g per lane) isolated from unorganized calli (Uc), embryogenic calli (Ec), and organogenetic calli (Oc) was separated on agarose gels containing formaldehyde, blotted, and hybridized with a $^{32}$P-labeled pRINO1 insert. Positions and sizes (in kb) of rRNAs are indicated. B, Total RNA isolated from roots (Rt) and shoots (Sh) 7 d after imbibition, from flowers (F), and from zygotic embryos 7 DAA (Em) were hybridized with the RINO1 probe as in A. C, Rice genomic DNA (5 $\mu$g per lane) digested with *Eco*RI (E), *Hin*dIII (H), or *Xho*I (X) were separated on an agarose gel, blotted, and hybridized with the RINO1 probe. Positions and sizes (in kb) of markers are indicated.

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Os   1 MFIESF---- ---RVESPHV RYGAAEIESD YQYDTTELVH ES-HDGASRW IVRPKSVRYN  52
Sp   1 ....K.---- ---.....N. K..DG....V .S.E.....- .-VRN.SYQ. V.K....Q.Q  52
Bn   1 ......---- ---K....N. K.TEN..H.V .D.E...V.. .N-VN..YQ. ..K..V.K.D  52
At   1 ......---- ---K....N. K.TEN..N.V .D.E...V.. .N-RN.TYQ. V.K..T.K.D  52
Pv   1 .....----- ---K....N. K.TEN..H.V .D.E....V. .-KTVN.TYQ. ..K..T.K.D  53
Sc   1 .TEDNIAPIT SVK.LVTDKC T.KDN.LLTK .S.ENAVV-- TKTAS.RFD- -.T.TVQD.V  56

Os  53 FRTT-TTVPK LGVMLVGWGG NNGSTLTAGV IANREGISWA TKDK---VQQ ANYYGSLTQA 108
Sp  53 .K.D-.R..R ..........  ..........  .........V ..E.----.. ...F.....S 108
Bn  53 .K.D-.R...  ..........  ..........  ...K...... ....---... ...F..... 108
At  53 .K.D-.R...  ..........  ..........  ...K...... ....---... ...F..... 108
Pv  54 .K.D-IR...  ..........  ..........  ...K...... ....---... ...F..... 109
Sc  57 .KLDFEKK.E KTRNYAH.VR WLQQW..LWP RYW.ISTM.S FQT.AKG.K. P..F..MT.C 116

Os 109 STIRVG-SYN GEEIYAPFKS LLPMVNPDDL VFGGWDISNM NLADAMTRAK VLDIDLQKQL 167
Sp 109 .S.....-.F. ..........  .........EI .........D. ......G... .......... 167
Bn 109 .S.....-.F. ...M......  .V........V .........D. ......G... .......... 167
At 109 .S....-... ..........  .........V .........D. ......A..R .......... 167
Pv 110 .S.....-.F. ..........  .........V .........D. ......A..R .......... 168
Sc 117 ..LKL.IDAE .NDV....N.  .....S.KHF .VS....N.A D.YE..Q.SQ ..EY..QR.  176

Os 168 RPYMESMVPL PGIYDPDVIA ANQGSRANNV IKGTKKE--- ------QMEQ IIKDIREFKE 218
Sp 168 ..........  ....N..F..  ..........  ...P..Q--- ------.VQR ..D..... 218
Bn 168 .....NI...  .......F..  ..........  .......--- ------.VD. ....M..... 218
At 168 .....N.I..  .......F..  .........S  .......--- ------.VDH ....M..... 218
Pv 169 .....NI...  .......F..  .........H  .......--- ------.VDH ....M..... 219
Sc 177 KAK.SLVK..  .S..Y..F..  ...DE...QC .NLDE.GNVT TRGKWTHLQR .RR.IQN... 236

Os 219 KSKVDKVVVL WTANTERYSN VCVGLNDTME NLLASVDKNE AEISPSTLYA IACVMEGIPF 278
Sp 219 RE..E.....  ..........D LV........  ....A.ERD. .......S.. L..I...V.. 278
Bn 219 .N........  ..........  .I........  ...MN...RD. S.......... ....L..... 278
At 219 .N....L...  ..........  .I.....T..  ......E.D. S.......... ....L..... 278
Pv 220 .N........  ..........  EV..M.....  D.ME...RD. .......... ....L..... 279
Sc 237 ENAL...I..  ........VE .SP.V.....  ...Q.IKNDH E..A...IF. A.SIL..V.Y 296

Os 279 INGSPQNTFV PGLIDLAIKN NCLIGGDDFK SGQTKMKSVL VDFLVGAGIK PTSIVSYNHL 338
Sp 279 V.........  ....EM...R .S........  ..........  .......... .......... 338
Bn 279 .........L  ....E...S.  .V........  ..........  .......... .......... 338
At 279 .........L  ....E...S.  .V........  .........W  .......... .......... 338
Pv 280 .....H....  .....M..R.  .V........  ..........  G......... .......... 339
Sc 297 ..........  ...VQ..EHE  GTF.A...L.  .....L....  AQ...D.... ....A..... 356

Os 339 GNNDGMNLSA PQTFRSKEIS KSNVVDDMVS SNAILYEL-- GEHPDHVVVI KYVPYVGDSK 396
Sp 339 ..........  ..........  ..........  ..G...P-- .......I.. .......... 396
Bn 339 ..........  ..........  .........A  ..G..F.P-- .......... ......A... 396
At 339 ..........  ..........  .........A  G..F.P-- .......... ......A... 396
Pv 340 ..........  ..........  .......VA  ..G..F.P-- .......... ......A... 397
Sc 357 .....Y....  .KQ.......  ..S.I..IIA  ..D...NDKL .KKV..CI.. ..MKP..... 416

Os 397 RAMDEYTSEI FMGGKSTIVL HNTCEDSLLA APIILDLVLL AELSTRIQLK A-----EGEE 451
Sp 397 ..........  .....I....  ..........  .......... ..........S 451
Bn 397 ..........  .....N...M  ..........  ..........  .I.....F. S-----.K.G 451
At 397 ..........  ....RN....  ..........  .........F  .-------..G 451
Pv 398 ..........  .....N...M  ..........  .........F  S-------.G 452
Sc 417 V.....Y..L  ML..HNR.SI  ..V.......  TAL.I..LVM T.FC..VSY. KVDPVK.DAG 476

Os 452 KFHSFHPVAT ILSYLTKAPL VPPGTPVVNA LAKQRAMLEN IMRACVGLAP ENNMILEYK-- 510
Sp 452 .........S .....S....  ..........  .S........ .L........ .........-- 510
Bn 452 ..........  K.........  ..........  .S........ .L........ .........-- 510
At 452 ..........  ..........  .....A....  .S...P.... .L........ .....M..--- 510
Pv 453 ..........  ..........  .....I....  .S........ .......... .....M.F--- 511
Sc 477 ..EN.Y..L.  F...WL....  TR..FHP..G  .N...TA... FL.LLI..PS Q.ELRF.ERLL 537
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**Figure 2.** Comparison of the deduced amino acid sequences of RINO1 (Os) with the amino acid sequences of I(1)P synthases from *S. polyrrhiza* (Sp), *B. napus* (Bn), Arabidopsis (At), *P. vulgaris* (Pv), and *S. cerevisiae* (Sc). Positions that are identical to the RINO1 sequence are indicated by dots, and gaps introduced for maximum alignments are indicated by dashes.

Since the pRINO1 clone was isolated by differential screening to isolate somatic embryogenesis-abundant genes, we expected the transcript to be accumulated preferentially in zygotic embryos in planta. We tested this expectation with northern analysis of total RNA isolated from shoots and roots of 7-d-old seedlings, from flowers just before heading, and from zygotic embryos 7 DAA. Figure 1B shows that the RINO1 probe detected an abundant transcript of the same size in zygotic embryos but not in the other tissues tested.

### Sequence Analysis of the Full-Length pRINO1 cDNA

Sequence analysis of the original pRINO1 insert indicated that the length of the cDNA (1111 bp) was shorter than the transcript size detected by northern analysis (1.9 kb). 5′-RACE was performed to obtain the full-length cDNA, and an additional 776 bases 5′ of the original pRINO1 insert were obtained. The total length of this cDNA was 1887 bp, which corresponds well with the size detected by northern analysis.

The DNA sequence of the full-length pRINO1 cDNA (accession no. AB012107) revealed a 510-amino acid open reading frame with a 109-bp 5′-untranslated region, a 225-bp

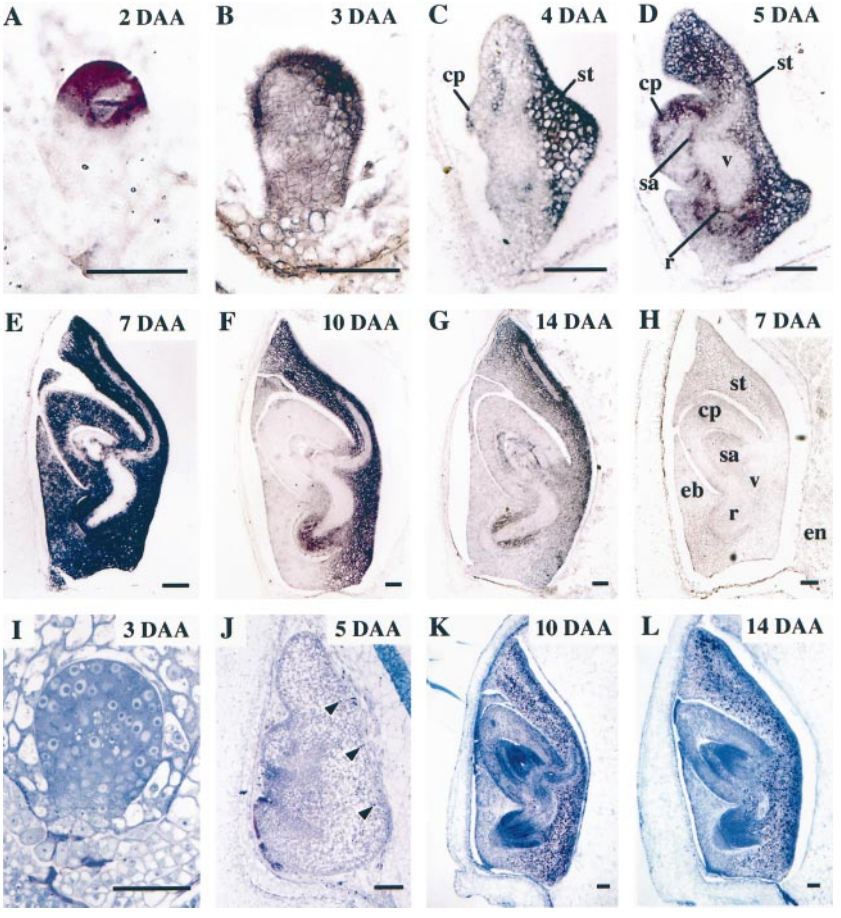


**Figure 3.** In situ localization of the RINO1 transcript detected by a DIG-labeled antisense probe (A–G) and globoids detected by toluidine blue staining (I–L) in developing embryos 2 (A), 3 (B and I), 4 (C), 5 (D and J), 7 (E), 10 (G and K), and 14 DAA (G and L). An embryo 7 DAA was also hybridized with a DIG-labeled sense probe (H). cp, Coleoptile; eb, epiblast; en, endosperm; r, radicle; sa, shoot apical meristem; st, scutellum; v, vascular procambium. Some of the globoids in J are marked with arrowheads. The dorsal side is to the right of each panel. Scale bars represent 50 $\mu$m.

3′-untranslated region, and a 20-bp poly($A^+$) tail. Alignment of the deduced amino acid sequence (Fig. 2) revealed an approximately 50% identity to *Ino1*, the I(1)P synthase gene from *Saccharomyces cerevisiae* (Johnson and Henry, 1989; accession no. L23520) and 86% to 88% identity to the I(1)P synthase cDNA from *Spirodela polyrrhiza* (Smart and Fleming, 1993; accession no. Z11693), *Brassica napus* (U66307), Arabidopsis (Johnson, 1994; accession no. U04876), and *Phaseolus vulgaris* (Wang and Johnson, 1996; accession no. U38920).

### Genomic Southern Analysis

Genomic Southern analysis was carried out to determine the complexity of the RINO1-encoding gene(s). Genomic DNA digested with restriction enzymes that did not digest the pRINO1 insert produced only one strongly hybridizing band in each digest (Fig. 1C). No additional band was visible in any of the digests, even with low-stringency hybridization (data not shown). These results suggest that a single gene encoding the pRINO1 insert is present in the rice genome.

### Accumulation of the RINO1 Transcript in Developing Seeds

In situ hybridization was performed to determine the accumulation pattern of the RINO1 transcript during the early embryogenesis of rice seeds. Cell division of the fertilized egg starts at about 20 h after anthesis (Satoh and Omura, 1986), and the embryo is globular until 3 DAA (Fig. 3, A and B). By 4 DAA the scutellum is distinct in that vacuolation and the emergence of the coleoptile can be recognized by a notch at the ventral surface of the embryo (Fig. 3C). Soon after this the shoot apical meristem is distinguishable just below the coleoptile and the radicle meristem is also established (Fig. 3D). By 7 DAA the scutellum, coleoptile, coleorhiza, vascular procambium, epiblast, and plumule are visible (Fig. 3, E and H), as can be seen in mature embryos. By 10 DAA morphological differentiation of the embryo is almost complete (Fig. 3, F and K), with seeds maturing at about 45 DAA.

The accumulation pattern of the RINO1 transcript in rice seeds was determined by in situ hybridization of longitudinal sections of seeds with a DIG-labeled antisense RNA probe (Fig. 3, A–G). The results showed that signals of the transcript first appeared in the globular stage embryo 2 DAA (Fig. 3A). The signal was found only in the upper half of the embryo. In the 3-DAA embryo, the stage before the appearance of the coleoptile, the signal was detected in the peripheral region of the dorsal side, where the scutellum is to be differentiated (Fig. 3B). The level of the transcript continued to increase until 7 DAA, when it began to decrease gradually (Fig. 3, C–G). In the 7-DAA embryo signals were detected in the scutellum, coleoptile, plumule,

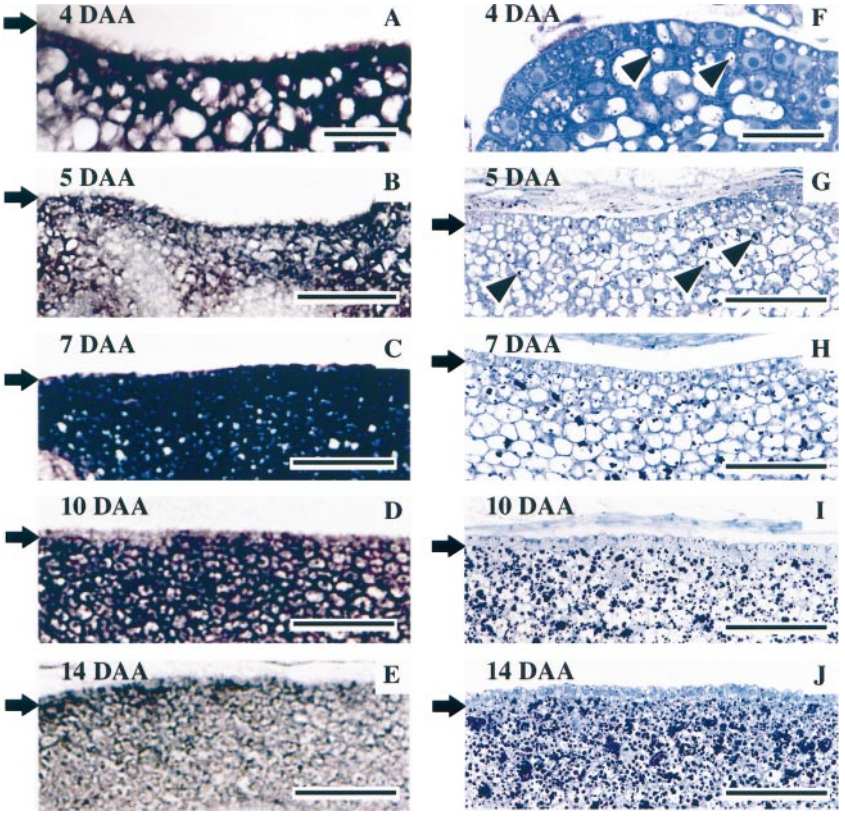


**Figure 4.** In situ localization of the RINO1 transcript detected by a DIG-labeled antisense probe (A–E) and globoids detected by toluidine blue staining (F–J) in the scutellum of embryos 4 (A and F), 5 (B and G), 7 (C and H), 10 (D and I), and 14 DAA (E and J). Arrows indicate the border between the scutellum (bottom) and epithelium (top). Some of the globoids in F and G are marked with arrowheads. Scale bars represent 10 $\mu$m for A and F and 50 $\mu$m for B to E and G to J.

radicle, and epiblast (Fig. 3E). Although the signal in the coleoptile, plumule, radicle, and the epiblast disappeared by 10 DAA, it could still be detected in the scutellum of 10- and 14-DAA embryos (Fig. 3, F and G). The RINO1 transcript could not be detected in the vascular procambium of either the scutellum or the embryo proper (Fig. 3, E–G).

Although the RINO1 transcript accumulated to a high level in the scutellum, closer investigation revealed that the transcript level was very low in the outermost cell layer of the scutellum (Fig. 4, A–E), the area that differentiates into the epithelium.

In addition to the embryos, signals were also detected in the outer cell layers of the endosperm (Fig. 5, A–E), which differentiate into the aleurone layer. The outer cell layers of the endosperm could be distinguished morphologically 5 DAA (Fig. 5, B and G). In mature seeds the aleurone layer consists of up to five cell files on the dorsal side and decreases to one cell layer on the ventral side. The signal first appeared 3 DAA in the outer cell layers of the dorsal side (Fig. 5A) and rapidly spread to the ventral side (data not shown). The level of the transcript increased until 7 DAA and then gradually decreased (Fig. 5, B–E). The expression level in the aleurone layer was lower than that in the scutellum. No signal could be detected in the inner endosperm cells at any stage (Fig. 5, A–E).

Accumulation of the RINO1 transcript was also observed in the shoot apical meristem of embryos during an early stage of development (Figs. 3E and 6). The shoot apical meristem could be identified visibly 5 DAA, and the accumulation of the RINO1 transcript was evident in a narrow region of the shoot apical meristem between 5 and 7 DAA (Fig. 6) but not 10 DAA (Fig. 3F).

### Accumulation of Globoids in Developing Seeds

To determine whether phytin synthesis in seeds is related to the accumulation of the RINO1 transcript, developing seeds 1 to 14 DAA were stained for globoids using toluidine blue (see "Materials and Methods"). Globoids first appeared 4 DAA in both the scutellum (Figs. 3J and 4F) and the aleurone layer (Fig. 5G). The size and number of globoids gradually increased in both tissues and also appeared in other embryo tissues such as the coleoptile, plumule, coleorhiza, and epiblast but not the vascular procambium (Fig. 3, K and L).

In the embryo the largest number and size of globoids were observed in the scutellum (Figs. 3L and 4J), although the outermost cell layer of the scutellum, the epithelium, contained a very small number of tiny globoids even 14 DAA (Fig. 4J). In contrast to the aleurone layer, no globoids were observed in the starchy endosperm cells, even 14 DAA (Fig. 5J).

Accumulation of globoids in the shoot apical meristem of developing seeds was not readily observable. However, a limited number of small globoids could be seen in a region several cell layers away from the shoot apical meristem (data not shown).

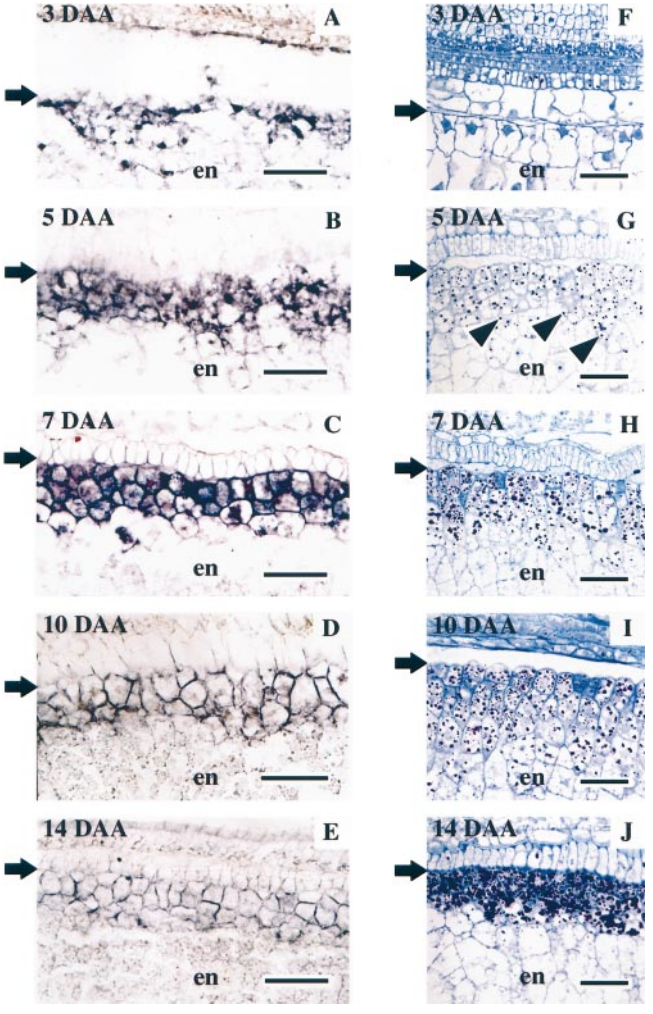


**Figure 5.** In situ localization of the RINO1 transcript detected by a DIG-labeled antisense probe (A–E) and globoids detected by toluidine blue staining (F–J) in the aleurone layer in the dorsal side of seeds 3 (A and F), 5 (B and G), 7 (C and H), 10 (D and I), and 14 DAA (E and J). Arrows indicate the border between the aleurone layer (bottom) and maternal tissues (top). en, Endosperm. Some of the globoids in G are marked with arrowheads. Scale bars represent 50 $\mu$m.

## DISCUSSION

We analyzed the expression pattern of a cDNA clone, pRINO1, which is highly homologous to the I(1)P synthase genes of yeast and plants. Localization of globoids, or phytin-containing particles, corresponded well with the accumulation pattern of the RINO1 transcript. Both globoids and the RINO1 transcript accumulated to high levels within the scutellum and the aleurone layer and much less in the outermost cell layer of the scutellum. We observed only few globoids and RINO1 transcripts in the starchy endosperm throughout seed development. In mature seeds of rice, globoids are observed in most of the embryo tissues and aleurone layer (Wada and Lott, 1997); however, the size varies depending on the tissue, and globoids larger than 1 $\mu$m in diameter are mainly observed in the scutellum and the aleurone layer (Wada and Lott, 1997). These two tissues are the ones in which the RINO1 transcript accumulated most abundantly and in which the duration of the transcript accumulation was the longest. These results demonstrated a close relationship between the RINO1 transcript and globoid accumulation, suggesting that I(1)P synthase plays a role in phytin biosynthesis in developing seeds of rice.

The accumulation of the RINO1 transcript started in the embryo and the aleurone layer 2 and 3 DAA, respectively. Globoids first appeared in these cells 4 DAA. Therefore, the accumulation of the RINO1 transcript precedes the appearance of globoids, supporting a role for I(1)P synthase in phytin synthesis. Our results also suggest that phytin synthesis is regulated at least in part at the level of I(1)P synthase mRNA accumulation.

Seed storage reserves generally accumulate in middle to late stages of seed development (Goldberg et al., 1989), following the completion of embryo morphogenesis. Glutelins and prolamins, the major seed storage proteins of rice, are detected 10 DAA but not 5 DAA (Li and Okita, 1993). We found that globoids started to accumulate as early as 4 DAA. In rice seeds cell division of the starchy endosperm ceases by 10 DAA (Hoshikawa, 1967), and the embryo morphogenesis is also completed by that time. Therefore, the onset of globoid accumulation is unusually early for a seed storage reserve. The accumulation of phy-

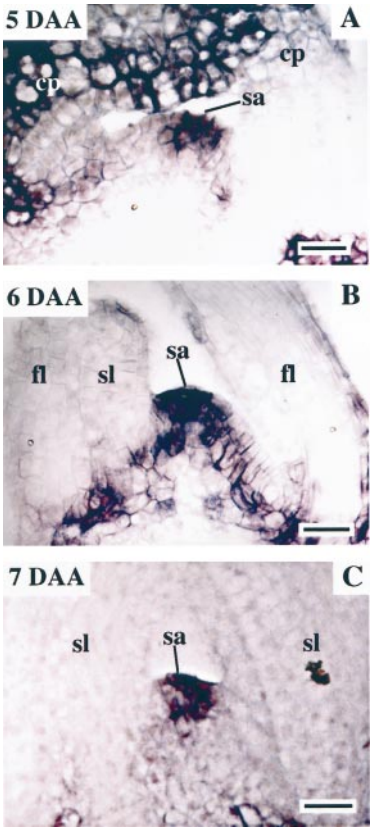


**Figure 6.** In situ localization of the RINO1 transcript detected by a DIG-labeled antisense probe in the shoot apical meristem region of embryos 5 (A), 6 (B), and 7 DAA (C). cp, Coleoptile; fl, first leaf; sa, shoot apical meristem; sl, second leaf. Scale bars represent 10 $\mu$m.

tin during the early stages of seed development must have a role not only after germination but also during seed development.

It is plausible that tissues accumulate phytin prior to a large demand for phosphorus, such as that of seed development. In fact, the shoot apical meristem also accumulated the RINO1 transcript during early embryogenesis. In support of this idea, the I(1)P synthase gene is expressed very soon after ABA-induced turion differentiation from growing fronds in the aquatic monocotyledonous plant *S. polyrrhiza* (Smart and Fleming, 1993). The fact that the pRINO1 cDNA was initially isolated from the embryogenic calli and that the transcript accumulated to a high level in the embryogenic calli but not in the unorganized calli before induction of somatic embryogenesis may also support this idea. However, it is still not known whether the accumulation of I(1)P synthase transcripts is accompanied by the accumulation of phytin in these cases.

A number of metabolites of *myo*-inositol and I(1)P play important roles in plant development (Bohnert et al., 1995). Although a close relationship between I(1)P synthase and the accumulation of phytin was shown in the present study, I(1)P synthase in rice could have roles other than phytin biosynthesis in seeds. Of the reports of the expression of I(1)P synthase in plants, only in the case of a halophyte was a role for this enzyme proposed. In *Mesembryanthum crystallinum*, I(1)P synthase, which is induced by salt stress, was suggested to have a role in the production of osmoprotectants that are methylated derivatives of I(1)P (Ishitani et al., 1996). Finer analyses of I(1)P synthase expression in tissues other than seeds will give further insight into the role of I(1)P synthase in rice. In situ analysis showed that the RINO1 transcript can be detected in very young leaves of 3-week-old plants (H. Koyama and K.T. Yoshida, unpublished data).

Whatever the role(s) of I(1)P synthase in rice seeds, the accumulation of the RINO1 transcript in the apical region of globular stage embryos 2 DAA and in the dorsal region 3 DAA indicates that RINO1 provides an early molecular marker for the establishment of both apical-basal and dorso-ventral patterns during rice embryogenesis. Jones and Rost (1991) reported that the cells of the apical two-thirds of the rice embryo at the end of the globular stage are vacuolated and are thought to be the progenitors of the scutellum. The accumulation of the RINO1 transcript in the apical region of embryos 2 DAA is likely to indicate the region expected to form the scutellum.

Phytin forms insoluble mixed salts with various mineral elements (Ashton, 1976) and reduces the bioavailability of phosphorus and minerals in monogastric animals, including humans. Supplementation of feedings with phytase, which hydrolyzes phytin into Pi and *myo*-inositol (Laboure et al., 1993; Barrientos et al., 1994; Hubel and Beck, 1996), improves the bioavailability of phosphorus (Simons et al., 1990). Recently, a direct application of this using phytase-producing transgenic plants was demonstrated (Pen et al., 1993; Verwoerd et al., 1995). Development of cultivars with low phytin content is another way to increase bioavailability. The reduction of phytin content in seeds is one of the major breeding objectives in several crops, including wheat (Batten, 1986), field beans (Griffiths and Thomas, 1981), soybean (Raboy et al., 1984), and pearl millet (Satija and Thukral, 1985). One problem, however, is that cultivars with a low phytin content often have low phosphorus and protein contents as well (Raboy et al., 1991). Studies of the expression of the I(1)P synthase gene and the transport of phosphorus in seeds will provide a means to genetically engineer plants with a low phytin content.

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