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The semidwarf gene, *sd-1*, of rice (*Oryza sativa* L.). II. Molecular mapping and marker-assisted selection

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Abstract To establish the location of the semidwarf gene, *sd*-1, the anthocyanin activator (*A*), purple node (*Pn*), purple auricle (*Pau*), and the isozyme locus, *EstI-2*, in relation to DNA markers on the molecular linkage map of rice, 20 RFLP markers, previously mapped to the central region of chromosome 1 (McCouch et al. 1988), were mapped onto an F_2 population derived from the cross Taichung 65 (*A,Pn,Pau*)/Taichung 65 (*sd*-1). *sd*-1 and *EstI*-2 were determined to be linked most tightly to RFLP markers RG 109 and RG 220, which cosegregated with each other. The distance between these RFLP markers and *sd*-1 was estimated to be 0.8 cM, based on an observed recombination value of 0.8%. The order of genes and markers in this region of chromosome 1 was determined to be *sd*-1 – (*EstI*-2 – RG220 – RG109) – RG381 – *A* – *Pn* – *Pau*. To test the efficacy of selection for *sd*-1 based on these linked markers, 50-day-old F_2 seedlings derived from another cross, Milyang 23/Gihobyeo, were analyzed for marker genotype. At this age, the semidwarf character could not be clearly detected based on phenotype. In addition, plant height was normally distributed in this population, making it difficult to unambiguously identify plants carrying *sd*-1. Thirteen seedlings homozygous for the *sd*-1-associated allele at *EstI*-2, RG220 and RG109, and 13 seedlings homozygous for the *Sd*-1-associated allele at all three marker loci were selected for further genetic analysis. At 20 days after heading, the culm lengths of these 26 plants were measured and the expected phenotype was confirmed in every case. These 26 plants were then selfed for four generations and F_6 lines were again evaluated to determine whether any recombination among the three molecular markers, or between these markers and the *sd*-1 gene, could be detected. No recombinants were identified, confirming the tight linkage of these loci and the usefulness of genotypic selection for this recessive semidwarf character prior to the time when it can be evaluated based on phenotype.

Key words Rice · Semidwarf gene (*sd*-1) · RFLP Molecular marker · Marker-assisted selection

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Introduction

The semidwarf gene (*sd*-1) in rice (*Oryza sativa* L.) is one of the most important single genes in the history of rice improvement. It was first identified in the Chinese variety Dee-geo-woo-gen (DGWG), and was first released in the Taiwanese variety Taichung Native 1 (TN-1) in 1956 (Aquino and Jennings 1966). This single, recessive gene causes reduced culm length and has been widely used to confer lodging resistance, high harvest index, responsiveness to nitrogen fertilizer, and favorable plant type, in the breeding of high-yielding rice varieties (Aquino and Jennings 1966).

The high-yielding rice variety 'Tongil' has played a vital role in achieving self-sufficiency for this staple food in Korea. This variety was developed in the late 1960s at the Rural Development Administration in Korea and at the International Rice Research Institute in the Philippines as a shuttle breeding from a cross involving both indica and japonica germplasm (IR8//Yukara/TN-1), and carries the *sd*-1 gene (Choi et al. 1974). Since the release of Tongil in Korea, a number of varieties with improved grain quality and disease and pest resistance have been released, but they

all carry the same single recessive semidwarf gene, *sd*-1 (Suh and Heu 1978).

At least 60 dwarfing genes, designated *d*-1 to *d*-60, have been identified in rice (Kinoshita 1990). Of these, *d*-47, otherwise known as *sd*-1, is the most useful for rice breeding. Many of the other dwarfing genes have been used as phenotypic markers in genetic studies, but they are not widely used in plant improvement because they are associated with floret sterility, incomplete panicle exsertion, or abnormal plant and grain development (Aquino and Jennings 1966). In the classical linkage map, *sd*-1 is known to be located on chromosome 1 (Suh and Heu 1978; Tsai 1991). It is linked to both a dominant and a recessive shattering habit (Yokoo and Saito 1986; Oba et al. 1990; Eun et al. 1991), as well as to the anthocyanin activator (*A*), purple pericarp (*Pp*), purple node (*Pn*), and purple auricle (*Pau*) (Suh and Heu 1978).

Molecular mapping of plant genomes has proceeded rapidly since Botstein et al. (1980) introduced the idea of constructing linkage maps in humans based on restriction fragment length polymorphism (RFLP) markers. In recent years, the development of new types of DNA markers offers an array of techniques that are useful in developing saturated genetic maps, in monitoring gene introgression in breeding programs, and in cloning genes based on their map position (Rommens et al. 1989; Tanksley et al. 1989; Martin et al. 1993). In rice, molecular mapping of *sd*-1 has been reported by several workers (Eun et al. 1991; Ogi et al. 1993; Yu 1991). However, the map positions reported by Eun et al. and by Yu were not consistent, despite the fact that a similar set of markers were used. The report by Ogi et al. was based on an independently constructed RFLP map of rice.

In this study, we have used 20 mapped clones as probes, based on an existing rice RFLP map (McCouch et al. 1988), and conducted experiments to identify RFLP markers that were tightly linked to the *sd*-1 and *EstI-2* loci, using segregating populations derived from a cross between a pair of NILs, Taichung 65 (*A,Pn,Pau*)/Taichung 65 (*sd*-1). We also evaluated the efficacy of marker-assisted selection in F_2 and F_6 plants derived from the cross Milyang23/Gihobyeo.

Materials and methods

Plant materials

'Taichung 65 (*sd*-1)' was a BC8 selection from the cross Taichung 65 /TN-1 (Tsai 1991). Taichung 65 (*A,Pn,Pau*) contains the linked morphological marker loci anthocyanin activator (*A*), purple node (*Pn*), and purple auricle (*Pau*). Taichung 65 (*sd*-1) was used as the pollen parent in a cross with its tall, isogenic mate 'Taichung 65 (*A,Pn,Pau*)', and the resulting F_2 and F_3 populations were used to analyse the linkage relationship between *sd*-1 and *EstI*-2 (Cho et al. 1994) and to clarify the relationships among molecular markers in this region of chromosome 1, the *sd*-1 gene, and the morphological marker loci, *A, Pn,* and *Pau.* Sixty of the F_2 lines were selected at random and DNA from their corresponding F_3 families was used in restriction fragment length polymorphism (RFLP) analysis.

F_2 and F_6 populations from the cross Milyang 23/Gihobyeo were used to determine whether molecular markers could be effectively used in in-vitro selection for semidwarf F_2 plants earlier than the heading stage. Two-hundred-and-three F_2 individuals were analyzed for molecular markers linked to *sd*-1. Twenty-six F_6 lines were derived from F_2 individuals that were homozygous at all linked marker loci.

DNA extraction, restriction digests, and Southern analysis

DNA was extracted from 5-week-old seedlings (parental lines and 60 bulks of at least 100 F_3 plants) by the procedure of McCouch et al. (1988), using an extraction buffer containing urea-phenol.Genomic DNA from the parental NILs, Taichung 65 (*sd*-1) and Taichung 65 (*A,Pn,Pau*), was digested with the seven restriction enzymes *Bam*HI, *Dra*I, *Eco*RV, *Sca*I, *Xba*I, *Eco*RI, and *Hin*dIII, and used to prepare parental survey filters as described by McCouch et al. (1988) with the exception that the alkali transfer method was used for Southern blotting according to the manufacturer's instructions (GeneScreen Plus, DuPont). Twenty rice genomic clones residing on chromosome 1 (McCouch et al. 1988) were selected for hybridization. Gel electrophoresis and Southern analysis were according to McCouch et al. (1988).

Evaluation of semidwarf plants

The height of 267 F_2 plants and ten plants per F_3 family was measured at 20 days after heading. Sixty of the F_3 families were selected at random for RFLP analysis.

Map construction

Linkage relationships and map distances (in cM; Kosambi 1944) were estimated using the program MAPMAKER II (Lander et al. 1987).

Results and discussion

Linkage relationship among chromosome-1 markers

A linkage map was developed for the RFLP markers analyzed in this study based on segregation analysis of the F_2 population derived from Milyang 23/Gihobyeo (Fig. 1). Previous work using 88 markers distributed throughout the genome in this population demonstrated an overall frequency of polymorphism of 87.5% (Cho 1992). Out of the 20 markers located on chromosome 1 that were surveyed for this cross, 12 (60%) detected polymorphism between the parents. This is slightly lower than that detected in the genome as a whole, but suggests that the region of chromosome 1 analyzed here does reflect the general level of polymorphism previously observed between indica and japonica genomes (McCouch et al. 1988; Wang et al. 1993), and is consistent with data suggesting that this level is higher than that observed between members of the same subspecies (Wang and Tanksley 1989; Wang et al. 1992).

Identification of clones putatively associated with the semidwarf gene (*sd*-1)

Out of 20 RFLP markers analyzed on NIL survey filters, three, RG109, RG220 and RG381, showed polymorphism between the isogenic parental lines Taichung 65

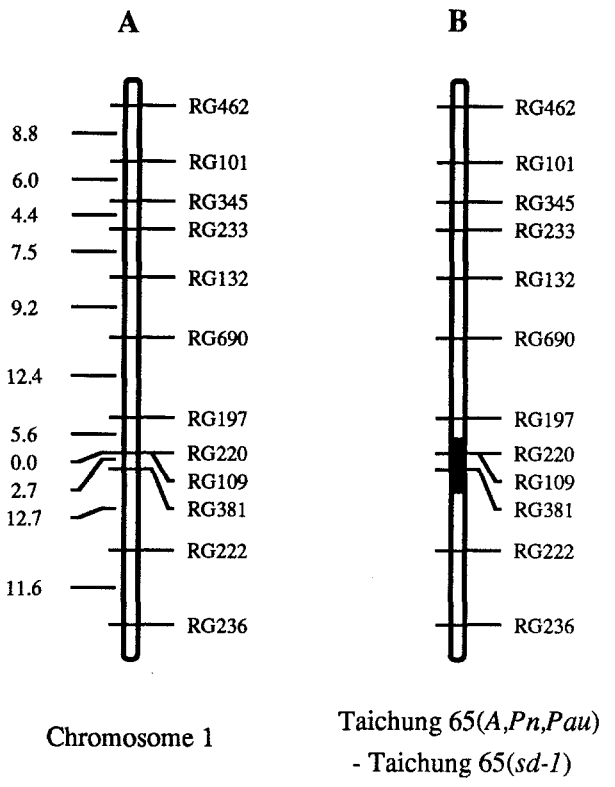

Fig. 1 **A** RFLP map of chromosome 1 of rice, showing the region near the *sd*-1 gene. The recombinational linkage map of chromosome 1 was developed by segregation analysis of RFLPs of RG220, RG109, RG381 and other nearby markers. All distances are given as cM and were derived from the cross Milyang 23/Gihobyeo. **B** The *dark region* represent the chromosomal segments in which recombinational events have occurred; the *plain area* represents segments derived from Taichung 65

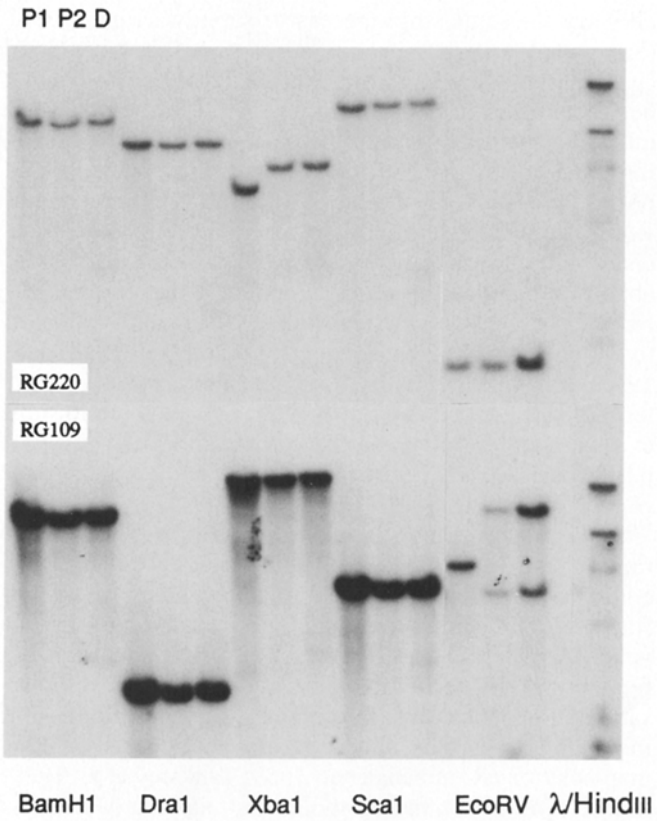

Fig. 2 Southern-blot analysis using clones of RG109 and RG220 with Taichung 65 (*A,Pn,Pau*), Taichung 65 (*sd*-1) and T(N)-1 (donor). The five restriction enzymes used for digestion were *Bam*H1, *Dra*I, *Xba*I, *Sca*I, and *Eco*RV. Results from three probes that gave positive RFLPs are presented here out of 20 rice genomic clones that were tested for the presence of RFLPs between near-isogenic lines (see Table 1). *P1*, Taichung 65 (*A,Pn,Pau*); *P2*, Taichung 65 (*sd*-1); *D*, donor, T(N)-1

Table 1 Restriction fragment length polymorphisms between Taichung 65 (*A,Pn,Pau*) and Taichung 65 (*sd*-1) using 20 clones and seven restriction enzymes

Clone	*Bam*HI	*Dra*I	*Eco*RV	*Sca*I	*Xba*I	*Eco*RI	*Hin*dIII
RG462	–	–	–	–	–	–	–
RG233	–	–	–	–	–	–	–
RG345	–	–	–	–	–	–	–
RG780	–	–	–	–	–	–	–
RG101	–	–	–	–	–	-	–
RG132	–	–	–	–	–	–	–
RG690	–	–	–	–	–	–	–
RG394	–	–	–	–	–	–	–
RG197	–	–	–	–	–	–	–
RG374	–	–	–	–	–	–	–
RG220	–	–	–	–	++	–	–
RG109	–	–	++	–	–	–	–
RG381	–	–	–	–	++	++	++
RG174	–	–	–	–	–	–	–
RG222	–	–	–	–	–	–	–
RG77	–	–	–	–	–	–	–
RG236	–	–	–	–	–	–	–
RG350	–	–	–	–	–	–	–
RG541	–	–	–	–	–	–	–
RZ14	–	–	–	–	–	–	–

(*A,Pn,Pau*) and Taichung 65 (*sd*-1) (Table 1). These markers defined a small, continuous region near the middle of chromosome 1 (Fig. 1). This result suggested that the polymorphic segment was introgressed from the donor of the *sd*-1 gene, TN-1, while the surrounding regions were derived from the recurrent parent, Taichung 65. The three polymorphic RFLP markers were considered putative positive markers associated with the *sd*-1 gene (Fig. 2).

χ^2-test for RFLP markers and agronomic traits

To clarify the representativeness of the sub-population used for RFLP analysis in this study, we used a χ^2-test to evaluate the segregation ratio of RG109, RG220, RG381, *Est1*-2, *sd*-1, *A*, *Pn*, and *Pau* (Table 2). All of these loci segregated in a 1:2:1 ratio, in accordance with the expected pattern for single genes. The lack of skewing among the 60 lines used for RFLP analysis suggested that these lines reflected a random gametic array, and that there was no bias inherent in the selection of this subset from the larger population.

Table 2 Segregation ratios of rice genomic clones *EstI-2*, *sd-1*, *A*, *Pn* and *Pau* of chromosome 1 in F_3 populations (1:2:1). A/A, homozygous for Taichung 65 (*A,Pn,Pau*) alleles; A/B, heterozygous; B/B, homozygous for Taichung 65 (*sd-1*) alleles

Locus	A/A	A/B	B/B	Total	$\chi^2(1:2:1)$	*P*
RG220	15	29	16	60	0.10^{NS}	0.97–0.95
RG109	15	29	16	60	0.10^{NS}	0.97–0.95
RG381	12	31	15	60	0.90^{NS}	0.75–0.50
EstI-2	15	29	16	60	0.10^{NS}	0.97–0.95
sd-1	15	30	15	60	0.10^{NS}	> 0.995
A	16	29	15	60	0.10^{NS}	0.97–0.95
Pn	17	28	15	60	0.40^{NS}	0.90–0.75
Pau	20	26	14	60	2.27^{NS}	0.90–0.25

Molecular mapping of the *sd-1* gene

Based on segregation data for RG109, RG220 and RG381, *EstI-2*, *A* (anthocyanin activator), *Pn* (purple node), *Pau* (purple auricle), and *sd-1* (culm length) evaluated among the 60 F_3 families from the cross Taichung 65 (*A,Pn,Pau*)/Taichung 65 (*sd-1*), linkage between the *sd-1* gene, the *EstI-2* allozyme, and the three RFLP markers, RG109, RG220, and RG381, on rice chromosome 1 was confirmed.

Among the 60 F_3 families evaluated for RFLPs, no recombination was observed between *EstI-2*, RG109, and RG220. One recombinant between these markers and *sd-1* was identified. The recombinant plant was homozygous at RG109, RG220, and *EstI-2*in the F_2 but segregated for the tall and semidwarf habit in the F_3 generation (Fig. 3). Three crossovers were observed between *sd-1* and RG381. The results from this study indicate that the *sd-1* and *EstI-2* genes (previously reported to be closely linked, Cho et al. 1994) are tightly linked to RFLP markers RG109 and RG220 on chromosome 1 in rice.

Suh and Heu (1978) reported that anthocyanin activator (*A*), purple pericarp (*Pp*), purple node (*Pn*) and purple auricle (*Pau*) were linked to the *sd-1* gene with recombination values of 24.8%, 35.1%, 40.9% and 42.9%, respectively, based on linkage analyses of various crosses between Tongil (*sd-1*) and tall varieties carring phenotypic markers, of which plant height was allelic to *sd-1*. In our study based on 267 F_2 individuals from the Taichung 65 (*A,Pn,Pau*)/Taichung 65 (*sd-1*) combination, two of these traits, *A* and *Pn*, show linkage with the *sd-1* gene, with recombination values of 34.4% and 43.0%, respectively. *Pau* is associated with *Pn* (on the far side of *sd-1*), with a recombination value of 15.4%. Figure 4 shows an RFLP map of rice chromosome 1, with the *sd-1* gene linked to *EstI-2*, RG109 and RG220 at a distance of 0.8 centiMorgans (cM). The loci analyzed in this cross combination appear to be arrayed along rice chromosome 1 in the order, *sd-1* – (*EstI-2* – RG220 – RG109) – RG381 – *A* – *Pn* – *Pau*.

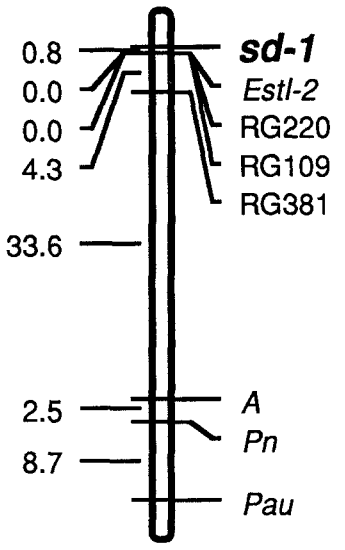

Fig. 4 Linkage map of rice chromosome 1 based on F_2 segregation of Taichung 65 (*A,Pn,Pau*)/Taichung 65 (*sd-1*). Kosambi cM to the left of the chromosome line; marker designations to the right of the chromosome line

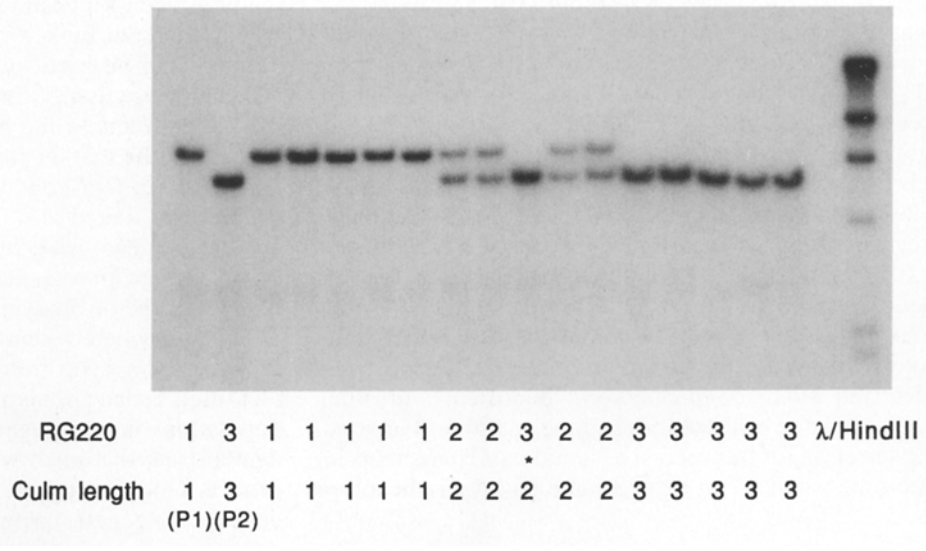

Fig. 3 Cosegregation of the *sd-1* gene and the RFLP marker RG220, in the F_3 families derived from the cross Taichung 65 (*A,Pn,Pau*)/Taichung 65 (*sd-1*). *Xba*I-digested DNAs were blotted onto the filter. Genotypes: *1*, homozygous for Taichung 65 (*sd-1*)(P1) allele; *2*, heterozygous; *3*, homozygous for Taichung 65 (*A,Pn,Pau*)(P2) allele; *, recombinant individual

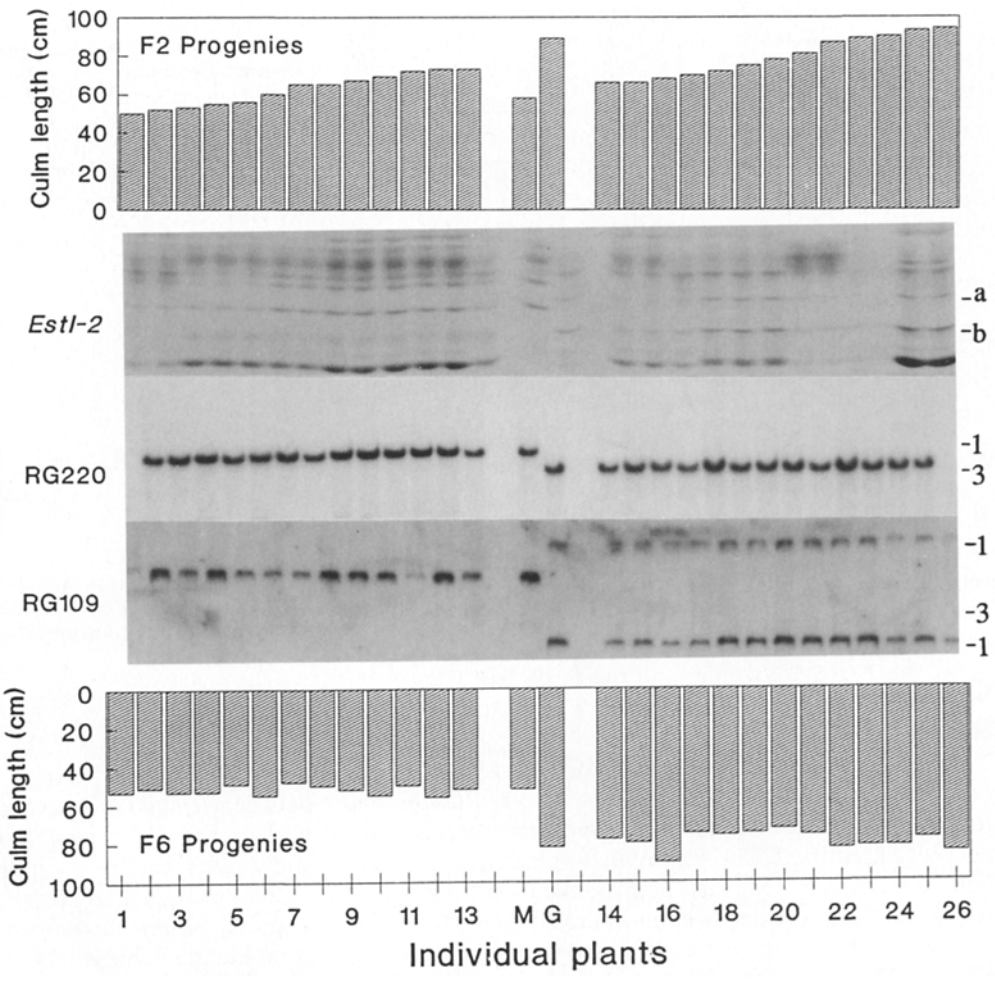

Fig. 5 Effect of in-vitro selection of plants at early stages, using molecular markers *EstI*-2, RG220 and RG109, on culm length at late stages of the F_2 generation and that of progenies in the F_6 generation of Milyang23/Gihobyeo F_6 plants were grown under greenhouse condition during winter time ('91/'92)

F_6 study

To test the efficacy of selection for *sd*-1 based on these linked markers, 203 50-day-old F_2 seedlings derived from the cross Milyang 23/Gihobyeo were analyzed for marker genotype. At this age, the semidwarf character could not be clearly detected based on phenotype. In addition, plant height was normally distributed in this population, making it difficult to unambiguously identify plants carrying *sd*-1. Thirteen seedlings homozygous for the *sd*-l-associated allele at *EstI-2*, RG220 and RG109, and 13 seedlings homozygous for the *Sd*-1-associated allele at all three marker loci were selected for further genetic analysis. At 20 days after heading, the culm lengths of these 26 plants were measured and the expected phenotype was confirmed in every case. These 26 plants were then selfed for four generations and F_6 lines were evaluated to determine whether any recombination among the three molecular markers, or between these markers and the *sd*-1 gene, could be detected. No recombinants were identified, confirming the tight linkage of these loci and the usefulness of genotypic selection for this recessive semidwarf character prior to the time when it can be evaluated based on phenotype (Fig. 5).

Discussion

The utility of molecular markers in plant breeding is based on finding tight linkages between markers and genes of interest. Such linkage permits one to indirectly select for the presence of a desirable gene by assaying for the molecular marker (Tanksley et al. 1989). Isozyme and RFLP markers can be used most effectively as selection tools when they are tightly linked, both genetically and physically. This reduces the probability of a cross-over event separating the marker from the gene. We report here that one isozyme, *EstI-2*, and two RFLP markers, RG109 and RG220, are linked at a distance less than 1 cM with the semidwarf gene, *sd*-1, on chromosome 1. These markers cosegregate with each other and are linked at a distance of 0.8 cM, based on analysis of 60 F_3 families.

In cases where a gene has been introduced into a wide array of germplasm from a single donor variety, markers identified in one population are likely to be useful in other populations that segregate for the gene. In this study, we demonstrate that even when the expression of the target gene is modified by the presence of other, unidentified genes in the genetic background of a variety, tightly-linked

markers can assist in accurately selecting for the target character. The markers, *EstI-2*, RG109 and RG220, were useful in predicting stature at a young age before the effect of the semidwarf gene could be clearly detected phenotypically, and also provided an efficient way of selecting for the presence of *sd*-1 in a population that showed continuous variation for plant height.

In addition, RFLP markers tightly linked to a gene of interest can be used to initiate a chromosome walk aimed at cloning the gene. We are currently using these markers to evaluate recombination in a larger population and to determine the relationship between genetic and physical distance in this region of chromosome 1, prior to attempting to clone *sd*-1.

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