PINE

Genetic Linkage Mapping of Genomic Regions Conferring Tolerance to High Aluminum in Slash Pine

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INTRODUCTION

Reports of reduced growth and vigor of forest trees in Europe and North America have been accumulating in recent years. In eastern

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North America, increased mortality and reduced radial growth rates have been noted for red spruce, frasier fir, and sugar maple. USDA Forest Service inventory data from permanent survey plots has revealed an unexpected reduction of radial growth (-50%) in natural pine forests over the past 30 years (Sheffield and Cost, 1987). Aluminum (Al) ions have been implicated as one of the main factors contributing to this decline on mineral soils at a pH below 5.5 (Johnson and Siccama, 1983). Elevated Al in the soil solution has been suggested as a possible result of acid rain that acts either directly at toxic levels to limit root development, or through selective inhibition of nutrient uptake at sub-toxic levels that results in nutrient imbalances and reduced growth (Taylor, 1991). Ameliorating extremely troublesome areas with lime or nutrient treatments is possible but difficult and expensive. A more promising solution is to breed Al-tolerant trees.

In contrast to annual crop species, little is known about the genetics and physiology of Al tolerance in perennial forest tree species. A number of forest tree species have been classified according to their ability to tolerate Al (Hutchinson et al., 1986; Kelly et al., 1990; Raynal et al., 1990). However, the extent of intraspecific variation in Al tolerance is only now being more fully explored. Geburek and Scholz (1992) noted significant differences in root and shoot growth among six provenances of Norway spruce (*Picea abies* Karst.) subjected to 1.68 mM Al, and suggest that the genetics of the chosen plant material strongly influenced the results of their experiment. Nowak and Friend (1995) report significant differences in various growth parameters among six full-sib loblolly pine (*Pinus taeda* L.) and six full-sib slash pine (*Pinus elliottii* var. *efliottii* Engel.) families subjected to 4.5 mM Al in solution culture. The fact that conifer families often exhibit considerable intraspecific variation in growth responses when grown under Al stress conditions make it a promising candidate trait for genetic dissection using molecular markers.

The objective of this study was to employ DNA-based molecular markers to examine the inheritance of Al tolerance in a single full-sib family of slash pine known to exhibit substantial variation for various growth parameters when grown under high Al conditions (Nowak and Friend, unpublished data).

METHODS

Plant Material

The slash pine family for which results are reported was derived from a cross between the genotypes 18-62(f) x 8-7(m). Both parents are part of a five-tree diallel at the Southern Institute of Forest Genetics (SIFG) in Saucier, Mississippi. These trees were originally selected for growth rate, form, and disease tolerance. Of six full-sib slash pine families originally evaluated for Al tolerance, this family exhibited the greatest variation in percent weight gain when subjected to 4.5 mM Al (Nowak and Friend, unpublished data). Open-pollinated (OP) seeds were also collected from 18-62 and 8-7 to assess the relative Al tolerance of the parents.

Aluminum Tolerance of Parents, and Effect of Aluminum on Family 18-62(f) x 8-7(m)

In order to determine the effect of Al on family 18-62(f) X 8-7(m), 96 progenies were screened, 48 under low (0.01 mM) and 48 under high (4.5 mM) Al. To assess the relative Al tolerance of the parents, a total of 108 and 106 OP progenies from parents 18-62 and 8-7, respectively, were screened under high Al. All seeds were stratified for 18 days at 4°C, planted at a depth of 5 mm in silica sand in leach tubes, and grown for seven weeks. The seedlings were subsequently transferred to hydroponic solutions of Nowak and Friend (1995), minus Al, for 10 days of acclimation. Al tolerance was assessed by replacing these solutions with one containing the addition of 4.5 mM AlC13. All seedlings were weighed prior to pretreatment, prior to Al treatment, and 21 days after Al treatment. The percent weight gain over the treatment period was determined and used as a metric of Al tolerance.

Mapping Population

A total of 235 additional progenies from the family 18-62(f) X 8-7(m) were screened under high Al. The protocol used to assess Al tolerance was similar to that stated above, except that the seedlings were acclimated for 18 days and then exposed to Al for 28 days. In an attempt to augment the detection of genomic regions conditioning Al

tolerance, we initially employed the method of selective genotyping described by Lander and Botstein (1989). A total of 27 progenies were selected from each tail of the percent weight gain distribution.

DNA Extraction and Random Amplified Polymorphic DNA (RAPD) Amplification

DNA was isolated from slash pine needles using a modification of the CTAB-based procedure outlined in Wagner et al. (1987). Oligonucleotide 10-mer primers were obtained from either Operon Technologies (Alameda, Calif., USA) or J.E. Carlson (Univ. of British Columbia, Vancouver, B.C., Canada). DNA amplification followed the protocol outlined in Nelson et al. (1994), modified by doubling the template DNA to 6.25 ng per reaction. Three hundred thirty-six primers were screened against both parents and six full-sib progenies (three from each tail of the percent weight gain distribution). A total of 120 primers were selected for mapping and data were collected for the remaining 48 individuals.

Segregation Analysis

JoinMap (version 2.0; Stam and Van Ooijen, 1995) was utilized to produce a comprehensive genetic linkage map for slash pine. Each polymorphism was tested for goodness of fit to its expected Mendelian segregation ratio using the chi-square (χ^2) test in the JoinMap single locus analysis (JMSLA) module. Two-point linkages were investigated using the JoinMap linkage group assignment (JMGRP) module and a log of the odds ratio (LOD) $\geq$ 4.0. For each of the suggested linkage groups, marker orders were determined using the JoinMap recombination estimation (JMREC) and map construction (JMMAP) modules.

Single-locus analysis of variance (ANOVA) models, in which the individual marker genotypes were used as class variables, were employed to investigate the co-inheritance of each marker locus with percent weight gain (Keim et al., 1990). The model for a straight line, $y = ax + b$ (where y = percent weight gain and x = marker genotype), was employed. An association between a marker and percent weight gain was considered significant if the probability of observing an F-value as large or larger than the observed value was ≤ 0.05. The

proportion of the phenotypic variance explained by segregation of the marker was determined by the r-square (R^2) value. Marker loci found to be significantly associated with percent weight gain using the selective genotyping approach were further characterized on an additional 132 randomly selected progenies. In an attempt to increase the probability of detecting secondary QTL, multiple marker models were constructed. A separate model was constructed for each linkage group allowing for the removal of variation associated with any other QTL that might be present on the same linkage group. The best multi-variable ANOVA model was determined by using both the stepwise and maximum R^2 improvement methods available in the statistical analysis software SAS® (version 6.12). A set of genome-wide models were then constructed that included all the significant markers located on different linkage groups.

RESULTS

Effect of Aluminum on Family 18-62(f) x 8-7(m) and Relative Aluminum Tolerance of Parents

Results for family 18-62(f) x 8-7(m), an OP family from parent 18-62, and OP family from parent 8-7, grown under low and/or high Al, are presented in Table 1. The effect of Al on the percent weight gain of progenies from family 18-62(f) x 8-7(m) was found to be highly significant (Prob. > F c 0.0001). No significant difference in

TABLE 1. Mean, standard deviation, and range of percent weight gain for slash pine family 18-62(f) x 8-7(m), OP family 18-62, and OP family 8-7 subjected to either low and/or high levels of Al for 21 days.

			Percent Weight Gain		
Family	Treatment[a]	NPA[b]	MPWTGN[c]	SD[d]	Range
18-62(f) x 8-7(m)	Low	48	95.6	12.5	67.5 to 120.7
18-62(f) x 8-7(m)	High	48	67.5	13.3	39.4 to 95.6
18-62 OP	High	108	80.7	17.6	40.8 to 128.6
8-7 OP	High	106	85.4	22.0	3.3 to 167.2

[a]High = 4.5 mM Al, Low = 0.01 mM Al
[b]NPA = number of progeny analyzed
[c]MPWTGN = mean percent weight gain
[d]SD = standard deviation of mean percent weight gain

mean percent weight gain was detected between OP families from the two parents (Prob. > F = 0.297). Bartlett's test of homogeneity of variances did, however, suggest that the variances of percent weight gain for the parents were significantly heterogeneous ($\chi^2_{obs} = 5.296 > \chi^2_{(0.05, 1)} = 3.84$).

Molecular Markers and Linkage Map Construction

A total of 159 RAPD markers were scored on 54 progenies from the mapping population. Of the 159 marker loci, 126 were segregating 1: 1 (62 were heterozygous in parent 18-62, and 64 were heterozygous in parent 8-7) and 33 were segregating 3:1 (heterozygous in both parents). Only nine markers (5.6%) were found to deviate significantly from their expected Mendelian inheritance ratio based on χ^2 analyses ($p \leq 0.05$). Of the 159 marker loci analyzed, 129 (81.1%) were linked to 17 groups (three or more loci) and 12 linked pairs at LOD > 4.0.

Molecular Evaluation of Aluminum Tolerance

The distribution of percent weight gain in the mapping population was determined to be normally distributed (n = 235) based on the Wilk-Shapiro test (Prob. < W = 0.7086). Of the 159 RAPD markers, a total of 14 were found to be significantly associated with percent weight gain based on single-marker ANOVAs and the selective genotyping approach. Only 13 of these markers were still significant after an additional 132 randomly selected progenies were included in the analyses (Table 2). Linkage group specific models support the existence of a single region on each of the groups C and E that appear to be conditioning an Al tolerance response. Genome-wide models support the existence of three putative regions; one on group C near marker 351_{2150}, one on group E near marker $Y10_{1125}$, and a third region near the unlinked marker $B08_{0850}$ (Figure 1). A model including all three marker loci as independent variables explained as much as 41.3% of the variation associated with percent weight gain using the selective genotyping approach (n = 50), but only 15.6% after an additional 132 randomly selected individuals were added to the analyses (n = 179). The mean and standard deviation of percent weight gain for individuals harboring different numbers of putative alleles for Al tolerance based on single marker and multiple marker genotypic classes are displayed in Table 3.

TABLE 2. Molecular markers significantly associated with tolerance to high levels of Al in the cross 18-62(f) x 8-7(m) based on single-marker ANOVA models.

Marker	LG[1]	NPA[2]	Prob. > F[3]	R-square[4]	NPA	Prob. > F	R-sauare
$G09_{0925}$	C	50	0.0131	0.119	179	0.0124	0.035
351_{2150}	C	53	0.0164	0.110	181	0.0067	0.040
5311050	C	53	0.0266	0.091	182	0.0089	0.037
6671600	C	53	0.0424	0.077	179	0.0307	0.026
$Y10_{1125}$	E	53	0.0013	0.182	185	0.0006	0.062
'871075	E	53	0.0019	0.171	183	0.0004	0.067
269_{0700}	E	53	0.0019	0.171	184	0.0007	0.053
597_{1025}	E	51	0.0019	0.177	179	0.0029	0.049
$J01_{1050}$	E	50	0.0039	0.158	181	0.0009	0.059
47' 0800	E	51	0.0476	0.076	178	0.0148	0.033
110_{2900}	LP	53	0.0384	0.080	181	0.0687	0.013
$B08_{0850}$	UL	52	0.0035	0.155	182	0.0004	0.067
269_{1850}	UL	53	0.0332	0.084	179	0.0199	0.030
452_{1700}	UL	52	0.0466	0.075	180	0.0272	0.027

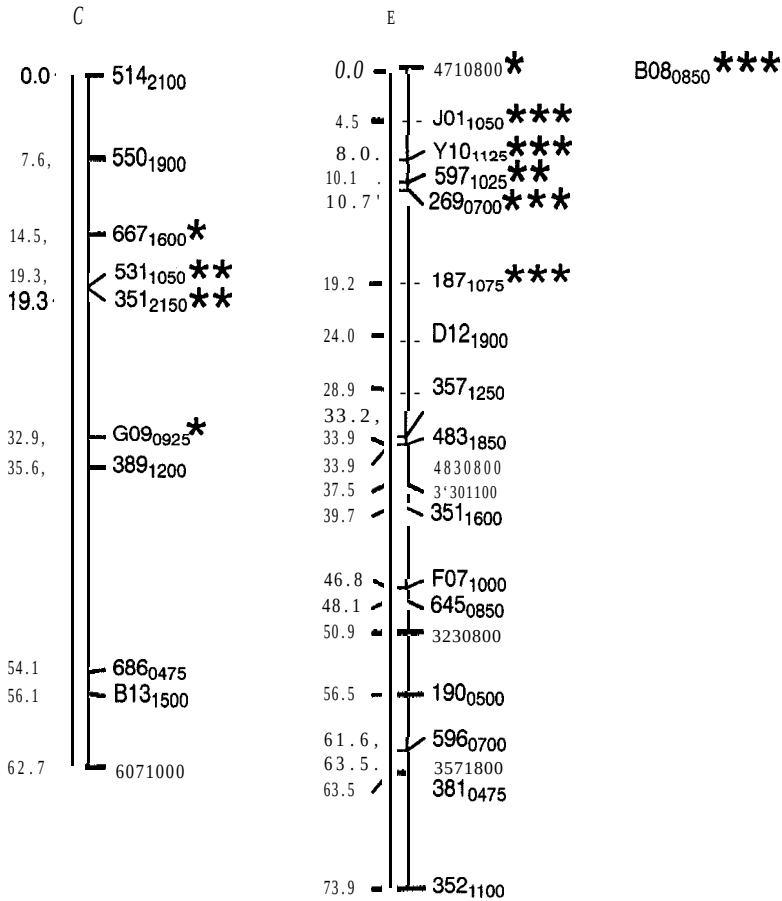
[1]LG = linkage group designation according to Doudrick 1996
[2]NPA = number of progeny analyzed
[3]Prob. > F = probability of a greater F value
[4]R-square = proportion of the phenotypic data explained by the marker locus

DISCUSSION AND CONCLUSIONS

Analyses of the RAPD marker and Al tolerance data suggest that three unlinked genomic regions appear to condition a response to high Al in the slash pine family 18-62(f) *x* 8-7(m). A partial RAPD linkage map for the parent 8-7 has been published affording us the opportunity to use the same linkage group designations reported previously for slash pine 8-7 (Doudrick, 1996). Results of this study are in accordance with studies of Al tolerance in other species, such as wheat, where much of the observed variability in Al tolerance can be explained by the existence of as few as one to three genes (Aniol and Gustafson, 1984; Aniol, 1990).

The fact that only three genomic regions were identified in slash pine, however, does not preclude the possibility that additional genomic regions influencing Al tolerance may exist. Genomic regions influencing Al tolerance may have eluded detection because they were not segregating in family 18-62(f) *x* 8-7(m), were located in genomic regions devoid of molecular markers, or had an effect below the threshold for detection. To address the latter two concerns, we are

Figure 1. Marker orders for slash pine linkage groups C and E. Markers IDs are provided on the right side of each group and genetic distance in Haldane cM are provided on the left. RAPD markers are identified by the manufacturer primer code corresponding to the IO-mer primer responsible for amplification followed by a subscript four digit number indicating the approximate product size in base pairs. Those markers followed by asterisks were associated with Al tolerance; * = P > F < 0.05, ** = P > F < 0.01, and *** = P > F < 0.001.



continuing to add markers to the 18-62(f) *x* 8-7(m) map, and have established all the full-sib progenies used in these analyses in a hedging orchard so that clonal propagules can be screened for Al tolerance. This additional data should afford us better genome coverage and allow us to obtain a more precise estimate (average) of percent weight gain.

TABLE 3. Mean and standard deviation of percent weight gain for individuals from the cross 18-82(f) x 8-7(m) harboring different numbers of putative alleles for Al tolerance based on molecular marker genotypes.

Marker	PNTA[b]e[a]	MNTA[c]	N[d]	MPWTGN[e]	SD[f]
Single markers					
aa	0	0	41	96.0	32.2
A-	1 or 2	1.5	141	113.5	37.1
bb	2	2	101	117.6	37.4
Bb	1	1	85	99.2	33.6
cc	2	2	49	124.9	42.0
C_	0 or 1	0.5	134	103.3	33.2
Three markers combined					
A_, bb, cc	5 or 6	5.5	26	133.9	41.4
A_, Bb, cc	4 or 5	4.5	9	128.8	48.9
aa, bb, cc	4	4	5	118.4	39.9
A_, bb, C_	3 or 5	4	52	116.6	33.6
aa, Bb, cc	3	3	8	103.0	28.8
A_, Bb, C_	2 or 4	3	52	97.8	29.9
aa, bb, C_	2 or 3	2.5	14	90.8	32.9
aa, Bb, C_	1 or 2	1.5	14	89.1	29.4

[a]A = tolerance-associated allele at marker 351_{2150}. b = tolerance-associated allele at marker $Y10_{1125}$. c= tolerance-associated allele at marker $B08_{0850}$
[b]PNTA = potential number of tolerance alleles
[c]MNTA = mean number of tolerance alleles
[d]N = number of individuals with particular marker genotype
[e]MPWTGN = mean percent weight gain of individuals with particular marker genotype
[f]SD = standard deviation of mean percent weight gain

Results of this study suggest that high levels of Al have an inhibitory effect on the growth of slash pine, but that tolerance mechanisms exist and appear to be genetically controlled. Potential gains in Al tolerance using marker-aided selection might be possible, provided that the effects of these genomic regions are indeed heritable. Our future goal is to confirm the inheritance of each of the three genomic regions in advanced generations.

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