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Research Report

Development of STS Markers Linked to the Major QTLs for Resistance to the Pepper Anthracnose Caused by *Colletotrichum acutatum* and *C. capsici*

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Abstract. Anthracnose (*Colletotrichum* spp.) causes significant yield losses in chili pepper (*Capsicum annuum* L.), and several loci conferring resistance to this disease have been identified. Seven and one amplified fragment length polymorphic (AFLP) markers were additionally located on LG 12 (*CaR12.2*, major locus resistant to *Colletotrichum acutatum*) and LG 9 (*CcR9*, major locus resistant to *C. capsici*), respectively, through AFLP analysis combined with extreme bulked segregant analysis (BSA). Among these, two AFLP markers, EtagMcgt04 (*CaR12.2*) and EtacMccg13 (*CcR9*), were converted into sequence tagged site (STS) markers (CaR12.2M1-CAPS and CcR9M1-SCAR, respectively), via sequencing analysis of internal and flanking regions of each AFLP marker. The selection efficiencies were 72% for CaR12.2M1-CAPS and 82.5% for CcR9M1-SCAR. These simple PCR-based markers will be useful for breeding cultivars with enhanced resistance to anthracnose, for pyramiding resistances to both *C. acutatum* and *C. capsici*, and for further characterization of the locus, including isolation of genes responsible for resistance.

***Additional key words*:** CAPS, *Capsicum*, major locus, QTL, SCAR

Introduction

Pepper (*Capsicum* spp.) anthracnose, which is caused by *Colletotrichum* spp. such as *C. capsici, C. acutatum*, and *C. gloeosporioides*, leads to significant yield losses in many Asian countries, including Korea, Thailand, Indonesia, and India (Than et al., 2008). The most significant causal pathogen of the disease in South Korea, *C. acutatum*, infects both immature (green) and mature (red) pepper fruits (Kang et al., 2005), whereas the primary causal pathogen in Thailand, *C. capsici*, mainly attacks pepper fruits at the red stage (Pakdeevaraporn et al., 2005; Park et al., 1990; Than et al., 2008).

The inheritance patterns of anthracnose resistance vary depending on the source of resistance and the pathogenic species used. For example, the resistance of *C. baccatum* 'PBC80' to an isolate of *C. acutatum* 'KSCa-1' is controlled by a dominant gene (Yoon and Park, 2005), whereas resistance of the *C. annuum* 'AR' line, which was derived from *C. chinense* Jacq. 'PBC932,' is inherited in a recessive manner (Kim et al., 2007). Similarly, the resistance of *C. annuum* '83-168' and 'Chungryong' to an isolate of *C. capsici* is controlled by a single dominant and a partially dominant gene, respectively (Lin et al., 2002; Park et al., 1990), while the resistance of *C. annuum* 'Daepoong-cho' and *C. chinense* 'PBC932' is inherited in a recessive fashion (Kim et al., 2008; Pakdeevaraporn et al., 2005). Recently, resistance of 'Daepoong-cho' and 'PBC932' to *C. capsici* isolates was found to be controlled by the same recessive gene (Kim et al., 2008).

In addition, some reports have indicated that resistance is controlled by polygenes. Voorrips et al. (2004) applied quantitative trait locus (QTL) mapping to analyze resistance in *C. chinense* 'PRI95030' to *C. gloeosporioides* and *C. capsici*. One major QTL (B1) and three minor QTLs (B2, H1, and D1) for resistance to *C. gloeosporioides* were detected, whereas one major (B1) and one minor (G1) QTL for resistance to *C. capsici* were detected (Voorrips et al., 2004). We previously analyzed QTLs for resistance to *C. acutatum* and *C. capsici* (Lee et al., 2010). Interestingly, the main-effect QTL (*CaR12.2*) for resistance to *C. acutatum* and the major QTL (*CcR9*) for *C. capsici* resistance are differently positioned; however, there are close links between the minor QTL *CcR12.2* and major QTL *CaR12.2*,

as well as the minor QTL *CaR9* and major QTL *CcR9*.

Simple PCR-based markers or high-throughput screening methods are needed for a marker-assisted selection (MAS) of mass populations for practical breeding programs. The amplified fragment length polymorphism (AFLP) technique, developed by Vos et al. (1995), has been widely used for identifying molecular markers linked to specific traits due to its high efficiency and reproducibility. However, it cannot be used directly for MAS because AFLP analysis involves time-consuming and laborious steps, such as polyacrylamide gel electrophoresis. Therefore, an AFLP marker that identifies a linkage with a specific trait needs to be converted into a breeder-friendly marker, such as sequence characterized amplified regions (SCAR; Paran and Michelmore, 1993) or cleavage amplified polymorphic sequences (CAPS; Konieczny and Ausubel, 1993) markers.

Here, we saturated the major QTL regions associated with anthracnose resistance through AFLP analysis combined with extreme bulked segregant analysis (BSA), converted the AFLP markers linked to the major QTLs (*CaR12.2* and *CcR9*) into STS markers, and estimated selection efficiency of the newly developed STS markers in a BC_1F_2 population.

Materials and Methods

Plant Materials

Capsicum baccatum 'PBC81' was used as a resistant parent to *C. acutatum* and *C. capsici*, while *Capsicum annuum* 'SP26' (Matikas) was used as a susceptible parent, which is partially compatible with 'PBC81,' based on an embryo rescue technique (Yoon et al., 2006), and the 'SP26' was also used as a recurrent parent due to hybrid male sterility. In total, 270 interspecific BC_1F_1 progenies (SP26/PBC81//SP26) were obtained. Among these, the highly resistant pepper '#99' was selfed to obtain BC_1F_2 progenies. In total, 87 individuals were planted and used for evaluation of anthracnose resistance.

Pathogens and Assessment of Anthracnose Resistance

Colletotrichum acutatum 'KSCa-1' and *C. capsici* 'ThSCc-1' isolates were used. The isolate 'KSCa-1' was collected from a naturally infected green or red peppers in Korean fields using the single-spore isolation method of Park and Kim (1992). 'ThSCc-1,' the Thai isolate, was obtained from S. M. Park (Seminis Thailand Co.). Inoculum preparation, inoculation, and incubation of post-inoculation followed the procedures of Yoon and Park (2001). The concentration of inoculum was adjusted to 5×10^5 conidia/mL. Artificial inoculation was performed on green and red fruits using the microinjection method developed by the Asian Vegetable Research and Development Center (AVRDC) with slight modifications (Yoon and Park, 2001). Overall lesion diameter, a millimeter value of infected sites among total inoculated sites, was used for assessment of disease reactions (Voorrips et al., 2004).

AFLP and Bulked Segregant Analysis

Genomic DNA was prepared from the fresh leaves according to the Miniprep method described by Prince et al. (1997). DNA concentration was adjusted to 100 ng·μL^{-1} by electrophoresis on 1.0% agarose gels, and 10 μL of DNA were used as template for AFLP analysis. AFLP analysis was performed as described by Vos et al. (1995) with the exception of visualizing PCR bands by silver staining instead of radioautography. Genomic DNA was digested with the restriction enzymes *Eco*RI and *Mse*I, ligated to *Eco*RI and *Mse*I adaptors, and then preamplified with four pairs of preselective primers for *Eco*RI and *Mse*I. Selective amplifications were performed using 256 pairs of selective primers, each with three additional nucleotides at the 3' ends (*Eco*RI + TNN and *Mse*I + CNN). The PCR products were electrophoresed in 5% denaturing polyacrylamide gels and visualized by silver staining using a Silverstar Staining Kit (Bioneer, South Korea) according to the manufacturer's instructions. For bulked segregant analysis (BSA), two bulk preamplicons were made from eight highly resistant and eight highly susceptible plants.

Linkage Mapping

Linkage maps were constructed using the software Mapmaker/EXP v. 3.0 (Lincoln et al., 1993). Distorted polymorphic markers were excluded by chi-squared tests ($P < 0.001$), and the identical markers were eliminated. Linkage groups were divided using LOD 4.0 and a maximum distance of 30 cM. The mapping function of Kosambi (1944) was used. Linkage maps were drawn using MAPCHART v. 2.1 (Voorrips, 2002).

Sequencing of Internal and Flanking Regions of AFLP Markers and Design of Primers

Polymorphic AFLP fragments were eluted from silver-stained gels through the crush and soak method (Sambrook and Russell, 2001) and then sequenced directly. Sequencing was conducted by the National Instrumentation Center for Environmental Management at Seoul National University, South Korea. Flanking sequences of AFLP fragments were obtained using the GenomeWalker™ Universal Kit (Clontech, CA, USA) according to instrument's protocols. To expand genomic regions flanking an AFLP fragment of interest, primer sets specific to the fragment were designed using the Primer 3 software (Genetics Computer Group Inc., WI, USA) and commercially synthesized by Bioneer Co. Ltd., South Korea.

SCAR and CAPS Analysis

Internal and flanking sequences from polymorphic AFLP fragments were compared using the program CLUSTAL X v. 1.83. The CAPS Designer software (http://soldb.cit.cornell.edu/tools/caps_designer/caps_input.pl) was used to design CAPS primer sets. The PCR program used was as follows: an initial denaturation at 95℃ for 5 min; 40 cycles of amplification, each consisting of 95℃ for 45 s, 66℃ for 45 s, and 72℃ for 1 min; and a final extension at 72℃ for 5 min. The PCR product in CAPS analysis was digested with the restriction enzyme *Bgl*II, and then separated on 1.2% agarose gels, while the PCR product in SCAR analysis was directly separated on 1.0% agarose gels.

Results

Additional AFLP Markers Linked to Major QTLs for Anthracnose Resistance

The major QTLs (*CaR12.2*, locus resistant to *Colletotrichum acutatum* on LG 12; *CcR9*, locus resistant to *C. capsici* on LG 9) for anthracnose resistance were found in a previous study (Lee et al., 2010). To saturate linkage maps LG 12 and 9 of the BC_1F_2 population (SP26/PBC81//SP26) derived from a cross between *Capsicum annuum* 'SP26' (susceptible) and *C. baccatum* 'PBC81' (resistant), 256 AFLP primer combinations were screened using two bulk preparations, each pooling with eight highly resistant and eight highly susceptible plants. In total, 51 AFLP markers were identified in 14 primer pairs selected by BSA-AFLP analysis. Seven (EtagMcgg05e, EtgcMcct03, EtagMcgt04, EacgMcgg02, EtaaMcga03, EtacMctg01, and EtacMccg11) and one (Etac Mccg13) AFLP markers were located on LG 12 and 9, respectively (Fig. 1). Among these, four (EtagMcgg05e, Etgc Mcct03, EtagMcgt04, and EacgMcgg02) and one (Etac Mccg13) markers were closely linked to the major QTLs *CaR12.2* and *CcR9*, respectively (Fig. 1).

Conversion of AFLP Markers into STS Markers

The major QTL *CaR12.2*, resistant to an isolate of *C. acutatum*, was positioned between HpmsE032 and Eata Mcgc01 (6.1 cM) on LG 12 (Fig. 1). We tried to convert eight QTL-linked AFLP markers, including EacgMcgg02,

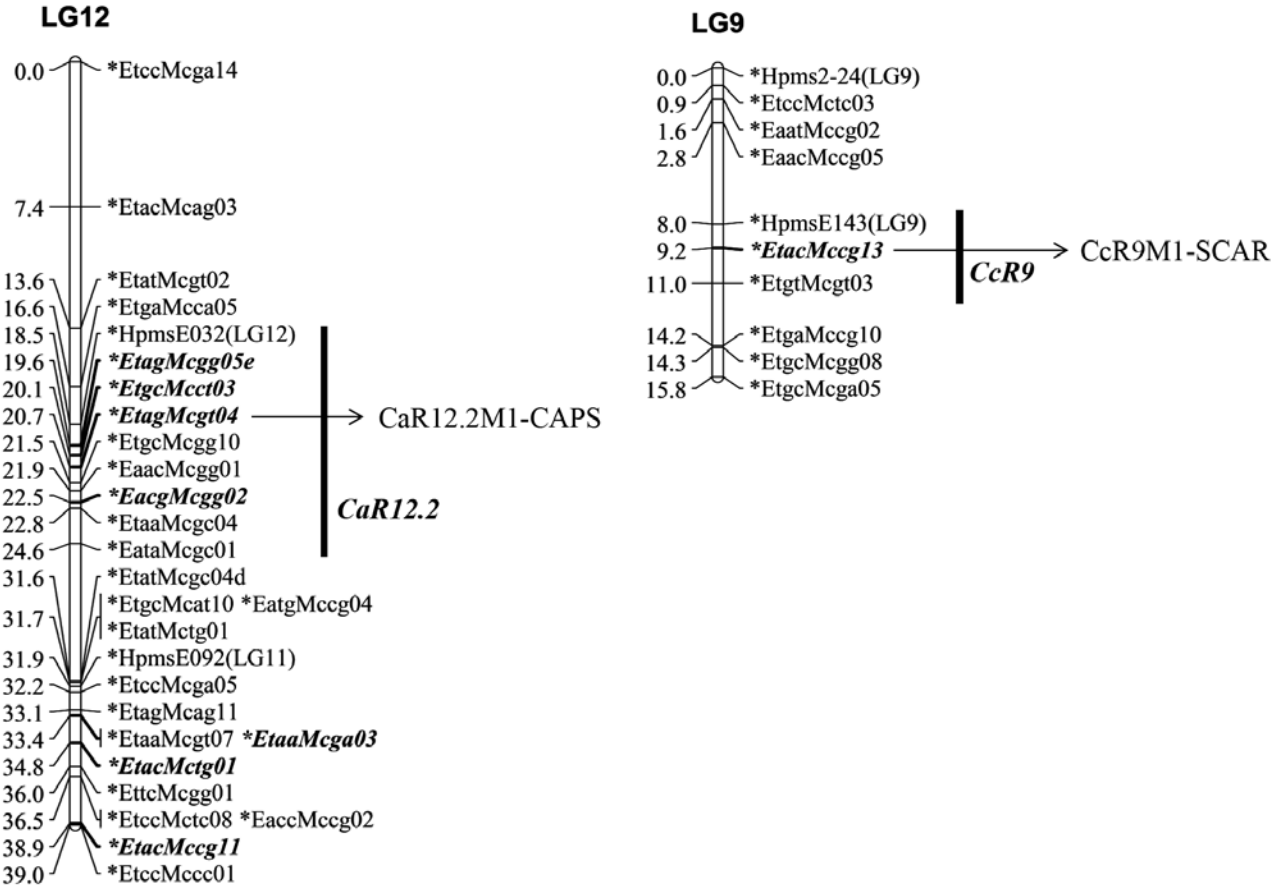

Fig. 1. Major QTL regions for resistance to pepper anthracnose (*C. acutatum* and *C. capsici*). *CaR12.2*, a major locus resistant to *C. acutatum* on LG 12; *CcR9*, a major locus resistant to *C. capsici* on LG 9; black bars, significant regions; bold and italic markers, AFLP markers additionally identified through BSA-AFLP analysis; left numbers, locations of markers (cM, Kosambi function); CaR12.2M1-CAPS, marker converted from the AFLP marker EtagMcgt04; CcR9M1-SCAR, marker converted from the AFLP marker EtacMccg13.

which had the highest LOD score, into STS markers (Fig. 1). The eight AFLP fragments (EtagMcgg05e, EtgcMcct03, EtagMcgt04, EtgcMcgg10, EaacMcgg01, EacgMcgg02, EtaaMcgc04, and EataMcgc01) were directly sequenced; however, no fragment was successfully sequenced. Therefore, the fragments were cloned into the pGEM-T Easy Vector (Promega, WI, USA) and then sequenced. The internal sequence (203 bp) of only one fragment (Etag Mcgt04) was obtained in this process (data not shown). In addition, a region (773 bp) flanking the *Eco*RI site of the fragment was also sequenced using the GenomeWalker™ Universal Kit (Clontech, CA, USA). Based on a total of 976-bp sequences, primer sets for the PCR reaction were designed using the software Primer3 v. 0.4.0 (http://frodo.wi.mit.edu/) to compare the sequences between resistant and susceptible plants. We found a polymorphic sequence on the *Bgl*II enzyme site that only cut resistance-linked sequences (Fig. 2). Finally, the EtagMcgt04 AFLP marker was converted to a codominant CAPS marker, CaR12.2M1-CAPS (Fig. 2).

The major locus *CcR9*, resistant to an isolate of *C. capsici*, is located between HpmsE143 and EtgtMcgt03 (Fig. 1). Using the methods described above, an attempt was made to convert the two AFLP markers (EtacMccg13 and EtgtMcgt03) into STS markers, and the internal sequences of EtacMccg13 were obtained by cloning into the pGEM-T Easy Vector. In total, ten clones were sequenced, but five different sequences appeared in the fragment of Etac Mccg13. Among these, the most frequently found sequence (six times, 129 bp) was used for further study. Although flanking sequences of the fragment were not obtained in the genome walker libraries of *Capsicum annuum*, the sequence (723 bp) flanking the *Eco*RI site of the fragment was obtained in libraries of *C. baccatum*. The primer pair for the PCR reaction was designed based on a total of 852-bp sequences. The PCR analysis using this primer pair revealed that the expected PCR products were amplified only in the resistance-linked sequence (Fig. 3). Finally, the EtacMccg13 AFLP marker was converted to a dominant SCAR marker, CcR9M1-SCAR (Fig. 3).

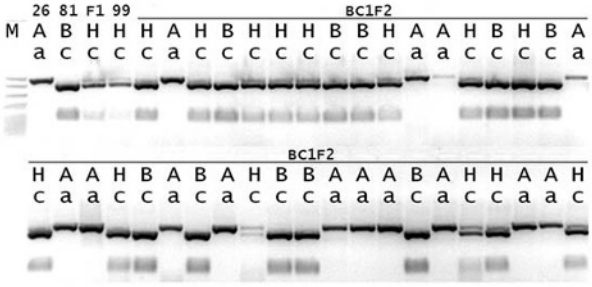

Fig. 2. Segregation patterns of a CaR12.2M1-CAPS marker converted from the AFLP marker EtagMcgt04. 26, susceptible parent 'SP26' (*C. annuum* 'Matikas'); 81, resistant parent 'PBC81' (*C. baccatum*); F1, hybrid between 'SP26' and 'PBC81'; 99, highly resistant individual in BC_1F_1 population (SP26/PBC81//SP26); BC1F2, segregating progeny selfed from '99'; capital letters (A, B, and H), scored data of CaR12.2M1-CAPS; lowercase letters (a and c), scored data of EtagMcgt04.

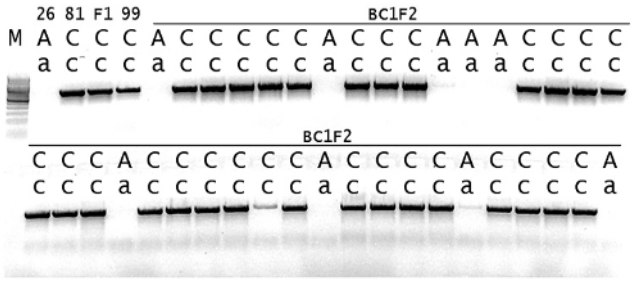

Fig. 3. Segregation patterns of a CaR9M1-SCAR marker converted from the AFLP marker EtacMccg13. 26, susceptible parent 'SP26' (*C. annuum* 'Matikas'); 81, resistant parent 'PBC81' (*C. baccatum*); F1, hybrid between 'SP26' and 'PBC81'; 99, highly resistant individual in BC_1F_1 population (SP26/PBC81//SP26); BC1F2, segregating progeny selfed from '99'; capital letters (A and C), scored data of CaR12.2M1-CAPS; lowercase letters (a and c), scored data of EtacMccg13.

Table 1. Selection efficiency of markers linked to major loci resistant to pepper anthracnose.

Marker[z]	Type	LG	ANOVA		Mean of resistance[y]			95% confidence interval for mean	Selection efficiency of marker			
			F value	Significance	AA[x]	AB	BB	Upper bound (marker type)	Total[w] (A)	R (B)	S	% (B/A*100)
HpmsE032	SSR	12	16.77	< 0.000	5.79	1.95	0.70	1.22 (BB)	23	18	5	78.3
CaR12.2M1	CAPS	12	18.50	< 0.000	5.66	1.91	0.75	1.21 (BB)	25	18	7	72.0
HpmsE143	SSR	9	17.50	< 0.000	8.52	1.30	0.57	1.49 (BB)	6	4	2	66.7
CcR9M1	SCAR	9	60.41	< 0.000	9.52	1.21		1.93 (AB or BB)	40	33	7	82.5

[z]HpmsE032 and HpmsE143: SSR markers previously reported by Yi et al. (2006); CaR12.2M1 and CcR9M1: STS markers newly developed from AFLP markers EtagMcgt04 and EtacMccg13, respectively.
[y]Resistance was evaluated by overall region diameter, as previously described by Voorrips et al. (2004).
[x]A, allele derived from the susceptible parent 'SP26' (*C. annuum*); B, allele originating from the resistant parent 'PBC81' (*C. baccatum*).
[w]Total: total number of BC_1F_2 progeny selected by each marker; R, resistant lines; S, susceptible lines.

Selection Efficiency of Major QTL-linked Markers

To test the validity of the molecular markers linked to major resistance loci to anthracnose, phenotype data in the BC_1F_2 population were compared to scoring data of four markers: two *CaR12.2*-linked (HpmsE032 and CaR12.2M1-CAPS) and two *CcR9*-linked (HpmsE143 and CcR9M1-SCAR) markers (Table 1). These markers were highly significant for resistance according to an ANOVA, and the allele (B in Table 1) originating from 'PBC81' (*C. baccatum*, resistant parent) increased the resistance (Table 1). The selection efficiencies were 78.3% for HpmsE032, 72% for CaR12.2M1-CAPS, 66.7% for HpmsE143, and 82.5% for CcR9M1-SCAR, using criteria of an upper boundary 95% confidence interval for BB or B_ mean (Table 1). The selection efficiency was calculated by the % value of phenotypic resistant plants among the total plants selected by the homozygous resistance markers (BB) in the segregating populations used in this study (Table 1).

Discussion

Detailed concepts and strategies for using molecular markers in QTL mapping for marker-assisted selection (MAS) have been comprehensively reviewed by Collard et al. (2005). This study shows mapping of additional markers linked to the major resistance loci to anthracnose and conversion of the AFLP markers closely linked to the loci into STS markers for use in MAS.

In total, 51 AFLP markers were identified through the extreme BSA-AFLP analysis. AFLP, a high-throughput marker technique that generates multiple loci per primer combination, is usually preferred for increasing marker density (Vos et al., 1995), and BSA is useful to identify additional markers linked to specific chromosomal regions (Giovannoni et al., 1991). Eight markers were successfully mapped on linkage groups LG 12 and LG 9, in which the major resistance loci were present, and five of these markers were closely linked to the resistance loci (Fig. 1).

Simple PCR-based markers are needed for breeders because the AFLP technique is complicated, time-consuming, laborious, and expensive. Conversion of AFLP markers to STS markers has been difficult (Meksem et al., 2001) because AFLP polymorphisms mostly result from SNPs on *Eco*RI or *Mse*I sites (Bradden and Simon, 1998). However, progress in AFLP fragment sequencing using T-vector ligation systems and sequencing of flanking regions using the universal genome walking system (Siebert et al., 1995) make conversion simpler. In this study, we developed two STS markers using a pGEM-T Easy Vector system (Promega, WI, USA) and the GenomeWalker™ Universal Kit (Clontech, CA, USA): a CAPS marker for a major resistance locus (*CaR12.2*) to an isolate of *C. acutatum* (Figs. 1 and 2), and a SCAR marker for a major resistance locus (*CaR9*) to an isolate of *C. capsici* (Figs. 1 and 3). These resistance loci, in which phenotypic variances were 20.46% for *CaR12.2* and 78.91% for *CcR9*, respectively, were identified and validated in a previous study (Lee et al., 2010). The selection efficiency of the markers located around *CaR12.2* locus was much higher than that of phenotypic variance of the locus in previous QTL analysis. It was due to that the different criteria used for those analyses. The selection efficiency was calculated by the qualitative-phenotypic criteria, resistance (R) or susceptible (S) but the QTL analysis used quantitative criteria from 0 to 100 in disease incidence index.

Development of codominant markers is important in pepper breeding for dominant disease resistance, since it is difficult to distinguish between homozygous and heterozygous resistant plants using the phenotype. Here, we could not convert a *CcR9*-linked AFLP marker (EtgtMcgt03) into codominant markers because several primer sets designed for amplifying the internal and flanking regions of Etgt Mcgt03 produced the expected PCR product in the resistant parent but not in the susceptible parent. This means that sequences of EtgtMcgt03 are only present in the resistant parent; therefore, we could only develop a dominant SCAR marker.

To increase the efficiency and effectiveness for selecting resistant plants, it is necessary that opposite flanking markers are converted to STS markers. Selection efficiency of each marker was more than 70% (Table 1), indicating that anthracnose resistance is controlled by a major resistance locus. However, minor QTL-linked markers must be applied to increase the accuracy of MAS. These simple PCR-based markers will be useful for breeding cultivars with enhanced resistance to anthracnose, for pyramiding resistances to both *C. acutatum* and *C. capsici*, and for the further characterization of the locus, including isolation of genes responsible for the resistance.

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