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A gene encoding a truncated large subunit of Rubisco is transcribed and salt-inducible in rice

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Abstract Using the rice salt-tolerant mutant 20 as material, a cDNA library was constructed and two salt-inducible clones, SIR5.5 and SIR8.1, were isolated by differential screening. Homology analysis revealed that the two clones together constituted a chimeric *rbcL* which encoded a truncated large subunit of Rubisco with 337 amino-acids, plus 64 amino-acids of unknown origin. The expressions of both the normal and the chimeric locus appeared to be developmentally regulated and salt-inducible in shoots of the salt-tolerant mutant 20 and its original variety 77-170. In roots, their expressions were salt-inducible in the salt-tolerant mutant 20 whereas no, or only premature, forms were present in the salt-treated original variety 77-170. Higher concentrations of salt reduced the expressions of both normal *rbcL* and the chimeric locus. ABA showed no effect on their expression.

Key words *Oryza sativa* · Rice salt-tolerant mutant · Chimeric *rbcL* · Rubisco · Salt stress

Introduction

Accumulation of salt in the soil causes deleterious effects and leads to a decline in rice production. In order to reduce the losses, scientists are currently attempting to improve the salinity tolerance of rice. A series of rice salt-tolerant mutants have been obtained in our laboratory by the anther culture method. Among them, one mutant line, 20, which was derived from Japonica variety 77-170, has stably inherited its salt-tolerant trait for nine generations (Chen 1988). RFLP analysis revealed that allelic differences occurred at two linked loci, RG711 and RG4, on chromosome 7, indicating that there were mutations at the DNA level in the mutant 20 (Chen et al. 1991 b). By means of two-dimensional electrophoresis, several new proteins were observed to be salt-induced in the leaves and roots of mutant 20 upon salt treatment (Chen et al. 1991 b). However, these proteins were not further studied.

There are a number of characterized proteins or genes that have been salt-induced in plants. The most extensively studied is osmotin (Singh et al. 1987; LaRosa et al. 1992). Others include SalT (Claes et al. 1990), TAS14 (Godoy et al. 1990), and Cit-SAP (Holland et al. 1993). So far, the exact functions of most of these salt-induced proteins are unknown, although it is assumed that they are essential to plant survival under salt stress.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a key enzyme in the photosynthesis of plants. It contains eight large subunits and eight small subunits. The genes for these subunits have been isolated and well studied (for review see Sugiura 1992). Their expressions were salt-inducible in salt-tolerant alfalfa cell lines (Winicov and Button 1991). Other well-known genes, like the PEPCase gene (Thomas et al. 1992), the proline biosynthesis-related genes (Chen et al. 1991 a; Williamson and Slocum 1992), and BADH gene (Weretilnyk and Hanson 1990), were also reported to accumulate the corresponding transcripts in salt-stressed plants. These genes play very important roles in plant physiological and biochemical processes.

In the present study, we have used the rice salt-tolerant mutant 20 (mutant 20) and its original variety 77-170 (170) to screen for salt-inducible clones by differential hybridization. Two clones were obtained and found to code together for a truncated large subunit of Rubisco. Their expressions were investigated in relation to salt stress.

Materials and methods

Plant material

Seeds of rice (*Oryza sativa* L., var. Japonica 77-170) and its salt-tolerant mutant 20 were imbibed in water for 2 days at 37 °C and ger-

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minated hydroponically on moistened cheesecloth at 27 °C with a photoperiod of 12 h. After 7 days of growth, seedlings were subjected to NaCl or ABA treatment with the solutions changed each day. At the appropriate times, shoots or roots of treated and controlled seedlings were harvested, immersed in liquid nitrogen, and stored at −70 °C until RNA extraction.

Preparation of total and poly $(A)^+$ RNA

Total RNA was extracted from frozen rice seedlings by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi 1987). Poly $(A)^+$ RNA was isolated by chromatography on an oligo (dT) cellulose column.

Construction and differential screening of cDNA library

Following the instructions of the cDNA synthesis kit (Boeringer, Germany), first-strand cDNA was synthesized from poly $(A)^+$ RNA isolated from shoots of mutant 20 seedlings treated with 0.5% NaCl for 4 days at 27 °C. After second-strand synthesis, *Eco*RI linkers were added and the cDNA fragments were size-fractioned and purified from excess linkers by the spun column method. The cDNAs were then cloned into the *Eco*RI site of lambda gt 10. Recombinant lambda DNAs were packaged in vitro and plated on *E. coli* C600hfl$^-$. This cDNA library was screened for salt-inducible cDNA clones by differential hybridization.

Recombinant phages were plated and plaques were transfered onto positively-charged nylon membranes (Hybond-N^+, Amersham) in replicas. These membranes were then treated according to standard procedures (Sambrook et al. 1989). Following prehybridization, one set of membranes was hybridized with ^{32}P-labelled first-strand cDNA synthesized using the poly $(A)^+$ RNA from shoots of mutant 20 seedlings which had been treated with 0.5% NaCl for 4 days at 27 °C. A second set was hybridized with similarly-labelled cDNA synthesized using the poly $(A)^+$ RNA from shoots of 170 control seedlings which had been maintained hydroponically without salt for 4 days at 27 °C. The labelled cDNA probes were prepared by the use of random primers and oligo (dT) primers. After hybridization, the membranes were washed at 65 °C successively with 2×SSC/0.1% SDS, 1×SSC/0.1% SDS and 0.5×SSC/0.1% SDS, for 20 min each, and autoradiographed with intensifying screens at −70 °C.

Putative cDNA clones which showed stronger hybridizations in mutant 20 were further purified by a second round of screening, and their cDNA inserts were isolated, subcloned into plasmid pUC19 and further characterized by Northern blotting.

Northern-blot analysis

Total RNA (30 g) was separated on 1.2% agarose gels containing formaldehyde, blotted onto nylon membranes, and hybridized to random-primed cDNA probes following standard protocols (Sambrook et al. 1989). The prehybridization, hybridization and washing procedures were performed exactly as described above under differential screening. The membranes can be stripped and reprobed with other probes. The 18*s* rRNA probe was used to detect the quality and quantity of the RNA. The signals on the autoradiograms were quantitated with a Flying Spot Scanner (Shimadzu). Northern blottings were repeated with at least two independent preparations of RNA and representative experiments are reported here. A commercial RNA ladder was used as a size marker.

DNA sequencing and data analysis

The sequences of both strands of the cDNA inserts were determined by the dideoxy nucleotide chain-termination method (Sanger et al. 1977) using a T7 DNA sequencing kit (Pharmacia, Sweden). The nucleotide and amino-acid sequences were compared with those in the EMBL, GenBank and DDBJ databases by using the analysis program of the BLAST Network Service.

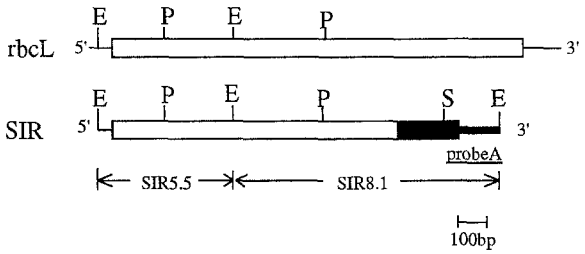

Fig. 1 The physical map of the SIR gene containing SIR5.5 and SIR8.1 in comparison with normal *rbcL*. *Boxes* represent open reading frames. The *filled box* and the *solid bar* in SIR8.1 represent the sequence different from normal *rbcL*. E: *Eco*RI, *P*: *Pst*I, *S*: *Sph*I. Probe A is the short *Sph*I/*Eco*RI fragment of SIR8.1

Results

Isolation and sequencing of the two cDNA clones

By differential screening of the cDNA library constructed in this study, several plaques were obtained which showed stronger hybridization to the probe synthesized from salt-treated mutant 20 seedlings. Northern-blot and cross-hybridization analyses resulted in two independent clones. The two clones were named as SIR (Salt-Inducible in Rice) 5.5 and SIR8.1 and further characterized.

SIR5.5 and SIR8.1 were sequenced and homology analysis revealed that together they encoded a truncated large subunit of Rubisco. Thus, two clones collectively constituted a SIR gene; the physical map is shown in Fig. 1. It can be seen that SIR5.5, a shorter *Eco*RI fragment, represented the 5′ part of the SIR gene, whereas SIR8.1, a longer *Eco*RI fragment, represented the 3′ part. The SIR gene was 1355 bp in length (Fig. 2) and contained an open reading frame of 1206 bp which encoded 401 amino-acid residues. The 337 amino-acid residues from the N-terminal of the deduced amino-acid sequence were extremely homologous with those of the normal large subunit of Rubisco (Moon et al. 1987; Nishizawa and Hirai 1987) except that two amino-acids in the 194th and 216th positions had changed from arginine and aspartic acid into alanine and glycine, respectively, in the normal large subunit. However, the amino-acid sequence of 64 residues beyond the 337th codon was completely different from the corresponding region of the normal large subunit of Rubisco (Fig. 3), although the nucleotide sequence of the poly (T) region from 1046 to 1084 in the SIR gene resembled that from 7 8395 to 7 8445 in the rice chloroplast genome (Hiratsuka et al. 1989). A similar clone, pCt-1, which coded for a truncated large subunit of Rubisco with 278 amino-acids, plus 33 amino-acid residues of unknown origin (Fig. 3), has been obtained by Moon et al. (1987).

The 5′ region of the SIR gene had only 34 bp, hence it was not available for promoter analysis. However, a 7-bp sequence, 5′-GGGAGGG-3′, located immediately upstream from the translation initiation codon ATG, was similar to the 'Shine-Dalgarno' sequence found in prokaryotic

Fig. 2 Nulceotide and deduced amino-acid sequences of the SIR gene. DNA bases and one-letter amino-acid codes are numbered at the right. *Eco*RI, *Pst*I and *Sph*I sites are *overlined*. *S.D.* represents the Shine-Dalgarno sequence and is *overlined*. The *asterisk* indicates the terminal codon. Direct or inverted repeats are indicated by *horizontal arrows*. The nucleotide and deduced amino-acid sequences have been submitted to GenBank as accession number L24073

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     EcoRI                                                S.D.
5'  GAATTCT TAA TTC ATG AGT TGT AGG GAG GGA CGT ATG TCA CCA CAA    46
                                                  M   S   P   Q     4

ACA GAA ACT AAA GCA AGT GTT GGA TTT AAA GCT GGT GTT AAG GAT TAT    94
 T   E   T   K   A   S   V   G   F   K   A   G   V   K   D   Y    20

AAA TTG ACT TAC TAC ACC CCG GAG TAC GAA ACC AAG GAC ACT GAT ATC   142
 K   L   T   Y   Y   T   P   E   Y   E   T   K   D   T   D   I    36

TTG GCA GCA TTC CGA GTA ACT CCT CAG CCG GGG GTT CCG CCC GAA GAA   190
 L   A   A   F   R   V   T   P   Q   P   G   V   P   P   E   E    52

         PstI
GCA GGG GCT GCA GTA GCT GCC GAA TCT TCT ACT GGT ACA TGG ACA ACT   238
 A   G   A   A   V   A   A   E   S   S   T   G   T   W   T   T    68

GTT TGG ACT GAT GGA CTT ACC AGT CTT GAT CGT TAC AAA GGC CGA TGC   286
 V   W   T   D   G   L   T   S   L   D   R   Y   K   G   R   C    84

TAT CAC ATC GAG CCC GTT GTT GGG GAG GAT AAT CAA TAT ATC GCT TAT   334
 Y   H   I   E   P   V   V   G   E   D   N   Q   Y   I   A   Y   100

GTA GCT TAT CCA TTA GAC CTA TTT GAA GAG GGT TCT GTT ACT AAC ATG   382
 V   A   Y   P   L   D   L   F   E   E   G   S   V   T   N   M   116

TTT ACT TCC ATT GTG GGT AAC GTA TTT GGT TTC AAA GCC CTA CGC GCT   430
 F   T   S   I   V   G   N   V   F   G   F   K   A   L   R   A   132

                    EcoRI
CTA CGT CTG GAG GAT CTG CGA ATT CCC CCT ACT TAT TCA AAA ACT TTC   478
 L   R   L   E   D   L   R   I   P   P   T   Y   S   K   T   F   148

CAA GGC CCG CCT CAT GGT ATC CAA GTT GAA AGG GAT AAG TTG AAC AAA   526
 Q   G   P   P   H   G   I   Q   V   E   R   D   K   L   N   K   164

TAC GGT CGT CCT TTA TTG GGA TGT ACT ATT AAA CCA AAA TTG GGA TTA   574
 Y   G   R   P   L   L   G   C   T   I   K   P   K   L   G   L   180

TCT GCA AAA AAT TAT GGT AGA GCA TGT TAT GAG TGT CTA GCC GGT GGA   622
 S   A   K   N   Y   G   R   A   C   Y   E   C   L   A   G   G   196

CTT GAT TTT ACC AAA GAT GAT GAA AAC GTA AAC TCA CAA CCA TTT ATG   670
 L   D   F   T   K   D   D   E   N   V   N   S   Q   P   F   M   212

CGT TGG AGG GGA CGT TTT GTC TTT TGT GCC GAA GCT ATT TAT AAA TCA   718
 R   W   R   G   R   F   V   F   C   A   E   A   I   Y   K   S   228

                                                        PstI
CAG GCC GAA ACC GGT GAA ATT AAG GGG CAT TAC TTG AAT GCG ACTGCAG   766
 Q   A   E   T   G   E   I   K   G   H   Y   L   N   A   T   A   244

 GT ACA TGC GAA GAA ATG ATT AAA AGA GCT GTA TTT GCG AGG GAA TTA   814
 G   T   C   E   E   M   I   K   R   A   V   F   A   R   E   L   260

GGG GTT CCT ATT GTA ATG CAT GAC TAC TTA ACC GGG GGG TTC ACC GCA   862
 G   V   P   I   V   M   H   D   Y   L   T   G   G   F   T   A   276
                         1
AAT ACT AGT TTG GCT CAT TAT TGC CGC GAC AAC GGC CTA CTT CTT CAC   910
 N   T   S   L   A   H   Y   C   R   D   N   G   L   L   L   H   292

ATT CAC CGA GCA ATG CAT GCA GTT ATT GAT AGA CAG AAA AAT CAT GGT   958
 I   H   R   A   M   H   A   V   I   D   R   Q   K   N   H   G   308

ATG CAT TTC CGT GTA TTA GCT AAA GCA TTG CGT ATG TCT GGG GGA GAT  1006
 M   H   F   R   V   L   A   K   A   L   R   M   S   G   G   D   324

CAT ATC CAC GCT GGT ACA GTA GTA GGT AAG TTA GAA GGG ATT TTT TTT  1054
 H   I   H   A   G   T   V   V   G   K   L   E   G   I   F   F   340

TTT TTT TTC TTT TTT TTT TTT TTT TTT TTA CGA AAA GAG CTA TAT GTA  1102
 F   F   F   F   F   F   F   F   F   L   R   K   E   L   Y   V   356

TGT GTA ATA ATC CAA AAC AAG CAC GTA GTA CAA GGA GTT GCA GTA CAT  1150
 C   V   I   I   Q   N   K   H   V   V   Q   G   V   A   V   H   372

GCA TGT AGA ATC TCT AGC TCT CTC TGG TAC ACG TAC CCC TCG AAA AGG  1198
 A   C   R   I   S   S   S   L   W   Y   T   Y   P   S   K   R   388

         SphI
AGC AAA ACG TAC GAG ACG AAC ACA CAA GAA GAA GAA AGC TAG CTA GCG  1246
 S   K   T   Y   E   T   N   T   Q   E   E   E   S   *           402

TGA CCG GGC CGA TCA ACC ATG GAT GCA TGG TGA TTG ATC GAG ACC TTA  1294

GCT AGC TCA TCT TTT ATT CCA GAA TAC AGA TAT GAT ACT ACT AGT ACT  1342

         EcoRI
GGA TAC AGA ATT C   3'                                          1355
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Fig. 3 Comparison of the deduced amino-acid sequence from the SIR gene with the rice normal large subunit of Rubisco (*RBCL*) and that from pCt-1 (Moon et al. 1987; Nishizawa and Hirai 1987). The amino-acid sequences are represented by one-letter codes and numbered at the left. Sequences before 279 are not listed because they are identical. The sequences different from RBCL are *underlined*.

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RBCL  (279) SLAHYCRDNG LLLHIHRAMH AVIDRQKNHG MHFRVLAKAL RMSGGDHIHA
SIR   (279) SLAHYCRDNG LLLHIHRAMH AVIDRQKNHG MHFRVLAKAL RMSGGDHIHA
pCt-1 (279) RREEGEERKM RVDGGKCGGR WGARFYCHGI AFR

RBCL  (329) GTVVGKLEGE REMTLGFVDL LRDDFIEKDR ARGIFFTQDW VSMPGVIPVA
SIR   (329) GTVVGKLEGI FFF FFFF FFF FLRKELYVCV IIQNKHVVQG VAVHACRISS

RBCL  (379) SGGIHVWHMP ALTEIFGDDS VLQFGGGTLG HPWGNAPGAA ANRVALEACV
SIR   (379) SLWYTYPSKR SKTYETNTQE EES

RBCL  (429) QARNEGRDLA REGNEIIRSA CKWSPELAAA CEIWKAIKFE FEPVDKLDS
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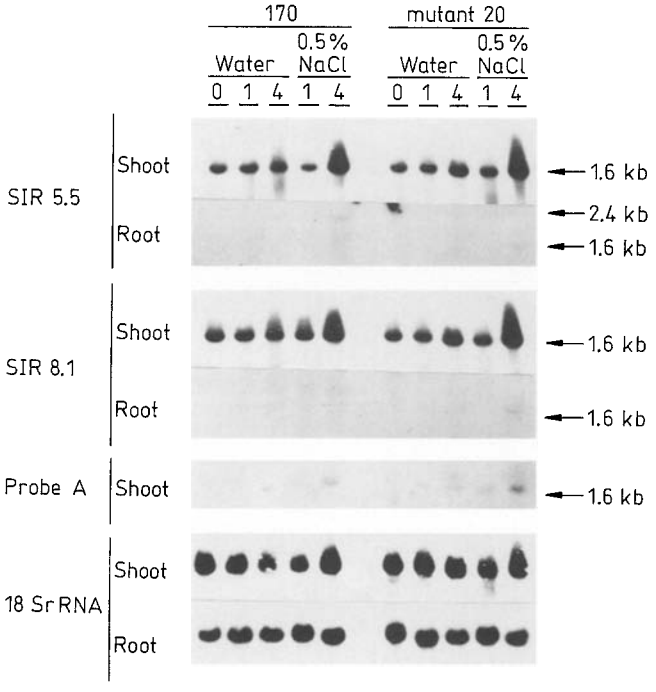

Fig. 4 Expression of the SIR gene in seedlings of rice salt-tolerant mutant 20 (mutant 20) and its original variety 77-170 (170). Rice seedlings (grown for 7 days) were treated with water or 0.5% NaCl for the indicated times. A total RNA (30 g) was loaded on each lane. The RNA blots were hybridized with SIR5.5, SIR8.1, probe A or the 18*s* rRNA probe. Exposure: 1 day for shoot blot, 7 days for root blot

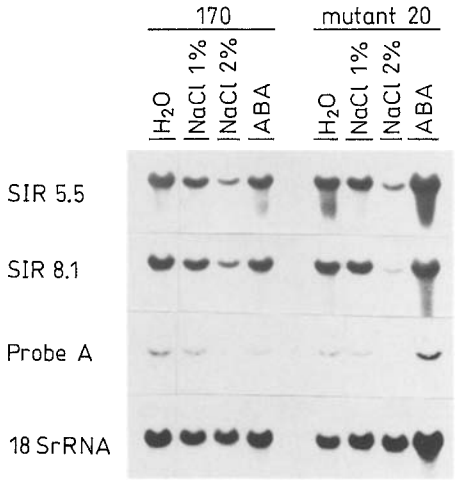

Fig. 5 Expressions of the SIR gene in shoots of rice mutant 20 and 170 upon salt or ABA treatment. Rice seedlings (grown for 7 days) were treated with 1% or 2% NaCl for 4 days, or 20 M ABA for 3 days. A total of 30 g of RNA was loaded on each lane. Other details are the same as in Fig. 4

mRNA (Shine and Dalgarno 1977) and may be the ribosome-binding site. The 3′ region contained 118 bp and had several short direct and inverted repeats (Fig. 2). These repeats are characteristic of chloroplast genes and might play a role in stabilizing the transcripts (Stern and Gruissem 1987).

Expressions of the SIR gene in response to salt stress

The expressions of the SIR gene were investigated in rice salt-tolerant mutant 20 and its original variety 77-170. The results (Fig. 4) showed that, in shoots of both 170 and mutant 20, the expression levels of SIR5.5 slightly increased in the 4-day period under control conditions, whereas its expression increased by 3–4-fold over the course of the 0.5% NaCl treatment for 4 days when compared with the controls (0 days) and the 18*s* rRNA levels. In roots of 170 and mutant 20, there was no, or only very weak, expression of SIR5.5 in the 4-day period under control conditions although after long exposure (7 days) its expression increased when the seedlings were treated with 0.5% NaCl for 4 days. However, the transcripts were different in size. In 170, roots contained a 2.4-kb transcript which may represent a precursor, while roots of mutant 20 had the mature form of 1.6 kb.

When the RNA blots were probed with the SIR8.1 clone, the patterns of expression in shoots of both 170 and mutant 20 were similar to that revealed by SIR5.5. However, the expression of SIR8.1 could only be observed in roots of salt-treated mutant 20 (4 days) and was not detected in roots of 170.

Because of the high homology with the normal gene for the large subunit of Rubisco (*rbcL*), what SIR5.5 or SIR8.1 detected should contain transcripts from the normal *rbcL* and from the present SIR gene (also referred as the chi-

meric *rbcL*). To verify the expression of the chimeric *rbcL*, a specific probe, A, was selected, which was the short *Sph*I/*Eco*RI fragment of SIR8.1 (Fig. 1) and differed completely in sequence from the normal *rbcL*. The result was presented in Fig. 4, shows that the chimeric *rbcL* did transcribe its mRNA especially in shoots of salt-treated 170 and mutant 20 (4 days). There were very low levels of the chimeric transtcripts in other treatments. This chimeric gene was hardly detectable in roots of either 170 or mutant 20 (data not shown).

When the seedlings of 170 and mutant 20 were exposed to higher concentrations of salt, the expressions of both SIR5.5 and SIR8.1 either showed no change, or else only slight decreases, at 1% NaCl but dramatic decreases at 2% NaCl (Fig. 5). The expressions of the chimeric *rbcL* detected with probe A showed a similar trend but at lower levels.

ABA has been reported to induce some salt-induced proteins (Claes et al. 1990; Godoy et al. 1990). However, it had almost no effect on the expressions of SIR5.5, SIR8.1, or the chimeric *rbcL*, when compared with the controls and the 18*s* rRNA levels (Fig. 5).

Discussion

Rubisco is a very important enzyme in plants. The rice large subunit of Rubisco has 477 amino-acids and the gene for it has been well studied (for a review see Sugiura 1992). By differential hybridization, two cDNA clones, SIR5.5 and SIR8.1, have been isolated from the cDNA library constructed in the present study. The two clones together constituted a chimeric *rbcL* which encoded a truncated large subunit of Rubisco with 337 amino-acids, plus 64 amino-acids of unknown origin. Similar results (pCt-1) were obtained by Moon et al. (1987) from chloroplast genomic DNA and it was shown that the normal and the truncated *rbcL* were located on different chloroplast DNA molecules, indicating that the chloroplast genome was heterogeneous. Variations in chloroplast DNA have also been observed in some other cases (Fitter and Rose 1993; Kanno et al. 1993). This variation and heterogeneity might be the result of intermolecular recombination of chloroplast DNA molecules in the presence of some short repeated sequence (Hiratsuka et al. 1989; Shimada and Sugiura 1989). In-vitro experiments have demonstrated the existence of such recombination (Cerutti and Jagendorf 1993). There are several regions in the chloroplast genome where many clustered pseudogenes or variations occur. Among them, the region downstream from *rbcL* appears to be one of the most variable regions within chloroplast DNA (Shimada and Sugiura 1989). Therefore, it is possible that the occurrence of recombination in this 'hot spot' region resulted in the formation of a chimeric *rbcL* which encoded a truncated large subunit of Rubisco.

The precise location of the present chimeric *rbcL* is not clear, although it is assumed to be in the chloroplast. However, there have been studies indicating the presence of chloroplast DNA in mitochondrial and nuclear genomes (Moon et al. 1987; Ayliffe and Timmis 1992).

The expression of the normal and the chimeric *rbcL* appeared to be both developmentally regulated and salt-inducible in shoots of rice 170 and mutant 20, as revealed by probing with SIR5.5, SIR8.1, and probe A. These results were consistent with those of Winicov and Button (1991). They reported the accumulation by salt-tolerant alfalfa cells of some photosynthesis gene transcripts, including *rbcL*s, in response to salt. Salinity-induced increases in photosynthetic capacity and/or Rubisco level were also observed in photosynthetic algae (Takabe et al. 1988). In the present study, the *rbcL* transcripts accumulated preferentially in roots of salt-treated mutant 20 while no transcripts (probed with SIR8.1) or only premature ones (probed with SIR5.5) appeared in roots of salt-treated 170, indicating that some salt-tolerance-related mechanisms existed in roots of mutant 20 in response to salt-stress.

From the results of Northern blots, it seemed that the chimeric *rbcL* transcripts represented only a small portion of the total *rbcL* transcripts. This may suggest that the chimeric *rbcL* had a very limited number of copies. The conclusion was possibly identical to that reported by Moon et al. (1987). They demonstrated that the copy number of the fragment containing a truncated *rbcL* (pCt-1) was only one-tenth that of the fragment containing the normal *rbcL* (pCt-3). Although the present chimeric *rbcL* can be transcribed, it remains to be determined whether it has any translation product or any function.

When rice 170 and mutant 20 were treated with higher concentrations of NaCl, the expressions of *rbcL* (normal and chimeric) were dramatically reduced. This apparently resulted from the detrimental effects that salt exerted on the plants. ABA has been reported to induce several salt-associated proteins or genes (Claes et al. 1990; Godoy et al. 1990). However, it had no influence on the expression of normal and chimeric *rbcL*, thus indicating that the induction of *rbcL* transcripts was different from that mentioned above.

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