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Full Length Research Paper

Conversion of the random amplified polymorphic DNA (RAPD) marker UBC#116 linked to *Fusarium* crown and root rot resistance gene (*Frl)* into a co-dominant sequence characterized amplified region (SCAR) marker for marker-assisted selection of tomato

Hai Thi Hong Truong[1,2], HakSoon Choi[1], Myoung Cheoul Cho[1] and Hye Eun Lee[1]*

[1]Vegetable Crop Research Division, Horticultural Department, National Institute of Horticultural and Herbal Science (NIHHS), Rural Development Administration (RDA), 540-41 Tap-Dong, Suwon, 441-440, Korea.
[2]Department of Horticultural Science, Agronomy Faculty, Hue University of Agriculture and Forestry (HUAF), 102 Phung Hung, Hue, Vietnam.

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***Fusarium* crown and root rot of tomato (*Solanum lycopersicum*) is the disease caused by the fungal pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL). The most effective way to control this disease is to plant resistant varieties. Markers tightly linked to *Fusarium* crown and root rot could be used in breeding programs to introgress crown rot resistance into new varieties. In this study, we converted the random amplified polymorphic DNA (RAPD) marker UBC#116, linked to the *Fusarium* crown and root rot resistance gene (*Frl)*, into a co-dominant sequence characterized amplified region (SCAR) marker. A fragment of about 480 bp, linked to the *Frl* gene, was polymerase chain reaction (PCR) amplified with the use of the UBC#116 primers, cloned and sequenced. A pair of primers were designed and PCR amplification was performed to develop a new SCAR marker for the *Frl* gene. The new marker was applied for the analysis of 96 tomato genotypes. The RAPD marker UBC#116 was also used and it revealed that the markers were equivalent to each other. However, the development of the new co-dominant SCAR marker has made marker-assisted selection (MAS) more practical, rapid and efficient.**

Key words: Fusarium oxysporum f. sp. radicis-lycopersicum (FORL), marker-assisted selection (MAS), Solanum lycopersicum, breeding.

INTRODUCTION

The soil-borne fungus, *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL), which causes *Fusarium* crown and root rot of tomato (*Solanum lycopersicum*), was first observed in the southern part of Japan in 1965. In 1994, the disease was reported in Korea (Lee et al., 1994). The disease is often referred to as 'crown rot' (Fazio et al., 1999) and attacks at least 36 other species (Menzies et al., 1990). To control the disease safely, several methods have been reported such as biocontrol (Liu et al., 2010) or soil solarization (Sivan and Chet, 1993); however, none of these methods were effective because airborne spores of FORL can re-infect the soil. Thus, genetic resistance represents the most promising method for controlling crown and root rot in tomatoes.

Crown rot resistant alleles were identified from the related wild species, *S. peruvianum*, such as plant introduction (PI) 126944, 128650 and 126926, and

*Corresponding author. E-mail: helee72@korea.kr. Tel: +82-31-290-6200. Fax: +82-31-290-6219.

introduced into *S. lycopersium* (Laterrot and Pecaut, 1969; Yamakawa and Nagata, 1975; Elkind et al., 1988). Berry and Oakes (1987) reported that crown rot resistance segregated as a single dominant gene and it was later confirmed and designated as the *Frl* gene (Vakalounkis, 1988). *Frl is* linked with both tobacco mosaic virus resistant genes, *Tm-1* (Elkind, 1988) and *Tm-2* (Laterrot and Couteaudier, 1989), which are alleles at the same locus. Laterrot and Moretti (1991) had performed allelic tests on three crown rot resistant sources and suggested that all three sources have the same *Frl* allele. Several studies have reported that the *Frl* gene is tightly linked with restriction fragment length polymorphism (RFLP) marker TG101, which is linked to *Tm-2*^{2}, on chromosome 9 (Young et al., 1988; Young and Tanksley, 1989; Laterrot and Moretti, 1991). However, studies of the genetic distance between *Frl* and *Tm-2* have been reported to be approximately 5.1 ± 1.07 cM in an F_3 population (Vakalounakis et al., 1988) and 10.6 cM in an F_5 population (Fazio et al., 1999). Thus, using markers linked to *Tm-2* for screening crown rot resistance is not precise or efficient. Markers tightly linked to the *Frl* gene could be used as breeding tools for the introgression of crown rot resistance into new varieties to avoid the inconsistent virulence of FORL caused by the influence of environmental factors on disease development (Jones et al., 1990). In addition, in the case of moderate resistance, using linked markers is most useful for the selection of resistant plants, for which phenotypic selection may be less effective. Thus, the efficiency of selection and flexibility of a breeding program will be increased.

Three RAPD markers, UBC# 116, 194 and 655, tightly linked to the *Frl* gene have been identified (Fazio et al., 1999). Of these, UBC# 116 and 194, are dominant markers and are not very easy to use in marker-assisted selection (MAS), because heterozygous plants cannot be distinguished from homozygous plants. UBC#655 is a co-dominant marker. However, the distance between the *Frl* gene and UBC#194, 116 and 655 is about 5, 7, and 8.5 cM, respectively (Fazio et al., 1999). UBC#194 is the most tightly linked marker; nevertheless, resistant fragment was not generated in our preliminary screening. Tanyolac and Akkale (2010) have used this primer to screen a set of tomato lines, but none of the samples showed the *Frl* linked marker. The objective of this study was to convert the RAPD marker UBC#116 tightly linked to *Frl* into a more suitable SCAR marker and use the markers for selection of tomato lines carrying the *Frl* allele from tomato germplasm.

MATERIALS AND METHODS

Plant and DNA extraction

Ninety-six (96) tomato genotypes were provided by National Institute of Horticultural and Herbal Science (NIHHS), Rural Development Administration (RDA), Korea. All genotypes belonged to *S. lycopersicum*. Genomic DNA was extracted from leaves of young seedlings (3 to 4 true leaves) using DNeasy Plant Kit (96-well format) from QIAGEN (Qiagen GmbH, Hilden, Germany). The DNA concentration was measured on a Nanovue spectrophotometer (GE Healthcare, U.K). The quality of the DNA was inspected using agarose gel electrophoresis and spectral absorbance (the A_{260}/A_{280} ratio).

Cloning and sequencing of RAPD fragments

RAPD analysis was performed by the methods of Fazio et al. (1999). Four replications were conducted. The RAPD fragments obtained from tomato line IT201665 and F_1 hybrid 'Wonmeongjinjuhwang' were excised from 1% agarose gels and purified with a QIAquick gel extraction kit (Qiagen, Germany). The fragments were cloned using TOPO TA cloning kit following the manufacturer instructions (Invitrogen, USA). Plasmid DNA was extracted using Core-one plasmid miniprep kit (Korea) and sent to sequencing company CoreBio (Korea) for sequencing. The sequences were cleaned and aligned using the program BioEdit 7.0 (Hall, 1999).

Primer design and SCAR marker analysis

A pair of primers were designed according to the sequence obtained using the program Primer3 4.0 (Rozen and Skaletsky, 2000). Oligonucleotide primers were synthesized by Bioneer Corp (Korea). Each PCR reaction was carried out in a total reaction volume of 25 µl containing 15 to 20 ng of genomic DNA, 200 µM deoxyribonucleotide triphosphate mix (Roche, Korea), 1 X PCR buffer, 1 U of DNA *Taq* polymerase (Roche, Korea) and 0.25 µM of each primer. PCR was performed on an Eppendorf Mastercycler Gradient (USA). The amplification profile consisted of an initial denaturation for 5 min at 94°C followed by 35 cycles of PCR amplification under the following parameters: 1 min at 94°C, 1 min at the annealing temperature of 60°C, and 1 min of primer elongation at 72°C. A final incubation at 72°C for 5 min was programmed to allow completion of primer extension. Amplified products were incubated with a 1:10,000 dilution of the SYBR Green I nucleic acid gel stain (Invitrogen, USA) for 20 min and separated on a 1.5% agarose gels using 0.5X TBE buffer for 2 h at 120 V and photographed under UV light. A 100 bp ladder was used as a molecular weight marker.

RESULTS

Screening tomato germplasm

Three RAPD markers, UBC#116, 194, 655, linked to *Frl* (Fazio et al. 1999), were initially screened on 96 tomato lines collected from NIHHS, RDA, Korea (Figure 1). Among these, 30 lines carried UBC#116-resistant fragment, four genotypes generated UBC#655-homozygous resistant fragment (Table 1), and none of tomato lines generated UBC#194-resistant fragment as described by Fazio et al. (1999). Heterozygous patterns were observed in 16 genotypes with the RAPD marker UBC#655 (Table 1).

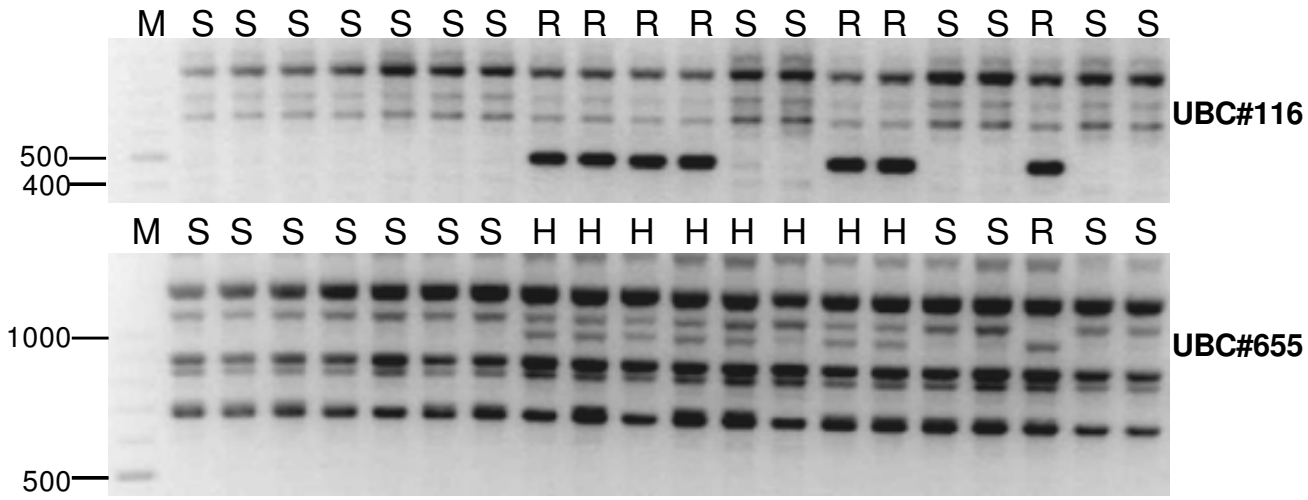

Figure 1. Agarose gel electrophoresis of selected tomato germplasm screened using RAPD markers UBC#116 and 655 markers. Lanes M, 100 bp molecular ladder; R, resistant; S, susceptible; H, heterozygous.

Table 1. Genotyping results of tomato germplasm tested.

Genotype	Origin	Genotyping/marker		
		UBC#116	SCU116	UBC#655
IT142186	NAC (National Agrobiodiversity Center)	S	S	S
IT142188	NAC (National Agrobiodiversity Center)	S	S	S
IT142190	NAC (National Agrobiodiversity Center)	S	S	S
IT142191	NAC (National Agrobiodiversity Center)	S	S	S
IT142195	NAC (National Agrobiodiversity Center)	S	S	S
IT156673	NAC (National Agrobiodiversity Center)	S	S	S
IT156677	NAC (National Agrobiodiversity Center)	R	H	S
IT160384	NAC (National Agrobiodiversity Center)	R	H	S
IT180572	NAC (National Agrobiodiversity Center)	S	S	S
IT189947	NAC (National Agrobiodiversity Center)	S	S	S
IT200624	NAC (National Agrobiodiversity Center)	S	S	S
IT201639	NAC (National Agrobiodiversity Center)	S	S	S
IT201642	NAC (National Agrobiodiversity Center)	S	S	S
IT201657	NAC (National Agrobiodiversity Center)	R	H	S
IT201665	NAC (National Agrobiodiversity Center)	R	R	R
IT203278	NAC (National Agrobiodiversity Center)	R	H	S
IT203280	NAC (National Agrobiodiversity Center)	S	S	S
IT203287	NAC (National Agrobiodiversity Center)	S	S	S
IT203291	NAC (National Agrobiodiversity Center)	S	S	S
IT203294	NAC (National Agrobiodiversity Center)	S	S	S
IT203296	NAC (National Agrobiodiversity Center)	S	S	S
IT203449	NAC (National Agrobiodiversity Center)	R	H	S
IT203452	NAC (National Agrobiodiversity Center)	R	H	S
IT203455	NAC (National Agrobiodiversity Center)	R	H	S
IT203466	NAC (National Agrobiodiversity Center)	S	S	S
IT203468	NAC (National Agrobiodiversity Center)	S	S	S
IT207036	NAC (National Agrobiodiversity Center)	S	S	S
IT207222	NAC (National Agrobiodiversity Center)	S	S	S
IT207244	NAC (National Agrobiodiversity Center)	S	S	S

Table 1. Continues

K044840	NAC (National Agrobiodiversity Center)	S	S	S
K052128	NAC (National Agrobiodiversity Center)	S	S	S
K060617	NAC (National Agrobiodiversity Center)	S	S	S
K060625	NAC (National Agrobiodiversity Center)	S	S	S
K060627	NAC (National Agrobiodiversity Center)	S	S	S
K060650	NAC (National Agrobiodiversity Center)	R	R	H
K060707	NAC (National Agrobiodiversity Center)	S	S	S
K100254	NAC (National Agrobiodiversity Center)	S	S	S
K100255	NAC (National Agrobiodiversity Center)	S	S	S
K100256	NAC (National Agrobiodiversity Center)	S	S	S
K100259	NAC (National Agrobiodiversity Center)	S	S	S
K100262	NAC (National Agrobiodiversity Center)	S	S	S
K100263	NAC (National Agrobiodiversity Center)	S	S	S
K100265	NAC (National Agrobiodiversity Center)	S	S	S
K100266	NAC (National Agrobiodiversity Center)	S	S	S
K100267	NAC (National Agrobiodiversity Center)	S	S	S
K101301	NAC (National Agrobiodiversity Center)	S	S	S
K121155	NAC (National Agrobiodiversity Center)	S	S	S
K124968	NAC (National Agrobiodiversity Center)	S	S	S
K125501	NAC (National Agrobiodiversity Center)	S	S	S
K125502	NAC (National Agrobiodiversity Center)	S	S	S
K126944	NAC (National Agrobiodiversity Center)	S	S	S
K130763	NAC (National Agrobiodiversity Center)	R	H	S
K133673	NAC (National Agrobiodiversity Center)	S	S	S
K146467	NAC (National Agrobiodiversity Center)	S	S	S
Wonmeonghwageoja	Commercial var, China	S	S	S
Yachesee	Commercial var, China	R	R	H
Sinkwanwoo	Commercial var, China	R	R	H
Damangolyo	Commercial var, China	R	R	H
Wonmeong (rkn-108)	Commercial var, China	R	R	H
Hapjak206	Commercial var, China	S	S	H
Wonmeongokyeohong	Commercial var, China	S	S	H
Sinkwan106	Commercial var, China	R	R	H
Taegong1	Commercial var, China	R	R	H
Shinbon-n88	Commercial var, China	R	H	S
Wonmeong-909	Commercial var, China	S	S	S
Wonmeongjinjuhwang	Commercial var, China	R	R	R
Daehongangdo	Commercial var, China	S	S	S
Alt516	Commercial var, China	S	S	S
Bizuhongka	Commercial var, China	S	S	S
Hapjak903	Commercial var, China	R	R	H
Hapjak906	Commercial var, China	R	H	S
Sinkwan1ho	Commercial var, China	R	R	H
Hapjak908	Commercial var, China	S	S	S
Hongjinjubunga	Commercial var, China	S	S	R
Shinbon-158	Commercial var, China	R	R	H
Hapjak919daehongbunga	Commercial var, China	R	R	H
Hapjak928bunga	Commercial var, China	R	R	H
Woonaehwangsija	Commercial var, China	R	H	S
Daejosija	Commercial var, China	S	S	S
Jukbooyou	Commercial var, China	S	S	S

Table 1. Continues

Daehwangsija	Commercial var, China	S	S	S
Angdobunga	Commercial var, China	S	S	S
Chumjuengbunga	Commercial var, China	S	S	S
Bukdoseoungsea	Commercial var, China	S	S	S
Americadaehong	Commercial var, China	S	S	S
T1-mobir	Local var, Uzbekistan	S	S	S
T2-riogranidu	Local var, Uzbekistan	S	S	S
T3-bargagradski	Local var, Uzbekistan	S	S	S
T4-timkaiyuorit22	Local var, Uzbekistan	S	S	S
T5-uzbekistan	Local var, Uzbekistan	R	R	H
T6-yousoofarboski	Local var, Uzbekistan	R	H	S
Galkandeu	Local var, Uzbekistan	R	R	R
Avemaria	Local var, Uzbekistan	R	H	S
Seyihum	Local var, Uzbekistan	S	S	S
Bohol	Local var, Uzbekistan	S	S	S
Arisenna	Local var, Uzbekistan	R	R	H

Cloning and sequencing of RAPD fragments

The UBC#116-resistant fragments generated from tomato line IT201665 and F_1 hybrid 'Wonmeongjinjuhwang' were used for cloning. The reason why these genotypes were selected was that both had UBC#116 and 655-resistant fragments. To avoid mismatch during amplification, four replications were conducted. The four amplified fragments from each genotype were cloned. At least, five different individual colonies with respect to a single RAPD clone of each replication were selected to confirm the presence and correct orientation of the insert. All the clones had the corrected insert (data not shown). Thus, one RAPD clone from each of the four replications was selected for sequencing. The length of the RAPD fragments isolated from IT201665 and 'Wonmeongjinjuhwang' was 482 bp (Figure 2) and homologous. The sequencing results were consistent among replications and the terminal 10 bases of 5'- and 3'-ends of the clones exactly matched the sequence of primer (Figure 3).

Amplification and applicability of the SCAR marker

A pair of 20-mer oligonucleotide primers was synthesized based on the sequence of the RAPD clone. The fragment amplified resistant and susceptible lines approximately 400 and 500 bp in size, respectively, as compared to the 482-bp fragment from the resistant lines using UBC#116. In heterozygous lines, the SCAR marker amplified both 400- and 500-bp fragments (Figure 4). This result indicates that dominant marker UBC#116 was converted into a co-dominant SCAR marker showing polymorphism. This new marker was designated as SCU116.

The applicability of SCU116 was tested using 96 tomato genotypes (Figure 5); of which 30 genotypes carried the resistant allele amplified by RAPD marker UBC#116. Of these 30 resistant genotypes, 17 genotypes amplified the 400 bp fragment of SCAR marker SCU116 only, while the rest were heterozygous. Therefore, RAPD and developed SCARs marker provided consistent results.

DISCUSSION

In tomato, comprehensive molecular maps have been developed (Foolad, 2007). The molecular makers adjacent to the genes of interest located on these maps will be available for use in MAS. In many cases, PCR based methodology would be more suitable than RFLP technique because of its lower cost and ease of use. Thus, PCR based markers will meet these challenges because the small amount of DNA required for PCR analysis makes it possible to analyze plants as early as the single leaf stage or prior to germination with half seeds (Chunwongse et al., 1993). This decreases the time, cost and growing space, and ensures that breeders are aware of individual genotypes before crosses are made. In this study, a SCAR marker was developed from a RAPD marker linked to the *Frl* gene (Fazio et al., 1999). One disadvantage of RAPD markers is that they are usually dominant; hence, does not allow the differentiation between heterozygous and homozygous plants. RAPD marker UBC#655 revealed a co-dominant pattern between FORL resistant and susceptible plants and can be used in MAS. However, this RAPD marker is loosely linked to the *Frl* locus when compared with RAPD UBC#116 (Fazio et al., 1999), and thus will be of limited

YDDVGDV*GVNPDVTLQIVMHVNLLIFIDF*WLKDPWDV*EVTWSVQKIWLFHTTLVFYLFNFLSYRVI*SISEIQKIKKCSRT
VNIVLHINQTFGV*MLGWSQ*ITPQSRINLY*SNVKETIILL*IVD*NSST*LSFNYIDRNINNKIFLLPIKVASS

Figure 2. Amino acid sequence of the UBC#116 fragment.

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                       10                                                        470         480
                  ....|....|....|....|....|....|....|....|....|....|....|....|..
UBC116-IT201665-1 TACGATGACGTCGGT.............................CAAAGTAGCGTCATCGTA
UBC116-IT201665-2 TACGATGACGTCGGT.............................CAAAGTAGCGTCATCGTA
UBC116-IT201665-3 TACGATGACGTCGGT.............................CAAAGTAGCGTCATCGTA
UBC116-IT201665-4 TACGATGACGTCGGT.............................CAAAGTAGCGTCATCGTA
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Figure 3. Selected sequence of UBC#116 fragment of four replications. Arrows indicate the terminal 10 bases of 5'- and 3'-ends of primer sequence.

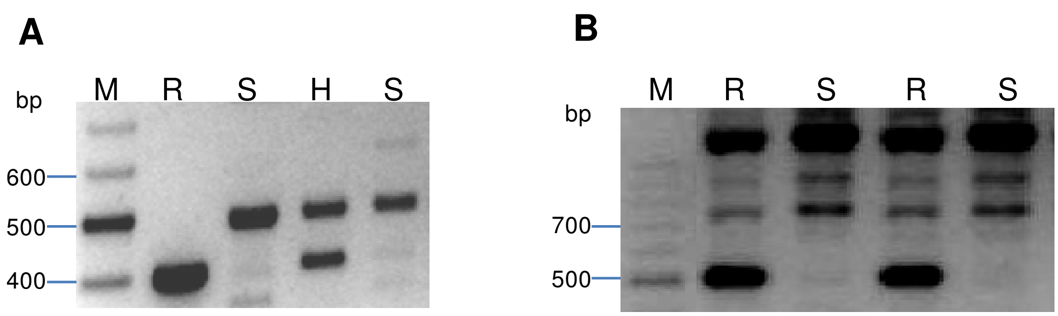

Figure 4. Amplified products of the new co-dominant SCAR marker SCU116 (A) when compared with those of RAPD marker UBC#116 (B). Lanes M, 100 bp molecular ladder; R, resistant; S, susceptible; H, heterozygous.

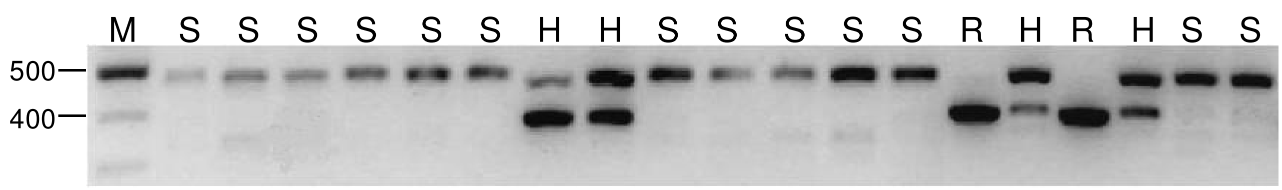

Figure 5. Agarose gel electrophoresis of 19 tomato germplasm screening using SCAR marker SCU116. Lanes M, 100 bp molecular ladder; R, resistant; S, susceptible; H, heterozygous.

use in MAS. The objective of this study was to develop a co-dominant marker that is more closely linked to the *Frl* locus to provide more effective selection and reduce error due to recombinants. Dominant RAPD marker UBC#194 is the most tightly linked to *Frl* locus (Fazio et al., 1999), but did not amplify in any of the 96 tomato genotypes which revealed the resistant fragment. This result is consistent with the screening result of Tanyolac and Akkale (2010). Marker UBC#116 is still over 7 cM from the *Frl* locus (Fazio et al., 1999), but to date, no markers completely linked to the *Frl* gene has been identified. Thus, the converted co-dominant SCAR marker SCU116 still offers breeders a better selection tool, because heterozygous plants can be detected and the need for screening plants with FORL, which can be unreliable due to the influence of environmental factors on disease evaluation (Jones et al., 1990), can be circumvented. The newly developed SCAR marker revealed heterozygous patterns in 13 genotypes that also showed resistance with the RAPD marker UBC#116. The remaining tomato lines showed consistent results between the amplifications using primers of RAPD marker UBC#116 and SCAR marker SCUB116. This result demonstrates the potential for application of converted co-dominant SCAR

marker for selection for *Fusarium* crown and root rot resistance in tomato.

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