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Molecular Cloning and Characterization of cDNAs Encoding Two Isoforms of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activase in Rice (*Oryza sativa* L.)

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Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activase catalyzes the activation of RuBisCO *in vivo*. Two full length cDNAs designated as *OsrcaA1* and *OsrcaA2* encoding two RuBisCO activase isoforms of 47 and 43 kDa, respectively, have been cloned and characterized. The two isoforms were 99% identical, the 47 kDa isoform having an additional 33 amino acids and a 5 amino acid substitution at the carboxyl terminus. The deduced amino acid sequences of *OsrcaA1* and *OsrcaA2* showed 73–89% identity with RuBisCO activase from other higher plants. Two highly conserved ATP binding sites were identified. The *Osrca* mRNAs, and the RuBisCO activase proteins of 43 and 47 kDa were specifically detected in leaf, but not in root or etiolated seedling tissues. During leaf development, the abundance of *Osrca* mRNAs increased from the 7th to the 3rd leaf, and reached a maximum in the 2nd leaf, although the amounts of the 43 and 47 kDa RuBisCO activase remained almost unchanged among the six leaves, indicating the involvement of post-transcription control in the regulation of RuBisCO activase expression in rice. The co-immunoprecipitation of RuBisCO LSU and SSU with RuBisCO activase suggests that RuBisCO activase interacts with RuBisCO *in vivo*.

Key words: rice, RuBisCO, RuBisCO activase.

The photosynthetic assimilation of CO_2 is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39). RuBisCO must be activated before it exhibits its catalytic activity, and this activation requires the ATP-dependent enzyme RuBisCO activase (*1*). RuBisCO activase was first discovered on analysis of a mutant of *Arabidopsis* that could grow only with an elevated CO_2 concentration (*2*). Subsequent analysis showed that the RuBisCO activase protein is present in all higher plants examined (*3*). RuBisCO activase changes the activity of RuBisCO by removing the otherwise inhibitory sugar phosphates, ribulose bisphosphate and, in some plants, 2-carboxyarabinitol-1-phosphate (*4*). RuBisCO activase is nuclear-encoded, synthesized in the cytosol as a precursor and processed into a mature polypeptide during import to the chloroplast stroma (*4*). RuBisCO activase cDNA clones have been isolated from many higher plants and green algae (*5–15*). In most plants examined to date, there are 2 isoforms of RuBisCO activase arising through alternative pre-mRNA splicing. Both isoforms catalyze the activation of RuBisCO and ATP hydrolysis *in vitro* (*16*). Recently, it was found that the larger isoform is more thermostable (*17*), and plays a unique role in the regulation of RuBisCO activity (*18*).

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Abbreviations: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; LSU, large subunit; SSU, small subunit; *Osrca*, *Oryza sativa* RuBisCO activase; GA, gibberellin.

In our previous study, a gibberellin (GA)-binding protein with a M_r of 47 kDa and a pI of 5.1 was identified in rice by means of a ligand-binding assay (*19*). Partial amino acid sequences of the purified GA-binding protein were determined from both the N-terminal and internal regions. A sequence homology search indicated that the amino acid sequence of the GA-binding protein was homologous to that of RuBisCO activase. Two proteins of 43 and 47 kDa cross-reacted with an antibody raised against the 47 kDa GA-binding protein (*19*). In this paper, the characterization of the cDNAs encoding two RuBisCO activase isoforms of 43 and 47 kDa in rice is described. The expression of the gene was analyzed at the mRNA and protein levels. Immunoprecipitation analysis showed that both the RuBisCO large subunit (LSU) and small subunit (SSU) co-immunoprecipitated with RuBisCO activase.

MATERIALS AND METHODS

Plant Material and Growth Conditions—Rice (*Oryza sativa* L. cv. Nipponbare) seedlings were grown in a growth chamber with 12 h of light and 12 h of darkness, and etiolated seedlings were grown in continuous darkness at 25°C for 2 weeks.

cDNA Library Construction and Screening—Total RNA was isolated from rice leaves by the method of Chomczynski and Sacchi (*20*). The mRNA was isolated from total RNA using a Quick Prep Micro mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). A cDNA library was constructed from mRNA using a λZAP II cDNA cloning kit according to the manufacturer's instructions (Strat-

agene, La Jolla, CA, USA). The probe for screening cDNAs encoding RuBisCO activase was prepared by reverse transcriptase–PCR (RT-PCR) using a StrataScript™ RT-PCR kit (Stratagene). Degenerate primers were designed based on the amino acid sequence of the 47 kDa GA-binding protein, which was characterized as RuBisCO activase (*19*). A sense primer corresponding to the amino acid sequence DEGKQTDQ was designed: 5′-GAY GAR GGN AAR CAR CAN GAY CAR-3′. An antisense primer corresponding to the amino acid sequence GNAGEPAK was used: 5′-YTT NGC NGG YTC NCC NGC RTT NCC-3′. The RT-PCR was performed for 30 cycles of 95°C for 1 min, 54°C for 2 min, and 72°C for 3 min. The PCR product was subcloned into the pT7 Blue T-vector and confirmed by nucleotide sequencing. Then a cDNA fragment of 388 bp was cut out from the PCR product with *Sac*I, labeled with digoxigenin-dUTP by random priming (Boehringer Mannheim, Mannheim, Germany), and then used as a probe to screen the cDNA library. Hybridization was performed at 47°C in a solution containing 50% formamide, 5 × SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 2% blocking reagent. Positive plaques were excised as pBluescript clones according to the manufacturer's instructions (Stratagene). Serial deletion constructs were generated using a kilo-deletion kit (TaKaRa, Osaka), and the cDNA clones were sequenced for both strands using a dideoxy dye terminator (Perkin-Elmer, Foster City, CA, USA). DNA sequences were analyzed using the GCG software package (Genetics Computer Group, Madison, WI, USA) and GENETYX-MAC 10.1 (Software Development, Tokyo).

Southern and Northern Blot Analyses—Rice genomic DNA was digested with restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, or *Hin*dIII), and then electrophoresed in 0.8% agarose gels. Total RNA was isolated from leaf blade, leaf sheath, root or etiolated seedling tissues, and then electrophoresed in 1.2% formaldehyde denatured agarose gels. DNA and total RNA were blotted onto positively charged nylon membranes (Boehringer Mannheim) and then hybridized with two digoxigenin-labeled cDNA fragments, one of 388-bp and the other of 464-bp cut out from the 3′ end of a cDNA (*OsrcaA1*) with *Xho*I. Hybridization was carried out under the same conditions as used for cDNA library screening.

Protein Extraction—All procedures were conducted at 4°C. A portion of leaf blade (50 mg), leaf sheath (100 mg), or etiolated (200 mg) tissues was homogenized in 1 ml extraction buffer containing 50 mM Tris-HCl (pH 7.8), 250 mM sucrose, 10 mM EGTA, 1 mM PMSF, and 1 mM DTT. The homogenate was centrifuged at 15,000 ×*g* for 10 min. The supernatant was used as the protein extract. To extract root proteins, root segments (500 mg) were homogenized in 1 ml extraction buffer. The homogenate was centrifuged at 15,000 ×*g* for 10 min, and the supernatant was precipitated with 10% trichloroacetic acid on ice for 20 min. Samples were then centrifuged at 15,000 ×*g* for 10 min, and the pellet was washed twice with extraction buffer and resuspended in 50 μl extraction buffer. The protein concentration was measured using a protein assay kit (Bio-Rad, Richmond, CA, USA) with BSA as a standard.

Western Blot Analysis—Protein extracts were separated by SDS-PAGE. The gels were either stained with Coomassie Brilliant Blue (CBB) or electroblotted onto polyvinylidene difluoride (PVDF) membranes (Pall BioSupport Division, Port Washington, NY, USA). The blots were cross-reacted with the antibodies raised against the 47 kDa GA-binding protein, RuBisCO LSU or SSU, and then visualized by incubation with horseradish peroxidase–conjugated goat anti–rabbit IgG followed by color development with 4-chloro-1-naphthol (Bio-Rad).

Immunoprecipitation—A protein extract (100 μl) prepared from rice leaves was incubated with 20 μl antibody raised against the 47 kDa GA-binding protein for 1 h at 4°C, and then with protein A-Sepharose (Sigma, MO, USA) for 1 h. The mixture was centrifuged at 15,000 ×*g* for 30 s, and then the pellet was washed 3 times with the buffer containing 50 mM Tris-HCl (pH 7.8), 250 mM sucrose, and 0.1% Triton X-100. Then the pellet was resuspended in 20 μl SDS sample buffer, boiled for 5 min and centrifuged at 15,000 ×*g* for 30 s. The supernatant was subjected to SDS-PAGE.

N-Terminal Amino Acid Sequencing—The proteins were subjected to SDS-PAGE, electroblotted onto PVDF membranes and then visualized by CBB staining. The protein was cut out, and the N-terminal amino acid sequence was determined by automated Edman degradation with a protein sequencer (model 492, Applied Biosystems).

Preparation of Polyclonal Antibodies—The proteins extracted from rice leaves were separated by two dimensional (2D)-PAGE (*21*), and then RuBisCO LSU and SSU were electroeluted from the 2D-PAGE gels. The protein solutions were dialyzed against deionized water for 2 d and then dried. Polyclonal antibodies, recognizing RuBisCO LSU and SSU, were raised in adult rabbits by the method of Bailey (*22*).

RESULTS

Isolation of cDNA Clones Encoding RuBisCO Activase—In our previous study, a GA-binding protein candidate was identified in rice (*19*). To clone genes encoding the GA-binding protein, degenerate primers were designed based on the amino acid sequences of the protein, which is homologous to RuBisCO activase, and were used to generate a hybridization probe by RT-PCR. The RCR product was used to screen a λ ZAP II cDNA library constructed from rice leaf mRNA. Seven positive λ clones were obtained and excised as pBluescript clones. The cDNA clones were named *Osrca*. The insert size of the clones was compared by digestion with restriction enzymes (*Bam*HI, *Pst*I, *Sac*I, *Acc*I, and *Apa*I), and the 5′ and 3′ ends of the longest clones, that ranged in size from 1.6 to 1.7 kb, were compared by sequencing. Based on the results, these clones were classified into two groups and two distinct cDNAs representative of each group were sequenced on both DNA strands. Two of these cDNAs, designated as *OsrcaA1* and *OsrcaA2* (GenBank accession numbers AB034698 and AB034748, respectively), are described. The two cDNAs differ in sequence in the inclusion of an 85-bp insert in *OsrcaA2* (Fig. 1, double lined). The coding sequences of *OsrcaA1* and *OsrcaA2* are GC-rich, *i.e.* about 59%, which is characteristic of many monocot genes (*23*). The deduced amino acid sequences of *OsrcaA1* and *OsrcaA2* are 99% identical, the larger isoform having an additional 33 amino acids and a 5 amino acid substitution at the carboxyl terminus (Fig. 1). A cDNA (*rcaII*) encoding a RuBisCO activase has been isolated from rice (*24*), and the deduced amino acid sequences of *OsrcaA2*

and *rcaII* are nearly completely homologous, *OsrcaA2* having one different (Ala^{127} instead of Ser^{127} in *rcaII*) and one additional (Ala^{190}) amino acid (Fig. 1, indicated by open triangles). The N-terminal residue was identical to Phe^{87} of the deduced amino acid sequence of *rcaII* (*24*), but, judging from our results (*19*), the transit peptide cleavage site lies

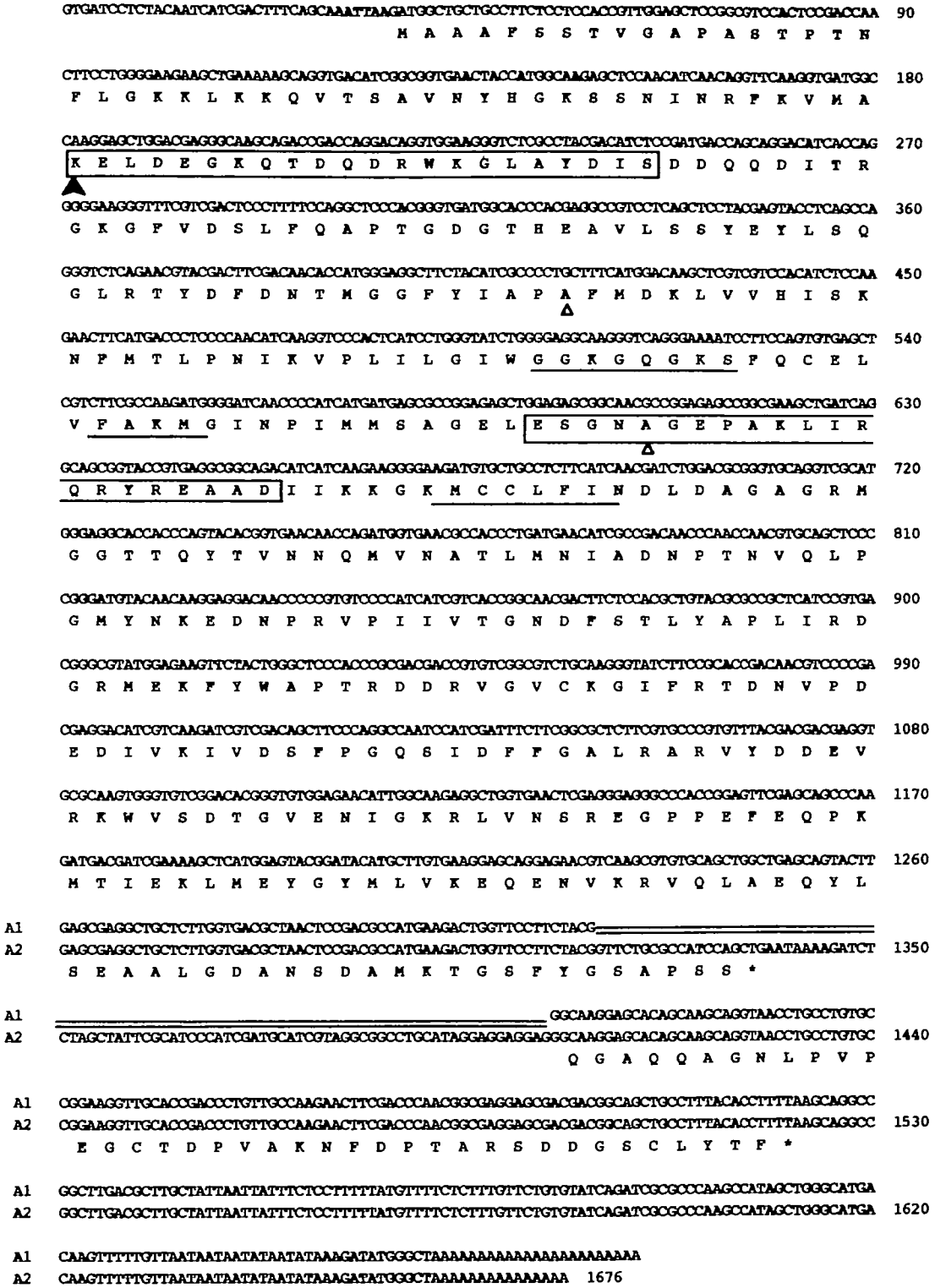

Fig. 1. **Nucleotide and predicted amino acid sequences of *OsrcaA1* and *OsrcaA2*.** The peptides corresponding to the sequences of the purified 47 kDa GA-binding protein are boxed. An arrowhead indicates the N-terminal residue. Lines indicate consensus ATP-binding sites. The double line indicates the 85-bp insert in *OsrcaA2*. Open triangles indicate the different amino acid residues deduced from *OsrcaA2* and *rcaII* (*24*). Asterisks indicate the stop codon. The accession Nos. for *OsrcaA1* and *OsrcaA2* are AB034698 and AB034748, respectively.

between Ala[48] and Lys[49] (Fig. 1). Therefore, the mature proteins encoded by *OsrcaA1* and *OsrcaA2* were calculated to be 46.5 and 42.9 kDa, respectively, which is in good agreement with our estimates on SDS-PAGE (47 and 43 kDa, respectively) (*19*). This is not consistent with that of To *et al.* (*24*), in that two RuBisCO activase polypeptides (RCAI and RCAII) were calculated to be 45 and 41 kDa, respectively. The transit peptide containing 48 amino acid residues encoded by *OsrcaA* (*A1* and *A2*) and barley *rcaA* are quite similar (*6*). Thirty-nine of the first amino acid residues are the same. This sequence is followed by one of little amino acid sequence similarity consisting of 6 residues. Finally, a conserved tripeptide sequence of Val-Met-Ala immediately proceeds the putative transit peptide cleavage site. Similar cleavage patterns were found for spinach and *Arabidopsis* (*5*). Two conserved ATP-binding sites, GGKG-QGKS (amino acid positions 157–164) and GKMCCLFIND (amino acid positions 212–221), were identified (Fig. 1), which is consistent with the ATP requirement for RuBisCO activase–mediated activation of RuBisCO *in vitro* (*25*). Two isoforms of rice RuBisCO activase exhibit 73–89% identity to the RuBisCO activase from higher plants including spinach and *Arabidopsis* (*5*), barley (*6*), cucumber (*7*), tobacco (*8*), apple (*9*), *Phaseolus vulgaris* (*10*), tomato (*11*), maize (*12*), and *Datisca glomerata* (*13*). The rice RuBisCO activase proteins are more closely related to those from a monocot plant, barley (86–89% identity), and also show about 62–63% identity to RuBisCO activase proteins from green algae including *Chlamydomonas* (*14*) and *Anabaena* (*15*).

Genome Analysis—Rice genomic DNA was digested with restriction enzymes whose recognition sites are not located in the coding regions of *OsrcaA1* and *OsrcaA2*, and Southern blot analysis was performed with two digoxigenin-labeled cDNA fragments as probes (Fig. 2). After *Eco*RV digestion, two bands corresponding to 8.8 and 6.0 kb hybridized to probe A, and a 6.0 kb band hybridized to probe B. With *Hin*dIII digestion, two bands, a strongly hybridizing one (14 kb) and a weakly hybridizing one (8.4 kb), were detected by probe B, but only a 14 kb band hybridized to probe A. On digestion of genomic DNA with *Bam*HI, two DNA bands were found to hybridize with both probe A (20 and 4.0 kb) and probe B (20 and 5.2 kb). This suggests that more than one *rca* gene is present in the rice genome.

Expression of RuBisCO Activase in Rice—Northern and Western blot analyses were performed to determine the patterns of accumulation of the mRNAs and proteins in various tissues (Fig. 3). Both RuBisCO activase mRNAs and proteins were highly expressed in leaf blade and sheath, but not in root tissues (Fig. 3A). The major band of *Osrca* mRNAs was estimated to correspond to about 1,700 nucleotides long (Fig. 3), a value that is consistent with the size of cDNAs (*OsrcaA1* and *OsrcaA2*). An additional faint band corresponding to about 3,500 nucleotides was also detected for leaf blade tissue (Fig. 3A). Two proteins of 43 and 47 kDa cross-reacted with the antibody raised against the 47 kDa GA-binding protein (Fig. 3A). The relative amounts of the two isoforms of RuBisCO activase are the same in leaf blade and sheath tissues. In contrast to the strong signals observed for light-grown seedlings (Fig. 3A, lanes S and L), RuBisCO activase mRNAs and proteins cannot be detected in dark-grown seedlings (Fig. 3A, lane E), This

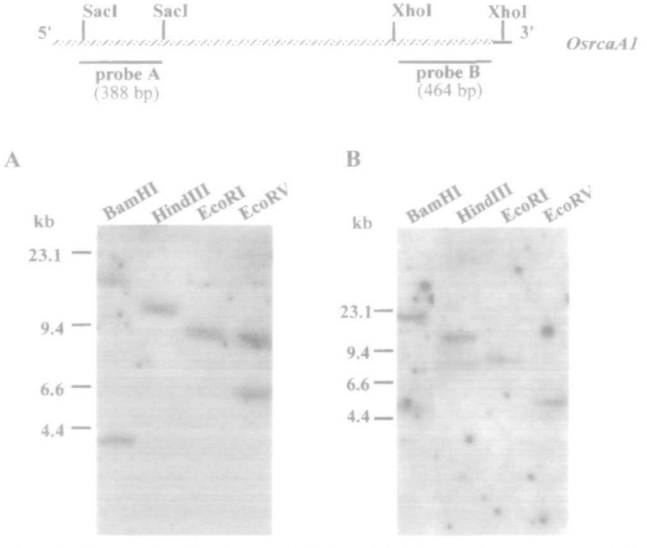

Fig. 2. **Genomic Southern blot analysis of the *rca* gene.** Rice genomic DNA was digested completely with *Bam*HI, *Hin*dIII, *Eco*RI, or *Eco*RV, separated by 0.8% agarose electrophoresis, and then blotted onto positively charged nylon membranes. The blots were hybridized to two partial cDNA fragments of 388-bp probe A (A) or 464-bp probe B (B), as indicated. Molecular size markers are indicated on the left.

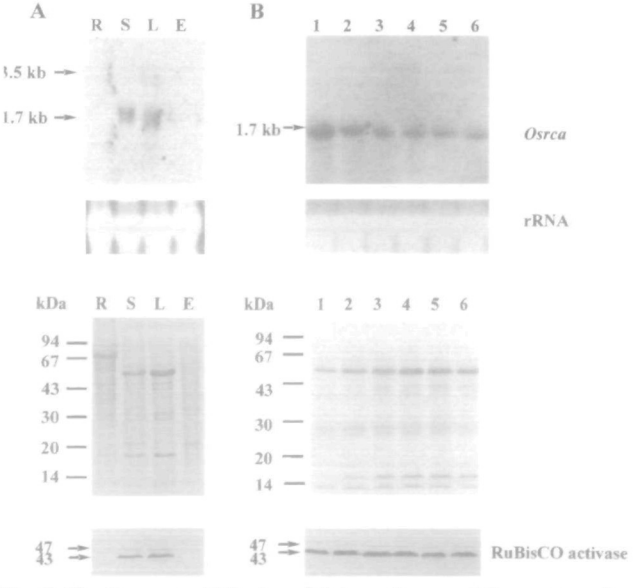

Fig. 3. **Northern and Western blot analyses of the expression of RuBisCO activase in rice.** (A) Tissue-specific expression of RuBisCO activase. (B) Expression of RuBisCO activase in different developed leaf tissues. Total RNA and soluble protein were extracted from leaf blade (lane L), sheath (lane S), root (lane R), or etiolated seedling (lane E) tissue, or the 2nd, 3rd, 4th, 5th, 6th, or 7th leaf blades of 30-d-old rice seedlings (lanes 1, 2, 3, 4, 5, and 6). RNA (30 μg) was probed with the digoxigenin-labeled 388-bp cDNA fragment. Staining with ethidium bromide is shown in the rRNA panel. For Western blot analysis, soluble proteins were separated by SDS-PAGE. Gels were blotted onto PVDF membranes and then probed with antibodies raised against the 47 kDa GA-binding protein. Extracts were assayed on the basis of an equal amount of soluble protein (A) or equal fresh weight of starting tissue (B). Staining with CBB is shown in panel.

shows that RuBisCO activase is specifically expressed in the photosynthetic tissues.

To investigate the expression of *Osrca* in different developed leaf tissues, 30-d-old rice seedlings, which consist of seven leaves, were used. Because the first leaves were dead, total RNA and protein were extracted from the 2nd to 7th leaf blades (base to top). The accumulation of *Osrca* mRNAs increases from the 7th to the 3rd leaf, and reaches a maximum in the 2nd leaf. In contrast, the amounts of the 43 and 47 kDa RuBisCO activase are almost the same among the six leaves (Fig. 3B).

Co-Immunoprecipitation of RuBisCO LSU and SSU with RuBisCO Activase—Immunoprecipitation analysis was performed to identify proteins interacting with RuBisCO activase *in vivo*. Antibodies raised against the 47 kDa GA-binding protein were added to the protein extract. The immunoprecipitated proteins were separated by centrifugation with protein A–Sepharose and then analyzed by SDS-PAGE. Two major proteins of 49 kDa (pp49) and 16 kDa (pp16) were co-immunoprecipitated with RuBisCO activase (Fig. 4). The N-terminal amino acid sequences of the 16 and 49 kDa proteins show homology to those of RuBisCO SSU (*26*) and LSU (*27*) from rice, respectively (Fig. 4C). Two proteins of 16 and 49 kDa cross-reacted with the antibodies raised against RuBisCO SSU and LSU, respectively (Fig. 4B). The results show that both RuBisCO SSU and LSU co-immunoprecipitated with RuBisCO activase.

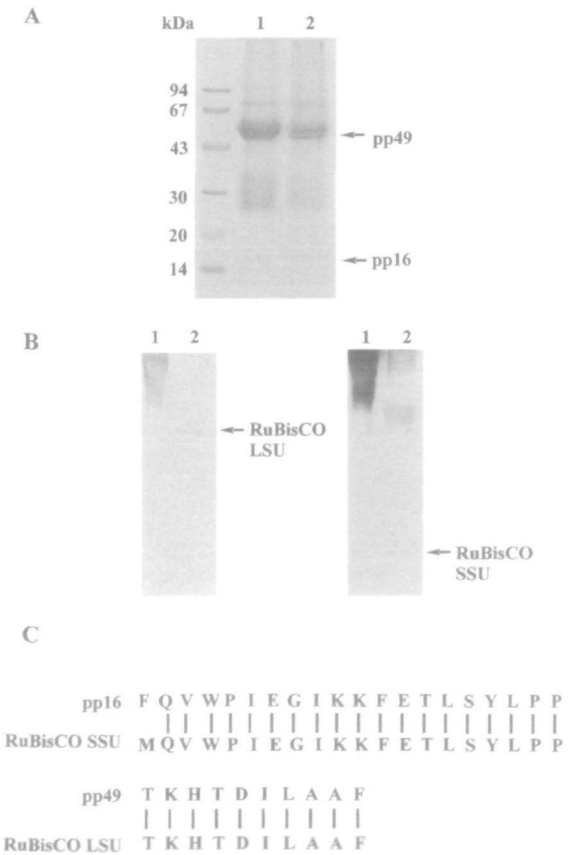

Fig. 4. **Co-immunoprecipitation of pp49 and pp16 associated with RuBisCO activase.** Antibodies raised against the 47 kDa GA-binding protein were added to a protein extract prepared from rice leaves (lane 2), or to extraction buffer saved as a control (lane 1). (A) Immunoprecipitated proteins were analyzed by SDS-PAGE and stained with CBB. (B) The gels were blotted onto PVDF membranes and then probed with antibodies against RuBisCO LSU or SSU. (C) The N-terminal amino acid sequences of pp16 and pp49 are homologous to those of RuBisCO SSU (*27*) and LSU (*26*), respectively.

DISCUSSION

RuBisCO activase is conserved in photosynthetic organisms across a wide range of genera, including lower eukaryotes. Two isoforms of RuBisCO activase with molecular weights of 43 and 47 kDa were found in rice, and two full-length cDNAs encoding the two isoforms were isolated and characterized in this study. It is evident that the two isoforms of RuBisCO activase arose through differential splicing of a common pre-mRNA in spinach, *Arabidopsis* and barley (*5*, *6*). In these plants, the last intron of the *rca* gene can be excised in two ways, including or excluding a 10-, 22-, and 50- bp insert for spinach, *Arabidopsis* and barley, respectively. The two cDNAs of *OsrcaA1* and *OsrcaA2* encoding the isoforms of RuBisCO activase differ in sequence in the inclusion of 85 bp in *OsrcaA2*, which results in the insertion of an in-frame termination codon (Fig. 1). As a consequence, *OsrcaA2* encodes 433 amino acids; and *OsrcaA1* encodes 466 amino acids, *i.e.* there are an additional 33 amino acids and a 5 amino acid substitution at the carboxyl terminus (Fig. 1), similar to that found in spinach, *Arabidopsis* and barley (*5*, *6*). These results suggest that the two cDNAs of *OsrcaA1* and *OsrcaA2* might also be generated through an alternative splicing mechanism. Recently, two different 3′ ends of *rca* mRNA were detected in rice on RT-PCR, and a partial genomic DNA fragment has also been cloned. Sequence comparison showed that an alternative splicing mechanism for a common *rca* mRNA precursor near the 3′ end exists in rice (*24*). Comparing the sequences of *OsrcaA1* and *OsrcaA2* with that of the partial genomic DNA (*24*), we concluded that the two mRNAs of *OsrcaA1* and *OsrcaA2* are generated from a common *Osrca* mRNA through alternative splicing as described by To *et al.* (*24*). On Northern blot analysis, two mRNAs of 1,700 and 3,500 nucleotides were detected (Fig. 3A). Consistent with the sizes of the cDNA clones, we concluded that the 1,700-nucleotide mRNA represented the mature message, and might contain the two mRNAs of *OsrcaA1* and *OsrcaA2*. The 3,500-nucleotide (Fig. 3A), whose level was very low and which could not be consistently detected, may be the pre-mRNA.

Genomic Southern blot analysis suggested that more than one *rca* gene is present in rice. Our result is not consistent with that of To *et al.* (*24*), in that only one band could be detected for *Eco*RI and *Hin*dIII digests. We notice that the bands detected for *Eco*RI and *Hin*dIII digests ranged from 4.4 to 23.1 kb in size (*24*), so it is difficult to conclude that there was only one band. In another monocotyledon, barley, RuBisCO activase proteins are encoded by two closely linked, tandemly oriented nuclear genes (*rcaA* and *rcaB*). *rcaA* produces two mRNAs, which encode polypeptides of 42 and 46 kDa through alternative splicing mechanism. *rcaB* is transcribed to a single mRNA, which encodes a mature peptide of 42 kDa. The *rcaA* mRNAs are 20–100-fold more abundant than *rcaB* mRNA, and among the 27 barley *rca* cDNA clones isolated, *rcaA*s sequences accounted for 25 clones, while the *rcaB* class was represented by two cDNA clones (*6*). We confirmed that the 7

Osrca cDNA clones isolated were generated from one gene through an alternative splicing mechanism. But, judging from the Southern blot results, we suggest that an addition genome gene and a third class mRNA are present in rice. This conclusion will be verified through the analysis of genomic clones and more cDNA clones.

As expected for a protein that functions in the photosynthetic carbon reduction cycle, the expression of RuBisCO activase in rice was found to be tissue-specific. Both *rca* mRNAs and proteins were predominantly detected in leaf blades and sheaths, the photosynthetic tissues, but were not detected in root tissue or etiolated seedlings (Fig. 3A). A light-inducible element of the *rca* promoter has been identified in spinach (*28*) and *Arabidopsis* (*29*). Although the effect of illumination of etiolated seedlings on the expression of RuBisCO activase in rice was not examined, it is reasonable to assume that the expression of *rca* in rice is regulated by light. The sizes of the two mRNAs encoding the 43 and 47 kDa RuBisCO activase are very close, thus they cannot be distinguished on Northern blot assaying with the 388-bp cDNA fragment as a probe (Fig. 3). But we noted that two RuBisCO activase proteins are present in different tissues (leaf blade and sheath) or different developed leaves in the same relative amounts, and the 43 kDa isoform was more immunologically stained than the 47 kDa isoform (Fig. 3). This suggests that either alternative splicing favors the production of *OsrcaA2* mRNA, or that the *OsrcaA2* mRNA can be preferentially translated. The accumulation of *OsrcaA* mRNA increased from the 7th to the 2nd leaf, in contrast, the amounts of the 43 and 47 kDa RuBisCO activase proteins showed no obvious differences among the six leaves (Fig. 3B), suggesting post-transcription control is involved in the regulation of RuBisCO activase expression in rice. Further experiments will be performed to analyze the accumulation pattern of the different mRNAs in different tissues and during leaf development using gene specific probes.

RuBisCO activase was considered to belong to the AAA^+ class, the proteins of this class often performing chaperone-like functions that assist in the assembly, operation or disassembly of protein complexes (*30*). There is evidence that RuBisCO activase may function as a molecular chaperone rather than a conventional enzyme in the activation of RuBisCO (*30, 31*). RuBisCO is conformed by LSU arranged as an octameric core and SSU in two layers of four, on opposite sides of the molecule (*32*). Both RuBisCO LSU and SSU were co-immunoprecipitated with the antibody against the GA-binding protein, which was identified as RuBisCO activase (Fig. 4), suggesting that RuBisCO activase associates with RuBisCO *in vivo* and may be involved in the assembly or protection of the holoenzyme RuBisCO. Further experiments are needed to confirm this and to clarify the mechanism of the interaction between the RuBisCO and RuBisCO activase.

RuBisCO activase is conserved in photosynthetic organisms and has no reported alternative functions. The two RuBisCO activases examined to date differ only in their carboxyl terminals, but recent evidence showed that the two isoforms play different roles in the regulation of RuBisCO activity (*18*). Although both isoforms can catalyze the activation of RuBisCO and ATP hydrolysis *in vitro* (*16*), the larger isoform exhibited minimal ATP hydrolysis and RuBisCO activation activity in comparison with the 43 kDa isoform with physiological ratios of ADP/ATP (*18*). Our early work showed that only the 47 kDa isoform has GA-binding activity (*19*). The amount of the protein was not affected by GA treatment (date not shown), but the 47 kDa protein was specificly phosphorylated with Ca^{2+}, Mg^{2+}, and ATP on GA treatment (*19*). Analysis of the function of the 47 kDa GA-binding protein in the GA signal transduction pathway is in progress.

Z. Zhang is a Japan Science and Technology Agency Fellow from the China Agricultural University in P.R. China.

REFERENCES

1. Portis, A.R., Jr., Salvucci, M.E., and Ogren, W.L. (1986) Activation of ribulosebisphosphate carboxylase/oxygenase at physiological CO_2 and ribulosebisphosphate concentrations by rubisco activase. *Plant Physiol.* **82**, 967–971
2. Somerville, C.R., Portis, A.R., Jr., and Ogren, W.L. (1982) A mutant of *Arabidopsis thaliana* which lacks activation of RuBP carboxylase *in vivo*. *Plant Physiol.* **70**, 381–387
3. Salvucci, M.E., Werneke, J.M., Ogren, W.L., and Portis, A.R., Jr. (1987) Purification and species distribution of rubisco activase. *Plant Physiol.* **84**, 930–936
4. Portis, A.R., Jr. (1990) Rubisco activase. *Biochim. Biophys. Acta* **1015**, 15–28
5. Werneke, J.M., Chatfield, J.M., and Ogren, W.L. (1989) Alternative mRNA splicing generates the two ribulosebisphosphate carboxylase/oxygenase activase polypeptides in spinach and *Arabidopsis*. *Plant Cell* **1**, 815–825
6. Rundle, S.J. and Zielinski, R.E. (1991) Organization and expression of two tandemly oriented genes encoding ribulosebisphosphate carboxylase/oxygenase activase in barley. *J. Biol. Chem.* **266**, 4677–4685
7. Preisig-Muller, R. and Kindl, H. (1992) Sequence analysis of cucumber cotyledon ribulosebisphosphate carboxylase/oxygenase activase cDNA. *Biochim. Biophys. Acta* **1171**, 205–206
8. Wang, Z.Y., Snyder, G.W., Esau, B.D., Portis, A.R., Jr., and Ogren, W.L. (1992) Species-dependent variation in the interaction of substrate-bound ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) and rubisco activase. *Plant Physiol.* **100**, 1858–1862
9. Watillon, B., Kettmann, R., Boxus, P., and Burny, A. (1993) Developmental and circadian pattern of rubisco activase mRNA accumulation in apple plants. *Plant Mol. Biol.* **23**, 501–509
10. Ryan, J.E., Andralojc, P.J., Willis, A.C., Gutteridge, S., and Parry, M.A.J. (1998) Cloning and sequencing of a *Phaseolus vulgaris* cDNA (Accession No. AF041068) encoding rubisco activase (PGR98-044). *Plant Physiol.* **116**, 1192
11. Zhu, Y., Lin, K.R., Huang, Y., Tauer, C.G., and Martin, B. (1998) A cDNA from tomato (*Lycopersicon pennellii*) encoding ribulose-1,5-bisphosphate carboxylase/oxygenase activase (Accession No. AF037361) (PGR98-053). *Plant Physiol.* **116**, 1603
12. Avala-Ochoa, A., Loza-Tavera, H., and Sanchez de Jimenez, E. (1998) A cDNA from maize encoding ribulose-1,5-bisphosphate carboxylase/oxygenase activase (Accesion No. AF084478) (PGR 98-207). *Plant Physiol.* **118**, 1535
13. Okubara, P.A., Pawlowski, K., Murphy, T.M., and Berry, A.M. (1999) Symbiotic root nodules of the actinorhizal plant *Datisca glomerata* express Rubisco activase mRNA. *Plant Physiol.* **120**, 411–420
14. Roesler, K.R. and Ogren, W.L. (1990) Primary structure of *Chlamydomonas reinhardtii* ribulose 1,5-bisphosphate carboxylase/oxygenase activase and evidence for a single polypeptide. *Plant Physiol.* **94**, 1837–1841
15. Li, L.A., Gibson, J.L., and Tabita, F.R. (1993) The Rubisco activase (*rca*) gene is located downstream from *rbcS* in *Anabaena* sp. strain CA and is detected in other *Anabaena*/*Nostoc* strains. *Plant Mol. Biol.* **21**, 753–764
16. Shen, J.B., Orozco, E.M., Jr., and Ogren, W.L. (1991) Expression

of the two isoforms of spinach ribulose 1,5-bisphosphate carboxylase activase and essentiality of the conserved lysine in the consensus nucleotide-binding domain. *J. Biol. Chem.* **266**, 8963–8968
17. Crafts-Brander, S.J., van de Loo, F.J., and Salvucci, M.E. (1997) The two forms of ribulose-1,5-bisphosphate carboxylase/oxygenase activase differ in sensitivity to elevated temperature. *Plant Physiol.* **114**, 439–444
18. Zhang, N. and Portis, A.R., Jr. (1999) Mechanism of light regulation of Rubisco: a specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f. *Proc. Natl. Acad. Sci. USA* **96**, 9438–9443
19. Komatsu, S., Masuda, T., and Hirano, H. (1996) Rice gibberellin-binding phosphoprotein structurally related to ribulose-1,5-bisphosphate carboxylase/oxygenase activase. *FEBS Lett.* **384**, 167–171
20. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
21. O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021
22. Bailey, G.S. (1985) The production of antisera in *Methods in Molecular Biology* (Walker, J.M., ed.) pp. 295–300, Humana Press, New Jersey
23. Cambell, W.H. and Gowri, G. (1990) Codon usage in higher plants, green algae, and cyanobacteria. *Plant Physiol.* **92**, 1–11
24. To, K.Y., Suen, D.F., and Chen, S.C.G. (1999) Molecular characterization of ribulose-1,5-bisphosphate carboxylase/oxygenase activase in rice leaves. *Planta* **209**, 66–76
25. Streusand, V.J. and Portis, A.R., Jr. (1987) Rubisco activase mediates ATP-dependent activation of rubisco bisphophate carboxylase. *Plant Physiol.* **85**, 152–154
26. Matsuoka, M., Kano-Murakami, Y., Tanaka, Y., Ozeki, Y., and Yamamoto, N. (1988) Classification and nucleotide sequence of cDNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase from rice. *Plant Cell Physiol.* **29**, 1015–1022
27. Hiratsuka, J., Shimada, H., Whittier, R., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C.R., and Meng, B.Y. (1989) The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. *Mol. Gen. Genet.* **217**, 185–194
28. Orozco, B.M. and Ogren, W.L. (1993) Localization of light-inducible and tissue-specific regions of the spinach ribulose bisphosphate carboxylase/oxygenase (rubisco) activase promoter in transgenic tobacco plants. *Plant Mol. Biol.* **23**, 1129–1138
29. Liu, Z., Taub, C.C., and McClung, C.R. (1996) Identification of an *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase/oxygenase activase (*RCA*) minimal promoter regulated by light and the circadian clock. *Plant Physiol.* **112**, 43–51
30. Neuwald, A.F., Aravind, L., Spouge, J.L., and Koonin, E.V. (1999) AAA$^+$: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res.* **9**, 27–43
31. Jimenez, E.S., Medrano, L., and Martinez-Barajas, E. (1995) Rubisco activase, a possible new member of the molecular chaperone family. *Biochemistry* **34**, 2826–2831
32. Chapman, M.S., Suh, S.W., Curmi, P.M., Cascio, D., Smith, W.W., and Eisenberg, D.S. (1988) Tertiary structure of plant RuBisCO: domains and their contacts. *Science* **241**, 71–74