Development of a Gene-based Marker for the non-ripening (nor) Gene in Cultivated Tomato

Thim Thi Nguyen[1] and Sung-Chur Sim[1,2]*

[1]Department of Bioresources Engineering, Sejong University, Seoul 05006, Korea
[2]Plant Engineering Research Institute, Sejong University, Seoul 05006, Korea

*Corresponding author: sungchur@sejong.ac.kr

Abstract

Long shelf-life is an important trait for providing fresh fruits to consumers in cultivated tomato (*Solanum lycopersicum* L.) The molecular mechanism of fruit ripening in tomato was previously defined by identifying several genes associated with abnormal ripening. Of these genes, *non-ripening* (*nor*) encodes a member of NAC domain family transcription factors that is important for diverse physiological processes, including fruit ripening. In this study, we investigated sequence variations associated with the *nor* mutation to develop a gene-based marker. Two varieties of *S. lycopersicum* with representative *nor* mutants (LA1793 and LA3013) and a wild-type variety (San Marzano) were used for sequence analysis of the *nor* gene. A total of eight primer sets were designed to sequence the full length of *nor* gene including a 2.8 kb open reading frame (three exons and two introns) as well as 2.0 kb region upstream from the start codon. Analysis of the 4.8 kb sequence for the *nor* gene found a 2 bp insertion and deletion (InDel) in the longest exon (725 bp) that results in a frame-shift mutation in the two mutant varieties. This InDel was used to genotype a collection of additional 81 varieties consisting of 30 inbred and 51 commercial F_1 hybrid varieties. Of these varieties, two inbred and one F_1 hybrid varieties had *nor* mutations that were in homozygous or heterozygous forms. These results will be useful for marker-assisted selection (MAS) for improving long shelf-life and quality of fruit in tomato breeding programs.

Additional key words: fruit ripening, insertion and deletion, long self-life, marker-assisted selection, vegetable

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely consumed fresh fruit vegetables in the world. Tomato fruits make a significant contribution to human nutrition due to their abundant vitamins, minerals, and antioxidants (Raiola et al., 2014; Perveen et al., 2015). As a climacteric and perishable vegetable, tomato fruit has a relatively short life after ripening due to biological processes affecting fruit quality during the postharvest stage (Zapata et al., 2008). The quality and nutritional value of ripening fruit are greatly reduced by postharvest handling and storage practices (Sablani et al., 2006), with a loss of fresh

tomato fruit that can be as much as 45.32% (Kasso and Bekele, 2016). Therefore, a number of strategies have been developed to improve fruit quality and shelf-life such as refrigeration, heat treatment, modified atmosphere packaging (MAP), and 1-methylcyclopropent (1-MCP) and calcium chloride ($CaCl_2$) application (Arah et al., 2016). Although effective in limiting postharvest losses, these methods can be costly and time-consuming, especially for small farms (Hoering, 2012). The use of ripening mutant genes, however, eliminates these disadvantages and is an effective strategy in tomato breeding programs.

Several tomato ripening genes have been reported including *ripening-inhibitor* (*rin*) (Robinson and Tomes, 1968; Vrebalov et al., 2002), *non-ripening* (*nor*) (Tigchelaar et al., 1973), *Colorless non-ripening* (*Cnr*) (Thompson et al., 1999; Eriksson et al., 2004; Manning et al., 2006), *Green-ripe* (*Gr*) (Kerr, 1958; Barry and Giovannoni, 2006), *Never-ripe* (*Nr*) (Lanahan et al., 1994), and *alcobaca* (*alc*) (Kopeliovitch et al., 1981). These genes participate independently and cooperatively in the fruit ripening process. For example, the *rin* mutation produces a partially deleted MADS-box protein that is a ripening-specific transcription factor and results in effectively blocking the fruit ripening process leading to green fruits (Vrebalov et al., 2002). The heterozygous mutation of the *rin* gene (*rin/Rin*) is often used to produce slow ripening and long shelf-life tomatoes (Giovannoni, 2007). Similarly, the mutation in the *nor* gene encoding a NAC transcription factor fails to initiate the normal ripening process and results in fruits that do not soften (Lincoln and Fischer, 1988; Giovannoni, 2004). The *nor* gene can be also used in the heterozygous form (*nor/Nor*) to improve shelf-life of tomato fruit. The NAC transcription factor family is important for diverse physiological process during development including stress response, flowering, and senescence (Martel et al., 2011). The *nor* mutation enhances disease resistance by up-regulating the expression of several genes associated with defense mechanisms that biotic stress, including the resistance of tomato fruit to *Botrytis cinerea* (Cantu et al., 2009).

Conventional plant breeding based on phenotypic selection is generally labor-intensive and time-consuming. Conversely, the use of molecular markers associated with traits of interest can be a cost-effective and accurate option to develop new elite varieties (Phan and Sim, 2017). Recent advances in next-generation sequencing technology have facilitated association mapping with genome-wide molecular markers such as single nucleotide polymorphism (SNP) and insertion and deletion (InDel). These efforts have led to the discovery of a number of markers associated with major genes and quantitative trait loci (QTL) (Truong et al., 2015; Devran et al., 2016; Veerappan et al., 2016). However, these markers are not always effective for breeding populations due to linkage disequilibrium (LD) decay. Gene-based markers can be more practical relative to the gene-associated markers for marker-assisted selection (MAS) in breeding programs. Although the molecular and biochemical basis of fruit ripening has been intensively studied in tomato, molecular markers for breeding have been less utilized in comparison to other traits such as disease resistance. Recently, a sequence characterized amplified region (SCAR) marker was developed for the *rin* mutation (Kim et al., 2013); however, few studies have developed molecular markers for the *nor* mutation, which is more effective for long shelf-life relative to the *rin* gene (Ng, 1976). Thus, the objectives of our study were to 1) detect sequence variations between the *nor* mutant and wild type varieties, 2) develop a gene-based marker for MAS to improve tomato fruit quality and long shelf-life, and 3) investigate the *nor* mutation in a collection of 81 inbred and commercial F_1 hybrid varieties based on our gene-based marker.

Materials and Methods

Plant Materials

Three tomato accessions, 'San Marzano' (wild-type allele, *Nor/Nor*), 'LA1793', and 'LA3013' (both mutant alleles, *nor/nor*) were provided by the C. M. Rick Tomato Genetics Resource Center at University of California, Davis, CA and used to investigate sequence variation in the *nor* gene. In addition, we used 81 other varieties to validate the resulting *nor* sequence variation(s). This germplasm panel consisted of 30 inbred and 51 commercial F_1 hybrid varieties from public and private breeding programs in South Korea. The inbred varieties were collected from the National Agrobiodiveristy Center (20 varieties) and National Institute of Horticultural & Herbal Science (10 varieties) at Rural Development Administration (RDA). For the F_1 hybrids, 51 varieties were derived from Bunong Seed (10 varieties), Monsanto-Korea (nine varieties), Nongwoo Bio (six varieties), Syngenta-Korea (five varieties), Takki-Korea, PPS (three varieties from each company), Jinheung Seed, NH-Nonghyup, Koregon, Asian Seed, and Konong (two varieties from each company); and the rest five varieties were from Sky Seed, Sakata-Korea, Dongwon Nongsan, Hyeondae Seed, and Gana Seed.

DNA Extraction

Genomic DNA was isolated from young leaves from seedlings using the CTAB method with minor modifications (Kabelka et al., 2002). Young leaf tissue was placed in a 2.0 mL micro-centrifuge tube containing 350 µL DNA extraction-lysis buffer and ground in a TissueLyser II (QIAGEN, Valencia, CA, USA) at 300 strokes per minute for 5 min. After incubation at 65°C for 20 min, 350 µL of chloroform: isoamyl (24:1) was added and then centrifuged at 5,000 g for 10 min. The supernatant was separated and mixed with 200 µL ice cold isopropanol, keep stable at 4°C for 1 h, followed by centrifugation at 5,000 g for 15 min. The DNA pellet was rinsed twice with 70% ethanol, dried at room temperature, and re-suspended in 100 mL TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). The quantity and quality of DNA sample were measured using agarose gel electrophoresis and diluted to 50 ng·µL⁻¹ for PCR amplification and high-resolution melting (HRM) analysis.

Sequencing the *nor* Gene and Polymorphism Detection

The full length of the *nor* gene (SGN Gene ID: Solyc10g006880) was retrieved from the tomato reference genome assembly version 2.50 (Tomato Genome Consortium, 2012; https://www.solgenomics.net). A set of primers was designed to amplify all of the exons and introns as well as 2 kb of the upstream region from the start codon using the Primer3Plus version 2.4.0 (Untergasser et al., 2012). PCR amplification was performed in a total reaction volume of 50 µL that contained 50-100 ng genomic DNA, 0.25 µM each primer, 0.2 mM dNTPs, 1X PCR buffer, and 0.5 U DNA *Taq* polymerase (Geneslabs, Seongnam, Korea). The amplification profile consisted of an initial denaturation for 3 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at an selected annealing temperature (50°C to 60°C depending on the melting temperature of primers), 45 s at 72°C, and one cycle of 7 min at 72°C as a final extension step. The amplified product was separated on a 1.5% agarose gel containing 1X RedSafe™ Nucleic Acid Staining Solution (20,000X) (JH Science, Lynnwood, WA, USA) in 1X TBE at 200 V for 1 h. The target DNA was collected and purified using the MinElute® Gel Extraction Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions and resulted in samples that were used for Sanger sequencing (Cosmogenetech, Seoul, Korea). The resulting DNA sequences were trimmed and aligned using the Staden Package software (Bonfield et al., 1995) and Clustal Omega tool (http://

www.ebi.ac.uk/Tools/msa/clustalo/). Sequence variations between the mutants ('LA1793' and 'LA3013') and wild-type ('San Marzano') varieties were identified by visual inspection of the sequence alignment.

High-resolution Melting Analysis in the Germplasm Collection

The primers for high-resolution melting (HRM) analysis were designed to produce an expected product size between 100-300 bp using the Primer3Plus version 2.4.0. The primer sequences were then analyzed using In Silico PCR at the SOL Genomic Network (https://solgenomics.net/tools/in_silico_pcr) to detect any possible unspecific products, ensuring the PCR amplification efficiency and HRM accuracy. HRM analysis was performed in a total volume of 20 μL using the LightCyler96 Real-Time PCR System (Roche Diagnostics, Indianapolis, IN, USA). The reaction mixture contained 50 ng qualified genomic DNA, 0.25 μM forward and reverse primers, and LightCycler® 480 HRM Master Mix 1X (Roche Diagnostics). The amplification was achieved by the following the protocol: 10 min initial denaturation at 95°C, 45 cycles of 10 s for denaturation at 95°C, 15 s at 55°C, 15 s at 72°C, followed by denaturation at 95°C for 15 s, 50°C for 10 s, 50°C for 50 s, 97°C for 1 s, with HRM ramping between 50°C and 97°C at 2.2°C/s increments.

Results and Discussion

Discovery of Sequence Variation in the *nor* Gene in Tomato

The *nor* gene originated from a spontaneous mutation in tomato on the short arm of chromosome 10 (Giovannoni et al., 1995). This gene has an open reading frame (ORF) consisting of three exons (356, 314, and 725 bp) and two introns (629 and 779 bp) (Fig. 1). Our eight primer sets produced PCR amplicons between 482-810 bp for the upstream and ORF regions of the *nor* gene in the two mutants 'LA1793' and 'LA3013', and a wild type 'San Marzano'. The 4.8 kb sequences (2.0 kb upstream and 2.8 kb ORF) were generated from these amplicons and aligned to identify single nucleotide polymorphism (SNP) and/or insertion and deletion (InDel). We detected a 2 bp InDel (AA/–) within the third exon, which was the longest exon of the *nor* gene (Fig. 2). This InDel causes a frame-shift that results in change of the polypeptide sequence from the glutamine at the amino acid 183 (Fig. 3). This mutation is the only sequence variation associated with fruit ripening in the *nor* gene. The *rin* mutation is also derived from a frame-shift in a MADS-box gene of the SEPELATTA clade (Hileman et al., 2006). For the *Cnr* gene, however, mutant phenotype is due to a spontaneous epigenetic change in the SBP-box (SQUAMODA promoter binding protein-like) promoter (Manning et al., 2006). The molecular mechanism of the *nor* gene for regulating fruit ripening still remains unclear compared to the *rin* and *Cnr* genes in tomato. Therefore, the InDel identified in our study can be useful to elucidate the *nor* gene function in the ripening process.
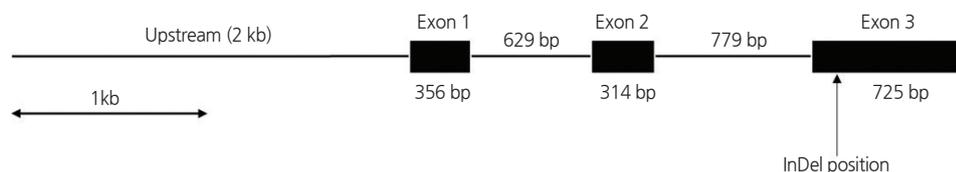
Fig. 1. A schematic of the *nor* gene (4.8 kb) sequenced in this study. The region includes the 2 kb upstream sequence and 2.8 kb open reading frame that consists of three exons (black boxes) and two introns (solid lines between exons).

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Heinz1706    tcttactccacactattttatttttattttttttgcagctagatgattgggtttttatgtcga
San Marzano  tcttactccacactattttatttttattttttttgcagctagatgattgggtttttatgtcga
LA1793       tcttactccacactattttatttttattttttttgcagctagatgattgggtttttatgtcga
LA3013       tcttactccacactattttatttttattttttttgcagctagatgattgggtttttatgtcga
             ************************************************************

Heinz1706    atttacaagaagaataacacacaaaggtccatagatgatttgcatgatatgttgggatcg
San Marzano  atttacaagaagaataacacacaaaggtccatagatgatttgcatgatatgttgggatcg
LA1793       atttacaagaagaataacacaca--ggtccatagatgatttgcatgatatgttgggatcg
LA3013       atttacaagaagaataacacaca--ggtccatagatgatttgcatgatatgttgggatcg
             **********************   ***********************************

Heinz1706    ataccacaaaatgtaccaaattcaatattacaaggaataaagccttcaaactatggtaca
San Marzano  ataccacaaaatgtaccaaattcaatattacaaggaataaagccttcaaactatggtaca
LA1793       ataccacaaaatgtaccaaattcaatattacaaggaataaagccttcaaactatggtaca
LA3013       ataccacaaaatgtaccaaattcaatattacaaggaataaagccttcaaactatggtaca
             ************************************************************

Heinz1706    atattgctcgaaaatgaatcgaatatgtacgatggaattatgaataacacgaacgatatt
San Marzano  atattgctcgaaaatgaatcgaatatgtacgatggaattatgaataacacgaacgatatt
LA1793       atattgctcgaaaatgaatcgaatatgtacgatggaattatgaataacacgaacgatatt
LA3013       atattgctcgaaaatgaatcgaatatgtacgatggaattatgaataacacgaacgatatt
             ************************************************************

Heinz1706    atcaacaataataatagatccattccacacaaatatcgtcaaagagaacgatgcatggaggt
San Marzano  atcaacaataataatagatccattccacacaaatatcgtcaaagagaacgatgcatggaggt
LA1793       atcaacaataataatagatccattccacacaaatatcgtcaaagagaacgatgcatggaggt
LA3013       atcaacaataataatagatccattccacacaaatatcgtcaaagagaacgatgcatggaggt
             ************************************************************
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Fig. 2. Sequence alignment of part of the 3rd exon of the *nor* gene between wild-type (Heinz 1706 and San Marzano) and mutant (LA1793 and LA3013) varieties. The "*" indicates identical nucleotides between these sequences. The 1st codon of the 3rd exon is indicated with bold letters and underlined. The gray boxes represent the HRM primer sequences.

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Heinz1706    MESTDSSTGTRHQPQLPPGFRFHPTDEELIVHYLKKRVAGAPIPVDIIGEIDLYKFDPWE
San Marzano  MESTDSSTGTRHQPQLPPGFRFHPTDEELIVHYLKKRVAGAPIPVDIIGEIDLYKFDPWE
LA1793       MESTDSSTGTRHQPQLPPGFRFHPTDEELIVHYLKKRVAGAPIPVDIIGEIDLYKFDPWE
LA3013       MESTDSSTGTRHQPQLPPGFRFHPTDEELIVHYLKKRVAGAPIPVDIIGEIDLYKFDPWE
             ***********************************************************

Heinz1706    LPAKAIFGEQEWFFFSPRDRKYPNGARPNRAATSGYWKATGTDKPVFTSGGTQKVGVKKA
San Marzano  LPAKAIFGEQEWFFFSPRDRKYPNGARPNRAATSGYWKATGTDKPVFTSGGTQKVGVKKA
LA1793       LPAKAIFGEQEWFFFSPRDRKYPNGARPNRAATSGYWKATGTDKPVFTSGGTQKVGVKKA
LA3013       LPAKAIFGEQEWFFFSPRDRKYPNGARPNRAATSGYWKATGTDKPVFTSGGTQKVGVKKA
             ***********************************************************

Heinz1706    LVFYGGKPPKGVKTNWIMHEYRVVENKTNNKPLGCDNIVANKKGSLRLDDWVLCRIYKKN
San Marzano  LVFYGGKPPKGVKTNWIMHEYRVVENKTNNKPLGCDNIVANKKGSLRLDDWVLCRIYKKN
LA1793       LVFYGGKPPKGVKTNWIMHEYRVVENKTNNKPLGCDNIVANKKGSLRLDDWVLCRIYKKN
LA3013       LVFYGGKPPKGVKTNWIMHEYRVVENKTNNKPLGCDNIVANKKGSLRLDDWVLCRIYKKN
             ***********************************************************

Heinz1706    NTQRSIDDLHDMLGS------IPQNVPNSILQGIK-PSNYGTILLENESNMYDGIMNNTN
San Marzano  NTQRSIDDLHDMLGS------IPQNVPNSILQGIK-PSNYGTILLENESNMYDGIMNNTN
LA1793       NTQVHRFAYVGIDTTKCTKFNITRNKAFKLWYNIARKIEYVRW--NYEHERY--------
LA3013       NTQVHRFAYVGIDTTKCTKFNITRNKAFKLWYNIARKIEYVRW--NYEHERY--------
             ***          .: :      * :*    .: .*    :*      : * : *

Heinz1706    DIINNNNRSIPQISSKRTMHGGLYWNNDEATTTTTTIDRNHSPNTKRFLVENNEDDGL-N
San Marzano  DIINNNNRSIPQISSKRTMHGGLYWNNDEATTTTTTIDRNHSPNTKRFLVENNEDDGL-N
LA1793       ---------YQQIHSTNIVKEND--------------------AWRFVLERRSNNNNNN
LA3013       ---------YQQIHSTNIVKEND--------------------AWRFVLERRSNNNNNN
                      ** *.. :: .                       : **::*...::. *

Heinz1706    MNNIS---RITNHEQSSS---------IANFLSQFPQNPSIQQQQQQQEEVLGSLNDGVV
San Marzano  MNNIS---RITNHEQSSS---------IANFLSQFPQNPSIQQQQQQQEEVLGSLNDGVV
LA1793       YEPFSKYKKVPCEQRGRWTHEYFANYKSTKLHCQFPEPVSSKSFDS------------T
LA3013       YEPFSKYKKVPCEQRGRWTHEYFANYKSTKLHCQFPEPVSSKSFDS------------T
             : :*     ::  .::.          ::: .***: * :. :.              .

Heinz1706    FRQPYNQVTGMNWYS---------------
San Marzano  FRQPYNQVTGMNWYS---------------
LA1793       TTTTTRRSIGISWGRLSTTLSSYWHELVLL
LA3013       TTTTTRRSIGISWGRLSTTLSSYWHELVLL
             .:  *:.*
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Fig. 3. Amino acid sequence alignment for the *nor* gene between wild-type (Heinz 1706 and San Marzano) and mutant (LA1793 and LA3013) varieties. The "*" indicates identical amino acid. The ":", ".", and no mark indicate high, low, and no similarity in the individual amino acids, respectively.

Table 1. The eight primer sets used to amplify the *nor* gene

Region	Primer name	Primer sequence (5′- 3′)[z]	Tm[y] (°C)	PCR product size (bp)
2.0 kb upstream sequence	*nor*-1	F: ACGTATCGCGAGGATTCATC	57	741
		R: CGTTATTAGGCTTTGAGAAGGAC	57	
	nor-2	F: GCTAATGACGACCTCCACTTC	59	810
		R: TTGTGTGGACATACTTTGATCG	59	
	nor-3	F: ACCACAAGAATCTCAAGACATG	56	780
		R: CGTAGTACGAGGTTGATAAATTCG	57	
2.8 kb open reading frame sequence	*nor*-4	F: GTTTATCATTTTCTCTCTTCCC	54	482
		R: AATTAGCAACGAAATTCGTC	55	
	nor-5	F: CAGCGATGAACAATTATTGTGTC	55	621
		R: AGGATATTTTCTATCTCTTGGAC	53	
	nor-6	F: CTTCTTATCATGTGTTTAACGC	55	666
		R: CCTCATATTAACCACGCGTA	56	
	nor-7	F: TACGAAATAGCCGAAAGAGGT	58	718
		R: TCGATCCCAACATATCATGCA	62	
	nor-8	F: TTTGCAGCTAGATGATTGGG	59	620
		R: ATCGATTGATTTTACAGGGCTA	58	

[z]F and R indicate forward and reverse primers, respectively.

[y]Tm = melting temperature.

Gene-based Marker Development and Genotyping in a Collection of Tomato Varieties

Based on the InDel in the 3rd exon, a primer set for a *nor*-InDel marker (forward: 5′-TGCAGCTAGATGATTGGGTTT-3′, reverse: 5′-ACCTCCATGCATCGTTCTCT-3′) was designed for HRM analysis. This primer set produced a 269 bp amplicon at an annealing temperature of 55°C and was used to genotype a collection of 83 tomato varieties including 'LA1793' and 'San Marzano'. The InDel marker clearly differentiated the mutant allele (*nor*) from the wild type allele (*Nor*) (Table 2). Among these varieties, the homozygous form of *nor* was found in the two inbreds, '14KT-5-24' and '14KT-6-10' derived from National Institute of Horticultural & Herbal Science (NIHHS) at Rural Development Administration (RDA). Among the 51 commercial F$_1$ hybrids, 'Gayachal Plus' from Syngenta-Korea had the heterozygous mutation (*nor/Nor*) (Table 2). The '14KT-5-24' variety produces yellow fruit, whereas the '14KT-6-10' and 'Gayachal Plus' varieties have pink fruit. Since abnormally ripened fruits that are green or yellow in color and common in the homozygous *nor* mutants (Tigchelaar et al., 1973), it was unexpected that the '14KT-6-10' variety has the *nor/nor* genotype. Although the '14KT-6-10' variety produces pink fruit, its fruit is firmer relative to wild type varieties with normally ripened fruit, which is similar to the other inbred '14KT-5-24' variety. Fruit firmness is an important factor affecting storage period and fruit quality after harvesting (Batu, 2004; De Ketelaere et al., 2004). This trait is mainly enhanced by cellulose and its cross-linkage with hemicellulose, pectin, and lignin (Vicente et al., 2007). The two cellulose synthases are more abundant in the *nor* mutant relative to a wild-type, indicating that the *nor* mutation has a positive effect to improve fruit firmness (Seymour et al., 2013). Tomato varieties with the heterozygous form of *nor* recovers normal fruit color but enhances fruit firmness. Thus, the heterozygous genotype is favorable for improving fruit quality and shelf-life in elite tomato varieties. Our result supports the effect of the *nor* mutation on fruit firmness and demonstrates that the *nor*-InDel marker is effective in detecting the *nor* mutation.

One of the main challenges in the tomato industry is to deliver fresh fruits of high quality (e.g. desirable taste, texture, and color). Fruit ripening is a complex process that involves numerous physiological and biochemical changes in color, carotenoid

Table 2. Genotypes of 83 tomato varieties via high-resolution melting (HRM) analysis using the gene-based marker developed for the *nor* gene

No.	Variety	Class	Source[z]	*nor* genotype[y]
1	LA1793	Inbred	TGRC	*nor/nor*
2	San Marzano	Inbred	TGRC	*Nor/Nor*
3	Cast Mart	Inbred	NAC	*Nor/Nor*
4	L385Dwart	Inbred	NAC	*Nor/Nor*
5	KA608-79L10743-1	Inbred	NAC	*Nor/Nor*
6	Serersanum	Inbred	NAC	*Nor/Nor*
7	Coloumbia	Inbred	NAC	*Nor/Nor*
8	Lucky Tm	Inbred	NAC	*Nor/Nor*
9	Leningradski	Inbred	NAC	*Nor/Nor*
10	Rougede Marmade	Inbred	NAC	*Nor/Nor*
11	Jubi	Inbred	NAC	*Nor/Nor*
12	L251	Inbred	NAC	*Nor/Nor*
13	C125F2	Inbred	NAC	*Nor/Nor*
14	Daehyungboksu	Inbred	NAC	*Nor/Nor*
15	Heungjin 101	Inbred	NAC	*Nor/Nor*
16	Heungjin 10	Inbred	NAC	*Nor/Nor*
17	84-4-428-2-1-1	Inbred	NAC	*Nor/Nor*
18	82-4-124-1-1-1-1	Inbred	NAC	*Nor/Nor*
19	82-4-65-1-1-1-1	Inbred	NAC	*Nor/Nor*
20	79-4-1-1-3-1-1	Inbred	NAC	*Nor/Nor*
21	77-4-25-1-2-1-1	Inbred	NAC	*Nor/Nor*
22	75-4-22-1-1-1-1	Inbred	NAC	*Nor/Nor*
23	14KT-6-5	Inbred	NIHHS	*Nor/Nor*
24	14KT-7-6	Inbred	NIHHS	*Nor/Nor*
25	14KT-8-1	Inbred	NIHHS	*Nor/Nor*
26	14KT-11-7	Inbred	NIHHS	*Nor/Nor*
27	AVTO1458	Inbred	NIHHS	*Nor/Nor*
28	14KT-5-24	Inbred	NIHHS	*nor/nor*
29	14KT-6-10	Inbred	NIHHS	*nor/nor*
30	14KT-9-4	Inbred	NIHHS	*Nor/Nor*
31	14KT-9-20	Inbred	NIHHS	*Nor/Nor*
32	15TG112	Inbred	NIHHS	*Nor/Nor*
33	Seowang	F$_1$ hybrid	Monsanto-Korea	*Nor/Nor*
34	SV0244TG	F$_1$ hybrid	Monsanto-Korea	*Nor/Nor*
35	Bacchus	F$_1$ hybrid	Monsanto-Korea	*Nor/Nor*
36	Styx TY	F$_1$ hybrid	Monsanto-Korea	*Nor/Nor*
37	Rafito	F$_1$ hybrid	Monsanto-Korea	*Nor/Nor*
38	Olleh TY	F$_1$ hybrid	Monsanto-Korea	*Nor/Nor*
39	Poseidon	F$_1$ hybrid	Monsanto Korea	*Nor/Nor*
40	Shincheonggang	F$_1$ hybrid	Monsanto-Korea	*Nor/Nor*
41	Unicorn	F$_1$ hybrid	Monsanto-Korea	*Nor/Nor*
42	Masecala	F$_1$ hybrid	Bunong Seed	*Nor/Nor*
43	TY Escot	F$_1$ hybrid	Bunong Seed	*Nor/Nor*
44	Black Eagle	F$_1$ hybrid	Bunong Seed	*Nor/Nor*
45	All Keeper	F$_1$ hybrid	Bunong Seed	*Nor/Nor*
46	Kstar	F$_1$ hybrid	Bunong Seed	*Nor/Nor*
47	Gold Sugar	F$_1$ hybrid	Bunong Seed	*Nor/Nor*
48	Vitamini	F$_1$ hybrid	Bunong Seed	*Nor/Nor*

Table 2. continued

No.	Variety	Class	Source[z]	*nor* genotype[y]
49	TY Candy	F$_1$ hybrid	Bunong Seed	*Nor/Nor*
50	Nice Gold	F$_1$ hybrid	Bunong Seed	*Nor/Nor*
51	TY Endorphin	F$_1$ hybrid	Bunong Seed	*Nor/Nor*
52	TY Altorang	F$_1$ hybrid	Nongwoo Bio	*Nor/Nor*
53	Sun Globe	F$_1$ hybrid	Nongwoo Bio	*Nor/Nor*
54	TY Sense Q	F$_1$ hybrid	Nongwoo Bio	*Nor/Nor*
55	Mini Maru	F$_1$ hybrid	Nongwoo Bio	*Nor/Nor*
56	Red Pang	F$_1$ hybrid	Nongwoo Bio	*Nor/Nor*
57	13T510	F$_1$ hybrid	Nongwoo Bio	*Nor/Nor*
58	Madison	F$_1$ hybrid	Syngenta-Korea	*Nor/Nor*
59	Dafnis	F$_1$ hybrid	Syngenta-Korea	*Nor/Nor*
60	Gayachal Plus	F$_1$ hybrid	Syngenta-Korea	*nor/Nor*
61	TP-7 Plus	F$_1$ hybrid	Syngenta-Korea	*Nor/Nor*
62	Landolino	F$_1$ hybrid	Syngenta-Korea	*Nor/Nor*
63	Dotaerang Dia	F$_1$ hybrid	Takki-Korea	*Nor/Nor*
64	Dotaerang Master	F$_1$ hybrid	Takki-Korea	*Nor/Nor*
65	TY Smartsama	F$_1$ hybrid	Takki-Korea	*Nor/Nor*
66	Prime Alexander	F$_1$ hybrid	PPS	*Nor/Nor*
67	Beta Tiny	F$_1$ hybrid	PPS	*Nor/Nor*
68	Tiara	F$_1$ hybrid	PPS	*Nor/Nor*
69	Sweety Redggoma	F$_1$ hybrid	Jinheung Seed	*Nor/Nor*
70	Blackrich	F$_1$ hybrid	Jinheung Seed	*Nor/Nor*
71	Super Ace	F$_1$ hybrid	NH-Nonghyup	*Nor/Nor*
72	Ruby Ball	F$_1$ hybrid	NH-Nonghyup	*Nor/Nor*
73	Super Dotaerang	F$_1$ hybrid	Koregon	*Nor/Nor*
74	B Blocking	F$_1$ hybrid	Koregon	*Nor/Nor*
75	Sugar Red	F$_1$ hybrid	Asian Seed	*Nor/Nor*
76	Sugar Yellow	F$_1$ hybrid	Asian Seed	*Nor/Nor*
77	Yoyo captain	F$_1$ hybrid	Konong	*Nor/Nor*
78	Yoyo	F$_1$ hybrid	Konong	*Nor/Nor*
79	TY Miracle	F$_1$ hybrid	Sky Seed	*Nor/Nor*
80	Rokusanmaru	F$_1$ hybrid	Sakata-Korea	*Nor/Nor*
81	Venus	F$_1$ hybrid	Dongwon Nongsan	*Nor/Nor*
82	Honggwang	F$_1$ hybrid	Hyeondae Seed	*Nor/Nor*
83	Jicored	F$_1$ hybrid	Gana Seed	*Nor/Nor*

[z]The inbred and F$_1$ hybrid varieties were collected from TGRC (Tomato Genetics Resource Center), NAC (National Agrobiodiversity Center), NIHHS (National Institute of Horticultural & Herbal Science), and seed companies.
[y]*Nor* indicates the wild-type allele and *nor* indicates the mutant allele for the *non-ripening* (*nor*) gene.

biosynthesis (Pék and Helyes, 2010), sugar content (Davies and Kempton, 1975), firmness, and pathogen resistance (Kramer et al., 1992; Wang et al., 2017). Therefore, the regulation of the ripening process is central to improve fruit quality in tomato. Many efforts have been made in the genetic dissection of the ripening process and a number of ripening genes have been identified. Of these genes, spontaneous frame-shift mutations in the *rin* and *nor* genes exhibit similar phenotypes in fruit ripening. The individuals that are heterozygous at these loci recover normal color and have firmer fruit relative to plants that are homozygous form of wild type alleles. With this knowledge, the use of these mutations has helped to develop elite tomato varieties in breeding programs. Among the 81 tomato varieties used in this study, only two inbred and one F$_1$ hybrid varieties have homozygous or

heterozygous forms of the *nor* mutation, suggesting that this mutation is not widely used in tomato breeding programs. This may be due to a lack of an efficient molecular tool to facilitate introgression of this mutation into elite breeding lines.

Selection based on phenotypes often requires intensive field evaluations to eliminate environmental variation. Marker-assisted selection (MAS) is an effective approach that can accelerate plant breeding because breeding lines with favorable alleles are selected based on their genotypes, which are neutral for environmental variation. The molecular mechanism of *rin* in the ripening process have been studied more relative to *nor* (Vrebalov et al., 2002; Yuan et al., 2016), and the SCAR marker associated with the *rin* mutation was also developed for MAS (Kim et al., 2013). In the present study, an InDel responsible for a frame-shift mutation was detected in the *nor* gene and used to develop a gene-based marker that can be applied to MAS for the identification of this mutation. With this marker, we determined the genotype of *nor* in a collection of 81 tomato varieties representing 30 inbreds and 51 commercial F_1 hybrids. Our results benefit the tomato research community, particularly breeders, by utilizing MAS for improving shelf-life and quality in tomato fruits.

Literature Cited

Arah IK, Ahorbo GK, Anku EK, Kumah EK, Amaglo H (2016) Postharvest handling practices and treatment methods for tomato handlers in developing countries: A mini review. Adv Agric 2016:1-8. doi:10.1155/2016/6436945

Barry CS, Giovannoni JJ (2006) Ripening in the tomato *Green-ripe* mutant is inhibited by ectopic expression of a protein that disrupts ethylene signaling. Proc Natl Acad Sci USA 103:7923-7928. doi:10.1073/pnas.0602319103

Batu A (2004) Determination of acceptable firmness and colour values of tomatoes. J Food Eng 61:471-475. doi:10.1016/s0260-8774(03)00141-9

Bonfield JK, Smith KF, Staden R (1995) A new DNA sequence assembly program. Nucleic Acids Res 23:4992-4999

Cantu D, Blanco-Ulate B, Yang L, Labavitch JM, Bennett AB, Powell AL (2009) Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires *NOR* but not *RIN* or ethylene. Plant Physiol 150:1434-1449. doi:10.1104/pp.109.138701

Davies JN, Kempton RJ (1975) Changes in the individual sugars of tomato fruit during ripening. J Sci Food Agric 26:1103-1110

De Ketelaere B, Lammertyn J, Molenberghs G, Desmet M, Nicolaï B, De Baerdemaeker J (2004) Tomato cultivar grouping based on firmness change, shelf life and variance during postharvest storage. Postharvest Biol Technol 34:187-201. doi:10.1016/j.postharvbio.2004.03.007

Devran Z, Göknur A, Mesci L (2016) D evelopment of molecular markers for the *Mi-1* gene in tomato using the KASP genotyping assay. Hortic Environ Biotechnol 57:156-160. doi:10.1007/s13580-016-0028-6

Eriksson EM, Bovy A, Manning K, Harrison L, Andrews J, De Silva J, Tucker GA, Seymour GB (2004) Effect of the *Colorless non-ripening* mutation on cell wall biochemistry and gene expression during tomato fruit development and ripening. Plant Physiol 136:4184-4197. doi:10.1104/pp.104.045765

Giovannoni JJ (2004) Genetic regulation of fruit development and ripening. Plant Cell 16 S170-180. doi:10.1105/tpc.019158

Giovannoni JJ (2007) Fruit ripening mutants yield insights into ripening control. Curr Opin Plant Biol 10:283-289. doi:10.1016/j.pbi.2007.04.008

Giovannoni JJ, Noensie EN, Ruezinsky DM, Lu X, Tracy SL, Ganal MW, Martin GB, Pillen K, Alpert K, et al (1995) Molecular genetic analysis of the *ripening-inhibitor* and *non-ripening* loci of tomato. Mol Gen Genet 248:195-206

Hileman LC, Sundstrom JF, Litt A, Chen M, Shumba T, Irish VF (2006) Molecular and phylogenetic analyses of the MADS-box gene family in tomato. Mol Biol Evol 23:2245-2258. doi:10.1093/molbev/msl095

Hoering U (2012) Lost harvests - Food losses and food insecurity: extent and causes, impacts and possible solutions. FDCL-Verlag, Berlin, Germany, p 24

Kabelka E, Franchino B, Francis DM (2002) Two loci from *Lycopersicon hirsutum* LA407 confer resistance to strains of *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology 92:504-510. doi:10.1094/PHYTO.2002.92.5.504

Kasso M, Bekele A (2016) Post-harvest loss and quality deterioration of horticultural crops in Dire Dawa Region, Ethiopia. J Saudi Soc Agric Sci 2016. doi:10.1016/j.jssas.2016.01.005

Kerr EA (1958) Mutations of chlorophyll retention in ripe fruit. Rep Tomato Genet Coop 8:22

Kim HJ, Lee HR, Hyun JY, Hong DO, Won DC, Harn CH (2013) A SCAR marker linked to *RIPENING-INHIBITOR* in tomato. Korean J Breed Sci 45:104-108. doi:10.9787/kjbs.2013.45.2.104

Kopeliovitch E, Rabinowitch H, Mizrahi Y, Kedar N (1981) Mode of inheritance of *alcobaca*, a tomato ripening mutant. Euphytica 30:223-225

Kramer M, Sanders R, Bolkan H, Waters C, Sheeny RE, Hiatt WR (1992) Postharvest evaluation of transgenic tomatoes with reduced

levels of polygalacturonase: processing, firmness and disease resistance. Postharvest Biol Technol 1:241-255. doi:10.1016/0925-5214(92)90007-C

Lanahan MB, Yen HC, Giovannoni JJ, Klee HJ (1994) The *Never ripe* mutation blocks ethylene perception in tomato. Plant Cell 6:521-530

Lincoln JE, Fischer RL (1988) Regulation of gene expression by ethylene in wild-type and *rin* tomato (*Lycopersicon esculentum*) fruit. Plant Physiol 88:370-374

Manning K, Tor M, Poole M, Hong Y, Thompson AJ, King GJ, Giovannoni JJ, Seymour GB (2006) A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nat Genet 38:948-952. doi:10.1038/ng1841

Martel C, Vrebalov J, Tafelmeyer P, Giovannoni JJ (2011) The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. Plant Physiol 157:1568-1579. doi:10.1104/pp.111.181107

Ng TJ (1976). Genetic and physiological characterization of the *rin* and *nor* non-ripening mutants of tomato (*Lycopersicon esculentum*, Mill.). PhD Diss., Purdue University, IN, USA

Pék Z, Helyes L (2010) Color changes and antioxidant content of vine and postharvest-ripened tomato fruits. HortScience 45:466-468

Perveen R, Suleria HA, Anjum FM, Butt MS, Pasha I, Ahmad S (2015) Tomato (*Solanum lycopersicum*) carotenoids and lycopenes chemistry; Metabolism, absorption, nutrition, and allied health claims. Crit Rev Food Sci Nutr 55:919-929. doi:10.1080/10408398.2012.657809

Phan NT, Sim SC (2017) Genomic tools and their implications for vegetable breeding. Hortic Sci Technol 35:149-164. doi:10.12972/kjhst.20170018

Raiola A, Rigano MM, Calafiore R, Frusciante L, Barone A (2014) Enhancing the health-promoting effects of tomato fruit for biofortified food. Mediat Inflamm 2014:139873. doi:10.1155/2014/139873

Robinson RW, Tomes M (1968) Ripening inhibitor: a gene with multiple effect on ripening. Rep Tomato Genet Coop 18:36-37

Sablani SS, Opara LU, Al-Balushi K (2006) Influence of bruising and storage temperature on vitamin C content of tomato fruit. J Food Agric Environ 4:54-56

Seymour GB, Chapman NH, Chew BL, Rose JK (2013) Regulation of ripening and opportunities for control in tomato and other fruits. Plant Biotechnol J 11:269-278. doi:10.1111/j.1467-7652.2012.00738.x

Thompson AJ, Tor M, Barry CS, Vrebalov J, Orfila C, Jarvis MC, Giovannoni JJ, Grierson D, Seymour GB (1999) Molecular and genetic characterization of a novel pleiotropic tomato-ripening mutant. Plant Physiol 120:383-389

Tigchelaar EC, Tomes ML, Kerr EA, Barman RJ (1973) A new fruit ripening mutant, *non-ripening* (*nor*). Rep Tomato Genet Coop 23:33

Tomato Genome Consortium (2012) The tomato genome sequence provides insights into fleshy fruit evolution. Nature 485:635-641. doi:10.1038/nature11119

Truong HTH, Kim S, Tran HN, Nguyen TTT, Nguyen LT, Hoang TK (2015) Development of a SCAR marker linked to bacterial wilt (*Ralstonia solanacearum*) resistance in tomato line Hawaii 7996 using bulked-segregant analysis. Hortic Environ Biotechnol 56:506-515. doi:10.1007/s13580-015-1050-9

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3-new capabilities and interfaces. Nucleic Acids Res 40:e115. doi:10.1093/nar/gks596

Veerappan K, Jung HJ, Hwang I, Kho KH, Chung MY, Nou IS (2016) Sequence variation in *SlMYB12* is associated with fruit peel color in pink tomato cultivars. Hortic Environ Biotechnol 57:274-279. doi:10.1007/s13580-016-0041-9

Vicente AR, Saladié M, Rose JKC, Labavitch JM (2007) The linkage between cell wall metabolism and fruit softening: looking to the future. J Sci Food Agric 87:1435-1448. doi:10.1002/jsfa.2837

Vrebalov J, Ruezinsky D, Padmanabhan V, White R, Medrano D, Drake R, Schuch W, Giovannoni J (2002) A MADS-Box gene necessary for fruit ripening at the tomato *Ripening-Inhibitor* (*Rin*) locus. Science 296:343-346. doi:10.1126/science.1068181

Wang W, Cai J, Wang P, Tian S, Qin G (2017) Post-transcriptional regulation of fruit ripening and disease resistance in tomato by the vacuolar protease *SlVPE3*. Genome Biol 18:47. doi:10.1186/s13059-017-1178-2

Yuan XY, Wang RH, Zhao XD, Luo YB, Fu DQ (2016) Role of the tomato *Non-ripening* mutation in regulating rruit quality elucidated using iTRAQ protein profile analysis. PLoS ONE 11:e0164335. doi:10.1371/journal.pone.0164335

Zapata PJ, Guillén F, Martínez-Romero D, Castillo S, Valero D, Serrano M (2008) Use of alginate or zein as edible coatings to delay postharvest ripening process and to maintain tomato (*Solanum lycopersicon* Mill) quality. J Sci Food Agric 88:1287-1293. doi:10.1002/jsfa.3220