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Identification of AFLP markers linked to a resistance gene against pine needle gall midge in Japanese black pine

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Abstract Bulked segregant and AFLP analyses of two mapping populations (R17 × S6 and R17 × S1) were used to identify markers linked to *Rpgm*, the only known gene responsible for resistance to pine needle gall midge in *Pinus thunbergii* Parl. *Rpgm* was found to be bracketed by *ACCC/CCTTT*$_{190}$ on one side at a distance of 6.6 cM and *ACGT/CCCGC*$_{250}$ at 15.3 cM on the other side. The segregation of these markers was analyzed in two other families in order to determine their phase and transferability. One of the two additional resistant parents carried *ACCC/CCTTT*$_{190}$ in the homozygous state while the marker was in coupling (plus marker allele linked with an *R* allele) in a resistant parent, R17. The marker *ACGT/CCCGC*$_{250}$ was in a repulsion phase in R17 and was not detected in the other two resistant pine trees. Out of four AFLP markers identified, only *ACGT/CCAAT*$_{290}$ was transferable in all resistant trees tested, although its phase was opposite for different trees. These results indicate that in applying those markers to select resistant trees, the phase state of the markers in each resistant tree with respect to *Rpgm* needs to be considered.

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Introduction

Japanese black pine (*Pinus thunbergii*) is one of the most important tree species in maritime forests of Japan and South Korea. The pine needle gall midge (PGM), *Thecodiplosis japonensis* Uchida et Inouye, is a major insect pest of *P. thunbergii*. PGM reduces the annual elongation of this pine tree by forming a gall on the basal part of current-year needles and eventually causes substantial losses of vigor (Sone 1986). The threat of PGM peaked in the 1970s when the insect population increased and was moving to the north along the Japan Sea, resulting in a large number of pine trees dying. Although the severity is decreasing over time, the disease is still a matter of concern, as damaged pine forests are reported occasionally.

Breeding programs to develop Japanese black pines that are resistant to PGM started in 1971 with the selection of resistant trees that grew with no damage in severely infected maritime forests (Tsuchiya 1978). Analysis of F_1 progenies generated by crossing resistant and susceptible trees showed that a dominant gene, *Rpgm*, confers PGM resistance, and that all the resistant trees carry the gene in the heterozygous state (Terada 1992). Field tests take several years as they involve sowing, raising seedlings, and transplantation. In addition, scoring for resistance necessitates the sampling of a large number of needles and careful observation of galls. As the phenotype-based test is technically demanding and time consuming, a rapid and reproducible method capable of identifying PGM resistance is highly desirable. Such a method would also contribute to reducing the time span involved in breeding forest trees that have longer life cycles than other cultivated plant species. PCR-based DNA markers linked to disease-resistance loci have been identified so far in forest trees (Benet et al. 1995; Devey et al. 1995; Lehner et al. 1995; Cervera et al. 1996; Newcombe et al. 1996; Wilcox et al. 1996; Kubisiak et al. 1997). Marker-assisted selection (MAS) has the potential to increase the efficiency of breeding programs for disease resistance.

In a previous work, three RAPD markers linked to *Rpgm* were identified using bulked segregant analysis (Kondo et al. 2000). The markers were, however, all located on the same side of *Rpgm* in the linkage group. For efficient selection, it is necessary to detect additional DNA markers that bracket *Rpgm* or that are tightly linked to it. AFLP techniques (Vos et al. 1995) are frequently employed in mapping because they generate reproducible markers with high index ratios (Travis et al. 1998; Marques et al. 1999; Remington et al. 1999), although radioactive elements or sequencing facilities are needed to resolve the markers. Therefore, a cheaper and safer technique was required that could be readily used by breeders. A very promising method in this respect is the high-efficiency genome scanning system (HEGS) devised in rice (Kawasaki et al. 2000; Murai et al. 2001). This system enabled the detection of markers linked to economic traits using the original method of AFLP, but safely and easily handled the electrophoresis system at reasonable cost.

We report here the identification of AFLP markers linked to *Rpgm* through bulked segregant analysis (Michelmore et al. 1991) using HEGS. The phase state of the markers was also determined in different resistant trees, and the potential usefulness of the markers is described. Since the mapping population used here was the one surveyed to detect RAPD markers linked to *Rpgm* in a previous study, we were able to compare the efficiency of AFLP and RAPD techniques in detecting markers linked to disease-resistant loci in conifer species.

Materials and methods

Plant materials

Thirty-five resistant and 27 susceptible individuals of a cross between a resistant female parent (R17) and a susceptible male parent (S6) were analyzed to identify AFLP markers linked to *Rpgm*. We also used this population in a previous *Rpgm* mapping study based on RAPD markers (Kondo et al. 2000). Since a larger sample size is required for constructing a fine linkage map, needles from a susceptible male parent (S1) and 67 individuals of an R17 × S1 cross were also collected and analyzed. In order to check the marker phase with respect to *Rpgm* in the resistant trees, needles from two resistant, female parents (R11 and R14) and 17–20 individuals from each of the two R11 × S6 and R14 × S6 crosses were also analyzed. The resistance gene was previously determined to be dominant, and was found to be in a heterozygous state (*Rr*) for R11, R14, and R17 and a homozygous recessive state (*rr*) for S1 and S6 (Terada 1992).

DNA extraction, AFLP, and RAPD analyses

DNA was extracted from the needles following procedures detailed in Kondo et al. (2000). HEGS was then used for the development and selection of AFLP markers, as described in the original protocol, except for changes in the number of selective nucleotides in the primers. Primers used in the first amplification cycle were complementary to the adapters for *Eco*RI and *Mse*I, with two additional 3′ selective nucleotides (AC and CC, respectively). Primer combinations containing the same sequences as those used in the first amplification and two (for the *Eco* primer) or three (for the *Mse* primer) 3′selective nucleotides were used in the second amplification cycle. After separating the fragments on 13% non-denaturing polyacrylamide gels, the banding patterns were developed by silver staining (Sil-Best Stain for Protein/PAGE; Nacalai Tesque, Kyoto). Since no data on the segregation of three RAPD markers previously shown to be linked to *Rpgm* ($OPC06_{580}$, $OPD01_{700}$, and $OPAX19_{2100}$) were available in R17 × S1, R11 × S6, and R14 × S6 crosses, RAPD analyses using corresponding primers were performed as described in Kondo et al. (2000).

Bulked segregant analysis

Bulked segregant analysis using the cross R17 × S6 was performed to identify AFLP markers linked to *Rpgm*. AFLP assessments were prepared using R17 and S6 parental DNAs and two bulked samples from 10 diseased and 10 non-diseased individuals (Michelmore et al. 1991). Markers linked to *Rpgm* were expected to distinguish between the two bulks and to show a positive banding pattern for R17 and a negative banding pattern for S6. Primer combinations showing these types of polymorphism were subjected to a second screening to remove false positives. In the second screening, assessments were prepared using eight bulked samples composed of two diseased individuals and eight bulked samples composed of two non-diseased individuals in each case. The AFLP markers that met either of the following criteria were selected for a final step: number of bands present was more than five in resistant bulks, while less than five in susceptible bulks; or less than five in resistant bulks, while more than five in susceptible bulks. In the final assay, the segregation of the selected AFLP markers was analyzed in 129 individuals of the two mapping populations, R17 × S6 and R17 × S1. The AFLP and RAPD markers were developed for the male parent S1 to determine whether the genotype was band present or band absent.

Linkage analysis

Chi-square tests based on Bailey's procedure (1961) were performed to estimate the likelihood of independence between the mode of PGM resistance and occurrence of markers obtained from AFLP and RAPD analyses in the two mapping populations (R17 × S6 and R17 × S1). Segregation data of RAPD and AFLP markers and the *Rpgm* gene were combined with those obtained for the 129 individuals. Recombination values and LOD (logarithm of odds) between loci were calculated, and a genetic linkage map of the region around *Rpgm* in R17 was constructed using the MAPMAKER/EXP 3.0 computer program (Lander et al. 1987; Lincoln et al. 1992). Marker phenotypes were reciprocally coded for each locus so that the program could calculate coupling- and repulsion-phase linkages from the arbitrary linkage-phase data. The "order" command was used to establish the order of markers and "compare" command was used to verify the order. A remaining marker was regarded as an accessory marker at its most likely interval. The Haldane map function was chosen to calculate distances between markers.

Results

Bulked segregant analysis using a cross (R17 × S6) was conducted to identify AFLP markers linked to *Rpgm* in a resistant tree, R17. One thousand twenty-four primer combinations were tested in the first screening. On average, each primer combination amplified 33.6 fragments. Of these, 2.3 (6.8%) on average were polymorphic between parents. For the next screening step, we chose 263 amplified fragments that distinguished between parents, giving positive banding patterns for R17 and

negative for S6 and also between two bulk segregants. In a second screening, the markers derived from 20 primer combinations were selected. The 20 combinations were subjected to a further assay of 129 individuals, representing two mapping populations derived from the same resistant, female parent, R17. Chi-square tests showed that four AFLP markers were linked with resistance loci against PGM (Table 1). The marker *ACCC/CCTTT*$_{190}$ was detected in 68 out of 72 resistant trees, while it was absent in 53 out of 57 susceptible individuals, indicating linkage in a coupling phase with respect to *Rpgm* (i.e., band present = resistance, band absent = non-resistance). In the analysis using the primer combination *Eco* + ACGT and *Mse* + CCCGC, two fragments were linked to *Rpgm*. A 250-bp fragment was absent in 64 out of 72 resistant individuals and present in 48 out of 57 susceptible individuals. Three RAPD markers identified in a previous work (Kondo et al. 2000) were also assayed in the mapping population R17 × S1, for which no segregation data were previously available. All seven markers were found to be band absent in the male parent S1, as well as in S6.

A linkage map of the region around *Rpgm* in R17 was constructed from the pooled segregation data for 129 individuals, representing the two crossed families, R17 × S6 and R17 × S1 (Fig. 1). The resistance gene was located between *ACGT/CCCGC*$_{250}$ and *ACCC/CCTTT*$_{190}$, the latter being nearest to *Rpgm* (6.6 cM). The RAPD marker *OPC06*$_{580}$ could not be located at confirmed map positions under the relative log-likelihood threshold of −2.0 of the maps containing the marker in all intervals in the order. It was placed at the most likely interval: the far side of *ACCC/CCTTT*$_{190}$, as an accessory marker.

The segregation of linked markers was scored in two other crosses to determine their phase with respect to *Rpgm* in resistant trees (Table 1). The AFLP analysis of the cross R11 × S6 using the primer combination *Eco* + ACCC and *Mse* + CCTTT suggested that R11 carries *ACCC/CCTTT*$_{190}$ in the homozygous plus state, since parent S6 showed a negative banding pattern, and all the R11 × S6 progeny showed a positive band. The phase of this marker in R14 could not be deduced because the segregation observed in the R14 × S6 cross did not fit ratios expected for either type of phase. The marker *ACGT/CCAAT*$_{290}$ was deduced as being in a coupling phase in the resistant tree R11, as in R17, since the marker was absent in parent S6 and all the susceptible individuals, but present in all the resistant individuals. In contrast, the AFLP analysis of R14 × S6 individuals indicated that the marker was in a repulsion phase with respect to *Rpgm* in R14, since it was present in 9 out of 10 susceptible individuals and absent in all resistant individuals. The marker *ACGT/CCCGC*$_{500}$ was found to be in a repulsion phase in R14, since nine resistant individuals were negative, and nine susceptible individuals were positive. Two susceptible individuals with negative banding patterns in these two marker analyses appeared to be recombinants. The *ACGT/CCCGC*$_{500}$ marker was not detected in R11. The marker *OPC06*$_{580}$ was found to

Table 1 Segregation of DNA markers linked to *Rpgm*, recombination fraction (*RF*), relative LOD scores, phenotypic, and phase state of the markers in three pine trees

Markers	RF	LOD	R17 × S6				R17 × S1				Total				R11 × S6				R14 × S6				Phase state of resistant pine trees		
			Resistant *n*=35		Susceptible *n*=27		Resistant *n*=37		Susceptible *n*=30		Resistant		Susceptible		Resistant		Susceptible		Resistant		Susceptible				
			+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	R17	R11	R14
ACCC/CCTTT$_{190}$	0.06	25.8	32	3	0	27	36	1	4	26	68	4	4	53	9	0	9	0	(3	5	6	3)[b]	C	H	N
ACGT/CCAAT$_{290}$	0.13	17.3	29	5	2	25	32	3	7	21	61	8	9	46	9	0	0	10	0	9	9	1	C	C	R
ACGT/CCCGC$_{250}$	0.13	17.0	3	32	27	0	5	32	21	9	8	64	48	9	–	–	–	–	–	–	–	–	R	N	N
ACGT/CCCGC$_{500}$	0.22	9.5	6	29	23	4	16	21	26	2	22	50	49	6	–	–	–	–	0	9	9	1	R	N	R
OPC06$_{580}$ [a]	0.08	23.6	32	3	0	27	36	1	4	26	68	4	4	53	10	0	0	10	10	0	1	9	C	C	C
OPD01$_{700}$ [a]	0.09	21.5	4	31	27	0	4	33	26	4	8	64	53	4	–	–	–	–	–	–	–	–	R	N	N
OPAX19$_{2100}$ [a]	0.12	17.8	6	29	25	2	3	34	25	5	9	63	50	7	–	–	–	–	–	–	–	–	R	N	N

[a] Segregation data in R17 × S6 were reported in Kondo et al. (2000)
[b] This fragment with 190 bp might be not the same marker that was detected in other crosses
+ Positive banding pattern, – negative banding pattern, ** significance level at 1%, C coupling, R repulsion, H homozygote, N band absent

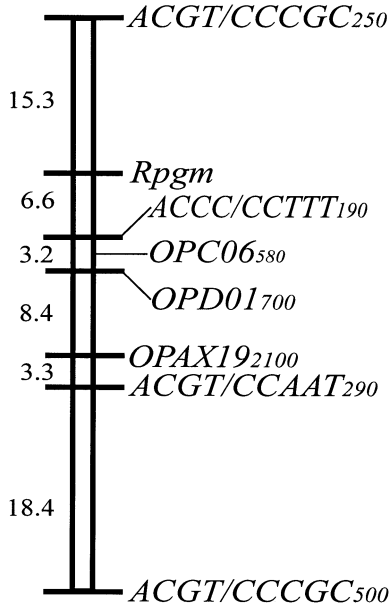

Fig. 1 Linkage map of a region around *Rpgm* using four AFLP markers identified in this study and three RAPD markers previously reported (Kondo et al. 2000). Map distances, in centiMorgans, are shown to the *left* of the *vertical line*

be in a coupling phase to *Rpgm* in all the resistant pine trees studied. The other two RAPD markers and *ACGT/CCCGC*$_{250}$ were not detected in either R11 or R14.

Discussion

HEGS consists of a compact, easily handled electrophoresis system, allowing several hundred individuals to be analyzed and thousands of fragments to be scanned per day. The apparatus required for this marker system is cheaper (costing approximately $1,500) than a sequencer for separation, and uses non-radioactive elements for detecting markers. The data can be scored manually, with no special needs for computer assistance. These features make it convenient for most breeders. The efficiency of the system for detecting polymorphism is also important, since mapping or bulked segregant analysis is required for evaluating economic traits of tree species. In this study, more than 256 lanes, each displaying more than 30 fragments, could be processed a day by combining HEGS with AFLP. With four samples per primer combination, 64 primer combinations were screened in each run. As 2.3 out of 33.6 fragments amplified showed polymorphism between parents on average, it was possible to scan more than 2,000 fragments and to detect as many as 140 polymorphisms a day: three times more than in the RAPD analysis we reported previously. Using the HEGS and AFLP approach, a single person was able to screen more than 1,000 primer combinations and identify the markers linked to *Rpgm* in a few months. Therefore, HEGS/AFLP is a suitable method for generating markers linked to useful traits in conifer species.

We identified here four AFLP markers linked to *Rpgm* in the tree R17 and constructed a linkage map around the gene using these markers together with three RAPD markers previously reported. The phases of the markers were determined using crosses between resistant and susceptible trees (Table 1). These results showed recombination events occurred in the region of *Rpgm* and these markers, and that the alleles in the region are in linkage equilibrium. Thus, it is necessary to consider the phase states of prospective resistant trees for breeding programs, and that more tightly linked DNA markers are required in order to use them among different crosses. For example, *ACCC/CCTTT*$_{190}$ was in the homozygous state in R11. Even though R11 has two copies of this marker allele, it does not carry two copies of the *Rpgm* gene. Then, the homozygosity in R11 makes *ACCC/CCTTT*$_{190}$ useless for MAS of resistant seedlings for progeny of R11, as it is impossible to discriminate between the resistant and the susceptible by use of this marker. The same is true for the markers *ACGT/CCCGC*$_{250}$, *OPD01*$_{700}$, and *OPAX19*$_{2100}$, which are absent in the two resistant trees. Although *ACGT/CCAAT*$_{290}$ was transferable in all resistant trees tested, opposite phases were found for different, resistant trees R11 and R14.

The marker *OPC06*$_{580}$ was consistently linked in a coupling phase to *Rpgm* in three resistant trees studied. A potential practical use for such a phase-consistent DNA marker is in quantifying the amount of resistant seedlings produced commercially, in addition to MAS of resistant trees derived from controlled crosses. Since plus trees planted in seed orchards are mostly susceptible to PGM (Terada 1992), and 20 trees randomly selected among them showed negative-banding patterns with this marker (data not shown), introducing resistant trees into the orchards would favor the production of resistant seedlings. The proportion of resistant seedlings, as determined by DNA marker analysis, can provide an indicator of the quality of seed in terms of resistance.

Segregation of *ACCC/CCTTT*$_{190}$ in R14 × S6 was significantly distorted from expected segregation ratios, assuming the marker is linked to *Rpgm* in either the coupling or repulsion phase. This fragment with 190 bp detected on this cross might be not the same marker that was detected in other crosses, but one that has a close molecular weight. This marker needs to be further evaluated using a much more sensitive technique, such as capillary electrophoresis and fluorescence-based detection.

Terada et al. (1992) reported the resistance locus is complementary at least among the resistant trees R11, R14, and R17. They developed crosses between the resistant trees and investigated degrees of damage in the progenies. The occurrence of diseased trees and non-diseased trees fitted an expected ratio of 3:1. Segregation data in the four progeny shown in Table 1 strongly supported Terada et al.'s results and indicated that all resistant trees studied here have a resistance locus that is located to the same chromosomal area. These findings, however, give no clue that the resistant trees all have the

same gene. While the very variable *R*-gene family is intensively studied, and the structure, function, and evolution of the *R*-gene cluster in crop species have been reported (Michelmore and Meyers 1998; Ellis et al. 2000), we have no information about the possible *R*-gene cluster and specificity for selective strains of PGM. Such knowledge is necessary to understand the mechanism of this disease resistance and must be obtained only with selective insect strains that can differentiate *R* genes.

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