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Development of a SCAR Marker Linked to Male Fertility Traits in 'Jinkyool' (*Citrus sunki*)

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In Citrus, an F_1 segregation population of 150 plants was constructed from a cross between 'Kiyomi' (*C. unshiu* × *C. sinensis*) carrying the male sterility trait and 'Jinkyool' (*C. sunki*). Sequence-related amplification polymorphism (SRAP) combined with bulked segregant analysis was used to develop markers linked to male fertility. In the F_1 population, 66 out of 150 seedlings had aborted anthers and the ratio of male sterile plants to fertile plants in the progenies matched the expected Mendelian segregation ratio of 1:1 (χ^2 =2.16 at p=0.05). From the profiling of the 197 SRAP primer sets, three SRAP primer sets (F4/R27, F39/R60, and F15/R37) that were closely linked to the target trait were identified and successfully converted into a sequence characterized amplified region (SCAR) marker for selection of male fertility in citrus. The SCAR marker, using the pMS 33U/pMS 1462L primer set specifically, produced a single 1.4-Kb fragment that was linked to male fertility. Our results suggested that this SCAR marker can be useful for marker-assisted selection of male sterile individuals in breeding F_1 progenies in Citrus.

Key words : Bulked segregant analysis, *Citrus unshiu*, male sterility, 'Satsuma' mandarin

Introduction

Citrus spp. at the diploid level produces seedy fruits by fertilization. The total number of seeds of almost all hybrids is less than the total ovules. Liu et al. [21] and Yamamoto et al. [44] showed that the average number of perfect seeds per citrus fruit was 0 to 62 due to a high genetic variation. Indeed, citrus fruit containing seeds is less acceptable to consumers in fresh fruit market since those seeds are the source of unfavorable aromatic compounds and bitterness in citrus juice [30]. Therefore, seedless hybrid is a very desirable trait for citrus growers, consumers, and related industries. The creation of sterile citrus cultivar producing seedless fruit may be one of the most important goals of citrus breeding programs.

In horticulture, parthenocarpic phenomenon induces naturally or artificially the production of fruit without fertilization of ovules. The citrus fruit is therefore seedless. Frost [10] assumed that various commercial citrus cultivars have some distinct degree of ovule or sterile pollens. Mukaku kishu-type seedless citrus is caused by arrested embryo development due to the maternal tissues [45]. In 'Satsuma' and 'Encore' mandarins, anther abortion resulting in the production of sterile stamen and no viable pollen, is associated with cytoplasmic-genic male sterility and is probably controlled by more than 2 major genes [25,41,43]. Those accessions have frequently showed maternal heredity due to aborted anthers without being affected by unfavorable weather conditions, diseases and injuries by insects during microspore development [4,6,22,27,37,41,43]. In brassica [2], *Nicotiana sylvestris* [13], maize [19], petunia hybrid [28], radish [29], and sunflower [18], the production of unviable pollens are similarly controlled by the expression of specific genes. The male sterile lines [5] have the ability to produce seedless fruits when cross-pollination is blocked. At present, several famous commercial citrus cultivars in Korea, such as the 'Satsuma' mandarin and 'Kiyomi' progenies are generally seedless in monoculture. 'Kiyomi' tangor is predominant for easy peeling and marketable fruit quality. 'Kiyomi' tangor was produced from a combination of 'Satsuma' mandarin cv. 'Miyagawa wase' and a sweet orange (*C. sinensis*) cv. 'Trovita' [27]. When 'Kiyomi' tangor is used as the female parent, the progenies are phenotypically distinct from the aborted pollen or fertile pollen types [15,24,25,26,42]. Therefore, the distinct floral trait may be a key phenotype

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in the breeding of seedless citrus cultivars. However, a long juvenility of citrus is a limiting factor for developing seedless citrus cultivars in conventional breeding since it may take as long as 5-20 years or longer [1,9,32,34].

With rapid advances in biotechnology, various types of molecular marker such as random amplified polymorphic DNAs (RAPDs) [39], amplified fragment length polymorphisms (AFLPs) [38], and several other polymerase chain reaction (PCR)-based markers have been applied to improve the efficiency of conventional citrus breeding. Li and Quiros [20] have developed the sequence-related amplification polymorphisms (SRAP) to amplify intragenic sites carrying AT or GC rich cores by using specific sets of primers. SRAPs are simple to run, and provide a moderate throughput rate. This marker type also reveals many co-dominant alleles originated from, open reading frames (ORFs) and allows simple isolation of PCR products for cloning and sequencing. Recently, Uzun et al. [36] and Gulsen et al. [12] constructed a *Citrus* linkage map based on SRAP markers and found that these markers were evenly spread on citrus genome. However, for the ease of use and rapid detection, SRAP marker needs to be converted into simple sequence characterized amplified regions (SCARs) marker. Paran and Michelmore [31] developed SCARs by designing locus-specific oligonucleotide primers based on RAPDs.

Molecular markers tightly linked to an important trait can be useful for marker-assisted selection (MAS). In citrus, the advantage of MAS is its ability to identify potential sterile individuals at the seedling stage by using DNA markers tightly linked to sterility. MAS is especially advantageous when selecting for traits that are expressed only at later stages of development, such as floral characteristics. Until now there have been no reports for DNA markers linked to anther type in Citrus.

Bulked segregant analysis (BSA) is a simple procedure employed to efficiently profile markers joined with specific regions of the genome. Generally in BSA, two bulked DNA samples composed of individuals that show two contrasting phenotypes for a targeted trait are developed, and molecular markers that are polymorphic between these DNA samples are screened [23].

The present research was conducted identifying SRAP markers for male fertility by BSA, and to convert the SRAPs into a robust SCAR marker for efficient MAS of male fertility in 'Jinkyool' breeding programs.

Materials and Methods

Plant materials

One hundred-fifty F_1 progenies from a cross of 'Kiyomi' (*C. unshiu* × *C. sinensis*) × 'Jinkyool' (*C. sunki*) were planted in the research orchard of the Citrus Experiment Station at the National Institute of Horticultural & Herbal Science, Rural Development Administration, Korea in 1994. F_1 progenies carrying aborted anthers were visually chosen based on the phenotypic characteristics like shrinking and discoloration of anthers at full bloom stage in 2009.

DNA extraction and preparation of bulked DNA

Young and healthy leaf samples were collected from 23 trees which were distinguished by their aborted or fertile anthers. Total gnomic DNA (gDNA) was extracted using a plant tissue DNA preparation cartridge (AS1030, Promega Corp, Madison, WI, USA) and an automatic DNA extractor MX-16 (Compacbio Sciences Co., Korea). The quality and quantity of the gDNA were determined using Spectrometer libra S35 (Biochrom Ltd., UK). For BSA, two separate DNA pools were constructed which is composed of male aborted (23) and male fertile (23) plants.

SRAP analysis

SRAP analysis (197 primer combinations used) was conducted as described by Li and Quiros [20]. The 20-μl PCR mixture was consisted of AccuPower PCR Premix (Bioneer, Daejen, Korea), containing 250 μM dNTP, 1.5 mM $MgCl_2$, 1.0 unit *Taq* DNA Polymerase, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, and 50 pmol of each forward and reverse primer, as detailed in Table 1, and 25 ng template DNA. PCR amplification was carried out using a Takara PCR thermal cycler (Takara, Japan). The PCR conditions were as follows: an initial cycle at 94°C for 5 min; 5 cycles of 94°C for 1 min; 35°C for 1 min, and 72°C for 2 min; 35 cycles of 94°C for 1 min; 50°C for 1 min; and 72°C for 2 min and a final elongation temperature of 72°C for 10 min [35]. The amplified DNA products were loaded on a 1.2% agarose gel and stained with 0.5 μg/ml ethidium bromide.

Conversion of SRAP marker into SCAR marker

A target DNA fragment was amplified in all male fertile types by PCR with the primer set F39/R60. A single PCR band of approximately 1.5 kb fragment was extracted from the gel using a gel extraction kit (5212, Nucleogen Co.,

Table 1. Characteristics of the SRAP and SCAR markers linked to male fertility and detected by bulked segregant analysis

Primer sets	Sequence (5′-3′)	Anneal temp. (°C) [a]	Size [b]	Trait
F4 (forward) R27 (reverse)	TGAGTCCAAACCGGAAT GACTGCGTACGAATTCGG	50	554	Fertility
F39 (forward) R60 (reverse)	TGAGTCCAAACCGGGCG GACTGCGTACGAATTTGT	50	1487	Fertility
F15 (forward) R37 (reverse)	TGAGTCCAAACCGGATG GACTGCGTACGAATTGCA	50	470	Fertility
pMS 33U (forward) pMS 1462L (reverse)	GGGCAAGTATCGCAACCCCTC CTTGAGAGGTGTAGTATAAGTG	53	1429	Fertility

[a] The annealing temperature (℃)
[b] Sizes of the polymorphic SRAP combinations and PCR products with the SCAR primer in base pairs

Korea) and cloned into Topo TA plasmid vector (K450001, Invitrogen Corp., Carlsbad, CA, USA). *Escherichia. coli* TOP10 was used for the cloning of the plasmid (C4040-03, Invitrogen Corp., Carlsbad, CA, USA). The cloned plasmid was extracted using Wizard® Plus SV Minipreps DNA purification system (A1460, Promega Corp., Madison, WI, USA). Nucleotide sequencing was performed using Bigdye™ Terminator Cycle Sequencing kits (PE Biosystems, Foster City, CA, USA) and an automated DNA sequencer (ABI 3100, Applied Biosystems, Rockville, MD, USA). Specific forward and reverse primer pairs for SCAR were designed and synthesized by Bioneer (Seoul, Korea) (Table 1). PCR with SCAR primers was performed with genomic DNA of the 'Kiyomi' × 'Jinkyool' population, and the amplified DNA fragment was visualized as described above.

Results and Discussion

Phenotyping of male sterility and fertility

Flowers of 150 F_1 progenies were evaluated for the presence or absence of pollen in May, 2008 and 2009. The number of male sterile and male fertile F_1 progenies was 66 and 84, respectively. A chi-square "goodness of fit" of the observed segregation ratio were not significantly different from the expected Mendelian segregation ratio of 1:1 (χ^2=2.16 at p=0.05). Hybrid seedlings with aborted anthers and male fertile anthers, as were observed in our experiments, arise from crosses of cultivars which have the cytoplasm of 'Kiyomi' tangor as seed parents [42]. This result was consistent with a previous study on male sterility that used a different cross of 'Kiyomi' and 'Kuchinotsu No. 20' [43]. The determination of male fertility can be achieved through the observation of anthers [25]. This study indicates that phenotype of anther can be efficiently used as a morphological marker for selecting male fertile plants. However, it may take approximately 4 years to screen new seedless/seedy hybrids between aborted anther types and normal anther types by using this morphological marker. BSA allows widespread application of profile markers linked to genetic traits [3,23]. BSA was easy to construct two pooled DNA samples of individuals from two segregating populations (male sterility/male fertility) using 'Kiyomi' tangor as female parent. It was also the same with the separation of male sterility to fertility which fitted the expected 1:1 ratio when 'Kiyomi' tangor was used as female parent [28]. DNA markers linked to the seedless/seedy trait identified in Ponkan mandarin (*C. reticulate* Blaco) [40].

SRAP markers linked to the male fertile trait by BSA

Three (F4/R27, F39/R60, and F15/R37) (Table 1) out of 195 SRAP primer pairs produced markers that were potentially linked to the male fertile locus. These primer sets showed polymorphic PCR bands between the two DNA pools. The polymorphic bands were amplified from the bulked DNA sample for male fertile plants (Fig. 1). However, perfect cosegregation between the phenotype of 23 individuals and the polymorphic PCR band was detected only from the primer set F39/R60 (Fig. 2). The previous work of Michelmore et al. [23], the number of individuals comprising each bulk carried from 14 to 20 plants. In this study, the number of individuals consisted of each bulk (23 individuals) was recognized quite larger and more strict than usually used bulked individuals in the experiments to detect linked markers. With dominant markers such as SRAPs, more individuals would need to be combined to en-

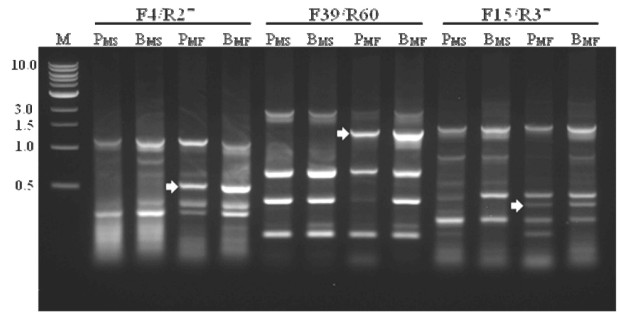

Fig. 1. Bulked segregant analysis with primers F4/R27, F39/R60 and F15/R37. P_{MS}=male sterile parent, P_{MF}=male fertile parent, B_{MS}=male sterile bulk, and B_{MF}=male fertile bulk. Primer combinations are indicated on top of the lanes. M=molecular weight marker (1 kbp ladder plus), Numbers on the left margin represent molecular weight markers in kb. Polymorphic bands are shown by the arrow.

sure it in the population as a whole.

Sequence analysis of SRAP markers

The male fertility-specific PCR band from the primer set F39/R60 was cloned and sequenced to be converted into a simple PCR-based SCAR marker. The resulting recombinant vector plasmids were amplified with the primer set F39/R60, and PCR products were examined on a sequencing gel to ensure that the correct target DNA fragments were inserted. The cloned DNA fragment was sequenced and found to be

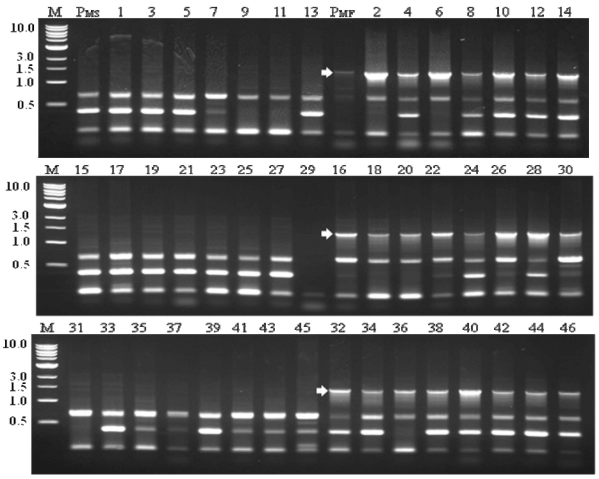

Fig. 2. Individual analysis of SRAP fragment F39/R60 in 'Kiyomi' (*C. unshiu* × *C. sinensis*) × 'Jinkyool' (*C. sunki*) F1 segregant populations. M=molecular weight marker (1 kbp ladder plus), P_{MS}=male sterile parent, P_{MF}=male fertile parent, odd numbers=male sterile F1 progenies, and even numbers=male fertile F1 progenies. Numbers on the left margin represent molecular weight markers in kb. Arrows on the left margin indicate F39/R60 SRAP fragments.

1,487-bp in length (Fig. 3). This sequence is available in the NCBI GenBank database (Accession number, HM135404.1). Blastn analysis was performed based on the sequence data of the marker, and high homology (99.0%) to 26s ribosome RNA gene of *C. aurantium* and *C. limon* revealed (Fig. 3) [17].

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   1 TGAGTCCAAA CCGGGCGGGG GGCCTCCCGA GGGGGCAAGT ATCGCAACCC CTCTCGACGA
  61 AACGGGAAAA ATAATGCGGG AGACCCTGGA CCCATTTTTC GGCCAAAAAG GGCCAAAATC
 121 CTTGCGCCAA ATTTCGAGTT TTCGGGCCGG CGTGGGATTT TTGGCTGATA TTGTTAGCCA
 181 CAGGCTCTTT GGCCAAATTT CGCCTTTGTG CCAGCCACTT AGATTTTGCA GAGAGTTAGC
 241 CTCATGAAAA AGCACAAGTC CACTCCGAGG CCCGAGCTAG CCTCCTTTCG GAGCCATCAC
 301 ACAAGTCTTT GTGCCAGAGT TAGCCTCAGG CATTGGAGCA AGTGTGCTGC CTTTGTGCCA
 361 GCCACTTAGA TTTTGCCGAG AGTTAGCCTC GTGAAAAAAC ACAAGTCCAC TCCGAGCCTT
 421 GGGCGGGGGC AGTCCGTGCG GGCGTGTGCG CCGCAGGGGG CTAACCTCCG GCGGGTTCCG
 481 AACAAGAGAA GACGGTGCGA GTGAGGGGGG AGGGACGAAT CTGAGCGACG TAGGGCTGAA
 541 TCTCAGTGGA TCGTGGCAGC AAGGCCACTC TGCCACTTAC AATACCCCGT CGCGTATTTA
 601 AGTCGTCTGC AAAGGATTCT ACCCGCCGCT CGATGGGAAT TACGATTCAA GGCGGCCGCC
 661 GCGGCCCTTC CGCCGCGGGG GCTTGGCCTA CGACACGTGC CTCTGGGGAC CGGGAGGTCC
 721 CTACTGCGGG TCGGCAAACG GGCGGCGGGC GCACGCGTCG CTCTAGCCCG GATTCTGACT
 781 TAGAGGCGTT CAGTCATAAT CCAGCGCACG GTAGCTTCGC GCCACTGGCT TTTCAACCAA
 841 GCGCGATGAC CAATTGTGCG AATCAACGGG TTCCTCTCGT ACTAGGTTGA ATTACTATTA
 901 CGACGCAGTC ATCAGTANGT AAAACTAACC TGTCTCACGA CGGTCTAAAC CCAGCTCACG
 961 TTCCCTATTG GTGGGTGAAC AATCCAACAC TTGGTGAATT CTGCTTCACA ATGATAGGAA
1021 GAGCCGACAT CGAAGGATCA AAAAGCAACG TCGCTATGAA CGCTTGGCTG CCACAAGCCA
1081 GTTATCCCTG TGGTAACTTT TCTGACACCT CTAGCTTCAA ATTCCGAAGG TCTAAAGGAT
1141 CGATAGGCCA CGCTTTCACA GTTCGTATTC GTACTGAAAA TCAGAATCAA ACGAGCTTTT
1201 ACCCTTTTGT TCCACACGAG ATTTCTGTTC TCGTTGAGCT CATCTTAGGA CACCTGCGTT
1261 ATCTTTTAAC AGATGTGCCG CCCCAGCCAA ACTCCCCACC TGACAATGTC TTTCGCCCGG
1321 ATCGGCCCCC GTGAGGGGGC CTTGGGTCCA AAAAGAGGGG CAGTGCCCCG CCTCCGATTC
1381 ACGGAATAAG TAAAATAACG TTAAAAGTAG TGGTATTTCA CTTTCGCCGT TTCCGGCTCC
1441 CACTTATACT ACACCTCTCA AGTCATTTCA CAAATTCGTA CGCAGTC
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Fig. 3. Nucleotide sequence of the fertility-specific DNA fragment amplified with the SRAP F39/R60 primer. PCR-specific primer sequences of pMS 33U and pMS 1462L are indicated in rectangle boxes. The primer for gene amplification was synthesized according to the nucleotide sequence of SRAP F39/R60. The size of this gene fragment is 1,487 bp.

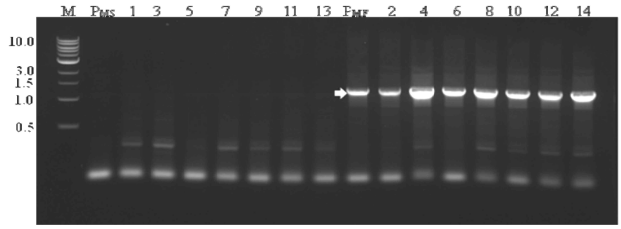

Fig. 4. Individual analysis of SCAR fragment pMS 33U/pMS 1462L in 'Kiyomi' (*C. unshiu* × *C. sinensis*) × 'Jinkyool' (*C. sunki*) F1 segregant populations. M=molecular weight marker (1 kbp ladder plus), P_{MS}=male sterile parent, P_{MF}=male fertile parent, odd numbers=male sterile F1 progenies, and even numbers=male fertile F1 progenies. Numbers on the left margin represent molecular weight markers in kb. The arrow indicates SCAR fragments.

The homology of our SCAR marker sequence to 26s rRNA gene of *C. aurantium* and C. *limon* provided valuable information on the relationship between the genetic function of rRNA and anther development. Kolosha and Fodor [17] reported that 26s rRNA in *C. limon* was present in the alignment with the large rRNA sequences of *Escherichia coli, Saccharomyces cerevisiae* and *Oryza sativa*. High degree of rearrangement in the flanking or coding regions of the 26s rRNA in Ogura mtDNA appeared to be a common feature of CMS plants in radish [7,8]. Therefore, a new transcriptional profiling study for the genic region of our SCAR marker sequence would be another interesting research subject.

Conversion of SRAP marker into SCAR marker

The SRAP marker F39/R60 was successfully converted into a simple SCAR marker (pMS33U/pMS 1462L) (Table 1). The individual F_1 plants with aborted normal anthers were screened by this SCAR marker and robust and reproducible PCR results were obtained (Fig. 4). Furthermore, the SCAR marker was tested 12 different cultivars. The results showed that the presence of the SCAR marker (1,429 bp) in 'Jinkyool' carrying normal anthers as expected from the sequence data, but absence in other seedless cultivars that were developed as male sterile parent producing abnormal anthers (Fig. 5). SCAR marker provides with potential benefits over universal primers like RAPD in that these markers amplify specific sites of the genome and prevent site-competition among primers [16,31]. This, in turn, indicated that SCAR markers are less sensitive to the PCR conditions [14]. SCAR markers also permits rapid and reproducible sample assessment [11], since the markers are generally associated with the amplification of a specific DNA fragment [33].

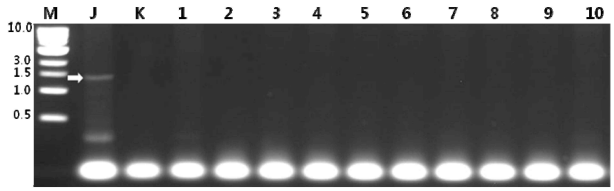

Fig. 5. Verification of SCAR marker linked to the normal anther in 'JinKyool'(J), and 'Kiyomi'(K), and male sterility of seedless citrus varieties, 'Miyagawa unshu'(1), 'Shiranuhi'(2), 'Setoka'(3), 'Reiko'(4), 'Tsunokaori'(5), 'Harumi'(6), 'Harehime'(7), 'Amakusa'(8), 'Seihou'(9), and 'Akemi'(10) ; The arrow refers to the amplification profiles for the primers pMS33u+1462L in specific SCAR Fragments (1,429 bp). M: 1kbbp size marker.

In conclusion, BSA constructed three SRAP primer sets (F4/R27, F39/R60, and F15/R37) that were linked to male fertility in citrus. In addition, we were able to convert this SRAP into a dominant SCAR marker, pMS 33U/pMS 1462L. This SCAR marker can be efficiently used for MAS as expected a reduction of the probability of some false positives/-negatives of male fertile lines in citrus breeding programs.

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초록 : '진귤' (*Citrus sunki*) 의 웅성가임 연관 SCAR 마커 개발

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감귤류 중 수술이 퇴화되어 웅성불임형질을 나타내는 '청견' 품종에 정상적인 수술의 형태를 가진 웅성가임인 '진귤' 품종을 교배하여 150개체의 F_1 집단을 구축하여 수술이 퇴화되는 개체와 정상인 개체를 분리하였다. 분리된 F_1 개체들을 사용하여 SRAP 기법과 집단 분리 분석법(BSA)을 조합하여 웅성 가임 연관 마커 개발에 활용하였다. F1 집단 내 150개체 중 66개체가 퇴화 수술을 갖고 있으며 웅성 가임성과 웅성 불임성의 분리비는 1:1이며 χ^2 값은 2.16(p=0.05)이었다. 197개의 SRAP 프라이머 조합들 중 웅성가임 특이밴드를 형성하는 3개의 SRAP 프라이머 조합(F4/R27, F39/R60, 및 F15/R37)을 선발하였으며, 이 중 F39/R60 프라이머에 특이적으로 증폭하는 DNA단편의 염기서열을 기본으로 하여 새롭게 작성한 양방향 프라이머 조합 중 웅성 가임 계통에서만 약 1.4 Kb의 특이밴드를 증폭하는 프라이머 조합, pMS 33U/pMS 1462L를 선발하여 SCAR 마커를 개발 하였다. 이러한 결과는 개발된 SCAR 마커로 무핵성 계통들의 육종 선발에 효율성을 높일 수 있을 것으로 기대된다.