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Characterization of a RAPD Fragment Unique to Species with Hairy Fruit Skin in the Genus *Actinidia*

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To develop a SCAR primer related to the hairy-fruit trait in the genus *Actinidia*, we took a PCR-RAPD approach using arbitrary 10-mer primers. PCR with the UBC 376 primer generated specific fragments from three species with hairy fruit skin. Those fragments were then cloned to determine their nucleotide sequences. Two SCAR primers were designed from the UBC 376 primer and nucleotide sequences were obtained from the PCR fragments. A SCAR primer, OKC385, specifically amplified a 385-bp fragment from one clone of *Actinidia eriantha*, four of *Actinidia chinensis*, and four of *Actinidia deliciosa*. Deduced amino acid sequences of this fragment showed high sequence homology with plant cellulose synthases, which are involved in the biosynthesis of cellulose, a major cell wall component. The 385-bp fragment was specifically detected only in the series *Perfectae* C.F. Liang of section *Stellatae* Li. This type has many hairs on the leaves, fruits, and stems, suggesting that the gene containing the PCR fragment is involved in hair formation in this phylogenetic group. Taken together, our results suggest that the SCAR primer, OKC385, can be used as a specific primer for early selection of the non-hair trait in breeding of the genus *Actinidia*.

Keywords: Actinidia, cellulose synthase, RAPD, SCAR primer

The genus *Actinidia*, or kiwifruit, includes more than 60 species of dioecious vines that originated in China and neighboring countries (Ferguson, 1990; Yan et al., 1997). Its fruit has been acclaimed for its nutritive and medicinal value as a very rich source of ascorbic acid. Kiwifruit breeding programs started globally in the late 1970s, and in Korea in the 1990s. New cultivars have mainly depended on selections from the wild, and have been developed by intra- or inter-specific crossbreeding of *A. chinensis* and *A. deliciosa*. Only one species, *A. deliciosa* var. *deliciosa*, is cultivated for commercial purposes. Diploid (2n=2x=58), tetraploid (2n=4x=116), and hexaploid (2n=6x=174) trees are found in the wild, and variations in ploidy exist even within individual species. In addition, it is difficult to identify the sex from the external form at the young stage, and *Actinidia* especially has a long juvenile period (Zhang, 1981; Fredrique et al., 1994; Ferguson et al., 1996).

Molecular markers have several advantages over traditional phenotypic markers. They can be detected in all phases of plant growth, whereas the latter can be identified only at a specific stage. The use of molecular markers can also improve the efficiency of crossbreeding because they are not modified by the environment. Their development has been greatly improved through the use of random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; This et al., 1997; del Rio and Bamberg, 2000), as well as restriction fragment length polymorphism (RFLP) (Zhong et al., 2003; Lízal and Relichová, 2004), amplified fragment length polymorphism (AFLP) (Lin and Kuo, 1995; Negi et al., 2000), and simple sequence repeats (SSR) (Powell et al., 1996). The RAPD technique, combined with bulked segregation analysis, can identify markers that are closely linked to economically important traits (Michelmore et al., 1991; Paran and Michelmore, 1993). To facilitate marker-assisted selection (MAS), RAPD markers have been converted into valuable sequence-characterized amplified region (SCAR) markers (Barzen et al., 1997; Gill et al., 1998; Kim et al., 2000; Vidal et al., 2000; Cao et al., 2001). Phylogenetic analyses of the genus *Actinidia* have been attempted using isozymes (Messina et al., 1991) and RAPD (Kim et al., 2003), sex-

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related SCAR markers via both RAPD (Harvey et al., 1997; Gill et al., 1998) and AFLP (Xiao et al., 1999), and chloroplast inheritance (Jung et al., 2003).

Although kiwifruit is the most nutrient-dense fruit in the world, hairs on its outer skin surface deter its selection by market consumers. Therefore, production of non-hairy fruit is an important aspect of any kiwifruit breeding program. The objective in our study was to develop a specific primer related to the hairy-fruit trait in order to improve the efficiency of breeding in the genus *Actinidia*.

MATERIALS AND METHODS

Plant Material and DNA Extraction

Leaf samples from eleven taxa in the genus *Actinidia* were collected in Korea and China from 1994 to 1998 (Table 1). All materials were immediately frozen in liquid nitrogen and stored at -70°C. Total DNA was purified from approximately 0.5 g of fresh leaves using the protein precipitation method of Dellaporta et al. (1983).

PCR Amplification and Purification of the PCR Products

RAPD analysis was performed with random decamer primers (Biotechnology Laboratory, University of British Columbia, Canada; UBC 101 to 120, UBC 301 to 420). Twenty nanograms of DNA was used as template in a total reaction volume of 25 μL that contained 2.5 μL of 10× reaction buffer, 2.5 mM $MgCl_2$, 400 μM of each dNTP, 4 pM of each primer, and 2.0 units *Taq* DNA polymerase (MBI Fermentas, Germany). Amplification was performed in a GeneAmp PCR System 9600 Thermal Cycler (Perkin Elmer Cetus, USA), with initial denaturation for 5 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 38°C, and 1.5 min at 72°C; followed by 5 min of final extension at 72°C. The PCR products were separated on 1.2% agarose gels at 100 V for 40 min using 1× Tris-borate-EDTA (TBE) buffer, and finally stained with ethidium bromide. After amplification, each RAPD marker fragment was purified with 1× TAE buffer from a low-melting-point agarose gel. After staining with ethidium bromide, the agarose block containing the DNA was excised from the gel under low-wavelength UV light. DNA was recovered using an Agarose Gel DNA Extraction Kit (Roche, Germany), according

Table 1. Taxa of *Actinidia* used in this study.

Taxon[a]	Lane no.	F (♀) · M (♂)	Source	Characteristics	
				Flesh color	Hair (leaf, pericarp)
Section *Leiocarpae*					
Series *Lamellatae*					
A. arguta (Sieb. et Zucc.) Planch Et Miq. I	1	(♀)	Bongae, Jeju, Korea	Green	None
A. arguta (Sieb. et Zucc.) Planch Et Miq. II	2	(♂)	Gwangdong, China	-	None
Series *Lamellatae*					
A. kolomikta (Maxim. Et Rupr.) Maxim.	3	(♀)	Gwangdong, China	Yellow Green	None
A. melanandra Franch	4	(♀)	Gwangdong, China	Green	None
Series *Solidae*					
A. polygama (Sieb. Et Zucc.) Maxim.	5	(♀)	Gwangdong, China	Yellow	None
A. macrosperma C.F. Liang	6	(♀)	Gwangdong, China	Purple	None
Section *Stellatae*					
A. eriantha Benth.	7	(♀)	Gwangdong, China	Dark Green	White (much)
A. chinensis Planch.	8	(♀)	Jiangxi, China	Yellow	Soft-haired (some)
A. chinensis Planch.	9	(♂)	Jiangxi, China	-	Soft-haired (some)
A. deliciosa (A. Chev.) C.F. Liang et A.R. Ferguson	10	(♀)	NISA[b] Jeju, Korea	Green	Stiff-haired (much)
A. deliciosa (A. Chev.) C.F. Liang et A.R. Ferguson	11	(♂)	NISA, Jeju, Korea	-	Stiff-haired (much)

[a]Taxonomic treatment follows Liang (1983), as revised by Ferguson (1990).
[b]NISA, National Institute of Subtropical Agriculture RDA.

to the manufacturer's protocol.

Cloning and Sequencing of the PCR Products

The DNA fragments were cloned into the pT-Adv vector, with the aid of an AdvanTAge™ PCR Cloning Kit (Clontech, USA), according to the manufacturer's instructions. Nucleotide sequences of the cloned fragment were determined using Cy5-labeled vector primers -- M13 -40 and M13 reverse -- and a Cy5™ AutoCycle™ Sequencing Kit (Pharmacia, USA) on an ALFexpress II DNA Sequencer. The samples were separated on 6% acrylamide-7 M urea gels in 0.5× TBE buffer at 1500 V for 700 min. Sequences were aligned with CLUSTAL W (Thompson et al., 1994) and then adjusted manually to align several conserved regions. Sites with missing data or gaps were excluded from all analyses.

SCAR Primer Design and Analysis

Four specific primers were designed from the sequences of the marker fragments. Each primer comprised a 10-base original RAPD primer sequence and an additional 9- to 12-base internal sequence. The PCR reaction mixture (25 μL) contained 2.5 μL

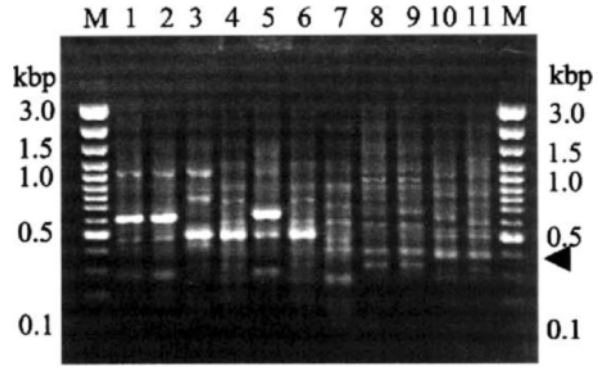

Figure 1. Accession-specific bands amplified by PCR-RAPD using UBC376 primer in *Actinidia* spp. Numbers represent the same as those assigned for 11 taxa in Table 1. Arrowhead indicates position of specific fragments amplified only in hairy-fruited vines of *A. deliciosa*, *A. chinensis*, and *A. erianthа*. M, DNA size marker (GeneRuler™ 100-bp DNA Ladder Plus).

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OKC385  CAGGACATCG CAGCCAATCC ATGCCAAACC TTATCTATGT ATCCAGACAG AAAA--GCAA
APP385  .......... .......... ........T. .......... .......... ....-.....
HAY385  .......... .......... .......... .......... .......... ....AC....
        ********** ********** ******** * ********** **********  ***  ****

OKC385  AACATCCCCA CATCATTTTA AGGCTGG-TG CTCTCAATGC CCCTGGTAAG CAAATCTATC
APP385  .......... .......... .......G.. .......... ..-....... ..........
HAY385  .....T.... .....C.... ....C..-.. .......... .T-....... .....A....
        ***** **** ***** **** **** ** ** ********** *  ******* ***** ****

OKC385  AAACGAATCC TACAAAAAAA -GGCTCATGT TTAGCTAATT ATCACTTCAT TAATTAAGTT
APP385  .......... .......... A......... .......... .......... ..........
HAY385  .......C.. .........- -......... .......... .......... ..........
        ******* ** *********   ********* ********** ********** **********

OKC385  AGGGTAAATT ATCACTGTAG CTCAGTGTTT AATACGAGGG AGCTTCTAAA TGCAAGCCAG
APP385  ......C... .......... .......... .......... ....C..... ..........
HAY385  G.A....... .......... .......... .......... ..A....... .........A
         * *** *** ********** ********** ********** ** * ***** *********

OKC385  GTGGGTCTCA TCACACCCC- AAAAAATGGG GAAAATATTA CAGTTTTATC CTCGAGTAAA
APP385  A......... .........C .........C CC........ .......... ..........
HAY385  A.A....... G...G....A .....T...G .......... .........G. ..........
         * ******* *** ****  *****  **    ******** ******** * **********

OKC385  ATTACCACAA AGGGCACAAT AGCCACCATG GCCCACAAAG GTTCTTGCGT AGAGAGGGAG
APP385  .......... ...-...... .......... C......... .......... ....G..A..
HAY385  .......... .......... .......... .......... ......T.A. .........C
        ********** *** ****** **********  ********* ****** * * **** ** *

OKC385  TGAGTGGTCT GTTGCATTGG CGATGTCCTG
APP385  ........G. .......... ..........
HAY385  A........C .......... ..........
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Figure 2. Multiple alignment of nucleotide sequences of PCR fragments by UBC376 primer from *A. chinensis* var. 'Okcheon' (OKC385), *A. chinensis* var. 'Apple' (APP385), and *A. deliciosa* var. 'Hayward' (HAY385). Nucleotide sequences are displayed 5' to 3'. Dots indicated same nucleotide as in OKC385; dashes represent gaps.

of 10 × reaction buffer, 2.5 mM $MgCl_2$, 400 μM of each dNTP, 4 pM of each primer, and 2.0 units *Taq* DNA polymerase. Amplification was performed in a GeneAmp PCR System 9600 Thermal Cycler, with initial denaturation for 5 min at 94°C; followed by 1 min at 94°C, 1 min at 48°C, and 1.5 min at 72°C (total of 30 cycles); with 5 min of final extension at 72°C. The PCR products were separated on 1.2% agarose gels at 100 V for 40 min using 1× TBE buffer before being stained with ethidium bromide.

RESULTS AND DISCUSSION

To facilitate an efficient breeding scheme for the fruits of eight species and eleven taxa in the genus *Actinidia*, we utilized a RAPD approach to select species-specific bands related to the hairy-fruit trait. In all, 140 primers were screened; among these, 12 detected polymorphism. Only one primer (UBC376) generated reproducible DNA fragments, which were amplified only in the hairy-fruited vines of *A. deliciosa*, *A. chinensis*, and *A. eriantha*. This primer produced a 385-bp fragment that we designated $UBC376_{385}$ (Fig. 1).

Because RAPD has poor reproducibility, RAPD markers must first be converted into SCAR markers. We cloned and determined the nucleotide sequences for our $UBC376_{385}$ fragments from *A. chinensis* var. 'Okcheon' (OKC385), *A. chinensis* var. 'Apple' (APP385), and *A. deliciosa* var. 'Hayward' (HAY385). When those sequences were compared using the multiple-alignment program, CLUSTAL W, OKC385 showed 93% identity with APP385 and 91% identity with HAY385. The OKC385 primer site was the same as that of APP385 (Fig. 2).

Our 19- and 23-mer SCAR primers were designed on the basis of the RAPD primer, UBC376, which amplified specific bands only from species with hairy fruit (Table 2). G+C contents of the forward and reverse OKC385 primers were 57.9 and 54.6%, respectively. The HAY385 primers had respective G+C contents of 60.0 and 56.5% (Table 2). We

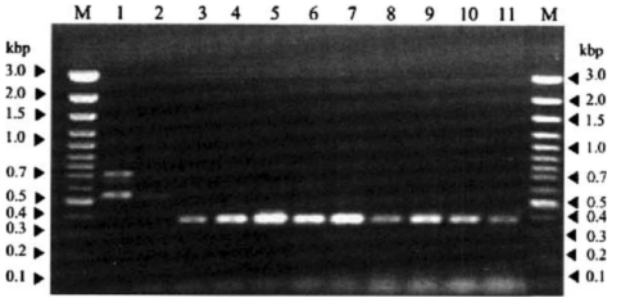

Figure 3. PCR using SCAR primer OKC385 in *Actinidia* spp. Lane 1, *A. arguta* (♂); Lane 2, *A. arguta* (♀); Lane 3, *A. eriantha* (♀); Lanes 4 and 5, *A. chinensis* lines (♂); Lanes 6 and 7, *A. chinensis* lines (♂); Lanes 8 and 9, *A. deliciosa* lines (♀); Lanes 10 and 11, *A. deliciosa* lines (♂). Bold-faced sequence indicates sequence of UBC376 primer. M, DNA size marker (GeneRuler™ 100-bp DNA Ladder Plus).

tested the SCAR primers for two clones of *A. arguta* (Lanes 1 and 2), one of *A. eriantha* (Lane 3), four of *A. chinensis* (Lanes 4 to 7), and four of *A. deliciosa* (Lanes 8 to 11). PCR data revealed that the OKC385 primer specifically amplified a 385-bp fragment only from the hairy-fruited vines, i.e., *A. eriantha*, *A. chinensis*, and *A. deliciosa* (Fig. 3). In contrast, our analysis of *A. arguta* generated 550- and 720-bp fragments from the male and a 550-bp smear fragment from the female (Fig. 3).

Liang (1984) has separated the genus *Actinidia* into the following four sections, according to the nature of their leaves and branches (slightly pubescent, hairy, or woolly): *Leiocarpae*, *Maculatae*, *Strigosae*, and *Stellatae*. *A. chinensis*, *A. deliciosa*, and *A. eriantha* are characterized by many hairs at the young fruit stage as well as numerous hairs or fuzz on their leaves and branches. These three species belong to the series *Perfectae* C.F. Liang of section *Stellatae* Li (Liang, 1983; Ferguson and Bollard, 1990; Kim et al., 2003). In contrast, *A. arguta* either lacks any hairs on its fruits and leaves, or has a few pubescences only at the young stage. This species belongs to the series *Lamellatae* C.F. Liang of section *Leiocarpae*. In the current study, we were able to use a SCAR primer, OKC385, to demonstrate that our results are in accordance with

Table 2. SCAR primers designed from sequences of PCR-RAPD fragments and UBC 376 primer.

Primer name	Primer sequences[a]	% GC	Expected band size (bp)
OKC385	F: 5'-**CAG-GAC-ATC-G**CA-GCC-AAT-C-3'	57.9	385
	R: 5'-**CAG-GAC-ATC-G**CC-AAT-GCA-ACA-G-3'	54.6	
HAY385	F: 5'-**CAG-GAC-ATC-G**CA-GCC-AAT-C-3'	60.0	385
	R: 5'-**CAG-GAC-ATC-G**CC-AAT-GCA-ACG-GA-3'	56.5	

[a]Bold-faced type indicates sequence of UBC376 primer.

Table 3. Similarity between deduced amino acid sequences of PCR fragment amplified with OKC385 primer and plant cellulose synthases.

Proteins (enzymes)	Plant sources	Identities	Positives	GenBank Accession No.
Cellulose synthase isolog	*Arabidopsis thaliana*	23[a]/25[b](92%[c])	24[a]/25[b](96%[c])	AC002343
Cellulose synthase isolog	*Arabidopsis thaliana*	23/25 (92%)	24/25 (96%)	AC002343
Cellulose synthase isolog	*Arabidopsis thaliana*	23/25 (92%)	24/25 (96%)	AY070072
Cellulose synthase catalytic subunit	*Gossypium hirsutum*	15/26 (57%)	19/26 (72%)	AF150630
Cellulose synthase catalytic subunit-like protein	*Arabidopsis thaliana*	15/26 (57%)	19/26 (72%)	NM_123770
Cellulose synthase catalytic subunit	*Nicotiana alata*	16/26 (61%)	19/26 (72%)	AF304374

[a]Number of amino acids exactly matched in homologous sequences of S2-385 sequence and GenBank search products.
[b]Number of total amino acids in homologous sequences of S2-385 sequence and GenBank search products.
[c]Number of homologous amino acids as percent of total amino acids in S2-385 sequence and GenBank search products.

the previous classification for the genus *Actinidia*, as suggested by Liang (1984).

We utilized the BLAST program (Altschul et al., 1997) to perform a homology search for deduced amino acid sequences of the PCR fragment amplified with the OKC385 SCAR primer against the NCBI database. The deduced amino acid sequences showed high sequence identity with plant cellulose synthases reported previously (Table 3). For example, the 50 amino-acid residues deduced from base pairs 21 to 173 had 96% identity with the cellulose synthase of *A. thaliana*, 80% with amino acids 246 to 270 of the *CSLH1* gene of *Oryza sativa*, and 79% homology with the cellulose synthase-like protein *OsCslE2* of *O. sativa*. Cellulose synthases are involved in the biosynthesis of cellulose, a major component in plant cell walls (Kimura and Kondo, 2002). Our result suggests that the cellulose synthase encoded by the fragment amplified with the OKC385 SCAR primer might play specific roles in mediating the biosynthesis of cellulose fibrils during hair development in hairy-fruited kiwifruit vines. Moreover, the development of unicellular trichomes or hairs in *Arabidopsis* is under the control of a regulatory circuit by MYB-related transcriptional factors such as *GLABROUS1* (*GL1*), *GLABROUS2* (*GL2*), *GLABROUS3* (*GL3*), and *TRANSPARENT TESTA GLABRA1* (*TTG1*) (Langdale, 1998; Szymanski et al., 2000; Kirik et al., 2001). Therefore, it would be interesting to see whether the expression of cellulose synthase genes in *Arabidopsis* is affected by mutation of those factors. Such a discovery might help elucidate the role of cellulose synthase during hair development in *Actinidia*.

In conclusion, the SCAR primer, OKC385, produced a specific fragment only in hairy-fruited vines of the kiwifruit, with high sequence identity being found for plant cellulose synthases. Therefore, we believe we have identified a candidate gene responsible for hair development in *Actinidia* fruits, leaves, and stems. This result is further supported by previous classification of the genus. Our report is presumably the first to demonstrate a SCAR marker that is closely related to the hairy-fruit trait. We are now cloning the full-length cellulose synthase gene and using it to transform kiwifruit in order to clarify its relationship with hair formation. Therefore, we propose that this SCAR primer, OKC385, will be a useful marker for early selection of the non-hairy trait in kiwifruit breeding programs.

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