SCAR MARKERS IN A LONGLEAF PINE × SLASH PINE F_1 FAMILY

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ABSTRACT

Sequence characterized amplified region (SCAR) markers were derived from random amplified polymorphic DNAs (RAPDs) that segregate in a longleaf pine × slash pine F_1 family. Nine RAPD fragments, five from longleaf pine and four from slash pine, were cloned and end sequenced. A total of 13 SCAR primer pairs, with lengths between 17 and 24 nucleotides, were developed. Nine (for SCAR loci FGP004, FGP005, FGE006, FGE007, FGP008, FGE009, FGP010, FGE011, and FGP012) were designed by extending the RAPD primers; three (for FGE001, FGP002, and FGE003) were based on the internal sequences of corresponding cloned RAPD fragments; and one (for FGP013) was based on the sequence of the original cloned RAPD fragment as well as the sequence of the cloned SCAR fragment amplified from the other parent. All SCAR primer pairs amplified bands of expected sizes. The primer pairs for FGP004, FGE006, and FGE007 amplified polymorphic bands between the parents. The primer pair for FGP013 revealed a polymorphism between the parents, but lost the within-tree polymorphism. The other nine primer pairs amplified monomorphic bands when separated on agarose gels. A polymorphism between the parents was identified for FGP005 by digesting the polymerase chain reaction (PCR) products with the restriction enzyme *SmaI*. FGP005 and FGP012 were found to be polymorphic when the PCR products were separated on a 3% acrylamide sequencing gel. The segregation of four of the six polymorphic SCARs was confirmed in 64 longleaf × slash F_1 individuals.

Keywords: sequence characterized amplified regions (SCARs), *Pinus palustris*, *Pinus elliottii*, polymerase chain reaction (PCR)-based genetic markers

INTRODUCTION

Polymerase chain reaction (PCR)-based genetic markers have become widely used markers for genome mapping, map-based cloning, and analysis of genetic variation. Since the first reports of random amplified polymorphic DNA (RAPD) markers by WILLIAMS *et al.* (1990) and WELSCH and MCCLELLAND (1990), their application has spread rapidly. Like restriction fragment length polymorphisms (RFLPs), RAPDs result from the transfer of nucleotide sequence polymorphisms into DNA fragment band polymorphisms. RAPDs use decamer nucleotides as primers to amplify a locus of template DNA, and nucleotide mismatches at the priming sites such as those caused by insertion, deletion of one or more base pairs, or translocation in the amplified region, may lead to a band polymorphism. RAPD markers are useful because the procedure is simple, fast, and uses trace amounts of template DNA. However, RAPD markers are usually dominant markers, and are sensitive to minor changes in reaction conditions during PCR amplification.

To improve the reliability of RAPDs and to convert them to codominant markers, PARAN and MICHELMORE (1993) developed sequence-characterized amplified regions (SCARs). SCARs are derived from RAPD markers by developing longer primers. After a RAPD fragment is cloned and end sequenced, a pair of primers, approximately 24 bases in length, are synthesized. These SCAR primers are used to amplify the specific regions of genomic DNA. SCAR markers are advantageous over RAPD markers because they usually detect only a single locus, their PCR amplification is less sensitive to reaction conditions, and they are more likely to be codominant markers. While SCAR primers usually amplify the RAPD locus in the source parent of a cross, they often amplify the locus from the other parent too. When these two fragments are monomorphic in length, they may be converted to polymorphic using various methods such as restriction digestion, or high resolution separation methods for PCR products (PARAN & MICHELMORE 1993). SCARs have been used for mapping genes of interest, map-based-cloning, or marker-assisted selection (MAS) on fungi (SCHILLING 1996) and several plants including lettuce (PARAN & MICHELMORE 1993; WITSENBOER *et al.*

1995), common bean (ADAM-BLONDON *et al.* 1994; GU *et al.* 1995), oak (BODENNES *et al.* 1997), and citrus (DENG *et al.* 1997).

The delay of early height growth (EHG), known as the "grass stage", and the susceptibility to brown-spot disease have been two important factors that limit artificial regeneration of longleaf pine (*Pinus palustris* Mill.) (SCHMIDTLING & WHITE 1989). The EHG of longleaf pine has drawn the attention of scientists since the 1950's. A previous strategy to improve the EHG of longleaf pine was to stimulate the plants with hormones (ALLEN 1958). However, regulation of the grass stage by introgression of genes for EHG from either loblolly pine or slash pine in a recurrent backcross breeding program may be a more direct and thorough solution to the problem. As part of backcross breeding programs, BROWN (1964) and DERR (1966, 1969) made crosses between longleaf pine and loblolly pine or slash pine. NELSON (personal communication) made crosses between longleaf pine and slash pine in 1990 and backcrosses to longleaf pine and slash pine in 1995. However, since EHG appears to be controlled by a small number of quantitative trait loci (QTLs) (BROWN 1964; NELSON unpublished data), a more efficient approach may be to map these loci with molecular markers, and then use the markers that are tightly linked in marker-assisted selection. As part of this approach, low to medium density RAPD marker linkage maps have been constructed for longleaf pine and slash pine (KUBISIAK *et al.* 1995; NELSON *et al.* 1993, 1994).

RAPD markers may be used for MAS, however, SCAR markers may be more reliable for large-scale marker-assisted selection. GU *et al.* (1995) have successfully used allele-specific associated primers (ASAPs or SCARs) to conduct large scale selection for the presence of photoperiod genes in common bean at a very low cost. The scoring of SCARs is more reliable and thus will enhance the reliability in indirect selection. The main goal of this research was to convert the RAPD markers putatively linked to QTLs controlling the EHG of longleaf pine × slash pine hybrids. In this paper we describe the development of 13 SCAR markers from RAPD markers and confirm Mendelian inheritance of four polymorphic SCARs in a longleaf pine × slash pine F_1 family. We also report the characteristics of SCARs and the conversion of monomorphic SCARs to polymorphic SCARs.

MATERIALS AND METHODS

Plant and DNA materials

DNA of 64 F_1 individuals from hybrids of 3-356 (longleaf pine) (♀) × H28 (slash pine) (♂) were used. The parents were selected for disease resistance by scientists of the USDA Forest Service, Southern Research Station, Southern Institute of Forest Genetics in Saucier, Mississippi. The cross was made in 1990. Seeds were harvested in 1991 and sown in 1992. Total DNA was extracted from the leaves of the F_1 seedlings in 1993 as described by WAGNER *et al.* (1987) except that spermine and spermidine were omitted from both the extraction and wash buffers.

RAPD analysis

Eight 10-mer primers that identified polymorphic loci in the longleaf pine × slash pine F_1 family (KUBISIAK *et al.* 1995) were purchased from Operon Technologies (Alameda, CA). Five of the eight primers amplified polymorphisms that were found to explain 5% or more (5.0–19.4%) of the total variation of the EHG in the F_1 population. These eight RAPD primers were used for RAPD analysis using the two parents and six F_1 individuals. Five of the RAPD primers amplified five bands of interest from longleaf pine and three of them amplified four bands of interest from slash pine. The numbering of the RAPD makers followed KUBISIAK *et al.* (1995). The RAPD analysis followed the procedure of NELSON *et al.* (1993).

Re-amplification of RAPD bands

Following PCR amplification, the products were separated on agarose gels stained with ethidium bromide and visualized under UV light. The band of interst was excised from the gel and placed into a microcentrifuge tube. Sterilized water (15 µl) was added to the tube. A small nick was made in the excised gel piece with a pipette tip and 5 µl water was pipetted in and out of the nick several times. The DNA was diluted one hundred fold and 2 µl was used as a template to re-amplify the RAPD band. The re-amplification was in 50 µl total volume with 1× *Taq*DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.1mM of each dNTP, 0.2 µM decamer primer, and 1 unit of *Taq*DNA polymerase (Promega). The temperature profile was: 94 °C for 3 min.; 35 cycles of 92 °C for 1 min., 35 °C for 2 min., and 72 °C for 2 min., followed by 72 °C for 10 min. The size of PCR products was verified by agarose gel eletrophoresis (1.5% gel, TAE).

Cloning and sequencing of RAPDs and SCARs

T-tailed vectors were constructed by a modified procedure described by MARCHUK *et al.* (1990). pUC18 plasmid was used instead of pBluescript and instead of incubating the tailing mixture at 70 °C for 2 hours, a

PCR-like temperature profile was used (93 °C for 3 min, 20 cycles of 93 °C for 1 min and 72 °C for 6 min, and 72 °C for 10 min). T-pUC18 and the re-amplified RAPDs were ligated in 2 µl 10x ligation buffer, 1µl 10mM ATP, 2 µl T_4 DNA ligase (4 U/µl) (STRATAGENE), 2 µl T-pUC18 (80 ng), and 2 µl RAPD fragment, totaling to 20µl volume. The ligation was incubated overnight at 15 °C and ligation products were used to transform competent *E. coli* DH5α (STRATAGENE). Probe was made from cloned fragments with the *Genius*™ 2 DNA Labeling Kit (Boehringer-Mannheim) and hybridized to Southern blots of corresponding RAPDs derived from the two parents and six F_1 individuals. The hybridization was detected with the *Genius*™ DIG Nucleic Acid Detection Kit (Boehringer-Mannheim). Each clone was end sequenced to approximately 300 bp with an ABI 373 DNA Sequencer System (Applied Biosystem, Foster City, CA) using PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit.

The cloning and sequencing for SCARs use the same strategy as for RAPDs. The PCR products of SCAR were used directly for ligation without re-amplification.

SCAR primer design and SCAR analysis

For each cloned RAPD, two oligonucleotides were developed to be used as SCAR primer pairs. The SCAR primers were designed using three methods. The first method used the computer program OLIGO Primer Analysis Software, version 5.0 for Windows by NBI (National Biosciences, Inc.; Plymouth, MN) to identify internal sequences suitable for PCR analyzsis. These internal sequences were used as the sequences for SCAR primer pairs. The second method was to extend the RAPD primer from its 3' end along the sequences of the cloned RAPD fragment. Each primer consisted of the original 10 bases of the RAPD primer and the next seven to 14 internal bases from the end. The third method is same as the first method, but it is based on the sequences of both the cloned RAPD fragment and the cloned SCAR amplified from the other parent using the SCAR primer pair derived from the same RAPD fragment. The SCAR primers were synthesized by either Operon Technologies Inc. (Alameda, CA) or LSU GENELAB (School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA).

SCAR amplification conditions followed the same procedures as RAPD analysis except that the annealing temperatures varied according to length and GC content of each primer pair. Each primer of a primer pair was included at a concentration of 0.2 µM. Each primer pair was tested for between parent polymorphism using total DNA from the two parents of the mapping population, longleaf pine 3-356 and slash pine H28, as templates. If the band of the expected size was amplified from the expected parent but not from the other parent, the locus was considered to be polymorphic between the two parents. And those identified to have a between parent polymorphism were further tested for within tree polymorphisms using total DNA from six F_1 individuals as templates. If the band of interest was amplified from one or more of the six F_1 individuals but not from all the six F_1 individuals, the locus was considered to be polymorphic within the parent. In order to lower the probability of mis-classification, any of the primer pairs that appeared monomorphic in the six F_1 individuals was tested further using 10 F_1 progenies.

Sequencing gel

SCAR primer pairs were labeled with ^{33}P as described in the Instruction Manual for AFLP™ Analysis System I, AFLP Starter Primer Kit (GIBCOBRL, LIFE TECHNOLOGIES) and PCR products were resolved in a 3% acrylamide sequencing gel.

RESULTS

Cloning and sequencing of the RAPDs and SCAR

Figure 1 illustrates the nine RAPD fragments that were cloned. These nine RAPDs were amplified using eight RAPD primers, C159, C258, C550, A12, E08, C242, B02, and G09. Primer G09 amplified two of the nine polymorphic RAPD fragments, and each of the other seven RAPD primers amplified one of the nine. The

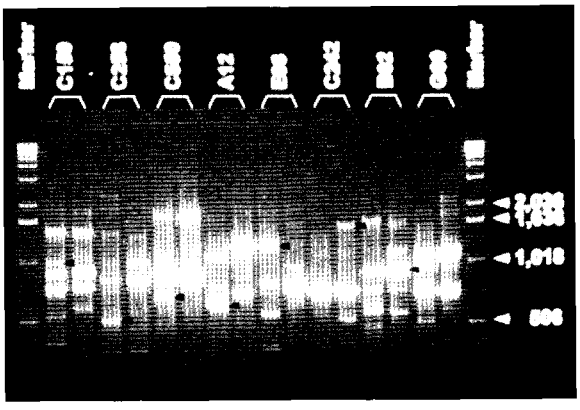

Figure 1. Identification of RAPD markers that were used for the production of SCAR markers. The original RAPD primers used are indicated. For each pair of lanes, the first contains products amplified from tree 3-356 (longleaf pine) and the second contains those from H28 (slash pine). Arrows indicate the fragments that were cloned.

Table 1. Summary of 13 SCARs for longleaf pine (3-356) and slash pine (H28).

SCAR loci	Cloned RAPD fragments	SCAR primer names	SCAR primer sequences	Annealing temperature (°C)	Expected product size (bp)	Design
FGE001	C242-1600	FGE001F	TGTAG ACCCA AGAGA TTGAC	60–62	1300	internal
		FPE001R	TTGTA AATGT GTAGG CAACT C			
FGP002	C550-670	FGP002F	TGAGG ATCTC GTTGG CATAC TTG	60–65	520	internal
		FGP002R	GGAAT TGTGT TTGGG ATGTT GTC			
FGE003	G09-750	FGE003F	GCCAT ATCAT CAAAG CAGTG A	58–62	420	internal
		FGE003R	AACAA CAAGA TCAGG CATAA GC			
FGP004	C550-670	FGP004F	GTCGC CTGAG CAGTA CATTG	67–68	670	extended
		FGP004R	GTCGC CTGAG GATCT CGTTG			
FGP005	E08-1200	FGP005F	TCACC ACGGT CACAC AAGCG	60–65	1200	extended
		FGP005R	TCACC ACGGT ACTAT CCAGG			
FGE006	G09-750	FGE006F	CTGAC GTCAC TTCTT CC	60–62	750	extended
		FGE006R	CTGAC GTCAC ACATC TG			
FGE007	G09-1150	FGE007F	CTGAC GTCAC TATCA TATAA GG	59–61	1150	extended
		FGE007R	CTGAC TGCAC CACCT ATGTA C			
FGP008	A12-600	FGP008F	TCGGC GATAG CGGAC ATC	54–60	600	extended
		FGP008R	TCGGC GATAG GTAGT AGC			
FGE009	C242-1600	FGE009F	CACTC TTTGC GACTC AATTT AAGG	58–62	1600	extended
		FGE009R	CACTC TTTGC ACAAT ATAAT GCCA			
FGP010	C258-500	FGP010F	GTCAC CGTTG GTAGA GGCCA C	60–65	500	extended
		FGP010R	GTCAC CGTTC TCAAT TTGGC TC			
FGE011	B02-900	FGE011F	TGATC CCTGG GGACA TATCG	60–69	900	extended
		FGE011R	TGATC CCTGG CCAAG TAGTC			
FGP012	C159-1000	FGP012F	GAGCC CGTAG ACCCA ATATA GG	60–68	1000	extended
		FGP012R	GAGCC CGTAG AGAGC AGGAA C			
FGP013	A12-600	FGP013F	TTAAA GGAGT TCAGC TAGC	56–58	550	internal
		FGP013R	CTATT TGTTG CATGC TTCG			

*) The range of temperature in which the same banding patterns with the bands of expected sizes were amplified.

RAPD markers C159-1000, C258-500, A12-600, B02-900, and G09-1150 were reported to explain 5.0%, 15.8% 19.4%, 5.0%, and 12.7% of the total variation of EHG in the longleaf pine × slash pine F_1 population (KUBISIAK 1994). Southern analysis indicated that the cloned fragments did correspond to the RAPD markers. The sequences of the two ends of the cloned RAPD fragments did not show any inverted repeats longer than the 10 bases comprising the primer binding sites for each RAPD. The RAPD primer G09 amplified two products, G09-750 (750 bp) and G09-1150 (1150 bp). Clones of these two products did not cross hybridize with each other and their sequences, except for the priming sites, were not similar.

SCAR primers

Initially, the three primer pairs (for FGE001, FGP002, and FGE003), which were internal to the original RAPD priming sites, were derived from the three cloned RAPD fragments C242-1600, C550-670, and G09-750, respectively. These three internal primer pairs amplified monomorphic SCARs FGE001, FGP-002, and FGE003. Then, one primer pair was derived from each of the nine cloned RAPD fragments by extending the RAPD primers. An additional primer pair for FGP013, which was also internal to the original RAPD priming sites, was derived from sequence data of the cloned RAPD fragment A12-600, as well as sequence data of the cloned SCAR amplified from the other parent. Table 1 summarizes the 13 SCAR primer pairs, the corresponding SCAR loci they amplified, and the cloned RAPD fragments from which they were derived.

Banding pattern of SCARs

All 13 SCAR primer pairs amplified bands of the expected sizes from the parents. Only four primer pairs (for FGP004, FGE006, FGE007, and FGP013) revealed polymorphisms between the two parents. The others primer pairs appeared to amplify bands of the

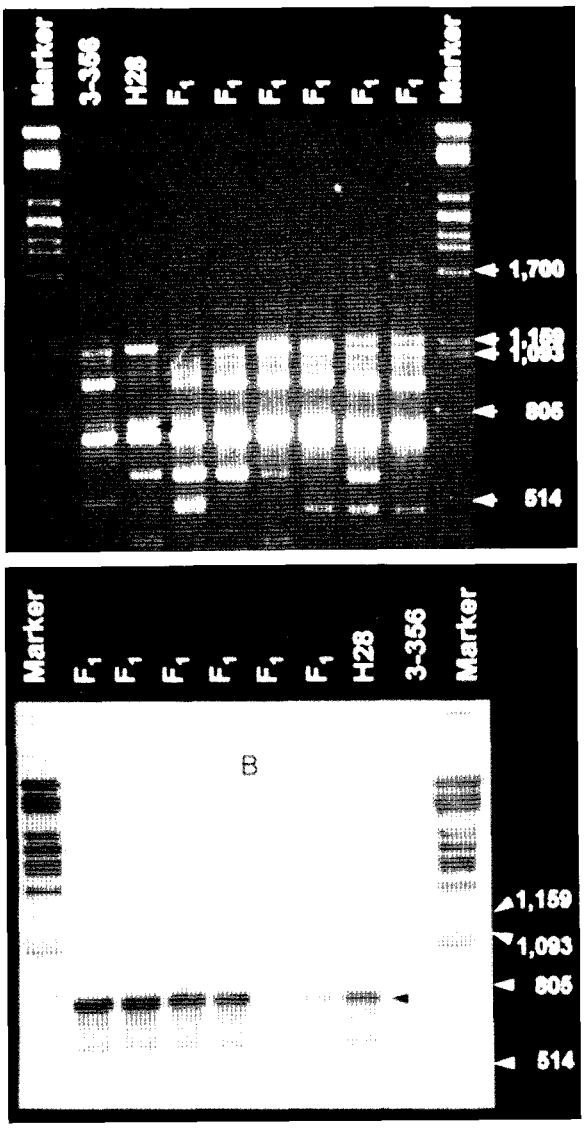

Figure 2. Identification of clone G09-750 using a non-radioactive labeling kit (*Genius*™ Boehringer Mannheim). A. RAPDs were amplified from DNA of the two parents (3-356 and H28) and six F_1 progeny with primer G09. B. Cloned RAPD fragment G09-750 was hybridized to the Southern blot of the agarose gel shown in "A". The position of bands in "B" are in mirror relation to those in "A".

same molecular weight in both parents based on agarose gel electrophoresis. At the upper limits of the annealing temperatures the primer pair for FGP004 amplified one band of expected size from 3-356 and another band of a different size from H28. The primer pair for FGE006 and FGE007 amplified a single band from H28. The primer pair for FGP013 amplified two bands from longleaf pine 3-356 and one band from slash pine H28. Comparing to four to fifteen bands amplified by the corresponding RAPD primers, the banding pattern amplified by the 13 SCAR primer pairs was simpler.

Effect of annealing temperature on SCAR banding pattern

SCARs were less sensitive to annealing temperature change than RAPDs. In our experiment, we amplified a very different banding pattern when the annealing temperature was changed by 1 °C for RAPDs. In contrast, we amplified the same banding pattern for each of the 13 SCAR primer pairs within a range of annealing temperature for PCR, with the widest range to be 10 °C for SCAR FGE011. High annealing temperature resulted in simpler banding patterns and low annealing temperature resulted in amplification of more bands. Table 1 shows the range of temperature that was tested to amplify the same banding pattern with bands of expected size and from expected parent. No bands were amplified at an annealing temperature beyond the upper limit of the range. The banding patterns for FGE006 and FGE007 varied with the annealing temperature during PCR. With a high annealing temperature (59–61 °C), a single band was amplified only from H28. With low annealing temperatures, bands of different sizes were amplified also from 3-356 (Figure 4). The SCAR markers will be codominant if these bands are alleles of the band amplified at high annealing temperature.

Polymorphism of SCARs

Each of the SCAR primer pairs was tested for polymorphisms between parents using DNA samples of the two parents at different annealing temperature. FGP004, FGE006, FGE007, and FGP013 were polymorphic between the two parents. These four SCARs were then tested for within-tree polymorphism using DNA samples from six F_1 individuals. FGP004, FGE006, and FGE007 segregated among the six F_1 individuals. However, FGP013 was amplified from all the six F_1 individuals. FGP013 was confirmed to be monomorphic in the further test using 10 more F_1 individuals. Two of the monomorphic SCARs, FGP005 and FGP012, were converted to polymorphic (described in the following paragraph). Table 2 presents some information for the polymorphic SCARs.

Conversion of monomorphic SCARs to polymorphic SCARs

Restriction sites in the sequences of the six cloned fragments that were used to develop SCARs (FGP005, FGP008, FGE009, FGP010, FGE011, and FGP012) were identified using the primer analysis software

Table 2. Polymorphic SCARs for longleaf pine (3-356) and slash pine (H28).

SCAR loci	Detecting methods	Polymorphism		Codominance potential	Segregation ratio present : absent	Number of bands conflicted with RAPD
		between parents	within parent			
FGP 004	agarose gel	yes	yes	yes	31 : 23	0
FGP 005	*Sma*I digestion + agarose gel or sequencing gel	yes	yes	yes	35 : 29	1
FGE 006	agarose gel	yes	yes	yes	35 : 29	3
FGE 007	agarose gel	yes	yes	yes	34 : 30	5
FGP 012	sequencing gel	yes	yes	–	–	–
FGP 013	agarose gel	yes	no	no	–	–

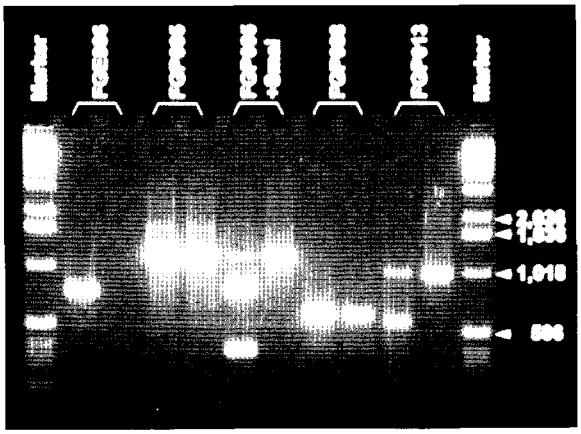

Figure 3. Amplification of SCAR markers in the two parents H28 (slash pine, on the left) and 3-356 (longleaf pine, on the right). The primer pair for FGE006 directly detected a polymorphic dominant marker. The polymorphism for FGP005 was obtained by digesting the PCR product with the restriction enzyme *Sma*I. FGP008 detected no polymorphism. The primer pair for FGP013, which were designed based on the sequences of the fragment amplified from the other parent H28 (slash pine) using FGP008 primers, detected a polymorphic band.

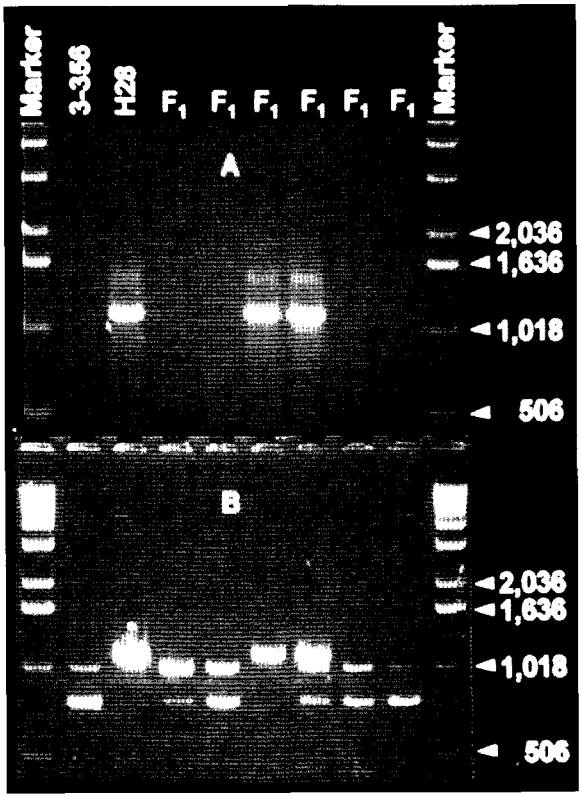

Figure 4. The banding pattern of SCAR FGE007 at different annealing temperatures. A. At 60 °C annealing temperature, FGE007 primers detected a dominant marker. B. At 56 °C annealing temperature, FGE007 primers amplified two additional fragments of different sizes from the other parent, which appear to segregate.

OLIGO. Based on the restriction sites, three restriction enzymes, *Sma*I, *Eco*RI, and *Taq*I, were chosen to digest the SCAR fragments separately. A polymorphism between the two parents was identified for FGP005 by digesting the PCR products with *Sma*I (Figure 3). FGP005 and FGP012 were converted to polymorphic SCARs by resolving the PCR products on a sequencing gel. The autoradiograph showed that, out of the primer pairs for six SCARs loci (FGP005, FGP008, FGE009, FGP010, FGE011, and FGP012), the two for FGP005 and FGP012 amplified bands with a difference of less than 10 bp in length from the two parents. And the other pairs of primers amplified bands of identical length from both parents (data not shown). FGP008 was developed based on the sequence of cloned RAPD fragment A12-600 that was amplified from 3-356. We cloned and sequenced the other monomorphic band amplified from H28. The differences between the sequences at the two ends were used to design a new primer pair FGP013 which amplified a polymorhpic band of about 550 bp from 3-356 and another band of about 1000 bp from both parents (Figure 3).

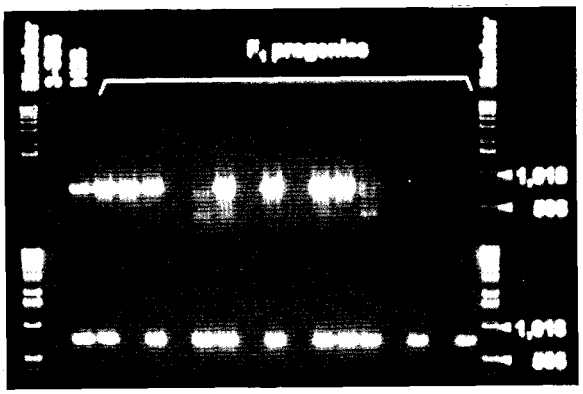

Figure 5. Segregation of SCAR FGE006 among 32 F_1 individuals of a longleaf pine × slash pine cross.

Segregation of SCARs among F_1 progeny

FGP004, FGP005, FGE006, FGE007, and FGP013, which showed polymorphisms in their PCR products or restriction digests of PCR products on agarose gel, were tested for Mendelian segregation of band presence using total DNA from 64 F_1 progeny of 3-356 × H28. Table 2 shows the segregation ratio for each of these SCARs. Chi square tests suggested that the segregation ratio for each SCAR was 1:1 ($P > 0.05$). FGP004 co-segregated exactly with the corresponding RAPD marker. FGP005, FGE006, and FGE007 conflicted with corresponding RAPDs in 1, 3, and 5 individuals, respectively. Seven of these nine apparent "errors" belonged to positive in the SCAR profiles and negative in the RAPD profiles and the other two belonged to negative in the SCAR profiles and positive in the RAPD profiles. The FGP013 did not segregate among the F_1 progeny. The 550-bp band was present in all F_1 progeny, indicating that FGP013 was homozygous in 3-356. FGP013 could not be used for mapping of parents, but may still be useful for mapping in backcrosses to H28. Figure 5 shows the segregation of FGP006 among 32 F_1 individuals.

DISCUSSION

We developed six polymorphic SCAR markers from nine RAPD markers in a longleaf pine × slash pine F_1 family. Differences in nucleotide sequence of template DNA at the priming sites have been suggested to be one of the causes of RAPD polymorphisms in the research conducted by PARAN and MICHELMORE (1993). Our research supports this contention. Out of nine extended primer pairs, six amplified bands of the same sizes from both parents. It is unlikely that these results were coincidences that there existed another locus in the alternate parent that gave rise to amplification of a product of the same size. It was most likely that amplification failed in RAPD analysis due to one or more nucleotide mismatches at the priming site in the alternate parent. And the amplification succeeded in SCAR analysis because the mismatches were tolerated by longer primers, or the mismatch position was shifted away from the 3' end of the primers.

Length of SCAR primers

Our research suggests that the length of the SCAR primers does not correlate with the number of detectable loci amplified when the primers were longer than 17 nucleotides. Both short primers and long primers could amplify one or more loci. FGP008F and FGP008R, which are 18 nucleotides long, amplified a single band at an annealing temperature of 54 °C; while FGE007F and FGP007R, which are 21 and 22 nucleotides long, amplified multiple bands at a 56 °C annealing temperature and only a single band at 62 °C. The genome size necessary to have one locus that matches an m base SCAR primer pair with n mismatches at both the forward and reverse priming sites can be estimated using the equation: $4^{2m} / [(\text{maximum PCR product size} - \text{minimum PCR product size}) \times (\binom{n}{m} \times 4^n)^2]$, assuming that the arrangement of bases in the genome is random and bases occur with equal frequencies. Thus, if we assume the maximum and the minimum PCR product sizes to be 2200 and 200 bp, respectively, the genome size necessary to have one locus that matches an 18 base SCAR primer pair with zero, one, or two mismatches at both forward and reverse priming sites can be estimated to be about 2×10^{18}, 4×10^{14}, 3×10^{11} bp, respectively. Since the genome size of longleaf pine is 33 to 57 pg (2C, equivalent to about 3×10^{10} bp for haploid) (OHRI & KHOSHOO 1986), the reason that SCAR primers amplify multiple loci is more likely that the primers are located in repetitive sequences, which is often the case for RAPDs, than due to the length of the primers.

Codominance

PARAN and MICHELMORE (1993) obtained four dominant SCAR markers out of the nine developed. In our research, of the 13, primer pairs for FGE006 and FGE007 amplified bands of different sizes from the two parents. However, whether or not these two SCARs are codominant cannot be determined at present time. Evidence for codominance inheritance needs to be confirmed using the gametes of the F_1 population.

Primer design methods and homology between longleaf pine and slash pine

Nine of the 13 SCARs were monomorphic before some

of them were converted to polymorphic. These results were not unexpected since longleaf pine and slash pine are closely related species and their genomes should be highly homologous. The high homology between the genomes of the two parents suggest that the "extended" method may be a better choice for SCAR primer design than "internal" method. All three "internal" SCAR primer pairs amplified only monomorphic SCARs. And all the three originally polymorphic SCARs were amplified by "extended" primer pairs. The "internal" primer pairs for FGP002 and FGE003 were derived from the same cloned RAPD fragments as the "extended" primer pair for FGP004 and FGE006. However, FGP002 and FGE003 were monomorphic and FGP004 and FGE006 were polymorphic. "Extended" SCAR primers, which included the RAPD primer sites, may preserve the cause of RAPD polymorphism. This may affect the rate of polymorphic SCARs for the two methods for primer design.

Conversion of monomorphic SCARs to polymorphic

Monomorphic SCARs that are not useful for mapping can be converted to polymorphic markers through several methods. In addition to restriction digestion, PARAN and MICHELMORE (1993) suggested four other methods to search for polymorphisms: (1) increasing the annealing temperature; (2) use of more genetically divergent lines as mapping parents; (3) use of higher resolution separation methods for resolving the PCR products; (4) sequencing the alternate band and using the sequence differences between the two fragments to develop a new primer pair. WILLIAMSON *et al.* (1994) converted a monomorphic SCAR to a polymorphic SCAR by digesting the monomorphic fragment with different restriction enzymes. Our research employed three of the methods and demonstrated they were effective. Depending on the size of the analyzed fragment, a sequencing gel can detect a difference as small as one base pair. FGP005 and FGP012 were monomorphic SCARs on agarose gels, but the bands amplified from the two different parents had differences of a few base pairs when they were separated on a sequencing gel. The resolution of separation for resolving PCR products can be further enhanced using the single-strand conformation polymorphisms (SSCP) strategy (ORITA *et al.* 1989). SSCP uses a denaturing gradient gel electrophoresis (DGGE) technique and can detect a single base pair difference, which results in conformation differences when denaturing, between two sequences of same length. SSCP may be a choice strategy in converting the remaining monomorphic SCARs (FGP008, FGE009, FGP010, and FGE011) into polymorphic SCARs in the future studies.

Segregation of SCARs among F_1 progeny

A total of nine disagreements in band presence/absence profiles between SCAR and RAPD analysis were found. The seven positives in the SCAR profiles and negatives in the RAPD profiles were most likely that the RAPDs were false-negatives. The two negatives in the SCAR profiles and positives in the RAPD profiles may be caused by contamination in RAPD analysis.

Marker-assisted selection using SCARs

The characteristics of SCAR markers make them favorable for MAS. In addition to fast identification shared by some other PCR-based markers, SCARs can be use to genotype individuals accurately and can be codominant. HITTALMANI *et al.* (1995) examined the accuracy of identifying rice individuals carrying a rice blast resistance gene by genotyping F_2 individuals that were selected for the linked specific amplicon polymorphism (SAP or SCAR) marker by progeny testing of their F_3 families for the blast disease responsiveness. The accuracy of identifying a resistant genotype was 97% when using a single linked marker and 100% when using two markers flanking the resistance gene. Our research shows that PCR amplification of the SCARs is reproducible and can be easily scored. If the SCARs developed are tightly linked with loci of interest, the genotyping of individuals for selection using these SCARs should be accurate and efficient.

SCARs amplify two types of banding patterns that are useful for mapping: (1) while maintaining the amplification of the fragment identical in size to the original RAPD fragment from one parent, they amplify another fragment of different size from the other parent; or (2) they maintain the original presence/absence polymorphism observed in RAPD analysis. In the first case, if the bands are allelic, the codominant SCAR can differentiate individuals that are homozygous at the original RAPD locus from those that are heterozygous. In the second case, if the SCAR amplifies a single band (which happens in most cases), it can be used for efficient genotyping (GU *et al.* 1995). In our research, although there was no data of genetic distance between the markers and the EHG QTLs, the RAPD markers corresponding to three polymorphic SCARs (FGE007, FGP012, and FGP013) did contribute significantly to the total EHG variation in QTL analysis using single marker method. These three SCARs could be considered to link to the EHG QTLs, hence they may be useful in MAS for improving the EHG of longleaf pine. Of these three SCARs, FGE007 was potentially codominant and could be also single-banded dominant. It may be used as a codominant markers (at low annealing temperatures) or as single-banded dominant markers (at high annealing tempera-

ture) for large-scale, cost-effective selections. However, the usefulness of FGE007 for the MAS in the backcross to longleaf pine is subject to further tests among more longleaf pine individuals that have been selected for the backcrosses.

CONCLUSION

We have successfully derived six polymorphic SCAR markers, with three of them linked to QTLs controlling the EHG, from RAPD markers mapped in a longleaf pine × slash pine F_1 family. SCARs were showed to be less sensitive to the changes in annealing temperature for PCR and to amplify simpler banding patterns than RAPDs. SCARs were also shown to have the potential to be codominant markers. Some monomorphic SCARs may be converted to polymorphic by various methods.

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