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(ガンマ線照射によって誘発されたイネ易変性細粒突然変異の遺伝)

2001

Masayoshi Teraishi

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Chapter I

Introduction

A slender glume mutant was induced by gamma-ray irradiation of seeds of the *japonica* rice variety Gimbozu (Fig.I-1A). This mutant character is probably controlled by a single recessive mutant gene. But the mutation has never been fixed genetically in spite of repeated self-propagation: in successive generations, not only normal plants but also plants chimeric for glume shape almost always appear with low frequency. The chimeras can be classified into three types: between-panicle chimeras, within-panicle (between primary branch) chimeras (Fig.I-1 B), and the mixed type. Such a phenomenon is likely to be due to the mutability of the mutant slender glume gene(s), which occasionally reverts to its wild-type (normal glume) state.

In higher plants, there are many reports on mutable traits especially for genes involved in pigmentation (Bonas et al. 1984; Fedoroff et al. 1984; Brown et al. 1989; Inagaki et al. 1994) and endosperm quality (Fedoroff et al. 1983). Recent molecular biological analyses have revealed that many such mutable traits are controlled by transposable elements (Fedoroff et al. 1983; 1984; Bonas et al. 1984; Brown et al. 1989; Inagaki et al. 1994) or result from epigenetic transformation (Vongs et al. 1993; Bender and Fink

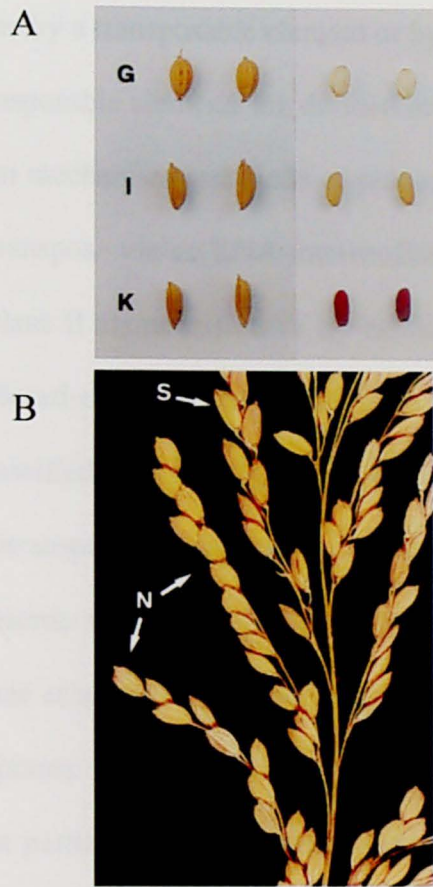


Fig.I-1 **A:** Glume shapes in the *japonica* rice variety Gimbozu, its slender glume mutant line IM294, and the *indica* rice variety Kasalath. Most *japonica* and *indica* varieties have such characteristic glume shapes. **B:** An example of a within-panicle chimera that appeared in the slender glume mutant line IM294.

1995). This suggests that the mutability of the slender glume may also be caused either by a transposable element or by epigenetic transformation.

Transposable elements are divided into two groups according to their transposition mechanism and mode of propagation: retrotransposons (class I elements) transpose via an RNA intermediate, while the DNA transposable elements (class II elements) move by excision and reintegration (Kunze et al. 1997). Based on the structures of DNA copies, elements of the former type are classified into long-terminal repeat (LTR) retrotransposons, non-LTR retrotransposons or long interspersed element (*LINE*)-like retrotransposons, short interspersed element (*SINE*)-like retrogenes, and others (Kunze et al. 1997). To date, more than 30 retroelements have been reported in plants. For the latter type (ClassII), as many as 40 elements have been at least partially analyzed at the molecular level, and those of the *Ac* superfamily (Muller-Neumann et al. 1984; Pohlman et al. 1984), the *En/Spm* superfamily (Pereira et al. 1986; Gierl et al. 1985), and the *Mutator* family (Chomet et al. 1991; Hershberger et al. 1991) have been especially well investigated. Although there are few reports of mutable characters in rice, many transposable elements are found in its genome (Hirochika et al. 1992; Mochizuki et al. 1992; Bureau and Wessler 1994; Motohashi et al. 1996). Most of them, however, transpose very infrequently in the intact plant. One of the retrotransposons, *Tos 17*, often transposes during callus induction *in vitro* (Hirochika et al. 1996), but there are no other effective ways to

stimulate the activity of transposable elements in rice. Transposable elements are useful for genetic engineering techniques in plants, such as transposon tagging (McLaughlin and Walbot 1987; Schmidt et al. 1987; Balcells et al. 1991; Gierl and Saedler 1992; Chuck et al. 1993; Biezen et al. 1996) and reverse genetics (Koes et al. 1995; Bensen et al. 1995), and for investigating the evolution of plant species (Mochizuki et al. 1993; Peterson 1993; Thompson et al. 1994; Huttley et al. 1995; Thatiparthi et al. 1995; Kunze et al. 1997). With the aim of applying the transposon tagging technique in rice, many researchers have attempted to introduce maize transposable elements, such as *Ac-Ds*, into the rice genome, but the efficiency of mobilization is still quite low (Izawa et al. 1991; Sugimoto et al. 1994). Concerning the roles of transposable elements in the evolution of plant species, there are two different views (Lönnig and Saedler 1997): some researchers regard transposable elements as selfish DNA without any phenotype function in the host organism (Doolittle and Sapienza 1980; Charlesworth and Langley 1989), others see them as a major source of variability in plant species (Alberts et al. 1994; Lewin 1994). It is thus still not clear whether transposable elements are deployed in eukaryotic organisms because of their selective advantage for their host organisms (Kunze et al. 1997). If so, successful cloning of the transposable element that presumably confers the mutability of *slg* will advance our knowledge of

rice genome evolution as well as facilitating the efficient genetic engineering of the rice genome.

Epigenetic transformation is also known to cause the mutability of genes. It is due to some genomic factor(s) modifying the methylation level of cytosine and thus the gene activity (Vong et al. 1993). But epigenetic transformation rarely induces mutation in other DNA regions. The author's preliminary experiment, however, showed that morphological and physiological mutants often appear in the selfed-progenies of revertants from the slender glume to its wild-type state. Therefore, there is little possibility that the mutability of the slender glume is caused by epigenetic transformation.

The ultimate goal is to understand whether the mutable slender glume mutation is associated with the insertion of a transposable element. This study was focused on understanding the genetic factor(s) controlling the slender glume phenotype and its mutability using conventional and molecular biological analyses. In this dissertation, the results and their implication are described in the following chapters; (II) Genetic analysis of the slender glume mutation, (III) Morphological analysis of glume-shape chimeras caused by the mutability of a mutant slender glume gene, (IV) RFLP (Restriction Fragment Length Polymorphism) mapping of the slender glume gene *slg*, (V) RLGS (Restriction Landmark Genomic Scanning) analysis

of *slg*, (VI) Identification of YAC (Yeast Artificial Chromosome) clones containing *slg*, and (VII) Summary.

Chapter II

Genetic analysis of the slender glume mutation

2.1. Introduction

A slender glume mutation induced by gamma-ray irradiation of seeds of the *japonica* rice cultivars Gimbozu has never been fixed in spite of repeated self-propagation: in successive generations not only normal plants but also plants chimeric for glume shape always appear with low frequency. Such a phenomenon is likely to be due to the mutability of the mutant slender glume gene(s) that occasionally reverts to its wild-type state.

In higher plants, many mutable traits have been reported, and many of them were found to be controlled by transposable elements (Bonas et al. 1984; Fedoroff et al. 1984; Brown et al. 1989; Inagaki et al. 1994) or epigenetic transformation (Vongs et al. 1983). This suggests that the mutability of the slender glume mutation may also be caused either by a transposable element or by epigenetic transformation. But there is little possibility that the mutability of the slender glume mutation is controlled by epigenetic transformation for reasons described in Chapter I.

In this chapter, as the first step to ascertain if the mutability of the slender glume mutation is controlled by a transposable element, the genetic factor controlling the slender glume mutation was first identified using

progenies from reciprocal crosses between the mutant line and its original variety. Subsequently, the effects of genetic background and backcrossing on the mutability were examined.

2.2. Materials and Methods

2.2.1. Inheritance of the slender glume mutation

The slender glume mutant (mutant line IM294) was crossed with four *japonica* rice varieties, Gimbozu, Koshihikari, Nipponbare, and Taichung 65. Reciprocal crosses were made with the parental variety. A total of 20 F₂ populations from five different cross combinations were subjected to genetic analysis for glume shape (cf Fig.I-1-A). Four F₂ populations derived from different parental slender glume plants (SGPs) from line IM294 were used for each cross combination. Each population consisted of 247 to 360 plants. A progeny test was conducted for the cross 'IM294/Gimbozu' in 1993 using 100 F₃ lines, which were derived from randomly selected F₂ normal glume plants (NGPs). Each F₃ line consisted of 30 plants. All the materials were grown in an experimental paddy field at Kyoto University, Kyoto.

2.2.2. Effects of genetic background and backcrossing

The results of the test crosses revealed that the slender glume phenotype was caused by a recessive mutation in a single gene. To examine the effect of genetic background on the mutability of this gene, the reverse mutation frequency (RMF) was calculated using the progenies of F₂, F₃, BC₁F₂, and BC₁F₃ SGPs that were all covered with plastic bags to prevent outcrossing. Backcrossing with parental varieties was carried out for two cross combinations, IM294/Gimbozu and IM294/Nipponbare. The F₃ and BC₁F₃ populations were grown in 1993, and the F₄ and BC₁F₄ populations in 1994. IM294 was grown as control in both years.

2.3. Results

2.3.1. Inheritance of the slender glume mutation

In all the F₂ populations, SGPs and NGPs were found to segregate. The proportion of SGPs varied considerably among F₂ populations (range: 10.5-21.9%, average: 15.0%) (Table II-1). All the F₂ populations except two showed ratios significantly lower than the 25% expected if the slender glume phenotype is governed by one recessive mutant gene. Preliminary experiments, however, suggested that SGPs were inferior to NGPs in germination ability and seedling viability. The slender glumes of IM294 and normal glumes of Gimbozu were mixed in a 1 : 3 ratio. A total of 3000 seeds were sown in nursery beds with field soil, and 1000 seedlings were

TableII-1 Segregation ratios of slender-glume plants in F₂ populations from crosses between the slender-glume mutant line IM294 and four varieties

Cross combination	No. of populations	No. of slender glume plants	No. of non-slender glume plants	No. of chimeric plants	No. of total plants	Segregation ratio ^a of slender glume plants (%)	χ^2 value ^b
Gimbozu/IM294	4	37	281	0	318	11.6	30.29 (P<0.001)
		50	275	0	325	15.4	16.03 (P<0.001)
		32	248	0	280	11.4	27.50 (P<0.001)
		40	247	0	287	13.9	18.73 (P<0.001)
	total	159	1051	0	1210	13.1 [*]	90.80 (P<0.001)
IM294/Gimbozu	4	48	273	0	321	15.0	17.28 (P<0.001)
		43	236	0	279	15.4	13.68 (P<0.001)
		46	229	0	275	16.7	10.04 (P=0.002)
		27	229	0	256	10.5	28.52 (P<0.001)
	total	164	967	0	1131	14.5 [*]	66.50 (P<0.001)
IM294/Koshihikari	4	48	230	1	279	17.2	8.84 (P=0.003)
		53	284	0	337	15.7	15.45 (P<0.001)
		54	193	0	247	21.9	1.29 (P=0.255)
		71	266	1	338	21.0	2.77 (P=0.096)
	total	226	973	2	1201	18.8 ^y	24.16 (P<0.001)
IM294/Nipponbare	4	51	309	0	360	14.2	22.53 (P<0.001)
		42	234	2	278	15.1	14.00 (P<0.001)
		35	286	0	321	10.9	34.02 (P<0.001)
		35	239	0	274	12.8	21.84 (P<0.001)
	total	163	1068	2	1233	13.2 [*]	90.63 (P<0.001)
IM294/Taichung65	4	31	246	0	277	11.2	28.17 (P<0.001)
		47	229	0	276	17.0	9.35 (P=0.002)
		46	230	0	276	16.7	10.22 (P=0.001)
		54	270	0	324	16.7	12.00 (P<0.001)
	total	178	975	0	1153	15.4 [*]	56.22 (P<0.001)

^a Different letters show the significant difference at the 5% level by pair-tests.

^b Test for one locus segregation (1:3).

transplanted to a paddy at the four- to five-leaf stage. After maturation, the proportion of slender glume plants was determined. The ratio of 14.2% did not significantly differ from the overall mean (13.8%) of two F₂ populations, IM294/Gimbozu and Gimbozu/IM294. Among the 100 F₃ lines derived from F₂ NGPs from the cross IM294/ Gimbozu, 65 exhibited almost the same segregation pattern as observed in the F₂ population, and 35 consisted of only NGPs. The ratio of 65 : 35 fits the 1:2 ratio expected for one-locus segregation ($\chi^2=0.125$, P=0.723). Since the RMF from slender glume to normal glume was at most 1% (Table II-2), reverse mutation was not regarded as the major factor determining the low frequency of F₂ SGPs. Thus, the reduced yield of SGPs in F₂ could be attributed to weak germination ability and/or weak seedling viability; this, in turn, supports the idea that the slender glume character is controlled by a single recessive mutant gene. The cross IM294/Koshihikari showed a significantly larger mean segregation ratio than others, all of which did not significantly differ from each other. This suggests that the germination ability and/or seedling viability might be influenced somewhat by genetic background, though the inheritance of the slender glume mutation is not markedly affected by genetic background or cytoplasmic factors. In conformity with the rules of gene nomenclature in rice, the mutant gene was designated *slg*.

2.3.2. Effects of genetic background on the mutability of slg

TableII-2 Reverse mutation frequency (RMF) for slender glume to non-slender glume in different genetic backgrounds

Cross combination	F ₃			F ₄		
	RMF(%)		Total	RMF(%)		Total
	Non-slender glume plants	Chimeric plants		Non-slender glume plants	Chimeric plants	
Gimbozu/IM294	0.23 (1)	0.00 (0)	439	0.53 (3)	0.71(4)	561
IM294/Gimbozu	2.30 (10)	0.23 (1)	435	0.63 (7)	0.54 (6)	1115
IM294/Koshihikari	0.07 (2)	0.00 (0)	2609	0.13 (2)	0.00 (0)	1581
IM294/Nipponbare	0.41 (7)	0.94 (16)	1695	0.53 (8)	0.40 (6)	1518
IM294/Gimbozu//Gimbozu	0.00 (0)	0.00 (0)	40	1.33 (11)	0.72 (6)	828
IM294/Nipponbare//Nipponbare	0.16 (1)	0.00 (0)	631	0.0 6 (1)	0.00 (0)	1661
		1993 (year)			1994 (year)	
IM294	0.84 (11)	0.31 (4)	1312	0.48 (6)	0.40 (5)	1249

Figure in parenthesis shows the number of plants.

The proportions of NGPs and chimeric plants (CPs) in progenies (F_3 and F_4) of F_2 SGPs are shown in Table II- 2. The proportions of NGPs and CPs in IM294 were also determined as a control. The frequencies of NGPs and CPs in IM294 in 1994 were 0.84 and 0.31%, respectively, while in 1995 the values were 0.48 and 0.40, respectively. In all the single cross combinations, NGPs and/or CPs appeared with low frequency, suggesting the occurrence of reversion from *slg* to its wild-type state in all the genetic backgrounds tested. The exact RMF could not be estimated due to the small number of plants examined, but the results indicated that the RMF was little affected by crossing or genetic background. The cross IM294/Gimbozu showed a slightly higher RMF than the reciprocal cross, and backcrossing to the original variety seem to reduce the RMF. Although a further analysis of this aspect will be needed, it is likely that backcrossing and cytoplasmic factors do not have noticeable effects on RMF and thus on the mutability of *slg*.

2.4. Discussion

The present study revealed that the induced slender glume mutation is caused by a single recessive, mutable gene *slg*, which occasionally reverts to its wild-type state. The reverse mutation frequency was little affected by crossing, backcrossing, genetic background, or cytoplasmic factors. The

appearance of chimeric plants is a clear indication of the occurrence of reverse mutation during mitosis as well as meiosis: *slg* is capable of reverting to its wild-type state throughout the whole growth stage of rice plants. In maize (*Zea mays*), morning glory (*Pharbitis nil*), and *Antirrhinum majus*, there are many reports on mutable genes (unstable alleles) and most of them were found to be caused by DNA transposable elements (Fedoroff et al. 1983; Bonas et al. 1984; Sommer et al. 1985; Pereira et al. 1986; Inagaki et al. 1994). Therefore, it is very probable that the mutability of *slg* is also caused by a DNA transposable element.

In the selfed progenies of NGPs, reversion in SGPs, and various kinds of morphological and physiological mutants, such as early or late-maturing, dwarf, and rolled leaf mutants, are often observed (data not shown). This fact also supports the idea that the mutability of *slg* might be caused by a DNA transposable element (Class II element). In this case, reversion could be caused by the excision of the transposable element inserted in the *slg* locus, while the novel mutations associated with the reversion may be induced by the insertion of the excised transposable element into other chromosomal regions. The change from the wild-type allele to *slg* could have been caused by the insertion of a transposable element that was activated and excised from some other chromosome region following the application of gamma-ray radiation. Such an effect of gamma-

ray irradiation is similar to that of the *in vitro* culture procedures employed by Hirochika et al. (1996).

Chapter III

Morphological analysis of glume-shape chimeras caused by the mutability of the slender glume gene *slg*

3.1. Introduction

Experimental results in Chapter II showed that the slender glume mutation is governed by a single recessive mutant gene *slg*, which frequently reverts to its wild-type state. Because of such mutability, the slender glume mutation has never been fixed genetically in spite of repeated self-propagation: in successive generations, not only normal plants but also plants chimeric for glume shape almost always appear with low frequency. The chimeras can be classified into three types: between-panicle chimeras, within-panicle chimeras, and the mixed-type. Such a phenomenon indicates that the reversion from *slg* to its wild-type state occurs during mitosis as well as meiosis. F₃ and F₄ progeny tests of chimeric plants in Chapter II revealed that the genotypes of slender and normal glumes of chimeric plants are *slg/slg* and *+/slg*, respectively. This indicates that chimeric plants for glume shape surely consist of genetically different cells.

Induced chimeric plants are considered as useful materials for investigating morphogenesis of higher plants (Szykowiak and Sussex 1996), because using mutant characters as markers follows up the

cell lineage throughout the whole growth stage of plants and thus makes it possible to construct fate maps of embryo and shoot apical meristem cells (McDaniel and Poethig 1988, Furnere and Pumfrey 1992, Irish and Sussex 1992).

It has been pointed out that transposable elements are the main natural sources to induce chimeric plants (Fedoroff et al. 1983, Fedoroff et al. 1984, Bonas et al. 1984, Brown et al. 1989, Inagaki et al. 1994). Experimental results in Chapter II showed that the mutability of *slg* is probably controlled by a DNA transposable element; therefore, analyzing the effect of *slg* on chimerism is considered to be very useful for investigating the reproductive organ development of rice.

In this chapter, chimeric plants caused by the mutability of *slg* were analyzed to disclose the developmental aspects of rice panicles.

3.2. Materials and Methods

The slender glume mutant (line IM294) was crossed with four *japonica* rice varieties, Gimbozu, Koshihikari, Nipponbare, and Taichung 65. Reciprocal crosses were made with the original variety. IM294 and the progenies of F₂, F₃, BC₁F₂, and BC₁F₃ slender glume plants (SGPs) were grown in a paddy field at Kyoto University, Kyoto in 1995. Backcrossing with the parental variety was carried out for two cross combinations,

IM294/Gimbozu and IM294/Nipponbare. Out of a total of 11856 plants tested, 48 were found to be chimeric for glume shape. At maturity, all the 48 chimeric plants were taken out from the paddy. After removing roots and soil carefully from plants, tillering order and glume shape of each panicle were examined. A progeny test was conducted for all the panicle-row lines of 26 chimeric plants to determine the genotype of their parental panicles for the *slg* locus. For chimeric panicles consisting of normal and slender glume branches (primary branch), a progeny test was conducted using primary-branch row lines.

3.3. Results

3.3.1. Morphological aspects of chimeric plants

Out of 48 chimeric plants, 40 were classified into three types based on the glume shape of panicle on the main culm: normal glume panicle (N-type ; 22 plants), chimeric panicle (C-type ; 10 plants) and slender-glume panicle (S-type ; 8 plants). Other eight plants accidentally lost their main panicles and could not be classified into any groups. The three types greatly differed in number of normal glume panicles per plant. To evaluate the relationship between the chimera-type and the mutated-sector size in plant, the relative size of mutated area (N-index) was calculated with the following formula:

$$\text{N-index} = (1 \times \text{No. of normal glume panicles} + 0.5 \times \text{No. of chimeric panicles}) / \text{Total No. of panicles per plant}$$

Most of the S-type and C-type plants showed a small N-index value, less than 0.4, while most of the N-type plants showed a large N-index value, over than 0.4 (Table III-1). The large value of N-index means that the reversion from *slg* to its wild-type state occurs at early growing stages. It has been pointed out that up to the third leaf primordia are already differentiated in embryo before dormancy, and that the primordia for lower primary tillers are already developed in embryo (Hoshikawa 1989). Under ideal growing conditions, the first primary tiller emerges from the second leaf axil (Hoshikawa 1989). Therefore, it is unlikely that most primordia of lower primary tillers are derived from a single cell mutated after germination. Thus, the reversion from *slg* to its wild-type state is likely to have occurred during embryogenesis in some of the N-type plants, especially in those with a large N-index value, exceeding 0.4. Contrary, in plants with a small N-index value, less than 0.4, the reversion seems to have occurred during seedling stage. For C-type and S-type plants, it is apparent that most of the reversion occurred after germination.

The chimerism of 39 plants is illustrated in Fig. III-1. In N-type (N-1 to N-22) plants, slender glume panicles appeared most frequently on the

Table III-1. Frequency distribution of N-index value in N-type, C-type and S-type chimeric plants

N -index	Type of chimeric plants		
	N-type	C-type	S-type
0.00-0.20	1	0	3
0.21-0.40	3	4	3
0.41-0.60	5	3	1
0.61-0.80	8	2	0
0.81-1.00	5	1	0
Total	22	10	7

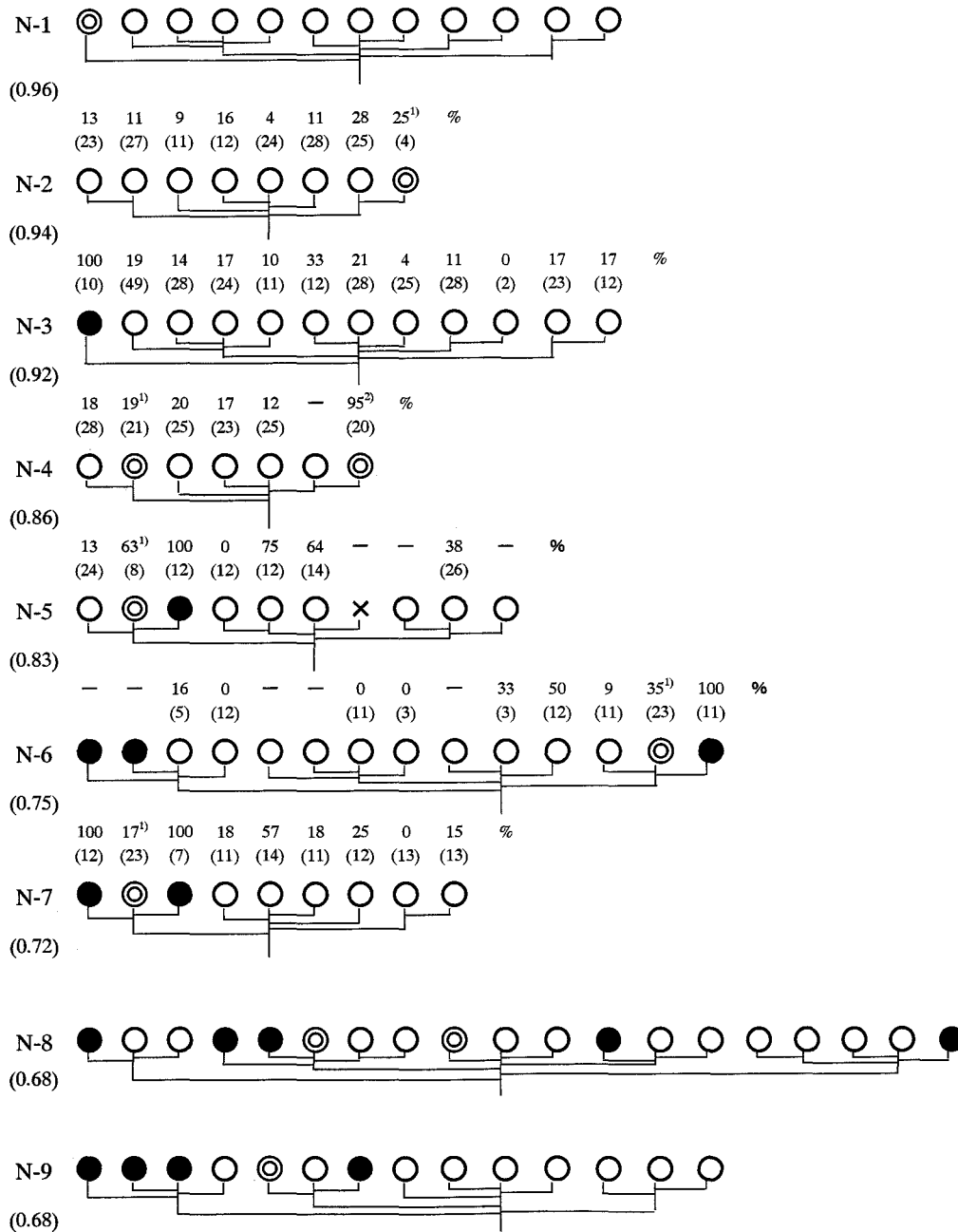


Fig.III-1 Tillering systems of plants chimeric for glume shape. Results of a progeny test for glume shape are also shown for each panicle.

●, ○, ⊙ : slender glume panicle, normal-glume panicle, and chimeric panicle, respectively.

Figures above the illustration of panicles indicate the segregation ratio of slender glume plants in their selfed-progenies. Figures in parentheses indicate the number of plant observed in the progeny test.

N-type plants (N-1 to N-22) have a normal panicle on their main stem. C-type plants (C-1 to C-10) have a panicle chimeric for glume shape on their main stem. S-type plants (S-1 to S-7) have a slender glume panicle on their main stem.

Fig.III-1(continued)

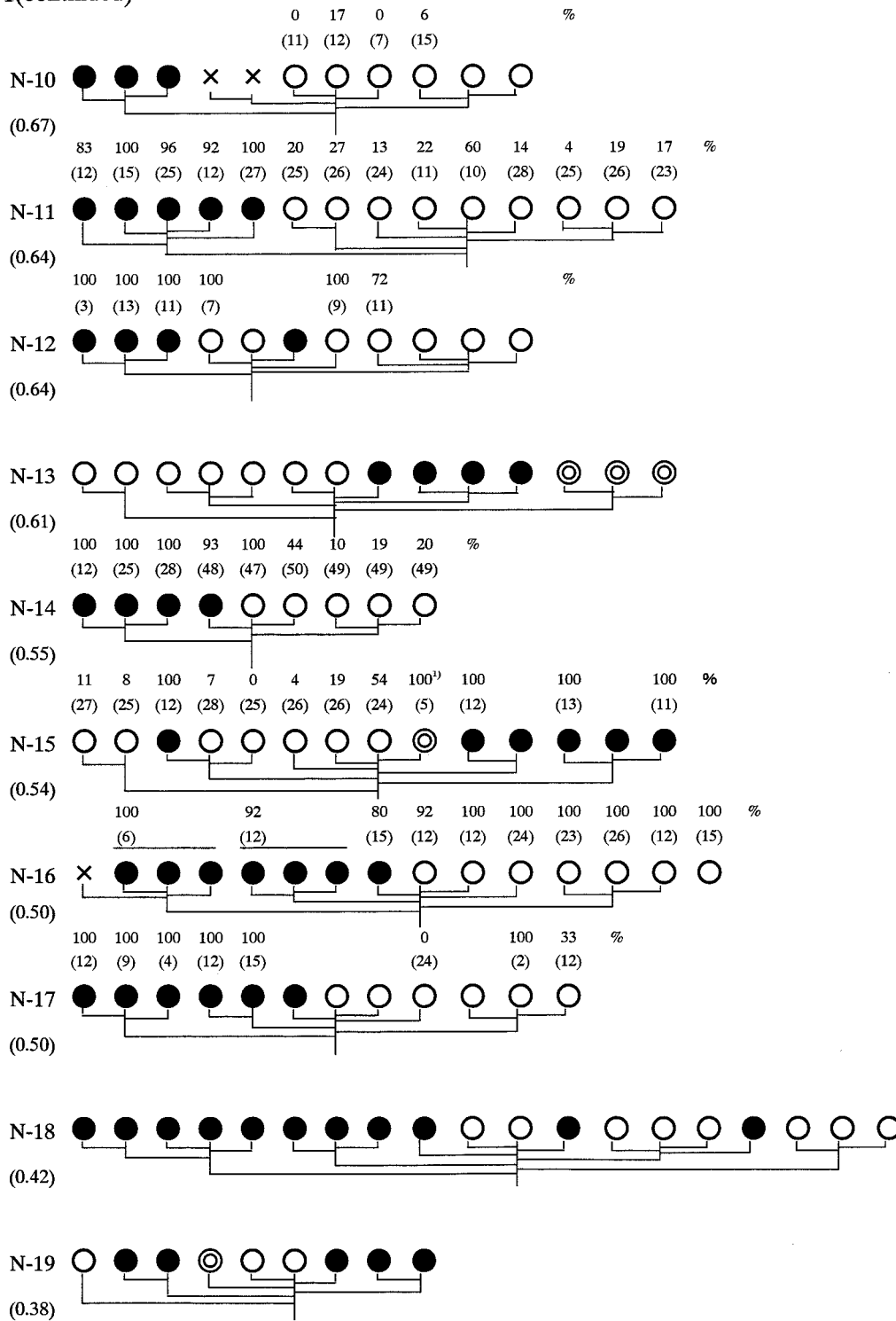
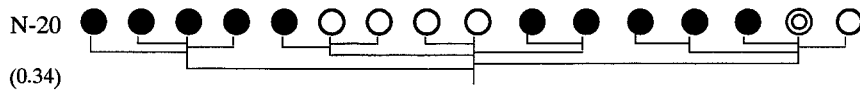
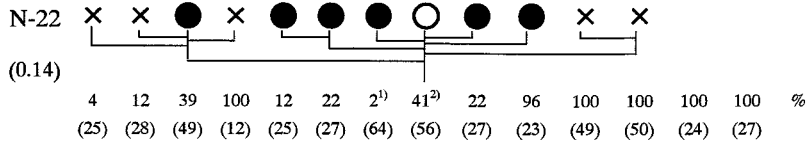
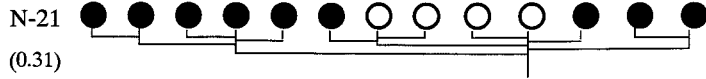


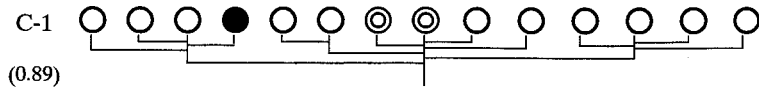
Fig.III-1(continued)



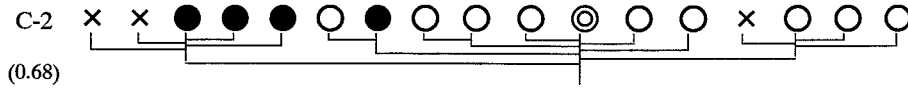
91 100 93 100 100 91 100 100 96 100 100 96 %
(11) (23) (15) (27) (22) (11) (26) (10) (24) (21) (24) (26)



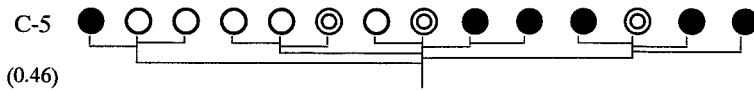
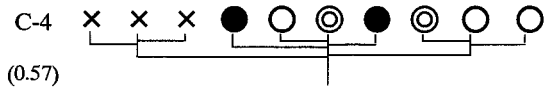
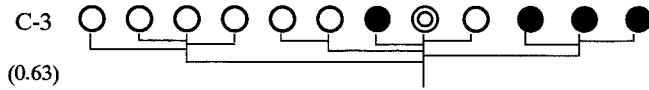
4 12 39 100 12 22 2¹⁾ 41²⁾ 22 96 100 100 100 100 %
(25) (28) (49) (12) (25) (27) (64) (56) (27) (23) (49) (50) (24) (27)



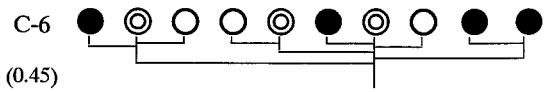
100 100 0 77 100 91 100 100 100 100
(12) (9) (3) (13) (12) (11) (26) (25) (26) (25)



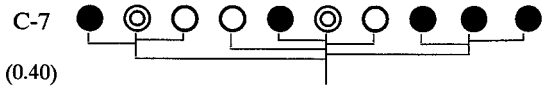
88 100 96 100 100 96 83 100¹⁾ 92 100 100
(25) (12) (25) (25) (15) (28) (12) (7) (12) (11) (11)



100 25¹⁾ 8 7 18²⁾ 100 13¹⁾ 31 100 100 %
(5) (29) (25) (27) (60) (12) (8) (26) (8) (13)



100 100 100 100 100 %
(15) (12) (27) (10) (11)



100 16¹⁾ 15 24²⁾ 22³⁾ 41⁴⁾ 100 33⁵⁾ 24 100 100 20 %
(24) (18) (26) (45) (81) (17) (13) (33) (25) (10) (7) (5)

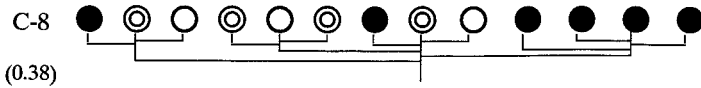
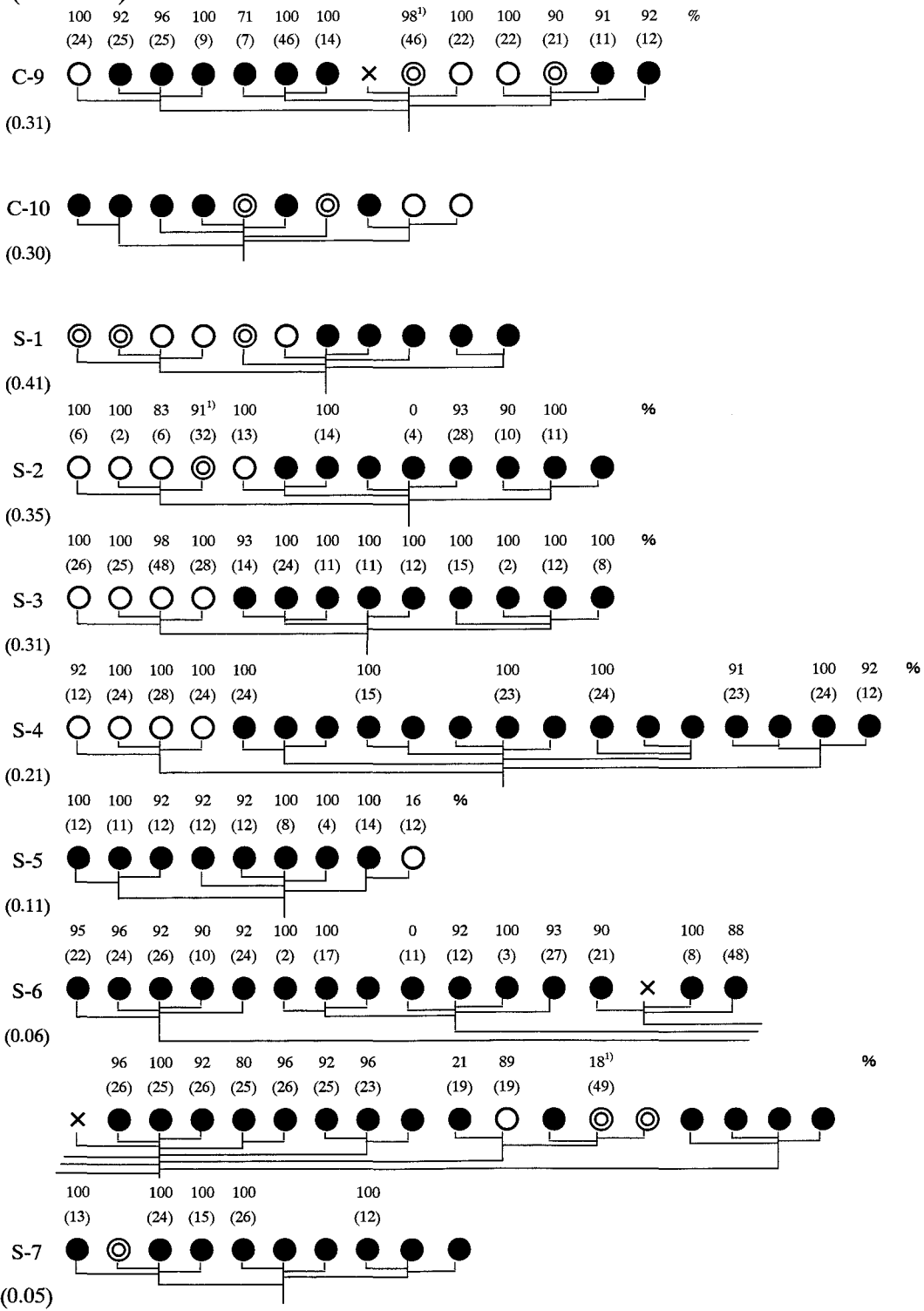


Fig.III-1(continued)



first primary tiller and also its secondary tiller(s). In N-type plants, all the cells in the crest of the shoot apical meristem appeared to be mutated to the wild-type state. The closer a cell is to the crest of shoot apical meristem, the later its progeny tissue will appear in development. Therefore, most of the panicles, which emerge in the later growing stage, were considered to be derived from mutated tissues. On the other hand, since the lower portion of the shoot apical meristem has an opportunity to retain non-mutated cells, slender glume panicles are frequently developed from such a portion.

In C-type plants (C-1 to C-10), slender glume panicles appeared not only on the first primary tiller but also on other primary tillers that emerge from upper nodes. In seven of ten C-type plants, only the last primary tiller bore the slender glume panicle. This suggests that the mosaic of shoot apical meristem is kept throughout the whole growing stage. Among the seven S-type plants (S-1 to S-7), those with normal panicles on lower tillers tend to show a large N-index value. Thus, in S-type plants, the mutated area becomes larger as the time of the reversion becomes earlier.

3.3.2. Progeny test

The results of the progeny test are also shown in Fig. III-1 and Table III-2. Numerical figures given above the illustration of panicles indicate the segregation ratio (%) of slender glume plants (SGPs) in their selfed-progeny lines. When the reversion occurs in a cell of genotype *slg/slg*, the mutated

TableIII- 2. Segregation ratio of slender glume plants (SGPs) in primary-branch-raw lines of panicles chimeric for glume shape among primary branches

Plant No.	Chimeric panicle No.	Glume shape of primary branch			
		Slender glume		Normal glume	
		Freq. of SGP(%)	No. ¹⁾	Freq. of SGP(%)	No. ¹⁾
N-2	1	25	4	0	0
N-4	1	0	2	21	19
	2	100	2	80	18
N-5	1	67	6	50	2
N-6	1	83	6	18	17
N-7	1	100	1	14	22
N-14*	1	100	4	100	1
C-1	1	78	9	11	54
	2	86	7	35	49
C-3*	1	100	2	100	5
C-6	1	100	5	15	20
	2	26	19	10	41
	3	0	0	13	8
C-8	1	0	0	17	18
	2	50	2	23	43
	3	100	9	13	72
	4	100	2	33	15
	5	56	18	73	15
C-9*	1	94	18	100	25
	2	100	4	88	17
S-2*	1	78	9	96	23
S-6	1	80	5	16	44

* Plants with periclinal chimeria (See text).

1) Number of plants observed.

cell is expected to have the genotype $+/slg$. Therefore, progeny lines of normal glume panicles are expected to involve SGPs in the ratio of 25%. As described in Chapter II, however, the overall mean segregation ratio of SGPs of F_2 populations, IM294/Gimbozu and Gimbozu/IM294, was 13.8%, lower than 25%, because of the weak germination ability and/or weak seedling viability of SGPs. In plants, N-2, -3, -4, -5, -7, -10, -11, -15, C-6, and -8, most of the progeny lines, each derived from a normal glume panicle, showed the segregation ratio expected if their parental panicles had the genotype $+/slg$. The progeny lines of slender glume panicles in these plants showed a high segregation ratio of SGPs (>90%), indicating that the genotype of their parental panicles was slg/slg . The appearance of a few normal glume plants (NGPs) in these lines is probably due to out-crossing and/or reversion from slg to its wild-type state. The progeny lines of normal glume panicles of N-12, -16, -21, C-1-, -2, -3, -7, -9, S-2, -3, -4, and -6 showed an unexpected high segregation ratio of SGPs (%), while those of slender glume panicles of these plants showed a high segregation ratio of SGPs. Therefore, while the genotype of germ cells of these normal glume panicles can be estimated slg/slg , the cells forming glume have the genotype $+/slg$. This implies that the reversion occurred only in the cells forming glumes. Such a phenomenon is called periclinal chimera. All the progeny lines of normal panicles of N-15 showed a higher segregation ratio than

expected (25%). This indicated that the periclinal chimera is stable throughout the whole growing stage.

The progeny lines of normal branches of chimeric panicles tended to show a high segregation ratio of SGPs, when periclinal chimera occurred in other panicles of the same plant (Table III-2). This indicates that chimeric panicles also harbor periclinal chimera. The progeny line of normal panicle on the main stem of N-5, -7, -11, and -15 showed a slightly higher segregation ratio of SGPs than expected, though other normal panicles showed the expected segregation ratio. This suggests that the main stems of these plants harbor an incomplete periclinal chimera: the inner apical cell layer forming germ cells consists of both mutated cells (*slg/+*) and non-mutated cells (*slg/slg*).

3.4. Discussion

In many dicotyledons, shoot meristem tissues consist of three apical cell layers, so-called L1, L2 and L3 (Szymkowiak and Sussex1996). Intensive investigation on cell lineages throughout the whole development stage of plants successfully constructed the fate map at apical meristem of mature seeds (Furner and Pumfrey 1992, Jegla and Sussex 1989, McDaniel and Poethig 1988). For monocotyledon, several species are known to have three apical cell layers (Stewart and Dermen 1979), and some only two

layers. In rice, Shimizu (1979) indicated the existence of cell layers in the apical meristem tissue through colchicine treatments following after Satina et al (1940). But final destination of these cell layers has not been genetically analyzed. Using ionizing radiation to produce chimeric rice plants, Osone (1963) investigated the developmental mechanism of mutated cells induced in rice seeds. He observed that: 1) the mutation frequency expressed by number of mutated M_1 panicles/total number of M_1 panicles tested is higher in earlier formed primary tillers, including the main stem, than in later formed tillers, and 2) F_2 segregation ratios of mutants in earlier formed primary tillers, including the main stem, are less than those in later formed primary tillers. He assumed that these results were highly associated with number of initial cells of each tiller at the time of irradiation: initial cells of panicles of the main stem and earlier formed primary tillers were superior to those of later formed primary tillers and secondary tillers in number at that time. Small number of initial cells brings forth a low mutation frequency and a high segregation ratio. Based on the segregation ratio of mutants, he estimated the initial cell number for primary tillers to be five to seven. However, he did not follow up the cell lineage to disclose the origin of reproductive organs. According to Stewart and Dermen (1979), germ cells of monocotyledon are also derived from L2 layer. In the present study, a progeny test for chimeric plants provided a clear evidence that germ and glume cells originate from different cell layers. Therefore, it is certain

that rice has at least two cell layers inside its shoot apex: the outer cell layer contributes to glume shape, while the inner cell layer contributes to germ cell. In our knowledge, this is the first report providing the functional differentiation of apical cell layers in rice.

In the present study, we could find no periclinal chimeras consisting of mutated inner layer and non-mutated outer layer. With this respect, two explanations will be given. First, we could not obtain such chimeric plants because they showed the same phenotype for glume shape as non-mutated slender glume plants. Second, the reverse mutation rate of *slg* gene is lower in the inner layer than in the inner layer. Related to this, Chaparro et al (1995) reported that the reverse mutation rate in the L2 layer was about four times lower than that in the L1 layer in peach.

Chapter IV

RFLP (Restriction Fragment Length Polymorphism) mapping of the slender glume gene *slg*

4.1. Introduction

Experimental results in Chapter II suggested that the mutability of the slender glume gene *slg* is probably caused by a DNA transposable element. To ascertain such a point, *slg* should be isolated and analyzed for its molecular biological function. But the gene product of *slg* has not yet been identified; therefore, orthodox gene-isolating techniques, such as use of its corresponding cDNA clone, could not be applied to *slg*.

Recently, various molecular biological techniques have been developed and applied in plant genetics. Consequently, gene isolation by chromosome walking from flanking molecular markers toward target genes has become possible (Mindrinos et al. 1994, Yoshimura et al. 1998, Alpert and Tanksley 2000). RFLP (Restriction Fragment Length Polymorphism) is a useful molecular marker and available for chromosome walking in many living organism, because its fine genetic map has been constructed in such organism (Tanksley et al. 1992, Kurata et al. 1994).

In this chapter, as the first step for map-based cloning of *slg*, the chromosomal location of *slg* locus was determined by RFLP analysis,

following trisomic and conventional linkage analysis. Southern blot analyses with known rice transposable elements were also performed to investigate whether the mutability of *slg* is caused by one of these elements.

4.2. Materials and methods

4.2.1. Trisomic and conventional linkage analyses

The primary trisomic series of the *japonica* rice variety Nipponbare, which was kindly provided by Dr. Iwata at Kyushu University, Japan, was crossed with IM294. Not all cross combinations could be obtained, and out of 11 kinds of cross combinations that produced F₁ trisomic plants, three for extra chromosomes 1, 2, and 3 were not suitable for analysis due to the extremely low seed fertility. Consequently, eight kinds of F₂ population, each derived from several F₁ trisomic plants, were subjected to analysis for glume shape. Since the results of the trisomic analysis suggested that the mutant gene was unlikely to be located on chromosomes 4, 5, 6, 8, 9, 10, 11, or 12. IM294 was crossed with seven conventional genetic marker lines for chromosomes 1, 2, 3, and 7 (Table IV-1). The F₂ populations used in the trisomic and conventional linkage analyses were grown in 1993 and 1995, respectively. The recombination value was estimated by the maximum likelihood method (Immer 1934; Allard 1956).

4.2.2. RFLP analysis

Table IV-1. Linkage relationships between *slg* and conventional marker genes

Marker line	Locus ^a	Chromosome	Normal glume		Slender glume		χ^2 value	Recombination fraction (%)
			Wild type	Marker type	Wild type	Marker type		
T41	<i>lax</i>	1	281	90	28	13	1.09	-
KL806	<i>gh-2</i>	2	288	81	48	15	0.11	-
TD12	<i>bc-1</i>	3	289	85	52	9	1.97	-
T46	<i>lg</i>	4	273	90	54	21	0.34	-
KL1002	<i>g-1</i>	7	250	124	56	15	1.77	41.0±3.9
KL1003	<i>Rc</i>	7	40	314	59	32	98.03 ^b	20.4±2.2
KL1008	<i>v-11</i>	7	369	143	86	2	26.97 ^b	18.9±3.9
KL1005	<i>rfs</i>	7	51	24	26	0	10.91 ^b	0.0±10.0
EG2	<i>el</i>	7	213	134	92	1	48.60 ^b	9.1±4.7

^a *lax*, lax panicle; *gh-2*, gold hull-2; *bc-1*, brittle culm-1; *g-1*, long sterile lemmas-1; *lg*, liguleless; *Rc*, brown pericarp and seed coat; *v-11* (t), virescent-11; *rfs*, rolled fine striped leaf; *el*, heading date-1

^b Significant at the 0.1% level

In the RFLP analysis, 75 F₂ plants from the cross IM294/ML17 were used. ML17 was derived from the cross Nipponbare/Kasalath (an *indica* cultivar)//Nipponbare, and has a Nipponbare-derived normal glume (*japonica*-type round glume) and Kasalath-derived RFLP alleles in the homozygous form at four loci that are tightly linked to the *rfs* (rolled fine stripe leaf) locus on chromosome 7. Total genomic DNAs were extracted from leaves sampled before flowering time using the CTAB method (Murray and Thompson 1980) with slight modifications. Extracted DNAs were digested with four restriction enzymes, *Bam*HI, *Bgl*III, *Eco*RV, and *Hind*III.

After electrophoresis, the DNAs were blotted onto a positively charged membrane (Hybond N+; Amersham) and were subjected to Southern hybridization. Four probes on chromosome 7, XNpb91, 20, 33, and 152, which were kindly provided by the Rice Genome Research Group at the National Institute of Agrobiological Resources, Japan, were used. Labeling of probes and Southern hybridization procedures were performed using the DIG DNA labeling kit and the DIG luminescent detection kit (Boehringer Mannheim), respectively. Linkage relationships were estimated with the MAPL program of Ukai et al. (1990).

Southern analyses with known rice transposable elements were performed to investigate whether *slg* was associated with the insertion of known rice transposable elements, retrotransposons of the Tos family or the

DNA element *RAc* (*Ac*-like element in rice) (Hirochika and Fukuchi 1992; Hirochika et al. 1992). Total genomic DNAs extracted from seedling leaves of IM294 and its parental variety with the CTAB method (Murray and Thompson 1980) were digested with the restriction enzymes *Bam*HI, *Bg*II, *Eco*RV, *Hind*III and *Xba*I, and were subjected to electrophoresis and capillary blotting to nylon membranes. Radioactive labeling of probes (Tos 1, 2, 3, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 17 and *RAc*) and Southern hybridization procedures were carried out according to the method of Sambrook et al. (1989).

4.3. Results

4.3.1. Linkage analysis

All the F₂ populations used for the trisomic analysis showed disomic segregation for glume shape (data not shown), suggesting that *slg* was unlikely to be located on chromosome 4, 5, 6, 8, 9, 10, 11, or 12. Based on these results, linkage analysis of *slg* was conducted using conventional marker genes assigned to chromosomes 1, 2, 3, and 7. In all the F₂ populations from crosses between IM294 and these marker lines, the observed segregation ratio of SGPs was less than 25% for the reasons described in Chapter II. But this does not bias the values for recombination between *slg* and marker genes. The linkage analysis showed that *slg* is linked to *Rc* (brown pericarp and seed coat), *v-11* (virescent-11), *rfs*, and *E1*

(heading date-1) on chromosome 7, with recombination values of 20.4, 18.9, 0.0 and 9.1%, respectively (Table IV-1). Thus, *slg* is tightly linked to, or represents the same locus as *rfs* on chromosome 7, though the latter possibility appears unlikely.

Based on the conventional linkage analysis, RFLP analysis using probes for the chromosomal regions near the *rfs* locus appeared feasible. But first we had to overcome the following difficulties. (1) Large numbers of RFLPs between *indica* and *japonica* rices are known (McCouch et al. 1988; Saito et al. 1991; Kurata et al. 1994), but they are rarely seen among *japonica* varieties (Zhang et al. 1992); hence, an *indica* variety must be used in the analysis. (2) Most *indica* varieties have slender glumes like the SGP, while most *japonica* varieties have normal (round) glumes (cf. Fig. I-1A); therefore, the segregation of glume shape in F₂ populations from crosses between SGP and such *indica* varieties are too complicated for analysis. To overcome such problems, we attempted to construct some *japonica*-type (round) glume lines having several homozygous *indica*-derived DNA segments near the *rfs* locus (Kishimoto et al. 1992). In the BC₁F₂ population from the cross 'Nipponbare/Kasalath//Nipponbare', we fortunately found a favorable line, ML17, having Kasalath-derived alleles in homozygous form at all the four known RFLP loci linked to the *rfs* locus.

The results showed that *slg* was located between *XNpb20* and *XNpb33*, with recombination values of 3.0 ± 2.1 and $3.2 \pm 2.3\%$, respectively

(Table IV-2). The location of *slg* on the RFLP map of Saito et al. (1991) is shown in Fig. IV-1.

4.3.2. Southern blot analyses with known rice transposable elements

The band patterns obtained by Southern analysis with known rice transposable elements, retrotransposons of the *Tos* family and the DNA element *RAc* were compared between IM294 and its parental variety Gimbozu. Various band patterns, from a single band to multiple bands, were observed (Fig. IV-2). If a known transposable element is inserted in *slg*, polymorphism in band should be observed between IM294 and Gimbozu. In most cases, however, such polymorphism was not detected, and none of the polymorphic bands observed corresponded to the segregation of the *slg* locus. This implies that the mutability of *slg* is caused by none of the known rice transposable elements tested.

4.4. Discussion

The southern analysis revealed that the element that confers the mutability of *slg* differs from the DNA transposable element *RAc* and from all the retrotransposons of the *Tos* family tested. So far, two LTR retrotransposon families, *Tos* and *RIRE1*, and the *SINE*-like element *p-SINE*, have been characterized in rice (Motohashi et al. 1997; Noma et al. 1997). Among the retrotransposons of the three families, only *Tos* 17

Table IV-2. RFLP mapping of *slg* RFLP probe F₂ segregation

RFLP probe	F2 segregation ^a						Recombination fraction(%)
	Normal glume			Slender glume			
	<i>AA</i>	<i>Aa</i>	<i>aa</i>	<i>AA</i>	<i>Aa</i>	<i>aa</i>	
<i>XNpb152</i>	22	35	7	1	3	7	20.0±5.7
<i>XNpb91</i>	22	37	5	0	4	7	14.9±4.9
<i>XNpb20</i>	22	42	0	0	2	9	3.2±2.3
<i>XNpb33</i>	18	44	2	0	0	11	3.0±2.1

^a *A* , Kasalath-type RFLP band; *a* , IM294-type RFLP band

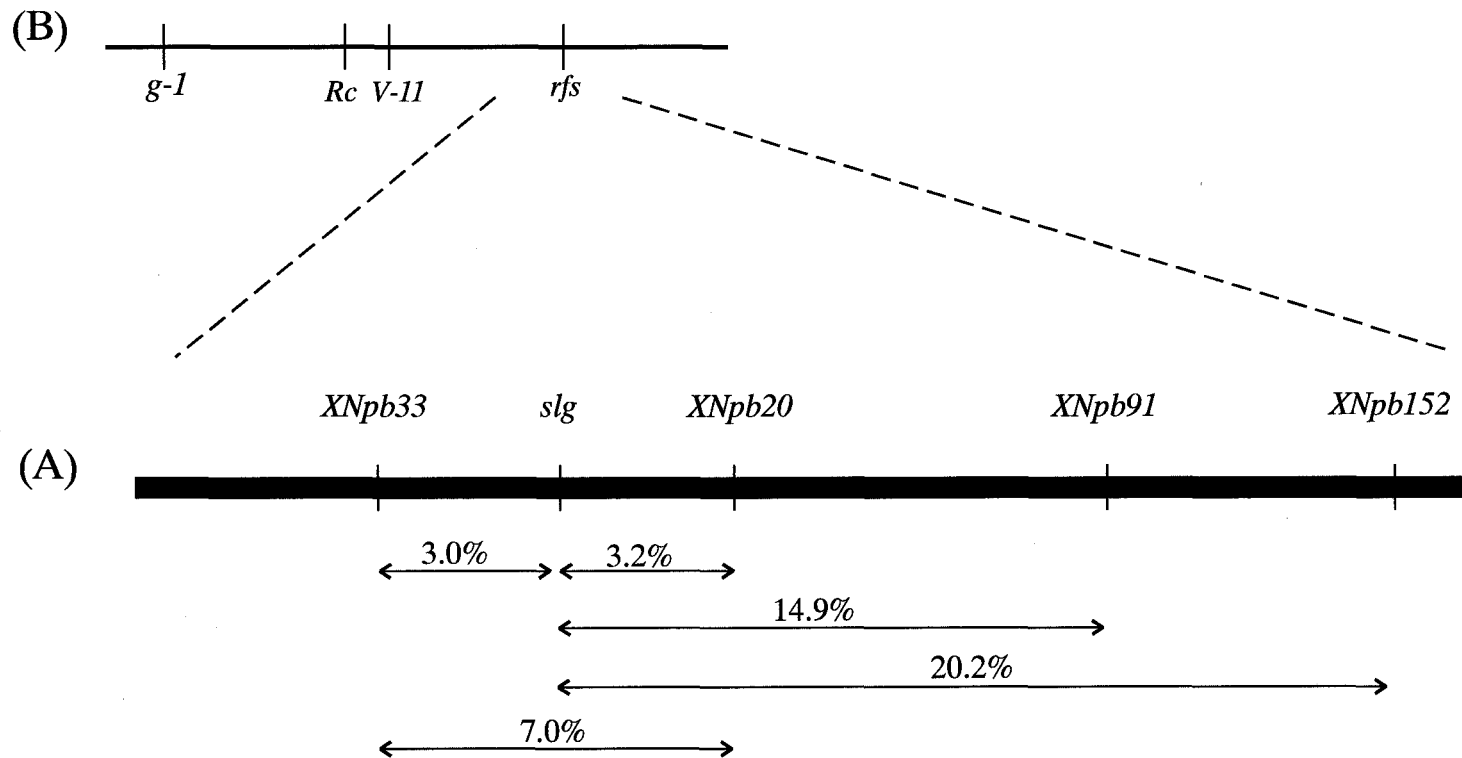


Fig. IV-1. Estimated location of the *slg* locus on the RFLP linkage map of Saito et al. (1991). The relationship between the RFLP map (A) and the conventional linkage map (B) is shown. According to Kishimoto et al. (1992), the *rfs* locus is located near the RFLP locus *XNpb20*.

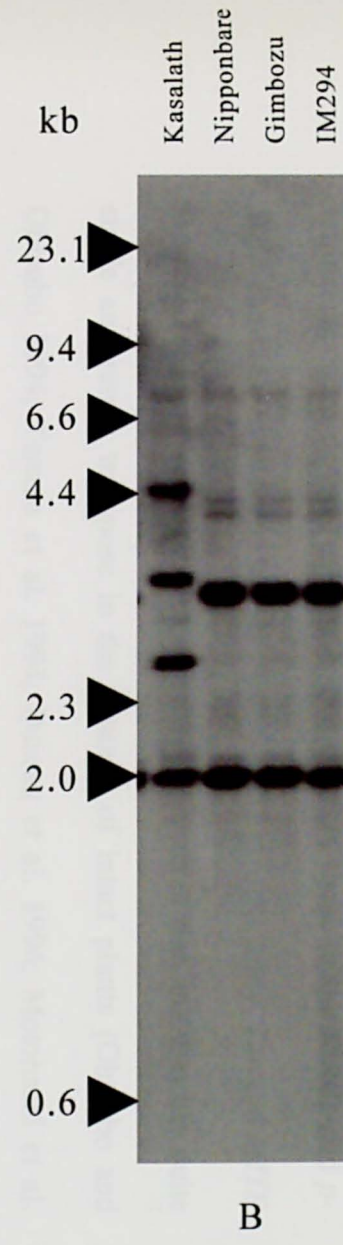


Fig.IV-2 Southern analysis using the retrotransposons *Tos 1*(A) and *Tos 17* (B), as probes. DNAs were digested with *Hind*III(A) and *Xba*I (B).

demonstrates transpositional activity, which is enhanced under *in vitro* culture stress, but *Tos* 17 never induces reverse mutations (Hirochika et al. 1996). Thus, the transposable element inserted in *slg* differs essentially from retrotransposons of the *Tos* family, and possibly those of the *RIRE1* and *p-SINE* families. In addition to *RAc*, tested in the present study, *Tnr* and *MITE* families are known as DNA transposable elements in rice, but they are quite stable and rarely transpose in the genome of intact plants (Ohtsubo and Ohtsubo 1994; Tenzen et al. 1994; Bureau et al. 1996; Motohashi et al. 1996). They do not differ in chromosomal localization between wild rice and cultivated rice, and thus were not influenced by the divergence of species in the genus *Oryza*. According to Hirochika and Fukuchi (1992), polymorphic hybridization band patterns were observed even among closely related rice varieties when *RAc-1* was used as a probe. Although mutable traits caused by *RAc* have not been found yet, this suggests that *RAc* could be activated frequently in the rice genome. Compared with *RAc*, however, the element inserted in *slg* appears to transpose frequently in the genome. This appears to be the first report of the possible presence of a DNA transposable element related to the mutability of a Mendelian gene in rice.

Two RFLP markers, *XNpb20* and *XNpb33*, were found to be linked to *slg*. But the genetic distances between *slg* and these markers are too large to permit the map-based cloning of *slg*. The author attempted to find RAPD markers more closely linked to *slg*. In spite of using 400 random primers,

however, no favorable RAPDs could be found. This indicates that other molecular mapping ways of *s/g* should be considered.

Chapter V

RLGS (Restriction Landmark Genomic Scanning) analysis of the slender glume gene *slg*

5.1. Introduction

In Chapter IV, the *slg* locus was found to be located between the RFLP (Restriction Fragment Length Polymorphism) loci, *XNpb20* and *XNpb33* on chromosome 7, with recombination values of 3.0 ± 2.1 and $3.2\pm 2.3\%$, respectively. But the genetic distances between *slg* and these two markers are too large to permit the map-based cloning of *slg*. The author attempted to find out RAPD (Randomly Amplified Polymorphic DNA) markers more closely linked to *slg*. In spite of using 400 random primers, however, no favorable RAPDs could be found.

The RLGS (Restriction Landmark Genomic Scanning) method, which has been developed recently, is a high-performance system in that a few thousand DNA fragments can be detected at the same time by performing twice or three-times restriction enzyme treatments and twice electrophoresis (Hatada et al. 1991). The RLGS is thus considered to be a convenient method for detecting DNA polymorphisms. Actually, 49 specific RLGS spots were detected out between *japonica* rice cultivars (Nipponbare and Koshihikari) with almost the same genetic background (Kawase 1994).

In this chapter, the author attempted to explore the DNA spots containing the *slg* locus or those tightly linked to this locus using the RLGS method. The efficiency of distinction of mutants was also discussed.

5.2. Materials and Methods

5.2.1. DNA Preparation

A slender glume mutant (line IM294) and its original variety Gimbozu were used for the RLGS analysis. BC₂F₂ plants of IM294/Gimbozu//Gimbozu were also used to check the segregation ratio of RLGS spots. Total genomic DNAs were extracted from leaves of each plant sampled before flowering time using the CTAB method (Murry and Thonpson 1987).

5.2.2. Procedure for two-dimensional gel electrophoresis

RLGS procedure was carried out as described below, according to Hatada et al (1991).

Genomic DNAs are usually cleaved at the preparation step and thus has nonspecific cleaved ends, nicks, and/or gaps. To prevent the incorporation of radioisotopes in the labeling process, these damaged sites were blocked with enzymatically incorporated new nucleotide analogues (ddATP[α S] and ddTTP[α S]), because these analogues prevent exonucleolysis and/or the additional incorporation of the nucleotide at

blocked ends. 5 μg of extracted DNA was allowed to react for 30min at 37°C with 10 units of DNA polymerase I in 0.33 μM dGTP[α S] and dCTP[α S] (Amersham), which can be incorporated into the cleavage site of *NotI* by fill-in reaction and 33 μM ddATP[α S] and ddTTP[α S] (TOYOBO), which cannot be incorporated into the cleavage site of *NotI* by fill-in reaction. Thereafter, the enzyme was inactivated at 65°C for 30min. The treated DNA was then digested with 100 units of *NotI*.

The cleavage ends were filled in with 20 units of Sequenase ver. 2.0 (USB) in the presence of 0.33 μM [α -³²P]dGTP and [α -³²P]dCTP, which can be incorporated into the cleavage site of *NotI* with Sequenase ver. 2.0(USB)(3000-6000 Ci/mmol;1Ci=37GBq) for 30min at 37 °C . To inactivate the enzyme, this reaction mixture was incubated at 65°C for 30min. Then, additional digestion was performed using *EcoRV*.

1 μg of the treated DNA was fractionated on a 50 \times 20 \times 0.1cm agarose gel (1.0% Seakem GTG agarose; FMC) and electrophoresed in 1 \times TAM buffer (50mM Tris-acetate, pH7.5/0.7mM magnesium acetate) at 4.5V/cm for 12h (Fig. IV-1).

The DNA-containing portion of the gel was excised as a strip and soaked in the reaction buffer appropriate for *MboI* (for 30min). Thereafter, DNA was digested in the gel with 1500 units of *MboI* for 2h.

The gel was fused with a 50×50×0.1cm polyacrilamide gel (5-6% polyacrylamide to acrylamide/bisacrylamide, 29:1) by adding Sea Plaque GTG agarose (FMC) to fill up the gap. Second-dimensional electrophoresis was carried out in 1×TBE buffer at 8V/cm for 6h (Fig. V-1).

The gel was dried with vacuum. An area 35×43cm of the original gel was then excised and autoradiographed for 3-10days on a film(XAR-5; Kodak) at -70°C using an intensifying screen (Quanta III; DuPont).

5.3. Results

RLGS profiles of IM294 and Gimbozu are shown in Figs. V-2 and V-3. Total spot number of each variety/line was approximately 2500, almost equivalent to that in the previous report in rice (Kawase 1994). Although almost all the spots were common between IM294 and Gimbozu, two (Fig. V-3, Spots A and B) and one (Fig. V-3, Spot C) specific spots were observed in Gimbozu and IM294, respectively. These three spots were located near to each other.

RLGS analysis was performed with seven normal glume and eight slender glume BC₂F₂ plants, which were derived from successive backcrossing using IM294 and Gimbozu as seed and recurrent parent, respectively. Two of seven non-slender glume BC₂F₂ plants and one of eight slender glume BC₂F₂ plants had Spot A. Therefore, it seemed that there was

A

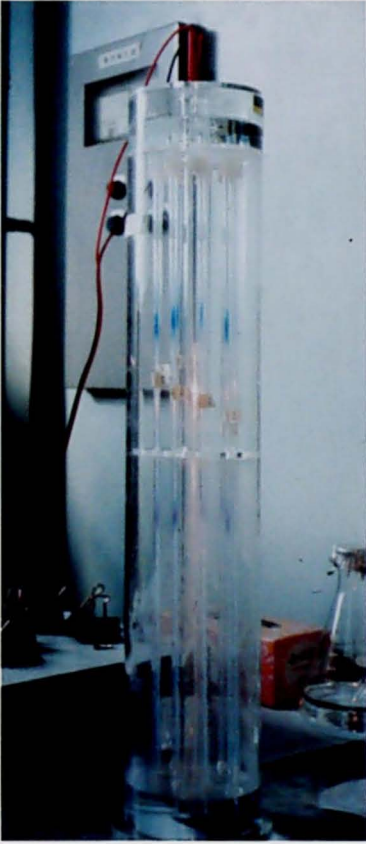
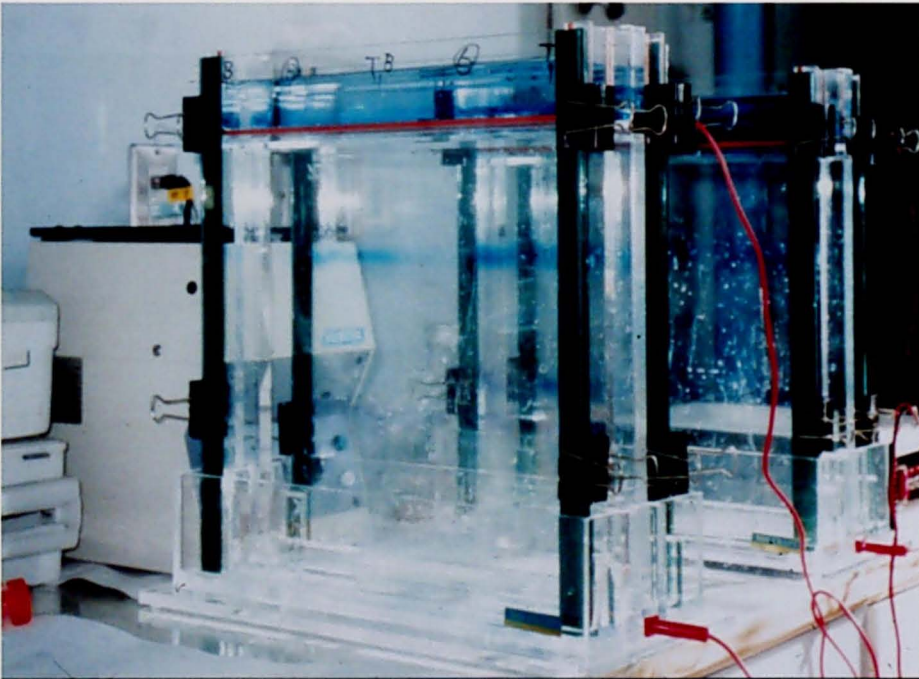


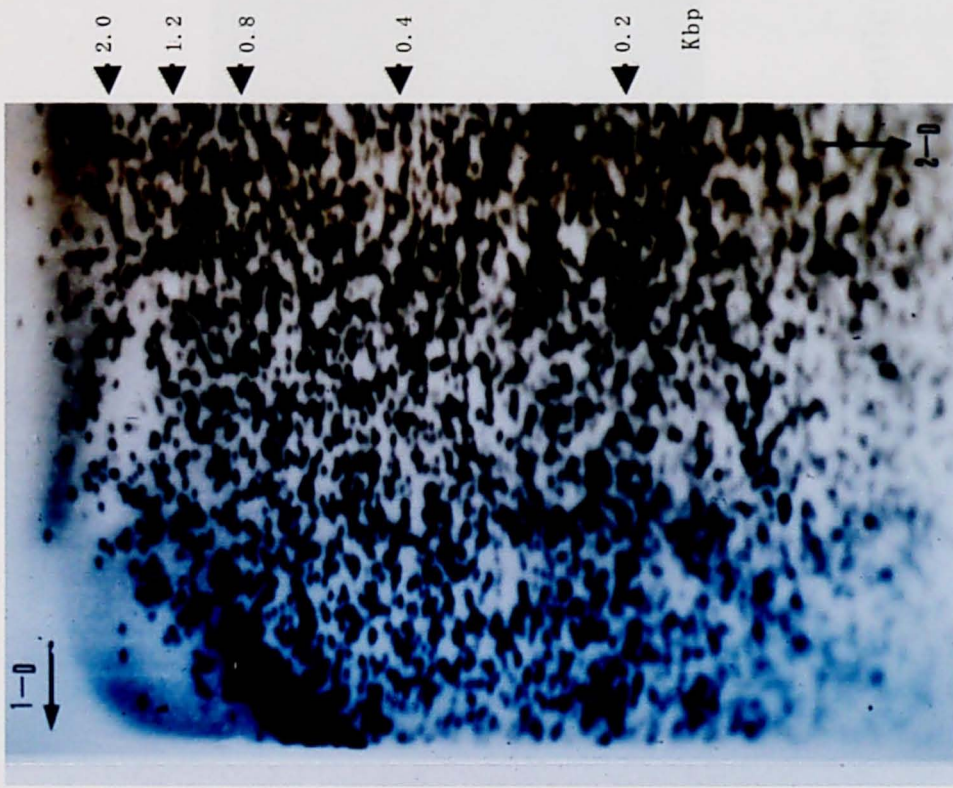
Fig.V-1 Electrophoresis apparatus for RLGS (Restriction Landmark Genomic Scanning) method

A) Electrophoresis equipment for the first dimension

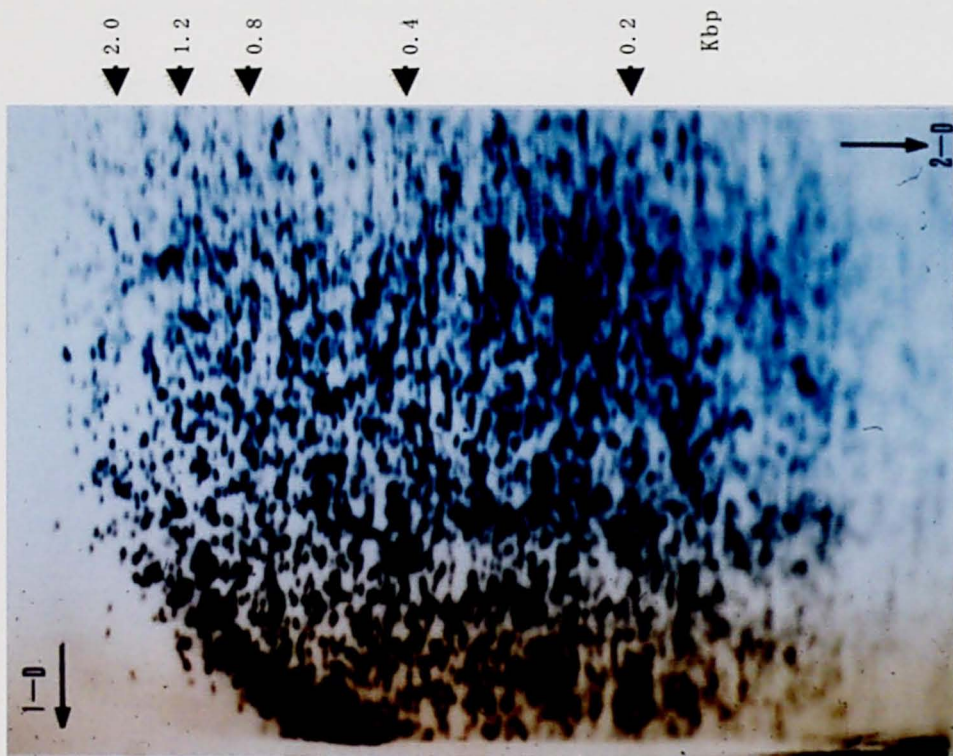
B) Electrophoresis equipment for the second dimension

B



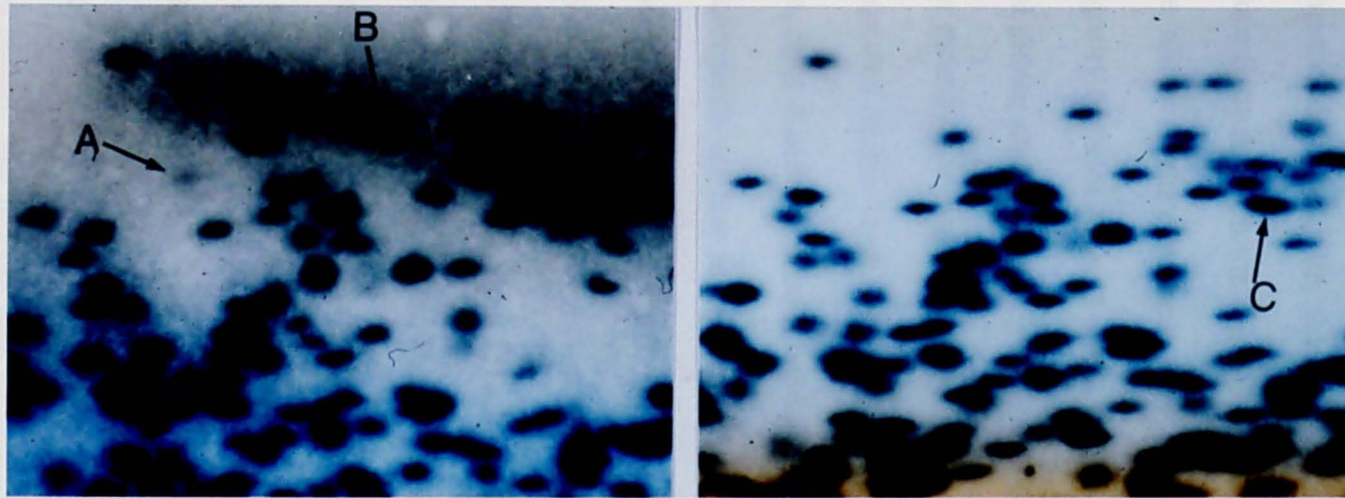


Gimbozu



IM294

Fig. V-2 RLGS profiles of IM294 and Gimbozu



Gimbozu

IM294

Fig.V-3 RLGS spots specific for Gimbozu and IM294, respectively

no relationship between Spot A and *slg*. But the plants with Spot A were superior to those without Spot A in number, irrespective of glume shape, suggesting the high possibility that the plants heterozygous for Spot A did not produce Spot A. Then the segregation ratio expected if Spot A and the wild-type allele at the *slg* locus was tightly linked to each other and heterozygosity for Spot A could not be detected was calculated as follows:

Normal plants; plants with Spot A : plants without Spot A=2.33:4.66=1:2

Slender glume plants; plants with Spot A : plants without Spot A=0:8

These expected ratios were well consistent with their observed ratios. Therefore, Spot A seemed to be related to the *slg* locus. But the plants used in this experiment are few in number. With this respect, therefore, further analysis will be needed. It was difficult to identify Spot B in BC₂F₂ plants, because Spot B often overlapped with a spot below. Spot C was recognized in all the BC₂F₂ plants. Thus Spot C had no relation to *slg*. But it seemed to be related to a cytoplasmic factor, because all the BC₂F₂ plants had this spot peculiar to Gimbozu used as the recurrent parent and pollinator.

5.4. Discussion

RLGS method has been developed mainly through the genome research of mouse. The linkage map of mouse with the RLGS method was constructed using a backcrossing population and RI (Recombinant Inbred)

lines (Hatada et al 1991). It took only two weeks to identify more than one thousand loci covering more than 90% of the mouse genome. Two approaches for detecting sequences of RLGS loci have been developed, one is direct cloning method from the RLGS gels and the other is contaminating method of *NotI* linking clones in genomic DNAs (Suzuki et al. 1994, Okuizumi et al. 1994, Okazaki et al. 1995). RLGS method is thus expected one of the core techniques of genome research in next generation as a high-speed DNA analyzing system.

DNA spots corresponding to and tightly linked to *slg* were not found out in this study. However, Gimbozu and IM294 were very close resembled in RLGS profiles. RLGS analysis is known to be a powerful tool for detecting DNA polymorphisms even between sibling lines. The author used only one combination of restriction enzymes for analysis, but other pairs of restriction enzymes will develop different RLGS profiles. For the future, a larger scale of experiment will be supplied for analysis of relationship between *slg* and the spot A, and screening of DNA spots specific to *slg* will be performed using other enzyme combinations.

Chapter VI

Identification of YAC (Yeast Artificial Chromosome) clones containing the slender glume gene *slg*

6.1. Introduction

Experimental results in Chapter II suggested that the mutability of the slender glume is probably caused by a DNA transposable element. Although many transposable elements have been found in the rice genome (Motohashi et al. 1996; Hirochika et al. 1992), they are quite stable and rarely transpose in the genome of intact plants (Ohtsubo and Ohtsubo 1994; Tenzen et al. 1994; Bureau et al. 1996; Motohashi et al. 1996), excepting for retrotransposon *Tos17* when grown under tissue culture conditions (Hirochika et al. 1996). Therefore, successful cloning of a transposable element that presumably confers the mutability of the slender glume will advance our knowledge of rice genome evolution as well as facilitating the efficient genetic engineering, such as transposon tagging, of the rice genome.

The ultimate goal is to understand whether the mutable slender glume is associated with the insertion of a transposable element utilizing map-based cloning. In Chapter IV, the chromosome location of the *slg* locus

on an RFLP (Restriction Fragment Length Polymorphism) genetic map was identified. Following a further RFLP analysis based on the results of the Chapter IV, the author attempts to construct a YAC (Yeast Artificial Chromosome) contig around the *slg* locus and identify the YAC clones containing this locus.

6.2. Materials and Methods

6.2.1. Plant material

The slender glume mutant (mutant line IM294), which was induced with gamma-ray irradiation to seeds of the *japonica* rice variety 'Gimbozu', was crossed with a line 'ML17'. The F₂ population was used for the linkage analysis to determine the location of the *slg* locus on the RFLP-based genetic map. There are large numbers of RFLPs between *indica* and *japonica* rices (McCouch et al. 1988; Saito et al. 1991; Kurata et al. 1994), but they are rarely seen among *japonica* varieties (Zhang et al. 1992), which indicates that an *indica* variety must be used as a cross parent in the RFLP analysis. Besides, most *indica* varieties have slender glumes, while most *japonica* varieties have normal (round) glumes. Therefore, the segregation of glume shape in F₂ from crosses between *indica* varieties is too complicated for analysis. To overcome such problems, we produced a *japonica*-type round glume line ML17 having several Kasalath-derived DNA segments near the *slg* locus. ML17 was derived from the backcross

involving 'Nipponbare' and 'Kasalath' (*japonica* and *indica* varieties, respectively). A total of 250 F₂ plants were grown at an experimental paddy field at Kyoto University, Kyoto, Japan, and were examined for glume shape at maturity.

6.2.2. RFLP analysis

Total genomic DNAs were extracted from leaves sampled before flowering time using the CTAB method (Murray and Thompson 1980). Extracted DNAs were digested with eight restriction enzymes, *Bam*HI, *Bgl*III, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, and *Xba*I. After electrophoresis, the DNAs were blotted onto a positively charged membrane (Hybond N+, Amersham), and were subjected to Southern hybridization. The *slg* locus has already been found to be located between two RFLP loci, *XNpb20* and *XNpb33*, on chromosome 7, with recombination values of 3.0 and 3.2%, respectively. In addition to these two RFLP loci, three additional RFLP loci, *R643*, *R1440*, and *R646*, all of which are located between *XNpb33* and *XNpb20*, were used as probes.

6.2.3. Physical mapping

First, colony hybridization was performed using two RFLP loci, *XNpb33* and *R1440*, as probes to select out candidate clones from a YAC library (a total of 7000 YAC clones) provided on five high density colony membrane filters. The YAC library used in the present study was

constructed from the genomic DNA of the *japonica* rice variety 'Nipponbare'. This is the only one YAC library so far constructed and characterized in rice (Umehara et al. 1995). The candidate YAC clones were isolated with a contour-clamped homogeneous electrophoresis (CHEF, BioRad), and were subjected to Southern hybridization with the RFLP probes to ascertain if they contained either of the two RFLP loci. The end fragments of the positive YAC clones thus selected were amplified on both sides by the inverse PCR (IPCR) method, as described by Umehara et al. (1995). For the chromosome walking toward the *slg* locus, the YAC end fragments isolated were used as probes for the linkage analysis of *slg*. The closer end to the *slg* locus of each YAC clone was used as a new probe for colony hybridization to identify the YAC clones much closer the *slg* locus. When YAC end clones isolated by the IPCR method did not show polymorphism between the parents, the plasmid rescue method of Hermanson et al. (1991) was applied to obtain longer fragments containing polymorphic regions between the parents.

6.3. Results

6.3.1. Fine genetic map of the *slg* locus

The *slg* locus proved to be located between the two RFLP loci, *XNpb33* and *XNpb20*, on chromosome 7. Although the marker density of the

chromosomal region between these two RFLP markers is quite low (Kurata et al. 1994), there are three other known RFLP loci, *R643*, *R1440*, and *R646*, between them. The linkage relationships of these three loci, in addition to *XNpb33* and *XNpb20*, with the *slg* locus were investigated. The results showed that the *slg* locus is located between *XNpb33* and *R1440*, with recombination values of 3.1 and 1.0%, respectively (Fig. VI-1).

6.3.2. Construction of a YAC contig for the *slg* locus

Since the two RFLP loci, *XNpb33* and *R1440*, were found to be located most near the *slg* locus, we performed colony hybridization using these two RFLP loci as probes, and selected out candidate YAC clones from the YAC library provided. After isolated by CHEF, the candidate YAC clones were blotted onto membranes to ascertain if they contained the DNA fragments corresponding to the above RFLP probes. The YAC clones that hybridized with the above RFLP probes were regarded as positive clones. We could select out one positive (Y4270) and four positive clones (Y5080, Y6038, Y0768, and Y4693) containing *XNpb33* and *R1440*, respectively (Fig. VI-1). Then, both end fragments of all five positive clones were amplified by the IPCR method. Most of the end fragments amplified showed clear polymorphism between the parents (Fig. VI-2).

The left end fragment of Y4270 (designated Y4270L) was found to be more closely linked to the *slg* locus than the right end fragment

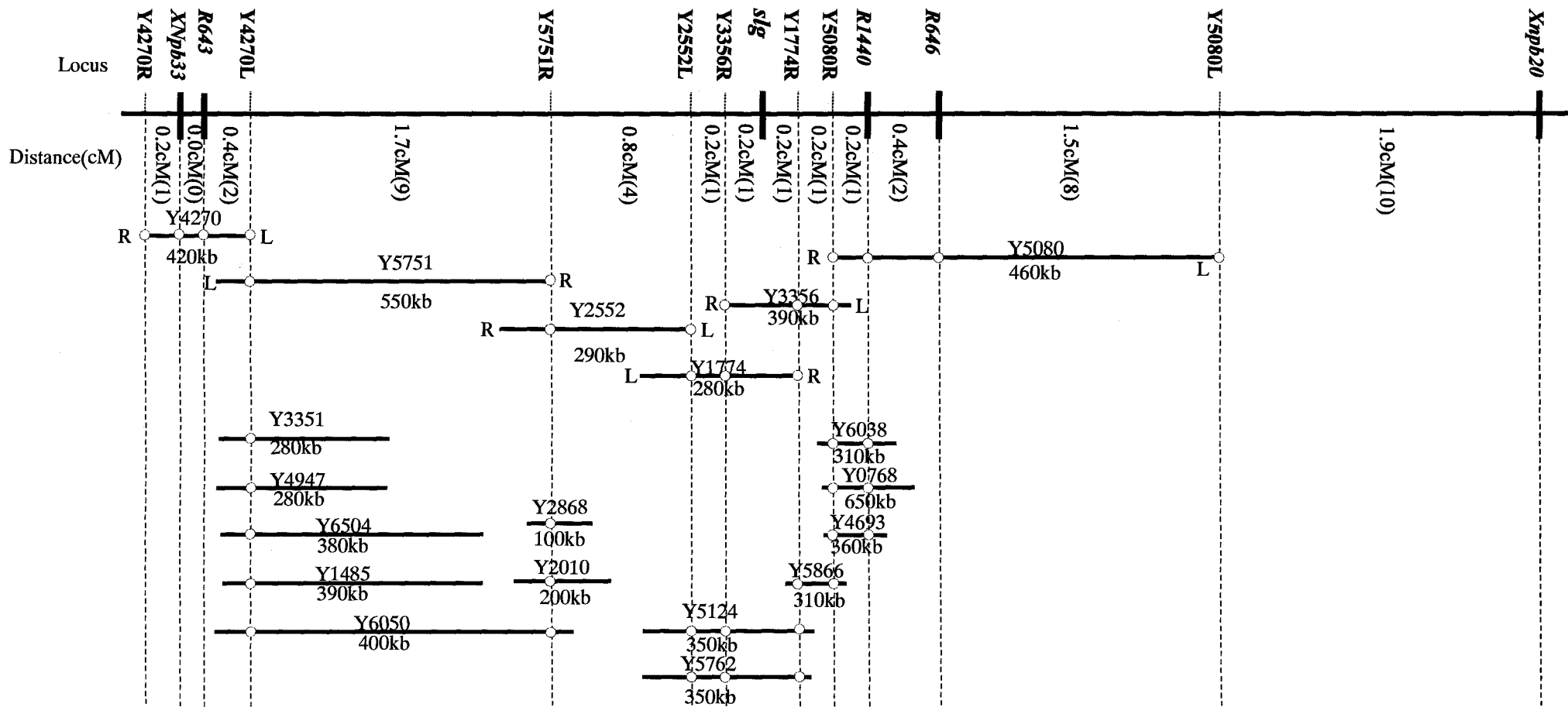


Fig.VI-1 YAC contig map of the region spanning the *slg* locus. Linkage relationships between the *slg* locus and surrounding RFLP markers are also shown

designated Y42703. Using Y42703 as a new probe, colony hybridizations were performed again. Consequently, six clones, Y1485, Y1501, Y4887, Y1751, Y1820, and Y1929 were found to hybridize with Y42703 (Fig. VI-2). After selecting Y1551, Y1802, and Y1910 with the same procedure, finally three YAC clones, Y1774, Y1824, and Y1762 were selected out by using the left end fragment of Y1551 as a probe.

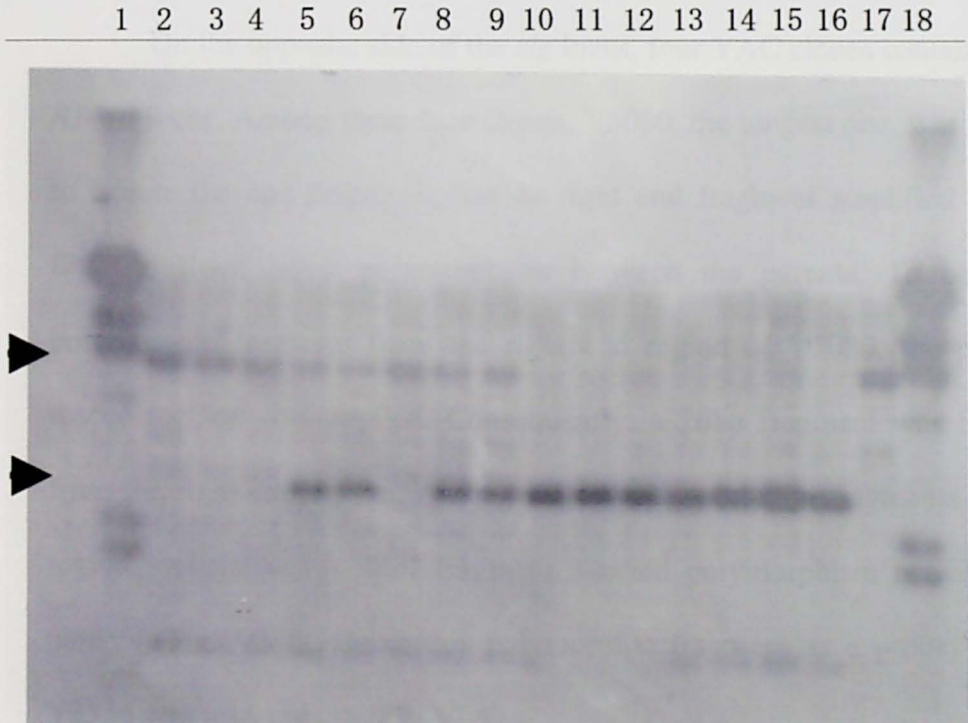


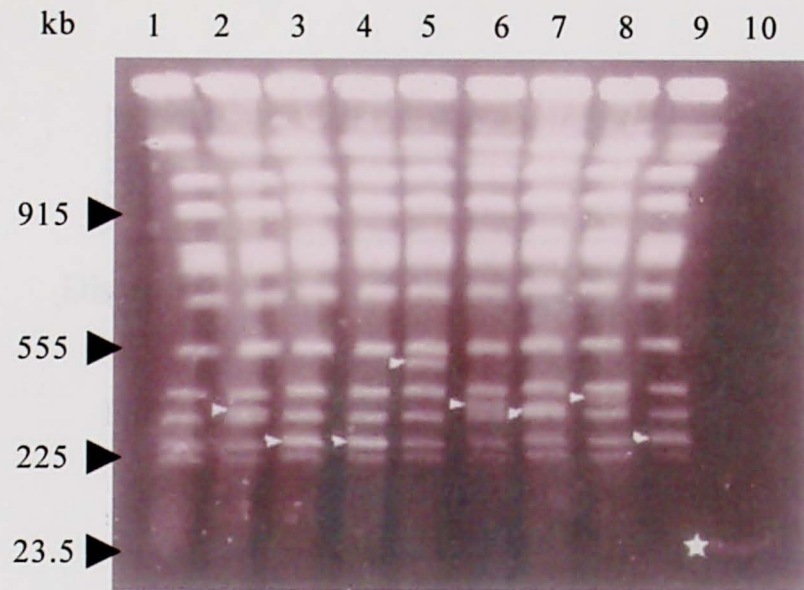
Fig. VI-2 An instance of Southern hybridization patterns with YAC end fragments. DNAs digested with *EcoRV* were hybridized with the right-end fragment of the YAC clone Y3356 amplified by IPCR. Lanes 1 and 18 are the molecular weight marker, λ DNA digested with *Hind*III. Lanes 2-9 are non-slender glume F₂ plants derived from the cross between IM294 and 'ML17'. Lanes 10-13 are slender glume F₂ plants derived from the same cross. Lane 14 is IM294, a slender glume mutable line of 'Gimbozu'. Lanes 15, 16, and 17 are 'Gimbozu', 'Nipponbare', and 'Kasalath', respectively. Arrowheads indicate RFLP bands tightly linked to the *slg* locus.

(designated Y4270R). Using Y4270L as a new probe, colony hybridization was performed again. Consequently, six clones, Y1485, Y3351, Y4947, Y5751, Y6050, and Y6504 were found to hybridize with Y4270L (Fig.VI-3). After selecting Y2552, Y2868, and Y2010 with the same procedure, finally three YAC clones, Y1774, Y5124, and Y5762 were selected out by using the left end fragment of Y2525 as a probe.

On the opposite side of the *slg* locus, four YAC clones contained the *R1440* locus. Among these four clones, Y5080, the longest one, was chosen to isolate the end fragment. But its right end fragment amplified by the IPCR did not show polymorphism between the parents. To obtain a polymorphic fragment from the right end region of Y5080, the plasmid rescue method was applied. Consequently, a 20kb fragment was isolated from the right end region. Among the several restriction fragments of this region, only a *SalI* - *XhoI* fragment showed polymorphism between the parents (Fig. VI-4). Using this polymorphic fragment as a probe, finally Y3356 was selected out (Fig VI-5).

Among the four YAC clones selected, Y3356 and Y1774 were amplified for their right end fragments. As a result of hybridization, it was found that Y3356R hybridized with Y1774, Y5124, and Y5762, while Y1774R hybridized with Y3356, Y5124, and Y5762. Clone Y5080 contained *R1440*, and Y5080L end was further from the *slg* locus than *R1440*, thus a YAC contig of about 6cM between Y4270R and Y5080L was

A



B

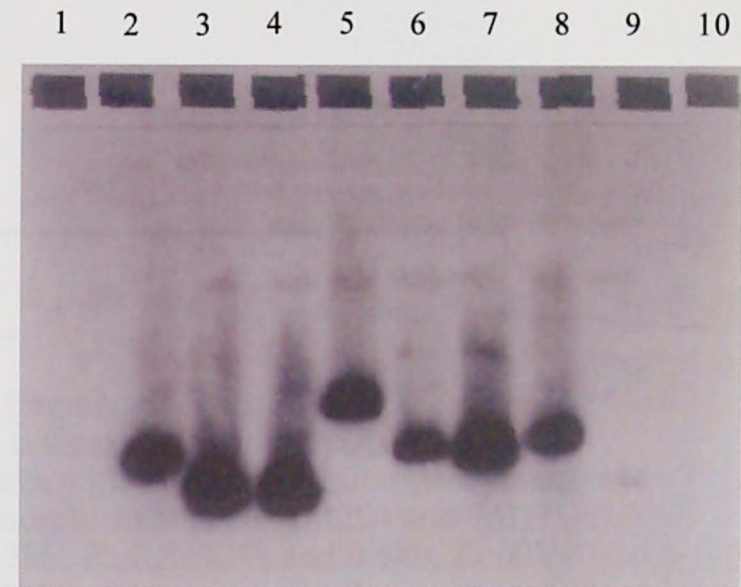


Fig. VI-3 Identification of yeast artificial chromosomes(YACs) containing left end clone of Y4270 (Y4270L). After YAC clones were separated with electrophoresis (A), they were blotted onto the membrane for the Southern analysis (B). **A:** Ethidium bromide-stained YAC DNA selected with Y4270L after separated by CHEF gel. Arrowheads indicate the position of YAC clones. YAC clones of Y3551, Y4947, Y6504, and Y1414 were not clearly separated from one of the yeast chromosomes under this electrophoresis conditions. An asterisk mark on Lane 10 indicates 23.5kb fragment of λ DNA digested with *Hind*III. **B:** Autoradiogram showing YAC clones hybridized with the Y4270L. Solid squares indicate well positions. No positive signal was detected on the Lane 9, Y1414. Lane 1 is *Saccharomyces cerevisiae* without YAC. Lane 2-9 are *S. cerevisiae* containing Y1485, Y3551, Y4947, Y5751, Y6050, Y6504, Y4270, and Y1414, respectively. Lane 10 is λ DNA digested with *Hind*III.

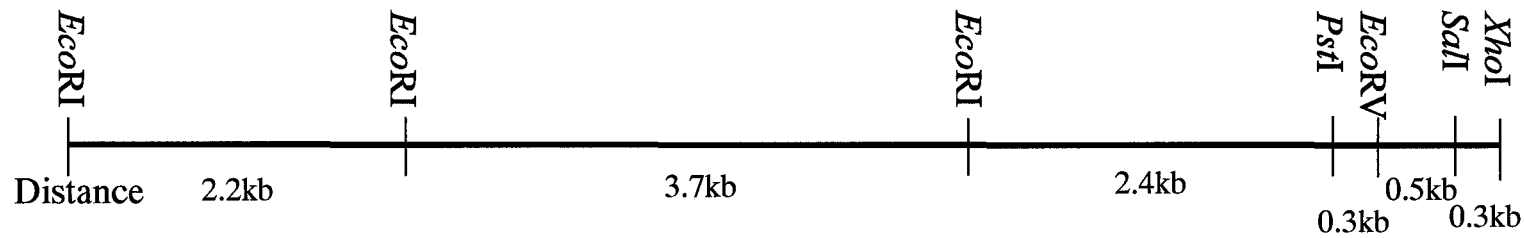


Fig. VI-4 The structure of the right-end fragment of Y5080. The fragment was acquired by the plasmid rescue method. The left end in this figure, *i.e.*, *EcoRI* site, is the right end of the YAC insert. The 2.2kb *EcoRI-EcoRI* fragment was divided into three pieces with 4-base recognition enzymes, and the hybridization with each piece produced no polymorphism between IM294 and 'M117'. Fragment of 3.7kb, *EcoRI-EcoRI*; 2.4kb, *EcoRI-PstI*; 0.3kb, *PstI-EcoRV*; and 0.5kb, *EcoRV-SalI* also did not show polymorphism. Only a 0.3kb *SalI-XhoI* fragment showed polymorphism.

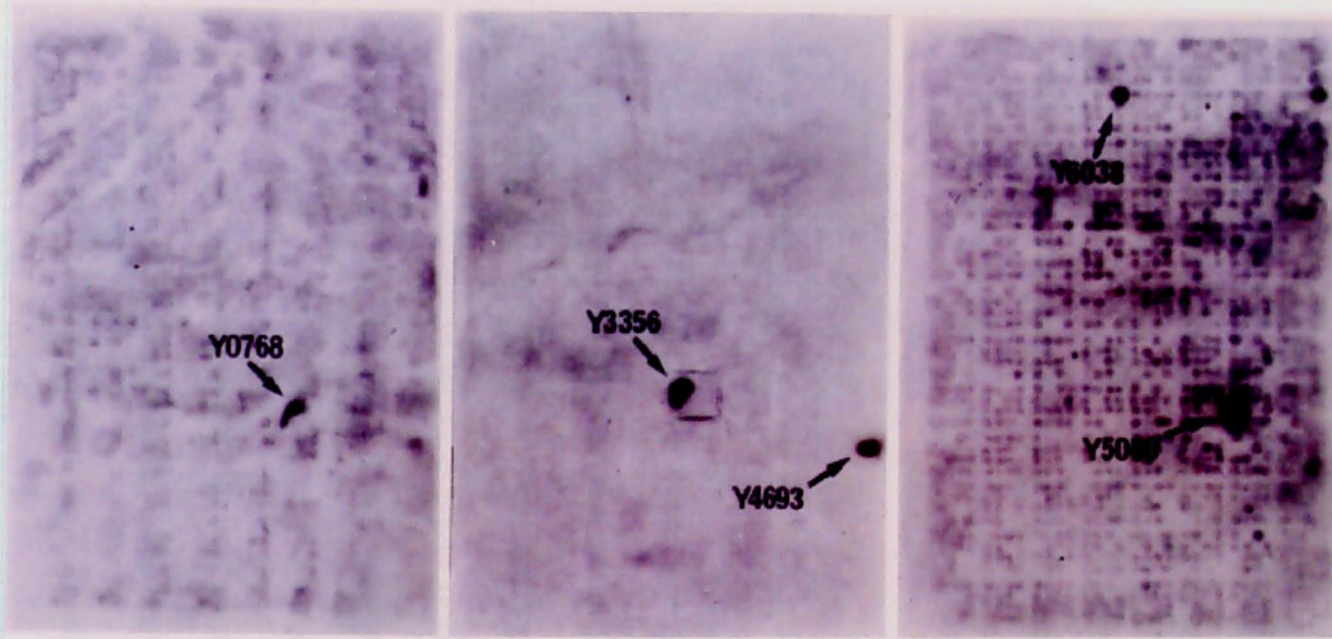


Fig.VI-5 Autoradiogram of high-density colony membrane filters that were probed with [32 P]-labeled Y5080R. Positive signals were observed at corresponding of Y0768, Y3356, Y4693, Y5080, and Y6038. The positive signal corresponding to Y5866 was not detected on this filter.

constructed (Fig. VI-1). The relative locations of the *slg* locus and its adjacent markers were as follows; Y2552L - Y3356R - *slg* - Y1774R - Y5080R. Since the four YAC clones, Y3356, Y1774, Y5124, and Y5762, all contain both Y3356R and Y1774R, it is obvious that the *slg* locus is present in all these clones. Among the four clones, Y1774 showed a minimum size of 280kb. Therefore, the location of the *slg* locus was narrowed down to the region with a physical distance of less than 280kb between Y3356R and Y1774R.

6.4. Discussion

Yoshimura et al. (1996) and Houten et al. (1996) demonstrated that the integration of a high resolution genetic map (Kurata et al. 1994) and a YAC library (Umehara et al. 1995) could greatly enhance the efficiency of map-based cloning of target genes in rice. In both the reports, the YAC clones involving the target gene were successfully identified through repeated colony hybridization using five membranes of a YAC library. Using these YAC clones as hybridization probes, Yoshimura et al. (1998) identified corresponding cosmid and/or cDNA clones constructed from the variety carrying the target gene *Xa1* (*Xanthomonas campestris* pv. *oryzae* resistance-1), which led to the successful cloning of this gene (Yoshimura et al. 1998). The author has already selected out about 100 cDNA clones that

hybridize with the YAC clone Y1774 from a cDNA library of the rice variety 'Nipponbare'. Since this variety is closely related to 'Gimbozu' and has normal glumes, some of these cDNA clones are expected to be available for selecting out cosmid clones containing *slg* and its revertant, from the cosmid libraries of the slender glume mutant and its revertants, respectively. Comparison of DNA sequence among the cosmid clones isolated for the mutant, revertant and wild-type alleles respectively will make it possible to understand if the mutability of the *slg* locus is conferred by the insertion of a transposable element.

According to Kurata et al. (1997) (cf. <http://bank.dna.affrc.go.jp:82/Publicdata.html>), there is a gap in YAC contigs between the two RFLP loci, *R643* and *R1440*, corresponding to the region of the author's interest. The marker density of this region (centromere region on chromosome 7) is rather lower than the average (a DNA marker per 190 kb). For that reason, unlike Yoshimura et al. (1996) and Hauten et al. (1996), the author could not construct a YAC contig simply by colony hybridization. To identify the target YAC clone, the author had to 'walk' on the chromosome several times. Fortunately, the density of repetitive sequences in this region of the rice genome is low enough to walk toward the *slg* locus. Most of the YAC end fragments isolated with the IPCR method showed polymorphism between the parents. Although many chimeric YAC clones are involved in this library (Umehara et al. 1995), most of the clones selected in the present

study were not chimeric. Therefore, the YAC library used in the present study is valuable for chromosome walking even when there are not adequate molecular markers tightly linked to the target gene.

From the genetic distance of Y4270 (420kb) and that of Y5080 (460kb), the ratios of physical distance to genetic distance (kb/cM) of those regions were estimated to be 700kb/cM and 210kb/cM, respectively. Umehara et al. (1995) demonstrated that the value of kb/cM largely depends on chromosomal region. The present study showed that even in a narrow region, there could be great positional differences.

Rice is considered as the model plant to study the genome structure of cereals because of its small genome size (ca. 430Mb) and low density of repetitive sequences. If the function and structure of rice genes are verified, the molecular biological analysis on their homoeologous genes present in cereals, such as wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.), will be much advanced. Therefore, the prompt development of effective methods for the functional analysis of rice genes is required. The map-based cloning method has already been utilized in rice, but it is still laborious and time-consuming (Yoshimura et al. 1998). Transposon tagging will be an alternative method because of its high efficiency. Actually, many genes have already been cloned and characterized using this method (Kunze et al. 1997). To date, many transposable elements have been reported in rice. Most of them, however, transpose very infrequently in the intact plant. Only

a retrotransposon, *Tos17*, often transposes during callus induction *in vitro* (Hirochika et al. 1996), but there are currently no other effective ways to stimulate the activity of transposable elements in rice. With the aim of applying the transposon tagging technique in rice, many researchers have attempted to introduce maize transposable elements, such as *Ac-Ds*, into the rice genome, but the efficiency of mobilization is still quite low (Izawa et al. 1991; Sugimoto et al. 1994). We have already shown that morphological and physiological mutants appear in the self-progenies of the revertants of the slender glume to the wild state. This strongly suggests that the mutability of *slg* is conferred by the insertion of a DNA transposable element. If true, successful cloning of the *slg* locus will advance the efficient genetic engineering of the rice genome.

7. Summary

A slender glume mutant (line IM294) was induced by gamma-ray irradiation of seeds of the *japonica* rice variety Gimbozu. This mutant character is probably controlled by a single recessive mutant gene. But the mutation has never been fixed genetically in spite of repeated self-propagation: in successive generations, not only normal plants but also plants chimeric for glume shape almost always appear with low frequency. Such a phenomenon is likely to be due to the mutability of the mutant slender glume gene(s), which occasionally reverts to its wild-type (normal glume) state. In higher plants, there are many reports on mutable traits especially for genes involved in pigmentation and endosperm quality. Recent molecular biological analyses have revealed that many such mutable traits are controlled by transposable elements or result from epigenetic transformation. This suggests that the mutability of the *slender glume* may also be caused either by a transposable element or by epigenetic transformation. Since reports on mutable morphological traits are few, the identification of genetic factor(s) controlling the mutability of the slender glume mutation could advance our knowledge of transposable elements or epigenetic transformation and of complicated morphogenetic processes in rice. From these points of view, the following experiments were performed to elucidate the inheritance of the slender glume mutation. First, the genetic

factor(s) controlling the slender glume phenotype and its mutability were investigated. Second, glume-shape chimeras caused by the mutability of the slender glume mutant gene were morphologically analyzed. Third, the chromosomal location of the slender glume mutant gene was determined by RFLP (Restriction Fragment Length Polymorphism) analysis, following trisomic and conventional linkage analysis. Southern blot analyses with known rice transposable elements were also performed to investigate whether the mutability of the slender glume mutation is caused by one of these elements. Fourth, DNA spots specific to the slender glume mutant gene were explored using RLGS (Restriction Landmark Genomic Scanning) analysis. Finally, following a further RFLP analysis, a YAC (Yeast Artificial Chromosome) contig around the slender glume mutant gene was constructed, and the YAC clones containing the mutant locus were identified.

1. Genetic analysis of the slender glume mutation

To investigate the genetic factor controlling the slender glume phenotype, progenies from crosses of the mutant line IM294 with several varieties were subjected to genetic analysis. Effects of crossing, backcrossing or cytoplasmic factors on the progeny of reversion from the mutation to its wild-type state were also examined. The results showed that the mutation is controlled by a single recessive, mutable mutant gene *slg*.

The frequency of reversion of *slg* to its wild-type state was little affected by crossing, backcrossing, genetic background or cytoplasmic factors.

2. Morphological analysis of glume-shape chimeras caused by the mutability of the slender glume gene *slg*

Chimeras for glume shape that appeared in the selfed-progenies of slender glume plants were morphologically analyzed. Experimental results showed that all the chimeras were caused by the reversion of the mutable slender glume gene *slg* to its wild-type state, which occur throughout the whole growing stage of plants. From the analyses on the relation between tillering order and the shape of glume on panicle, it was found that periclinal chimera occurred in some of the slender glume plants, which was confirmed by progeny tests. In periclinal chimera plants, glume shape was normal, but embryo has non-mutated genotype *slg/slg*. Since the chimerism is maintained quite stable, it was assumed that the apical cell layers are rigidly differentiated in rice. Thus, chimeric plants were found to be good materials for investigating cell-to-cell communication in rice.

3. RFLP (Restriction Fragment Length Polymorphism) mapping of the slender glume gene *slg*

The chromosomal location of the mutant gene *slg* was investigated by RFLP analysis, following trisomic and conventional linkage analysis. Southern blot analyses with known rice transposable elements were also performed. Conventional trisomic and linkage analyses revealed that the *slg*

locus was located close to the *rfs* (rolled fine stripe leaf) locus on chromosome 7. In a subsequent RFLP analysis, *slg* was found to be located between the two RFLP loci *XNpb20* and *XNpb33*, with recombination values of 3.0 and 3.2%, respectively. Southern analysis indicated that the mutability of *slg* is caused by none of the known transposable elements in rice. From these results, it is inferred that *slg* has a novel transposable DNA insert in its vicinity, which was possibly activated by gamma-ray irradiation.

4. RLGS (Restriction Landmark Genomic Scanning) analysis of the slender glume gene *slg*

The RLGS method was applied to identify the DNA fragments specific to or closely linked to *slg* necessary for the positional cloning method of *slg*. IM294, a slender glume line (*slg/slgl*), its original variety Gimbozu, and the F₂ population following two-times backcrosses of Gimbozu to the F₁ from IM294/Gimbozu (BC₂F₂) were analyzed. About 2,500 DNA spots were observed each in IM294 and Gimbozu. Most of the DNA spots detected proved to be common to both the parents, but two spots (A and B) were specific to Gimbozu and one spot (C) to IM294. The comparison of these three spots between slender-glume BC₂F₂ plants and non-slender-glume BC₂F₂ plants suggested that Spot A was linked to *slg*. However, any relationships between the other two spots and *slg* were not found out. These result indicate the necessity of confirming the relationship between *slg* and Spot A as well as that of detecting the polymorphic RLGS

spots linked to *slg* using different kinds of combinations of restriction enzymes.

5. Identification of YAC (Yeast Artificial Chromosome) clones containing the slender glume gene *slg*

The final goal was to understand whether the slender glume mutation was associated with the insertion of a transposable element, utilizing map-based cloning techniques. A further RFLP analysis revealed that the *slg* locus was located between two RFLP loci, *XNpb33* and *RI440*, on chromosome 7 with recombination values of 3.1% and 1.0%, respectively. Using these two RFLP loci as probes, five YAC clones containing either of these two loci were selected from a YAC library. Subsequently, both end fragments of these YAC clones, amplified by the inverse PCR (IPCR) method, were used to select new YAC clones more closely located to the *slg* locus. After repeating such a procedure, a 6-cM YAC contig was successfully constructed, and four overlapping YAC clones, Y1774, Y3356, Y5124, and Y5762, covering the *slg* locus were identified. The chromosomal location of the *slg* was narrowed down to the region with a physical distance of less than 280 kb between the right-end fragments of Y1774 and Y3356.

The result of the present study showed the high possibility that a DNA transposable element is inserted in the slender glume gene *slg* induced

by gamma-ray irradiation. In rice, many transposable elements so far have been reported, but most of them do not have transposition activity indispensable for transposon tagging technique. A transposable element inserted in *slg*, if so, has strong transposition activity; therefore, use of this transposable element could open a way for the efficient gene cloning system and could advance our knowledge of the rice genome. In this sense, the cloning of *slg* appears to be the most urgent necessity. But it will be realized in near future, because four YAC clones containing *slg* locus were identified in the present study. The result of the morphological analysis of chimeric plants induced by the mutability of *slg* indicated that the shoot apical meristem of rice was differentiated into two cell layers. This suggests that the *slg* is a useful material for investigation of morphogenesis of the rice plants.

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9. Summary (in Japanese)

和文摘要

ガンマ線照射によって誘発されたイネ易変性細粒突然変異の遺伝

イネ品種銀坊主のガンマ線種子照射によって誘発された細粒突然変異体の後代系統（細粒系統）は、自殖を繰り返しても固定せず、毎世代若干数の非細粒個体および細粒と非細粒からなる粒形キメラ個体を分離する。このような細粒突然変異の易変性は、細粒突然変異遺伝子に挿入されたトランスポーザブルエレメントによる可能性がきわめて高いが、これを証明するためには細粒突然変異の遺伝様式を明らかにするとともに、関与遺伝子をクローニングしその分子構造を解析する必要がある。本研究は、このような観点から、以下に述べる5つの実験を行った。

1. 細粒突然変異形質の遺伝解析

細粒突然変異に関与する遺伝因子を分析するために、細粒突然変異体（IM294 系統）と銀坊主、コシヒカリ、日本晴および台中 65 号との交雑 F_2 集団および戻し交雑 F_2 集団を供試して細粒個体の分離比を調査した。その結果、細粒の分離比は 10~22%（総平均 15%）となり、細粒性が 1 劣性遺伝子に支配されているときに期待される値 25%より低かった。しかしながら、細粒個体は非細粒個体に比べ発芽能力および幼苗時の活性が劣ること、IM294 と銀坊主を 1:3 の比で混合した種子集団で細粒個体の分離頻度が 15%程であったこ

と、 F_2 非細粒個体の次代系統では非細粒個体のみの系統と細粒と非細粒が分離する系統の比が 1:2 であったことから、細粒形質は1つの劣性遺伝子 *slg* によって支配されていることが明らかになった。また、その易変性は戻し交雑、遺伝的背景の影響を受けないことが明らかになった。ついで、上記 F_2 集団のうち、細粒個体の後代系統 (F_3 , F_4 細粒固定系統) を供試して、非細粒個体および粒形キメラ個体の分離出現頻度を調べたところ、どちらの頻度も 1% 未満であった。これらの結果と、細粒から正常粒への復帰突然変異に伴って様々な突然変異が誘発されることから、細粒突然変異遺伝子 *slg* の易変性は DNA 型トランスポーザブルエレメントが関与している可能性がきわめて高いと考えられた。

2. 細粒遺伝子の易変性に伴って生じる粒形キメラの形態学的解析

細粒個体の自殖後代に出現した粒形キメラについて形態学的な分析を試みた。その結果、キメラはすべて細粒遺伝子 *slg* の正常遺伝子への復帰変異によるものであり、その変異は全生育期間にわたって生じていることが明らかになった。粒形キメラの後代を供試して、分けつと粒形の関係について調べたところ、周縁キメラがいくつかのキメラ個体で起こっていることが認められた。これら周縁キメラはすべて粒形が正常である (復帰変異している) にもかかわらず、胚の遺伝子型は未変異 (*slg/slg*) であった。これらのことから、粒形キメラはイネの細胞間の相互関係を調査する上で好適な材料であると考えられた。

3. 細粒遺伝子 *slg* の RFLP (制限酵素断片長) 分析

従来の遺伝子クローニング法は、蛋白質やRNAなどの遺伝子産物を同定したのち、それを目印にして cDNA ライブラリーから目的とする遺伝子を含むクローンを単離するという手順を経た。しかしながら、近年様々な分子遺伝学的手法が確立され、遺伝子産物に関する情報が無くても染色体上を歩行すること、いわゆる map-based クローニングにより遺伝子を単離することが可能になった。そこで、トリソミック分析および形質マーカーを用いた連鎖分析により *slg* 座が染色体7の *rfs* 座近傍に位置することを明らかにした後、RFLP分析を行い、RFLP連鎖地図上への *slg* のマッピングを行った。イネのRFLP分析では、通常、多型が多く観察されるようにインディカとジャポニカ間のような亜種間の交雑 F_2 を用いる。しかし、 F_2 において細粒系統由来の細粒性とインディカ品種由来の細粒性を判別することが困難であったため、日本晴/カサラス//日本晴の後代の中で日本晴型由来の粒形 (ジャポニカ型、短粒) をもち、染色体7の *rfs* 座近傍にカサラス由来のDNA断片をもつ個体 (ML17) を選抜し、これと IM294 との交雑 F_2 集団を用いて RFLP 分析を行った。*rfs* 座近傍の RFLP マーカーをプローブにして RFLP 分析を行ったところ、*slg* 座は RFLP マーカー遺伝子座 *XNpb33* と *R1440* との間に座乗していることが明らかとなった。次に、*slg* と既知のイネトランスポザブルエレメントとの関係を明らかにするために、*Tos* レトロトランスポザブルエレメント群および *RAc* 因子

をプローブとしてサザン解析を行い、IM294 と原品種間で多型の存在を調べた。もし *slg* 遺伝子内にこれらの既知トランスポーザブルエレメントが挿入されていれば IM294 と原品種間で多型が、また *slg* 座近傍にトランスポーザブルエレメントが存在すればこれと連鎖する多型が検出されるはずである。しかし、シングルバンドからマルチプルバンドまで様々なバンドパターンがみられたが、*Tos17* と制限酵素 *XbaI* の組み合わせで *slg* と連鎖するバンドが検出されたほかは多型を全く検出することができなかった。また、 F_2 集団を供試して *Tos17* をプローブにして RFLP 分析を試みたが、泳動距離が短いこともあり多型を検出することができなかった。すなわち、*slg* 遺伝子は今回用いたトランスポゾン因子とは関係のないことが明らかになった。

4. 細粒遺伝子 *slg* の RLGS 分析

RFLP や RAPD 等の DNA 多型検出技術は遺伝子をマッピングに広く利用されているが、これらの技術では、遺伝的背景が非常に似かよった材料間では多型を検出することが難しく、一工程で観察することのできる遺伝子座の数も少ない。近年開発された RLGS 法 (Restriction Landmark Genomic Scanning Method) は、同時に 2000~3000 個の DNA スポットを検出・比較でき、極めて高い DNA 多型検出能をもつ。そこで IM294 と原品種を供試して RLGS 分析を行い、*slg* 座特異的 DNA 部位の獲得を試みた。銀坊主と IM294 との間で RLGS 像を比較したところ、双方ともスポッ

ト総数は約2500であり、従来イネで観察されているスポット数とほぼ一致した。RLGS像を詳細に調べたところ、ほぼ全てのスポットがIM294と銀坊主間で共通であったが、銀坊主に特異的なスポットが2個、IM294に特異的なスポットが1個確認された。これらスポットが*slg*座と連鎖するかどうか調べるために、銀坊主とIM294との交雑F₂から非細粒個体7個体および細粒個体8個体を選んでDNAを抽出し、RLGS分析を行ったところ、これらDNAスポットの1つと*slg*座との間に連鎖関係のあることが示唆された。今後は、交雑F₂の個体数を多くしてこの連鎖関係を詳細に解析するとともに、本実験とは異なる制限酵素を用いて*slg*座に特異的なDNAスポットを探索する必要があると考えられた。

5. 細粒遺伝子*slg*を含むYAC（人工酵母染色体）の同定

イネYACライブラリーの利用による*slg*のmap-basedクローニングを意図して、*slg*座近傍の細密連鎖地図の作成を試みた。*slg*座は染色体7のRFLP遺伝子座*XNpb33*および*R1440*との間に位置していることが判明している。そこで、まず*XNpb33*もしくは*R1440*を含むYACクローンをスクリーニングし、さらにこれらクローンと重なり合うYACクローンをスクリーニングしていくという手順によって、*slg*座を含む*XNpb33-R1440*間をYACクローンで埋めていくことを試みた。その結果、この領域が少なくとも6つのYACクローンによりつながることが明らかになった。各YACクローンの挿入ゲノム断片長は100~700kb、平均400kbであった。IPCRで

獲得された Y A C クローン末端はプローブとしてサザン分析に用いてもほぼ全てがシングルバンドを示し、かつインディカージャポニカ間で多型を示した。唯一 IPCR 法で得られた Y5080 の右末端が多型を示さなかったが plasmid rescue 法により多型を示す断片を得ることができた。この領域は反復配列の少ない領域であると考えられた。IM294 と銀坊主との交雑 F₂ 250 個体を用いて RFLP 分析を行ったところ、*slg* 座は Y3356 右末端と Y1774 右末端の間に座乗し、*slg* 座との遺伝的距離はどちらも 0.2 cM であった。以上より、*slg* 座は Y3356 と Y1774 がオーバーラップしている領域 (280 kb 内) に存在することが判明した。今後はこの領域に存在する cDNA クローンをスクリーニングして *slg* 遺伝子そのものを探索するか、もしくはコスミドゲノムライブラリーを構築することによって更に細密な地図を作成し、領域を狭めていく必要があると考えられた。

本研究の結果、ガンマ線照射によって誘発されたイネ細粒遺伝子 *slg* には DNA 型トランスポゾン因子が挿入されている可能性がきわめて高いことが示された。イネにおいてはこれまで多数のトランスポゾン因子が発見されているが、これらはいずれも転移活性をもたないことから、それらを利用するトランスポゾンタギング技術はまだ確立されていない。*slg* に挿入されていると考えられるトランスポゾン因子は明らかに転移活性をもつ。したがって、これを利用したトランスポゾンタギングはイネの遺伝子単離並びにゲノム研究にきわめて有効な手法になると考えられる。そのためには、*slg* のクロ

ーニングを急がねばならぬが、*slg* を含むYAC（人工酵母染色体）クローンを同定することができたことから、*slg* のクローニングは時間の問題と考えられる。また、*slg* 遺伝子の易変性によって誘発されたキメラ個体の形態学的観察から、イネの頂端分裂組織には少なくとも二つの細胞層が存在することが明らかになった。したがって、*slg* はイネの形態形成に関する研究にもきわめて有用な材料になると考えられた。

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