九州大学学術情報リポジトリ
Kyushu University Institutional Repository

Map-based cloning of Dwarf 1 gene in rice (Oryza sativa L.)

芦苅, 基行
九州大学農学研究科遺伝子資源工学専攻

https://doi.org/10.11501/3150676

出版情報：九州大学, 1998, 博士（農学）, 課程博士
バージョン：
権利関係：

Map-based cloning of *Dwarf* 1 gene in rice (*Oryza sativa* L.)

Motoyuki Ashikari

1999

Map-based cloning of *Dwarf 1* gene in rice (*Oryza sativa* L.)

Motoyuki Ashikari

1999

Contents

General Introduction ---------- 1

Chapter 1. RFLP linkage mapping of *d1* locus

Introduction ---------- 6
Materials and Methods---------- 7
Results ----------13
Discussion ----------20

Chapter 2. Physical mapping of *D1* gene

Introduction ----------23
Materials and Methods----------24
Results ----------28
Discussion ----------36

Chapter 3. Cloning of *D1* gene

Introduction ----------39
Materials and Methods ----------40
Results ----------43
Discussion ----------49

Summary and Conclusion----------54

Acknowledgment ----------57

References ----------59

Appendix ----------67

General Introduction

Rice (*Oryza sativa* L.) is the most important food crop in the world, since it provides the main source of food for 50 % of the world's population. Especially in Asia, rice has become the staple food and major cultivated crop for Asian people in long period because it is an excellent food source that grows favorably in warm and humid climate. In fact more than 90 % of all rice is produced and consumed in Asia. But now, Asian population is growing at 1.7 % a year (53 million annually) in 1990s. During the 1990-2025 period, the demand for rice is projected to be increased by another 70 %, which will require an increase in production from 480 million ton to 810 million ton by 2025 (Hossain 1995). Therefore, it is necessary to increase the rice production for supplying the future demand. Since rice production is still greatly affected by adverse environmental factors including low temperature, drought, wind, disease and harmful insects so that elucidating the basic mechanisms of rice plant growth at molecular level and innovative improvements are necessary to ensure increasing rice production.

Information obtained from the studies of higher-plant genetics is essential for molecular genetics and for application of techniques of molecular genetics to crop improvement. Knowledge of fundamental genetics of rice lagged behind that of other major crops such as maize, barley and tomato. However, during the last decade, rapid advances of molecular genetics have made rice a model crop for genomics. There are some factors as the reasons why rice is chosen as a model crop. Rice is diploid, has a relative small genome,

can be regenerated from protoplasts, and can be transformed by exogenous DNA. In addition, rice has a vast germplasm of cultivated and wild species. With the recent progress in rice genomics, a great number of cDNA clones have been isolated and sequenced. High density linkage map with molecular markers have been constructed (Causse et al. 1994, Kurata et al. 1994, Harushima et al. 1998), and YAC (Yeast Artificial Chromosome) (Umehara et al. 1995) and BAC (Bacterial Artificial Chromosome) (Wang et al 1995, Zhang et al. 1996) libraries were constructed to develop physical map of large region of genomic DNA. The progress of rice genomics such as construction of the linkage map and physical map made it possible to isolate the genes such as *Xa21* and *Xa1*, both resistance genes to rice bacterial blight disease, by map-based cloning strategy (Song et al. 1995, Yoshimura et al. 1998).

In Plant Breeding Laboratory, Kyushu University, dwarf chimeric rice plant has been maintained for more than 50 years. This mutant shows chimeric morphology about dwarfism in many parts of the plant body. Allelic and progeny tests of this mutant revealed that the chimerism frrequently occured at *d1* locus (Ashikari 1997). To elucidate a mechanism of this chimerism, a cloning of *D1* gene allelic to recessive allele *d1* is essential. With understanding the above, cloning of *D1* gene through map-based cloning strategy was conducted in this study. A rice dwarf mutant Daikoku carrying *d1* gene was first identified as a spontaneous mutant that exhibited not only short plant height, but also broad, dark green leaves, compact panicles, and small round grains (Akemine 1925; Fig. 1). These phenotypes were all induced by a recessive allele *d1*. Dwarf mutant in plants is crucial for elucidating the mechanisms for plant growth and

development. Isolating and characterizing the rice *D1* gene will also contribute to elucidating the physiological mechanisms of plant growth and development. It is also important for rice-breeding program to understand dwarfism, because dwarfism is one of the most important agricultural traits.

Fig.1-1. Plant type of *d1* mutant, Daikoku.
left; Daikoku dwarf, right; wild-type (Nipponbare).

(1)

(2)

Fig.1-2. Phenotypes of panicle and seeds in *d1* mutant, Daikoku.
(1) Panicle. left; Compact panicle in Daikoku dwarf. right; Normal panicle in wild-type (Nipponbare). (2) Seeds. left; Small round grains in Daikoku dwarf. right; Normal grains in wild-type (Nipponbare).

Chapter 1

RFLP linkage mapping of *d1* locus

Introduction

The first rice linkage map consisting of 12 linkage groups were constructed based on morphological markers, corresponding to the haploid number of rice chromosomes (Nagao and Takahashi 1963, Takahashi and Kinoshita 1968). The locus of *d1* was belonged to linkage group VI. The translocation and trisomic analysis revealed that *d1* was located on chromosome 2 (Iwata et al. 1970). In the decade of 1970, the correspondence between chromosomes and linkage groups was elucidated. The linkage group VI was agreed to chromosome 2 (Iwata and Omura 1971, Iwata and Omura 1975). Since the numbering system of chromosomes and linkage groups was established (The Second International Rice Genetics Symposium in 1990), the *d1* gene was assigned to chromosome 5.

In 1988, the first rice RFLP (Restriction Fragment Length Polymorphism) linkage map was constructed in rice (McCouch et al. 1988). Now, a total of about 3500 DNA markers with mainly RFLP markers have been mapped on several rice linkage maps (McCouch et al. 1988, Saito et al. 1991, Causse et al. 1994, Kurata et al. 1994, Chen et al. 1997, Harushima et al. 1998). The results made it possible to isolate the genes such as *Xa21* and *Xa1*, both resistance genes to rice bacterial blight disease, by map-based cloning strategy (Song et al. 1995, Yoshimura et al. 1998). For map-based cloning in plants, assignment of the

exact map position of a target gene (high-resolution RFLP linkage map), construction of physical map of the region including the gene and accumulation of molecular markers tightly linked to the gene are required. The construction of high-resolution linkage map is essential for construction of physical map in the second step of map-base cloning.

In this chapter, construction of a high-resolution RFLP linkage map of *d1* locus was described. As the first step of map construction, map position of the *d1* was approximately determined using the small and large-scale population. Secondly, the exact map position of the *d1* was determined by high-resolution mapping.

Materials and Methods

Mapping population and sampling

1. Mapping population for a small scale mapping

Ninety-seven F_2 plants derived from the cross between an Indica variety IR24 and a Japonica marker line FL423 carrying *d1* were used for analyzing the linkage between *d1* gene and 14 RFLP markers located on chromosome 5 (Saito et al.1991, Kurata et al. 1994). For DNA extraction, ten mature leaves from each plant were collected and frozen in liquid nitrogen.

2. Mapping population for a large scale mapping

The *d1* plants that were selected from F_2 population derived from the cross between IR24 and FL423 (*d1*) was backcrossed with IR24. The resultant

BC_3F_2 population (Appendix 1) was used for a large scale mapping of *d1* gene. Out of approximately 2600 BC_3F_2 seeds sown in nursery, 640 dwarf ones (homozygous recessive) were selected at the seedling stage and transplanted in the paddy field. In every five plants, wooden labels were stood to discriminate each pool (Fig. 2-1). To reduce the laborious work for DNA extraction, pooled sampling method (Churchill et al. 1993) was adopted and the procedures are shown in Appendix 2-1 and 2-2. Two leaves of each individual were collected. Leaves of five plants were combined as one pool. Total 128 pooled leaves were frozen in liquid nitrogen and used for DNA extraction.

3. Mapping population for a high-resolution mapping of *d1* gene

F_2 population from the cross of *d1* (Japonica) and Kasalath (Indica) showed large variations of phenotypes and seed sterility (Fig. 2-2). In this population, it is difficult to identify whether the plant carries *d1* gene or not. It is also difficult to obtain a large amount of F_3 seeds due to sterility. Therefore, this population is not good to use for high-resolution mapping. On the other hand, F_2 population derived from the cross between FL2 carrying *d1* and a substitution line (SL18) which replaced of chromosome 5 of Kasalath for Nipponbare (Japonica) (Appendix 3) showed clear phenotypic differences and high seed fertility (Fig. 2-3). In this population, the plants carrying *d1* gene could be easily recognized. Each plant in the population produced normal sets of F_3 seeds. Based on these observations, the F_2 population from the cross of FL2 and SL18 was used for a high-resolution mapping of *d1* gene.

(1)

(2)

(3)

Fig.2.(1). Dwarf plants were selected and transplanted in paddy field. Wooden labels were stood in every five plants. (2). Phenotypic variation in F_2 population derived from the cross of the FL2 and Kasalath. (3). Phenotypic variation in F_2 population derived from the cross of the FL2 and SL18.

About 13000 F_2 seeds were sown in a nursery. At the seedling stage, 3185 dwarf plants were selected and transplanted in a paddy field. Pooled sampling method that was described in large-scale mapping was employed. There were 638 pools of leaves, which were frozen in liquid nitrogen and used for DNA extraction.

DNA extraction

DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as described by Murray and Thompson (1980) with small modifications (Appendix 4).

RFLP marker

RFLP markers located on chromosome 5 in the linkage map (Harushima et al. 1998) were used for linkage analysis of *d1*. RFLP markers were indicated in italic in figures, not in text.

Southern hybridization

Total genomic DNA was digested with six restriction enzymes (*Eco*RI, *Eco*RV, *Hind*III, *Bam*HI, *Bgl*I and *Dra*I). Two micrograms of each digested DNA was loaded on a 0.8% agarose gel, run for 16 h at 20 V and blotted onto a nylon membrane. Southern hybridization (Southern 1975) was done with ^{32}P labeled RFLP markers or horseradish-peroxidase-labeled (HPL) RFLP markers

according to the protocol for the Enhanced Chemiluminescent (ECL) Direct Nucleic Acid Labeling and Detection System (Amersham). The banding pattern of ^{32}P labeled RFLP markers was detected on X-ray film at -80 ℃ for three days. The banding pattern of HPL labeled RFLP markers was detected on X-ray film at room temperature for three hours.

Linkage analysis

In small scale mapping, the recombination values were calculated by the maximum likelihood method (Allard 1956) and converted into genetic distances (cM) using the Kosambi function (Kosambi 1944). In large scale mapping and high resolution mapping, map distance was calculated based on the following formula; $r = -(1/2k)\ln[1-(yA/n)]$, r : the approximate maximum likelihood estimator of genetic distance from the target locus, k : the number of individuals per pool, yA: a total number of recombinant pools, n : the number of pools (Churchill et al. 1993). In these experiments, the number of individuals per pool (k) was five. The number of pools (n) was 128 in large scale mapping and 637 in high resolution mapping. Total numbers of recombination between *d1* gene and RFLP markers were counted as the recombinant bands of Southern hybridization with the RFLP markers around *D1* locus (Fig. 3). Recombinant bands in pools indicated by arrowheads were observed at the same size of SL band.

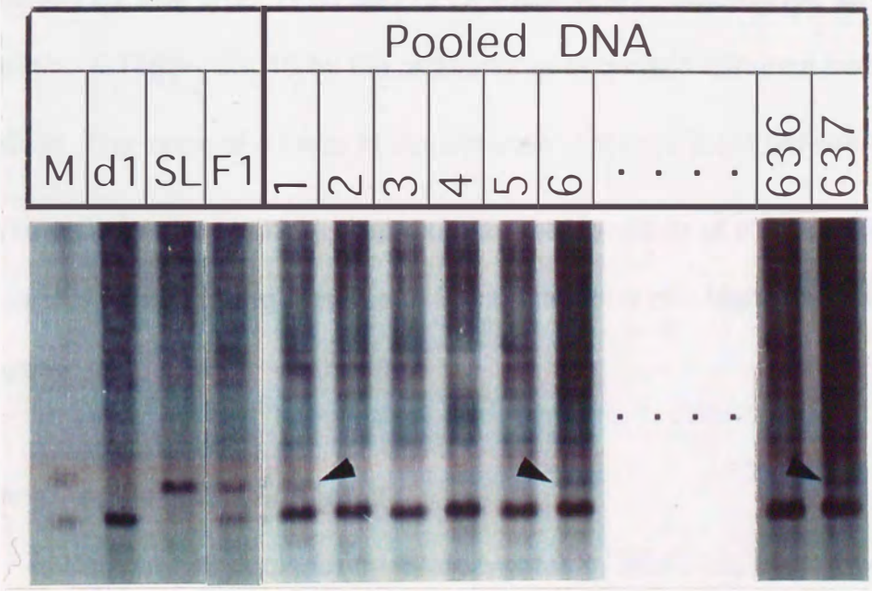


Fig.3. Southern hybridization in pools including the recombinant plants. Recombinant bands in pools were indicated by arrowheads. M : λ marker, d1 : FL423 (*d1*), SL : Substitution line 18, F1 : F1 plant derived from cross of FL423 and SL18.

Results

A small-scale mapping of *d1*

DNA of 97 F_2 plants derived from the cross between IR24 and marker line FL423 with *d1* was extracted individually and RFLP analysis was done with 16 RFLP markers located on chromosome 5 (Saito et al. 1991, Kurata et al. 1994). RFLP markers C309, Npb251, G1458 and G3010 were linked to *d1* at 3.9 ±2.0 cM, 7.1±2.7 cM, 6.5±2.5 cM and 14.9±3.9 cM, respectively (Fig. 4 and Appendix 5). The order of *d1* and RFLP markers was determined as C309 - *d1*- Npb251 - G1458 - G3010 by the relationship of genetic distance between all markers. The locus of *d1* was in the distance of 8.8±2.2 cM between RFLP markers C309 and Npb251. Approximate map position of *d1* gene determined by small scale mapping was useful for construction of a high-resolution map of *d1* gene.

A large-scale mapping of *d1*

DNA of 128 pools for *d1* homozygotes in BC_3F_2 population was extracted and RFLP analysis was performed with three RFLP markers C309,S2351 and G1458. Out of 128 pools, 10 recombinant pools were detected for RFLP marker C309 and the genetic distance between the *d1* gene and the marker was 0.8 cM. In the same manner, 10 and 41 recombinant pools were detected for RFLP marker S2351 and G1458, respectively.

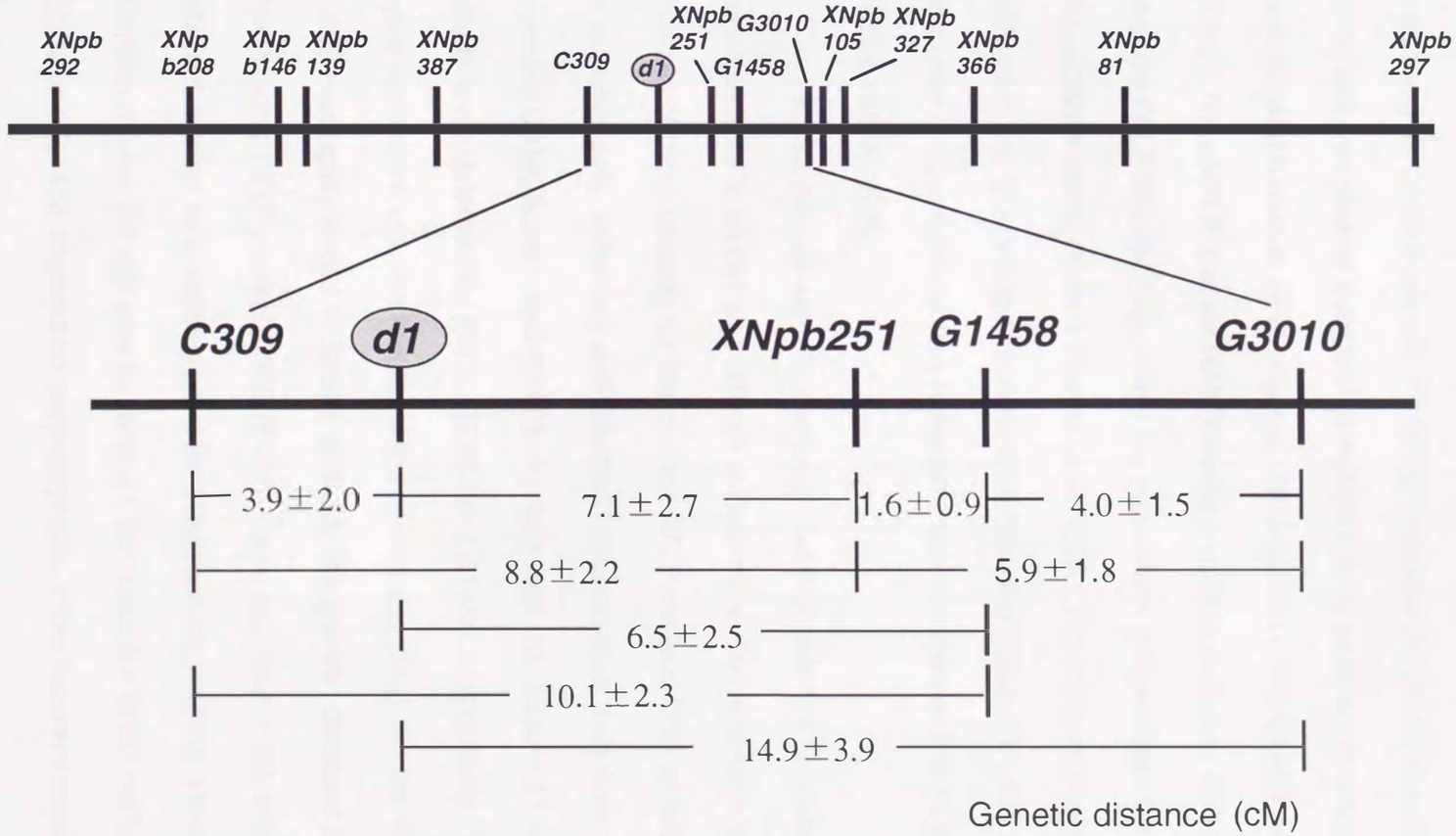


Fig.4. Small scale linkage map of *d1* with RFLP markers on chromosome 5.

Linkage relationship of *d1* with all analyzed RFLP markers were shown above. The linkage map in the vicinity *d1* was indicated below. The genetic distance was indicated under the map in thevicinity of *d1*.

The genetic distances between the *d1* gene and the markers S2351and G1458 were 0.8 cM and 3.9 cM, respectively. RFLP marker G1458 instead of Npb251 was used for pooled-sample mapping, because Southern hybridization pattern of G1458 was clearer than that of Npb251. In large scale mapping, the *d1* gene was located between RFLP marker C309 (S2351) and G1458 with 4.7 cM (Fig.5). Nine RFLP and six RAPD markers existed between C309 and G1458 on the rice RFLP linkage map using the population derived from the cross of Nipponbare and Kasalath (Kurata et al. 1994). But these markers could not be utilized in the BC_3F_2 population derived from the cross of FL423 and IR24, because of no polymorphism between Japonica marker line FL423 (*d1*) and Indica variety IR24.

In this pooled-sample mapping, determination of an exact number of recombinants in a pool was difficult to determine. To determine the exact number of recombinants for the closest RFLP marker, DNA of 640 dwarf plants was individually extracted and Southern hybridization was done using RFLP markers C309, S2351 and G1458. As the result, 10, 10 and 41 recombinant plants were detected for C309, S2351and G1458, respectively. Those results were consistent with the results of the pooled sampling. As the RFLP marker C309 was tightly linked to S2351 at 0 cM. The genetic distance between *d1* and the flanking RFLP marker C309 (S2351) was less than 1 cM, the C309 was efficient marker as a starting point for chromosome walking. However, the other flanking marker G1458 was far from *d1*. Because the RFLP markers between C309 and G1458 showed no polymorphism, these markers could not be used for this mapping.

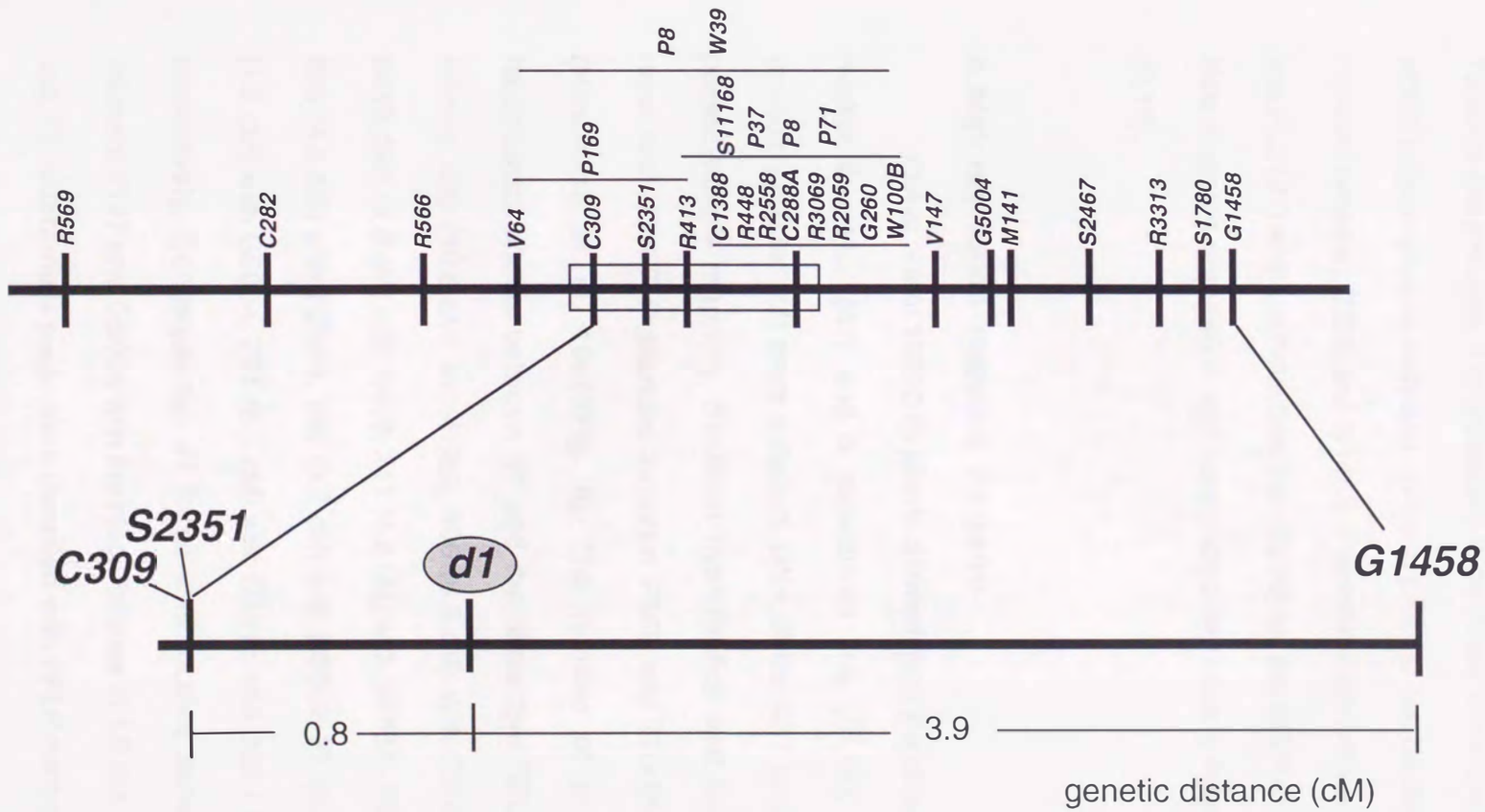


Fig.5. Large scale linkage map of *d1* gene.

The high density RFLP linkage map of centromere region of chromosome 5. (Harushima et al.1998) was shown in above. Centromere region was indicated in box. Large scale linkage map of *d1* gene constructed by using BC_3F_2 population was shown below.

To solve this problem, it is necessary to construct other mapping population in which polymorphisms between crossing parents can be detected for RFLP markers between C309 and G1458.. Polymorphism between a Japonica marker line FL2 (*d1*) and a substitution line (SL18) for the RFLP markers was observed. New mapping population was constructed by crossing these two lines (FL2 and SL18).

A high-resolution mapping *d1* gene

Out of about 13000 F_2 plants derived from the cross between Japonica marker line FL2 (*d1*) and a substitution line (SL18), 3185 dwarf plants (homozygous for *d1*) were selected. DNA of the 637 pools was extracted for pooled-sample mapping, Southern hybridization and linkage analysis were done with 13 RFLP markers between R569 and G1458 on chromosome 5 (Harushima et al. 1998)(Fig. 6). The number of pools containing the recombinant alleles between *d1* and the respective RFLP markers were as follows; 420 (10.8 cM) with R569, 306 (6.5 cM) with C309, 255 (5.1 cM) with R413, 243 (4.8 cM) with R448, 241 (4.8 cM) with C288A, 235 (4.6 cM) with G260, 225 (4.4 cM) with R2059, 198 (3.7 cM) with R3069, 37 (0.6 cM) with V147, 71 (1.2 cM) with G5004, 291 (6.1 cM) with R3313 and 365 (8.5 cM) with S1780, respectively. Consequently, *d1* locus was located between flanking RFLP markers V147 and G5004 with the map distance of 1.8 cM. Out of 637 pools, 37 and 71 recombinant pools were identified with RFLP markers V147 and G5004, respectively.

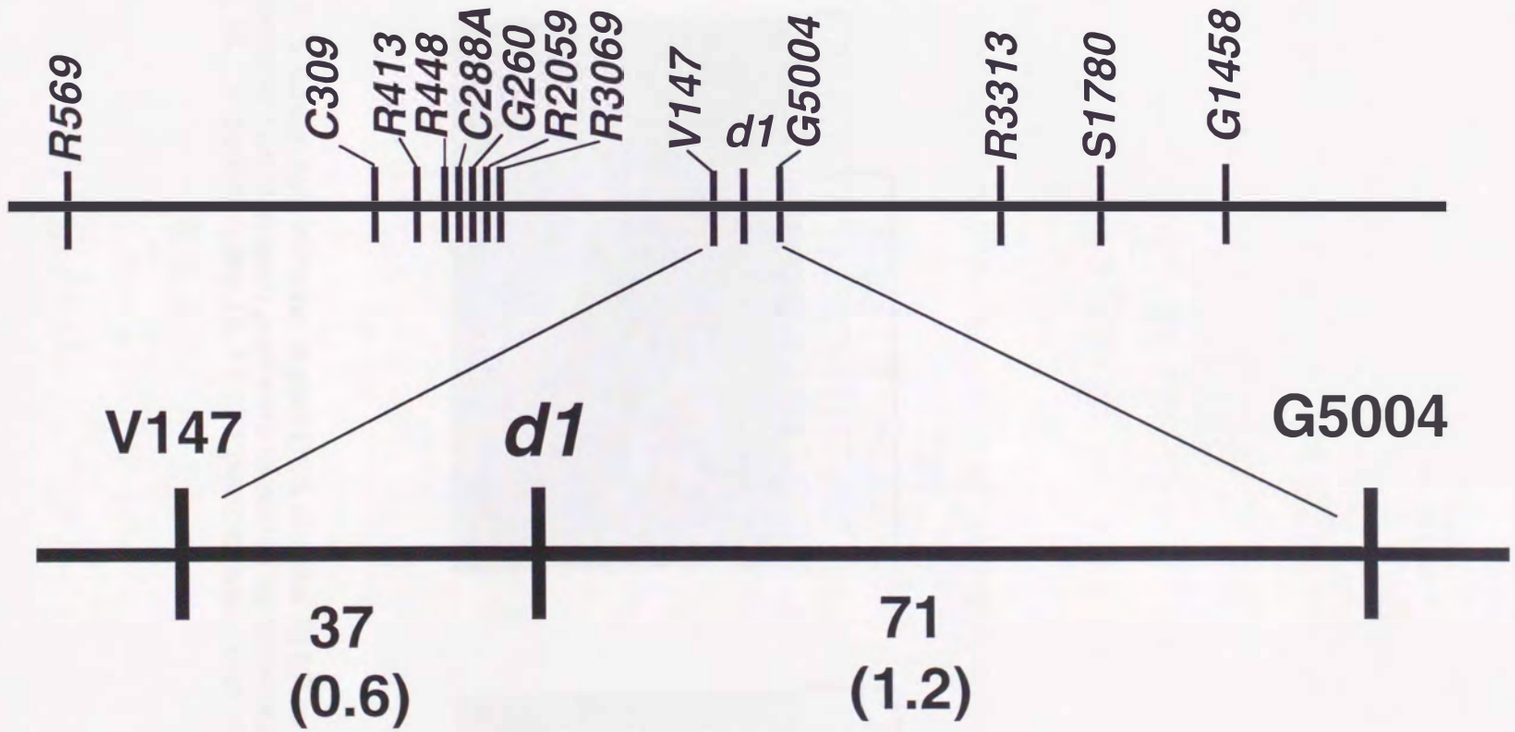


Fig.6. High resolution mapping of *d1* gene.

High resolution RFLP linkage map of *d1* was shown in above. The linkage map between V147 and G5004 was focused in below. The number of recombinant plant between *d1* and RFLP markers were indicated under the map. Genetic distances between adjacent markers are shown in parentheses.

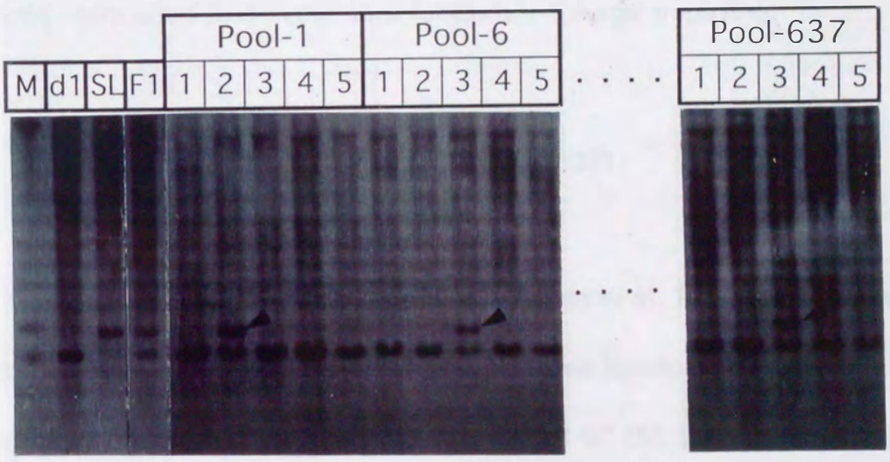


Fig.7. Southern hybridization in pool DNA with the RFLP marker V147. Recombinant plant in each pool were indicated by arrowheads. d1 : FL423 (d1), SL : Substitution line 18, F1 : F1 plant derived cross of FL423 and SL18.

To select the individual recombinant plants from each pool, DNA was extracted from individual 540 plants (5 plants ×108 pools). Southern hybridization was done with the marker V147 (Fig. 7). Recombinant plants in the pools were detected by RFLP fragment from SL18. Thirty-seven recombinant plants heterozygous for the locus V147 and 71 recombinant plants heterozygous for the locus G5004 were identified. Total recombinant plants (108) in the pooled sampling method were the same as the total recombinant plants in the 540 individual sampling method. The plants in which the recombination were occurred between V147 and G5004 were confirmed in 3185 F_2 plants. These plants were used and significant for further linkage mapping.

Discussion

In integrated linkage map of rice (Ideta et al. 1996) *d1* gene was mapped very roughly on chromosome 5. It was located between RFLP markers Npb387 and Npb105 with the distance about 40 cM. Small- scale mapping in this study revealed that the *d1* gene was mapped between C309 and Npb251 with the distance about 8.8 cM. The order of the *d1* gene and the RFLP markers in this small–scale map was verified.

Large scale mapping made possible to precisely map *d1* between C309 (S2351) and G1458. Though 15 markers existed in the region between C309 (S2351) and G1458, no polymorphisms were observed in the population when we used many kinds of restriction enzymes. This suggested that *d1* region of

both parents FL2 and IR24 might be identical.

The *d1* gene was mapped between V147 and G5004 with the distance 1.8 cM in the high-resolution mapping. Of the 3185 plants, 37 recombinant plants between *d1* and RFLP marker V147 (0.59 cM), and 71 recombinant plants between *d1* and G5004 (1.2 cM) were observed. Many plants in which the recombination occurred between tightly linked RFLP markers sandwiching *d1* (recombinant plants) were obtained. They were very useful for chromosome walking described in chapter 2.

The *d1* plants were easily distinguished from normal plants when we used the substitution line as a parent of the mapping F_2 population. In a high-resolution mapping, the substitution lines were useful materials for high-resolution mapping. High-resolution mapping is depended on the size of mapping population. It is difficult to estimate optimum size of mapping population, but the population size of this high-resolution mapping were consequently adequate as seen in chapter 2.

Handling more than 1000 F_2 plants for high-resolution RFLP linkage map is very laborious work. To facilitate the procedure of high-resolution mapping, utilization of PCR markers should be considered. Once we find the tightly linked RFLP markers sandwiching a target gene using small scale mapping population (about 100 F_2 plants), replace the two RFLP markers by PCR markers. The replacement of RFLP markers by PCR markers can be achieved by designing PCR primers based on the sequence of the RFLP markers and subsequent digestion analysis with many restriction enzymes. Using the PCR markers, the recombinants between the two markers are

selected in more than 1000 F_2 plant from cross of the mutant and appropriate SL line and only the recombinants are used for high-resolution mapping. The procedure mentioned above would economize consuming time and planting space.

Chapter 2

Physical mapping of *d1* gene

Introduction

For map-based cloning in plants, construction of the high-resolution RFLP linkage map is essential (described in chapter 1). In addition, accumulation of molecular markers tightly linked to a target gene and construction of physical map which consists of continuously overlapping contigs of a large insert genomic DNA covering the target gene are also required. In recent years, yeast artificial chromosome (YAC) libraries have been constructed for human (Burke et al. 1987), *Arabidopsis* (Ward and Jen 1990, Grill and Somerville 1991), tomato (Martin et al. 1992), maize (Edwards et al. 1992), barley (Kleine et al. 1993) and rice (Umehara et al. 1995). An another bacterial artificial chromosome (BAC) system to clone large DNA fragments, was described (Shizuya et al. 1992), and rice BAC libraries were also constructed (Wang et al. 1995, Zhang et al. 1996, Zhang and Wing 1997). Those YAC and BAC libraries provide powerful tools to generate molecular physical maps for gene tagging.

A YAC library constructed by RGP (Rice Genome Research Program) containing 6934 clones with an average insert size of 350 kb and covered six times of the haploid genome of rice (Umehara et al. 1995). The YAC library contributed to product of physical map of the large region containing the gene

(Antonio et al. 1996, Shimokawa et al. 1996, Saji et al. 1996, Wang et al. 1996, Umehara et al. 1996, Tanoue et al. 1997 and Koike et al. 1997).

This chapter described the construction of a physical map of *d1* region using YAC library, the identification of the YAC clones carrying *d1* gene and the accumulation of molecular makers around *d1* .

Materials and Methods

DNA extraction from YAC clones

DNA was extracted from YAC clones cultured for three days in AHC medium. (Imai and Olsen 1990) (Appendix 6).

A YAC library and DNA markers

A YAC library (Umehara et al. 1995) was used for construction of physical map of *d1* region. All YAC clones were blotted on five filters and multiplied on many replica filters. DNA markers including RFLP, sequence-tagged site (STS) and YAC-end clones, which are located around *d1* gene on chromosome 5, were used for screening of a YAC libraly.

Screening of YAC library

1. For Colony hybridization

The replica filters blotted with YAC clones were hybridized with 10 ng / ml of a probe labeled by enhanced chemiluminescent (ECL) hybridization system (Amersham). YAC clones hybridized to the probe (Fig. 8) were selected,

cultured for three days in AHC medium and DNA was extracted from the clones. The DNA was digested with the some restriction enzymes, electrophorested overnight and transferred to nylon membranes. These DNAs was hybridized with the specific probe to confirm the YAC clones containing the same sequences of the RFLP marker in each candidate YAC clone.

2. Three-step PCR screening

A screening system of three-step polymerase chain reaction (PCR) to isolate YAC clones with STS markers (Jones et al. 1994) was carried out with modification (Umehara et al. 1996, Wu et al. 1997). This system was employed for the screening of YAC clones using YAC-end DNA fragments. The procedures were illustrated in Appendix 7. The 20 bp primers were designed from the sequences of YAC-end DNA fragments. In the first screening, a PCR reaction (2 minutes at 96℃ and followed 25 cycle of 1 minutes at 94.5℃, 2 minutes at 58℃ and 3 minutes 72℃) was done with the nine W superpools of YAC DNA, DNA of yeast host strain AB1380 and DNA of a rice variety Nipponbare. Each W superpools contained DNA mixture of about 800 YAC clones. In the second screening, 29 subpools (8 X pools, 12 Y pools and 9 Z pools) of each W pools that showed positive in the 1st PCR were screened by the 2nd PCR. The YAC clones screened through 1st and 2nd PCRs were considered as candidates, finally, the positive clones were confirmed by 3rd PCR using the individual YAC clone DNA as a template.

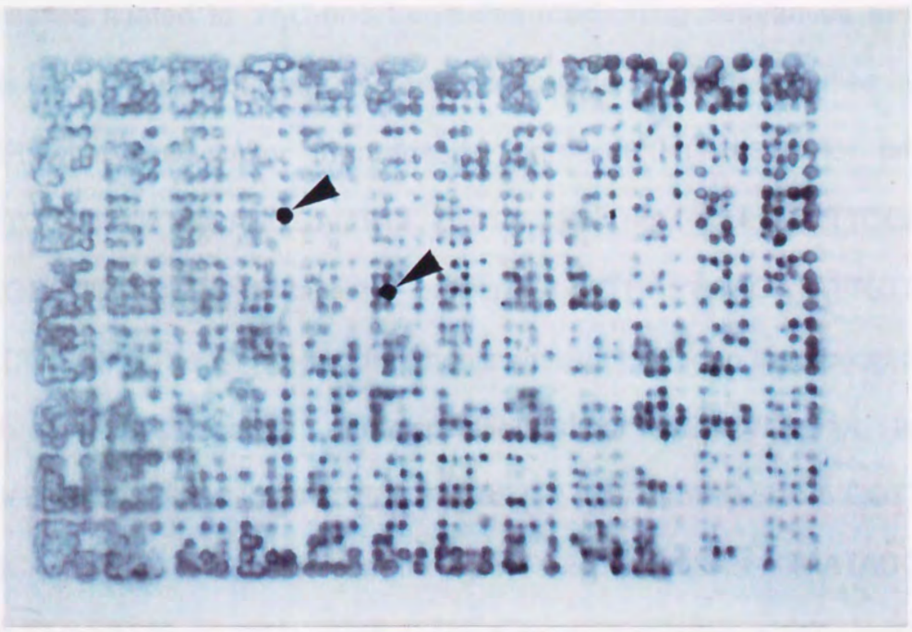


Fig.8. Screening of YAC clones by colony hybridization.
Positive YAC clones were indicated by arrowheads.

Isolation of YAC end DNA fragment

YAC end DNA fragments were obtained by a cassette-PCR method with slight modification (Isegawa et al. 1992). The procedures were illustrated in Appendix 8. The inserted rice genomic DNA including yeast genomic DNA was extracted (described above) and digested with restriction enzyme (*Alu*I, *Hae*III, *Dra*I, *Eco*RV) at 37℃ for 45 minutes. An Oligo cassette adapter of double strand was designed: 5'GTACATATTGTCGTAGAACGCGTAATACGACTCAC TATAGG GAGA3'. The cassette adapter was ligated to digested YAC DNA fragments. The cassettes ligated to YAC-end fragments (containing sequences at both the outsides of YAC vector and the cassette fragments) were amplified in the first PCR with YAC vector arm specific primer S1 (TVL319 for left arm : 5'TTCGCTACTTGGAGCCACTA3', R120 for right arm: 5'TTCGCTCTTG GAGCCACTA3') and cassette primer C1 (C1: 5'GTACATATTGTCGTTAG AACGC G3'). Then YAC-end fragments were amplified in the successive PCR with the YAC vector arm specific primer S2 (VL3 for left arm: 5'GGTTTAAGGCGCAAGACTGTA3', R521 for right arm: 5'GGTTTAAGG CGCAAGACTGTA3') and cassette primer C2 primer (C2: 5'TAATACGACTCA CTATAGGGAGA3'). PCR reaction was done for 2 minutes at 96℃ and followed 25 cycle of 1 minutes at 94.5℃, 2 minutes at 58℃ and 3 minutes at 72℃. Amplified DNA fragments was purified and cloned to TA cloning vector (Invitrogen).

Similarity search

Computer searches for homologous sequences were carried out against the public databases Genpept, PDB, Spupdate and PIR using the BLAST algorithm (Altschul et al. 1990, Altschul and Lipman 1990).

EST mapping

In RGP the large-scale rice EST mapping has been carried out (Wu et al. 1997). Primer pairs were designed from the 3'-end (3'UTR) sequences of random and selected cDNA and the PCR screening was done by 3-step PCR screening method (described above).

Results

A rice yeast artificial chromosome (YAC) library was screened for construction of YAC contigs covering the *d1* region. Colony hybridization was done with the RFLP markers V147 and G5004 as probes. YAC clones carrying the sequence of markers (V147 and G5004) were identified as positive clones on the X-ray film. Four YACs (Y5483, Y3401, Y4480 and Y4336) were identified using the RFLP marker V147, and two YACs (Y2287 and Y4611) were identified using the RFLP marker G5004. Southern hybridization using V147 and G5004 as probes confirmed that these YAC clones apparently contained the sequences of the markers (Fig. 9).

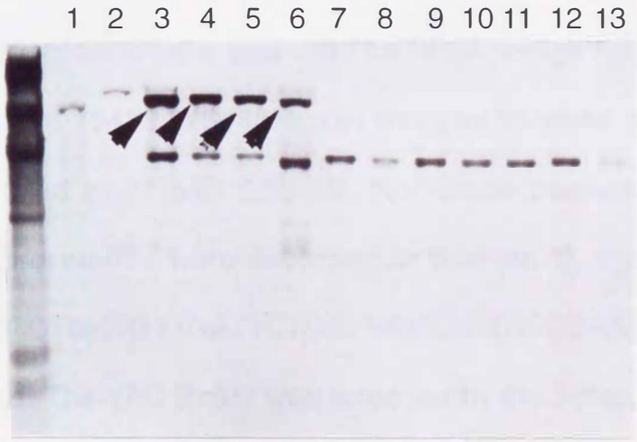


Fig.9 Southern hybridization of YAC clones using V147 as a probe. 1;λ/*Hin*dIII 2; Nipponbare, 3; Yeast, 4; Y3401, 5; Y4480, 6; Y5483, 7; Y4336, 8; Y1988, 9; Y3745, 10; Y4480, 11; Y2287, 12; Y4611, 13; Y5074,13; Y5078,

Four YACs (Y5483, Y3401 Y4480 and Y4336) showed the band at the same size of the Nipponabare band with the V147. Similarly, two YACs (Y2287 and Y4611) showed the band at the same size of Nipponbare with G5004. Both end sequences of these YACs were amplified by the cassette-PCR method. Both of the end DNA fragments of four YAC clones containing V147 sequences (Y5483, Y3401, Y4480, and Y4336) did not hybridize to two YAC clones containing G5004 sequences (Y2287 and Y4611) and vice versa. These results indicated these YAC clones did not cover the region between V147 and G5004. Both of the end DNA fragments were also used for RFLP linkage mapping. The right end DNA fragment of Y5483 (Y5483R) was mapped between *d1* and G5004 and was tightly linked to *d1* with 0.03 cM. Nucleotide sequence of Y5483R was determined (procedures were described in chapter. 3), and the oligo primers (5483U: CCTGCTGGTGTTGATTCTGG, 5483L: GTACCGAAGGGGATCT TCAG) were designed. The YAC library was screened by the 3-step PCR method using these primers. Three YACs (Y1988, Y3745 and Y4488) were newly identified (Fig. 10). In the first PCR, three positive superpools (W3, W5 and W7) were identified and the 2nd and the 3rd PCR screenings appeared four YAC clones (Y1988, Y3745, Y4488 and Y5483) carrying the sequence of Y5483R. DNA of four YAC clones was extracted and Southern hybridization was performed using Y5483R as a probe (Fig. 11). Four YACs (Y1988, Y3745, Y4488 and Y5483) showed the band at the same size of the Nipponabare, indicating that these YAC clones contained the Y5483R sequences.

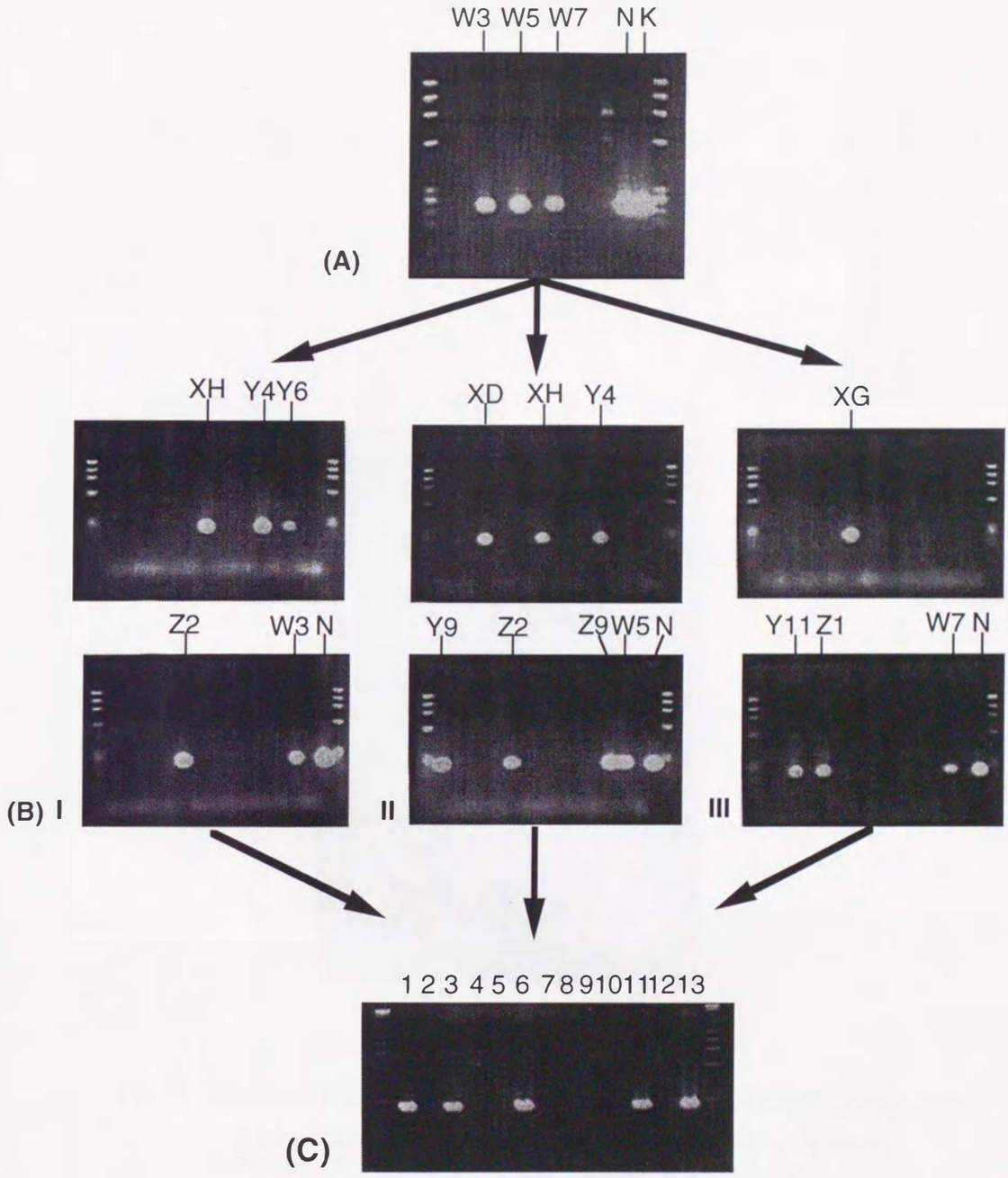


Fig.10 Identification of YAC clones by 3-step PCR method.

A) 1st PCR. Three super pool (W3,W5,W7) contained the positive YAC clones. **B)** 2nd PCR. **I**; In W3 pool, XH, Y4, Y6, Z2 pools contained the positive YAC clones. **II**; In W5 pool, XD, XH, Y4, Y9, Z2, Z9 pools contain the positive YAC clones. **III**; In W7 pool, XG, Y11, Z1 pools contain the positive YAC clones. **(C)** 3rd PCR, 1: Nipponbare, 2: yeast DNA, 3: Y1988, 4: Y1990, 5: Y3745, 6: Y3740, 7: Y3776, 8: Y3793, 9: Y4440, 10: Y4445, 11: Y4488, 12: Y4493, 13: Y5483. N: Nipponbare, K: Kasalath.

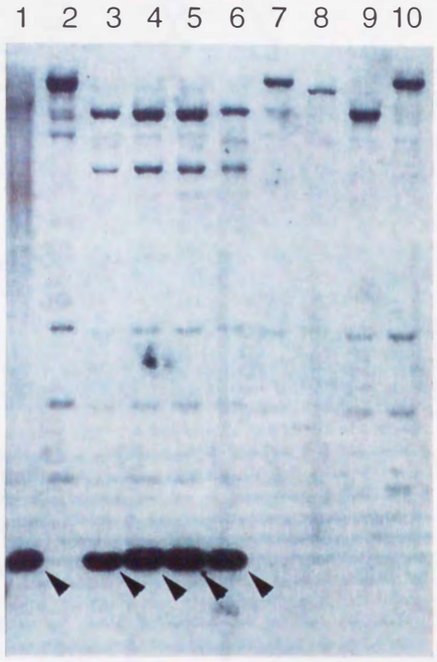


Fig.11 Southern hybridization of YAC clones using Y5483R as a probe.
1; Nipponbare, 2; Yeast, 3; Y5483, 4; Y1988, 5; Y3745,
6; Y4488, 7; Y6083, 8; Y6183, 9; Y6645,10; Y6683.

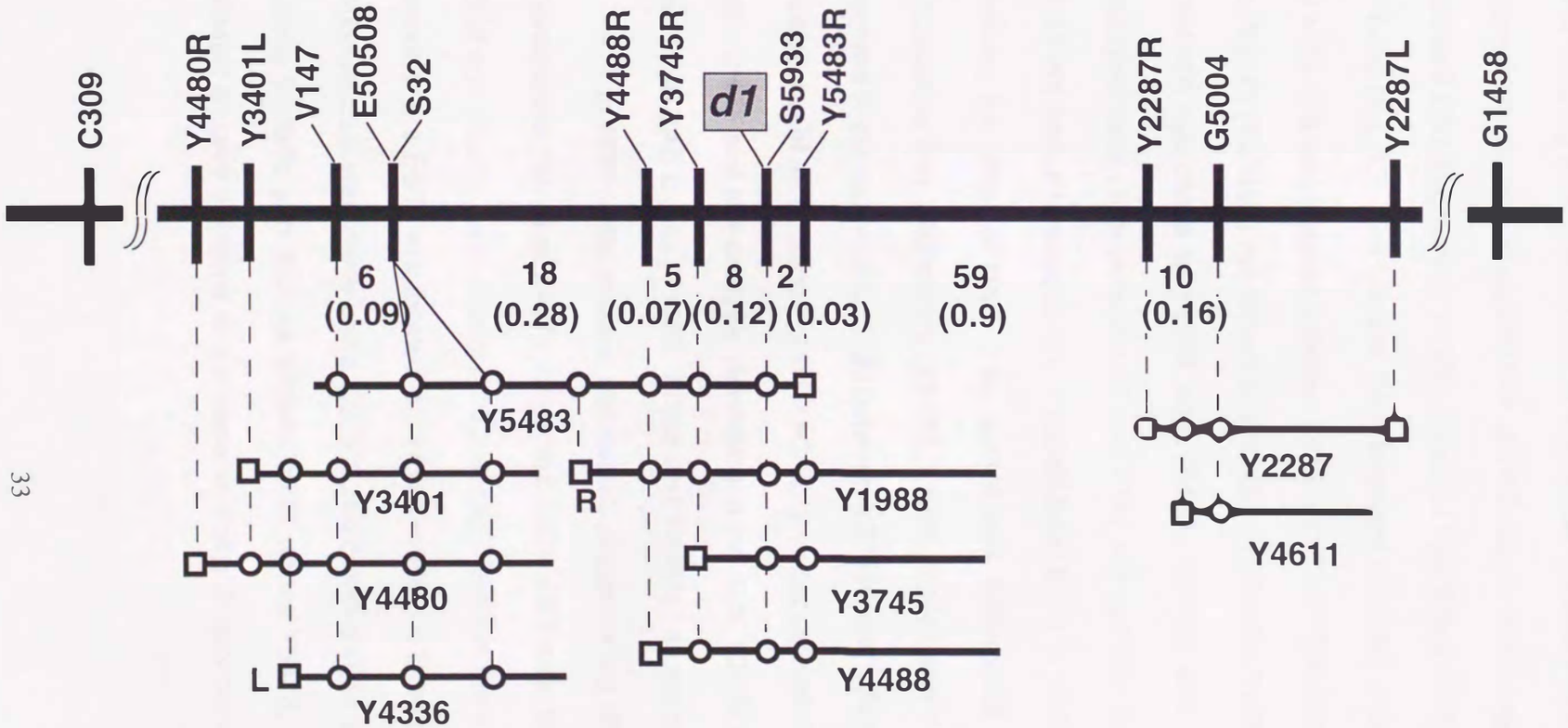


Fig. 12. High-resolution RFLP linkage map and YAC clone contigs at the *d1* locus on chromosome 5.
RFLP markers are shown above the linkage map, and the numbers of recombinants are under it. Genetic distances between adjacent markers are shown in parentheses. □: End-fragment DNA of YAC clone. ○: RFLP markers or YAC end-fragment DNA are contained in the YAC clone.

For ordering each YAC clones in this region, Southern hybridization was done using both the end-fragment DNA of YAC clones in this region as a probe. The left end DNA fragment of Y4336 (Y4336L) hybridized to YAC clone Y3401 and Y4480 (Fig. 12). The left end DNA fragment of Y3401 (Y3401L) hybridized to Y4480 and was mapped between C309 and V147. The right end DNA fragment of Y3745 (Y3745R) hybridized to YAC clones (Y4488, Y1988 and Y5483). And Y4488R hybridized to Y1988 and Y5483. Y3745R and Y4488R were both mapped each other between *d1* and V147 with genetic distances of 0.12 and 0.19 cM from *d1*, respectively. Y1988R hybridized to Y5483. Based on these results, the order of these YAC clones was determined. And *d1* gene was included in four YAC clones (Y5483, Y1988, Y3745 and Y44889) and at least located in the region of 0.15 cM between Y3745R and Y5483R (Fig. 12). In RGP, insert size of all of clones of the YAC library was estimated by electrophoresis with CHEF and blot-analysis. According to the data, Y3745 was 350 kb in length, but three YAC clones (Y5483, Y1988 and Y4488) had not been determined.

In RGP, a large-scale expressed sequence tag (EST) map has been constructed (Wu et al. 1997). According to the EST map, three ESTs (E50508, S32 and S5933) were onto the YAC clone (Y5483) in the *d1* region. To confirm weather the ESTs are located on YAC clones in *d1* region or not, Southern hybridization was done using E50508, S32 and S5933 as probes (Fig. 13). Using E50508 and S32 as probes, YAC clones Y5483, Y1988, Y3745 and Y4480 showed the same size of band as that of Nipponbare.

Fig.13 Southern hybridization of YAC clones located on *d1* region using ESTs as probes.

Probes **A**. S32, **B**. E50508, **C**. S5933

1; Nipponbare, 2; Yeast, 3; Y3401, 4; Y4480, 5; Y5483, 6; Y4336, 7; Y1988, 8; Y3745, 9; Y4480, 10; Y2287, 11; Y4611, 12; Y5074, 13; Y5078, 14; Y6553

While in S5933 as a probe, YAC clones Y5483, Y1988, Y3745 and Y4488 showed the same size of band as that of Nipponbare. These results indicated that each YACs (Y5483, Y3401, Y4480 and Y4336) carried the sequences of E50508 and S32. On the other hand, the sequence of S5933 was included in YACs (Y5483, Y1988, Y3745 and Y4488) (Fig. 12). The three ESTs were used for genetic mapping in the population of high-resolution mapping described in chapter 1. As the results, E50508 and S32 were mapped with 0.5 cM between *d1* and V147. The S5933 was cosegregated with *d1* in the 3185 *d1* segregants. The cDNA S5933 (350-bp insert) was derived from green shoots, and its amino acid sequence completely coincided with the sequence from Phe^{332} to Thr^{380} of the α subunit of rice GTP-binding protein *RGA1* (Seo et al. 1995, Ishikawa et al. 1995, Iwasaki et al. 1997).

Discussion

The YAC library contained a lot of chimeric clones by insertion of two segment of genomic DNA or deletion of a part of inserted genomic DNA. Although 40 % of clones in the YAC library were estimated as chimeric, most YAC clones with *Eco*RI fragment below 400 kb were intact colinear clones (Umehara et al. 1995). Four YAC clones (Y5483, Y1988, Y3745, and Y4488) carrying the *D1* gene were identified and *D1* gene was located between Y3745R and Y5483R within 0.15 cM. Because Y3745R and Y5483R that were very tightly linked and both sides of *D1* locus, the connection of two fragments of

genomic DNA between Y3745R and Y5483R was improbable. The deletion between Y3745R and Y5483R was possible very low frequency. Because the four YACs (Y5483, Y1988, Y3745, and Y4488) contained both of Y3745R and Y5483R, These YACs seemed to carry the *D1* gene. Based on this physical map, three EST appeared to be located on around *D1* locus. One of the EST S5933 was cosegragated in 3185 *d1* segragants by a high-resolution mapping.

The EST S5933 indicated the high homology to α subunit of heterotrimeric GTP-binding protein *TGA1* in tomato (Ma et al. 1991) and *GPA1* in *Arabidopsis* (Ma et al. 1990, Weiss et al. 1993). Hetrotrimeric GTP-binding protein consists of α, β and γ subunits. In recent years a lot of evidence that the protein plays an important role of signal transduction has been accumulated in animals and microbes (Gilman 1987, Kaziro et al. 1991, Neer 1995, Hamm 1998). α subunit of GTP-binding protein is converted into active form that bound GDP to be replaced with GTP and dissociated to β and γ dimer. Then the activated α subunit was transmitted as the first signal from receptor to effector. Heterotrimeric GTP-binding proteins have been identified in several plant species (Terryn et al. 1993, Ma 1994). Gibberellins (GAs) are a large family of tetracyclic diterpenoid plant growth hormones and are associated with regulation of α-amylase expression in the cereal aleurone layer (Baulcombe and Buffard 1983, Nolan and Ho 1988). Since *d1* mutant did not produce alpha-amylase in cereal aleurone layer with application exogenous GA, the mutant was classified into gibberellin insensitive mutant (Mitsunaga et al. 1994).

It was speculated that *d1* mutant lost the function of GA signal transduction. The function of GTP-binding protein associated with signal transduction fit well the speculated function of *d1* gene. The EST S5933 might encode the *D1* gene.

Accumulating the DNA markers around the target gene and locating the target gene between the DNA markers, as minimum genetic distances as possible were important for map based cloning. In this study, several DNA markers tightly linked to *d1* gene could be identified. Because large number of plants (3185 dwarf plants) were used for linkage analysis, *d1* gene could be mapped between two DNA markers Y3745R and Y5483R with 0.15 cM, precisely. In addition, the EST S5933 cosegregated to *d1* gene was appeared as candidate of *D1* gene. To confirm that the EST S5933 encodes *D1* gene, Southern and Northern analysis of a series of *d1* mutants using S5933 as a probe are described in chapter.3.

Chapter 3

Cloning of the *D1* gene

Introduction

High-resolution RFLP linkage mapping and physical mapping in the *d1* region revealed a possible candidate EST of *D1* gene in chapter 2. The EST cosegregated 3185 *d1* segregants and encoded α subunit of GTP binding protein. The GTP-binding protein plays the very important role of signal transduction of adenyl cyclase in mammal (Gilman 1987). Recent evidences that the protein also has the role of the same function in plants has been reported (Ma 1994). Since the *d1* mutant was classified into gibberellin insensitive mutant (Mitsunaga et al. 1994), the *D1* gene is possibly to encode protein associate with GA signal transduction. The role of GTP-binding protein and predicted function of *d1* gene seem to be associated mutually. For elucidating the association between the EST and *D1* gene, analyzing the gene structure and the expression of EST in *d1* mutants were required. In Kyushu University, nine *d1* mutant lines have been maintained. In this chapter, Southern analysis and Northern analysis in the *d1* mutant lines were performed, and the region of the EST was partially sequenced. The association between the EST and GA signal transduction was also discussed in this chapter.

Materials and Methods

Plant materials

Nine *d1* mutant lines, FL2, HO532, HO533, HO537, HO538, HO541, HO552, CM392 and CM1729 maintained in Kyushu University, were used. Wild-type varieties, Nipponbare and Kasalath were employed in the experiment.

Extraction of total RNA

Rice total RNA was extracted from frozen two-weeks-old seedlings using the single-step method (Chomczynski and Sacchi 1987) with modifications.

Seven grams of seedlings were ground in liquid nitrogen with a mortal and a pestle and. Ground well after addition of 50 ml guanidium thiocyanate solution. Subsequently 25 ml TE-saturated phenol and 25 ml of CIA (chloroform and isoamyl alcohol; 24:1) were added to the homogenized sample and it was centrifuged at 5,000 rpm under room temperature for 10 minute. After addition of 60 ml PCIA (TE saturated phenol and CIA; 1 : 1), the upper aqueous phase was shaken and centrifuged at the same condition. This step was repeated again. To the upper aqueous phase,160 µl acetic acid, 5 ml of 3 M NaOAC (pH 5.2) and 30 ml of isopropyl alcohol were added and the tube was centrifuged at 7,500 rpm and 4 ℃ for 30 minutes. The pellet was suspended in the 6 ml

guanidiumthiocyanate solution. After addition of 2.4 ml PCI to the tube, the sample was shaken and centrifuged at 15000 rpm under room temperature for 15 minutes. After addition of 0.8 ml CIA to the upper phase, the sample was shaken and centrifuged at 15,000 rpm under room temperature for 5 minutes. The upper aqueous phase, 300 μl 3M sodium acetate (pH 5.2) and 7.5 ml of 99.5% ethanol was added to it and it was centrifuged at 15,000 rpm under room temperature for 15 minutes. The pellet was dried up in air and resolved 2 ml of diethylpyrocarbonate (DEPC) water.

Purification of messenger RNA

Messenger RNA was purified from total RNA by Oligotex-dT30 super (Takara). 500 μl The Elution buffer (10 mM Tris-HCL (pH 7.5), 1 mM EDTA and 0.1% SDS) was added to ajust 250 μl of total RNA (2 μg/ml). After 500 μl Oligotex-dT30 super was added to it, incubated 67 ℃ for 7 minutes and placed on ice for 3 minutes. After 250 μl 5M NaCl was added to it, the sample was shaken gently at 37 ℃ for 10 minutes and centrifuged at 5,000 rpm under room temperature for 5 minutes. The pellet was washed with 1 ml washing buffer (10 mM Tris-HCL (pH 7.5), 1 mM EDTA, 0.5M NaCl and 0.1% SDS), centrifuged at 5,000 rpm under room temperature for 5 minutes. And 500 μl DEPC water was added to the pellet and the sample was mixed, the mixture was incubated 67 ℃ for 7 minutes and placed on ice for 3 minutes. The sample was centrifuged at 5,000 rpm under room temperature for 5 minutes. The supernatant was

transferred to a new tube and 1 ml of 99% ethanol and 40 μl of 3M NaOAc (pH 5.2) were added to it and the sample was centrifuged at 15,000 rpm under 4 ℃ for 5 minutes. The pellet was washed with 70% ethanol and resolved in 20 μl DEPC water.

Northern hybridization

Three micrograms of mRNA was electrophoresed on 1.0% agarose containing 18 % formaldehyde for 3 hours at 75 V and blotted onto a nylon membrane. Northern hybridization was done in the rapid hybridization buffer (Amersham) at 65 ℃ overnight with ^{32}P-labeled cDNA as a probe. The nylon membrane was washed twice in 1×SSC buffer containing the 1% SDS 65 ℃ for 20 minutes, then twice in 0.1×SSC buffer containing the 1% SDS 65 ℃ for 30 minutes. The banding patterns of labeled cDNA s were detected on X-ray film at -80 ℃ for 1 week.

DNA Sequencing

Nucleotide sequencing of the cloned DNA was performed by the dideoxy chain-termination method using the Taq dye primer cycle sequencing kit (Amersham) with an Applied Biosystem 377 automatic DNA sequencer.

DNA extraction and Southern hybridization

These procedures were described in chapter. 1

Results

To confirm the allelic relationships among the nine *d1* mutants FL2, HO532, HO533, HO537, HO538, HO541, HO552, CM392 and CM1729, allelic tests were done. All of F_1 hybrids from the crosses between FL2 and each of HO532, HO533, HO537, HO538, HO541, HO552, CM392, and CM1729 showed *d1* dwarf phenotype. These results confirmed that 9 mutant lines were caused by the mutation occurred in the *d1* locus.

Genomic Southern hybridization analysis of these *d1* mutant lines and two wild-type varieties, Nipponbare (Japonica) and Kasalath (Indica), was done using S5933 as a probe (Fig. 14). The 10.4 kb band was observed in the two wild types, but there was no polymorphism between them. On the other hand, five different banding patterns were observed in 9 *d1* mutant lines ; the 6.8 kb bands were observed in FL2, HO533, HO537 and HO538, a 6.2 kb band in HO532, a 9.6 kb band in HO541 and a 5.2 kb band in HO552, 10.4 kb bands in CM392 and CM1729. In general, RFLPs were easily observed between Japonica variety and Indica variety, but hardly detected among Japonica varieties. These results strongly suggest that several types of structural variations had occurred in the region of S5933 in the *d1* mutant lines.

Messenger RNAs were extracted from the two wild types, Nipponbare, Kasalath and the four *d1* mutants FL2, HO532, HO541 and HO552 that showed different types of banding patterns in genomic Southern hybridization with S5933 as a probe.

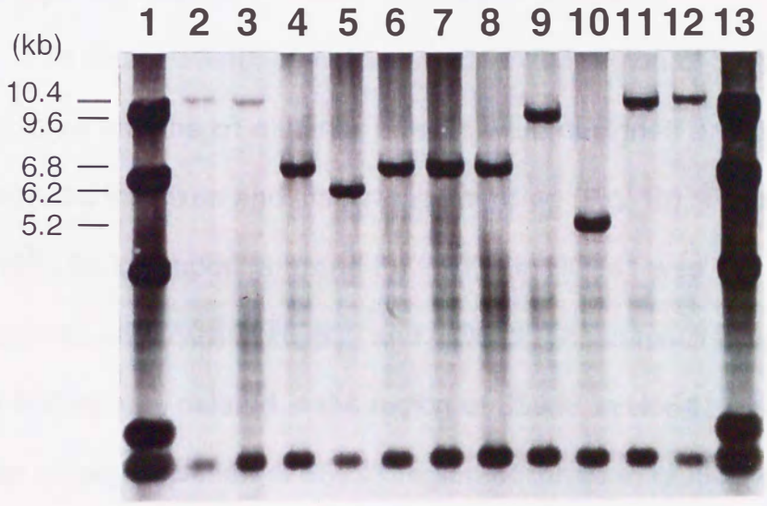


Fig. 14 Genomic Southern hybridization analysis of nine *d1* mutants using ST5933 as a probe.

Lane 1; λ /*Hind*III, 2; Nippnbare, 3;Kasalath, 4; FL2, 5; HO532, 6; HO533, 7; HO537, 8; HO538, 9;HO541, 10; HO552, 11; CM392, 12; CM1792, 13; λ / *Hind*III. 2 ug total DNA was digested with *Hind* III.

Northern analysis was done with S5933 as a probe (Fig. 15). As a positive control, S14002 (actin) was also used as a probe. The translated product was observed with equal density in Nipponbare, Kasalath and the four *d1* mutants when probed with S14002 (Fig. 15-B), indicating that volume of messenger RNAs on the filter were all equal. On the other hand, a 1.8-kb translated product was observed in Nipponbare and Kasalath but not in the four *d1* mutant lines using S5933 as a probe (Fig. 15-A). The size of the product was in accordance with size for mRNA of the α subunit of rice heterotrimeric GTP-binding protein (Seo et al. 1995, Ishikawa et al. 1995).

To detect differences of sequence in the region of S5933 among Nipponbare and the *d1* mutants, primers were designed and the sequence between the first exon and intron was amplified (Fig. 16). The amplified product was 1550 bp in Nipponbare and FL 2. That in HO541 was 700 bp. No amplified product was observed in HO532 and HO552. These results suggested that about 850 bp was deleted in the region of S5933 in Ho541 and primer site change or large deletion of S5933 region occurred in HO532 and HO552. The structural mutation of another region of S5933 may be occurred in FL2. The 1550 bp amplified product in Nipponbare and the amplified products of 700 bp in HO541 were inserted to TA vector and sequenced. Nucleotide between the first exon and intron in HO541 was 833 bp shorter than Nipponbare (Fig. 17). This result coincides well with the genomic Southern hybridization pattern that used S5933 as a probe; about 800-bp difference in length was observed between Nipponbare (10.4 kb) and HO541 (9.6 kb) (Fig. 14).

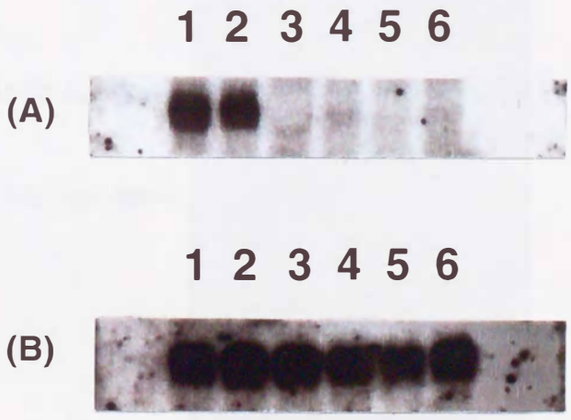


Fig. 15. Northern hybridization analysis using as probes (A) S5933 or (B) S14002 (actin) as probes. Lane. 1 : Nipponbare, 2 : Kasalath, 3 : FL2, 4 : HO532, 5 : HO541, 6 : HO552.

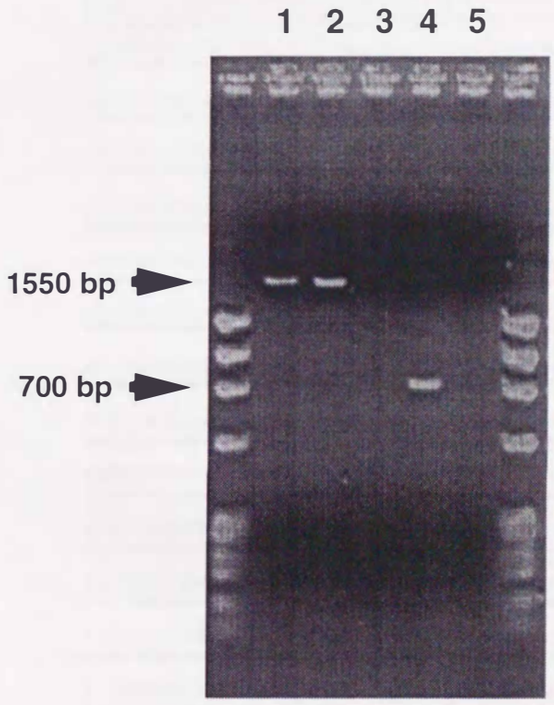


Fig. 16. Amplification of the genomic region between the first exon and the first intron in S5933.
lane 1: Nipponbare, 2: FL2, 3: HO532, 4: HO541, 5: HO552.

```
Nipponbare GGAAGAAGATTGCTAGTTGGCTTGTGGGTTCCACGTGGGCCCTGCACTGACTCAGCCACC
HO541(d1)  GGAAGAAGATTGCTAGTTGGCTTGTGGGTTCCACGTGGGCCCTGCACTGACTCAGCCACC

           ACGTCAACAAAACCGGAAAGCAATACTACCTTGGGATTAAAAGTGGACCGTTTTGCAAGA
           ACGTCAACAAAACCGGAAAGCAATACTACCTTGGGATTAAAAGTGGACCGTTTTGCAAGA

           TTAGGGGTTCAGGGCCGTTTAAAACTCAACGACAAGATAAGGGACCTTAGATGAACTTTT
           TTAGGGGTTCAGGGCCGTTTAAAACTCAACGACAAGATAAGGGACCTTAGATGAACTTTT

           TTGGGAGATGAGGCGCCTGTGCATATGACCCATAGGCAGGCGACAACGCGGCAGCAGAGC
           TTGGGAGATGAGGCGCCTGTGCATATGACCCATAGGCAGGCGACAACGCGGCAGCAGAGC

           GCTGCACGACCTCCGCCTCCCCAATCCGTCCCGTCTCCTCGCCCGCCGCCCTCGACTCCC
           GCTGCACGACCTCCGCCTCCCCAATCCGTCCCGTCTCCTCGCCCGCCGCCCTCGACTCCC

           CTCCTGCTCCCGCCGCATTCGCATCCTAATCCATCCCATCCGGCCTCGGCCTCGAATTTC
           CTCCTGCTCCCGCCGCATTCGCATCCTAATCCATCCCATCCGGCCTCGGCCTCGAATTTC

           GTCTCCTCGCCCGCCGCTTGCGCCGATCCCCATCCTGCGAGCAGAGGTGGCGCCCGGCGT
           GTCTCCTCGCCCGCCGCTTGCGCCGATCCCCATCCTGCGAGCAGAGGTGGCGCCCGGCGT

           CCTCCTTCTTCCACCGCATCGTCGCTCGCCGGTGAGCATCCCTCCCTACCCACACCTCAT
           CCTCCTTCTTCCACCGCATCGTCG

           CAGGTATTCTGCGTTATCCCTCAAAAAGTTTGGGGGTTCAACCGAAACAGGGTTAGATCT

           GCCTCTGCCCAGCTCCACCTACTTTCAAGTTGTTAATTTTGATTTCCGTTTTTAGCAGAT

           GTAATTTGGTTATTGAATCTCATCCAAACACCCTTAGAACGAAAAATGTAAGCTCACATA

           ATTTGTACTCATCCAAACTCACATAAATTTCTTAAGAAGAATCTAGACCACAAACTACCC

           ATAGTCACACAAAATATCTTTTTATCAAGGTATATTCTTATAAGTAATTTTTATTCCACA

           AAATTTTCTAAAGGACATACTGTGACACTTGGCATGAATCTAATTTAACATTCTTTCTTT

           ATCAGGTTATAAATTGAAACCATTGAACGTATTTTGTCTTCCCGTCCCTAAAACATTGTG

           ATATATCTGTAATATTTTAATATTTTTAATTTTAAAAATAATTTAGACTAATAATATAGA

           TATTTAAATTAGGTAATTAGTAAATTCAAATCAACTATAGTTTGGAATAAAAAAACAAAA

           TGATATATGCCAAGTACAATCTAATAACAATAGAATGCACTTTTGGTTAATGTTTACTCC

           TAATTTCTTAATTTCTAGTTTGGCTCCCCCCACCGTTATGGTAAAAAATCGTTATGTTTT

           AACTATTTATCTGCTAGAGCATATGTAATATTTATGTAAACTATGTTCTTTATTACCAAT

           CAAAATAGACACCTTACCTACTTTCATCAAAAGAAATAAAAAATGTAGTAGGTATTATTC

           TGGAATACTCCTCGTCGGAAACATATGGAACGCATTTCCCTCAAAACGCAAAAACCGGGG
                                GAAACATATGGAACGCATTTCCCTCAAAACGCAAAAACCGGGG

           CCCCGAGCCCTCGACACCAACCCCGTCAGATGTGAAGCCGACGGCCCAAATGGAGGCCAG
           CCCCGAGCCCTCGACACCAACCCCGTCAGATGTGAAGCCGACGGCCCAAATGGAGGCCAG

           CGGTGCCAATGCGTAGGCCACGCGTGCCTCTGCGCAAATCTTATCTCCCGGGAGGTTCGC
           CGGTGCCAATGCGTAGGCCACGCGTGCCTCTGCGCAAATCTTATCTCCCGGGAGGTTCGC

           TCCACTTCCCTCCCCGAGTCACTCAAACCATCCAATCCCCCCACCTTCCCTTTCCTTGCC
           TCCACTTCCCTCCCCGAGTCACTCAAACCATCCAATCCCCCCACCTTCCCTTTCCTTGCC

           CCCATTCCCCACCGTCGCAAGGAAAAATATTATTAATAAACAATGATGTTGATGTATTAT
           CCCATTCCCCACCGTCGCAAGGAAAAATATTATTAATAAACAATGATGTTGATGTATTAT

           TATTAATCAATGATATTGATGTATTTCTAAATATTTTCACGTATTATTTATTAAGTGTCA
           TATTAAACAATGATATTGATGTATTTCTAAATATTTTCACGTATTATTTATTAAGTGTCA

           GTGATTGAAATGGATCCTGGTCGGAAATGAATCCTGGTCGGAAAATGGTTAC
           GTGATTGAAATGGATCCTGGTCGGAAATGAATCCTGGTCGGAAAATGGTTAC
```

Fig. 17. Nucleotide sequence of the genomic region between the first exon and intron of the α subunit of rice G-protein in a *d1* mutant line (HO541) and Nipponbare. Potential CAAT and TATA boxes are underlined. Exon 1 is boxed.

These results may suggest a *d1* mutant HO541 is caused by lack of expression of GTP-binding protein by a structural deficiency in the gene.

Discussion

A sries of d1 mutant lines as well as wild type variety were analyzed by Southern and Northern hybridization analysis using the EST (S5933) which was a possible candidate gene of *D1* as a probe. Southern analysis showed that five different polymorphisms among the*d1* mutant lines (Fig.14). By Northern analysis, a 1.8-kb translated product was observed in wild type varieties, was not observed in the four *d1* mutant lines (Fig.15-A). In addition, the sequencing analysis indicated that the region between the first exon and first intron of S5933 in a *d1* mutant HO541 lacked 833 bp sequence. Based on Southern, Northern and sequencing analysis of *d1* mutant, S5933 encodes *D1* gene and transcription of S5933 is not be transcribed in four *d1* mutant lines. To verify strictly whether the EST S5933 encode the *D1* gene or not, complementation test is required. Therefore, the cosmid clones carrying the *D1* gene is screened from the cosmid library of Nipponbare, and the clones is transformed into the *d1* mutant. Subsequently, observation of transformants showing normal phenotype or not is required.

Rice dwarf mutant *d1* shows the dwarf phenotype as well as shows various phenotypes of dark green leaves, compact panicles and short round grains. As these characters are all induced by the *d1* gene, the rice α subunit of heterotrimeric GTP-binding protein that *D1* encodes is likely to play a key role in

controlling growth and development. In addition to the genetic and molecular evidence described above, the modulation technique of gene expression supports our conclusion that *d1* is caused by a structural deficiency in the α subunit of GTP-binding protein; that is, the antisense cDNA of wild-type *RGA1* caused a dwarf phenotype (Y. Iwasaki, personal communication).

Since *d1* mutant in rice was classified into gibberellin insensitive dwarf mutant (Mitsunaga et al. 1994), *D1* gene may associate with gibberellins (GAs) signal transduction. Gibberellins (GAs) are large families of tetracyclic diterpenoid plant growth-hormones, It involved in a number of plant growth and development processes such as seed germination, stem elongation, flowering and fruit development (Crozier 1983). Gas also regulate expression of alpha-amylase in the cereal aleurone layer (Baulcombe and Buffard 1983, Nolan and Ho 1988). There were many mutants with respected to gibberellins. They were classified into several types of dwarf mutant with GA deficient; non-response to GA, reduced response to GA, weak response to GA, and over sensitive response to GA (constitutive response and enhanced response)(Ross et al. 1997). GA biosynthetic pathways have been investigated well by characterizing GA-deficient mutant plants, and several genes coding enzymes catalyzing steps in GA biosynthesis were isolated from these mutants (Sun et al. 1992, Sun and Kamiya 1994, Xu et al. 1995, Chiang et al. 1995, Winkler and Helentjaris 1995). For example, in GA-sensitive dwarf mutant *le* in pea, first identified by Gregor Mendel, phenotype is reversed by treatment GA_1 and the mutant was shown to be deficient in gibberellin-3-beta-hydroxylase which converts GA_{20} to GA_1 in the

GA biosynthetic pathway (Lester et al. 1997, Martin et al. 1997).

On the other hand, since insensitive, constitutive and enhanced GA mutants possibly lack the signal or made errors in the signal transduction pathway of GAs, and therefore analysis of these mutants can open the cue to clarify molecular mechanisms of signal transduction by GAs. Several mutants were defined as non response GA mutants, for example, *GAI* in *Arabidopsis* (Koornneef et al. 1985, Peng and Harberd 1993), *D8* and *D9* in maize (Harberd and Freeling 1989, Winkler and Freeling 1994), *Rht3* in wheat (Ho et al. 1981), as constitutive GA respond mutants *spy* and *rga* in *Arabidopsis* (Jacobsen and Olszewski 1993, Silverstone et al. 1998), *sln* in barley (Chandler 1988, Lanahan and Ho 1988). *GAI* gene (gibberellin insensitive) and *spy* gene (elongated constitutive activating GA signal) were isolated in *Arabidopsis* (Jacobsen et al. 1996, Peng et al. 1997). *Spy* containing a tetratricopeptide (TPC) repeat region acts to suppress GA signal transduction, and it is epistatic to *gai* included *Spy* acting on downstream of *GAI* (Jasobsen et al. 1996). But the mechanisms of signal transduction triggered by GAs are still not clear. Because the rice mutant *d1* has been characterized as GA-insensitive (Mitsunaga et al. 1994), the identification of the gene responsible for the phenotype might contribute to the elucidation of the signal transduction pathways related to GA if the dwarfing is caused by a deficiency in these pathways. Very recently, it has been shown that heterotrimeric G-proteins are associated with inducing the expression of the alpha-amylase in oat aleurone by gibberellin (Jones et al. 1998). This result is very significant, because alpha-amylase isregulated by gibberellic acids (GAs)

in the cereal aleurone layer (Baulcombe and Buffard1983, Nolan and Ho1988). Recently, a putative G-protein-coupled receptor which influences sensitivity to one of the cytokinins (GPCR or GCR) has been identified in *Arabidopsis* (Josefsson and Rask 1997, Dymock et al. 1998). Since heterotrimeric GTP-binding protein plays an important role in signal transduction in animals and microbes (Gilman 1987, Neer 1995, Hamm 1998), in several plant species (Terryn et al. 1993, Ma 1994), the G-protein encoded by *D1* gene in rice may associate with GA signal transduction.

In this study, it is concluded that rice dwarf *D1* gene encodes α subunit of heterotrimeric GTP-binding protein and the protein plays an important role of cellular growth, differentiation and development in plant. As the result of lack of the signal, *d1* mutants may show abnormal phenotype such as dwarf, compact panicle and small round grain. Author suggested the α subunit of heterotrimeric GTP-binding protein in rice was associated with GA signal transduction. Characterization of the GTP-binding protein that definition the mutation caused by GA-insensitivity and analyses of the structure of plausible plant hormone receptors and the biochemical interactions between the receptor and G-proteins are required to clarify the G-protein-mediated signal transduction system in plants.

Dwarf mutants in plants are crucial for elucidating the mechanisms for plant growth and development. Dwarf mutants have been isolated in many plant species and have been extensively analyzed for their mode of inheritance and their response to plant hormones (Reid 1993, Hooley 1994, Ross et al. 1997). In

rice, at least 60 dwarf mutants were known (Kinoshita 1995), but only the 2 mutants, *d18* and *d35*, are known to have a deficiency in gibberellin biosynthetic pathways (Suge and Murakami 1968, Murakami 1995, Futsuhara and Kikuchi 1995). In rice dwarf mutants, there might be more gibbrellin associated mutants, screening and analysis of them should be required for elucidation the mechanism of signal transduction of GAs.

Summary and Conclusion

The past rice genetic studies have led to the establishment of a linkage map of rice chromosomes based on morphological markers. With the recent progress in biotechnology, DNA can now be manipulated easily so molecular markers have been propagated as a useful tools for rice genetics. These progresses of rice genomics such as the construction of rice RFLP linkage maps and genomic libraries made it possible to isolate the genes by map-based cloning strategy.

A dwarf chimeric rice plant has been maintained for more than 50 years in Kyushu University. This mutant showed chimeric morphologies about dwarf and normal in many parts of a plant body. Allelic and progeny test revealed that the chimerism appeared to occur at the *D1* locus. To elucidate the mechanism to generate the chimerism, cloning of *D1* gene was essential. The cloning *D1* locus from the dwarf chimeric rice plant was very difficult by means of map-based cloning strategy, because the phenotypes were unstable. Thus, instead of the dwarf chimeric rice plant, the rice dwarf mutant Daikoku carrying *d1* gene was used as materials for map-based cloning of *D1* locus. Daikoku carrying the *d1* gene was first identified as a spontaneous mutant that exhibited not only short plant height but also broad, dark green leaves, compact panicles, and small round grains. All of phenotypes were induced by a recessive allele (*d1*) of the *D1* gene.

In this study, the mapping population consisting of 3182 F_2 plants

derived from the cross of a*d1*mutant and a substituted line was constructed at first. The high-resolution RFLP linkage map using this population and the physical map was constructed. The linkage analysis revealed that a EST encoding the α-subunit of GTP-binding protein cosegregated with *d1* in 3185 *d1* segregants. Southern hybridization analysis using the EST as a probe exhibited different band patterns in several *d1* mutant lines. Northern analysis was done with the EST as a probe. A 1.8-kb translated product was observed in two wild types Nipponbare and Kasalath, but not in the four*d1* mutant lines. Sequencing analysis of genomic region of S5933 in Nipponbare and a *d1* mutant line (HO541) revealed that 833-bp deletion had occurred in the *d1*mutant allele, which resulted in an inability to express GTP-binding protein. Based on these results, Author concluded that the rice *Dwarf 1* gene encodes the α -subunit of GTP-binding protein and the protein plays an important role in plant growth and development in the plant.

The *d1* mutant was characterized as gibberellin-insensitive and the wild*D1* gene may associate with Gibbrellin signal transduction. Since GTP-binding protein was well known as the most important protein in signal transduction in animal cells, author suggested that the GTP-binding protein encoded by the *D1* gene might be associated with GA signal transduction of GAs. GAs is important plant hormones as well ethylene, auxin, abscisic acid, and cytokinin, which regulate and control plant growth and development. To know the molecular mechanism of GAs to function as plant hormone is crucial to understand the plant physiology as a whole. In spite of many and long-time

efforts to elucidate the physiological function of GAs at molecular level, no clear results are still obtained. However, using tools and information produced by the recent rice genome research, Author succeeded in the identification of the *D1* gene associated with signal transduction of GAs.

In future works, the complementation test of the gene should be required for confirmation that *D1* gene encoded the α -subunit of GTP-binding protein. In addition, characterizing the structure, transcription and translation of the *D1* gene in dwarf chimeric rice plant will be also required for elucidation mechanism of chimerism.

Acknowledgment

I would like to express my deepest appreciation to my adviser, Dr. Atsushi Yoshimura, Professor of the Plant breeding laboratory, Faculty of Agriculture, Kyushu University, for his invaluable advice and guidance throughout this work and for reviewing this thesis.

I offer my thanks to Dr. Hikaru Satoh, Professor of the laboratory Plant Genetic Resources, Faculty of Agriculture, Kyushu University for critically reviewing and precious suggestions.

I also express to Dr. Hiroshi Fujii, Professor, the laboratory of Insect genetic Resources, Faculty of Agriculture, Kyushu University for critically reviewing and valuable advice.

I would like to express my appreciation Dr. Nobuo Iwata, former professor of the Plant breeding laboratory, Faculty of Agriculture, Kyushu University

I greatly appreciate to Dr. Takuji Sasaki, head of Rice Genome Research Program (RGP) in Japan for directions and management.

I also greatly appreciate to Dr. Masahiro Yano sub leader of RGP for instruction and valuable suggestions.

I offer my thanks to Dr. Hideshi Yasui, Associate professor of the Plant breeding laboratory, Faculty of Agriculture, Kyushu University for valuable suggestions.

I also express my most sincere thanks and appreciation to students of

Plant laboratory in Kyushu University and colleges in RGP.

Lastly, I would like to appreciate profoundly my father Masaaki, my mother Suzuko and my wife Tomoko for supporting me.

References

Akemine, M. 1925. On the inheritance of dwarlfism in rice. Nippon gakujyutsukaihou 1 : 308-314. (in Japanese)

Allard, R. W. 1956. Formulas and tables to facilitate the calculation of recombination values in heredity. Hilgardia 24 : 235-278.

Altschul, S. F and D. J. Lipman. 1990. Protein database searches for multiple alignments. Proc. Natl. Acad. Sci. USA. 87 : 5509-5513.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215 : 403-410.

Antonio, B. A., M. Emoto, J. Wu, I. Ashikawa, Y. Umehara, N. Kurata and T. Sasaki. 1996. Physical mapping of rice chromosomes 8 and 9 with YAC clones. DNA research. 3 : 393-400.

Ashikari, M., O. Ideta, A. Yoshimura and N. Iwata. 1997. Genetic analysis a d1 chimeric rice plant. International Rice Research Notes. 2 : 12-13.

Baulcombe, D. C and D. Buffard. 1983. Gibberellic-acid-regulated expression of α-amylase and six other genes in wheat aleurone layers. Planta 157 : 493-501.

Burke, D. T., G. F. Carle and M. V. Olson. 1987. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science. 236 : 806-812.

Causse, M. A., T. M. Fulton, Y. G. Cho, S. N. Ahn, J. Chunwongse, K. Wu, J. Xiao, Z. Yu, P. C. Ronald, S. E. Harrington, G. Second, S. R. MaCouch and S. D. Tanksley. 1994. Saturated molecular map of the rice genome based on an interspecific backcross population. Genetics. 138 : 1251-1274.

Chandler, P. M. 1988. Hormonal regulation of gene expression in the slender mutant of barley (*Hordeum vulgare* L.). Planta. 175 : 115-120.

Chen, X., S. Temnykh, Y. Xu, Y. G. Cho, S. R. McCouch. 1997. Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). Theor. Appl. Genet. 95 : 553-567.

Chiang, H-H., I. Hwang and H. M. Goodman. 1995. Isolation of the arabidopsis *GA4* locus. Plant cell. 7 : 195-201.

Chomczynski, P and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochem. 162 : 156-159.

Churchill, G. A., J. J. Giovannoni and S. D. Tanksley. 1993. Pooled-sampling makes high-resolution mapping practical with DNA markers. Proc. Natl. Acad. Sci. USA. 90 : 16-20.

Crozier, A. 1983. The biochemistry and physiology of gibbrellins. Vol. 2. (Praeger, New York).

Dymock, S. P., D. Dymock and R. Hooley. 1998. A highr plant seven-transmembrane receptor that influences sensitivity to cytokinins. Curr. Biol. 8 : 315-324.

Edwards, K. J., H. Thompson, D. Edwards, A. D. Saizieu, C. Sparks, J. A. Thompson, A. J. Greenland, M. Eyers and W. Schuch. 1992. Construction and characterization of a yeast artificial chromosome library containing three haploid maize genome equivalents. Plant. Mol. Biol. 19 : 299-308.

Futsuhara, Y and F. Kikuchi. 1995. Dwarf characters. Science of Rice plant. Vol.3, Genetics. pp. 300. T.Matsuo, K. Kumazawa, R. Ishihara and H. Hirata,Eds. (Food and Agriculture Policy Research Center, Tokyo).

Gilman, A. G. 1987. G proteins: Transducers of receptor-generated signals. Ann. Rev. Biochem. 56 : 615-649.

Grill, E and C. Somerville. 1991. Construction and characterization of a yeast artificial chromosome library of *Arabidopsis* which is suitable for chromosome walking. Mol. Gen. Genet. 226 : 484-490.

Hamm, H. E. 1998. The many faces of G protein signaling. J. Biol. Chem. 273 : 669-672.

Harberd, N. P and M. Freeling. 1989. Genetics of dominant gibberellin-insensitive dwarfism in maize. Genetics. 121 : 827-838.

Harushima, Y., M. Yano, A. Shomura, M. Sato, T. Shimano, Y. Kuboki, T. Yamamoto, S. Y. Lin, B. A. Antonio, A. Parco, H. Kajiya, N. Huang, K. Yamamoto, Y. Nagamura, N. Kurata, G. S. Khush and T. Sasaki. 1998. A high-density rice genetic linkage map with 2275 markers using a single F_2 population. Genetics. 148 : 479-494.

Ho, T-H. D., R. C. Nolan and D. E. Shute. 1981. Characterization of a gibberellin-insensitive dwarf Wheat, D6899. Plant Physiol. 67 : 1026-1031.

Hooley, R. 1994. Gibberellins: perception, transduction and responses. Plant. Mol. Biol. 26 : 1529-1555.

Hossain, M. 1995. Economic prosperity in Asia : implications for rice research. Rice Genetics. III : 3-16.

Ideta, O., A. Yoshimura and N Iwata. 1996. An integrated linkage map of rice.Rice Genetics III. : 47-56.

Imai, T and M. V. Olsen. 1990. Secon-generation approch to the construction od yeast artificial chromosome libraries. Genomics. 8 : 297-303.

Isegawa, Y., J. Sheng, Y. Sokawa, K .Yamanishi, O. Nakagomi and S. Ueda. 1992. Selective amplification of cDNA sequence from total RNA by cassette-ligation mediated polymerase chain reaction (PCR): application to sequencing 6.5 kb genome segment of hantavirus strain B-1. Mol. Cell. Probes. 6 : 467-475.

Ishikawa, A., H.Tsubouchi, Y. Iwasaki and T. Asahi. 1995. Molecular cloning and characterization of a cDNA for the _ subunit of a G protein from rice. Plant. Cell. Physiol. 36 (2) : 353-359.

Iwasaki, Y., T. Kato, T. Kaidoh, A. Ishikawa and T. Asahi. 1997. Characterization of the putative _ subunit of a heterotrimeric G protein in rice. Plant. Mol. Biol. 34 : 563-572.

Iwata, N., T. Omura and M. Nakagahra. 1970. Studies on the trisomics in rice plants (*Oryza sativa* L.), I. Morphological classification of trisomics. Japan. J. Breeding. 20 : 230-236.

Iwata, N and T. Omura. 1971. Linkage analysis by reciprocal translocation method in rice plant (*Oryza sativa* L.), I. Linkage groups corresponding to the chromosome 1. 2. 3 and 4. (in Japanese with English summary). Japan. J. Breed. 21 : 19-28.

Iwata, N and T. Omura. 1975. Studies on the trisomics in rice plants. (*Oryza sativa* L.), III. Relation between trisomics and genetic linkage groups. Japan. J. Breed. 25 : 363-368.

Jacobsen, S. E and N. E. Olszewski. 1993. Mutations at the *SPINDLY* locus of Arabidopsis alter gibberellin signal transduction. Plant Cell. 5 : 887-896.

Jacobsen, S. E., K. A. Binkowski and N. E. Olszewski. 1996. SPINDLY, a tetratricopeptide repeat protein involved in gibberellin signal transduction in *Arabidopsis*. Proc. Natl. Acad. Sci. USA. 93 : 9292-9296.

Jones, M. H., O. S .A. Khwaja, H. Briggs, B. Lambson, P. M. Davey, J. Chalmers, C-Y. Zhou, E. M. Walker, Y. Zhang, C. Todd, M. A. Ferguson-Smith and N. A. Affara. 1994. A set of ninety-seven overlapping yeast artificial chromosome clones spanning the human Y chromosome euchromatin. Genomics. 24 : 266-275.

Jones, H. D., S. J. Smith, R. Desikan, S. P. Dymock, A. Lovegove and R. Hooley. 1998. Heterotrimeric G protein are implicated in gibberellin induction of _-amylase gene expression in wild oat aleurone. Plant. Cell. 10 : 245-253.

Josefsson, L. G and L. Rask. 1997. Cloning of a putative G-protein-coupled receptor from Arabidopsis thaliana. Eur. J. Biochem. 249 : 415-420.

Kaziro, Y., H. Itoh, T. Kozawa, M. Nakafuku and T. Satoh. 1991. Structure and function of signal-transducing GTP-binding proteins. Annu. Rev. Biochem. 60 : 349-400.

Kinoshita, T. 1995. Report of committee on gene symbolization, nomenclature and linkage groups. Rice. Genetics Newsletter. 12 : 9-153.

Kleine, M., W. Michalek, A. Graner, R. G. Herrmann and C. Jung. 1993. Construction of a barley (*Hordeum vulgare* L.) YAC library and isolation of a *Hor1*-specific clone. Mol. Gen. Genet. 240 : 265-272.

Koike, K., K. Yoahino, N. Sue, Y. Umehara, I. Ashikawa, N. Kurata and T. Sasaki. 1997. Physical mapping of rice chromosome 4 and 7 using YAC clones. DNA research. 4 : 27-33.

Koornneef, M., A. Elgersma, C. J. Hanhart, E. P. van. Loenen-Mortinet, L. van. Rijn and J. A. D. Zeevaart. 1985. A gibberellin insensitive mutant of *Arabidopsis thaliana.* Physiol. Plant. 65 : 33-39.

Kosambi, D. D. 1944. The estimation of map distances from recombination values. Ann. Eugen. 12 : 172-175.

Kurata, N., Y. Nagamura, K. Yamamoto, Y. Harushima, N. Sue, J. Wu, B. A. Antonio, A. Shomura, T. Shimizu, S-Y. Lin, T. Inoue, A. Fukuda, T. Shimano, Y. Kobuki, T. Toyama, Y. Miyamoto, T. Kirihara, K. Hayasaka, A. Miyao, L. Monna, H. S. Zhong, Y. Tamura, Z-X. Wang, T. Momma, Y. Umehara, M. Yano, T. Sasaki and Y. Minobe. 1994. A 300 kilobase interval genetic map of rice including 883 expressed sequences. Nature Genetics 8 : 365-372.

Lanahan, M. B and T-H. D. Ho. 1988. Slender barley : A constitutive gibberellin-respnse mutant. Planta. 175 : 107-114.

Lester, D. R., J. J. Ross, P. J. Davies and J. B. Reid. 1997. Mendel's stem length gene (*Le*) encodes a gibberellin 3B-hydroxylase. Plant Cell. 9 : 1435-1443.

Ma, H. 1994. GTP-binding proteins in plants : new members of an old family. Plant. Mol. Biol. 26 : 1611-1636.

Ma, H., M. F. Yanofsky and E. M. Meyerowitz. 1990. Molecular cloning and characterization of *GPA1*, a G protein _ subunit gene from *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA. 87 : 3821-3825.

Ma, H., M. F. Yanofsky and H. Huang. 1991. Isolation and sequence analysis of *TGA1* cDNA encoding a tomato G protein _ subunit. Gene. 107 : 189-195.

Martin, G. B., M. W. Ganal and S. D. Tanksley. 1992. Construction of a yeast artificial chromosome library of tomato and identification of cloned segments linked to two disease resistance loci. Mol. Gen. Genet. 233 : 25-32.

Martin, D. N., W. M. Proebsting and P. Heedden. 1997. Mendel's dwarfing gene: cDNAs from the *Le* alleles and function of the expressed proteins. Proc. Natl. Acad. Sci. USA. 94 : 8907-8911.

McCouch, S. R., G. Kochert, Z. H. Yu, Z. Y. Wang, G. S. Khush, W. R. Coffman and S. D. Tanksley. 1988. Molecular mapping of rice chromosomes. Theor. Appl. Genet. 76 : 815-829.

Mitsunaga, S., T. Tashiro and J. Yamaguchi. 1994. Identification and characterization of gibberellin-insensitive mutants selected from among dwarf mutants of rice. 1994. Theor. Appl. Genet. 87 : 705-712.

Murray, M. G and W. F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic. Acids. Research. 19 : 4321-4325.

Murakami, Y. 1995. Gibbrellins. Science of Rice plant. Vol.2. Physiology. pp. 182-189. T.Matsuo, K. Kumazawa, R. Ishihara and H. Hirata,Eds. (Food and Agriculture Policy Research Center, Tokyo).

Neer, E. J. 1995. Heterotrimeric G proteins: Organizers of transmenbrane signals. Cell. 80 : 249-257.

Ngao, S and M. Takahashi. 1963. Trial construction of twelve linkage groups in japanese rice. (Genetical studies on rice plant, XXVII). Journ. Facul. Agr., Hokkaido Univ., Sapporo. 53 : 72-130.

Nolan, R. C and T-H. D. Ho. 1988. Hormonal regulation of gene expression in barley aleurone layers. Planta. 174 : 551-560.

Peng, J and N. P. Harberd. 1993. Derivative alleles of the Arabidopsis gibberellin-insensitive (*gai*) mutation confer a wild-type phenotype. Plant Cell. 5 : 351-360.

Peng, J., P. Carol, D. E. Richards, K. E. King, R. J. Cowling, G. P. Murphy and N. P. Harberd. 1997. The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. Gene Develop. 11 : 3194-3205.

Reid, J. B. 1993. Plant hormone mutants. J. Plant. Growth. Regul. 12 : 207-226

Ross, J. J., I. C. Murfet and J. B. Reid. 1997. Gibberellin mutants. Physiol. Plant. 100 : 550-560.

Saito, A., M. Yano, N. Kishimoto , M. Nakagahra, A. Yoshimura, K. Saito, S. Kuhara. Y. Ukai, M. Kawase, T. Nagamine, S. Yoshimura, O. Ideta, R. Ohsawa, Y. Hayano, N. Iwata and M. Sugiura.1991. Linkage map of restriction fragment length polymorphism loci in rice. Japan. J. Breed. 41 : 665-670.

Saji, S., Y. Umehara, N. kurata, I. Ashikawa and T. Sasaki. 1996. Construction of YAC contigs on rice chromosome 5. DNA research. 3 : 297-302.

Seo, H-S., H-Y. kim, J-Y. Jeong, S-Y. Lee, M-J. Cho and J-D. Bahk. 1995. Molecular cloning and characterization of *RGA1* encoding a G protein _-subunit from rice (*Oryza sativa* L. IR-36). Plant. Mol. Biol. 27 : 1119-1131.

Shimokawa, T., N. Kurata, J. Wu, Y. Umehara, I. Ashikawa and T. Sasaki. 1996. Assignment of YAC clones spanning rice chromosomes 10 and 12. DNA research. 3 : 401-406.

Shizuya, H., B. Birren, U-J. Kim, V. Mancino, T. Slepak, Y. Tachiri and M. Simon. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. USA. 89 : 8794-8797.

Silverstone. A. L., C. N. Ciampaglio and T. Sun. 1998. The arabidopsis *RGA* gene encodes a Transcriptional regulator repressing the gibberellin signal transduction pathway. Plant Cell. 10 : 155-169.

Song, W-Y. G-L. Wang, L-L. Chen, H-S. Kim, L-Y. Pi, T. Holsten, J. Gardner, B. Wang, W-X. Zhai, L-H. Zhu, C. Fauquet and P. Ronald. 1995. A receptor

kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270 : 1804-1806.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98 : 503-517.

Suge, H and Y. Murakami. 1968. Occurrence of a rice mutant deficient in gibberellin-like substances. Plant and cell Physiol. 9 : 411-414.

Sun, T-P., H. M. Goodman and F. M. Ausubel. 1992. Cloning the Arabidopsis *GA1* locus by genomic subtraction. The plant cell. 4 : 119-128.

Sun, T-P and Y. Kamiya. 1994. The Arabidopsis *GA1* locus encodes the Cyclase *ent*-kaurene synthetase A of gibberellin biosynthesis. Plant Cell. 6 : 1509-1518.

Takahashi, M and T. Kinoshita. 1968. Present status of rice linkage map. Genetical studies on rice plant, XXXI. (in Japanese with Enblish summary). Res. Bull. Univ. Farm Hokkaido Univ. 16 : 33-41.

Tanoue, H., T. Shimokawa, J. Wu, N. Sue, Y. Umehara, I. Ashikawa, N. Kurata and T. Sasaki. 1997. Ordered YAC clone contigs assigned to rice chromosomes 3 and 11. DNA Research. 4 : 133-140.

Terryn, N., M. V. Montagu and D. Inze. 1993. GTP-binding proteins in plants. Plant. Mol. Biol. 22 : 143-152.

Umehara, Y., A. Inagaki, H. Tanoue, Y. Yasukouchi, Y. Nagamura, S. Saji, Y. Otsuki, T. Fujimura, N. Kurata and Y. Minobe. 1995. Construction and characterization of a rice YAC library for physical mapping. Mol. Breed. 1 : 79-89.

Umehara, Y., H. Tanoue, N. Kurata, I. Ashikawa, Y. Minobe and T. Sasaki. 1996. An ordered yeast artificial chromosome library covering over half of rice chromosome 6. Genome Reserch. 6 : 935-942.

Yoshimura, S., U. Yamanouchi, Y. Katayose, S. Toki, Z-X. Wang, I. Kono, N. Kurata, M. Yano, N. Iwata and T. Sasaki. 1998. Expression of *Xa1*, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. Proc. Natl. Acad. Sci. USA. 95 : 1663-1668.

Ward, E. R and G. C. Jen. 1990. Isolation of single-copy-sequence clones from a yeast artificial chromosome library of randomly-sheared *Arabidopsis thaliana* DNA. Plant. Mol. Biol. 14 : 561-568.

Wang, G-L., T. E. Holsten, W-Y. Song, H-P. Wang and P. C. Ronald. 1995. Construction of a rice bacterial artificial chromosome library and

identification of clones linked to the *Xa-21* disease resistance locus. Plant. J. 7 : 525-533.

Wang, Z-X., A. Idonuma, Y. Umehara, W. V. Houten, I. Ashikawa, Y. Minobe, N. Kurata and T. Sasaki. 1996. Physical mapping of rice chromosome 1 with Yeast artificial chromosomes (YACs). DNA research. 3 : 291-296.

Weiss, C. A., H. huang and H. Ma. 1993. Immunolocalization of the G protein _ subunit encoded *GPA1* in Arabidopsis. Plant Cell. 5 : 1513-1528.

Winkler, R. G and T. Helentjaris. 1995. The maize *Dwarf 3* gene encodes a cytochrome P450-mediated early step in gibberellin biosynthesis. Plant Cell. 7 : 1307-1317.

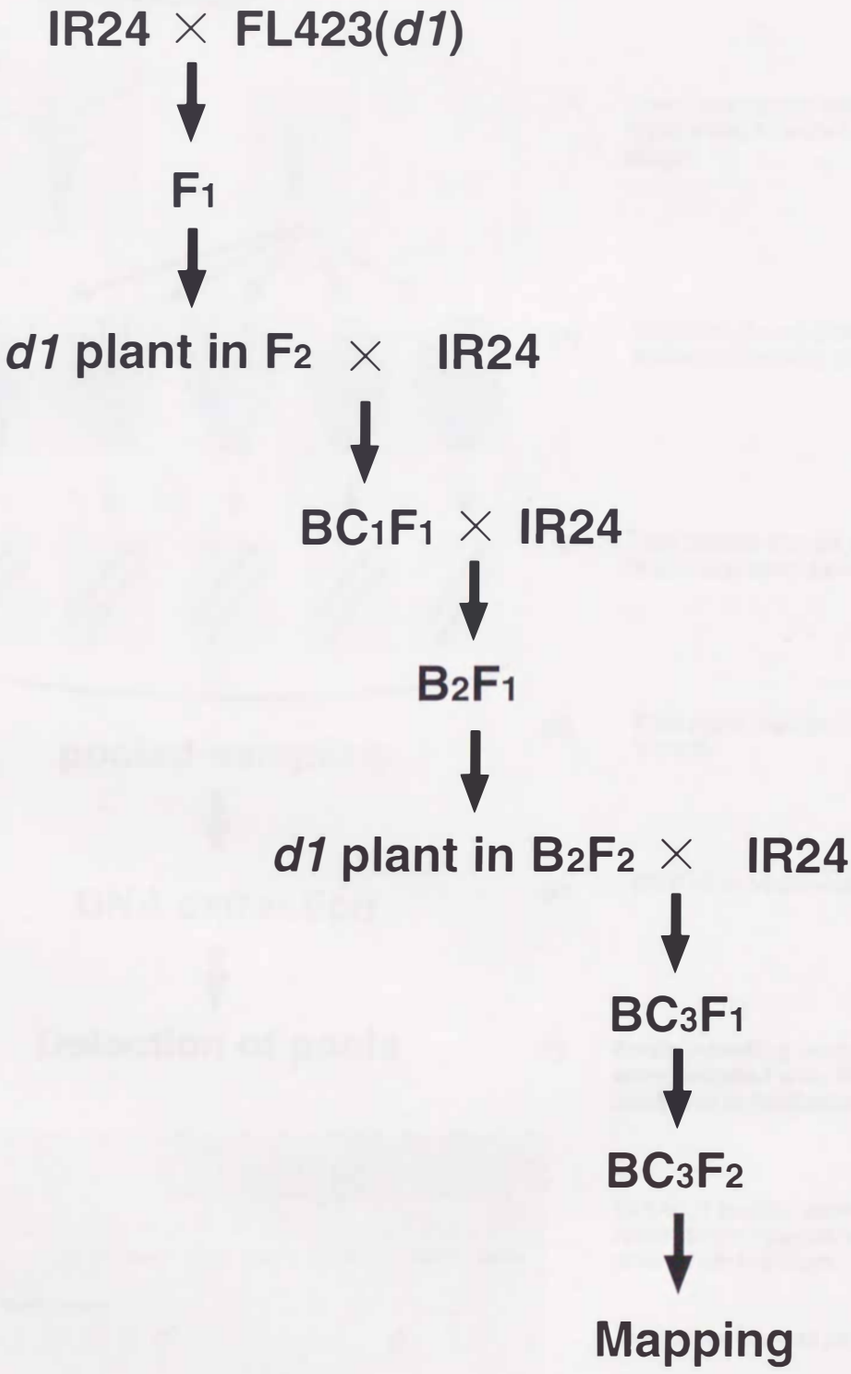
Winkler, R. G and M. Freeling. 1994. Physiological genetics of the dominant gibberellin-nonresposive maize dwarfs, *Dwarf 8* and *Dwarf 9*. Planta. 193 : 341-348.

Wu, J., T. Shimokawa, T. Maehara, K. Koike, Y. Umehara and T. Matsumoto. 1997. Mapping rice ESTs onto YAC clones. Toward the establishment of a comprehensive rice expression map. Rice Genome 6 : 2-3.

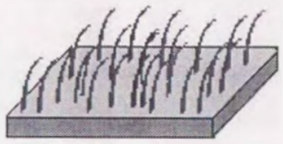
Xu, Y-L., L. Li, K. Wu, A. J. M. Peeters, D. A. Gage and J. A. D. Zeevaart. 1995. The *GA5* locus *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase : Molecular cloning and functional expression. Proc. Natl. Acad. Sci. USA. 92 : 6640-6644.

Zhang, H-B., S. Choi, S-S. Woo, Z. Li and R. A. Wing. 1996. Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. Mol. Breed. 2 : 11-24.

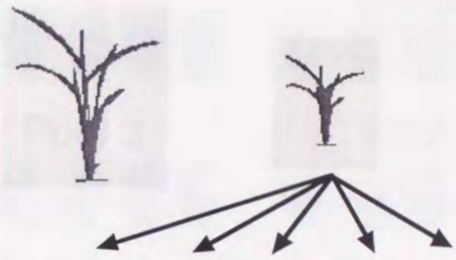
Zhang, H-B and R. A. Wing. 1997. Physical mapping of the rice genome with BACs. Plant. Mol. Biol. 35 : 115-127.



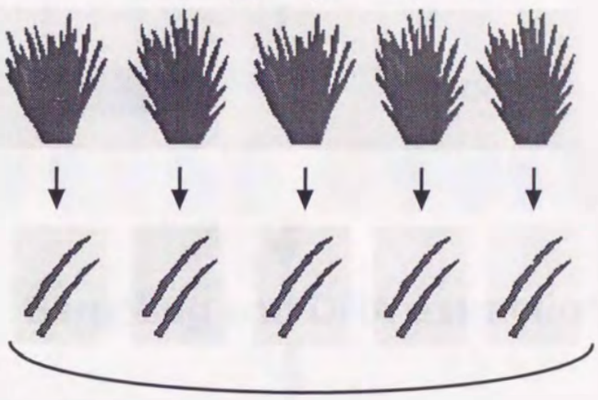
Appendix 1. Preparation for large scale mapping population.
BC_3F_2 population was used for large scale mapping of *d1* gene.



(1) F_2 seeds were sown in seed bed.



(2) Dwarf plants (*d1* recessive homo type) were selected at the seedling stage.



(3) Selected dwarf plants were transplanted into a research field.

(4) Two mature leaves of each individual were collected.

(5) Five plant leaves were bulked for 1 pool.

pooled-sampling

↓

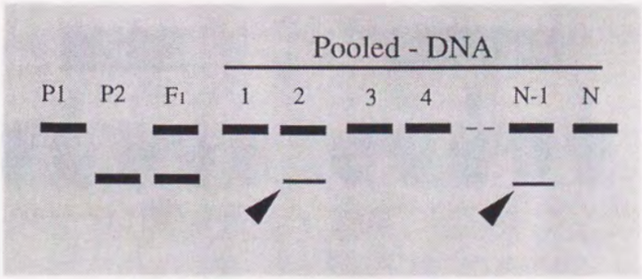
DNA extraction

↓

Detection of pools

(6) DNA of N pools were extracted.

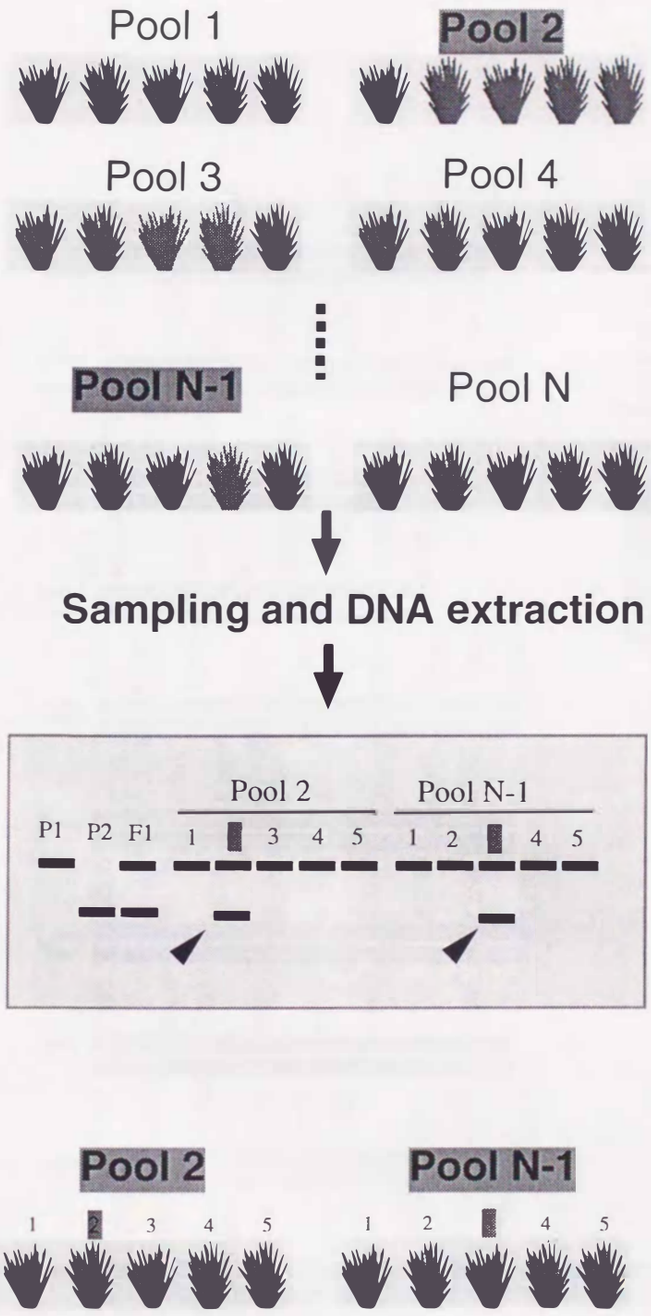
(7) Pools including recombinant plants were detected with RFLP markers by Southern hybridization.



Out of N pools, pools including recombinant plants were identified with RFLP markers.

Map distance was calculated.

Appendix 2-1. Scheme of pooled-sampling method-1.



(8) The pools including recombinant plants were identified from the information of Southern hybridization.

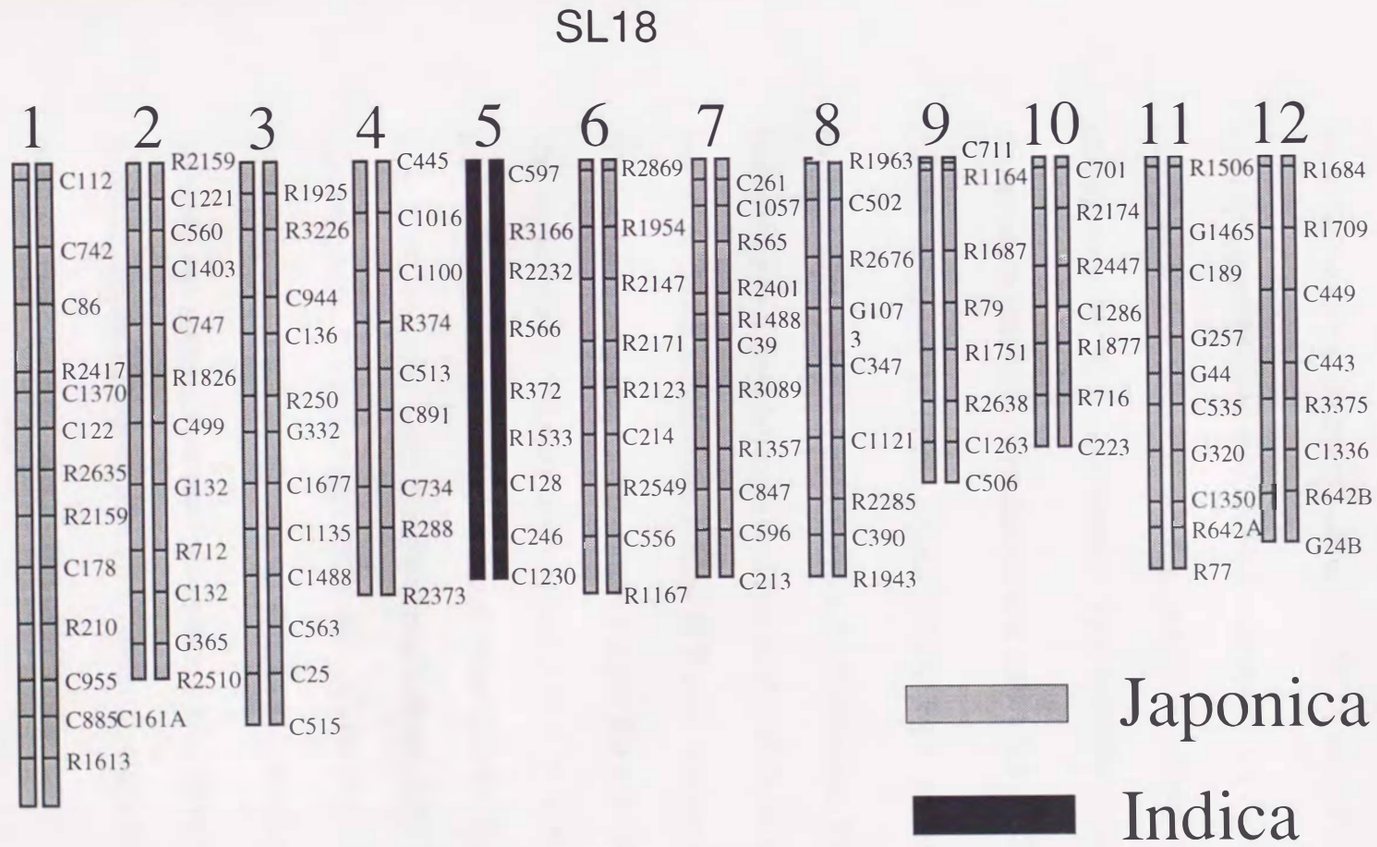
Pools including recombinant plants are shown in gray.

(9) To select the individual recombinant plants from the pools, mature leaves were collected from the pools that including the recombinant plants. And DNA was extracted

(10) Detection of recombinant plants in the pools by Southern hybridization.

(11) Identify the reconbinants plant in the pools using the information of Southern hybridization.

Appendix 2-2. Scheme of pooled-sampling method-2.



Appendix 3. Graphical genotype of SL18.

A substitution line (SL18) which substitutes of chromosome 5 of Kasalath for Nipponbare (Japonica). Japonica chromosomes are shown in gray and Indica chromosomes in black.

Appendix 4. DNA extraction method

Two g of rice leaves is ground until to be very fine powder in liquid nitrogen with hiscotron homogenizer. The powder is transferred into a 13 ml polypropylene centrifuge tube. 3.5 ml of extraction buffer (2×CTAB) was added to it and mixed well. After incubated at 56 ℃ for 5 minutes, 3.5 ml CIA (chloroform and isoamyl alcohol; 24:1) is added to it and the sample is incubated at 56 ℃ for 30 minutes. Spin the tube at 2600 rpm for 5 minutes. The supernatant solution is transferred to a new tube (A). 2 ml extraction buffer (1× CTAB) is added to lower phase, mixed well and incubated at 56 ℃ for 5 minutes. Spin the tube at 2600 rpm for 5 minutes. The supernatant solution is transferred to a new tube (A) and blended well. 6 ml of CIA and 1/10 volume of the 10 % CTAB solution are added to it and incubated at 56 ℃ for 5 minutes. Shake the tube gently for 15 minutes. Spin the tube at 2600 rpm for 5 minutes. The supernatant solution is transferred to a new tube. An equal volume of precipitation buffer is added to it and mixed gently. Keep it at room temperature until CTAB/nucleic acid complex precipitates fully. Collect the complex by centrifugation. 3 ml high-salt TE buffer with RNase is added and incubated 56 ℃ for overnight. An equal volume of isopropyl alcohol is added to it and mixed by inversion until nucleic acid precipitates fully. Spin the tube at 2600 rpm for 20 minutes. Wash the pellet with 70 % ethanol. Spin the tube at 2600 rpm for 20 minutes. Resolve the pellet in 200 μl TE buffer.

Appendix 5 Linkage

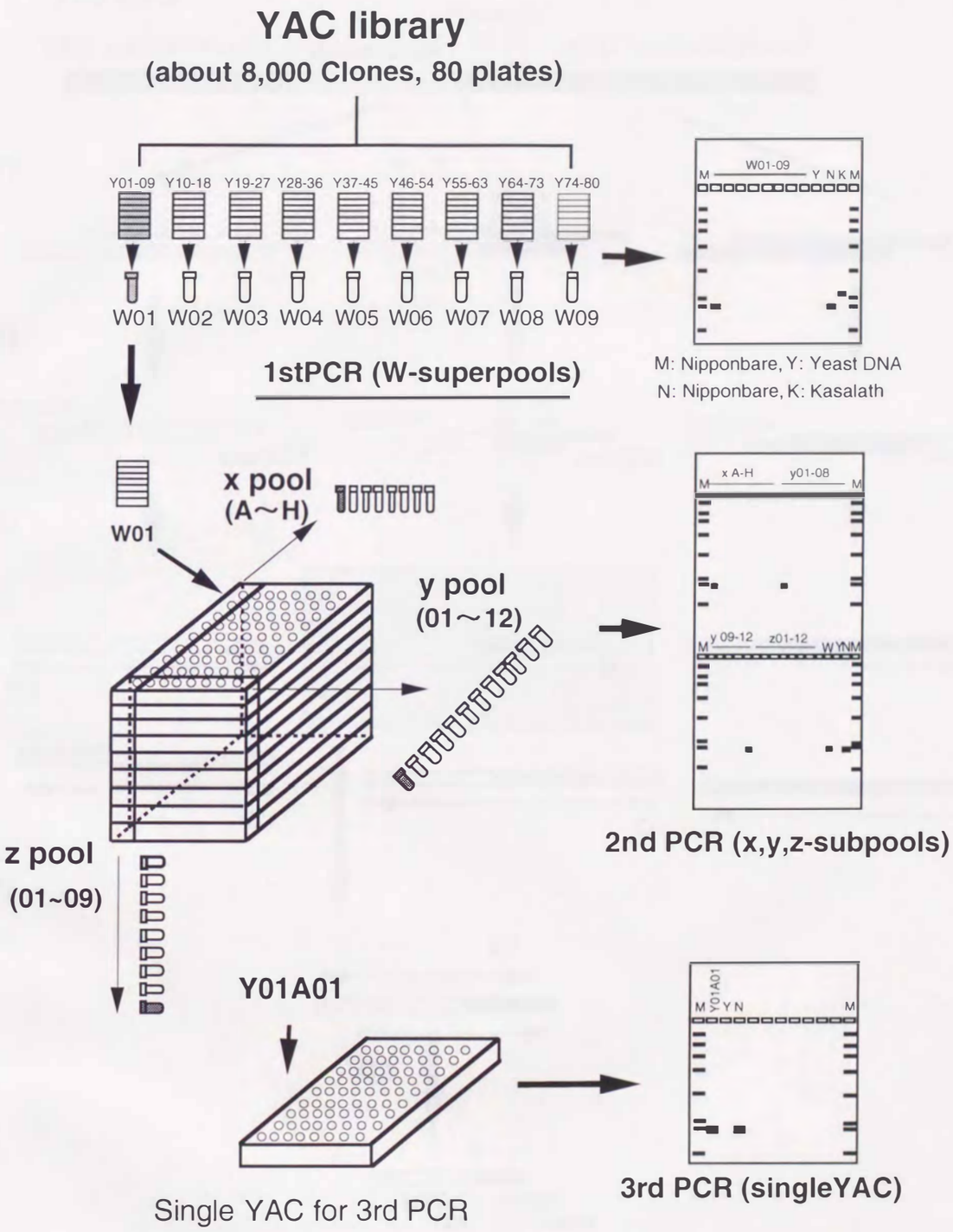
Gene pair		Segregation mode										χ^2	Recombination	Genetic distance
A(a)	B(b)	AABB	AABb	AAbb	AaBB	AaBb	Aabb	aaBB	aaBb	aabb	Total	(linkage)	Value	(cM)
d-1	X292	12	30	23	0	0	0	5	12	9	91	0.011	49.530% ± 6.419%	133.884 ± 6.455
d-1	X387	19	29	17	0	0	0	1	10	14	90	12.689	31.371% ± 5.708%	36.857 ± 5.733
d-1	X139	16	34	17	0	0	0	1	13	16	97	13.887	31.136% ± 5.481%	36.472 ± 5.503
d-1	X208	49	0	18	0	0	0	16	0	14	97	6.104	37.274% ± 6.471%	48.136 ± 6.508
d-1	X251	17	41	6	0	0	0	0	1	25	90	84.785	7.135% ± 2.796%	7.184 ± 2.799
d-1	X146	16	34	17	0	0	0	1	13	16	97	13.887	31.136% ± 5.481%	36.472 ± 5.503
d-1	X105	18	41	7	0	0	0	0	9	20	95	43.800	15.961% ± 4.047%	16.539 ± 4.056
d-1	X81	19	35	13	0	0	0	4	10	16	97	15.866	31.389% ± 5.499%	36.888 ± 5.522
d-1	X327	18	40	8	0	0	0	0	8	21	95	47.253	15.822% ± 4.030%	16.384 ± 4.039
d-1	X366	10	44	11	0	0	0	6	6	15	92	21.652	33.349% ± 5.787%	40.265 ± 5.813
d-1	X297	19	33	15	0	0	0	8	14	7	96	0.021	49.548% ± 6.250%	134.871 ± 6.282
d-1	X1458	18	43	5	0	0	0	0	2	28	96	98.688	6.553% ± 2.596%	6.591 ± 2.598
d-1	X309	21	44	2	0	0	0	0	2	26	95	96.375	3.959% ± 2.033%	3.967 ± 2.034
d-1	X3010	20	40	6	0	0	0	0	7	17	90	38.467	14.519% ± 3.970%	14.949 ± 3.978
X292	X387	11	6	0	7	23	10	1	10	21	89	51.045	21.458% ± 3.563%	22.943 ± 3.569
X292	X139	11	6	0	4	31	7	1	8	23	91	70.407	16.034% ± 3.011%	16.620 ± 3.015
X292	X208	17	0	0	38	0	4	7	0	25	91	74.648	10.894% ± 3.427%	11.072 ± 3.433
X292	X251	5	6	5	5	22	13	7	13	12	88	3.659	44.388% ± 5.232%	70.562 ± 5.251
X292	X146	11	6	0	4	31	7	1	8	23	91	70.407	16.034% ± 3.011%	16.620 ± 3.015
X292	X105	5	8	4	5	26	11	8	13	11	91	4.780	44.940% ± 5.162%	73.297 ± 5.181
X292	X81	5	8	4	4	21	17	10	15	7	91	6.648	52.038% ± ?0or-	±
X292	X327	5	8	4	5	26	11	8	12	12	91	6.341	43.720% ± 5.121%	67.573 ± 5.139
X292	X366	3	9	4	5	26	11	8	14	10	90	2.644	48.685% ± 5.265%	107.959 ± 5.285
X292	X297	6	6	5	8	25	9	9	15	8	91	2.868	50.000% ± ?0or-	±
X292	X1458	5	8	4	6	22	14	7	13	11	90	2.733	44.624% ± 5.181%	71.699 ± 5.200
X292	X309	7	5	5	7	22	13	7	15	9	90	3.422	45.683% ± 5.212%	77.461 ± 5.231
X292	X3010	5	8	4	7	25	9	8	13	10	89	3.315	46.182% ± 5.254%	80.664 ± 5.274
X387	X139	17	3	0	0	37	2	0	4	27	90	146.867	5.119% ± 1.689%	5.137 ± 1.689
X387	X208	20	0	0	35	0	4	6	0	25	90	77.874	10.195% ± 3.335%	10.340 ± 3.340
X387	X251	9	8	1	6	19	14	2	14	14	87	18.862	31.357% ± 4.459%	36.834 ± 4.471
X387	X146	17	3	0	0	37	2	0	4	27	90	146.867	5.119% ± 1.689%	5.137 ± 1.689
X387	X105	8	12	0	6	21	12	4	14	13	90	14.533	33.669% ± 4.561%	40.845 ± 4.574
X387	X81	6	9	5	8	20	11	6	14	11	90	2.044	44.066% ± 5.162%	69.082 ± 5.180
X387	X327	8	12	0	6	20	13	4	14	13	90	14.733	34.157% ± 4.597%	41.748 ± 4.610
X387	X366	4	13	2	5	22	12	7	13	11	89	5.517	43.442% ± 5.168%	66.416 ± 5.186
X387	X297	5	10	5	10	18	11	9	16	6	90	0.956	52.772% ± ?0or-	±
X387	X1458	9	11	0	6	19	14	3	13	14	89	19.607	31.944% ± 4.455%	37.814 ± 4.467
X387	X309	12	7	1	7	21	11	2	14	14	89	25.697	28.522% ± 4.179%	32.408 ± 4.189
X387	X3010	9	11	0	7	21	11	4	14	12	89	14.011	33.390% ± 4.566%	40.338 ± 4.579
X139	X208	17	0	0	45	0	2	3	0	30	97	115.165	4.513% ± 2.147%	4.526 ± 2.148
X139	X251	8	6	1	7	24	15	2	12	15	90	19.067	29.543% ± 4.240%	33.949 ± 4.250
X139	X146	17	0	0	0	47	0	0	0	33	97	210.649	0.000% ± -	0.000 ±
X139	X105	7	10	0	7	27	12	4	13	15	95	16.642	31.142% ± 4.251%	36.482 ± 4.261
X139	X81	5	8	4	10	23	14	8	14	11	97	1.330	46.100% ± 5.031%	80.109 ± 5.048
X139	X327	7	10	0	7	27	12	4	11	17	95	22.789	29.497% ± 4.123%	33.878 ± 4.132
X139	X366	3	12	1	6	25	14	7	13	11	92	6.065	43.016% ± 5.066%	64.729 ± 5.083
X139	X297	5	7	5	12	24	11	10	16	6	96	1.208	54.138% ± ?0or-	±
X139	X1458	8	9	0	8	23	16	2	13	17	96	23.250	29.658% ± 4.114%	34.126 ± 4.123
X139	X309	10	6	1	9	25	13	2	15	14	95	19.779	29.811% ± 4.148%	34.363 ± 4.157
X139	X3010	8	9	0	9	25	11	3	13	12	90	13.467	31.524% ± 4.398%	37.111 ± 4.409
X208	X251	15	30	16	0	0	0	2	12	15	90	11.422	33.137% ± 5.837%	39.884 ± 5.863
X208	X146	17	45	3	0	0	0	0	2	30	97	115.165	4.513% ± 2.147%	4.526 ± 2.148
X208	X105	14	38	12	0	0	0	4	12	15	95	12.418	33.041% ± 5.674%	39.714 ± 5.699
X208	X81	15	32	18	0	0	0	8	13	11	97	1.241	47.482% ± 6.204%	91.409 ± 6.236
X208	X327	14	38	12	0	0	0	4	10	17	95	18.902	30.432% ± 5.485%	35.338 ± 5.507
X208	X366	9	40	13	0	0	0	7	10	13	92	9.768	42.063% ± 6.243%	61.275 ± 6.276
X208	X297	17	32	16	0	0	0	10	15	6	96	0.854	54.726% ± ?0or-	±
X208	X1458	16	32	17	0	0	0	2	13	16	96	12.354	32.966% ± 5.640%	39.580 ± 5.664
X208	X309	19	30	16	0	0	0	2	16	12	95	8.067	36.307% ± 5.879%	46.026 ± 5.907
X208	X3010	17	35	10	0	0	0	3	12	13	90	11.622	31.223% ± 5.697%	36.614 ± 5.722

X251	X146	8	7	2	6	24	12	1	15	15	90	19.067	29.543% ±	4.240%	33.949 ± 4.250
X251	X105	15	2	0	3	37	2	0	7	24	90	112.889	8.094% ±	2.128%	8.166 ± 2.129
X251	X81	10	5	2	8	25	9	3	12	16	90	25.333	28.137% ±	4.124%	31.841 ± 4.133
X251	X327	15	2	0	3	36	3	0	7	24	90	109.756	8.693% ±	2.207%	8.782 ± 2.208
X251	X366	6	9	1	4	31	7	6	7	17	88	32.341	27.873% ±	4.148%	31.456 ± 4.158
X251	X297	5	9	3	12	17	13	8	17	6	90	2.244	50.000% ±	?0or-	±
X251	X1458	17	0	0	1	40	0	0	2	29	89	178.551	1.699% ±	0.977%	1.699 ± 0.977
X251	X309	16	1	0	5	34	3	0	6	24	89	113.472	8.777% ±	2.230%	8.869 ± 2.231
X251	X3010	17	0	0	3	38	1	0	6	22	87	122.678	5.919% ±	1.848%	5.947 ± 1.849
X146	X105	7	10	0	7	27	12	4	13	15	95	16.642	31.142% ±	4.251%	36.482 ± 4.261
X146	X81	5	8	4	10	23	14	8	14	11	97	1.330	46.100% ±	5.031%	80.109 ± 5.048
X146	X327	7	10	0	7	27	12	4	11	17	95	22.789	29.497% ±	4.123%	33.878 ± 4.132
X146	X366	3	12	1	6	25	14	7	13	11	92	6.065	43.016% ±	5.066%	64.729 ± 5.083
X146	X297	5	7	5	12	24	11	10	16	6	96	1.208	54.138% ±	?0or-	±
X146	X1458	8	9	0	8	23	16	2	13	17	96	23.250	29.658% ±	4.114%	34.126 ± 4.123
X146	X309	10	6	1	9	25	13	2	15	14	95	19.779	29.811% ±	4.148%	34.363 ± 4.157
X146	X3010	8	9	0	9	25	11	3	13	12	90	13.467	31.524% ±	4.398%	37.111 ± 4.409
X105	X81	12	5	1	9	30	11	0	10	17	95	39.947	21.735% ±	3.473%	23.284 ± 3.479
X105	X327	18	0	0	0	48	2	0	0	27	95	175.168	1.058% ±	0.746%	1.059 ± 0.746
X105	X366	9	8	0	3	38	7	4	4	19	92	57.217	18.270% ±	3.211%	19.155 ± 3.215
X105	X297	7	7	4	14	24	11	4	16	7	94	3.489	43.587% ±	5.034%	67.013 ± 5.051
X105	X1458	15	3	0	3	39	7	0	2	25	94	115.000	8.317% ±	2.111%	8.395 ± 2.112
X105	X309	14	4	0	7	32	10	0	8	18	93	57.237	16.971% ±	3.070%	17.672 ± 3.074
X105	X3010	16	2	0	4	44	0	0	1	23	90	135.889	3.972% ±	1.487%	3.981 ± 1.487
X81	X327	12	9	0	5	29	11	1	10	18	95	42.768	21.636% ±	3.464%	23.162 ± 3.470
X81	X366	9	10	0	4	33	8	3	7	18	92	42.587	21.534% ±	3.511%	23.037 ± 3.517
X81	X297	16	7	0	8	28	9	3	12	13	96	35.708	24.712% ±	3.711%	27.082 ± 3.718
X81	X1458	10	10	3	6	25	13	2	10	17	96	24.313	29.355% ±	4.090%	33.662 ± 4.099
X81	X309	10	9	4	5	30	9	6	7	15	95	24.095	32.072% ±	4.322%	38.031 ± 4.333
X81	X3010	10	10	0	8	28	6	2	9	17	90	37.489	23.142% ±	3.695%	25.045 ± 3.701
X327	X366	9	8	0	3	37	7	4	5	19	92	54.413	18.926% ±	3.273%	19.917 ± 3.277
X327	X297	7	7	4	14	24	10	4	16	8	94	3.979	42.560% ±	4.993%	63.023 ± 5.009
X327	X1458	15	3	0	3	38	6	0	3	26	94	119.043	8.308% ±	2.110%	8.386 ± 2.111
X327	X309	14	4	0	7	31	10	0	9	18	93	55.796	17.580% ±	3.128%	18.363 ± 3.132
X327	X3010	16	2	0	4	43	0	0	2	23	90	132.222	4.553% ±	1.592%	4.566 ± 1.593
X366	X297	7	5	4	12	27	11	4	15	7	92	4.152	42.354% ±	5.038%	62.285 ± 5.055
X366	X1458	6	5	5	10	33	7	1	6	18	91	36.714	25.941% ±	3.917%	28.736 ± 3.925
X366	X309	6	4	6	11	31	7	3	9	14	91	19.505	34.136% ±	4.570%	41.710 ± 4.583
X366	X3010	7	5	4	10	37	1	2	5	18	89	53.742	21.412% ±	3.559%	22.887 ± 3.565
X297	X1458	5	14	8	10	18	18	3	13	6	95	2.642	50.000% ±	?0or-	±
X297	X309	5	14	8	10	23	13	6	9	7	95	0.811	52.038% ±	?0or-	±
X297	X3010	5	16	3	11	20	13	4	11	7	90	4.156	45.039% ±	5.194%	73.815 ± 5.213
X1458	X309	16	2	0	5	36	4	0	7	24	94	105.787	10.046% ±	2.324%	10.185 ± 2.326
X1458	X3010	18	0	0	1	42	1	0	5	22	89	135.494	4.015% ±	1.503%	4.024 ± 1.504
X309	X3010	16	5	0	4	33	6	0	9	17	90	68.222	14.332% ±	2.854%	14.745 ± 2.857

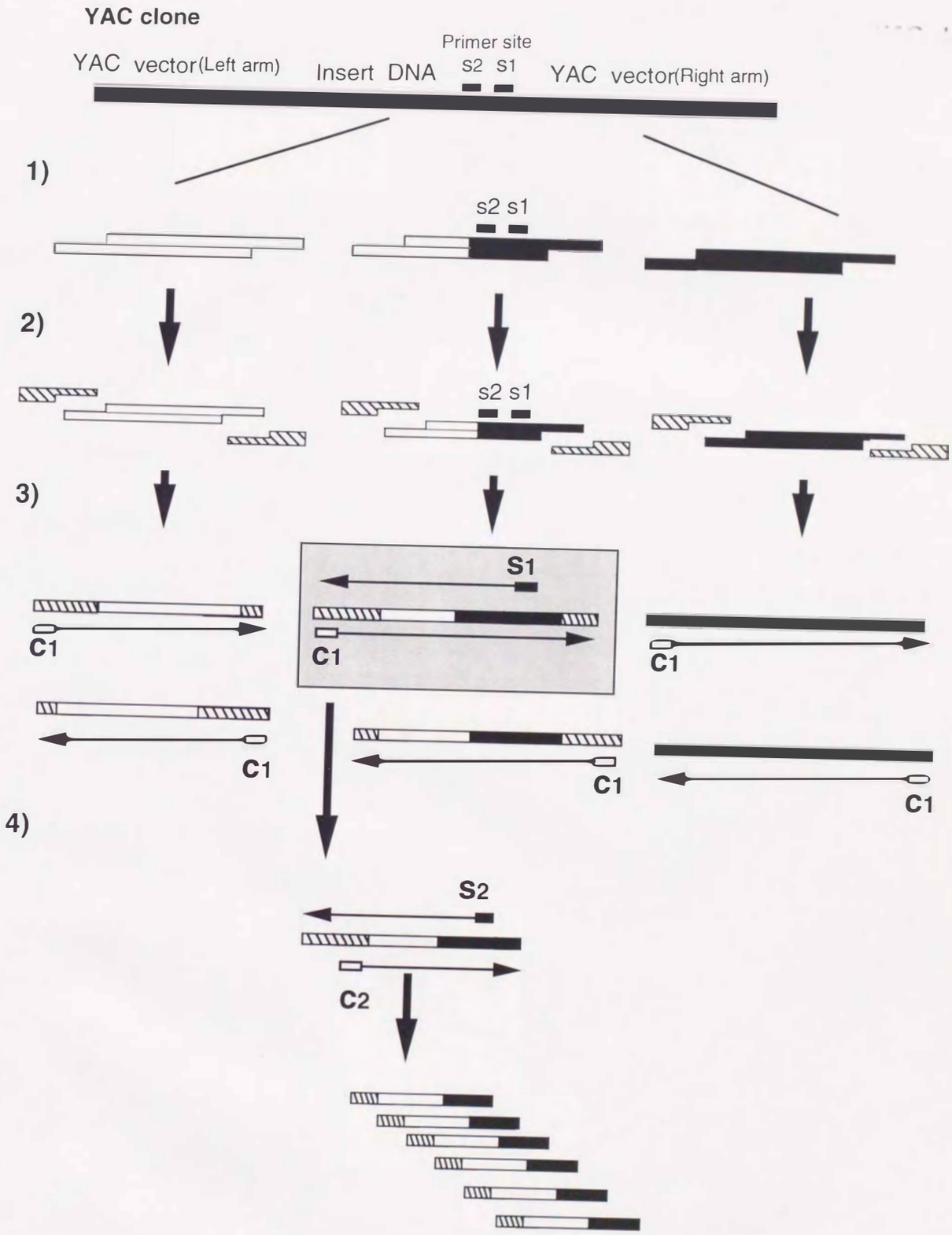
X indicates *XNpb*

Appendix 6. YAC DNA extraction

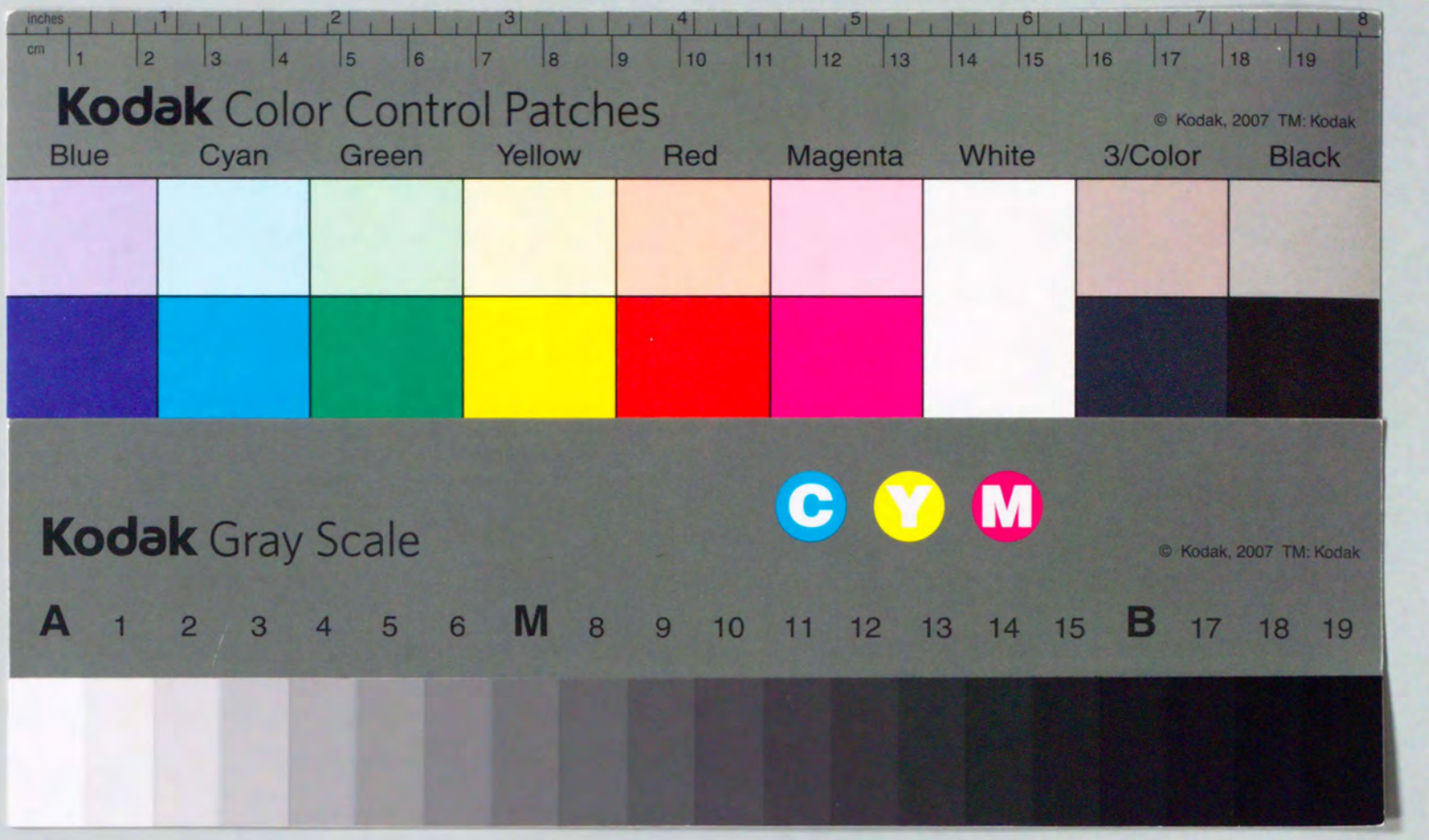
YAC clones are cultured in AHC medium at 30 ℃ with shaking for three days. YAC clones are collected by centrifugation. 1 ml of 50 mM EDTA (pH 8.0) is added to the pellet and mixed well. The tubes are spun at 5,000 rpm and 4 ℃ for 1 minute. 40 μl of SCEDZ (1M solbitol, 100 mM sodium citrate, 0.5M EDTA, 2M DTT, 1 mg zymorase) is added and Incubated at 37 ℃ for 1 hour. The tubes are spun at 5,000 rpm and 4 ℃ for 1 minute. Pellet is resolved in 50 mM Tris-HCL (pH 7.5), 20 mM EDTA (pH 8.0), 1% salcosil and Incubated at 65 ℃ for 30 minute. 40 μl of 5M KOAc is added to it and mixed well. Placed on ice for 60 minute. The tubes are spun at 50,00 rpm and 4 ℃ for 1 minute. The supernatant is transferred to a new tube. 150 μl isopropyl alcohol is added to it and mixed gently. The tube is spun at 12,000 rpm and 4 ℃ for 5 minute. Dissolved in 100 μl TE. 100 μl PCIAA (25 phenol : 24 chloroform : 1 isoamyl alcohol) is added and centrifuged at 10,000 rpm, 4 ℃ for 5 minute. The upper phase is transferred to a new tube, added 100 μl chloroform to it and centrifuged at 10,000 rpm and 4 ℃ for 5 minute. Upper phase is transferred to new tube and added 100 μl isopropyl alcohol and 10 μl 3M NaOAC (pH 5.2). Centrifuge at 12000 rpm and 4 ℃ for 15 minute. The pellet is washed with 500 μl of 70% ethanol, dried and dissolved in 30 μl TE buffer.



Appendix 7. Scheme of PCR-based screening of the YAC library



Appendix 8. Scheme of isolation of YAC end fragment DNA by cassette PCR method.
1) Digestion YAC clone by restriction enzyme. 2) Ligation of oligo cassette. 3) 1st PCR with the vector-specific primer (S1) and cassette-specific primer (c1). 4). 2nd PCR with the vector-specific primer (S2) and cassette-specific primer (c2).


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
C
Y
M
Kodak Gray Scale
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19