Molecular Linkage Analysis of Brown Planthopper Resistance Genes in Rice

村田, 和優

(Degree)
博士（農学）

(Date of Degree)
1998-03-31

(Date of Publication)
2009-05-19

(Resource Type)
doctoral thesis

(Report Number)
甲1782

(JaLCDOI)
https://doi.org/10.11501/3141125

(URL)
https://hdl.handle.net/20.500.14094/D1001782

Doctoral Dissertation

Molecular Linkage Analysis of Brown Planthopper Resistance Genes in Rice

分子マーカーによるイネトビイロウンカ抵抗性遺伝子の連鎖分析

Kazumasa Murata

The Graduate School of Science and Technology
Kobe University

January, 1998

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CHAPTER 1 General introduction

In the late of this century, population explosion has become one of the most serious problems, besides forest destruction, environmental pollution and shortage of natural resources. It has demanded more food production, hence plant breeders have tried to improve major crops for high yielding ability. The breeders' efforts have partly been fulfilled in Green Revolution - the development and spread of high-yielding varieties of two major cereal crops, rice and wheat, revolutionized agricultural production. Introduction of semi-dwarf and high tillering plant type, such as 'IR8' of rice and 'Mexican varieties' of wheat, greatly increased the yields. By changing long culm and heavy panicle type to the short culm and large number panicle type, more dense cultivation became possible with the improved mechanization of field works. Green Revolution thus greatly contributed to increase in food production.

Green Revolution, however, brought about some serious troubles and negative effects. The complete display of the high yielding ability needed the intensively controlled cultivation system such as full irrigation system and heavy use of agrochemical including nitrogenous fertilizer and pesticides. Such controlled cultivation system so far has been difficult to adopt in many under-developing countries, and only the countries with developed agricultural technology could effectively cultivate new developed varieties. As the result, the difference in the capacity of agricultural production among countries has become larger, and the agro-economical superiority in food production has been established. Furthermore, the great changes in the field ecosystem disturbed balance between crops and microbial pests and insect pests (Smith 1972, Nickel 1973). The high-yielding plant types promoted rapid spread of plant diseases and insect pests, and also advent of new pests adaptive to such varieties. Rice brown planthopper is one of the most representative insect pests stimulated by Green Revolution.

At present, brown planthopper (abbreviated as BPH), Nilaparvata lugens stål, is regarded as one of the most virulent insect pests of rice, and is widely distributed throughout the tropics and the temperate areas in east to south Asia, Micronesia and Australia (Dyck and Thomas 1979, Saxsena and Barrion 1985). Before 1970's, outbreaks

of BPH occurred infrequently only in east temperate Asia including Japan, Korea and a part of China. The wide spread of many tillering rice varieties supplied good environments for BPH, because BPH grow gregariously and survive on rice stems just near the ground. BPH sucks the sap of mostly from the phloem of rice plants and causes severe damage symptom, commonly referred as hopper-burn (Saxsena and Barrion 1985). BPH also transmits rice grassy stunt virus (Rivera et al. 1966) and rice rugged stunt virus (Ling et al. 1977). Both of these direct feeding damage and the transmitted viral diseases cause considerable yield losses in rice production (Heinrich 1979).

Protection against pest insects including BPH has depended mostly on insecticides in the pest. But the development of tolerance to insecticides has often been recognized. In the case of BPH, its insecticide tolerance was firstly detected in the late 1960's (Nagata and Moritani 1974, Endo 1996). In the middle of 1970's, BPH developed its tolerance to some organic phosphoric insecticides, and the LD values became four to twenty times higher than those in 1967 (Endo 1996). In 1979, tolerance to carbamate insecticides became apparent (Kilin et al. 1981). In addition, resurgence, a phenomenon of pest population increase after application of insecticides (Suenaga and Nakatsuka 1958, Heinrichs et al. 1982), has often been reported. Though the safety of agrochemical has increased, there may be a danger of polluting and damaging natural environments by their excessive use. Considering these negative effects, plant protection solely by insecticides should be criticized and the protection system not depending on agrochemical is needed (Chelliah and Heinrichs 1980). In view of this point, the natural host plant resistance should be searched for and the resistant varieties should be bred and utilized as an important alternative in the integrated pest management.

As the first step for breeding of insect resistant plant, genetic analysis has always been essential. On rice, genetic analysis of insect resistance begun after the Second World War and some Japanese researchers reported resistance to stem borer and stem maggot. The most prominent example was the study on resistance to rice stem maggot. Yuasa (1952) concluded that resistance to rice stem maggot was controlled by an incomplete dominant gene, and its primary mechanisms antibiosis so that the larvae died after incursion into the culm of rice. But, as the damage by these pests decreased with the

development of effective insecticides, research interest in insect resistance declined in Japan. However, in the southeast Asia, International Rice Research Institute (IRRI) was founded in the Philippines in 1960, and started extensive research on pest insects and plant diseases. Pathak (1969) reported that a rice variety TKM6 was found to be resistant to stem borers. He bred IR20, a high yielding variety resistant to this pest insect, using TKM6 as the donor of resistance (IRRI 1970). Following this first attempt, the research and breeding for BPH resistance was strengthened.

The concept of host plant resistance to pest insects was proposed by Painter (1941). He classified the host resistance into the following three categories, i. e. non-preference, antibiosis and tolerance. 'Non-preference' was defined as the negative action of insects in approaching, feeding and oviposition. 'Antixenosis' later was used as a term in crop plants (Kogan and Ortman 1978). 'Antixenosis' is caused by plant morphology such as plant form, color and tissue characteristics. 'Antibiosis' was defined as the plant function affecting insect growth and reproductive ability. 'Antibiosis' is mostly displayed by chemical substances and in some cases in special organs or secreted on epiderms to catch larvae. 'Tolerance' was used in the case that plants are damaged a little , in spite of the infestation with the numbers of insect pests that severely damages susceptible plants. The host plant resistance is mediated by host reaction against infestation or virulence of insect pests, and is expressed as a result of interrelation of various factors. Physiological conditions of host plants and infestivity of the insect pest including its growth stages and living conditions are most important among them. On the resistance tests in the laboratory, for example, insufficient light weakens expression of resistance against wheat stem sawfly (Cephus cinctus) in wheat (Platt 1941), and against aphid (Myzus persicae) in sugar beet (Lowe 1974, Tingey and Singh 1980). Therefore, we should pay much attention to these factors in studies of mechanisms of pest resistance (Kaneda 1987).

Genetic analysis of BPH resistance was first conducted by Athwal et al. (1971) in IRRI. After development of bulk seedling test for BPH resistance, many BPH resistant varieties were identified. Athwal et al. (1971) reported that BPH resistance in an Indian local variety Mudgo and two other breeding lines CO22 and MTU15 was controlled by a single dominant gene, and the resistance in a breeding line ASD7 by a single recessive

gene. These genes were considered to be closely linked or allelic because no recombinants were detected (Athwal et al. 1971, Athwal and Pathak 1972, Ikeda and Kaneda 1981). The dominance gene was referred to Bph1 and the recessive gene bph2 (Table 1). BPH resistance was observed in indica varieties but not in japonica varieties at all. IR747-B2-6, a BPH resistant variety found in the field of IRRI, was bred from a cross of two susceptible parents. Martinez and Khush (1974) suggested that TKM6, one parent of IR747 and susceptible to BPH, possessed both Bph1 and I-Bph1 (inhibitor of Bph1), because TKM6 could produce a few resistant F2 from crosses with some susceptible varieties such as IR8. In 1974, the first BPH resistant cultivar IR26 with Bph1 was released, but its genetic resistance was broken down within three years after the release due to the occurrence of a new BPH biotype in the Philippines and other countries (Feuer 1976, Anonymous 1975, Stapley 1975). Another BPH resistant cultivar IR36 bred to carry bph2 was also succumbed to more virulent BPH biotype (IRRI 1982).

To cope with such a problem of resistance breakdown associated with outbreaks of new biotypes, additional genetic sources were searched for widening the genetic base. Two new BPH resistance genes were identified, i. e., Bph3 in a Sri Lanka local variety Rathu Heenati, and bph4 in another Sri Lanka local variety Babawee that is closely linked or allelic to Bph3 (Lakshminarayana and Khush 1977, Sidhu and Khush 1978, 1979, Ikeda and Kaneda 1981). Khush et al. (1985) further identified bph5 in ARC10550 which is resistant to Bangladesh BPH biotype. This gene was not effective against BPH wild type in east Asia and independent from 4 genes, Bph1 to bph4. Kabir and Khush (1988) identified Bph6 in Swarnalata, and bph7 in T12 together with bph5. Ikeda and Kaneda (1986) and Nemoto et al. (1989a), using three different biotypes and two resistant varieties that carry Bph3 and bph4, identified a new recessive resistance gene bph8 in TC5 (Thai Collection 5), TC10 and Chin Saba, and a new dominant gene Bph9 in three Sri Lanka local varieties, Pokkali, Balamawee and Kaharamana. Furthermore, a wild relative of rice, O. australiensis, also provided a dominant resistance gene Bph10(t), that was introgressed into an indica breeding line (Ishii et al. 1994).

It has been considered that there will be no possibility, in Japan, of appearance of new BPH biotypes through cultivation of resistant varieties, because BPH cannot survive

Table 1-1 Characters of BPH resistance genes

Resistance gene	Year of identification	Note	Trisomic analysis	RFLP analysis
Bph1	1967	closely linked or allelic to bph2	[a]chr. 4	chr. 12
bph2	1971	linked to d2, recombnation value of 39.4%	chr. 4	
Bph3	1977	closely linked or allelic to bph4	chr. 10	
bph4	1977	linked to rk2, recombnation value of 30.3%	chr. 10	
bph5	1988	resistant only South Asian biotype		
Bph6	1988	resistant only South Asian biotype		
bph7	1988	resistant only South Asian biotype		
bph8	1986			
Bph9	1986			
Bph10(t)	1994	identified in wild relatives, oryza australiensis		chr. 12

[a] chr. indicates chromosome

winter season in Japan. However, as BPH migrates to Japan from southern parts of China or southeast Asia in rainy season every year (Kisimoto 1971, Kisimoto 1976), there remains a danger that other biotypes migrate to Japan (Nishiyama et al. 1975). In fact in 1991, a biotype which can attack IR26 with Bph1 was found in a BPH population in Kyushu (Sogawa 1992). Therefore, we need to prepare a wider genetic base of BPH resistance for probable invasion of new biotypes (Kaneda 1971, Kaneda et al. 1977). To date, four BPH resistant lines have been bred in National Agriculture Research Center and registered by the Ministry of Agriculture, Forestry and Fisheries. They were Norin-PL3 with Bph1 from an Indian local variety Mudgo (Kaneda et al. 1985), Norin-PL4 with bph2 from a line IR1154-243 (Kaneda et al. 1986), Norin-PL7 with bph4 from a Sri Lanka local variety Babawee (Nemoto et al. 1988), and Norin-PL10 with Bph3 from another Sri Lanka local variety Rathu Heenati (Nemoto et al. 1989b).

The linkage relationship of BPH resistance gene to some other agronomically important traits was observed in the process of introgression of Bph1 in Mudgo (Kaneda 1984). The plants showing more japonica-type characteristics among the segregating population exhibited a lower possibility to be resistant randomly selected plants. The genes for low fertility, long culm and unfavorable quality of hulled rice were strongly linked to Bph1. Among them, long culm was the most difficult problem in breeding of Norin-PL3 (Kaneda et al. 1985).

Identification of the rice factors controlling BPH resistance has been attempted for a long time. Sogawa and Pathak (1970) reported that BPH on resistant variety Mudgo could pierce and penetrate its stylet to suck as frequently as BPH on susceptible variety, but could excrete much smaller amount of honey dew on resistant varieties. The difference between susceptible variety and Bph1-carrying resistant variety, has been considered to be not due to structural differences of tissues but at least partly due to differences in some chemical compounds in the phloem sap. The rice substances as control factors of BPH sucking were classified divided into three categories, i. e. substances that stimulate probing (probing stimulants) and promote or prevent sucking (sucking stimulants and sucking inhibitors). Probing is a BPH action to search for sucking spot. Probing stimulants may be specific flavonoid compounds such as salicylic acid (Sogawa 1974,

Sogawa 1976, Kim 1979). Sucking stimulants include glucose, a major nutrient translocating in the phloem (Sakai and Sogawa 1976), seven amino acids, i. e. aspartic acid, glutamic acid, alanine, serine, leucine, asparagin and valine (Sogawa 1970, Sogawa 1972), and organic acids such as succinic acid and malic acid (Sogawa 1982). Kim (1979) extracted transaconit acid as a sucking inhibitor against BPH from millet. Yoshihara et al. (1979) showed that resistant varieties had higher concentrations of oxalic acid to serve as sucking-prevention factor than the susceptible varieties. However, transaconit acid can be detected at a very low concentration in rice plants and oxalic acid is a very common organic acid found in many plants. Kim et al. (1994) suggested that the sucking prevention was caused by more than these two compounds. The chance of contamination of foreign substances might not also be ruled out. Therefore, it seems to be difficult to study real substances in the translocation system. In fact, wheat translocation substances extracted by one method in the phloem saps, particularly amino acids, was much different from those extracted by other methods (Simpson and Dalling 1981). But from sucking insects, more pure phloem sap could be obtained. During insect sucking, an application of laser beam can cut their stylets penetrating to the phloem, thus the phloem sap overflowing through stylets could be collected. Using this method, Shigematsu et al. (1982) analyzed phloem saps from a pair of near isogenic rice lines carrying Bph1 and susceptible, and found that the resistant lines translocated higher concentrations of β-sitosterol and lower concentrations of aspartic acid. But, it is difficult to conceive that one gene Bph1 could control components of two factors, β-sitosterol and aspartic acid.

In spite of much efforts, it has been hard to determine the most essential and critical factor of BPH resistance. The cloning and expression analysis of BPH resistance genes, therefore, will throw one additional light on this problem. As the first step toward cloning, chromosomal assignment was attempted for five BPH resistance genes. Ikeda and Kaneda (1983) tried to identify the chromosomes carrying two BPH resistance genes, Bph1 and bph2. They reported that Bph1 and bph2 were linked to 'ebisu' dwarf gene, d2, on chromosome 4 with a recombination value of 39.4%. This chromosome assignment agreed with the result by the trisomic analysis (Ikeda and Kaneda 1983). Trisomic analysis also suggested that Bph3 and bph4 are located on chromosome 10, and a linkage

of bph4 to a round kernel gene, rk2, was indicated with a recombination value of 30.3% (Ikeda 1985). However, Bph1 from a breeding line TKM6 and from an Indian local variety Mudgo was lately mapped on chromosome 12 by linkage analyses using RFLP (restriction fragment length polymorphism) markers (Hirabayashi and Ogawa, 1995, Tooyama et al. 1995). Bph10(t) from O. australiensis was also mapped on chromosome 12 by RFLP mapping (Ishii et al. 1994).

As mentioned above, only two genes so far have been tagged and/or mapped by RFLP markers. Therefore, in this study a mapping of four other BPH resistance genes was attempted. To do this, genetic analyses of BPH resistance genes were also conducted using the segregating populations.

CHAPTER 2 Analysis of BPH resistance gene in PL7

1. Introduction

In our country, the oldest record of BPH damages on rice can be traced back to the year 694. From that time BPH outbreaks occurred sporadically until now. Kyoho-famine, in which over one million people died for starvation, occurred by a large outbreak of BPH in 1732 (Suenaga and Nakatsuka 1958). An another outbreak in 1897 caused yield losses of 960,000 t, and outbreak in 1966 caused damages over 780,000 ha and yield losses of 350,000 ton (Kisimato 1980).

Four BPH resistant lines have been bred in our country (Table 2). First, introduction of Bph1 from an Indian local variety Mudgo into japonica cultivar was attempted in National Agriculture Research Center in Japan : the breeding was initiated using an original cross of a japonica variety Hoyoku and Mudgo in 1968 (Kaneda et al. 1979, 1980). For sixteen years, backcrosses with susceptible varieties and self pollinations followed by selection for BPH resistance were carried out. The first BPH resistant line, Norin-PL3 (hereafter abbreviated as PL3), was bred and registered by the Ministry of Agriculture, Forestry and Fisheries in 1984 (Kaneda et al. 1985). Following this first attempt, introgression breeding for three other BPH resistance genes were initiated and performed simultaneously. An IRRI breeding line IR1154-243 was used as the donor of bph2, crossed with a japonica variety Asominori in 1973, and Norin-PL4 (PL4) was bred and registered in 1985 (Kaneda et al. 1986). Two Sri Lanka local varieties Rathu Heenati and Babawee were used as the donors of Bph3 and bph4, respectively. They were crossed both with a japonica variety Tsukushibare in 1976, and Norin-PL10 (PL10) with Bph3 and Norin-PL7 (PL7) with bph4 were bred and registered in 1988 and 1987, respectively (Nemoto et al. 1988, 1989b).

Except for IR1154-243, all donor indica varieties have very strong photoperiod sensitivity. Particularly, all Sri Lanka varieties do not differentiate panicles at all in Japan. All of these donors also show hybrid sterility in crosses with japonica varieties. Red kernels, very long leaves and culms, etc. are their additional undesirable traits. In the breeding of PL3, because the elimination of inferior traits from Mudgo was very difficult,

Table 2-1 BPH resistant lines bred in Japan

Resistance gene (pedigree)	Resistant line	[a]Donor variety	Initiation of breeding	Registration
Bph1	Norin-PL3	Mudgo	1968	1984
([b]$F_6$324/Akitsuho//Tsukushibare)				
bph2	Norin-PL4	IR1154-243	1973	1985
(Asominori*3/IR1154-243)				
Bph3	Norin-PL10	Rathu Heenati	1976	1988
(Tsukushibare///Tsukushibare*3/Rathu Heenati//Tsukushibare)				
bph4	Norin-PL7	Babawee	1976	1987
(Tsukushibare*2/Babawee)				

[a]Donor variety: Mudgo is a local variety in India, IR1154-243 is a breeding line of IRRI, and Rathu Heenati and Babawee are local varieties in Sri Lanka.

[b]$F_6$324; Hoyoku/mudgo//Kochikaze///IR781-1-94/4/Hoyoku

five susceptible varieties were used as recurrent parents. Although other three breeding lines had only one variety as recurrent parents, some backcrosses were needed for their breeding. These introgression lines so far could be considered as BPH resistant japonica lines, and utilized as near isogenic lines.

As mentioned above, only two genes, Bph1 and Bph10(t), have been mapped, and cloning of BPH resistance genes have not yet been achieved. Some preliminary study showed that PL7 expressed stronger and more stable BPH resistance than PL10, so that BPH resistance gene in PL7, supposedly bph4, was first analyzed. Since bph4 was reported to be allelic or closely linked to Bph3, the analysis of bph4 was expected to provide information on Bph3.

In this chapter the results of molecular mapping of bph4 with RFLP (restriction fragment length polymorphism) markers (McCouch 1988, Saito et al. 1991, Kurata et al. 1994) or RAPD (random amplified polymorphism DNA) markers (Williams et al. 1990) are reported.

2. Genetic analysis

1)Materials and Methods

Plant materials

A cultivar 'Tsukushibare' was used as a susceptible parent in the analysis of BPH resistance gene in PL7. Tsukushibare is a high-yielding, widely planted cultivar in Kyushu, and used as a recurrent parent of PL7. In the progenies derived from crosses of Tsukushibare with PL7, the expression of BPH resistance gene was expected to be stable, thus they provide a critical material to test for BPH resistance.

In 1991, first crosses were made in the National Agriculture Research Center. Four crosses of Tsukushibare/PL7 designated as K5 to K8, and the F_2 seeds were harvested. Harvested seeds were dried for 5 days, and heat-treated at 50°C for a week to break seed dormancy. Individuals of F_2 seedlings were transplanted in the rice field of Kobe University; a single seedling per hill with spacing of 25.0 x 15.0 cm. A compound fertilizer (12:12:12 = N: P: K) was added before seeding at 40 g/m^2.

Culture of BPH

An original colony used in this experiment was a mixture of BPH populations obtained from Kyushu Agriculture Experiment Station and Hyogo Prefectural Agricultural Institute. Both of these BPH colonies were predominantly of biotype 1. BPH was cultured in a growth chamber under 15 h light: 9 h dark at and day-night temperatures of 30-25°C. About 2,500 to 3,500 BPH nymphs at the second to fourth instar stages were infested for the bulk seedling test. As it was desirable to use BPH nymphs of the same stage for the bulk seedling test, 150 to 200 adults of BPH were selected for oviposition on new seedling trays.

Bulk seedling tests of BPH resistance

As the third instar nymphs were reported to be the most adequate for the bulk seedling test (Kaneda 1975a, 1975b), 18-day-old BPHs after oviposition were used for the testing. About 30 seeds per entry were germinated in a petri dish at 30°C in the dark. The rapid growing lines were kept under cooler conditions to adjust their growth. Seventeen germinated seeds at the similar growth stage were transplanted with the line space of 0.8 cm in a seedling tray of 29.0 x 29.0 x 2.5 cm with sterilized soil, and seedlings were grown in the biochamber. BPH nymphs were infested to the seedlings at the rate of 7 to 10 nymphs per seedling.

At 9 to 10 days after infestation, damages on the seedlings of each row were compared with the control rows to judge their phenotypes. PL7 and Tsukushibare were used as the resistant and susceptible varieties, respectively. Generally, seedlings shorter than 4 cm, with thin stem, withered leaves and dark brown leaf color, were judged susceptible. While seedlings with over 8 cm in height, thick stem and green leaf color were regarded as resistant. F_2 individuals were also tested directly under the same conditions for their resistance.

Allelism tests

For the allelism test of resistance genes in PL7 and PL4, PL4 possessing bph2 was crossed with PL7 and a Bph1 carrier, PL3. The cross of PL3 and PL7 was not successful.

In the crosses, emasculation was made by soaking panicles of seed parents in the hot water of 43°C for 7 minuets just before flowering. After pollination, each ear was covered with a sack of vitriol paper to prevent contamination of other pollens. Hybridization was done in a very humid condition because pollens could be easily damaged by drying (Yamada 1987).

2) Results

BPH resistance segregation was determined in Tsukushibare/PL7 by two different tests. In the first test, genotypes of 191 F_2 individuals derived from crosses K5 to K8 were determined based on the segregation of resistance in F_3 lines (Tables 2-2, Appendix-1). In the second test, genotypes of 1,138 F_2 individuals were determined directly (Table 2-2). The results of these analyses clearly demonstrated that PL7 carries a dominant gene for BPH resistance. Since the F_3 ratio fitted to 1RR: 2RS: 1SS, the frequency distribution of resistant progenies in the segregating F_3 lines was analyzed. The analysis showed a wide range of the frequency distribution with a mode of 65-75% resistant progenies (Fig. 2-1). The frequency distribution was skewed towards the lower percentages of resistant progenies than that expected based on the complete dominance.

Considering very weak resistance of Bph3 in PL10, it was postulated that the dominant resistance gene in PL7 was identical to Bph1. Therefore, PL7 was crossed with PL4 (a carrier of bph2) and the genotypes of F_2 progenies were determined. The result of this allelism test, when compared with the result obtained between PL3 (a carrier of Bph1) and PL4, strongly indicated that the resistance gene introgressed in PL7 is in fact Bph1 (Table 2-3).

3. RFLP analysis and mapping of resistance gene in PL7

1)Material and Method

Plant materials

Ninety F_3 lines derived from K5 to K8 of Tsukushibare/PL7 (described in 2-2-1) were used as a segregating population in RFLP analysis. Genotypes of BPH resistance

Table 2-2 Segregation of BPH resistance in F_2 individuals and F_3 lines derived from a cross of Tsukushibare/PL7

F_2 test	Number of F_2 individuals		
	Resistant (RR, Rr)	Susceptible (SS)	Total
Tsukushibare/PL7	850	288	1138[a]
Tsukushibare	0	68	68
PL7	67	0	67
F_3 test	[b]Number of F_3 lines		
	RR	Rr	SS	Total
Tsukushibare/PL7	40	101	50	191[c]

[a] χ^2 for 3:1=0.06 (P>0.75)

[b] An average of 61 individuals per line were assayed for BPH resistance in more than three times of tests. RR includes F_3 lines with more than 85% resistant progenies and SS includes lines with less than 5% resistant progenies (see Fig. 2-1).

[c] χ^2 for 1:2:1=1.68 (p>0.45), for 3R: 1S=0.14 (p=0.72)

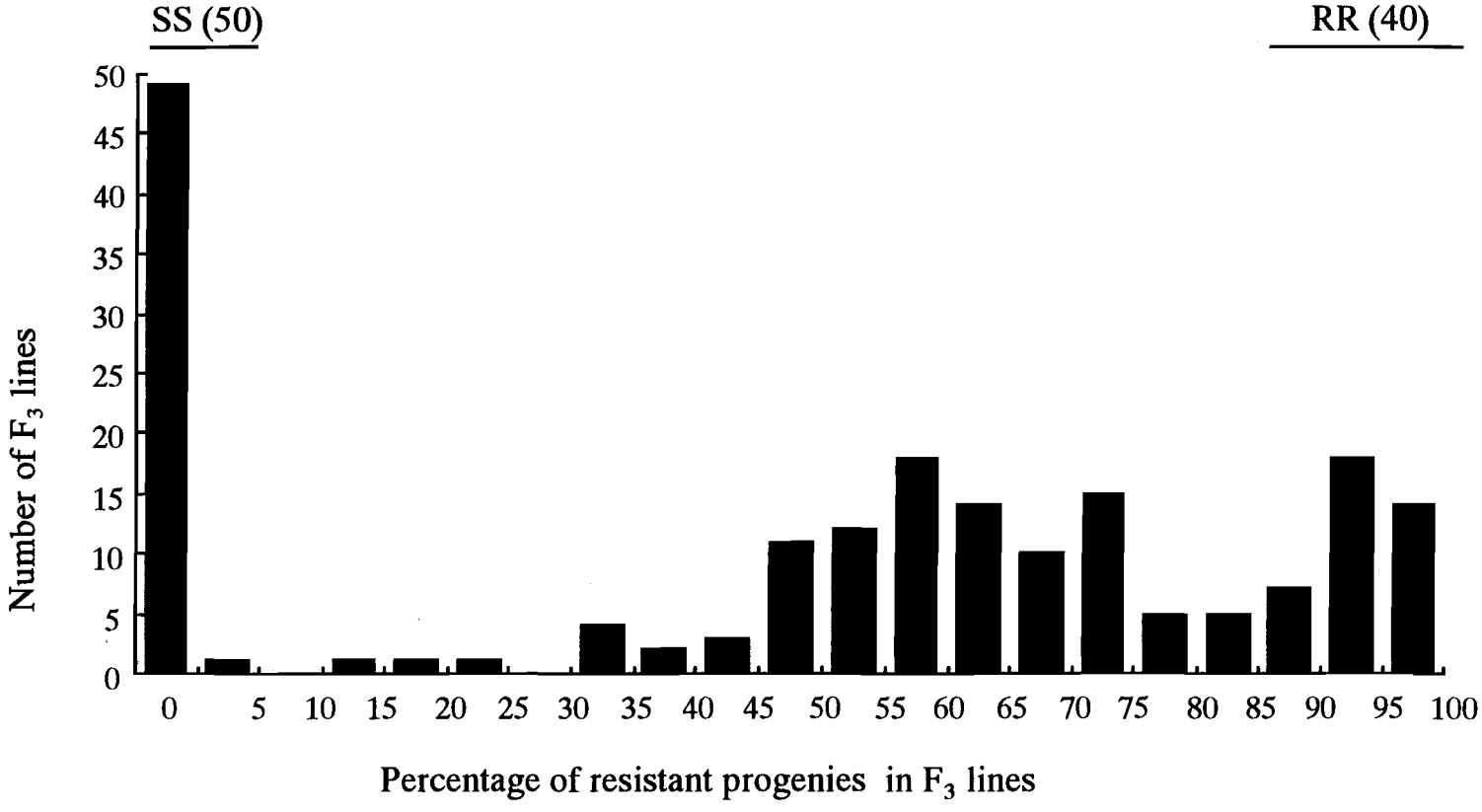

Fig. 2-1 The frequency distribution of resistant progenies in 191 F3 lines derived from a cross of Tsukushibare/PL7

Table 2-3 Allelism tests of the resistance gene in PL7 with Bph1 of PL3 and bph2 of PL4

Cross	Number of F_2 individuals		
	Resistant	Susceptible	Total
PL7/PL4	116	2	118
PL3/PL4	99	1	100
PL7 self	95	1	96
PL3 self	90	0	90
PL4 self	182	0	182

gene were determined by bulked seedling test (described in 2-2-2) : the population was determined either as resistant homozygotes and, heterozygotes and susceptible homozygotes. As control materials, DNAs of PL3, No.53, Aichi97, Hoyoku and the two resistance donor varieties, Babawee and Mudgo, were subjected to the analysis. No.53 was an earlier breeding line for Bph1, Aichi97 was a recent breeding line for Bph1, and Hoyoku was a BPH susceptible japonica cultivar and one of the recurrent parents used in the breeding for PL3.

DNA preparation

After genotypes of the F_2 individuals were determined, total DNAs from F_3 lines and the both parents were isolated in bulks of about 20 seedlings per line. Their total genomic DNA was extracted from seedlings of each F_3 line whose genotypes of BPH resistance were pre-determined. A CTAB method (Murray and Thompson 1980) was applied on a large and a small scale.

A large scale procedure for more than 1 g leaves was applied when a large amount of pure DNA was needed, e. g. for bulked segregant analysis, southern hybridization, and/or mapping analysis with RFLP markers. Average amount of 80 to 90 μ g of DNA could be extracted from 1 g weight fresh leaves on large scale extraction. A small scale procedure was simpler and could be finished in a shorter time, although the quantity was less.

Southern hybridization

All procedures were the same as the methods by Kurata et al. (1994). Total DNA was digested with 8 restriction enzymes (BamHI, BglII, EcoRV, HindIII, ApaI, DraI, EcoRI and KpnI). Digested DNA was electrophoresed through 0.6% agarose gel and blotted onto nylon membrane (Boehringer Mannheim) in 0.4 N NaOH. The membrane was washed in 2 x SSC, air-dried and baked at 120°C for 20 min. DNA probes used for Southern hybridization were obtained after amplification by PCR. Southern hybridization and signal detection were made by ECL™ direct nucleic acid labeling and detection system (Amersham). Recombination values between the resistance gene and RFLP markers were

calculated by MAPMAKER Version 3.0 (Lander et al. 1987) and converted into genetic map distance (Kosambi 1944).

Bulked segregant analysis

Bulked segregant analysis (Michelmore 1991) provides a method to determine the chromosomal regions of interest. Also it is a method for rapidly locating chromosomal regions which are linked with the gene of interest in populations used to generate the genetic map.

RFLP markers are usually considered as co-dominant markers. By mixing pooled DNA samples, except the region closely linked to the genes of interest, there is little difference in the hybridization patterns among the progenies. This procedure efficiently identifies markers linked to the genes of interest, allowing their rapid placement on a genetic map.

Bulked DNAs were prepared by pooling DNA samples from 10 individuals with the same genotype identified in the resistance tests. In the first screening, DNA samples of each parent and the two bulked genotypes (resistant homozygous and susceptible homozygous) were used for hybridization, and the hybridized patterns were compared. In the second screening, the polymorphism on each of the pooled samples was analyzed.

2) Results

Based on the results of the above segregation analyses and allelism tests together with an available knowledge about the carrier chromosome of Bph1 (Hirabayashi and Ogawa, 1995, Tooyama et al., 1995), a polymorphism survey was carried out using 34 RFLP probes on chromosome 12 and DNAs from PL7, Tsukushibare, and two F_3 bulks representing resistant homozygous and susceptible homozygous derived from a cross of Tsukushibare x PL7. The study showed that 8 probes, G148, R1709, R2708, R3106, R643, C2808, S2545 and C751, were polymorphic between susceptible and resistant materials (Fig. 2-2). They were further hybridized with DNAs from 90 F_3 lines, and all showed co-segregation with the resistance gene (Fig. 2-3). A few recombinants can be detected in Fig. 2-3, i. e. 1, 1 and 2 recombinants in resistant homozygous, susceptible

homozygous and heterozygous lines, respectively. The linkage analysis showed that the resistance gene in PL7 locates distal to 6 markers, G148, R1709, R2708, R3106, R643 and C2808, on the Nipponbare/Kasalath map developed by the National Institute of Agrobiological Resources, Japan (Kurata et al., 1994). No recombinants were detected among these markers covering 5.7 cM, but the resistance gene was located at 1.7 cM from the closest marker G148 that is 16.1 cM from one arm end (Fig. 2-4). Two other proximal markers, C751 and S2545, were located at 3.2 cM from the resistance gene: the distance was much smaller than that (17.0-22.7 cM) between these and the above 6 markers on the Nipponbare/Kasalath map.

To confirm the source of the resistance gene in PL7, the presence/absence of the sequence detected by the closely linked RFLP marker G148 was studied in PL7, PL3, No. 53, Aichi97, Hoyoku and the two resistance donor varieties, Babawee and Mudgo. The presence of the homologous sequences in Bph1 carriers, PL7, No. 53, Aichi97 and Mudgo, but the absence in a bph4 carrier, Babawee, unequivocally demonstrated that the resistance gene in PL7 was derived from Mudgo (Fig. 2-5).

4. RAPD analysis

1)Material and Method

Plant materials

DNAs from 135 F_3 lines derived from K5 to K8 of Tsukushibare/PL7 (described 2-2-1), including 32 resistant homozygous, 68 resistant heterozygous and 35 susceptible plants were surveyed for RAPD analysis. Tsukushibare, four parent lines and their donors except for IR1154-243 were used as control.

DNA preparation and bulked segregant analysis

Template DNAs for RAPD-PCR were extracted by the same procedures as described in 2-3-1. Three different bulked DNA samples were further prepared by mixing the DNA samples from three representative genotype groups consisting of 10 resistant, 17 segregating, and 15 susceptible F_3 lines respectively.

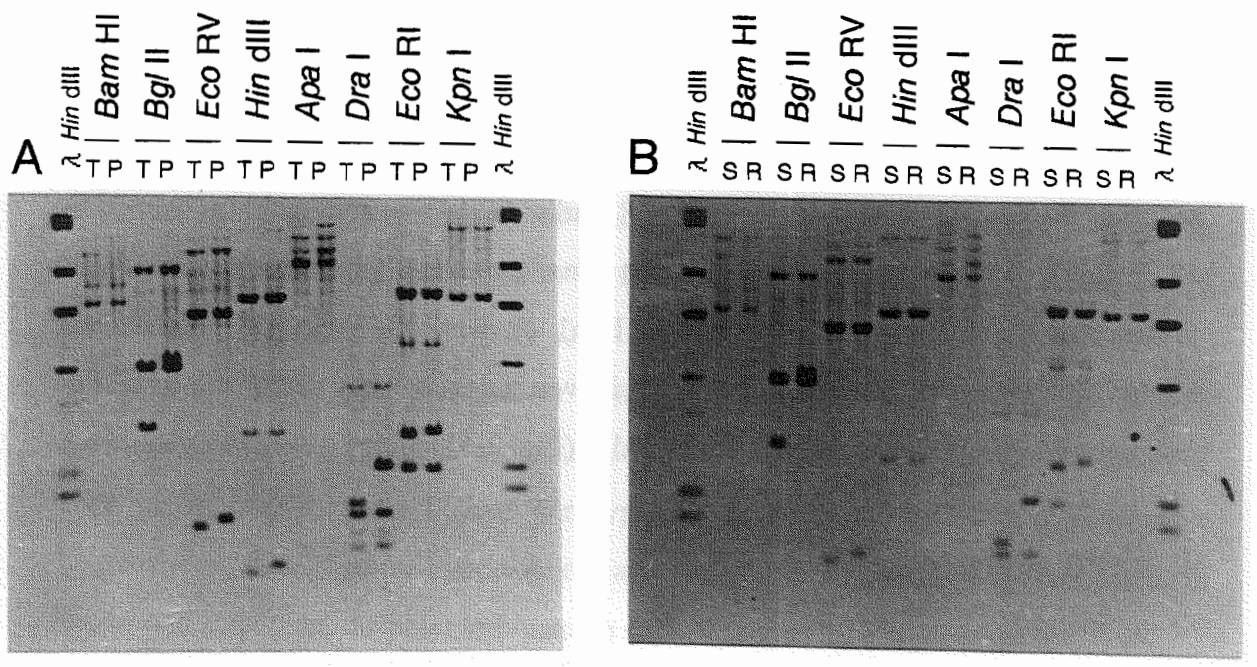

Fig. 2-2 Bulked segregant analysis of BPH resistance gene in PL7. DNAs of;
A) PL7 (P) and Tsukushibare (T), and B) F_3 bulks from susceptible homozygotes and resistant homozygotes were digested by 8 enzymes and probed with R643.

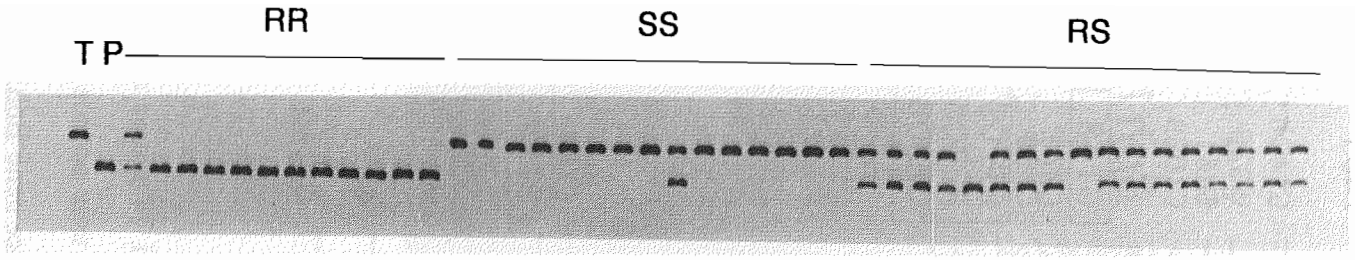

Fig. 2-3 Linkage analysis of BPH resistance gene in PL7. DNAs of F_3 lines from resistant homozygotes (RR), susceptible homozygotes (SS), resistrant heterozygotes (RS), PL7 (P) and Tsukushibare (T) were digested by BamHI and probed with S2545.

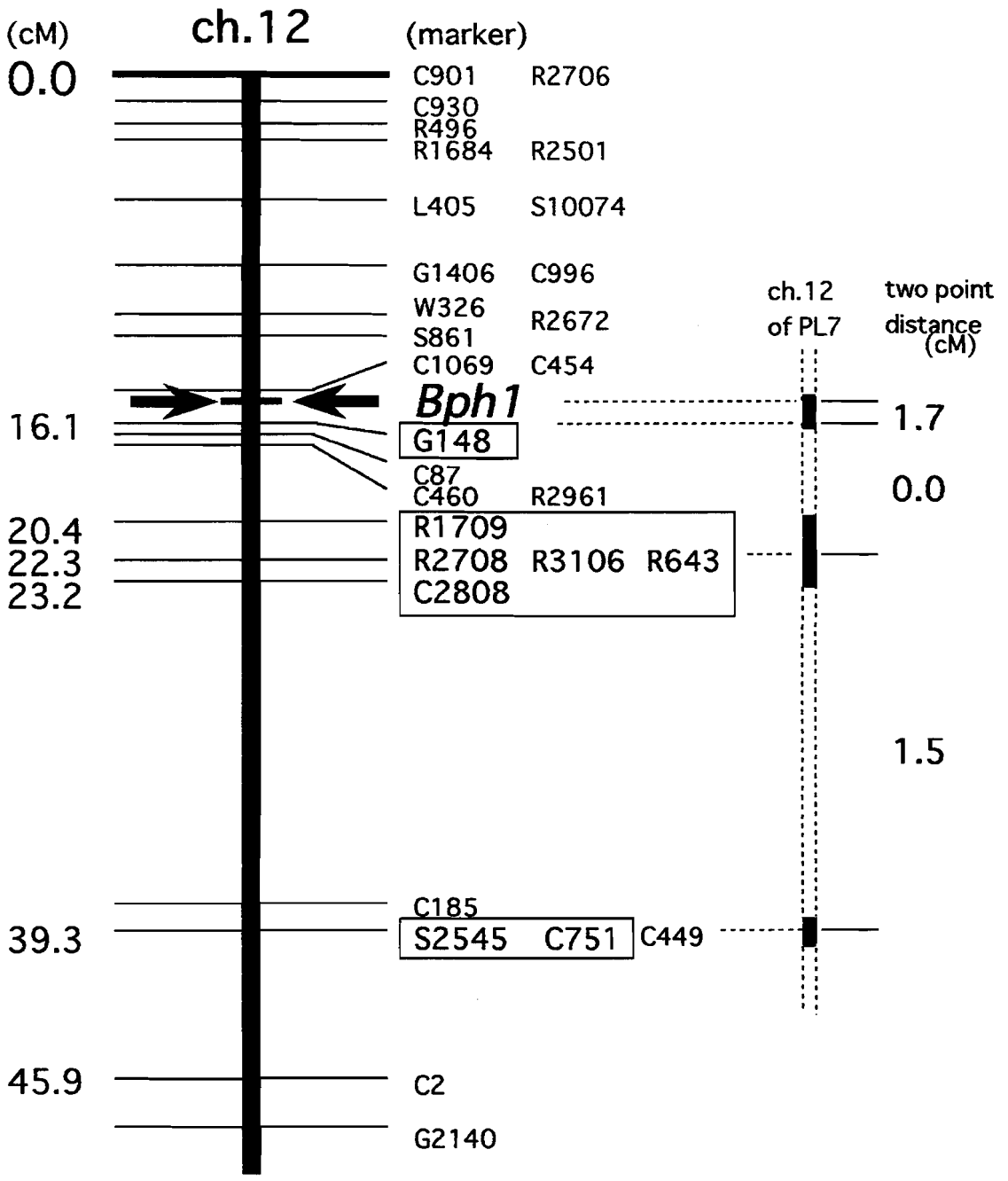

Fig. 2-4 A location of the resistance gene Bph1 in PL7 on the Nipponbare/Kasalath map and deduced structure of chromosome 12 of PL7. Solid bars indicate chromosome segments derived from PL7.

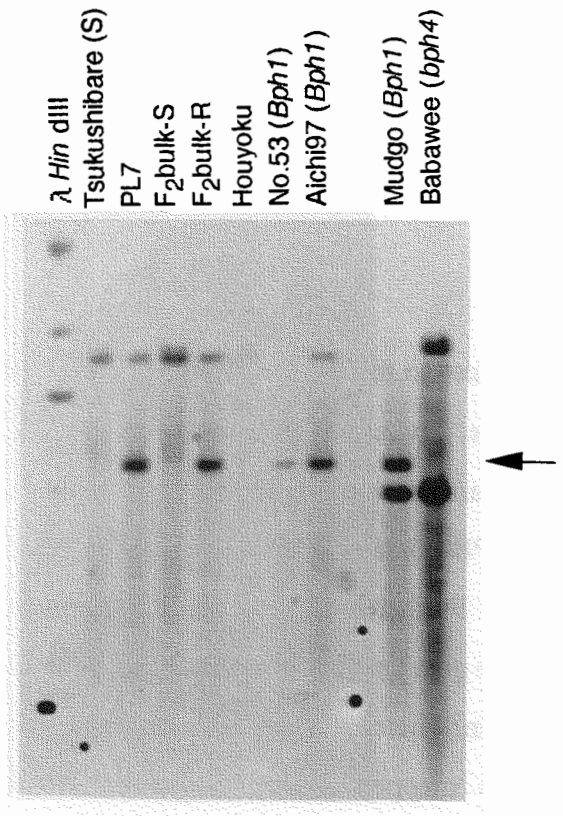

Fig. 2-5 Determination of the source of BPH resistance gene present in PL7. RFLP patterns of DNAs from various sources after digestion with DraI and probing by a marker G148.

RAPD-PCR reaction

A total of 540 random 10-mer primers (Operon Technologies Inc.) were surveyed for detecting RAPD markers linked to the resistance genes, and also to determine its donor of the mis-introgressed Bph1. Amplification was performed in Perkin Elmer Cetus Gene Amp PCR System 9600 programmed at 93 °C for 30 sec, followed by 40 cycles of denaturation at 93 °C for 20 sec, fastest transition to 50 °C, transition to 40 °C with a ramp of 70 sec, to 36 °C with a ramp of 100 sec, and primer annealing at 36 °C for 60 sec, followed by primer extension at 73 °C for 120 sec. A post-extension was at 73 °C for 120 sec. (Kaneda et al. 1996).

2)Results

RAPD markers linked to the BPH resistance gene in PL7 were searched for by the bulked segregant analysis. Twenty-nine primers among 540, that amplified polymorphic fragments between the parents, were used in RAPD analysis of DNA samples from 135 F_3 lines. One primer, OPB-15, amplified a fragment ($OPB15_{550}$) that co-segregated with susceptibility (Fig. 2-6). Further it was shown that the marker is linked to susceptibility (in trans to the resistance gene) with a map distance of 4.8 cM in a homozygous resistant population and 3.0 cM in a heterozygous resistant population (Table 2-4). The marker, however, could not be mapped on the Nipponbare/Kasalath map, because it was monomorphic in the two parents.

The presence of $OPB15_{550}$ was also studied in other resistant breeding lines and their resistance donors (Fig. 2-6). The marker was present in two resistant lines, PL4 with bph2 and PL10 with Bph3, but absent in PL3 with Bph1. The marker was absent in all donor varieties except for IR1154-243, a donor of bph2 in PL4.

4. Discussion

In this study, two serious problem arose that should be avoided or solved in any introgression breeding. The first problem was the mis-introgression. The work was aimed at mapping of one recessive BPH resistance gene bph4 that was supposed to be introgressed in a breeding line Norin-PL7 from a Sri Lanka local resistant variety

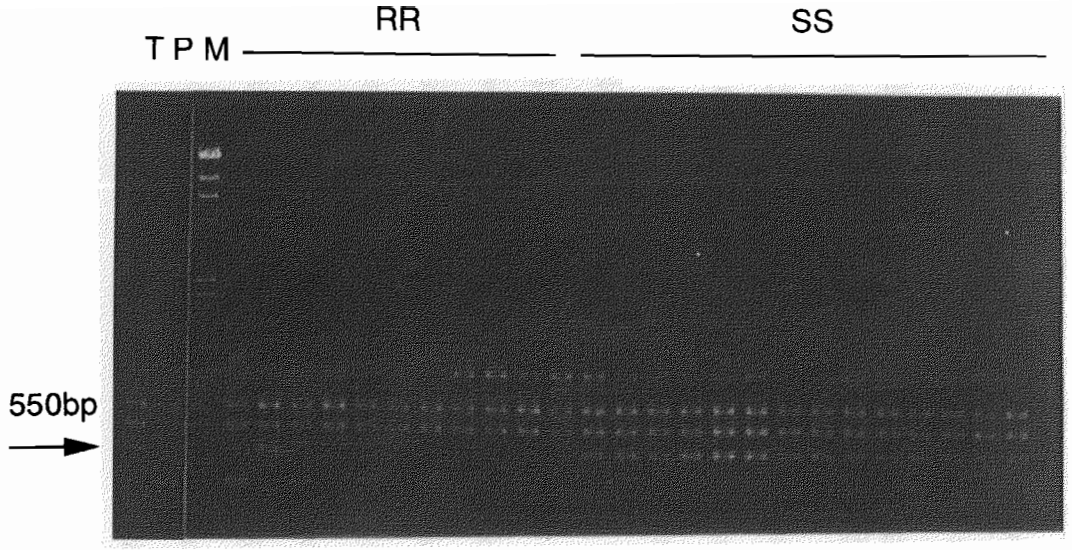

Fig. 2-6 A linkage analysis of a RAPD marker amplified by primer OPB-15. Segregation of the marker was studied in F_3 lines derived from a cross of Tsukushibare x PL7. T: Tsukushibare, P: PL7, RR: resistant homozygotes, SS: susceptible homozygotes, M: λ HindIII marker.

Table 2-4 Linkage analysis of $OPB15_{550}$ with the resistance/susceptibility gene in PL7

F_3 group	$OPB15_{550}$			Genetic
(genotype)	Present	Absent	Total	distance (cM)[a)]
Resistant homozygous (RR)	2	29	31	3.3
Segregating (Rr)	42	28	70	2.4
Susceptible homozygous (SS)	28	0	28	<0.08

[a)] Calculated according to Kosambi (1944)

Babawee. In a series of genetic analysis, however, it was found that the resistance in PL7 is governed by a single dominant gene (Tables 2-2, Appendix-1). Allelism tests further indicated that the resistance gene in PL7 is identical to Bph1 derived from an Indian local resistant variety Mudgo (Table 2-3). Furthermore, an analysis using a tightly linked RFLP marker G148 (Fig. 2-5) unequivocally demonstrated that not Babawee but Mudgo is a source of the resistance gene. The mis-introgression resulting in the complete elimination of genetic contribution of Babawee could have occurred at any stages of the breeding program, perhaps by simple mistagging. Chance of mistagging might be large in many different breeding lines are grown simultaneously in the nearby environment. However, recognition and correction of such mistakes require much efforts. Therefore, to avoid such mis-introgression, DNA markers closely linked to the resistance genes concerned are needed. BPH biotypes distinguishing resistance genes (Ikeda and Kaneda, 1986) could also be used effectively for this purpose, although unfortunately such biotypes had been lost. A lesson from this result is that molecular markers are not only useful but also necessary in monitoring introgression of agronomically important genes.

Despite the above difficulties several promising results were obtained in the present mapping study of BPH resistance genes. Linkage of Bph1 was already reassigned from chromosome 4 to chromosome 12 and the gene was mapped in the following two independent studies. The first mapping was done by Hirabayashi and Ogawa (1995), and a marker XNpb248 was located at a map distance of 10.7 cM on the Kasalath/FL134 (SS) map (Saito et al., 1991). Tooyama et al. (1995) also located Bph1 in a region spanning 17.4 cM between two markers W326 and G148 on the Nipponbare/Kasalath map. In the present study, a tighter linkage with 6 RFLP markers was detected with a closest marker G148 being at 1.7 cM from Bph1 (Fig. 2-4). The map distances between these markers estimated in Tsukushibare/PL7 were highly condensed as compared with those on Nipponbare/Kasalath map, indicating the presence of Mudgo-derived chromosomal region(s) acting as a large block of crossing-over suppresser. However, since a recombinant between Bph1 and G148 was detected, a line homozygous for Bph1 locating on a short chromosomal segment can be selected later. Furthermore, if the marker is generally polymorphic against japonica, it is expected to provide a useful molecular tag in

breeding of BPH resistant japonica cultivars.

Recently Bph1 has been mapped again using another breeding line on the Cornell University map (Huang et al. 1997). The map position, however, could not be directly compared with the presently determined map position because of the different nature of the maps. Integration of differently constructed maps (construction of the consensus maps) is necessary for future map-based cloning of BPH resistance genes.

CHAPTER 3 Analysis of BPH resistance gene in PL4

1. Introduction

It was demonstrated that a breeding line PL7, in which a recessive gene bph4 was supposed to be introgresed, possessed a dominant gene Bph1 from Mudgo. Despite this unfortunate mis-introgression, Bph1 in PL7 was mapped on chromosome 12 with more closely linked marker than those reported in other Bph1 mapping studies. The mapping of bph2 in PL4 was therefore next attempted.

bph2 was earlier reported to be linked to ebisu-dwarf gene d2 on chromosome 4 (Kaneda and Ikeda 1976, Ikeda and Kaneda 1977, Ikeda and Kaneda 1983). But their recombination value of 39.4% was quite large. A trisomic analysis conducted also showed that bph2 located on chromosome 4 (Ikeda and Kaneda 1983). Since bph2 was reported to be either allelic or closely linked to Bph1 (Athwal et al. 1971), on chromosome 12, bph2 was expected to be located also on chromosome 12. In this study, the results of mapping of bph2 and its genetic behavior were described.

2. Genetic analysis

1)Materials and Methods

Plant materials

Genetic analysis was conducted in the segregating populations (F_2, F_3) derived from a cross of Tsukushibare/PL4. Tsukushibare and PL4 were used as control. In 1994, Tsukushibare was crossed with PL4 in Kobe University, and the F_1 plants were grown in the next summer. The F_2 segregating population was planted in December 1995 in Hyogo Prefactural Agricultural Institute, and their F_3 seeds were harvested in 1996.

Culture of BPH and bulk seedling tests

All procedures were the same as described in Chapter 2. In the bulked seedling tests, PL4 was used as a resistant standard, and Tsukushibare as a susceptible control.

2) Results

Segregation of BPH resistance was studied in F_2 individuals and F_3 lines derived from a cross of Tsukushibare/PL4. Although the number of F_2 individuals tested was small, nearly 10,000 individuals were tested for resistance in 159 F_3 lines derived from this cross. The result showed that the segregation of resistance did not significantly deviate from 3R: 1S in both cases, demonstrating the dominant nature of bph2 (Tables 3-1, Appendix-2). However, since the F_3 ratio did not fit to 1RR: 2RS: 1SS, the frequency distribution of

resistant progenies in the segregating F_3 lines was further analyzed. The analysis showed a wide range of the frequency distribution with a mode of 65-75% resistant progenies (Fig. 3-1). The frequency distribution was further skewed towards the lower percentages of resistant progenies than that expected based on the complete dominance.

3. Graphical genotyping and linkage analysis of PL4 with RFLP markers

1)Materials and methods

Plant materials

For the graphical genotyping (Young and Tanksley 1989) of PL4, the parental varieties, Asominori and IR1154-243, were used as references. Tsukushibare that was used to produce the segregating population was also used as japonica control. For the linkage analysis, 90 F_3 lines derived from a cross of Tsukushibare/PL4 were used.

Graphical genotyping and linkage analysis

All procedures of DNA preparation and Southern hybridization were the same as described in Chapter 2. Graphical genotyping of PL4 was conducted using 98 RFLP markers randomly chosen from 12 chromosomes of the rice genome (Kurata et al. 1994). Recombination values between the resistance gene and RFLP markers were calculated by MAPMAKER Version 2.0 (Lander et al., 1987) and converted into genetic map distance (Kosambi 1944).

Table 3-1 Segregation of BPH resistance in F_2 individuals and F_3 lines derived from a cross of Tsukushibare/PL4

F_2 test	Number of F_2 individuals		
	Resistant (RR, Rr)	Susceptible (SS)	Total
Tsukushibare/PL4	35	17	52[a]
PL4	51	0	51
Tsukushibare	0	51	51
F_3 test	[b]Number of F_3 lines		
	RR	Rr	SS	Total
Tsukushibare/PL4	28	96	35	159[c]

[a] χ^2 for 1R:3S=49.0 ($p<0.001$), for 3R:1S=1.64 ($p=0.18$)

[b] An average of 61 individuals per line were assayed for BPH resistance in triplicated tests. RR includes F_3 lines with more than 90% resistant progenies and SS includes lines with all susceptible progenies (see Fig. 3-1).

[c] χ^2 for 1RR:2RS: 1SS=7.47 ($p=0.025$), for 3R:1S=0.76 ($p=0.38$).

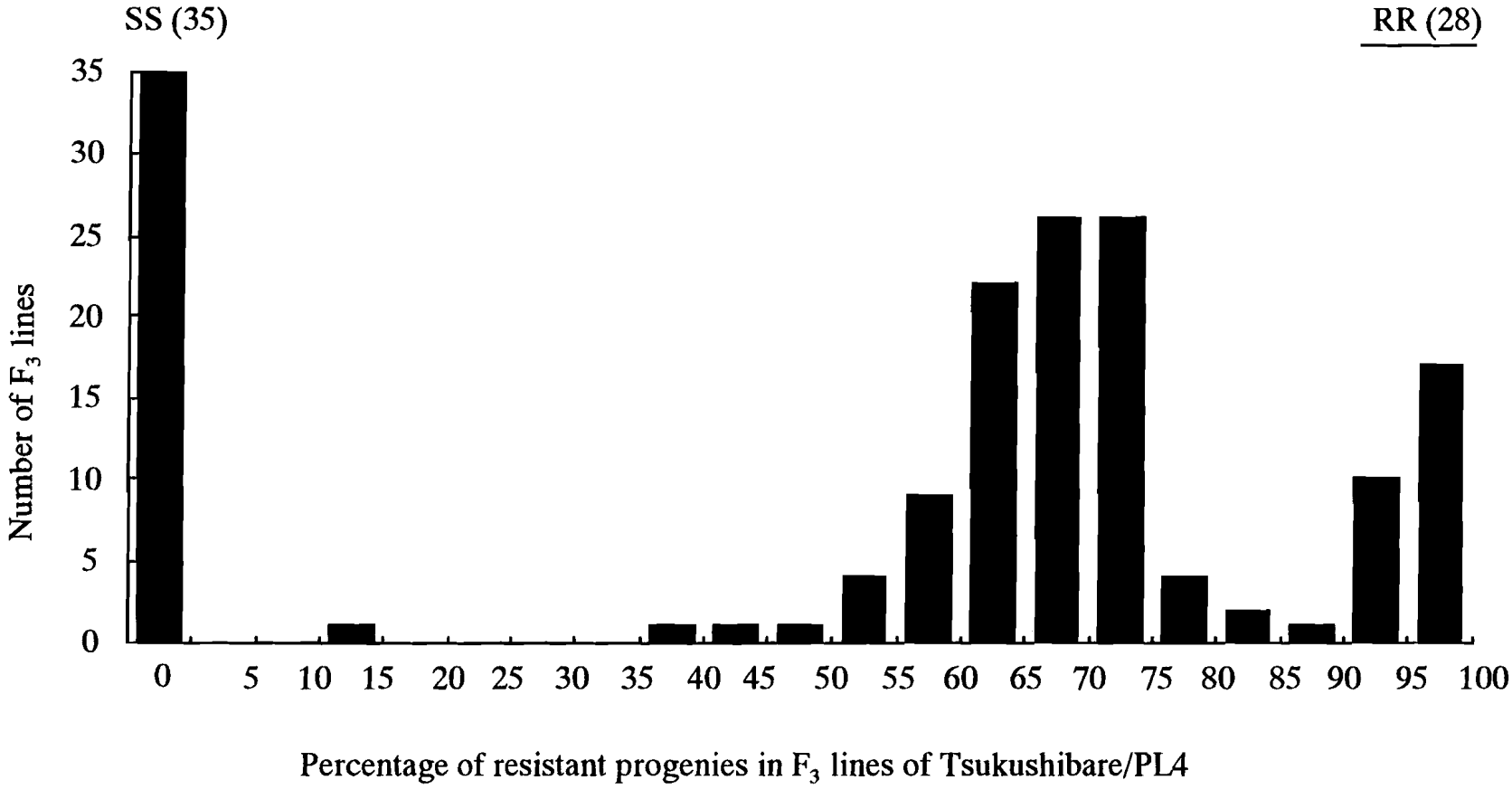

Fig. 3-1 The frequency distribution of resistant progenies in 159 F_3 lines derived from a cross of Tsukushibare/PL4.

2) Results

Graphical genotyping of PL4

To confirm that the resistance gene introgressed in PL4 is bph2 derived from the resistance donor, IR1154-243, a graphical genotyping of PL4 was conducted using 98 RFLP markers randomly chosen from 12 chromosomes of the rice genome. A comparison of RFLP patterns among PL4, IR1154-243, Asominori and Tsukushibare showed that a large region spanning ca. 60 cM on chromosome 12 in PL4 was derived from IR1154-243 (Fig. 3-2). Six polymorphic markers were detected in this region (Fig. 3-3A). Four other regions were also derived from IR1154-243 (Fig. 3-3B). Three of them on chromosomes 1, 4 and 11 were polymorphic between Tsukushibare and PL4 and between Asominori and IR1154-243, but a region on chromosome 2 was monomorphic among the resistant lines and Tsukushibare and polymorphic with Asominori. Regions defined by 15 markers were monomorphic among all the lines and the other regions were from Asominori. The result clearly demonstrated that IR1154-243 was the donor of the resistance gene, thus the resistance gene in PL4 was identical to bph2.

Linkage analysis

Based on the confirmation of the identity of bph2 in PL4, a linkage analysis was conducted using 90 F_3 lines derived from a cross of Tsukushibare/PL4. Six RFLP markers on chromosome 12 co-segregated with BPH resistance, while markers on four other chromosomes segregated independently (Fig. 3-4). bph2 was mapped at 3.5 cM from the closest RFLP marker, G2140, on chromosome 12 (Fig. 3-5). The map position of bph2 was ca. 30 cM apart from that of Bph1 previously determined on Nipponbare/Kasalath map (Murata et al. 1997).

4. Pyramiding of Bph1 and bph2

1) Plant materials and genetic analysis

The crosses of Bph1/bph2 were also performed in 1994 summer season. The F_1 plants were planted in 1995, and F_2 individuals in 1996. Genetic analysis was conducted

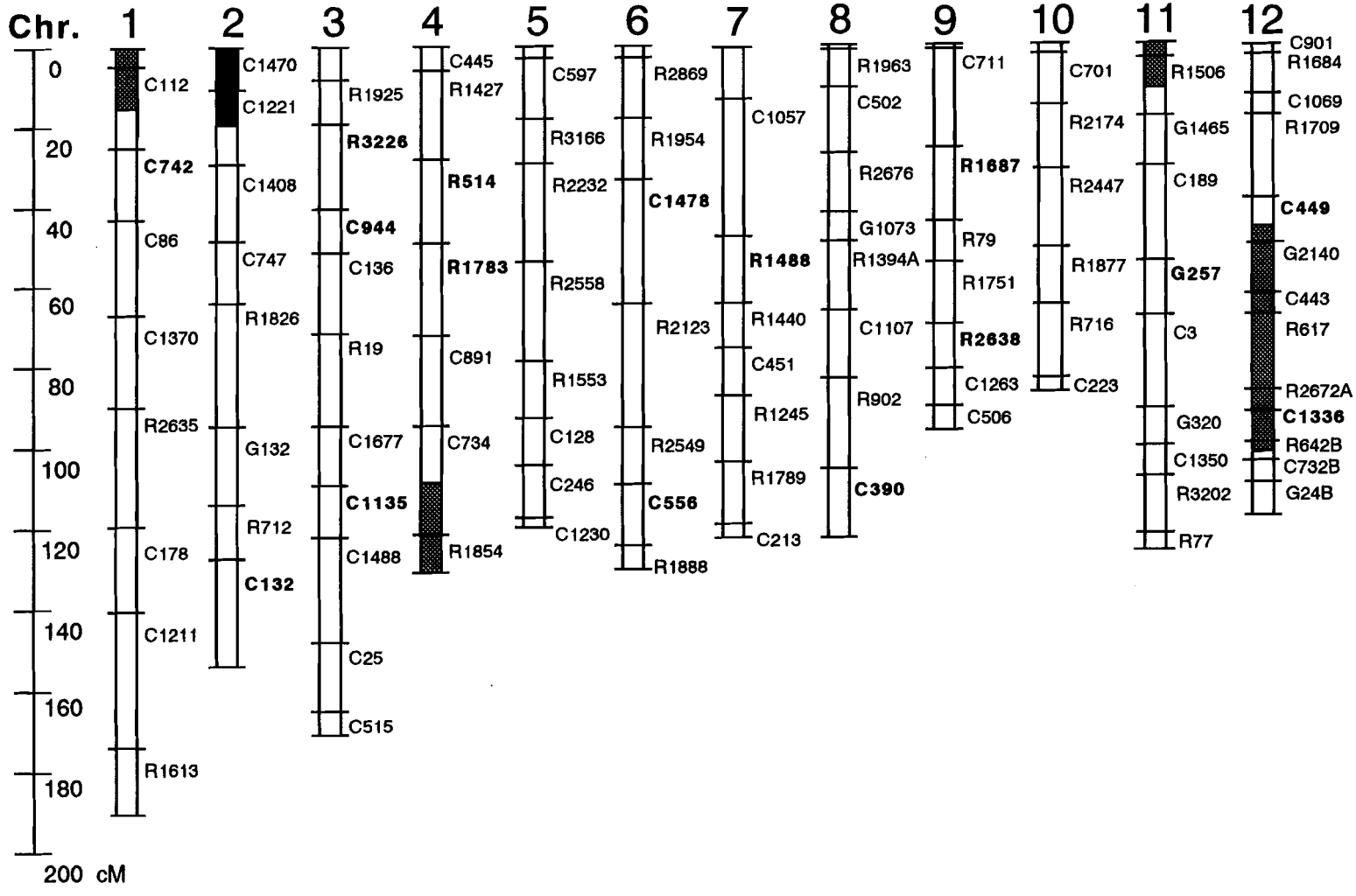

Fig. 3-2 A graphical genotype of PL4. Hatched boxes on chromosomes 1, 4, 11 and 12 indicate the regions commonly polymorphic between Tsukushibare and PL4 and between Asominori and IR1154-243, but monomorphic between IR1154-243 and PL4. A block box on chromosome 2 indicates the region monomorphic among the resistant lines and Tsukushibare and polymorphic with Asominori. Bold-faced markers were monomorphic among all the lines. Among 7 RFLP markers in the region on chromosome 12, C1336 was monomorphic among the lines. Proximal ends of the boxed regions are arbitrarily placed at the middle of the two flanking markers.

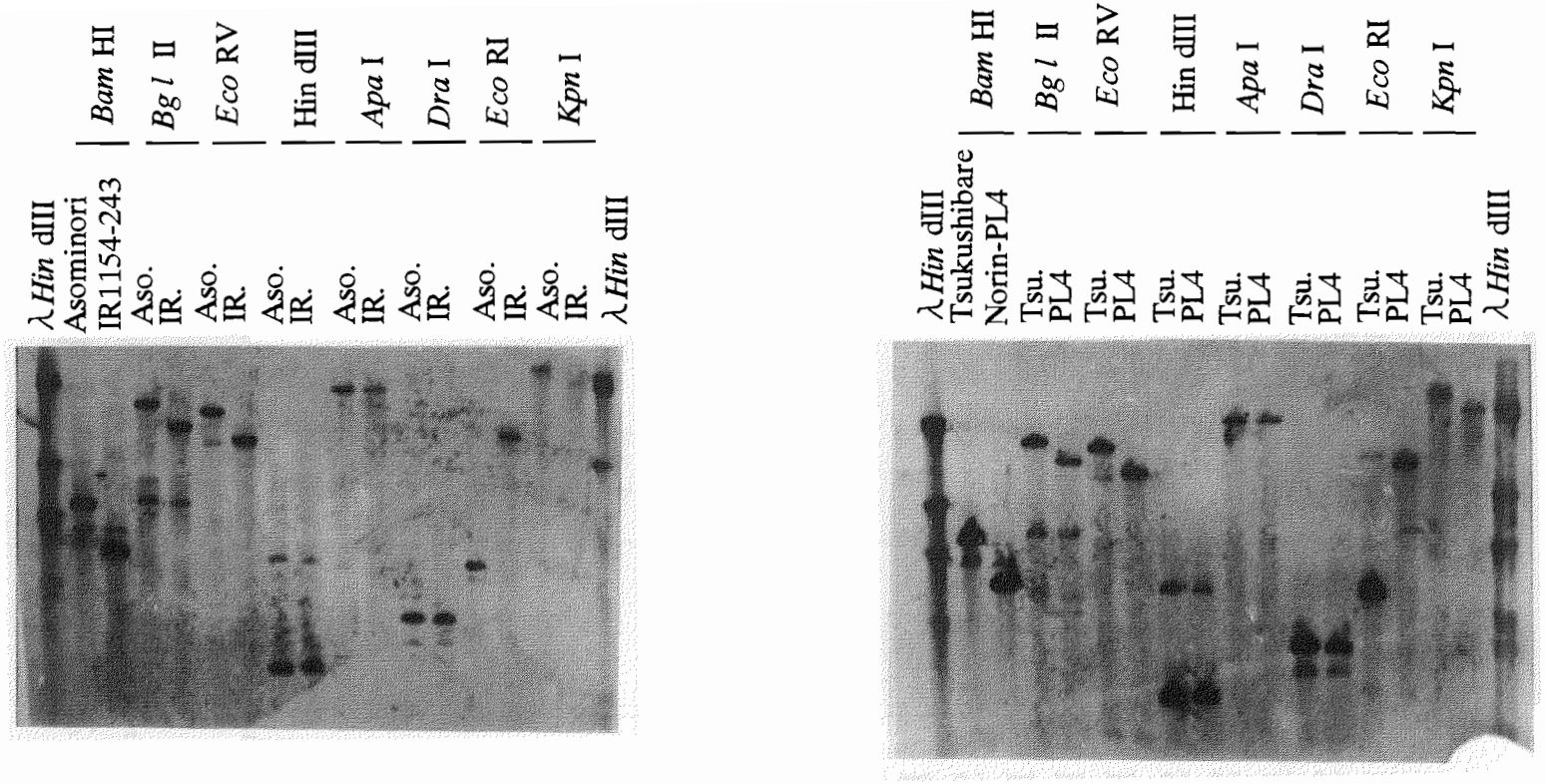

Fig. 3-3 RFLP pattems with parents of Norin-PL4. DNAs of;

A) Asominori (Aso.) and IR1154-243 (IR.), and B) Tsukushibare (Tsu.) and Norin-PL4 (PL4) were digested by 8 enzymes and probed with G2140 on chromosome 12 .

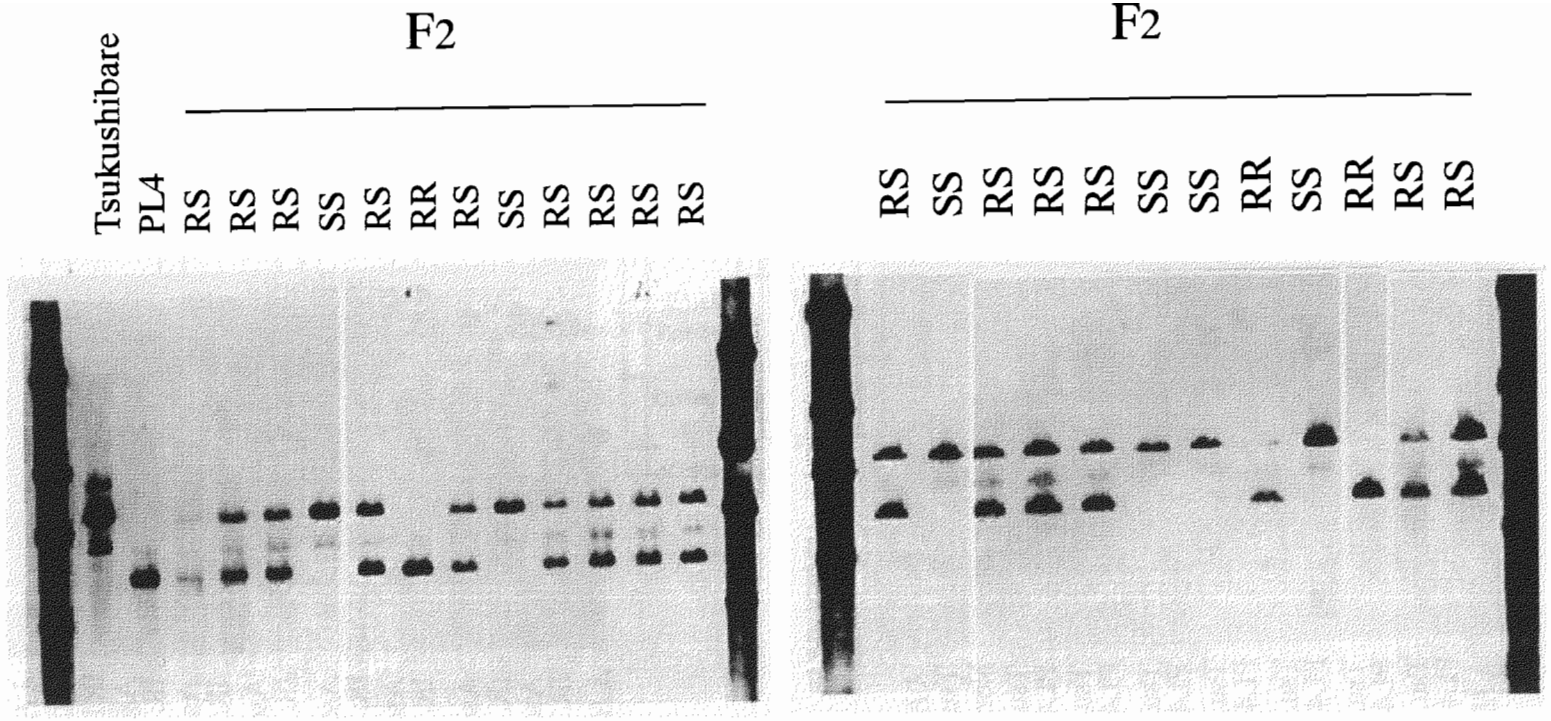

Fig. 3-4 Linkage analysis of BPH resistance gene in PL4.
DNAs of F_2 individuals from resistant homozygotes (RR), susceptible homozygotes (SS), resistrant heterozygotes (RS), Tsukushibare and PL4 were digested by BamHI and probed with G2140.

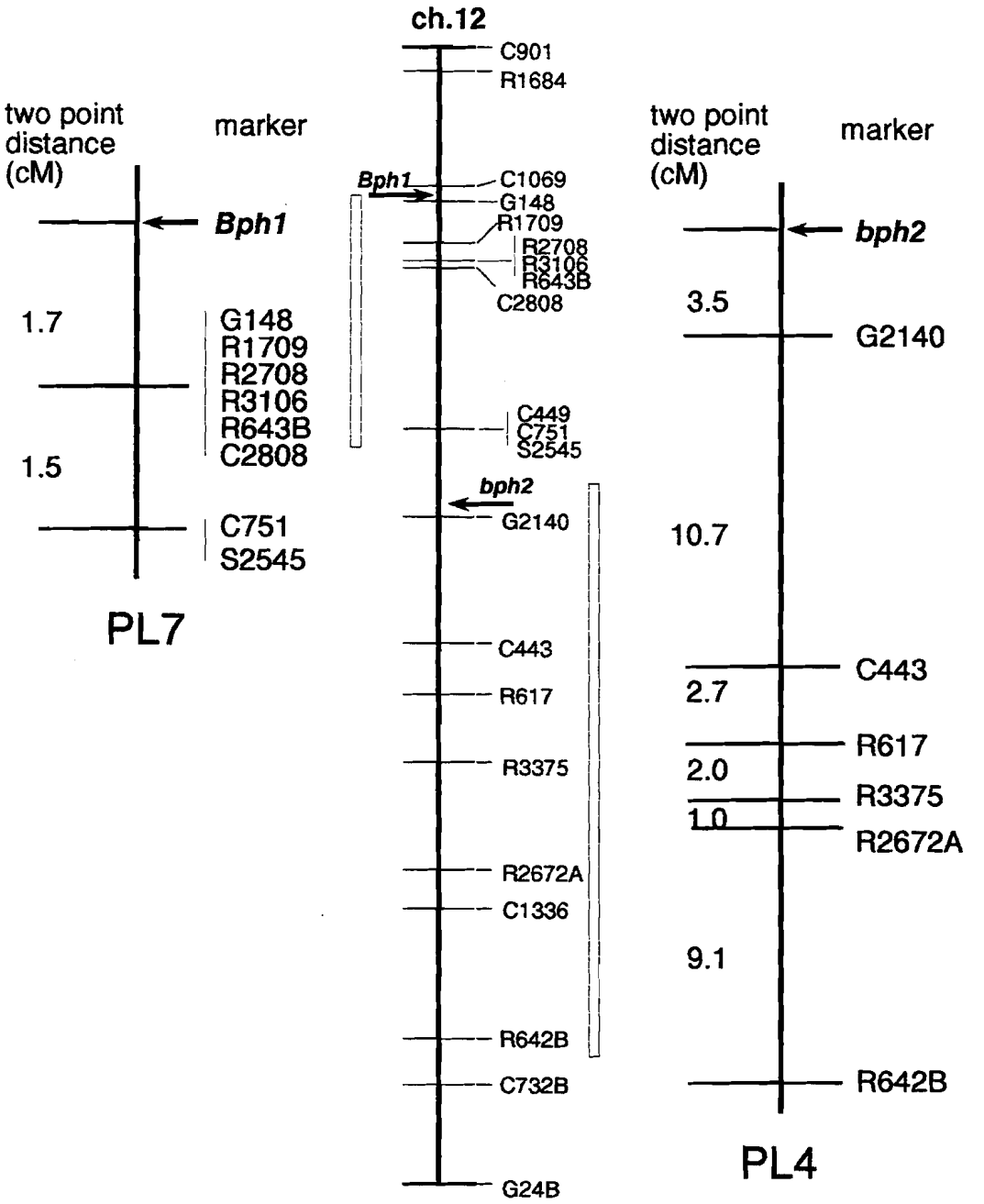

Fig. 3-5 Map positions of the resistance genes, Bph1 and bph2. The central map of chromosome 12 is from Nipponbare/Kasalath map. Maps and boxes on both sides of this standard map indicate the chromosomal regions introgressed from the resistance donors to the breeding lines, PL7 (Bph1) and PL4 (bph2). The map position of Bph1 is from Murata et al. (1997).

in F_2 population (see Table 2-3) and 573 F_3 lines derived from crosses of PL3/PL4 and PL7/PL4, were subjected to the BPH resistance tests.

2) Results

Bph1 and bph2 were tried to be pyramided by crossing two introgression lines of Bph1 (PL3 and PL7) with PL4 and resistance in F_3 lines was analyzed (Table 3-2). Contrary to the expectation based on the considerable map distance between these two BPH resistance genes, neither susceptible F_3 lines nor lines segregating susceptible progenies were detected after bioassays of ca. 8,600 F_3 individuals from the crosses.

5. Discussion

The segregation analysis of BPH resistance in F_2 individuals and F_3 lines from the cross of Tsukushibare/PL4 demonstrated that the resistance gene in PL4 behaved as a major dominant gene (Tables 3-1, Appendix-2 and Fig. 3-1). The resistance gene in two japonica breeding lines derived from PL4 was also reported to be a dominant gene (Takita, 1996). To prove that the resistance gene in PL4 is identical to bph2, we conducted graphical genotyping of PL4. The study clearly demonstrated the presence of a large chromosomal segment that was derived from the donor of bph2, i.e. IR1154-243 (Fig. 3-2). Moreover, this region co-segregated with BPH resistance in the mapping population. So far two dominant BPH resistance genes have been introgressed from indica rice into japonica rice breeding lines. The location of Bph1 is ca. 30 cM from bph2 (Fig. 3-5). Another dominant gene, Bph3, as well as a recessive gene, bph4, have recently been mapped on chromosome 4 (Khush, personal communication; Murata Chapter 6). It is thus conclusive that the gene introgressed in PL4 is bph2.

bph2 was first found in ASD7 (Athwal et al., 1971). A study on the resistance segregation in a large number of F_2 and F_3 progenies from the cross of Taichung Native 1 (TN1)/ASD7, clearly demonstrated the recessive nature of bph2 (Athwal et al., 1971). Later, a line IR1154-243 that was derived from a cross involving two susceptible cultivars, IR8 and Zenith, was found to possess bph2 (Martinez and Khush, 1974). Because of the susceptibility of both parents, they postulated the presence of a dominant inhibitor gene,

Table 3-2 Segregation analysis of BPH resistance in F_3 lines from crosses of PL3 and PL7 (introgression lines of Bph1)/PL4

Cross	Number of F_3 lines			
	Resistant (RR)	Segregating (Rr)	Susceptible (SS)	Total[a]
PL3/PL4	309	0	0	309
PL7/PL4	264	0	0	264

[a] An average of 15 individuals per line were assayed in duplicate tests in both crosses.

perhaps in Zenith, on the analogy of the case of 'TKM6' which was first found as a susceptible Bph1 carrier (Martinez and Khush, 1974).

It is well known that many recessive genes control resistance to pathogens and insect pests. Among 10 BPH resistance genes so far identified, 4 have been reported to be recessive. A fundamental question is how the recessive genes can confer the host-specific resistance. According to the gene-for-gene theory, host-specific resistance is controlled by the complementary genes of the functional race-specific host resistance gene and the functional pest avirulence gene (Flor, 1971). Recently one novel resistance mechanism has been demonstrated in that defective, recessive alleles of the gene Mlo are conferring the resistance on barley plants against powdery mildew (Buschges et al., 1997). The monogenic resistance mediated by the recessive mlo alleles confers a broad spectrum resistance to almost all known pathogen isolates and is durable in the field despite extensive cultivation (Jorgensen, 1992). However, this is apparently not the case in BPH resistance conferred by recessive BPH resistance genes.

If the resistance is controlled by a functional allele encoding a functional protein, the criterion for the dominance or recessiveness should solely depend on the phenotype of the heterozygotes, i. e., if the heterozygotes show resistant phenotype the gene is referred to dominant, while if they show susceptibility the gene is said to be recessive. The dominance or recessiveness might well be determined by the penetrance of the gene involved. In fact, many cases have been reported where recessive resistance genes behave as dominant genes under different environments, particularly under different temperature conditions, in different genetic background and with different pathotypes (races) or biotypes. Because of this, the Catalogue of Gene Symbols for Wheat, for example, recommends to use capital letters to designate all resistance genes irrespective of dominance or recessiveness (McIntosh 1988). Our analysis on the frequency distribution of BPH resistant progenies in F_3 lines derived from a cross of Tsukushibare/PL4 suggested the incomplete dominance of bph2 in PL4 (Fig. 3-1). Also bph2 might behave differently in indica and japonica backgrounds. The problem of dominance/recessiveness of bph2 addresses an interesting hypothesis that a given single resistance gene behaves differently under different environment, different genetic background, and/or against BPH

biotypes with different virulence property. Genetically defined biotypes and resistant hosts are undoubtedly the most critical requirement to test this hypothesis.

Our mapping study indicated that Bph1 and bph2 locate at a considerable distance (ca. 30 cM) on chromosome 12 on Nipponbare /Kasalath map (Fig. 3-5). The segregation analysis in a large number of F_3 lines derived from crosses of two Bph1 carriers and PL*4*, however, did not show any susceptible lines and lines that segregated susceptible progenies (Table 3-2). The result of no recombinations between the two resistance genes confirmed the previous report (Athwal et al, 1971). The map distances between RFLP markers in the regions carrying Bph1 and bph2 in our study were much shorter than those in the corresponding regions on Nipponbare/Kasalath map (Fig. 3-5). Although the physical distance remains unknown, it can be suggested that some structural or functional constraint prevents crossing-overs in the region covering the two BPH resistance genes. For pyramiding Bph1 and bph2, some other strategy has to be adopted.

CHAPTER 4 Analysis of BPH resistance gene in Pokkali

1. Introduction

The virulence of BPH against rice varieties can differentiate in many biotypes. Biotype 1 defined as the predominant wild type in southeast and east Asia can feed only on varieties without any resistance gene. Biotype 2 and biotype 3 can feed on Bph1 and bph2 carriers, respectively, and susceptible varieties (Kaneda 1990). The biotypes in south Asia differ from those in southeast Asia. For example, most of the varieties carrying Bph1 and bph2 are susceptible to BPH in south Asia including India and Sri Lanka (Khush 1984). Lakshminarayana and Khush (1977) identified Bph3 in a Sri Lankan local variety Rathu Heenati and bph4 in another Sri Lankan local variety Babawee. They are resistant against all of Asian biotypes. Following that, bph5, Bph6 and bph7 were identified using Bangladesh biotypes.

While in Japan, biotypes 2 and biotype 3 were selected by continuous culturing of the wild type BPH (predominantly biotype) on Mudgo carrying Bph1 and ASD7 carrying bph2, respectively (Kaneda and Kisimoto 1979, Ito and Kisimoto 1981). Kaneda et al. (1981) classified many varieties into three groups, i. e. susceptible varieties, Bph1 carriers and bph2 carriers, by using the selected biotypes. But, because Bph3 and bph4 were both resistant against both biotypes, they could not be classified only by biotype reaction. So that, integrated identification system combining test cross and biotype reaction was designated. Ikeda and Kaneda (1986) demonstrated that, among the resistance varieties to all of three biotypes, Balamawee, Kaharamana and Pokkali had unidentified dominant gene, and Collection 5 Thailand (Thai Col. 5), Thai Col. 11 and Chin saba had unidentified recessive gene. Nemoto et al. (1989a) reported that Thai Col. 11 and 5 and Chin saba possess a same recessive gene, bph8, and that another three varieties including Balamawee, Kaharamana and Pokkali possess a same dominant gene Bph9, after allelism tests among them. However, breeding of resistance lines possessing bph8 and Bph9 have not been conducted, and no analysis for their chromosome assignment have yet been made.

In this chapter, mapping of Bph9 in Pokkali by RFLP analysis is described. The

result on the identification of tightly linked RAPD markers was also described.

2. Chromosome assignment and mapping of Bph9 by RFLP analysis

1) Materials and Methods

Plant materials

Segregating populations for Bph9 were derived from a cross of Pokkali and Norin-PL9. Pokkali is a Sri Lankan local variety carrying Bph9. Norin-PL9 (hereafter abbreviated as PL9) has cross compatibility with indica rice and is susceptible to BPH. Considering possible hybrid sterility in indica-japonica crosses, PL9 was used as a female parent.

In 1992, first crosses of PL9/Pokkali were made in Kobe University. The number of harvested F_2 seeds, however, were too small to carry out genetic analysis in the next year. Therefore, for the genotype determination of F_2 individuals, not only F_3 lines but also F_4 lines were used.

Bulk seedling tests of BPH resistance, DNA preparation and Southern hybridization

All procedures were the same as described in Chapter 2.

DNAs from 41 lines whose BPH resistance genotypes were determined were prepared for the mapping analysis. DNAs from F_3 lines and both parents were isolated in bulks of about 10 seedlings per line.

Recombination values between the resistance gene and RFLP markers were calculated by MAPMAKER Version 2.0 (Lander et al. 1987) and converted into genetic map distance (Kosambi 1944).

Bulked segregant analysis

Bulked DNAs were prepared by pooling DNA samples from 4 F_3 individuals with the same genotype identified in the resistance tests. In the first screening, the DNA samples of each parent and the two bulked genotypes (resistant homozygous and

susceptible homozygous) were used for hybridization, and the hybridized patterns were compared.

2) Results

The bulked segregant analysis survey was conducted using 106 RFLP markers randomly chosen from 12 chromosomes and DNAs from PL9, Pokkali, and two F_3 bulks representing resistant and susceptible homozygotes derived from the crosses of PL9/Pokkali. A comparison of RFLP patterns among them showed that only 9 probes on chromosome 12, R1709, R643, R3106, C2808, C449, S2545, G2140, C443, and R617, were polymorphic between susceptible and resistant materials (Fig. 4-1 and Fig. 4-2). This region spanned ca. 45 cM on Nipponbare/Kasalath map. Except for R643 and C449, they were further hybridized with DNAs from 65 F_3 lines, and all showed co-segregation with the resistance gene (Fig. 4-3). As the loci of R643 and C449 were mapped at the same loci of R3106 and S2545, respectively, they were not used for mapping. The linkage analysis showed that the Bph9 in Pokkali locates between S2545 and G2140 with a genetic distance of 11.6 and 13.0 cM, respectively (Fig. 4-4).

3. RAPD analysis

1) Materials and Methods

Plant materials

Segregating F_3 individuals derived from crosses of PL9/Pokkali were surveyed by RAPD analysis. F_4 lines were used for BPH resistance tests, and their genotypes were determined.

DNA preparation and bulked segregant analysis

Template DNAs for RAPD-PCR were extracted by the same procedures described in 2-3-1. Each of the two bulked DNA samples for both resistant and susceptible homozygotes were further prepared by mixing the pooled DNA samples.

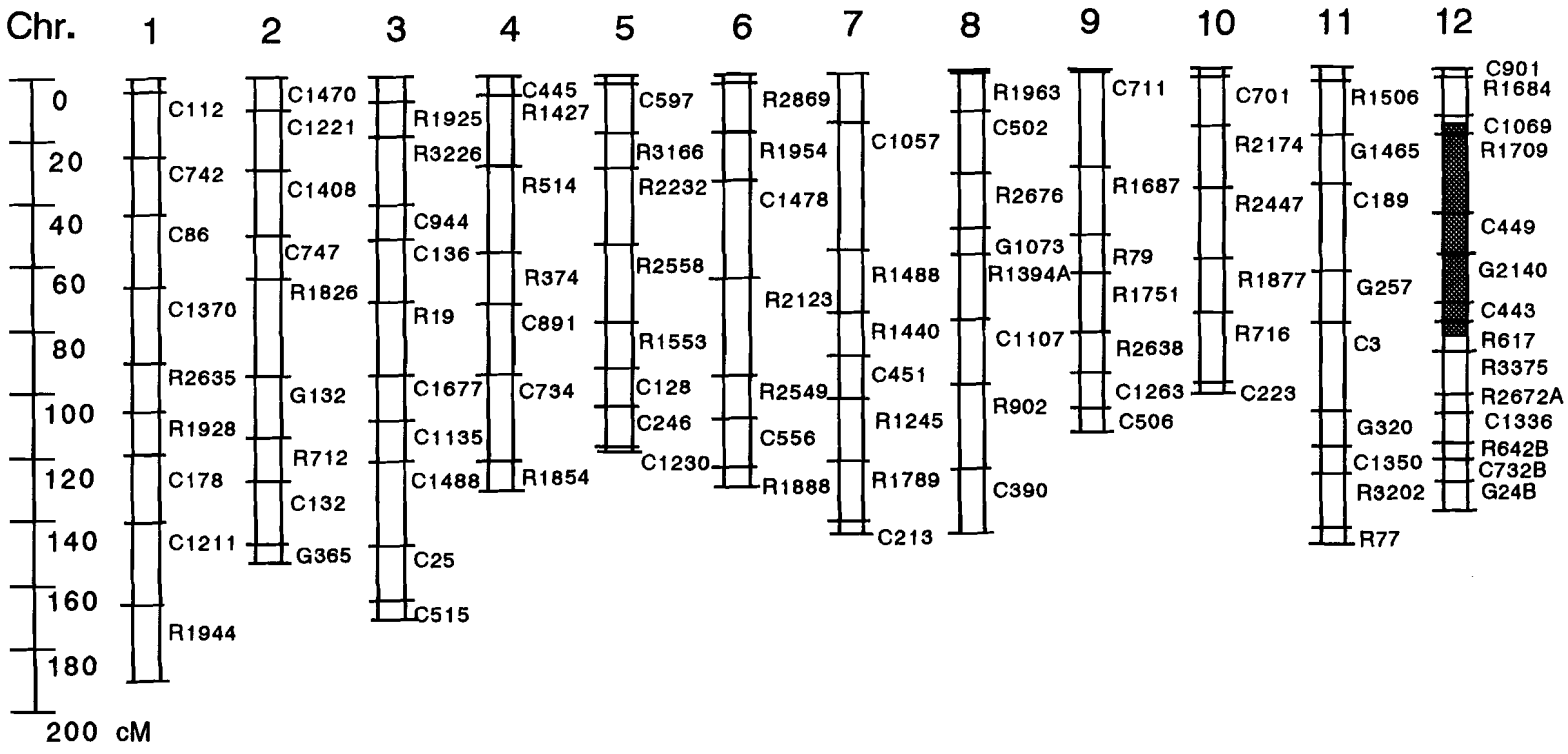

Fig. 4-1 Bulked segregant analysis for Bph9. A hatched box on chromosomes 12 indicates the region commonly polymorphic between Norin-PL9 and Pokkali possessing Bph9 and between F_3 bulks from susceptible and resistant homozygotes. Proximal ends of the boxed regions are arbitrarily placed at the middle of the two flanking markers.

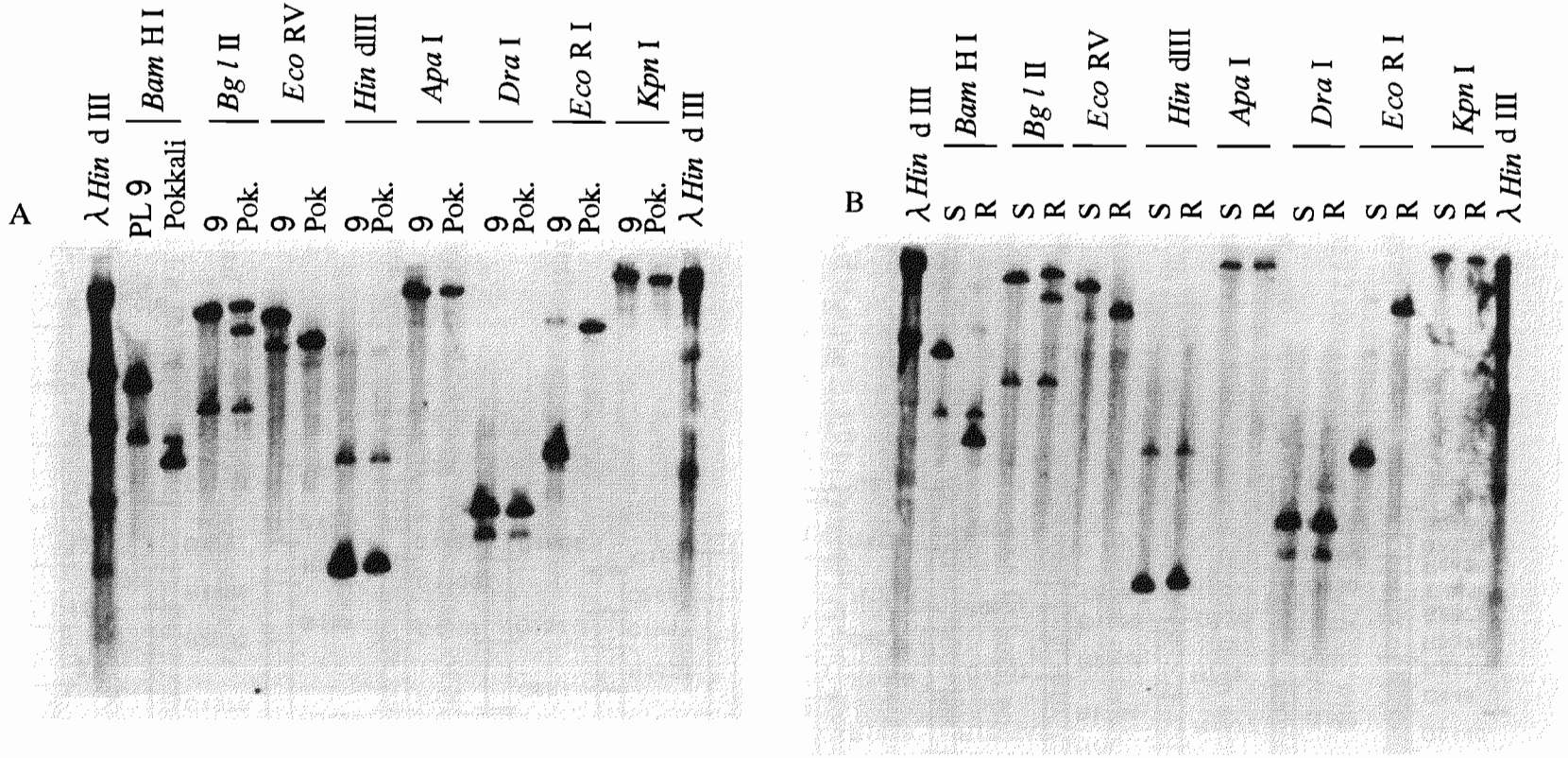

Fig. 4-2 Bulked segregant analysis of BPH resistance gene of Bph9 in Pokkali.
DNAs of; A) PL9 (9) and Pokkali (P), and B) F_3 bulks from susceptible homozygotes (S) and resistant homozygotes (R) were digested by 8 enzymes and probed with G2140.

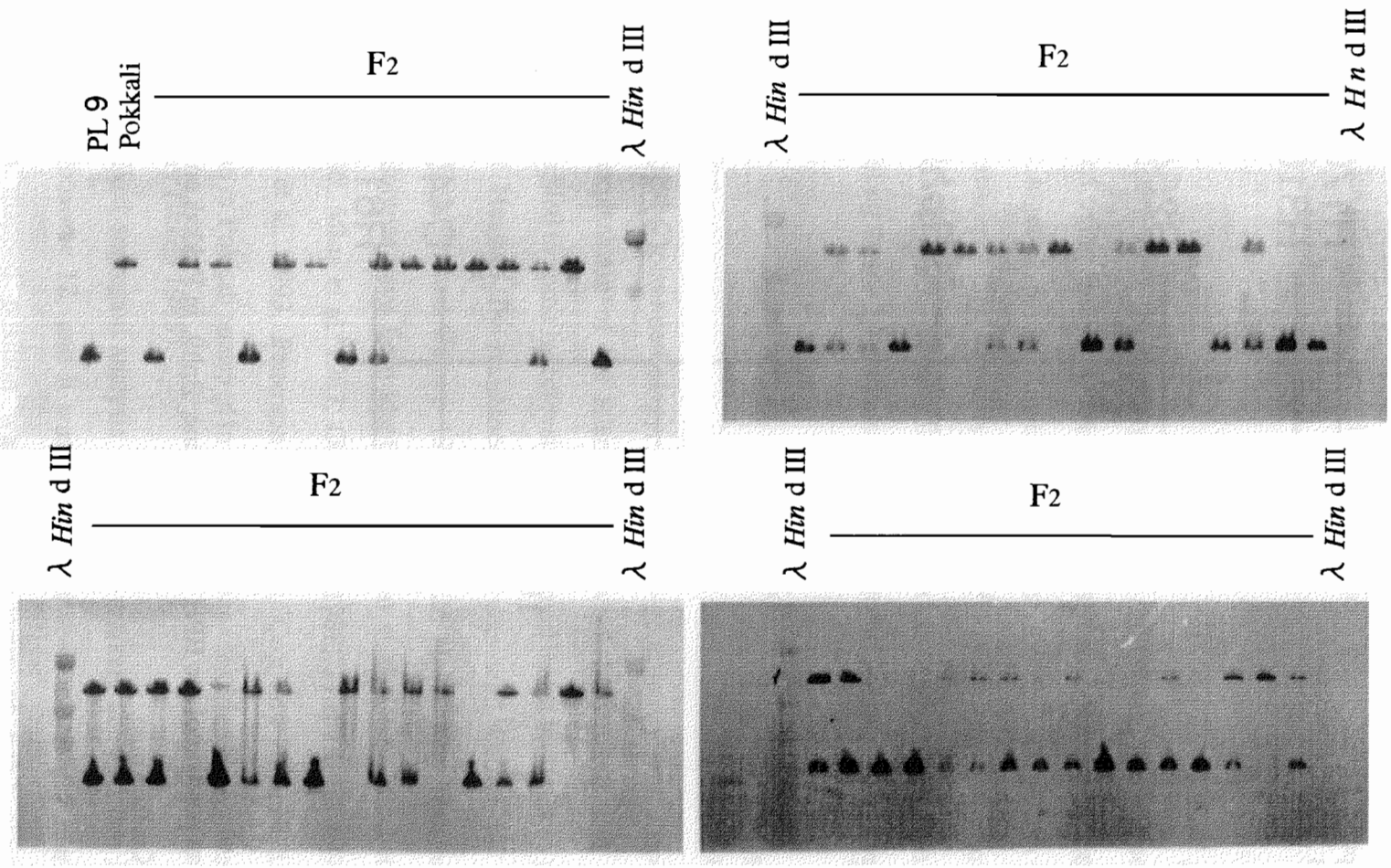

Fig. 4-3 Linkage analysis of BPH resistance gene Bph9 in Pokkali. DNAs of F_2 individuals, PL9 and Pokkali were digested by EcoRI and probed with G2140.

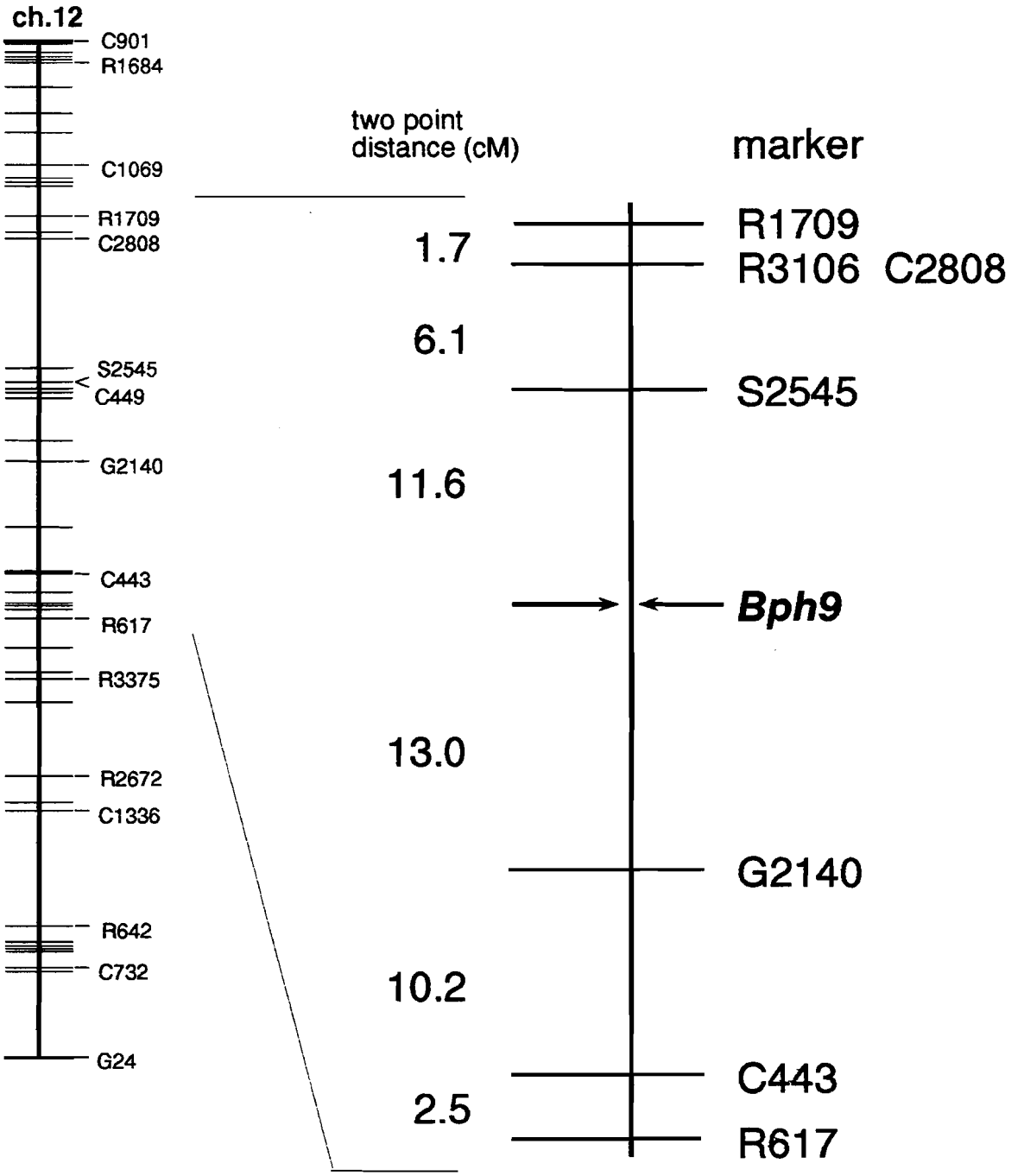

Fig. 4-4 Map position of the BPH resistance gene, Bph9, in Pokkali on the Nipponbare/Kasalath map.

RAPD-PCR reaction

A total of 240 random 10-mer primers (Operon Technologies Inc.) were surveyed for detecting RAPD markers linked to the resistance gene. Amplification condition was described in 2-4-1.

2) Results

After determining the genotypes of 32 F_3 individuals, bulked segregant analysis was performed using bulked DNAs from 10 resistant homozygotes and 9 susceptible homozygotes together with parental DNAs. Polymorphic fragments associated with resistance or susceptibility was surveyed by RAPD-PCR with 240 primers, resulting in the detection of 22 RAPD markers linked either in cis or in trans to Bph9 (Table 4-1 and Fig. 4-5). Among these, 13 markers were found to be polymorphic between Nipponbare and Kasalath.

4. Discussion

In the field of plant pathology, the gene-for-gene theory was proposed as the most fundamental explanation of the interrelation between host plants and pathogens. This theory was applied to some insect resistance, assumed insect biotypes as pathogen races. The study of Hessian fly resistance in wheat was well known as a model of the interrelation (Hatchett and Gallum 1970). Now, 26 Hessian fly resistance gene and 14 Hessian fly biotypes have been identified (Carlson et al. 1978, Hatchett and Gill 1983, Gill et al. 1986, Maas et al. 1987, Obanni et al. 1988, Friebe et al. 1990, Sharma et al. 1992, Raupp et al. 1993, Patterson et al. 1994, Cox and Hatchett 1994). The BPH resistance gene in Pokkali analyzed in this study was identified by application of the gene-for-gene theory (Ikeda 1985).

The bulked segregant analysis with RFLP markers showed that Bph9 in Pokkali was on chromosome 12, and it was mapped between two RFLP markers S2545 and G2140. This mapped position was near the locus of bph2 already mapped on chromosome 12 (Murata et al. 1997b). Bph9 was considered to be difference gene from Bph1 and bph2 because of the resistance reactions to both BPH biotype 2 and 3 (Ikeda

Table 4-1 A list of RAPD markers linked in cis and in trans to Bph9

[a]cis linkage	Band size	[b]trans linkage	Band size
OPA-07	0.9 kbp	OPA-02	1.3 kbp
OPB-08	2.4*	OPC-10	1.8*
OPC-14	0.7	OPC-12	1.3
OPE-11	1.6*	OPD-03	2.0*
OPE-14	1.9*	OPD-04	1.1*
OPP-04	1.6*	OPD-11	0.8*
OPR-02	2.2	OPD-18	0.7
OPR-20	0.6	OPF-15	1.1*
OPT-04	0.6	OPQ-01	1.7*
OPU-01	0.5*	OPR-04	1.2*
		OPU-09	0.7
		OPU-12	1.1*

[a] cis linkage means that the marker and Bph9 locate on the same chromosome.

[b] trans linkage means that they locate on the two homologous chromosoem.

*indicates polymorphic markers between Nipponbare aned Kasalath.

1985, Nemoto et al. 1989). Although Ikeda (1985) demonstrated that the F_2 populations of Pokkali(Bph9)/Rathu Heenati (Bph3) and Pokkali/Babawee (bph4) segregated into expected ratio of 15 resistant to 1 susceptible and 13 resistant to 3 susceptible, respectively, Pokkali have not been crossed with Bph1 and bph2 varieties.

It is very interesting that 4 resistance genes, Bph1, bph2, Bph9 and Bph10(t) among 10 BPH resistance genes identified have been mapped on same chromosome 12. In the study of Hessian fly, Ohm et al. (1995) reported that 8 resistance genes, H3, H6, H9, H10, H12, H15, H16 and H17 among 26 resistance genes identified have been mapped on same chromosome 5A. To clarify whether the genes on the same chromosome have similar sequence, and express similar function or not, clonings of these genes have to be accomplished.

10 RAPD markers were shown to be co-segregating with the resistance gene and 12 other trans-linked markers have been detected (Table 4-1 and Fig. 4-5). since 13 such markers were found to be polymorphic between Nipponbare and Kasalath. These will be mapped and provide useful molecular tags in introgression breeding for Bph9 in Pokkali.

CHAPTER 5 Analysis of BPH resistance gene in PL10

1. Introduction

The first BPH resistant semidwarf cultivar, IR26 with Bph1, was released by IRRI in 1973. Its source of resistance was from TKM6. Following IR26, four lines of IR28, IR29, IR30 and IR34 were bred as high yielding semidwarf varieties, all possessing Bph1. IR32 was also bred to possess bph2. In those days, the breeding program for resistance to BPH was based on these two genes, i. e. Bph1 and bph2. However, resistance in IR26 was broken down within a few years after the release; in 1975 in Philippines and Solomon Islands (Anonymous 1975, Stapley 1975) and in 1977 in Indonesia (Harahap 1979). The occurrence of new biotypes, therefore, necessitated the identification of new genes for BPH resistance. Lakshminarayana and Khush (1977) identified a single dominant gene conveying BPH resistance in a Sri Lankan local variety Rathu Heenati, and a single recessive gene in another Sri Lankan local variety Babawee. The dominance gene segregated independently of Bph1 and was designated as Bph3. Similarly, the recessive gene that segregated independently of bph2 was designated as bph4.

The varieties possessing Bph1 and bph2 were resistant to southeast and east Asian biotypes but not to south Asian biotypes. However, as both Bph3 and bph4 expressed resistance to all of these biotypes, a danger in breakdown of resistance was expected to be small as far as these resistance genes are concerned (Nemoto et al. 1989b). PL10 (Tsukushibare/// Tsukushibare *3/Rathu Heenati//Tsukushibare), a japonica introgression line of Bph3 from Rathu Heenati, was bred in National Agricultural Research Center (Nemoto et al. 1989b). Although introgression of a recessive gene bph4 in Babawee was tried at the same time, a breeding line PL7, unfortunately, was found to possess Bph1 from Mudgo instead of bph4 (Murata et al. 1997a). In this chapter, the results of genetic analysis of a resistance gene in PL10 are described. Since some preliminary study showed that PL10 expressed much weaker BPH resistance than any other japonica introgression lines, the results of preference tests supplementary conducted are also reported.

2. Genetic analysis and preference tests

1). Materials and Methods

Plant materials

The plant materials were prepared similarly to the analysis of BPH resistance gene in PL7 described in Chapter 2. Four crosses of Tsukushibare/PL10 designated as K1 to K4, and the F_2 seeds were harvested in National Agriculture Research Center. Individuals of F_2 seedlings were transplanted in the rice field of Kobe University (see Chapter 2).

Culture of BPH and bulk seedling tests

All procedures were the same as described in Chapter 2. In the bulked seedling tests, PL10, PL7 and Rathu Heenati were used as resistant standards, and Tsukushibare as susceptible control.

Preference tests of BPH

To confirm the level of resistance in PL10 and the donor variety, preference tests were conducted in the following manner. Five or seven seedlings of the same stage were line-planted in a seedling tray with 3.0 x 3.0 cm space, and infested with nymphs at the second to fourth instar. Then, the number of nymphs on plants was recorded every 12 hours. A total of 5 tests were conducted with different combinations of varieties and different numbers of infested nymphs at different seedling stages (Table 5-1).

2) Results

Genetic analysis

A total of 96 F_3 lines of K1 to K4 were tested for determining BPH resistance genotypes. Most of the lines did not show clear segregation. Moreover, PL10, the standard resistant variety with Bph3, did not indicate clear resistance reaction to BPH. Also in F_2 populations of K1 to K4, the segregation of resistance did not indicate the presence of a dominant resistance gene in PL10 (Table 5-2).

Table 5-1 The conditions of BPH preference tests for PL10

Test No.	Tested varieties (x lines) R: resistant, S: susceptible	BPH nymphs/ seedlings	Seedling stage
1	R: PL7, PL10, Babawee, Rathu Heenati S: Tsukushibare x 2	210/30	second
2	R: PL7, PL10, Babawee, Rathu Heenati S: Tsukushibare x 3	420/35	third
3	R: PL3, PL4, PL7, PL10 S: Tsukushibare x 4	480/40	third
4	R: PL3, PL4, PL7, PL10 S: Nipponbare x 3	600/49	fourth
5	R: PL3, PL4, PL7, PL10 Babawee, Rathu Heenati S: Tsukushibare x 3	700/70	[a] fourth (only stem)

[a] Test after cutting all leaf blades off at the fourth leaf stage.

Table 5-2 BPH resistance tests on F_2 individuals derived from cross of Tsukushibare/PL10

F_2 test	Number of F_2 individuals			
	Resistant	Intermediate	Susceptible	Total
Tsukushibare/PL10	11	48	212	271
PL10	29	3	1	33
PL7	34	0	0	34
Rathu Heenati	44	0	0	44
Tsukushibare	0	1	33	34

Preference tests

Preference tests were conducted after obtaining the results of resistance tests of K1 to K4. Their progenies did not show any clear segregation of BPH resistance. Therefore, tests were made to determine experimental conditions in that resistant plants with Bph3 are clearly distinguished from susceptible ones.

Figures 5-1A to 5-1E show the results of five preference tests. The preference is the proportion (%) of BPH on the plants among the total number of BPH. The percentage of susceptible check was divided by the number of replicated rows. Figs. 5-1A, -1B and -1E showed that the introgression line PL10 was more easily infested than indica resistance varieties. In all tests, the percentage preference of PL10 was relative higher than those of other resistance varieties. The difference between resistant and susceptible varieties started to appear at 24 hours after infestation. At around 60 hours after infestation at the second and third leaf stage, and at around 80 hours after infestation at the fourth leaf stage, seedlings of the susceptible check began yellowing, those of resistance except for PL10 not yellowing but growing. But PL10 did not begin yellowing, and not growing, wilting with green leaves.

3. Discussion

BPH resistance did not express in the progenies of Tsukushibare/PL10; the resistant individuals could not be clearly distinguished from susceptible ones. This was contrasting to the clear-cut resistance in the progenies of Tsukushibare/PL7. In the bulked seedling tests, susceptible check plants generally stop growing around 24 hours after BPH infestation, and became yellowish and wilted around 5 days after infestation. On the contrary, resistant check plants like PL7 continued to grow and developed leaves, thus they were not apparently affected by BPH infestation.

In the case of PL10, however, leaf development was hindered and leaves became withered but did not turn yellowish and wilted. In the F_2 population of the cross Tsukushibare/PL10, most of the progeny plants appeared to be susceptible or intermediate. In F_3 lines, it was too hard to distinguish resistant homozygous lines from resistant heterozygous ones. Researchers in Aichi Agricultural Research Center reported that,

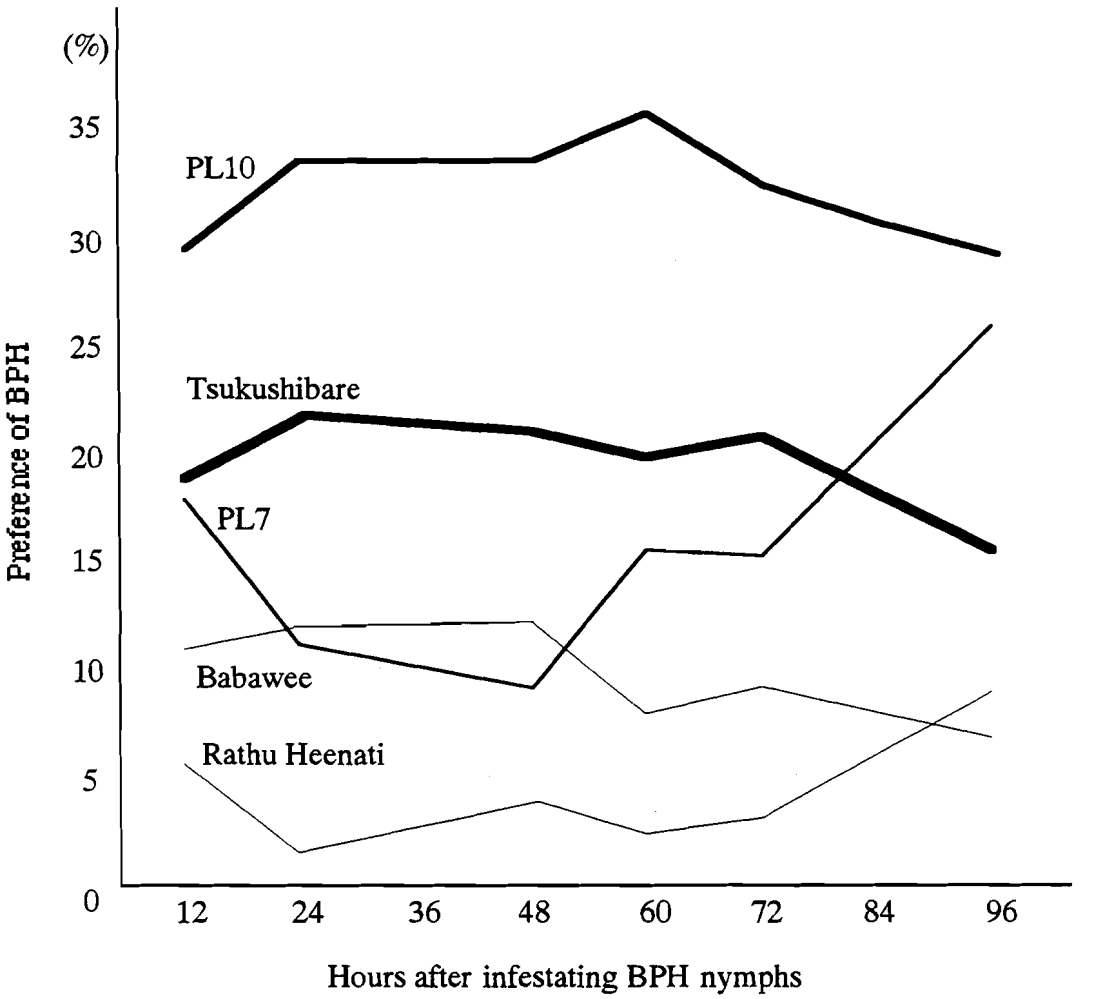

Fig. 5-1A Preference test 1. (See Table 5-1)

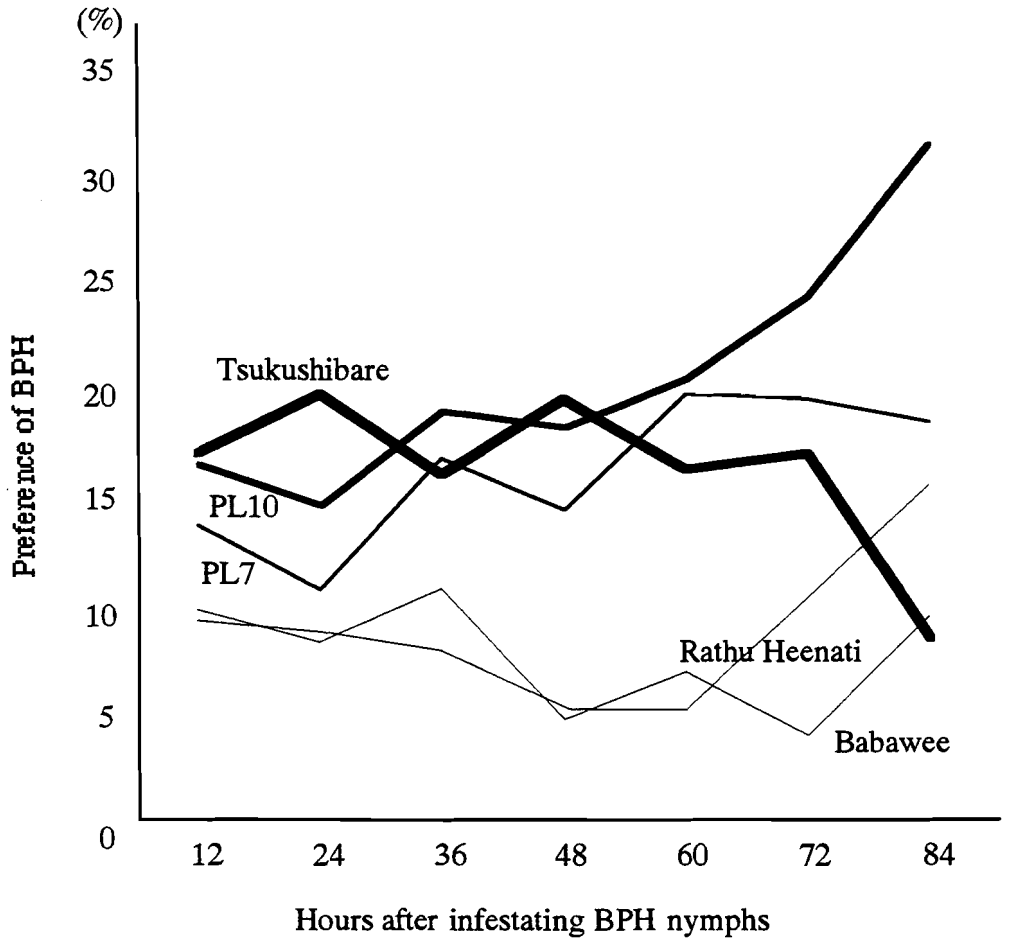

Fig. 5-1B Preference test 2. (See Table 5-1)

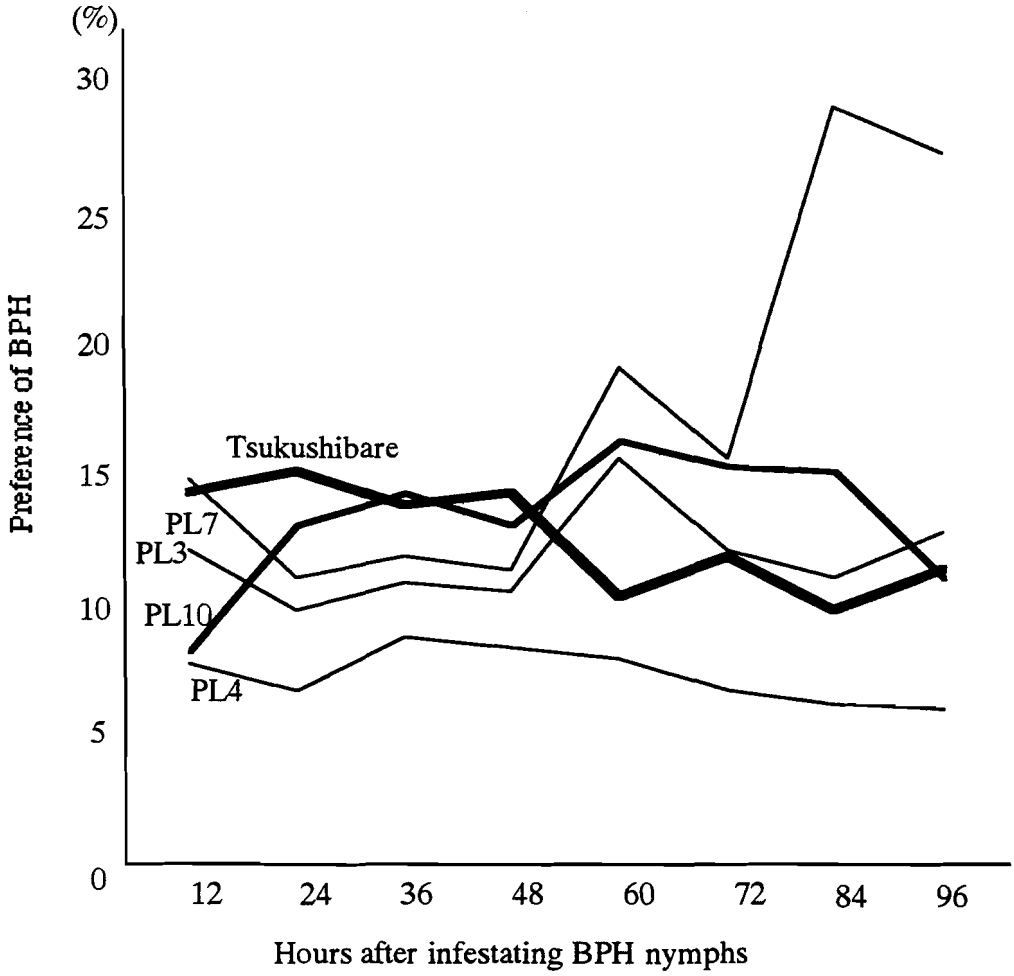

Fig. 5-1C Preference test 3. (See Table 5-1)

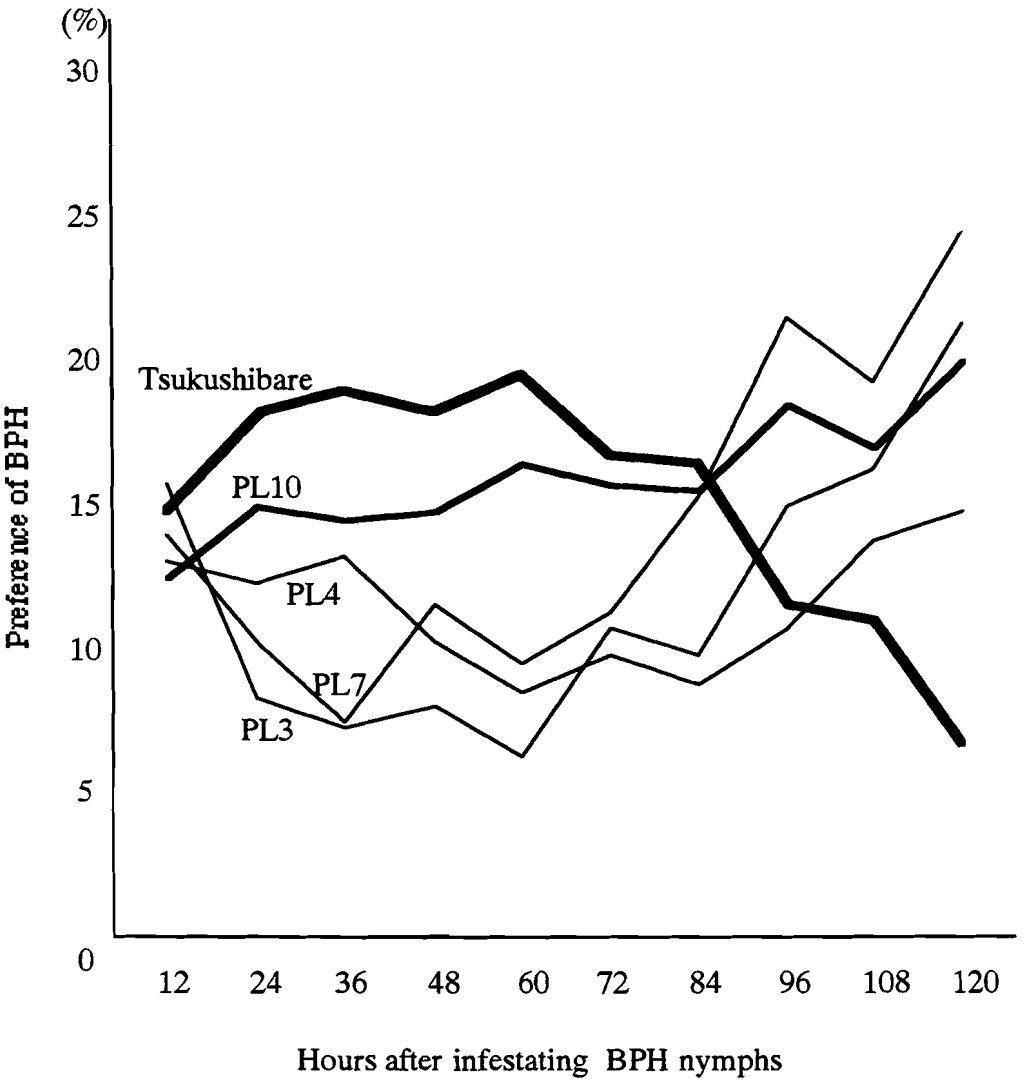

Fig. 5-1D Preference test 4. (See Table 5-1)

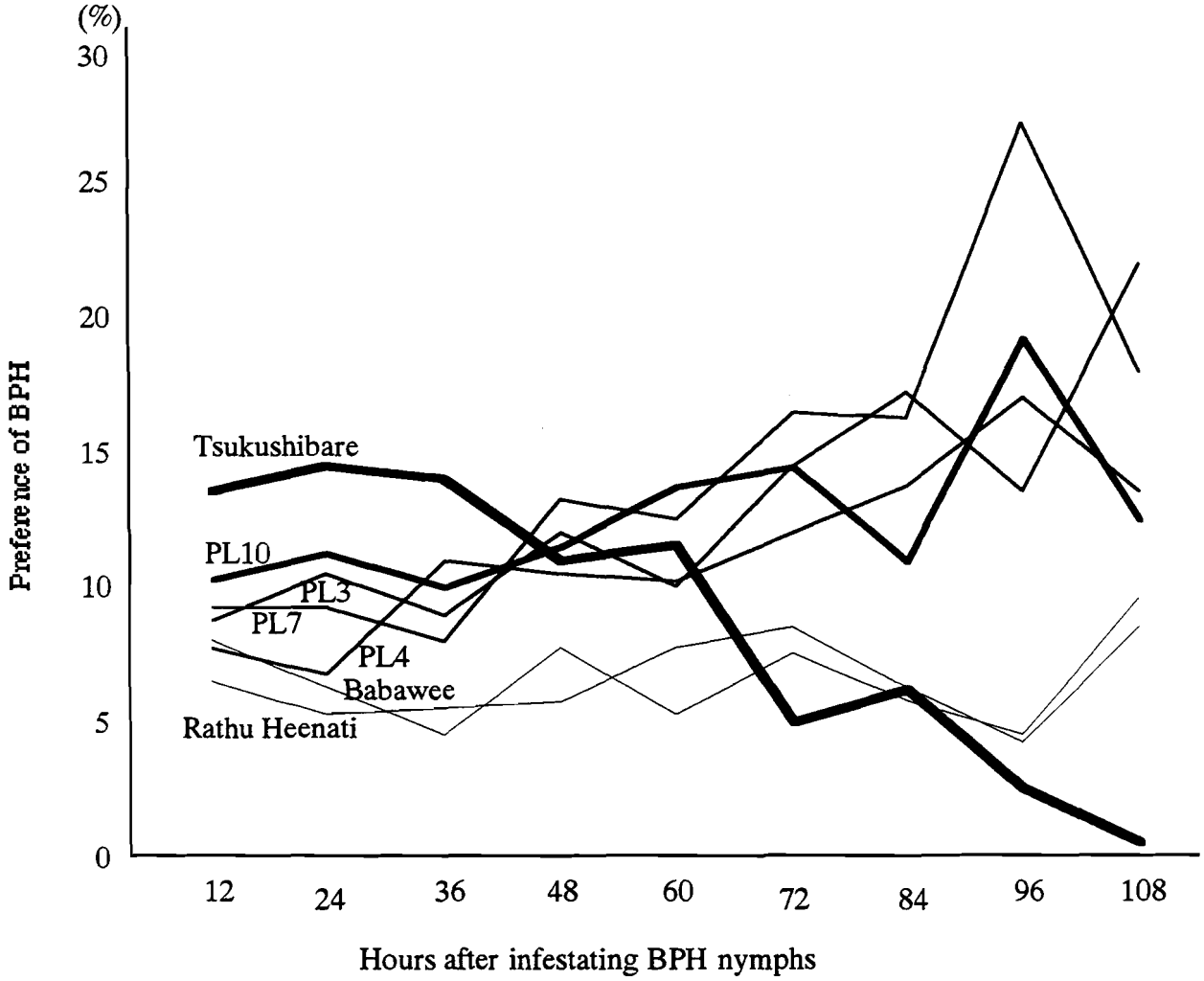

Fig. 5-1E Preference test 5. (See Table 5-1)

although they tried to introgress Bph3 of PL10 to superior breeding lines, no resistance could be identified after some backcrosses (Crop Research Institute, Aichi Agricultural Research Center 1994). They suggested that the level of resistance in PL10 was not high enough. But alien fragments seemed to be derived from Rathu Heenati were detected in the genome of PL10 by our RAPD analysis (Fig. 5-2).

Results of preference tests except in test 1 indicated that PL10 was resistant to BPH based on the comparison at 24 hours after infestation (Figs. 5-1B to 5-1E). But it was considered that its resistance was relatively weak, because PL10 was generally more preferred than other resistant lines in the younger seedling stage. The number of BPH nymphs on PL10 rapidly increased when that on the susceptible variety started to decrease. This suggest that because the level of antibiosis is not high enough in PL10, PL10 will suffer from BPH migrating from susceptible plants which had already become poor hosts. Therefore, the time for rating resistance must be set earlier, using seedlings of the later growth stages, as suggested by preference test (Figs. 5-1C and -1D). A fewer number of nymphs to be infested will also help clear the rating.

In this study, molecular analysis of Bph3 was not conducted because of unclear determination of genotypes of segregating populations. Improvement of methods to identify genotypes of resistance is essential for molecular analysis of Bph3 in PL10. PL10 was bred by five times crosses with Tsukushibare. Considering that BPH resistance in Rathu Heenati was also controlled by additional minor genes, the replicated backcrosses might eliminated them. Additional materials were made after the cross of Tsukushibare /Rathu Heenati in 1994. These materials might provide alternative means for testing this resistance gene.

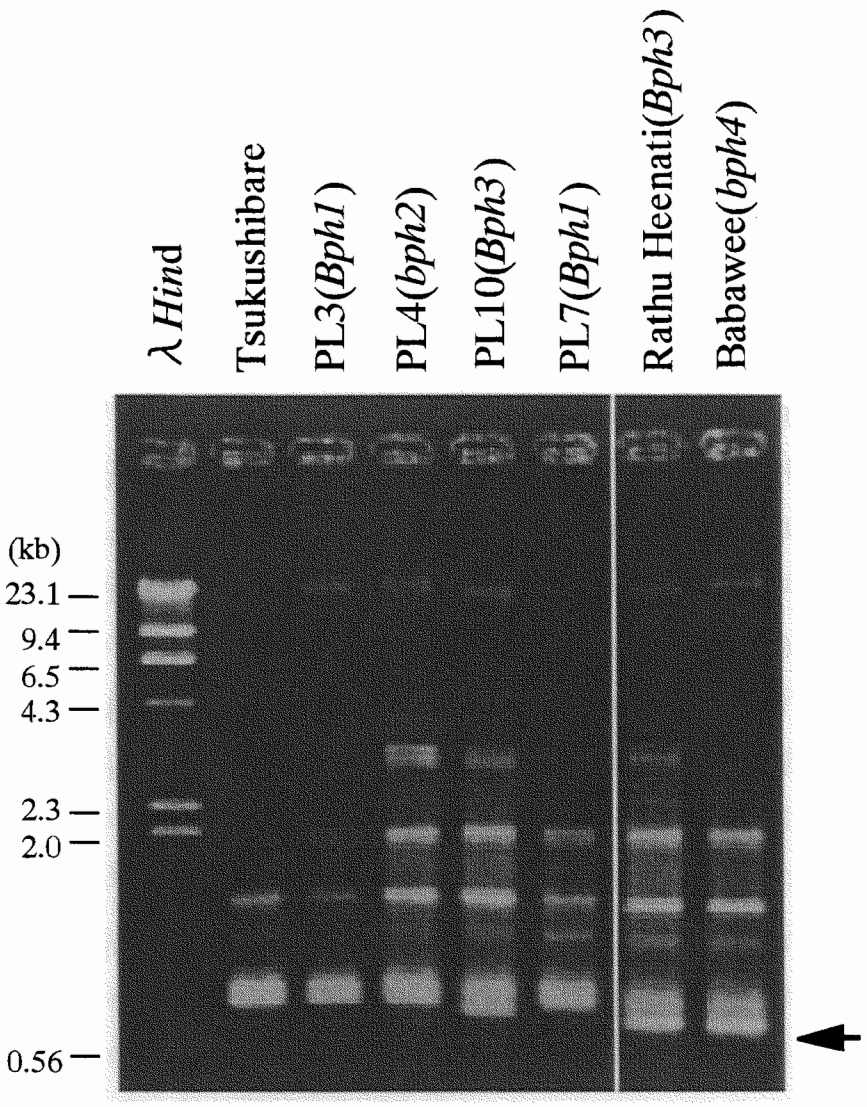

Fig. 5-2 A RAPD marker amplified by primer OPF-2. A polymorphic band (←) was commonly detected between PL10 and Rathu Heenati, a *Bph3* donor variety, but not on other resistant breeding lines and Tsukushibare which was the only recurrent parent of PL10. Both of Rathu Heenati and Babawee are Sri Lankan local varieties.

CHAPTER 6 Genetic analysis and RFLP analysis of BPH resistance in the progenies from original crosses involving Bph3 and RFLP analysis of bph4 carriers

1. Introduction

A series of mapping analysis of BPH resistance genes in japonica introgression lines showed that Bph1 in PL7 and bph2 in PL4 locate on chromosome 12. Neither Bph3 nor bph4, however, could be mapped because of some troubles in the introgression lines. New breeding program was needed for widening genetic base of BPH resistance and for mapping analysis. Therefore, some original crosses between japonica susceptible varieties and indica resistance varieties carrying Bph3 and bph4 were conducted. And also, IR24 was crossed with Babawee carrying bph4 to observe the difference between indica-japonica cross and indica-indica cross.

In this chapter, the results of genetic analysis on F_2 populations, and F_3 segregating lines derived from two crosses, Tsukushibare/Babawee and IR24/Babawee, are described.

2. Genetic analysis on F_2 populations and F_3 segregation lines derived from crosses involving Bph3 and bph4 carriers

1) Materials and Methods

Plant materials

Varieties carrying Bph3 and bph4 including Sri Lankan local varieties Rathu Heenati, Muthumanikam and Horana Mawee as Bph3 carriers, and Babawee, Kalkurwee and Vellai Illankali as bph4 carriers (Sidhu and Khush 1979) were tested. Three japonica varieties, Tsukushibare, Nipponbare and Hinohikari, and an indica breeding line, IR24, were prepared as BPH susceptible plant materials. These susceptible varieties were randomly crossed with the resistance varieties as pollen parents. All of F_1 plants were short-day treated, and F_2 seeds harvested were used in genetic analysis. Fertility of each F_1 also was investigated.

Culture of BPH and bulk seedling tests

All procedures were the same as described in Chapter 2. Rathu Heenati and Babawee were used as resistance controls, and Tsukushibare and IR24 as susceptible control.

2) Results

In order to obtain more plant materials for further genetic studies, crosses were made as shown in Table 6-1. F_1 plants possessing Bph3 were produced by three japonica-indica crosses. F_1 plants possessing bph4 were derived from four japonica-indica crosses and one indica-indica cross. All of these F_1 plants height over 120 cm, being much taller than susceptible plants. Among showed plant susceptible varieties used as mother parents, cross compatibility and hybrid fertility of Hinohikari was extremely low, and the number of harvested F_2 seeds was so small that their progenies could not be analyzed.

All F_1 plants were short-day treated at 15 hours dark for harvesting F_2 seeds. Table 6-1 showed the fertility of F_1 plants and the results of genetic analysis of BPH resistance in F_2 populations derived from 6 japonica-indica and one indica-indica crosses. Segregation ratios of BPH resistant and susceptible progenies were significantly different among three populations of Bph3 from the expected 3R: 1S. No susceptible plants were observed in F_2 population derived from Tsukushibare/Horana Mawee. Also, only two susceptible plants were detected in Tsukushibare/Rathu Heenati. The fertility of F_1 plants from which these two F_2 populations were derived was equivalent to that of two control varieties, Rathu Heenati and Babawee. Contrary to this, a F_2 population derived from Nipponbare/Muthumanikam segregated in 2 resistant: 1 susceptible. The fertility of F_1 from this cross was nearly one half of those of the control varieties.

While a F_2 population derived from Nipponbare/Vellai Illankali showed the expected segregation of 1R: 3S. However, others showed significant differences from the expected ratio. Particularly, a. F_2 population derived from Tsukushibare /Kalkurwee segregated 3 resistant: 1 susceptible, despite that the resistance gene in this variety was reported to be a recessive bph4. Two F_2 populations crossed with Babawee segregated nearer 1 resistant: 1 susceptible. F_1 plants derived from crosses with Tsukushibare with

Table 6-1 The fertility of F_1 plants and genetic analysis of F_2 populations derived from several original crosses for BPH resistance

Cross	Resistance gene	Number of F_1 plants	[a]Fertility (%) ([b]harvested seeds)	Number of F_2 individuals			[d]x^2 value (probability)
				Resistant	Susceptible	Total	
Tsukushibare/Rathu Heenati	Bph3	1	73.1 (365)	30	2	32	6.0 (P=0.015)
Tsukushibare/Horana Mawee	Bph3	3	71.5 (1314)	40	0	40	13.3 (p<0.001)
Nipponbare/Muthumanikam	Bph3	2	34.2 (388)	39	20	59	2.49 (p=0.11)
Tsukushibare/Kalukurwee	bph4	1	76.1 (485)	112	37	149	200.0 (p<0.001)
Tsukushibare/Babawee	bph4	8	62.0 (2869)	85	150	235	15.6 (p<0.001)
IR24/Babawee	bph4	9	NA[c]	112	146	258	46.6 (p<0.001)
Nipponbare/Vellai Illankali	bph4	9	38.9 (3067)	71	191	262	0.62 (p=0.45)
Hinohikari/Vellai Illankali	bph4	1	0.42 (3)	NA[c]			
Rathu Heenati	Bph3	3	74.6 (1704)				
Babawee	bph4	3	70.9 (1719)				
Tsukushibare	S	5	91.2 (4104)				

[a] Fertility is based on the proportion (%) of ripe seeds among total of caryopses in the plant.

[b] Harvested seeds were the total number of seeds from all F_1 plants on same cross combination.

[c] NA indicates not analyzed.

[d] χ^2 values were caluculated with expected ratio of 3 resistant : 1 susceptible in the case of Bph3, and 1 resistant: 3 susceptible in the case of bph4.

bph4 carriers expressed a higher fertility than cross with Nipponbare.

3. Genetic and RFLP analysis of bph4 in Babawee

1) Materials and Methods

Plant materials

Segregating populations for bph4 were derived from two crosses of Tsukushibare/Babawee and IR24/Babawee (see 6-2-1). All F_2 individuals derived from IR24/Babawee were placed under short-day condition of 15 hours dark at two to four tillers stage for four weeks. F_2 individuals derived from Tsukushibare/Babawee were same short-day treatment for five weeks.

Genetic analysis of BPH resistance by bulk seedling tests and the investigation of fertility

All procedures were the same as described in Chapter 2.

Thirty-two F_3 lines derived from a cross of IR24/Babawee and 80 F_3 lines derived from a cross of Tsukushibare/Babawee were surveyed for genetic analysis. Furthermore, 106 F_3 lines of IR24/Babawee were tested (Kawaguchi et al. 1997) . In the bulked seedling tests of two types of F_3 lines, PL3 (carrier of Bph1) and Babawee were used as resistant control, and Tsukushibare and IR24 as susceptible control. As the fertility of each F_2 individual of Tsukushibare/Babawee, the proportion (%) of ripe seeds among total of caryopses every plant was investigated.

DNA preparation and Southern hybridization

All procedures were the same as described in Chapter 2.

DNAs from 64 F_2 plants of IR24/Babawee and from 72 F_2 plants of Tsukushibare /Babawee, whose BPH resistance genotypes were determined, were prepared for the mapping analysis. DNAs from three parents were isolated in bulks of about 5 plants.

Bulked segregant analysis and linkage analysis with RFLP markers

Bulked segregant analysis was conducted using F_3 lines of IR24/Babawee. Bulked DNAs were prepared by pooling DNA samples from 8 F_2 individuals of IR24/Babawee with the same genotype identified in the resistance tests. In the first screening, the DNA samples of each parent and the two bulked genotypes (resistant homozygous and susceptible homozygous) were used for hybridization to 97 RFLP markers randomly chosen from 12 chromosomes of the rice genome, and the hybridized patterns were compared.

A linkage analysis was conducted with F_3 lines of Tsukushibare/Babawee, whose genotypes were determined, using RFLP markers detected as co-segregation with BPH resistance gene in Babawee. Also, 106 genotypes of F_3 lines of IR24/Babawee determined by Kawaguchi were surveyed.

Recombination values between the resistance gene and RFLP markers were calculated by MAPMAKER Version 2.0 (Lander et al. 1987) and converted into genetic map distance (Kosambi 1944).

2) Results

Genetic analysis

The results of genetic analysis using two F_3 lines derived from IR24 /Babawee and Tsukushibare/Babawee are shown in Figure 6-1 (and Table Appendix-3) and Figure 6-2 (Table Appendix-4), respectively. Both of the two histograms did not show clear borders of regions of resistant homozygous (RR) and heterozygous (RS) progenies and susceptible homozygous progeny (SS). Especially, in F_3 lines of Tsukushibare/Babawee, the distribution was continuous (Fig. 6-2). Another set of F_3 lines of IR24/Babawee was tested by Kawaguchi (Kawaguchi et al. 1997). The result shown in Figure 6-3 demonstrated that the distribution is also continuous. To explain the possible involvement of segregation distortion, the fertility of F_3 lines of Tsukushibare/Babawee was investigated (Fig. 6-4 and Table Appendix-4).

Bulked segregant analysis and linkage analysis

The bulked segregant analysis survey was conducted using 97 RFLP markers

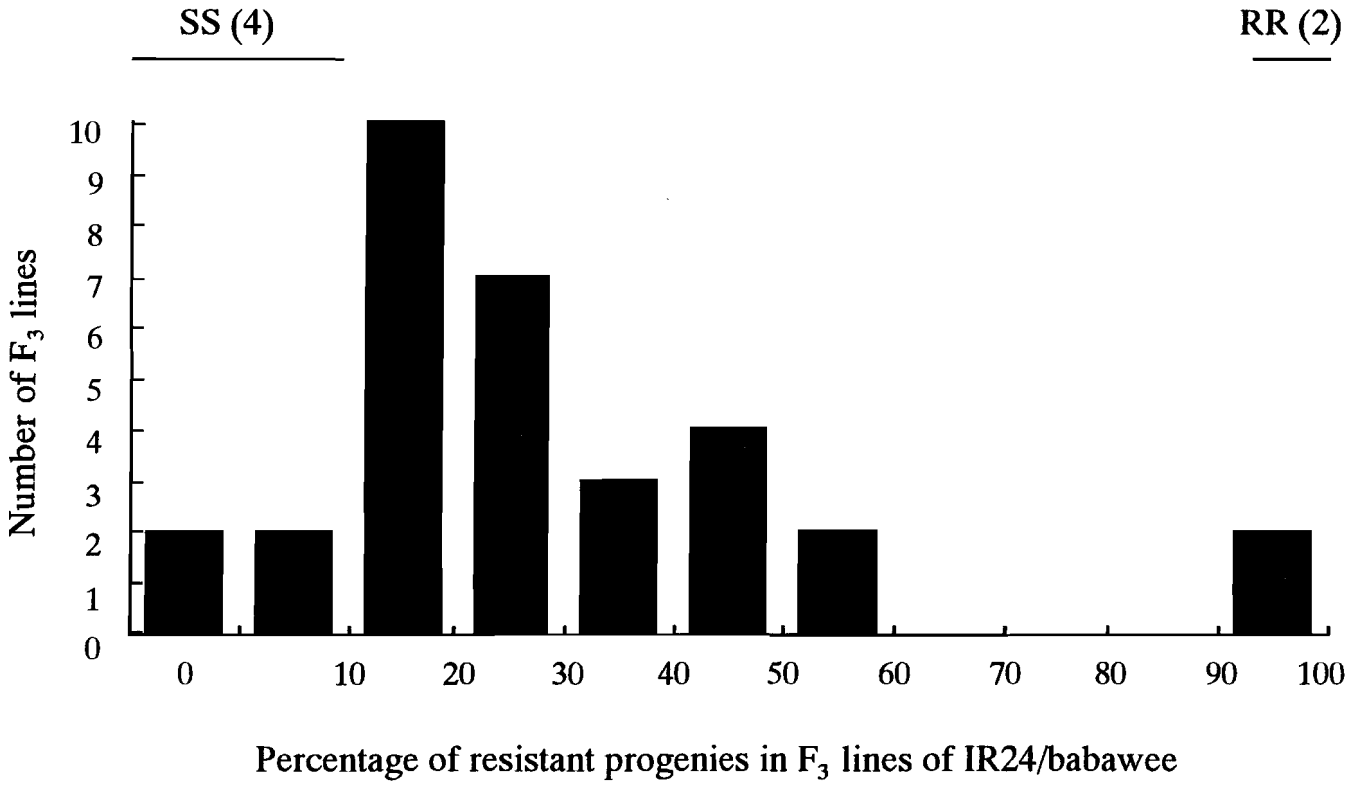

Fig. 6-1 The frequency distribution of resistant progenies in 32 F_3 lines derived from a cross of IR24/Babawee.

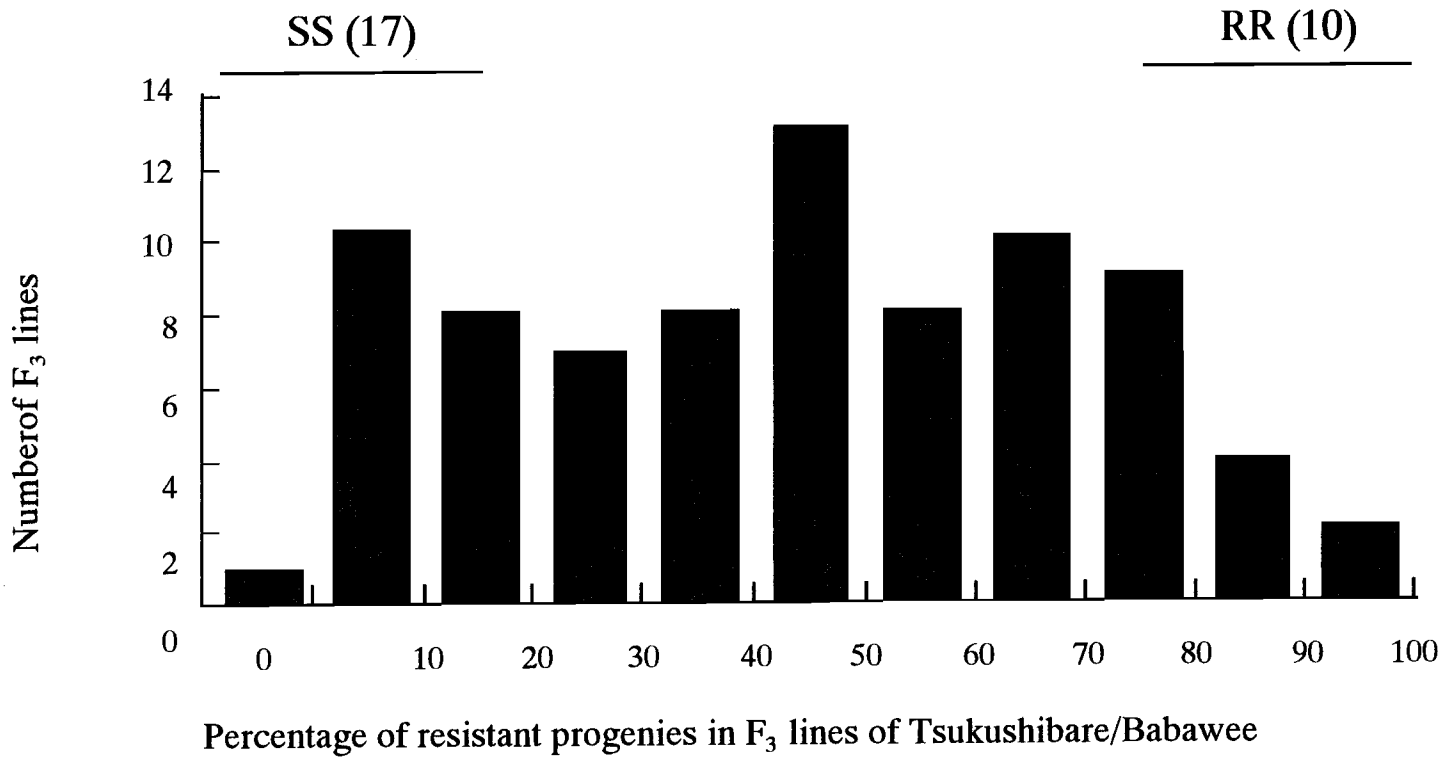

Fig. 6-2 The frequency distribution of resistant progenies in 80 F_3 lines derived from a cross of Tsukushibare/Babawee.

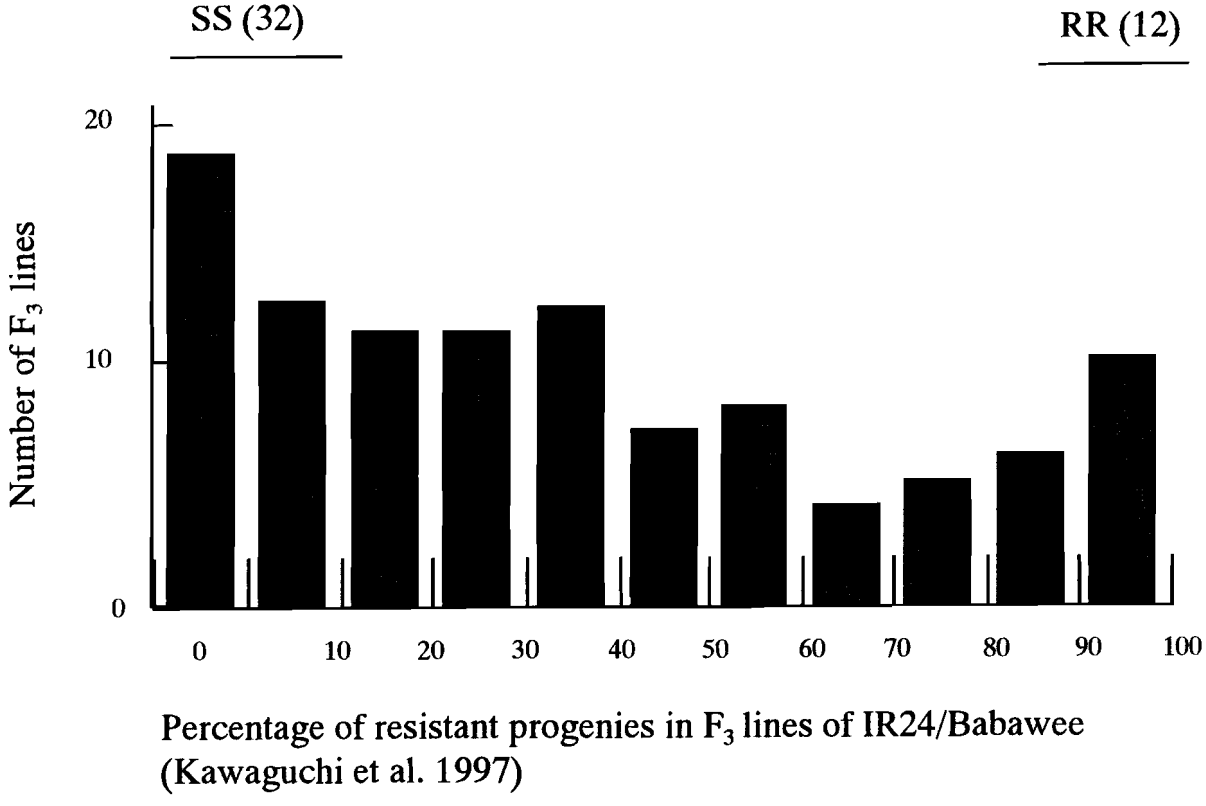

Fig. 6-3 The frequency distribution of resistant progenies in 106 F_3 lines derived from a cross of IR24/Babawee (Kawaguchi et al. 1997).

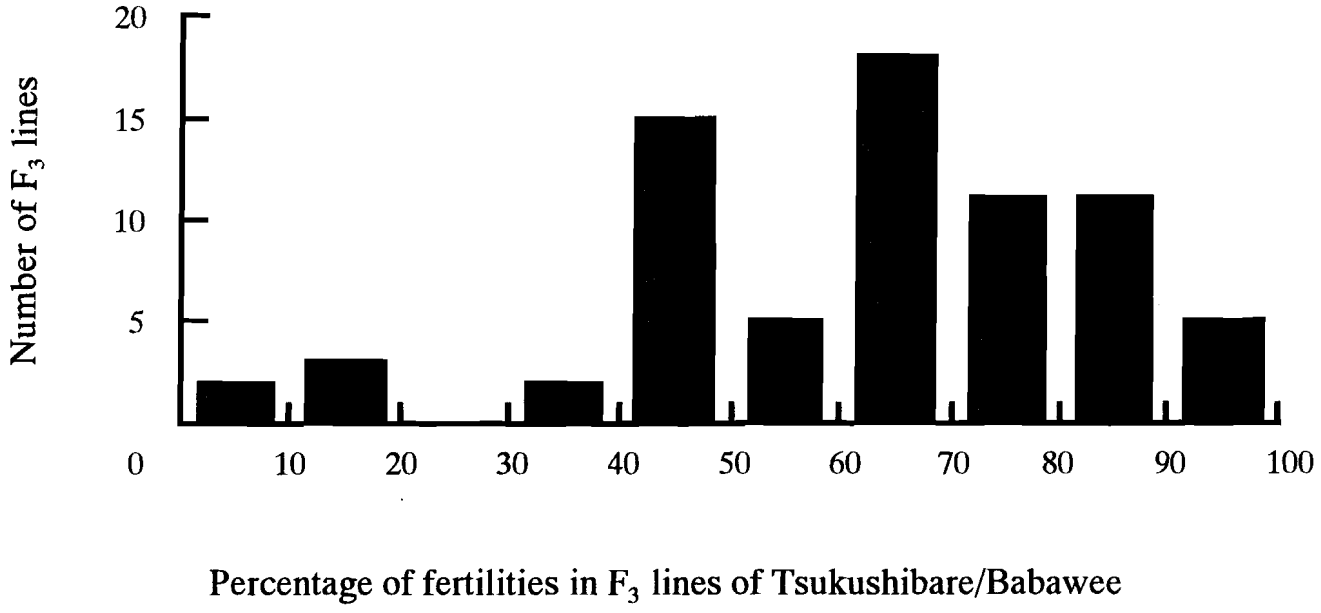

Fig. 6-4 The frequency distribution of fertility in 72 F_3 lines derived from a cross of Tsukushibare/Babawee.

randomly chosen from 12 chromosomes and DNAs from IR24, Babawee, and two F_3 bulks representing resistant and susceptible homozygotes derived from crosses of IR24/Babawee. A comparison of RFLP patterns among them showed that only one marker on chromosome 4, C891, was polymorphic between susceptible and resistant materials (Fig. 6-5), although 39 RFLP markers among of 97 were monomorphic.

Since BPH resistance gene bph4 in Babawee was strongly suggested to be on chromosome 4, mapping of the gene was conducted using two F_3 lines with some RFLP markers on chromosome 4. In the survey using F_3 lines of Tsukushibare /Babawee, the linkage of four markers, C1100, R1783, C335 and C513 to bph4 was suggested, the closest RFLP marker C513 being linked to bph4 with a map distance of 36.2 cM (Fig. 6-6A). However, in the survey using F_3 lines of IR24/Babawee, in which bulked segregant analysis were conducted, the map distance between bph4 and C891 was so far 40.4 cM (Fig. 6-6B).

4. Discussion

Two BPH resistance genes, Bph3 and bph4, were considered to be allelic or closely linked (Lakshminarayana and Khush 1977, Sidhu and Khush 1978, 1979, Ikeda and Kaneda 1981). The mappings of these genes were attempted using two japonica introgression lines, it became impossible to carry out the original plan for some troubles in these introgression lines. PL7 supposed to carry bph4 in Babawee possessed Bph1 from Mudgo, and PL10 was found not to express stable BPH resistance. Because of these some indica varieties with Bph3 and bph4 were crossed with some japonica susceptible varieties.

In japonica-indica crosses, F_1 plants from crosses with Tsukushibare showed a considerately high self fertility (Table 6-1). The fertility of those F_1 plants was similar to those of indica controls, Rathu Heenati and Babawee. However, the fertility of F_2 individuals used for linkage analysis differed quite significantly among them. The fertility of F_1 plants from crosses with Nipponbare was lower than those of all parents. Furthermore, F_1 plants from a cross with Hinohikari was mostly sterile, and even cross hybridization with Hinohikari was so difficult. In a breeding process of PL3 (Bph1), it

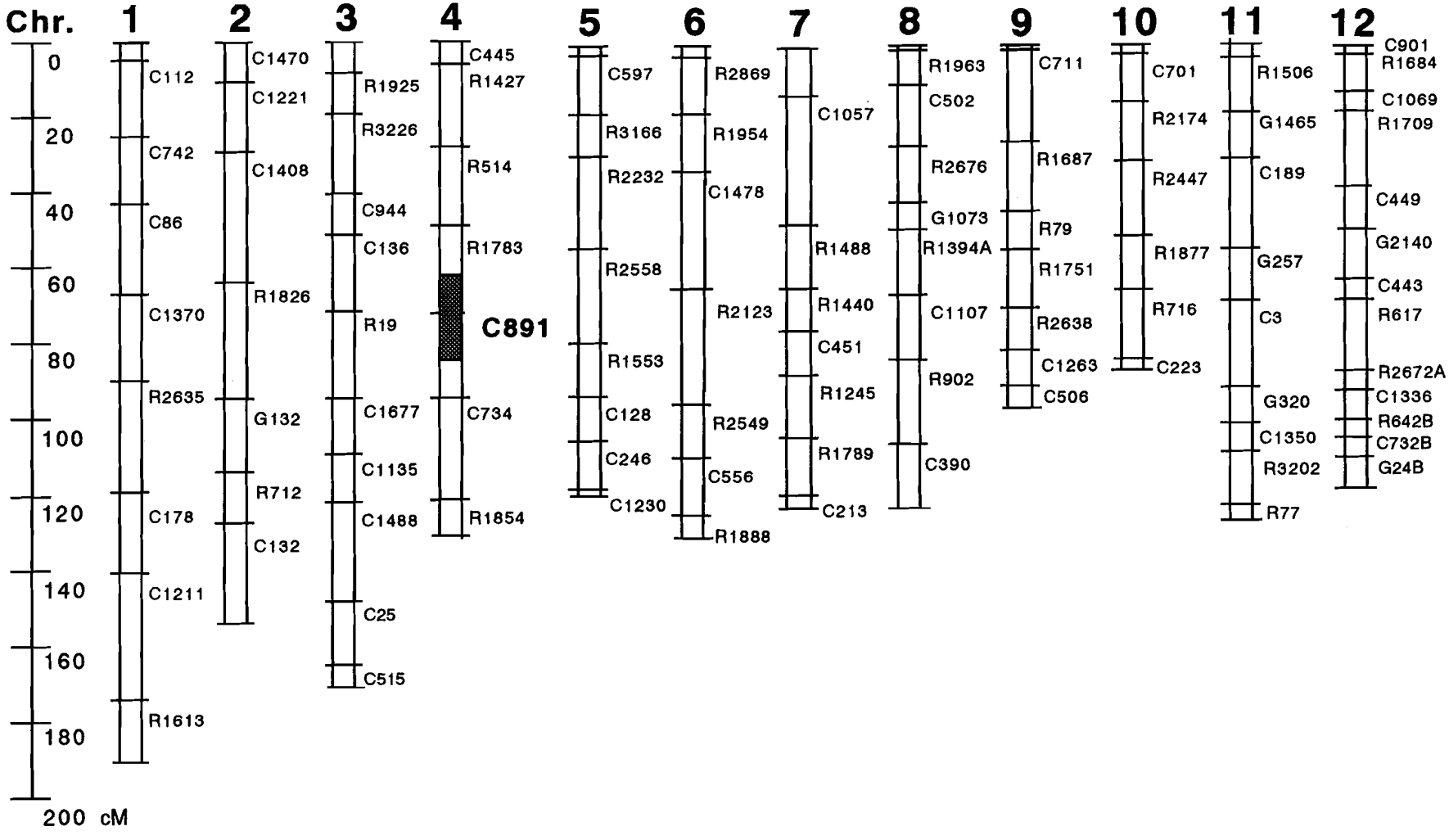

Fig. 6-5 Bulked segregant analysis for bph4. A hatched box on chromosomes 4 indicates the region commonly polymorphic between IR24 and Babawee possessing bph4 and between F_3 bulks from susceptible and resistant homozygotes. Proximal ends of the boxed regions are arbitrarily placed at the middle of the two flanking markers.

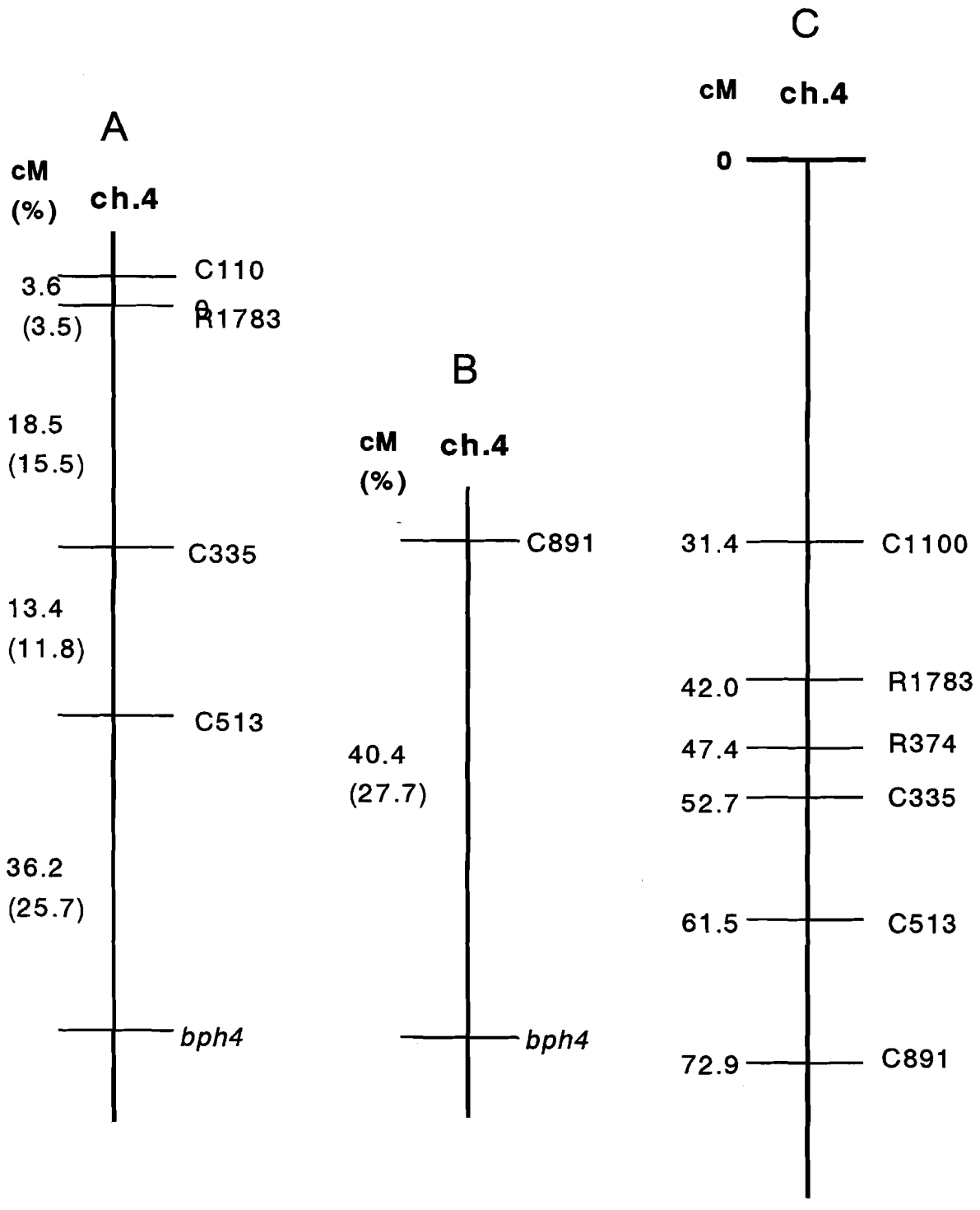

Fig. 6-6 Map positions of the resistance gene, bph4, in Babawee.
The map of chromosome 4 is from A: Tsukushibare/Babawee, B: IR24/Babawee (Kawaguchi et al. 1997) and C: Nipponbare/Kasalath map.
Two RFLP markers R374 and C891 are monomorphic between Tsukushibare and Babawee. Only one RFLP marker C891 is polymorphic between IR24 and Babawee.
cM and % indicate the genetic distance and the recombination value, respectively.

was reported that unfavorable traits including low fertility were excluded from the progenies of a cross with Tsukushibare after a few generations (Kaneda 1984). An advantage of Tsukushibare as parent material was thus supported in the present study. The fertility of F_1 plants of IR24/Babawee was lower than both parents, despite of indica-indica cross (date not shown).

The factor affecting the fertility seemed to affect the segregation of BPH resistance, as non-transmission of specific chromosomal region. As Babawee possesses a recessive BPH resistance gene bph4, F_2 population from the cross with susceptible plant was expected to segregate 1 resistant and 3 susceptible. But the frequency of BPH resistance in both F_3 lines of crosses with Tsukushibare and IR24 did not fit to this ratio showed continuously distribution (Fig. 6-1 and Fig. 6-2). F_3 lines of crosses with IR24 judged by Kawaguchi (Kawaguchi et al. 1997) also showed continuous distribution of segregation (Fig. 6-3). So that, all of three sets of F_3 lines from crosses with Babawee distorted from expected segregation ratios.

Since, some F_3 lines could be determined as resistance or susceptible homozygous, bulked segregant analysis was conducted using these lines. The results demonstrated that bph4 was on chromosome 4 because only one RFLP marker C891 on chromosome 4 co-segregated with BPH resistance (Fig. 6-5).

According to this information of bph4 , i. e. it locates on chromosome 4, the linkage analyses were conducted using two F_3 lines, determined. In the case of using F_3 lines from crosses of IR24/Babawee, the map distance between bph4 and C891 was so far 40.4 cM, recombination value was 27.7 % (Fig. 6-6B). While in the case of using F_3 lines from crosses of Tsukushibare/Babawee, the bph4 was mapped at 36.2 cM from the closest RFLP marker C513. The distance between bph4 and C891 and C513 were much longer than, the distance between C513 and C891 (11.4 cM) (Fig. 6-6C).

Segregating distortion caused by non-transmission of specific chromosomal region and/or expression of lethal factors should be considered. However, to carry out more precise mapping analysis, breeding lines such as recombinant inbred lines (RIL) or near isogenic lines (NIL) should be made and utilized.

The progenies from the five types of F_1 plants possessing Bph3 or bph4 will be

subjected more detailed genetic and linkage analyses. No susceptible plants were detected on the F_2 population of Tsukushibare/Horana Mawee, and the F_2 population of Tsukushibare/Kalukurwee showed segregation that fitted to a dominant gene (Table 6-1). Their analysis will appeared the linkage relationship of Bph3 and bph4 and/or the degree for confidence of gene identification by some biotypes reactions.

Chapter 7 General discussion

So far, 10 major resistance genes and some virulent biotypes have been identified in BPH-rice interaction (Table 1-1). The first attempts of chromosome assignment were conducted by trisomic analysis and test crosses with morphological marker traits. By these methods, bph2 in PL4 and bph4 in PL7 were assigned to chromosome 4 and 10, respectively (Ikeda and Kaneda 1983, Ikeda 1985). However, these linkage assignments were corrected by the later linkage analyses using molecular (RFLP) markers (Hirabayashi and Ogawa 1995, Murata et al. 1997a). Although trisomic analysis is one of the effective means of chromosome assignment especially in diploid species (Iwata and Omura 1975, 1976), there is a danger of mis-identification due to variable transmission rates of the extra chromosomes through male and female gametes and possible distortion of chromosome transmission under various environments. Although caution has to be paid for segregation distortion in any types of linkage analysis, molecular linkage analysis is more effective to determine linkage relationship.

In this study, molecular mappings of BPH resistance genes were attempted using several japonica introgression lines. However, these BPH resistant introgression lines gave some serious problems. The first problem was the apparent mis-introgression of Bph1 for bph4 in PL7. The mis-introgression must have occurred at some stage of the breeding program, and resulted in the complete elimination of bph4 from PL7. Although it was strongly suggested that PL7 carries Bph1, it is nearly impossible to determine the nature of mistake. And more importantly, its recognition and correction required much time and efforts. The result should emphasize the necessity of molecular markers in monitoring introgression of agronomically important genes.

The second problem concerns with the genetic nature of the resistance gene, i. e., dominance or recessiveness. The segregation analysis of a recessive resistance gene bph2 in PL4, showed that it behaves as a major dominant gene (Tables 3-1, Appendix-2 and Fig. 3-1). bph2 was originally found in a line 'IR1154-243' that was derived from a cross involving two susceptible cultivars, 'IR8' and 'Zenith' (Martinez and Khush, 1974).

perhaps in Zenith, was postulated. If such a dominant and independent inhibitor gene was present in Zenith and other susceptible parents, crosses involving these and IR1154-243 should have resulted in a segregation of 13S: 3R in F_2. Martinez and Khush (1974) reported a segregation of 3S: 1R in all combinations of crosses involving 'IR1154-243' and susceptible indica varieties. Our result on bph2 in PL4, however, did not show the presence of such inhibitor at least in the japonica parent, Tsukushibare. It should be interesting to examine if some or all indica rice varieties possess dominant inhibitor gene(s) against bph2. Another possible explanation for this discrepancy may be that a single resistance gene behaves differently with BPH biotypes having different virulence activity. Genetically defined biotypes are undoubtedly the most critical requirement in the reliable bioassays for BPH resistance. Segregation distortion or preferential transmission and/or elimination of the resistance gene or its carrier chromosome can also explain the discrepancy. In a case of bph4 possessed by three indica varieties (Babawee, Vellai Illankali and Kalukurwee), segregation of resistance in crosses involving these and japonica and indica susceptible cultivars deviated significantly from the expected 1R: 3S ratio depending on the cross combinations (Table 6-1). In some cases, the deviation occurred in a direction towards higher numbers of resistant individuals. Molecular markers and bigger segregating population should provide a dependable means to detect possible segregation distortion independently from the bioassays for BPH resistance.

The third problem is the degree of BPH resistance. Bulk seedling tests showed that BPH resistance in PL10 (Bph3) was so weak that genotypes of F_2 and F_3 progenies of Tsukushibare/PL10 could not be clearly determined. However, Rathu Heenati, a Bph3 donor of PL10, expressed strong BPH resistance (Table 5-2). The breeding process involved five backcrosses of Tsukushibare/Rathu Heenati with Tsukushibare for exclusion of some unfavorable traits. It was considered that minor genes to promote or support expression of the major gene (Bph3) in Rathu Heenati were excluded together with unfavorable traits during this process. Kaneda (1984) observed, in the breeding of PL4, that after the second backcross antibiosis of the introgressed bph2 became weaker. To test the polygenc nature of BPH resistance in Rathu Heenati, an original cross was made again between Tsukushibare/Rathu Heenati. BPH bioassay showed that in F_2

population of this cross strong resistance and susceptibility clearly segregated (Table 6-1). Therefore, QTL analysis will be needed to determine the presence of minor genes for expression of BPH resistance. Some segregating populations carrying Bph3 prepared in this study should provide a useful experimental materials.

Hessian fly resistance in wheat is one of the most famous models attesting to the gene-for-gene relationship between biotypes of pest insects and resistance genes of host plant (Hatchett and Gallum 1970). To date, 23 dominant, 2 incomplete dominant and 1 recessive Hessian fly resistance genes were identified in wheat together with 14 different biotypes (Carlson et al. 1978, Hatchett and Gill 1983, Gill et al. 1986, Maas et al. 1987, Obanni et al. 1988, Friebe et al. 1990, Sharma et al. 1992, Raupp et al. 1993, Patterson et al. 1994, Cox and Hatchett 1994). The chromosome assignment of Hessian fly resistance genes was mostly accomplished by monosomic analysis (Gallun and Patterson 1977, Gill et al. 1986). The expression of some Hessian fly resistance genes was reported to be affected by high temprature. The temprature response also enables to classify Hessian fly resistance genes (Sosa and Foster 1976, Maas et al. 1987). For example, a wheat cultivar Abe carrying H5 was resistant to some Hessian fly biotypes at a temprature less than 20°C but lost its resistance completely at 24°C, while the genes, H9 and H10, were 100% effective even at 24°C (Maas et al. 1987). The gene H18 in Marquillo expressed resistance to Hessian fly biotype D at 16±2°C, but the gene was ineffective at 20°C (Maas et al. 1987, Obanni et al. 1988). In the case of BPH resistance of rice, bph2 was reported to be affected by low light condition (Kaneda 1987). The gene-for-gene relationship between BPH virulence genes and rice plant resistance genes might well be affected by environmental factors such as temperature and light.

A fundamental question is if the gene-for-gene relationship can be applicable to rice-BPH interaction. It is for sure thet rice genome possesses major BPH resistance genes and some minor genes as well e. g. in case of IR64 (Cohen et al. 1997). On the other hand, it remains to be answered if BPH virulence in controlled by major genes or by polygenes. It is conceivable that infestation ability and virulence of BPH against rice plants with major resistance genes are controlled by polygenes. The work by Cheng et al. (1979) indicating gene-for-gene could not be repeated by others. The results of Sogawa

(1981) was inconclusive; he found a possible major gene for one biotype but polygenic inheritance for another. den Hollander and Pathak (1981) clearly demonstrated the polygenic basis of BPH virulence. In our laboratory a BPH colony was selected which shows virulence against bph8 carriers, I. e. Thai Col. 11 and Thai Col. 5 (Ketipearachchi 1998). The effectiveness of the selection of BPH virulence against a given major resistance gene might suggest that the number of selectable genes involved in determining the specificity of BPH virulence is rather small. Apparently, more research is necessary to find an answer to this important problem.

Although the present study encountered with some serious problems mentions above, the loci of four BPH resistance genes, Bph1, bph2, Bph9 and bph4, were determined on rice linkage maps. Hirabayashi and Ogawa (1995) already mapped Bph1 at a position 10.7 cM from one RFLP marker XNpb248 on the Kasalath/FL134 (SS) map constructed by Saito et al. (1991). We detected a closer marker G148 that locates at 1.7 cM from Bph1 (Fig. 2-4). The map distance covering these markers in Tsukushibare/PL7, however, was highly condensed as compared with that on Nipponbare/Kasalath map, indicating the presence of 'Mudgo'-derived chromosomal region(s) acting as a large block of crossing-over suppresser. A recombination between Bph1 and G148 led to the selection of a line homozygous for Bph1, having a shorter chromosome segment introgressed from 'Mudgo' (Fig. 2-4).

bph2 was, for the first time, mapped on chromosome 12 at a position 3.5 cM from the closest RFLP marker G2140 (Fig. 3-5). bph2 was previously reported to be either allelic or closely linked to Bph1 (Athwal et al. 1971). The present mapping study showed that these two genes locate at a considerable distance on rice chromosome 12. Surprisingly, however, no F_3 lines segregating susceptible individuals (recombinants) were detected after screening of a large number of F_3 progenies derived from crosses of PL3 x PL4 and of PL7 x PL4 (Table 3-2). A reason for this remains unknown, but our result at least suggests the presence of some structural or functional constraint that prevents crossing-overs in the region covering the two BPH resistance genes.

In this study, Bph9 in Pokkali, was also mapped for the first time on chromosome 12 with two interposing markers, S2545 and G2140 (Fig. 4-4). This map position

appeared to be very close to that of bph2. As Bph9 resistance was easily distinguish from bph2 using BPH biotype 3, these genes have not yet been subjected to the allelism tests. Four BPH resistance genes including Bph10(t) thus have been mapped on the same chromosome 12. Analyzing the progenies derived from crosses among lines with these resistance genes, will provide further knowledge for linkage relationship. Additional pyramided lines possessing more than two resistance genes may also be obtained from such crosses.

bph4 in Babawee was mapped on chromosome 4 using the two segregating populations. But the map distance between bph4 and the closest RFLP marker C513 was 36.2 cM, if estimated in F_2 segregating population of Tsukushibare/Babawee (Fig. 6-6A). In the F_2 population of IR24/Babawee, bph4 was located on chromosome 4 at a map distance of 40.4 cM from one RFLP marker C891 (Fig. 6-6B). These results are consistent with the information that Bph3, a partner of bph4, has recently been mapped on chromosome 4 (Khush personal communication). In the present study, it was noted that the mapping of bph4 might likely be affected by segregating distortion as suggested by low fertility. Some lethal factors or preferential non-transmission of the carrier chromosome or chromosomal region might also be involved. For more precise mapping of bph4 some isogenic lines would be necessary.

Lately, a cloning of rice blight disease resistance gene, Xa1, was accomplished (Yoshimura 1997). Transformation by the isolated Xa1 conferred resistance on the susceptible variety Nipponbare. Complete length of Xa1 is 5,910bp, and the deduced amino acid sequence revealed a nucleic acid binding site and leucine rich repeats. No insect resistance genes, however, have yet been cloned. To achieve map-based cloning of BPH resistance genes, much closer markers should be detected. A marker systems such as AFLP will hopefully be helpful in this attempt.

Acknowledgments

I wish to express my sincere thanks to Former Professor Chukichi Kaneda and Present Prof. Chiharu Nakamura of Plant Genetics Laboratory, Faculty of Agriculture in Kobe University, who have kindly provided me with many advices throughout this study and for reviewing the manuscript of this thesis. I wish to acknowledge Prof. Shigeyuki Mayama, Laboratory of Plant Pathology, Prof. Osamu Kamijima, Laboratory of Plant breeding, Faculty of Agriculture, Kobe University, for their kind review of this manuscript and for valuable comments and criticisms. I am also very thankful to Associate Prof. Naoki Mori and Assistant Prof. Shigeo Takumi, Laboratory of Plant genetics, Faculty of Agriculture, Kobe University, for appropriate advices and kind encouragement for me to complete this study.

I would like to extend my appreciation to Dr. Noboru Horisue and Dr. Kiyoaki Maruyama of National Agriculture Research Center who prepared plant materials; Kyushu Agricultural Experiment Station and Hyogo Prefectural Agricultural Institute for providing BPH cultures; Dr. Hideyuki Hirabayashi of Kyushu Agricultural Experiment Station and Dr. Takamichi Tooyama of Crop Research Institute, Aichi Agricultural Research Center who gave various informations; and Dr. Takuji Sasaki, Dr. Masahiro Yano and their associates, National Institute of Agrobiological Resources, for a kind help in RFLP mapping.

I thank Mr. Manabu Fujiwara and Mr. Mitsuhiro Kawaguchi, Laboratory of Plant Genetics, Faculty of Agriculture, Kobe University, for giving me important information. I also thank Dr. Yasaratna Ketipearachchi, Dr. Nobuaki Asakura, Dr. Kazue Kasai and other all members of Laboratory of Plant Genetics, Faculty of Agriculture, Kobe University, for their continuous encouragement, valuable assistance and kindly friendship.

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Table Appendix-1 Resistance tests of F_3 lines derived from Tsukushibare/PL7

Cross No.	Line No.	[a]Number of plants				[b]Res. rate	[c]F_2 genotype
		Res.	Int.	Sus.	Total		
K5	1	51	0	17	68	0.75	Rr
	2	52	2	28	82	0.65	Rr
	3	0	3	116	119	0	rr
	4	61	8	33	102	0.65	Rr
	5	58	0	27	85	0.68	Rr
	6	87	5	27	119	0.76	Rr
	7	48	5	14	67	0.77	Rr
	8	65	3	0	68	1	RR
	9	40	6	20	66	0.67	Rr
	10	49	1	18	68	0.73	Rr
	11	118	0	1	119	0.99	RR
	12	0	0	102	102	0	rr
	13	82	2	1	85	0.99	RR
	14	25	8	17	50	0.6	Rr
	15	113	2	4	119	0.97	RR
	16	0	1	101	102	0	rr
	17	94	1	4	99	0.96	RR
	18	96	3	3	102	0.97	RR
	19	35	2	31	68	0.53	Rr
	20	99	0	3	102	0.97	RR
	21	44	2	20	66	0.69	Rr
	22	119	4	8	131	0.94	RR
	23	42	6	20	68	0.68	Rr
	24	80	3	2	85	0.98	RR
	25	0	4	81	85	0	rr
	26	58	3	24	85	0.71	Rr
	27	22	9	36	67	0.38	Rr
	28	2	4	62	68	0.03	rr
	29	21	5	21	47	0.5	Rr
	30	33	5	12	50	0.73	Rr
	31	47	9	27	83	0.64	Rr
	32	98	4	0	102	1	RR
	33	1	4	114	119	0.01	rr
	34	52	10	4	66	0.93	RR
	35	39	9	18	66	0.68	Rr
	36	0	4	43	47	0	rr
	37	34	8	26	68	0.57	Rr
	38	0	10	92	102	0	rr
	39	50	0	0	50	1	RR
	40	85	0	0	85	1	RR
	41	63	3	1	67	0.98	RR
	42	0	4	64	68	0	rr
	43	98	4	0	102	1	RR
	44	60	2	19	81	0.76	Rr
	45	52	4	9	65	0.85	Rr
	46	65	2	1	68	0.98	RR
	47	69	3	13	85	0.84	Rr
	48	38	3	10	51	0.79	Rr

Table A-1. (continued)

Cross No.	Line No.	Number of plants				Res. rate	F2 genotype
		Res.	Int.	Sus.	Total		
K6	1	78	4	3	85	0.96	RR
	2	56	10	2	68	0.97	RR
	3	43	5	2	50	0.96	RR
	4	68	4	12	84	0.85	Rr
	5	0	0	17	17	0	rr
	6	0	0	47	47	0	rr
	7	0	0	51	51	0	rr
	8	19	14	18	51	0.51	Rr
	9	0	0	51	51	0	rr
	10	0	0	50	50	0	rr
	11	16	0	18	34	0.47	Rr
	12	49	2	0	51	1	RR
	13	22	1	12	35	0.65	Rr
	14	0	0	84	84	0	rr
	15	11	14	8	33	0.58	Rr
	16	13	11	10	34	0.57	Rr
	17	0	0	68	68	0	rr
	18	76	4	20	100	0.79	Rr
	19	66	0	3	69	0.96	RR
	20	19	2	13	34	0.59	Rr
	21	17	1	17	35	0.5	Rr
	22	20	0	14	34	0.59	Rr
	23	43	3	21	67	0.67	Rr
	24	83	1	0	84	1	RR
	25	32	3	16	51	0.67	Rr
	26	61	1	23	85	0.73	Rr
	27	3	4	77	84	0.04	rr
	28	98	0	2	100	0.98	RR
	29	10	2	39	51	0.2	Rr
	30	36	11	38	85	0.49	Rr
	31	2	0	66	68	0.03	rr
	32	46	3	35	84	0.57	Rr
	33	0	0	51	51	0	rr
	34	0	0	51	51	0	rr
	35	17	9	7	33	0.71	Rr
	36	34	0	0	34	1	RR
	37	18	5	11	34	0.62	Rr
	38	23	5	7	35	0.77	Rr
	39	0	0	51	51	0	rr
	40	14	8	12	34	0.54	Rr
	41	49	6	13	68	0.79	Rr
	42	39	5	37	81	0.51	Rr
	43	16	9	26	51	0.38	Rr
	44	49	7	29	85	0.63	Rr
	45	63	3	0	66	1	RR
	46	46	2	3	51	0.94	RR
	47	0	0	85	85	0	rr
	48	0	0	85	85	0	rr

Table A-1. (continued)

Cross No.	Line No.	Number of plants				Res. rate	F_2 genotype
		Res.	Int.	Sus.	Total		
K7	1	73	6	40	119	0.65	Rr
	2	0	0	101	101	0	rr
	3	76	6	37	119	0.67	Rr
	4	52	4	29	85	0.64	Rr
	5	69	6	43	118	0.62	Rr
	6	83	0	2	85	0.98	RR
	7	63	7	45	115	0.58	Rr
	8	0	0	84	84	0	rr
	9	67	12	39	118	0.63	Rr
	10	59	5	21	85	0.74	Rr
	11	45	8	32	85	0.58	Rr
	12	0	0	66	66	0	rr
	13	0	0	102	102	0	rr
	14	0	0	101	101	0	rr
	15	32	2	16	50	0.67	Rr
	16	52	4	29	85	0.64	Rr
	17	52	0	15	67	0.78	Rr
	18	77	4	21	102	0.79	Rr
	19	18	4	12	34	0.6	Rr
	20	0	0	102	102	0	rr
	21	63	2	3	68	0.95	RR
	22	no seed					
	23	0	0	102	102	0	rr
	24	0	0	85	85	0	rr
	25	0	0	34	34	0	rr
	26	0	0	34	34	0	rr
	27	47	8	13	68	0.78	Rr
	28	62	4	1	67	0.98	RR
	29	45	3	17	65	0.73	Rr
	30	0	1	50	51	0	rr
	31	48	3	0	51	1	RR
	32	0	0	51	51	0	rr
	33	0	0	51	51	0	rr
	34	50	4	14	68	0.78	Rr
	35	1	0	67	68	0.01	rr
	36	47	1	3	51	0.94	RR
	37	28	1	22	51	0.56	Rr
	38	39	1	11	51	0.78	Rr
	39	30	5	13	48	0.7	Rr
	40	24	0	27	51	0.47	Rr
	41	33	3	15	51	0.69	Rr
	42	30	5	15	50	0.67	Rr
	43	28	2	20	50	0.58	Rr
	44	0	1	49	50	0	rr
	45	30	3	18	51	0.63	Rr
	46	16	4	28	48	0.36	Rr
	47	26	3	22	51	0.54	Rr
	48	25	5	16	46	0.61	Rr

Table A-1. (continued)

Cross No.	Line No.	Number of plants				Res. rate	F_2 genotype
		Res.	Int.	Sus.	Total		
K8	1	39	5	25	69	0.61	Rr
	2	26	1	17	44	0.6	Rr
	3	22	0	12	34	0.65	Rr
	4	21	0	12	33	0.64	Rr
	5	0	1	33	34	0	rr
	6	28	0	6	34	0.82	Rr
	7	14	1	2	17	0.88	Rr
	8	19	2	30	51	0.39	Rr
	9	18	1	15	34	0.55	Rr
	10	0	0	34	34	0	rr
	11	20	2	27	49	0.43	Rr
	12	12	6	15	33	0.44	Rr
	13	34	1	33	68	0.51	Rr
	14	5	0	28	33	0.15	Rr
	15	10	0	24	34	0.29	Rr
	16	0	0	34	34	0	rr
	17	2	1	31	34	0.06	rr
	18	22	0	10	32	0.69	Rr
	19	17	1	16	34	0.52	Rr
	20	30	0	4	34	0.88	RR
	21	51	2	15	68	0.77	Rr
	22	24	0	10	34	0.71	Rr
	23	0	0	34	34	0	rr
	24	33	0	1	34	0.97	RR
	25	0	0	34	34	0	rr
	26	32	1	2	35	0.94	RR
	27	0	0	34	34	0	rr
	28	0	0	34	34	0	rr
	29	22	4	6	32	0.79	Rr
	30	29	2	3	34	0.91	RR
	31	22	0	12	34	0.65	Rr
	32	18	1	15	34	0.55	Rr
	33	22	4	8	34	0.73	Rr
	34	24	1	9	34	0.73	Rr
	35	26	2	6	34	0.81	Rr
	36	31	1	2	34	0.94	RR
	37	0	0	34	34	0	rr
	38	32	2	0	34	1	RR
	39	0	0	34	34	0	rr
	40	27	2	5	34	0.84	Rr
	41	0	0	34	34	0	rr
	42	0	0	34	34	0	rr
	43	10	5	2	17	0.83	Rr
	44	16	1	0	17	1	RR
	45	17	0	0	17	1	RR
	46	15	0	2	17	0.88	Rr
	47	17	0	0	17	1	RR
	48	9	1	7	17	0.56	Rr

Table A-1. (continued)

Control varieties	Number of plants				Genotype
	Res.	Int.	Sus.	Total	
PL7	1102	62	108	1272	RR
Tsukushibare	19	28	1450	1497	rr

[a] Res., Int. and Sus. indicate resistant, intermediate and susceptible, respectively.

[b] Resistant rate is calculated by Res. / (Res.+Sus.)

[c] F_2 genotypes were estimated on the assumption that PL7 has a dominant resistance gene (R; dominant, r; recessive).

Table Appendix-2 Resistance tests of F_3 lines derived from Tsukushibare/PL4

Line No.	[a]Number of plants				[b]Res.	[c]F_2
	Res.	Int.	Sus.	Total	rate	genotype
33001	48	1	21	70	0.7	Rr
33002	33	1	23	57	0.59	Rr
33003	41	2	22	65	0.65	Rr
33005	49	2	30	81	0.62	Rr
33006	54	0	26	80	0.68	Rr
33007	47	0	23	70	0.67	Rr
33008	0	0	22	22	0	rr
33009	82	2	39	123	0.68	Rr
33011	88	3	41	132	0.68	Rr
33015	49	2	20	71	0.71	Rr
33016	46	0	15	61	0.75	Rr
33017	36	0	0	36	1	RR
33018	53	1	22	76	0.71	Rr
33019	0	0	80	80	0	rr
33020	0	0	39	39	0	rr
33021	53	0	16	69	0.77	Rr
33022	46	1	25	72	0.65	Rr
33023	97	5	29	131	0.77	Rr
33026	89	2	32	123	0.74	Rr
33027	41	0	29	70	0.59	Rr
33030	153	1	3	157	0.98	RR
33031	44	1	39	84	0.53	Rr
33032	102	1	4	107	0.96	RR
33033	17	0	0	17	1	RR
33035	57	0	22	79	0.72	Rr
33036	0	0	51	51	0	rr
33037	35	2	39	76	0.47	Rr
33038	63	1	16	80	0.8	Rr
33039	61	0	0	61	1	RR
33040	153	2	6	161	0.96	RR
33041	0	0	33	33	0	rr
33042	56	0	1	57	0.98	RR
33043	50	0	20	70	0.71	Rr
33044	0	1	113	114	0	rr
33045	0	0	54	54	0	rr
33046	51	0	25	76	0.67	Rr
33047	0	0	95	95	0	rr
33048	0	0	34	34	0	rr
33049	37	1	23	61	0.62	Rr
33050	0	0	47	47	0	rr
33051	51	1	26	78	0.66	Rr
33053	53	0	11	64	0.83	Rr
33055	84	1	0	85	1	RR
33056	38	2	21	61	0.64	Rr
33057	68	1	1	70	0.99	RR
33058	46	0	15	61	0.75	Rr
33059	86	0	23	109	0.79	Rr
33060	49	0	0	49	1	RR

Table A-2. (continued)

Line No.	[a]Number of plants				[b]Res.	[c]F_2
	Res.	Int.	Sus.	Total	rate	genotype
33061	1	1	93	95	0.01	rr
33062	60	3	12	75	0.83	Rr
33063	0	0	104	104	0	rr
33064	0	0	74	74	0	rr
33065	0	0	35	35	0	rr
33066	50	0	14	64	0.78	Rr
33068	67	2	18	87	0.79	Rr
33069	0	0	98	98	0	rr
33070	3	0	17	20	0.15	rr
33071	60	1	16	77	0.79	Rr
33072	38	0	0	38	1	RR
33073	0	0	69	69	0	rr
33074	59	1	15	75	0.8	Rr
33075	43	0	14	57	0.75	Rr
33076	49	0	16	65	0.75	Rr
33077	40	1	10	51	0.8	Rr
33078	21	0	13	34	0.62	Rr
33079	0	0	16	16	0	rr
33080	55	0	23	78	0.71	Rr
33081	60	0	17	77	0.78	Rr
33082	55	0	23	78	0.71	Rr
33083	51	1	20	72	0.72	Rr
33084	0	1	60	61	0	rr
33086	48	0	27	75	0.64	Rr
33087	67	0	22	89	0.75	Rr
33088	0	0	74	74	0	rr
33089	43	0	1	44	0.98	RR
33090	33	0	0	33	1	RR
33091	0	0	88	88	0	rr
33092	36	1	11	48	0.77	Rr
33093	35	2	12	49	0.74	Rr
33101	66	2	0	68	1	RR
33102	47	0	14	61	0.77	Rr
33103	0	0	67	67	0	rr
33104	49	1	18	68	0.73	Rr
33105	46	1	21	68	0.69	Rr
33106	33	0	14	47	0.7	Rr
33107	0	0	64	64	0	rr
33108	26	0	8	34	0.76	Rr
33109	50	0	18	68	0.74	Rr
33110	34	0	0	34	1	RR
33111	44	0	23	67	0.66	Rr
33112	51	1	16	68	0.76	Rr
33113	0	0	68	68	0	rr
33114	29	0	5	34	0.85	Rr
33115	68	0	0	68	1	RR
33116	25	0	9	34	0.74	Rr
33117	49	0	19	68	0.72	Rr

Table A-2. (continued)

Line No.	[a]Number of plants				[b]Res.	[c]F_2
	Res.	Int.	Sus.	Total	rate	genotype
33118	0	0	60	60	0	rr
33119	45	0	23	68	0.66	Rr
33120	53	0	15	68	0.78	Rr
33121	29	0	5	34	0.85	Rr
33122	47	2	19	68	0.71	Rr
33123	23	0	12	35	0.66	Rr
33124	49	0	19	68	0.72	Rr
33125	46	0	22	68	0.68	Rr
33126	62	0	0	62	1	RR
33127	51	0	17	68	0.75	Rr
33128	0	0	34	34	0	rr
33129	68	0	0	68	1	RR
33131	67	0	1	68	0.99	RR
33132	44	1	23	68	0.66	Rr
33133	0	0	68	68	0	rr
33134	46	1	20	67	0.7	Rr
33137	48	0	20	68	0.71	Rr
33138	49	0	19	68	0.72	Rr
33139	45	0	20	65	0.69	Rr
33140	52	0	16	68	0.76	Rr
33142	53	0	15	68	0.78	Rr
33143	51	0	18	69	0.74	Rr
33144	49	1	17	67	0.74	Rr
33145	0	0	68	68	0	rr
33146	67	0	1	68	0.99	RR
33148	27	0	7	34	0.79	Rr
33150	52	0	16	68	0.76	Rr
33152	47	0	20	67	0.7	Rr
33153	0	0	30	30	0	rr
33154	49	1	15	65	0.77	Rr
33155	23	0	11	34	0.68	Rr
33156	68	0	0	68	1	RR
33157	46	0	22	68	0.68	Rr
33158	68	0	0	68	1	RR
33160	66	1	1	68	0.99	RR
33161	39	0	29	68	0.57	Rr
33162	11	0	15	26	0.42	Rr
33163	67	1	0	68	1	RR
33164	34	1	26	61	0.57	Rr
33166	36	0	22	58	0.62	Rr
33167	34	0	0	34	1	RR
33168	47	0	21	68	0.69	Rr
33169	67	0	1	68	0.99	RR
33170	21	0	13	34	0.62	Rr
33171	28	0	6	34	0.82	Rr
33172	22	0	12	34	0.65	Rr
33173	34	0	0	34	1	RR
33174	0	2	29	31	0	rr

Table A-2. (continued)

Line No.	[a]Number of plants				[b]Res.	[c]F_2
	Res.	Int.	Sus.	Total	rate	genotype
33175	0	0	34	34	0	rr
33176	25	0	9	34	0.74	Rr
33177	0	0	34	34	0	rr
33178	0	0	34	34	0	rr
33179	24	0	10	34	0.71	Rr
33181	22	0	11	33	0.67	Rr
33182	25	0	9	34	0.74	Rr
33183	23	0	11	34	0.68	Rr
33184	0	0	34	34	0	rr
33185	24	1	8	33	0.75	Rr
33186	22	0	8	30	0.73	Rr
33187	24	1	9	34	0.73	Rr
33188	47	0	4	51	0.92	RR
33189	0	1	33	34	0	rr
33190	23	0	11	34	0.68	Rr

[a] Res., Int. and Sus. indicate resistant, intermediate and susceptible, respectively.

[b] Resistant rate is calculated by Res. / (Res.+Sus.)

[c] F_2 genotypes were estimated on the assumption that the resistance gene in PL4 functions as a dominance resistance gene (R; dominant, r; recessive).

Table Appendix-3 Resistance tests of F_3 lines derived from IR24/Babawee

Line No.	[a]Number of plants				[b]Res.	[c]F_2
	Res.	Int.	Sus.	Total	rate	genotype
2902	8	4	59	71	0.12	RS
2906	9	0	68	77	0.12	RS
2910	12	0	63	75	0.16	RS
2913	18	6	55	79	0.25	RS
2918	31	6	33	70	0.48	RS
2923	25	3	50	78	0.33	RS
2927	18	4	54	76	0.25	RS
2928	10	6	61	77	0.14	RS
2936	7	3	65	75	0.1	RS
2944	12	1	59	72	0.17	RS
5710	8	5	59	72	0.12	RS
5731	8	5	56	69	0.13	RS
5741	9	3	66	78	0.12	RS
5803	5	3	61	69	0.08	SS
5806	0	2	74	76	0	SS
5812	20	8	39	67	0.34	RS
5817	8	1	66	75	0.11	RS
5821	16	7	55	78	0.23	RS
5903	27	3	48	78	0.36	RS
5905	43	3	30	76	0.59	RS
5911	0	1	77	78	0	SS
5912	17	3	51	71	0.25	RS
5914	26	4	35	65	0.43	RS
5915	33	7	34	74	0.49	RS
5928	16	8	53	77	0.23	RS
5936	36	6	32	74	0.53	RS
5938	73	0	2	75	0.97	RR
5956	20	8	48	76	0.29	RS
5962	30	8	40	78	0.43	RS
5966	7	11	59	77	0.11	RS
5968	76	2	1	79	0.99	RR
5970	17	2	56	75	0.23	RS

[a] Res., Int. and Sus. indicate resistant, intermediate and susceptible, respectively.

[b] Resistant rate is calculated by Res. / (Res.+Sus.)

[c] F_2 genotypes were showed resistant homozygotes, heterozygotes and susceptible homozygotes as RR, RS and SS, respectively.

Table Appendix-4 Plant height and fertility of F_2 plants, and resistance tests of F_3 lines, derived from Tsukushibare/Babawee

Line No.	Plant height (cm)	Ripe seeds	Empty seeds	Total	[a]Fertility	[b]Number of plants				[c]Res. rate	[d]F_2 genotype
						Res.	Int.	Sus.	Total		
10301	122	90	37	127	0.71	9	5	62	76	0.13	SS
10302	103	109	77	186	0.59						
10303	93	123	21	144	0.85	25	6	35	66	0.42	RS
10304	117	108	103	211	0.51	83	2	13	98	0.86	RR
10305	106	94	32	126	0.75	45	3	20	68	0.69	RS
10307	117	57	68	125	0.46	10	1	28	39	0.26	RS
10308	115	61	109	170	0.36	5	0	3	8	0.63	RS
10309	105	50	31	81	0.62	26	1	12	39	0.68	RS (SS)
10310	91	151	22	173	0.87	2	1	97	100	0.02	SS
10311	91	61	40	101	0.6						
10312	109	75	78	153	0.49	35	3	26	64	0.57	RS
10313	102	91	55	146	0.62	19	0	21	40	0.48	RS
10314	118	45	292	337	0.13				0		
10315	96	124	26	150	0.83	3	0	58	61	0.05	SS
10316	91	51	10	61	0.84						
10317	60	194	110	304	0.64	56	2	9	67	0.86	RR
10318	105	346	115	461	0.75	48	0	13	61	0.79	RS (RR)
10319	119	41	75	116	0.35						
10320	89	40	3	43	0.93						
10321	115	72	117	189	0.38	3	0	33	36	0.08	SS
10322	87	115	23	138	0.83	1	0	49	50	0.02	SS
10323	111	69	33	102	0.68	28	0	20	48	0.58	RS
10324	115	112	56	168	0.67	31	2	25	58	0.55	RS
10325	100	99	50	149	0.66	10	1	27	38	0.27	SS (RS)
10326	123	24	93	117	0.21						
10327	98	176	20	196	0.9	24	2	11	37	0.69	RR (RS)
10329	113	108	63	171	0.63	18	0	21	39	0.46	RS
10330	106	91	6	97	0.94	14	1	25	40	0.36	RS (SS)
10331	102	215	40	255	0.84	24	1	7	32	0.77	RS (RR)
10332	111	110	30	140	0.79	17	1	18	36	0.49	RS
10333	114	45	52	97	0.46						
10334	93	105	10	115	0.91	16	1	22	39	0.42	RS
10335	106	13	85	98	0.13						
10336	121	67	63	130	0.52						
10338	123	19	48	67	0.28						
10339	73	120	16	136	0.88						
10340	60	103	9	112	0.92						
10341	144	21	130	151	0.14						
10342	117	43	30	73	0.59						
10343	124	39	33	72	0.54						
10344	61	85	131	216	0.39						
10345	130	58	74	132	0.44						
10346	69	65	39	104	0.63						
10347	91	75	49	124	0.6						
10348	130	70	97	167	0.42						
10349	129	64	75	139	0.46						
10350	104	82	59	141	0.58						

Table Appendix-4. (continued)

Line	Plant	Ripe	Empty	Total	[a]Fertility	[b]Number of plants				[c]Res.	[d]F_2
No.	height (cm)	seeds	seeds			Res.	Int.	Sus.	Total	rate	genotype
10351	99	15	148	163	0.09						
10352	102	118	78	196	0.6						
10354	112	84	70	154	0.55						
10355	85	163	65	228	0.71						
10356	112	69	22	91	0.76						
10357	72	112	11	123	0.91						
10358	113	124	82	206	0.6						
10359	126	85	79	164	0.52						
10360	117	120	77	197	0.61	3	1	33	37	0.08	SS
10501	122	25	142	167	0.15	2	2	21	25	0.09	SS
10502	128	111	102	213	0.52	5	2	50	57	0.09	SS
10503	79	101	33	134	0.75	1	2	62	65	0.02	SS
10504	130	89	100	189	0.47	15	0	11	26	0.58	RS
10505	115	138	13	151	0.91	36	3	18	57	0.67	RS (RR)
10506	47	152	15	167	0.91	27	3	47	77	0.36	RS
10507	118	131	191	322	0.41	13	5	59	77	0.18	RS
10508	145	106	178	284	0.37	13	5	57	75	0.19	RS
10509	112	124	52	176	0.7	4	1	59	64	0.06	SS
10510	112	78	55	133	0.59	42	1	25	68	0.63	RR (RS)
10511	55	118	53	171	0.69	51	2	20	73	0.72	RR (RS)
10512	46	1	74	75	0.01						
10513	118	229	82	311	0.74	37	2	21	60	0.64	RS (RR)
10514	107	129	75	204	0.63	27	2	33	62	0.45	RS
10516	118	3	86	89	0.03	0	0	3	3	0	
10517	120	84	33	117	0.72	4	3	65	72	0.06	SS
10518	126	2	213	215	0.01	0	0	2	2	0	
10519	105	101	46	147	0.69	45	6	23	74	0.66	RS (RR)
10520	125	9	122	131	0.07						
10521	112	79	46	125	0.63	32	3	35	70	0.48	RS
10522	119	43	64	107	0.4	7	1	15	23	0.32	RS
10523	104	15	96	111	0.14	0	0	15	15	0	SS
10524	110	106	84	190	0.56	30	2	45	77	0.4	RS
10525	85	97	119	216	0.45	50	1	20	71	0.71	RR (RS)
10526	104	117	28	145	0.81	37	2	27	66	0.58	RS
10527	109	47	66	113	0.42	16	2	19	37	0.46	RS
10528	114	79	76	155	0.51	43	3	23	69	0.65	RR (RS)
10529	129	150	85	235	0.64	16	1	60	77	0.21	RS
10530	114	176	48	224	0.79	10	3	59	72	0.14	SS (RS)
10531	60	202	10	212	0.95	37	3	38	78	0.49	RR (RS)
10532	106	62	33	95	0.65	33	1	10	44	0.77	RS (RR)
10533	116	143	71	214	0.67	64	0	8	72	0.89	RR
10534	115	32	58	90	0.36						
10535	88	39	25	64	0.61						
10536	131	99	141	240	0.41	21	2	50	73	0.3	RS
10537	122	116	149	265	0.44	67	6	17	90	0.8	RR (RS)
10538	128	68	69	137	0.5	12	0	32	44	0.27	RS
10539	67	58	19	77	0.75	14	1	28	43	0.33	RS (SS)
10540	107	106	14	120	0.88	43	6	42	91	0.51	RS

Table Appendix-4. (continued)

Line No.	Plant height (cm)	Ripe seeds	Empty seeds	Total	[a]Fertility	[b]Number of plants				[c]Res. rate	[d]F_2 genotype
						Res.	Int.	Sus.	Total		
10541	129	18	59	77	0.23						
10542	126	46	45	91	0.51	7	0	20	27	0.26	RS
10543	106	13	98	111	0.12						
10544	111	81	117	198	0.41	9	3	56	68	0.14	RS
10545	125	58	60	118	0.49	12	1	28	41	0.3	SS (RS)
10546	101	161	95	256	0.63	27	2	23	52	0.54	RS
10547	67	140	74	214	0.65	29	3	63	95	0.32	RS
10548	101	74	99	173	0.43	23	2	38	63	0.38	RS
10549	101	221	48	269	0.82	9	5	77	91	0.1	SS
10550	89	107	54	161	0.66	33	4	22	59	0.6	RS
10551	105	36	51	87	0.41						
10552	130	5	78	83	0.06						
10553	45	74	43	117	0.63	26	0	4	30	0.87	RR
10554	48	21	13	34	0.62						
10555	88	132	137	269	0.49	67	4	18	89	0.79	RR (RS)
10556	115	14	22	36	0.39						
10557	113	60	57	117	0.51	26	4	20	50	0.57	RS
10558	61	151	32	183	0.83	30	1	44	75	0.41	RS
10559	134	43	231	274	0.16						
10560	106	113	318	431	0.26	11	2	27	40	0.29	RS
10561	117	92	136	228	0.4	64	1	4	69	0.94	RR
10562	68	24	349	373	0.06						
10563	95	99	16	115	0.86	2	4	64	70	0.03	SS
10564	107	32	45	77	0.42						
10565	116	34	37	71	0.48						
10566	84	115	23	138	0.83	15	1	61	77	0.2	RS
10567	69	34	43	77	0.44						
10568	100	23	2	25	0.92						
10569	111	220	57	277	0.79	32	0	35	67	0.48	RS
10570	86	66	7	73	0.9						
10571	96	56	27	83	0.67						
10572	116	123	221	344	0.36	56	3	7	66	0.89	RR
10573	73	21	5	26	0.81						
10574	64	37	173	210	0.18	12	2	20	34	0.38	SS (RS)
10575	100	159	75	234	0.68	67	1	26	94	0.72	RS
10576	123	105	16	121	0.87	27	1	10	38	0.73	RS

[a] Fertility is calculated by the number of Ripe seeds / Total

[b] Res., Int. and Sus. indicate resistant, intermediate and susceptible, respectively.

[c] Resistant rate is calculated by the number of Res. / (Res.+Sus.)

[d] F_2 genotypes were showed resistant homozygotes, heterozygotes and susceptible homozygotes as RR, RS and SS, respectively.