

Molecular Characterization of the *waxy* Locus of Rice (*Oryza sativa*)

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The gene at the *waxy* (*wx*) locus in rice was cloned and the nucleotide sequence of the coding region was determined. The *wx*⁺ gene from rice was composed of a number of exons and introns. The deduced amino acid sequence indicated that the predicted polypeptide is composed of a putative transit peptide of 77 amino acids and a mature protein region of 532 amino acids. The putative transit peptide, which may be essential for transporting the product of the gene at the *wx* locus (*wx*⁺ protein) into the amyloplast, has some characteristic features similar to those of the transit peptide associated with proteins to be transported into the chloroplast. An antiserum against the rice *wx*⁺ protein (anti-WXP serum) was prepared and the expression of the *wx* locus was examined by the Northern blotting analysis using DNA cloned from the rice *wx* locus and by the immunoblotting analysis using the anti-WXP serum. The expression of the *wx*⁺ gene was regulated in a tissue-specific manner, namely, it was expressed only in the endosperm and pollen. The *wx*⁺ gene was active at the early and middle stages and was almost inactive at the late stage of seed development. During active transcription of the gene, the *wx*⁺ protein appears to accumulate linearly.

Key words: Endosperm — *Oryza sativa* — Starch synthesis — Tissue specificity — Transit peptide — *wx* locus.

The *wx* locus controls amylose synthesis in the rice endosperm. The amylose content (relative to the total amount of starch) is about 14–28% in wild-type strains of rice and is genetically regulated in each individual strain. In most *wx* mutants, the starch is composed almost exclusively of amylopectin. The gene product (*wx*⁺ protein) at the *wx* locus was identified to be a protein of 60-kDa, which binds strongly to starch granules (Sano 1984).

The maize *wx* locus has been well characterized with advantages for genetic research, such as the pollen analysis (Nelson 1962, 1987). The same approach is available for studies of the *wx* locus in rice (Li et al. 1968). In addition, the rice *wx* locus provides a unique model for elucidation of regulation at the molecular level, since the expression of the locus is regulated quantitatively depending on the particular alleles at the locus (Sano 1984, Sano et al. 1986) and is responsive to low temperature (Sano et al. 1985). In addition to these characteristic features of the rice *wx* locus, the tissue-specific regulation of gene expression in rice is

also of interest. The level of the *wx*⁺ protein is positively correlated with the amylose content in nonwaxy strains of rice (Sano 1985). Since the amylose content affects the quality of rice grains and its cooking properties, studies of the *wx* locus are not only of interest in understanding gene regulation but they are also important for agricultural applications.

The genes at the *wx* locus have been cloned from maize (Shure et al. 1983, Schwarz-Sommer et al. 1984) and rice (Okagaki and Wessler 1988). The nucleotide sequence of the maize *wx*⁺ gene has been determined (Klößen et al. 1986), but a detailed analysis of gene expression has not yet been reported in either plant. Similarities in the function of the *wx* locus and in the properties of the gene product between rice (Sano 1984) and maize (Shure et al. 1983) suggest that the rice *wx* locus encodes the same enzyme, ADP-glucose starch glycosyl transferase, as that encoded by the maize *wx* locus (Nelson and Rines 1962, Tsai 1974, MacDonald and Preiss 1985). To elucidate the molecular mechanism of the unique gene regulation at the rice *wx* locus, we cloned a *wx*⁺ gene from rice using the *wx*⁺ DNA from maize (Shure et al. 1983) and prepared an antiserum against the rice *wx*⁺ protein (anti-WXP serum). In this article, we report the complete nucleotide sequence of the

Abbreviations: anti-WXP serum, an antiserum against the rice *wx*⁺ protein; DAP, days after pollination; IgG, immunoglobulin G; PCR, polymerase chain reaction; SSC, saline sodium citrate; T65, Taichung 65.

protein-coding region from the rice *wx* locus and the analysis of gene expression with reference to the tissue specificity and the development of seeds.

Materials and Methods

Materials—Rice strains (*Oryza sativa*) used were Taichung 65 (T65; *wx*⁺, Japonica), Peiku (*wx*⁺, Indica), and T65wx (a near-isogenic line that carries the mutant *wx* allele from Kinoshitamochi (Japonica) on the genetic background of T65). The clone of the maize *wx* locus (pWx5) was kindly provided by Dr. N. Fedoroff (Shure et al. 1983) and a 2.1-kb *Sal*I fragment (#2, Wessler and Varagona 1985) of pWx5 was subcloned into pBluescript vector (Stratagene) and used for the initial Southern blotting experiments and screening of plaques.

Cloning and sequencing of the *wx*⁺ gene of rice—General techniques for manipulation of recombinant DNA were carried out as described by Maniatis et al. (1982) or followed the recommendations of commercial suppliers. Genomic DNA was isolated from rice seedlings as described by Hirano et al. (1989). A partial genomic library was constructed using the lambda vector (λDASH, Stratagene) and the genomic DNA isolated from T65. *Hind* III fragments corresponding to a single band (about 15-kb) that hybridized with the maize *wx*⁺ DNA were eluted from the agarose gel, purified by column chromatography on DE52 (Whatman), and used for construction of a genomic library. About 50,000 recombinant phages were screened and more than ten positive clones were identified. Three of them were analyzed by digestion with restriction enzymes and shown to be identical clones. An *Eco*R I fragment of 6-kb in length (Fig. 1) was subcloned into pBluescript (pRWB101) and a deletion series of DNA fragments was constructed. The nucleotide sequence was determined by the dideoxy chain-termination method by following the protocol provided by the suppliers using the Sequenase kit (U.S.B.).

Northern blotting analysis—Seeds were harvested, frozen in liquid N₂ after removal of hulls, and stored at −80°C. Sampling was only carried out once per rice plant, because physiological changes are assumed to occur in the remaining seeds as a result of removal of seeds nearby. The isolation of RNA from seeds was carried out as described by Naito et al. (1988). Northern blotting experiments followed the protocols described by Maniatis et al. (1982). RNA was separated on a 1% agarose gel that contained formaldehyde and transferred to a nylon membrane (Gene Screen Plus, NEN) by the capillary method. Hybridization was carried out with the *Dra* I fragment (Fig. 1) labeled with ³²P under the stringent condition in a solution containing 50% formamide, 10% dextran sulfate, 100 μg μl^{−1} salmon sperm DNA and 1% SDS. The membranes were washed at 65°C for 30 min twice in a solution

of 0.1 × SSC and 1% SDS.

Preparation of antiserum against the rice *wx*⁺ protein

—The *wx*⁺ protein was purified from the mature seeds of Peiku as a major protein bound to starch granule (Sano 1984) by the procedures described by Shure et al. (1983). A polypeptide of about 58-kDa was finally purified on a polyacrylamide gel. A polyclonal antiserum against the purified rice *wx*⁺ protein was raised commercially in a rabbit (IBL, Fujioka, Japan).

Immunoblotting analysis—Sampling for immunoblotting analysis was carried out using the same procedures as those used for Northern analysis. Seeds were ground in a mortar and pestle, and the protein was extracted in a sample buffer [2% SDS, 100 mM dithiothreitol, and 60 mM Tris-HCl (pH 6.8)] by boiling for 5 min. After removal of the pellet by centrifugation (15,000 rpm for 10 min), the extract was used for estimation of protein concentration and immunoblotting analysis as described by Harlow and Lane (1988). Total protein was separated on a 10% polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell). Detection was carried out using peroxidase-labeled anti-rabbit IgG and an ECL detection kit from Amersham.

Results

Cloning of the *wx*⁺ gene from rice—More than ten positive clones were identified by screening of a partial genomic library prepared from rice with the maize *wx*⁺ DNA. Three clones, further analyzed, were shown to generate the same patterns (Fig. 1) by restriction enzyme analysis, indicating that they were identical.

A 2.4-kb transcript was detected in a *wx*⁺ strain (T65) but not in a *wx* strain (T65wx) in the Northern blotting experiment with a cloned rice DNA (Fig. 2). Ambiguous minor bands detected in a *wx* strain may be mutant transcripts as described by Okagaki and Wessler (1988) or due

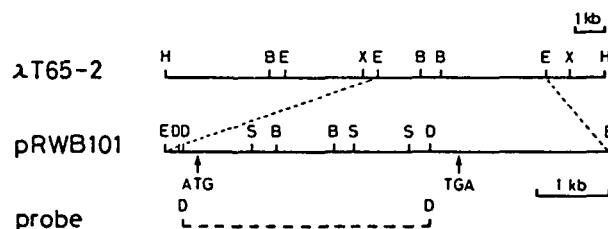


Fig. 1 Physical maps of the recombinant lambda DNA (λT65-2) selected from the partial genomic library and the subcloned DNA (pRWB101) that contained the coding region of the *wx*⁺ gene. The dashed line indicates the *Dra*I fragment used as a probe in Northern blotting analysis. The positions of the initiation (ATG) and termination codons (TGA) are shown. Restriction enzymes: B, *Bam*H I; D, *Dra* I; E, *Eco*R I; H, *Hind* III; S, *Sal* I; X, *Xba* I.

to the mismatched hybridization with the intron DNA contained in a probe because the same bands were occasionally detected in the wild type strain in other Northern blotting analyses (data not shown). Since the presence or absence of a 2.4-kb transcript that hybridized to the rice cloned DNA coincided with the *wx*⁺ or *wx* phenotype of rice plants, the rice DNA, which was selected by use of the maize DNA, should be derived from the *wx* locus of rice. The results of restriction mapping of the rice *wx*⁺ gene, as determined in this study (Fig. 1), agreed closely with those reported previously for another rice strain by Okagaki and Wessler (1988).

Nucleotide sequence of the *wx*⁺ gene of rice—The nucleotide sequence of the coding region of the rice *wx*⁺ gene was determined (Fig. 3). From a comparison with the nucleotide sequence of the maize *wx*⁺ DNA (Klösigen et al. 1986), the structure of the rice *wx*⁺ gene could be predicted (Fig. 3, 4). The rice *wx*⁺ gene was divided into a number of exons with introns as is the maize *wx*⁺ gene. The smallest exon was 64-bp in length and the largest intron was 357-bp in length in the rice gene. The junctions of each intron consisted of 5'-GT and 3'-AG consensus

sequences, which are associated with the splicing (Breathnach and Chambon 1981).

To elucidate the structure of the rice *wx*⁺ gene in the region in which the first intron is located in the case of the maize gene, PCR amplification experiments were carried out using the genomic DNA and poly A⁺ RNA from rice as templates (the primers used are indicated in Figures 3 and 4). Since the DNA fragments of equal length were amplified from both genomic DNA and poly A⁺ RNA (data not shown), the putative "second exon" of the rice *wx*⁺ gene is longer than that of the maize gene. If the putative "first intron" exists in the rice gene, it may be located at a more distant site. The number and position of the other exons and introns of the rice *wx*⁺ gene are the same as those of the maize gene. The 5th and 8th introns of the maize gene are not present in the gene from barley (Rohde et al. 1988).

Deduced amino acid sequence and transit peptide—A long open reading frame was identified in the DNA sequence constructed by combining the exons of the rice *wx*⁺ gene. The initiation and termination codons in the open reading frame of the rice *wx*⁺ gene are located at the same positions as those of the *wx*⁺ genes from maize and barley. The amino acid sequence deduced from this open reading frame indicated that rice *wx*⁺ gene encoded a polypeptide composed of 609 amino acids.

The sequence from the 78th to the 94th amino acid in the deduced amino acid sequence is completely identical with that of the 17 amino acids at the N-terminus of the rice *wx*⁺ protein (Uematsu et al. 1990). This indicates that the predicted polypeptide includes a sequence of 77 amino acids in addition to the mature protein (Fig. 5). The additional peptide may be a transit peptide, which is essential for transporting the protein into the amyloplast. The cleavage site of the precursor protein of rice is at the same position as that of maize (Klösigen et al. 1986). The putative transit peptides of the *wx*⁺ proteins of rice, maize and barley are different in size (77, 72 and 70 residues, respectively) and the extent of sequence homologies among them is lower than that among the mature proteins.

The mature *wx*⁺ protein of rice is composed of 532 amino acids, whereas those of maize and barley are composed of 533 amino acids. The homologies in the amino acid sequences of the mature *wx*⁺ proteins of the three plants are 88% (rice-maize), 86% (rice-barley), 86% (maize-barley). The predicted molecular weight (58.5-kDa) of the mature product of the rice *wx*⁺ gene is consistent with that estimated in a previous study (60-kDa; Sano 1984) and in this study (58-kDa) by immunoblotting analysis (Fig. 7A).

Tissue-specific expression—Northern analysis showed that a 2.4-kb transcript of the *wx* locus in rice was present only in the seed, and not in the leaf, stem, or whole seedling without seeds (Fig. 6). Thus, the *wx*⁺ gene in rice is expressed in a tissue-specific manner.

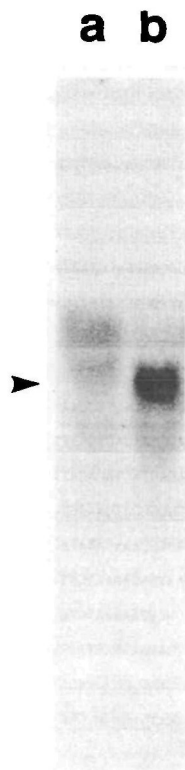


Fig. 2 Northern blotting analysis of the expression of the *wx* locus in *wx*⁺ and *wx* rice strains. The RNA was isolated from seeds on the 11th day after pollination and 10 µg of total RNA were used for analysis. The rice strains used were (a) T65*wx* (*wx*) and (b) T65 (*wx*⁺).

Fig. 3 Nucleotide sequence of the *wx*⁺ gene of rice. The position 1 is the *EcoR* I site used for subcloning. The initiation (ATG) and termination (TGA) codons are indicated by boxes. The putative exons are underlined. The initiation site of the precursor and mature proteins are shown with hooked arrows. The primer sequences used for PCR amplification are indicated by horizontal arrows.

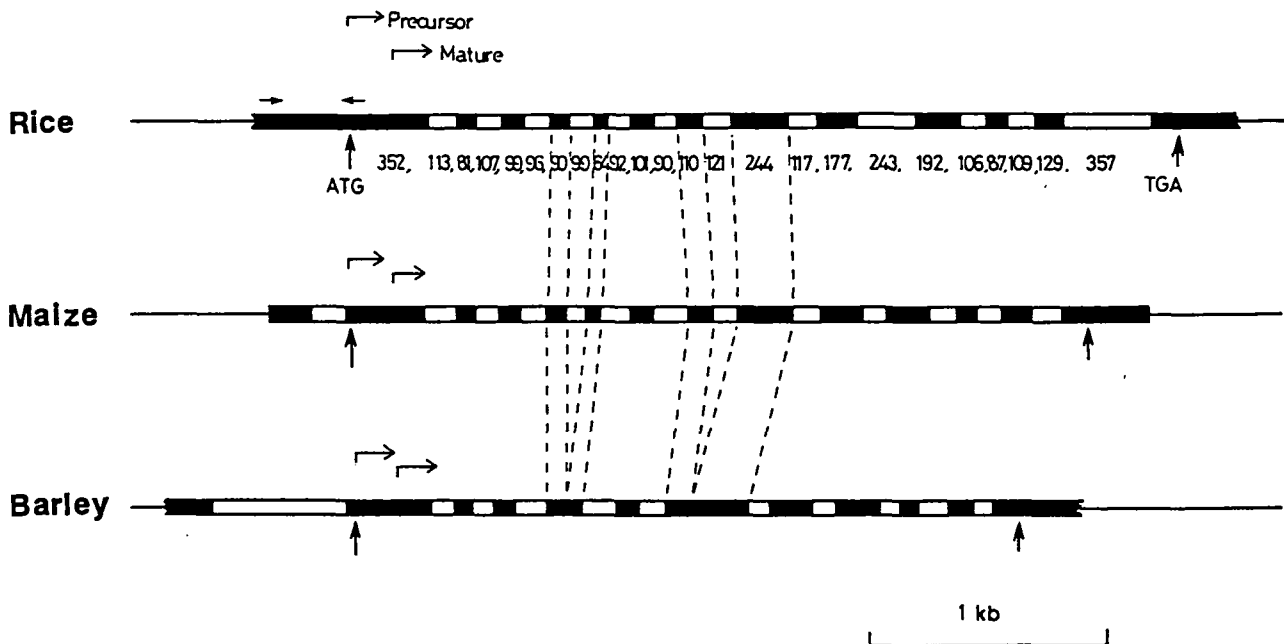


Fig. 4 Comparison of the structures of the *wx*⁺ genes of rice, maize and barley. The exons and introns are indicated as black and white regions, respectively. The numbers indicate the lengths (in bp) of exons and introns in rice *wx*⁺ gene. The small horizontal arrows show the positions of the primers used for PCR amplification.

The anti-WXP serum reacted specifically with the 58-kDa protein in the seed of *wx*⁺ plants, and no band was detected in the case of *wx* seeds (Fig. 7, lanes a and h). In wild-type rice, a faint band was detected at the 58-kDa position in the case of anthers, as well as in the case of seeds (Fig. 7, lane d). Reaction with iodine (I_2/KI) stained only the endosperm and pollen but did not stain other tissues in the seed and anther (data not shown). Therefore, the *wx*⁺ protein may be expressed specifically in the endosperm and

pollen. Since the intensities of the band in the lanes a and d (Fig. 7) are nearly equal, the amount of the *wx*⁺ protein in the pollen was about 2% of that in the endosperm. A few minor bands were observed in the analysis of leaf, stem, and seedling (Fig. 7). Since these bands were detected also in the *wx* rice (data not shown) and their sizes were different from that of the protein in the endosperm and pollen, the detected proteins in the leaf, stem and seedling may not necessarily be products of the *wx* locus.

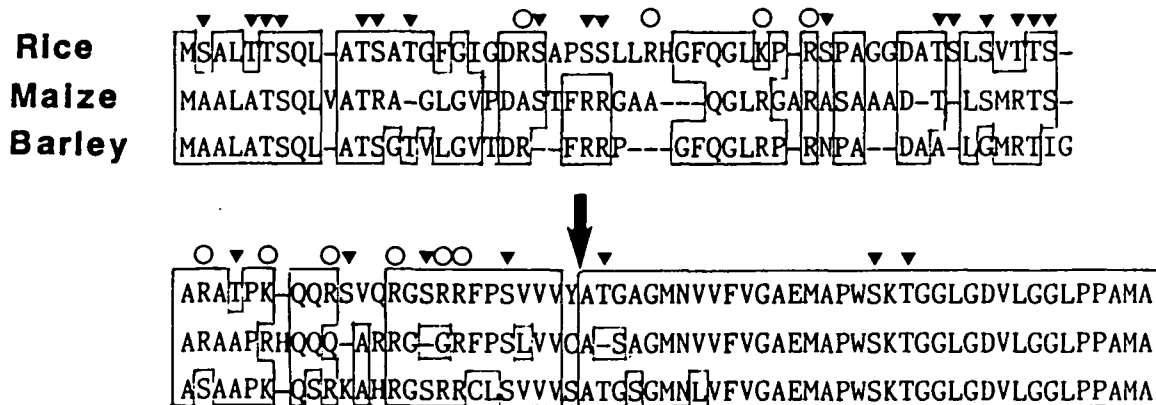


Fig. 5 Deduced amino acid sequences of the transit peptides of the *wx*⁺ precursor proteins of rice, maize and barley. The amino acid sequences of the N-termini of the mature *wx*⁺ proteins of rice and maize were as determined by amino acid sequencing. The cleavage site is indicated by an arrow. Symbols indicate basic (○) and hydroxylated (▼) amino acids rich in the transit peptide.

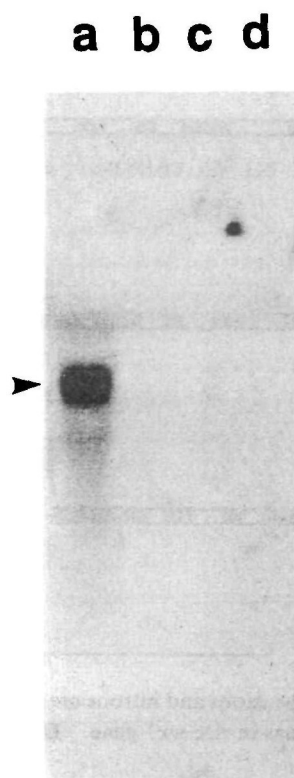


Fig. 6 Northern blotting analysis of tissue specificity. Total RNAs (10 μ g) isolated from seeds, leaves and seedlings of wild-type rice (T65) were analyzed: a, seed; b, leaf; c, stem; d, seedling. The seedlings contained leaf, stem and root, but the seed was removed.

Expression of the wx^+ gene during seed development
—The expression of the wx locus during the development of rice seeds was examined (Fig. 8). The accumulation of the wx^+ transcript and the wx^+ protein was estimated from the intensity of signals on X-ray films and the amounts of total RNA or total protein (Fig. 9). The transcript of the wx^+ gene was already produced at a low level on the 4th day after pollination. The relative level of the wx^+ transcript as a proportion of the total RNA increased until the 13th day (Fig. 8A) and the amount of wx^+ transcript per seed increased rapidly together with the increase in the total amount of RNA (Fig. 9A). The wx^+ transcript was most abundant in seeds in the period from the 13th to the 18th day. After a rapid decrease, the level of the wx^+ transcript was very low on the 23rd day. Thus, the expression at the wx locus is temporally regulated during seed development. The wx^+ protein accumulated linearly from the 4th to the 23rd day. The period during which the level of the wx^+ protein increases in amount corresponds to that during which the wx locus is actively expressed. Abundant wx^+ mRNA from the 13 to the 18th day may be required for the large-scale protein synthesis, namely, the turnover

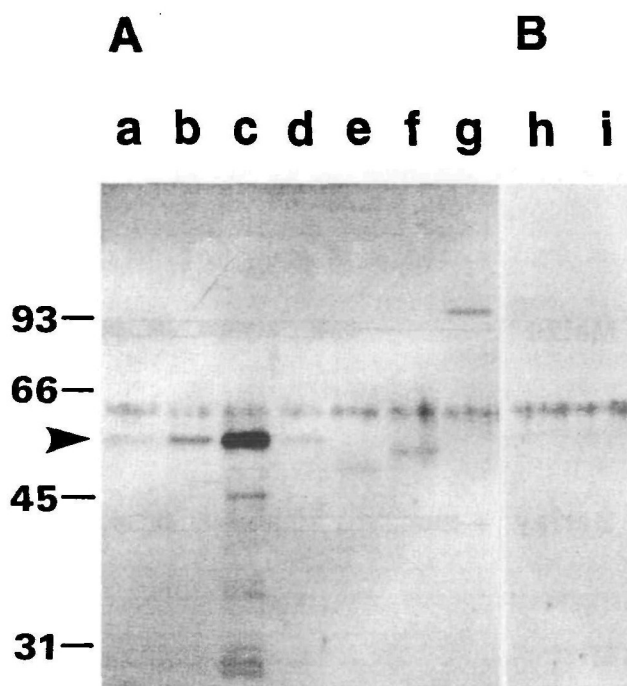


Fig. 7 Immunoblotting analysis of tissue specificity with the anti-WXP serum. Numbers indicated the size of molecular weight standard proteins in kDa. The tissues and the amounts of protein in extracts were as follows. (A) A wild-type rice (T65): a, seed (0.04 μ g); b, seed (0.2 μ g); c, seed (2 μ g); d, anther (2 μ g); e, leaf (2 μ g); f, stem (2 μ g); g, seedling (2 μ g). (B) A mutant rice (T65 wx): h, seed (2 μ g); i, anther (2 μ g).

of the wx^+ protein that has accumulated and the synthesis of the additional wx^+ protein to be newly synthesized.

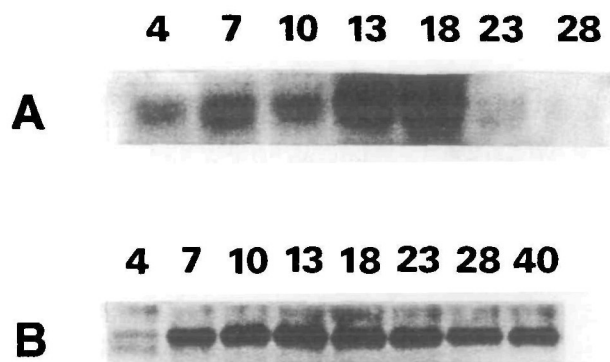


Fig. 8 Expression at the wx locus during seed development. (A) Northern blotting analysis. RNA was isolated from the seeds 4, 7, 10, 13, 18, 23 and 28 days after pollination. Total RNA (10 μ g) was analyzed by Northern blotting. (B) Immunoblotting analysis. Total protein was extracted by boiling from seeds harvested on the days indicated, as described Materials and Methods. Two micrograms of protein was used for immunoblotting.

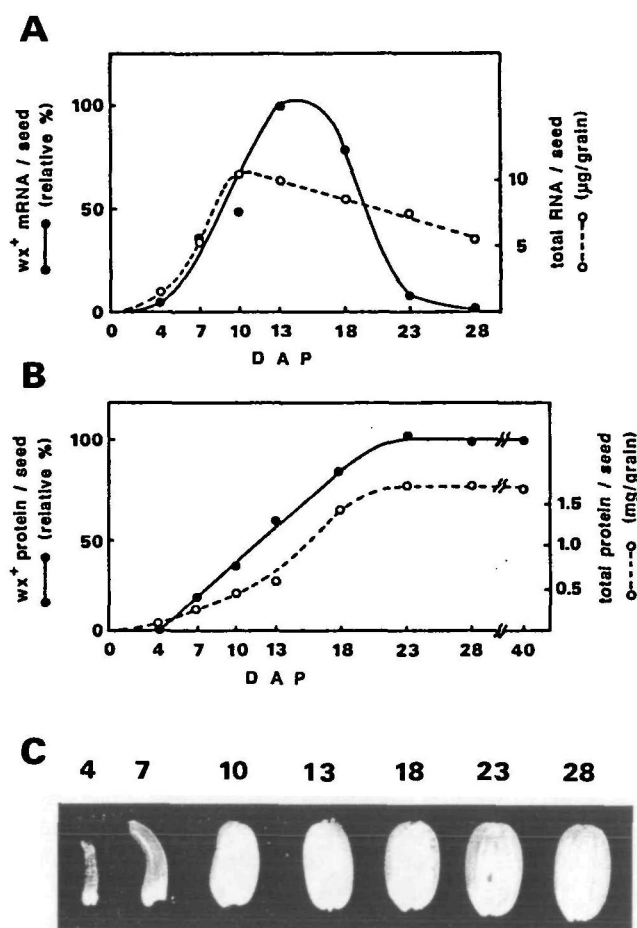


Fig. 9 Accumulation of the transcript of the *wx*⁺ gene (A) and the *wx*⁺ protein (B) during seed development. The amounts of the *wx*⁺ transcript and the *wx*⁺ protein per seed were estimated from the intensity of the signals on X-ray film (Fig. 8) and the total RNA and protein in the seed. The amount of total RNA was determined by measuring the absorbance at 260 nm of the final solution of RNA (1 A₂₆₀ unit being taken equivalent to 40 µg). The amount of total protein in extracts was estimated by use of a standard protein (bovine γ-globulin; Bio-rad). (C) Developing rice seeds, with days after pollination indicated.

The total starch from seeds remained in the water-insoluble fraction after extraction of proteins by boiling and accounted for a large portion of the fraction. The water-insoluble fraction accumulated during almost the same period as the *wx*⁺ protein and the rate of accumulation of the two was nearly equal (data not shown).

Discussion

Rice is one of the best plant materials for molecular biological study. A number of genes in rice have been mapped on the chromosomes (Kinoshita 1987). The rice

genome is comparatively small among those of major crops (Iyenger et al. 1979). Furthermore, techniques for transformation and regeneration have been established (Shimamoto et al. 1989). Moreover, from the viewpoint of practical application, rice is a major carbon source for human beings. Despite its agronomic importance, the starch synthesis in rice is poorly understood at the molecular level.

The *wx* locus is responsible for the synthesis of amylose in the rice endosperm. The rice *wx*⁺ gene was cloned by use of the maize *wx*⁺ gene as a molecular probe. As judged from the following evidence, the cloned DNA is considered to be derived from the *wx* locus in rice. (i) The function of the *wx* locus is similar in rice and maize, namely, the locus controls the amylose synthesis in the endosperm and pollen and encodes the major protein that binds to starch granules; moreover, the molecular weight of the protein is nearly equal in rice and maize (Shure 1983, Sano 1984). (ii) The transcript is detected only in wild-type rice (*wx*⁺) but not in a mutant (*wx*) rice in Northern blot using cloned rice DNA. (iii) The molecular weight of the protein predicted from the deduced amino acid sequence is consistent with that of the *wx*⁺ protein of rice. The *wx* locus of rice should encode ADP-glucose starch glycosyl transferase as does that of maize because of the high degree of sequence homology between deduced amino acid sequences from the two plants.

The results from analysis with various restriction enzymes and the comparison of the intensity of the signal with that of control DNA for copy number estimation in Southern blotting experiments (data not shown) suggested that the *wx*⁺ gene is present as a single copy in the rice genome. This inference is supported by the result that three clones selected from the rice genomic library were identical. Moreover, this hypothesis is confirmed by the analysis of one of the mutant strains of rice, which was generated by a single mutagenetic event. The mutant synthesized a protein encoded by the *wx* locus but the protein was a little larger than the *wx*⁺ protein (Sano et al. 1991). If the *wx*⁺ gene is present at more than one copy per rice genome, a protein of the same size as the wild-type should be present, in addition to the mutant protein, in the mutant strain. Since only a protein with altered size was found in the mutant, the *wx*⁺ gene seems to be a single copy. The fact that the gene of interest is a single copy represents a great advantage for molecular genetic studies.

The structure of the rice *wx*⁺ gene was predicted from that of the maize *wx*⁺ gene. Since the putative introns of rice followed the GT-AG rule and the amino acid sequence deduced from the combined putative exons exhibited a high homology with those of maize and barley, the predicted structure is probably correct. The 5th and 8th introns of the maize *wx*⁺ gene are present in the rice *wx*⁺ gene but not in the barley *wx*⁺ gene. This result is

consistent with the evolutionary relationship among these plants (Wolfe et al. 1989).

Recently the nucleotide sequence of the gene at the *wx* locus in rice was registered in the EMBL data base (Wang et al. 1990). Base change and insertion were detected at five positions between the sequences of two rice *wx*⁺ gene but no amino acid change were occurred. However, the authors' comments (Wang et al. 1990) are not consistent with our results nor their experimental result (i.e., sequence). Firstly, they referred that the rice *waxy* gene had 13 introns, but the number of introns indicated in their sequence was 12. Secondly, they pointed out a putative TATA box and Agga box at the region apart from the ATG site by about 270- and 310-bp. But our result from the PCR amplification experiment indicated that this region was also included in mRNA. Thus, the correct structural features of upstream region of the *wx*⁺ gene remain to be studied.

The predicted protein encoded by the *wx*⁺ gene contained an additional polypeptide, which might be the transit peptide required for transporting the *wx*⁺ protein into the amyloplast. The sequence similarity of the transit peptide from rice, maize and barley, was high in the N- and C-terminal regions but was relatively low in the internal regions. There are a number of basic amino acids (K and R), hydroxylated amino acids (S and T), and small hydrophobic amino acids (A and V), but only a few acidic amino acids (Fig. 5). This striking feature is similar to that of the transit peptides of proteins transported into the chloroplast (Keegstra et al. 1989) and of α -glucan phosphorylase (Nakano et al. 1989), which is functional in the amyloplast. Since the amyloplast and chloroplast originate from proplastids, similar mechanisms of protein transport are expected for both types of organelle. In fact, a fused precursor protein consisting the transit peptide of the maize *wx*⁺ protein and β -glucuronidase was shown to be transported into the chloroplast in vitro (Klöggen et al. 1989) and in transgenic plants (Klöggen and Weil 1991). The above mentioned features common to the transit peptides of the amyloplast and chloroplast proteins may be involved in the mechanism for transport of proteins.

The expression of the rice *wx* locus was shown to be regulated in a tissue-specific manner. The gene was expressed only in the endosperm and pollen, and the regulation was at the transcriptional level. Of great interest to us is the molecular mechanism of gene regulation in two distinctly different, differentiated tissues, the function, developmental origin and ploidy of which are different. In immunoblotting analysis, bands cross-reacting with anti-WXP serum were detected in analysis of leaf, stem and whole seedling. Since these bands were detected in those tissues of *wx* rice, the proteins may be unrelated to the *wx* locus. If antibodies in the anti-WXP serum recognize a common portion of proteins, for example the active center

or substrate-binding site, it is possible that these proteins are the enzyme responsible for the synthesis of assimilated starch (amylose) in those organs. It is noteworthy that a protein of the same size as that in the stem (Fig. 7) was present at the early stage of seed development (Fig. 8B) and disappeared as the green color of the seed disappeared (Fig. 9C).

The *wx*⁺ gene is developmentally regulated. The rice seed reaches to maturity in about 40 days. The *wx*⁺ gene was active from the early to the middle stage of seed development and the *wx*⁺ transcript was present at the maximum level between the 13 and 18th day after pollination. Over the next 20 days, the *wx* locus was not expressed. In contrast, the gene for a seed storage protein, a 13-kDa prolamins, maintains the high level of expression during the late stages of seed development (Masumura et al. 1990). The slight decrease in the level of the *wx*⁺ protein relative to total protein after the 23rd day (Fig. 8A) suggests that the active synthesis of seed storage proteins continues after that time.

The *wx* locus of rice has some particular advantages for genetic research. The genes affecting expression of the *wx* locus in *trans* have been identified and some of the genes have been mapped on the chromosomes (Sato and Amano 1987). The fine mapping of the *wx* locus in rice can be performed by the pollen analysis, which has been a powerful technique for studies of the maize *wx* locus (Nelson 1962). Moreover, techniques for production of fertile transgenic plant has been established in rice (Shimamoto et al. 1989). With these advantages and techniques, the molecular mechanism of the regulation of the *wx*⁺ gene should be amenable to detailed elucidation.

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