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Characterization of IQ Domain Gene Homologs as Common Candidate Genes for Elongated Fruit Shape in Cucurbits

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Abstract

The *SUN* gene is responsible for an elongated fruit shape in tomato and belongs to the IQ domain (IQD) gene family, which is involved in growth and development in plants. In the present study, IQD gene homologs were evaluated for their roles in determining fruit shape in cucurbit crops. A total of 151 IQD homologs and their chromosomal locations in *Arabidopsis*, tomato, and three cucurbit species, watermelon, melon, and cucumber, were compared based on their genomic information. A phylogenetic dendrogram of these IQD homologs revealed putative orthologous and paralogous relationships among these genes, and showed that previously reported candidate fruit shape IQD genes in watermelon (*ClSUN8*), melon (*CmSUN14*), and cucumber (*CsSUN2*) were clustered under the same node with a similarity coefficient of 0.97. A comparison of the physical locations of the IQD homologs with fruit shape QTLs in genetic maps indicated that *ClSUN8*, *CmSUN14*, and *CsSUN2* were co-localized with the major QTLs for the fruit shape index (FSI). This co-localization further indicated that minor QTLs for FSI were also associated with the IQD gene family. We used early developmental stages of immature fruits to conduct a morphological assay and characterize *ClSUN8* in watermelon. Histological analysis indicated that elongated or round fruit shape is determined at the early stage of ovary formation, at least earlier than -4 days after fertilization (-4 DAF), and fruit elongation is due to increased cell division in the longitudinal direction. Quantitative RT-PCR showed that the highest expression of *ClSUN8* occurred at -4 days after fertilization and gradually decreased; however, there was no direct relationship between the gene expression level and fruit shape. RACE-PCR revealed a non-synonymous SNP between the *ClSUN8* alleles of elongated- and round-fruited watermelon accessions, suggesting that the SNP might be a causative mutation affecting fruit shape.

Additional key words: allelic variation, Cucurbitacea, molecular marker, paraffin section, phylogeny

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Introduction

Fruit shape is an important horticultural trait that is a parameter in sensory evaluation for breeding. The size and shape of the fruit determine yield, consumer preference, packaging, and transportation. The

molecular basis for the diversity in fruit shapes remains unclear. Cucurbitaceae crops, including watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), cucumber (*Cucumis sativus*), and squash plants (*Cucurbita* spp.) (Pitrat, 2008; Sadrnia et al., 2007), have a diverse array of fruit shapes and are ideal for studying fruit shape.

Intensive genetic studies of fruit morphology have been performed in melon, watermelon, and cucumber. At least five genetic maps have been constructed for melon fruit shape, and a total of 42 QTLs, demonstrating a range of R^2 values from 10.0% to 52.2%, have been detected. Among these QTLs, three major QTLs with R^2 values higher than 30% (*FSQQ2.2*, 52.2%; *FSQS8.1*, 41%; *FSQA9.1*, 33%) have been found in three different chromosomes (Chr. 2, 8, and 9, respectively) (Périn et al., 2002; Monforte et al., 2004; Eduardo et al., 2007; Paris et al., 2008; Harel-Beja et al., 2010; Díaz et al., 2014). Similarly, at least 13 QTLs for fruit shape have been detected in watermelon from five genetic maps. The R^2 values for these QTLs ranged from 2.4% to 79.7% and three major QTLs located on Chr. 3 and 11 showed R^2 values higher than 30% (*fsi3.1*, 79.7%; *FSI3.2*, 38.22%; *fsi11.1*, 56.6%) (Sandlin et al., 2012; Ren et al., 2014; Kim et al., 2015; Cheng et al., 2016; Liu et al., 2016). One major QTL (*fsi3.1*) in watermelon was highly correlated with a homolog (*Cla011257*) to the *SUN* gene responsible for elongated fruit shape in tomato (Rodríguez et al., 2011; Huang et al., 2013). This *SUN* homolog was reported as a candidate gene for the fruit shape index (FSI) trait in watermelon (Kim et al., 2015). With the exception of tomato, no genes identified for directly conferring fruit shape-related traits have been cloned in major fruit crops, including cucurbits (Xiao et al., 2008).

The four fruit-shape genes cloned in tomato include: *SUN* for elongated fruit shape (Xiao et al., 2008), *OVATE* for pear and ellipse fruit shape (Liu et al., 2002), and *LC* and *FAS* for locule number (Cong et al., 2008).Various fruit shapes found in tomato germplasm are highly related to the modification of these four cloned QTLs and their composition in individual genotypes (Huang et al., 2013). The *SUN* gene for fruit length elongation belongs to the IQ67 domain gene family. The IQ67 domain is a plant-specific domain of 67 conserved amino acids and is characterized by an IQ domain (IQD) (Abel et al., 2005). The IQD mediates calmodulin (CaM) retention in a Ca^{2+}- independent manner (Bähler and Rhoads, 2002; Hoeflich and Ikura, 2002) and regulates the growth and metabolism of plants (Feng et al., 2014; Abel et al., 2005). The IQD gene family has been analyzed across the plant kingdom, including *Arabidopsis thaliana* (Bürstenbinder et al., 2013), *Glycine max* (Feng et al., 2014), *Oryza sativa* (Abel et al., 2005), *Populous trichocarpa* (Ma et al., 2014), and *Moso bamboo* (Wang et al., 2016).

In this study, we identified IQD gene homologs in major cucurbit crops and assessed the phylogenetic relationships among them. Further, we co-localized these homologs with the fruit shape QTLs to explore their roles in determining fruit morphology. Sequences and gene expression of two IQD genes in watermelon and melon were characterized, and the results are discussed herein.

Materials and Methods

Phylogenetic Analysis and Co-localization of QTL with IQD genes

The sequences of Arabidopsis IQD genes were obtained from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org). Homologs of these IQD genes in other species were found using BLASTP searches (E-value of 10^{-5}) in the genome databases of the International Cucurbit Genomics Initiative (ICuGI) (http://www.icugi.org) for watermelon and cucumber, Melonomics (http://www.melonomics.net) for melon, and Sol Genomics Network (http://www.solgenomics.net) for tomato. The pairwise genetic similarity coefficients among the genes were estimated based on the protein

sequences using the Jones-Thornton-Taylor (JTT) method (Jones et al., 1992). The phylogenetic dendrogram was constructed for IQD proteins using MEGA 5.0 (Tamura et al., 2011) based on the neighbor-joining method and bootstrap analysis was conducted using 1,000 replicates.

For co-localization of the IQD genes, we searched for fruit shape QTLs based on previous QTL mapping studies in watermelon (Sandlin et al., 2012; Kim et al., 2015; Cheng et al., 2016; Liu et al., 2016), melon (Díaz et al., 2011; Díaz et al., 2014), and cucumber (Weng et al., 2015). PCR primer sequences flanking the fruit shape index (FSI) QTLs at the closest vicinity were obtained from previous publications (Périn et al., 2002; Monforte et al., 2004; Eduardo et al., 2007; Paris et al., 2008; Harel-Beja et al., 2010; Sandlin et al., 2012; Díaz et al., 2014; Ren et al., 2014; Kim et al., 2015; Cheng et al., 2016; Liu et al., 2016) and BLAST searches to the watermelon (97103 genome version 1), melon (DHL92 genome version CM3.5), and cucumber (Chinese long genome version 2) reference genomes to identify their physical genomic locations. Similarly, the physical locations of the IQD homologs identified as described above were compared for co-localization with the QTLs for FSI in the three cucurbit crops.

Full-length cDNA Cloning of *Cla011257*

Transcript sequence characterization of the candidate IQD gene *Cla011257* for fruit shape was carried out with two watermelon accessions 'Arka Manik' (AM, round Crimson-type) and 'TS34' (TS, elongated Jubilee-type) that were the parental lines in our previous QTL mapping study (Kim et al., 2015). Each plant was grown in a growth chamber and fully expanded first true leaves were collected, directly frozen in liquid nitrogen, ground into powder, and stored at -80°C until use. Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Germantown, MD, USA) and checked for quality using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). The full-length cDNA of *Cla011257* was cloned by rapid amplification of cDNA ends (RACE) PCR using the Smarter RACE 5′/3′ Kit (Clontech Laboratories, Mountain View, CA, USA) following the user manual. Plasmids were purified using the Exprep Plasmid kit (GeneAll, Seoul, Korea). Purified plasmids were digested by *Eco*RI and *Hin*dⅢ (10,000 U·mL^{-1}, New England BioLabs Inc., Ipswich, MA, USA) and the insert DNA was checked on a 1.0% agarose gel. The insert DNAs were sequenced based on the dye-termination method by Genotech (Daejeon, Korea). Full-length cDNA sequences obtained were aligned using ClustalX 1.83 (Thompson et al., 1997).

Transcriptome Analysis of *Cla011257*

Seedlings of four watermelon accessions, SGR (highly elongated), TS (elongated), AM (round), and SBA (round), were grown in a growth chamber under the conditions 35°C / 14 h day and 22°C / 10 h night. Seedlings at the third or fourth true-leaf stage were transplanted in a plastic greenhouse at Pusan National University (Miryang, Korea) until their fruit was harvested. Immature fruits (ovaries) were harvested at different developmental stages: -4 and -2 (four and two days before fertilization), and 0, 2, 4, and 6 days after fertilization (DAF). Due to the difficulties in determining the precise time of flowering, -4 and -2 DAF were determined based on the green and canary-yellow buds on the ovary, respectively, whereas the time of hand pollination was designated as 0 DAF. Immature fruits harvested at each developmental stage were immediately frozen in liquid nitrogen and stored at -75°C until use. Total RNA extraction was conducted as described in the previous section. cDNA syntheses from the RNA samples was conducted using the Hyperscript RT premix kit (GeneAll, Seoul, Korea) following the user manual.

For reverse transcription quantitative PCR (RT-qPCR), primers were designed from the coding sequence of *Cla011257* (Table 1). The primers for the reference gene [β-Actin (Actin)] were obtained from Kong et al. (2014). RT-qPCR analysis was carried out using a Light Cycler 480 Real-Time PCR System (Roche Life Science, Basel, Switzerland) with the Light Cycler 480 SYBR Green I Master kit. All RT-qPCRs were performed in a total volume of 5 μL containing 1.25 μL of cDNA, 0.5 μL of forward and reverse primers (10 pm·μL^{-1}), 2.5 μL master mix buffer, and 0.75 μL ddH_2O. RT-qPCR conditions were: 1 cycle for pre-incubation at 95°C for 5 min; 45 cycles for amplification at 95°C for 10 s, annealing temperature of each primer set for 20 s, and extension at 72°C for 1 min; and 1 cycle for melting curve analysis at 95°C for 5 s, 65°C for 1 min, and continuous 97°C, followed by cooling at 40°C for 10 s. All RT-qPCRs were conducted with three replications and the average values were used for further analysis.

Analysis of Allelic Variation Between *ClSUN8* (*Cla011257*) and *CmSUN14* (*MELO3C015418*)

Partial genomic DNA sequencing of *ClSUN8* (*Cla011257*) was carried out for four watermelon accessions "11-3" (elongated), "12-6" (elongated), "12-7" (round), and "42-2" (elongated). In our previous study (Jin et al., 2017), these inbred accessions showed failed PCR amplifications when tested by the *Cla011257* gene-based dCAPS marker (Kim et al., 2015) and were speculated to carry another mutant allele (s) for *Cla011257*. For partial sequencing of *Cla011257*, new PCR primers (Table 1) were designed to encompass the primer locations of the dCAPS marker. In addition, genomic DNA sequencing of *MELO3C015418* in melon was conducted from two melon accessions CM-P01 (oval cantaloupe melon) and MM-P02 (round musk-net melon). To clone *MELO3C015418*, the genomic DNA sequence was retrieved from the Melonomics database and PCR primers (Table 1) were designed at approximately 500 bp upstream and downstream of the gene.

All PCRs were performed in a total volume of 50 μL containing 5 μL of genomic DNA (10 ng·μL^{-1}), 2.5 μL each of forward and reverse primers (10 pm·μL^{-1}), 5 μL of 10X PCR buffer, 1 μL of dNTPs (10 mM each), and 0.5 μL of Taq polymerase (5 U·μL^{-1}) (SolGent, Daejeon, Korea). For plasmid vector cloning, the PCR amplicons were agarose-gel electrophoresed,

Table 1. Primers used for gene expression, genotyping, RACE-PCR, and PCR cloning of IQD gene homologs in this study

Purpose	Primer set name	Primer sequence (5'-3')	Enzyme	Product size (bp)
Gene expression	Cla011257-qRT	F: AAGATTCAAACTTGCTTCAGAG		207
		R: AAGTCTTGTTTCGTCGTTTATG		
	Cla007792-qRT	F: CCATGTATGTTGCCATCCAG		135
		R: GGATAGCATGGGGTAGAGCA		
Genotyping	Cla011257-dCAPS	F: CCTATTTCACCAAACTCTCTCG	*Eco*R Ⅰ	345/373
		R: TCCACTAAGACTACTTCTCGATTCCATGAAT		
	Cla011257-SCAR	F: ATGACACAAAAAGCGAACACAC		573/414
		R: GAGAACAAGATCTCTGCATTCTAACT		
RACE-PCR	Cla011257-RACE	5': CGCCGCCGCACCAGTCAGAGTCAT		
		3': AACTCTCTCGTCTCCACTCCCTTGCC (for AM)		
		3': AGCTCAAGCCACCGTTCGATCTCAGA (for TS)		
TA-cloning	MELO3C015418P1-1-TA	F: TCATCTTCTTATCTTCCTCAGTC		790
		R: GTGTTCGCTTTTTGTGTCCTC		
	MELO3C015418P1-2-TA	F: ATTTAGGAGAGATTTGAGGACA		876
		R: AAAACAGGGGAGATAGAGAAA		

gel-purified using the Expin Gel kit (GeneAll, Seoul, Korea), and ligated using pGEM-T Easy Vector System I (Promega, Madison, WI, USA) following the manufacturers' instructions. Cell transformation of the recombined plasmid vectors was carried out using HIT competent cells-DH5α (RBC, Taiwan, China) following the manufacturer's instructions. Transformed cell colonies were amplified in liquid LB medium. Plasmid purification and subsequent detection and sequencing of the insert DNA were carried out as described in the section 'Full-length cDNA cloning of *Cla011257*' except that purified plasmids were digested by *Eco*RI (10,000 $U \cdot mL^{-1}$, New England BioLabs Inc., Ipswich, MA, USA).

Morphological Analysis of Watermelon Fruit

The immature fruit samples were harvested from the same four watermelon accessions [SGR (highly elongated), TS (elongated), AM (round), and SBA (round)] at -4, -2, 0, 2, and 4 DAF. Harvested fruit samples (three fruits per accession) were scanned using a SAMSUNG SL-C483W printer and the length and diameter of the fruits were measured using a vernier caliper (Mitutoyo, Kawasaki, Japan).

Paraffin sectioning was used for histological analysis of the fruits at different developmental stages. Harvested fruit tissues were fixed in FAA (5 mL formaldehyde, 5 mL acetic acid, 90 mL 50% ethanol, and 5 mL glycerol) for 24 h and then cleaned in 50% ethanol for 10 min. Cleaned samples were then sequentially soaked in 35%, 50%, 70%, 85%, 95%, and 100% (three times) ethanol and then finally in 100% xylene (three times) for 3 h each. The samples were then incubated in paraffin (Paraplast, Leica Biosystems, Solms, Germany) at 61°C for 3 h for three times, embedded in paraffin, and then sliced into 6μm sections using a Leica RM2235 microtome (Leica, Solms, Germany). The paraffin was removed using xylene and hydrated with 100%, 70%, and 35% ethanol, and then ddH_2O for 3 min, and the tissues were stained using 0.1% toluidine blue solution (0.1 g toluidine blue, 0.5 mL acetic acid, and ddH_2O in a total volume of 100 mL). Finally, the tissues were dehydrated and sealed with Canada balsam (Sigma, Steinheim, Germany). A DM2500 microscope with a DFC295 camera (Leica, Solms, Germany) was used to observe the sections.

Results and Discussion

Phylogeny of the IQD Family Genes in Cucurbits

The IQD gene homologs in the three cucurbits and tomato were searched in BLASTP using protein sequences of 33 IQD genes of Arabidopsis. A total of 31, 29, 28, and 30 genes containing the IQ67 domain were retrieved for watermelon, cucumber, melon, and tomato, respectively. Previously reported IQD genes reported to be putatively responsible for fruit shape [*Solyc10g079240.1.1* (*SUN*) for tomato (Xiao et al., 2008); *Cla011257* for watermelon (Kim et al., 2015); and *Csa1G575000* for cucumber (Pan et al., 2016)] were also confirmed to be among this gene family.

A phylogenetic dendrogram of the IQD genes was constructed by MEGA (Fig. 1). Each group showed a tendency to be composed of the IQD genes of different plant species. Considering that there is a high level of synteny among the genomes of cucurbit crops (Garcia-Mas et al., 2012), the genes of different species in the same clade may be orthologous and may have evolved from a common ancestral origin. In addition, a possible paralogous relationship was shown among the genes of the same species clustered under the same node. Further studies are necessary to explain the detailed evolutionary relationships among these IQD genes. Importantly, we identified from the dendrogram that two genes proposed as fruit-shape genes in

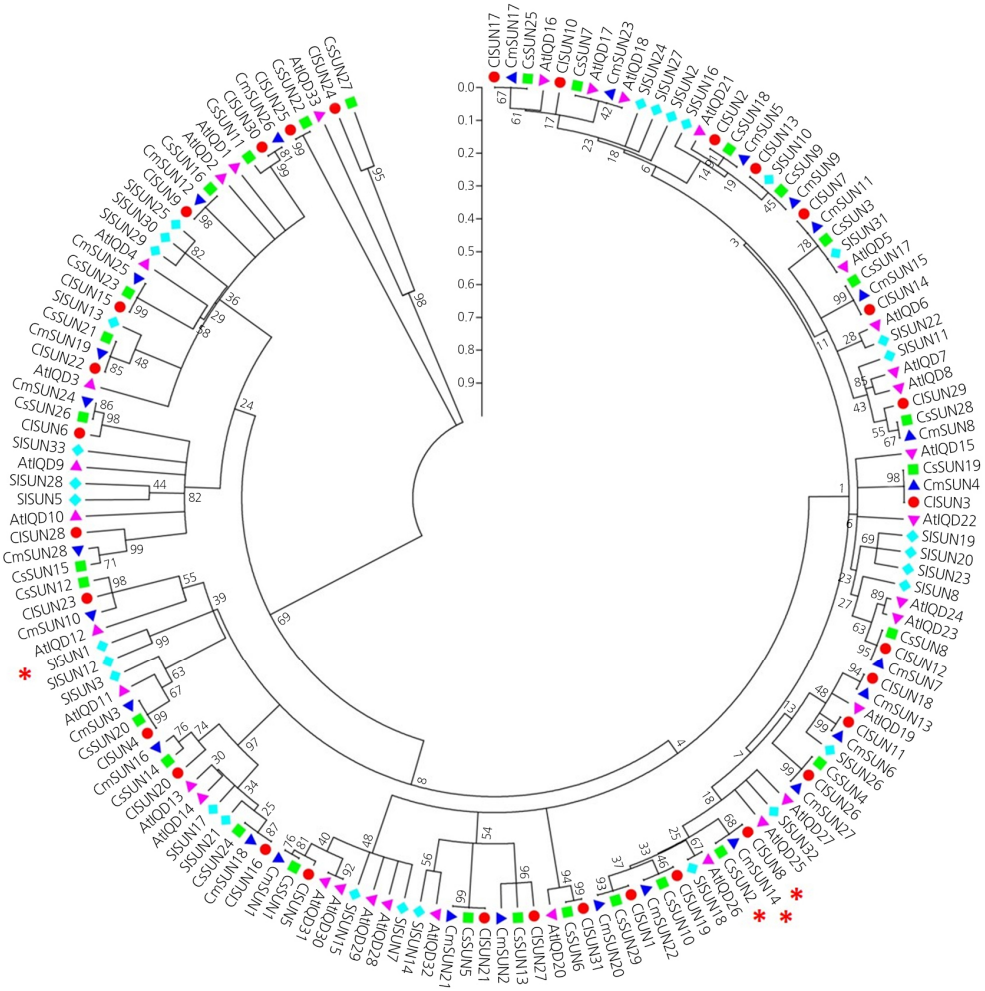

Fig. 1. A phylogenetic tree of the IQD genes (SUN homologs) in *Arabidopsis* (purple triangle), watermelon (red circle), cucumber (green square), melon (blue triangle), and tomato (light blue diamond). The unrooted tree was constructed from full-length amino acid sequences using MEGA 5.0 based on the neighbor-joining method. The red asterisks indicate candidate IQD genes for fruit shape revealed by QTL mapping in tomato, watermelon, melon, and cucumber.

watermelon [ClSUN8 (Cla011257)] and cucumber [CsSUN2 (Csa1G575000)], and one melon IQD gene [CmSUN14 (MELO3C014258)] were grouped at the same node with a similarity of 0.97 (Fig. 1). However, the tomato fruit-shape gene [SUN (Solyc10g079240.1.1)] was distantly located from the genes in cucurbits. Subsequently, the molecular characteristics were analyzed for these four proteins.

Co-localization of Fruit-shape QTLs with the IQD Family Genes

The physical genomic locations of the IQD homologs in watermelon, melon, and cucumber are displayed on a schematized chromosome map (Fig. 2). The positions of watermelon IQD homologs were scattered on all chromosomes except for Chr. 11. The number of genes on each chromosome ranged from 1 for Chr. 3 to 7 for Chr. 5. Similarly, in melon, the genes were spread across all 12 chromosomes, with the number of genes ranging from 1 for Chr. 8 and Chr. 10 to 6 for Chr. 6.

The QTLs for fruit shape were found in previous QTL mapping studies on watermelon (Sandlin et al., 2012; Kim et al., 2015; Cheng et al., 2016; Liu et al., 2016), melon (Diaz et al., 2011; Díaz et al., 2014), and cucumber (Yuan et al., 2008; Bo

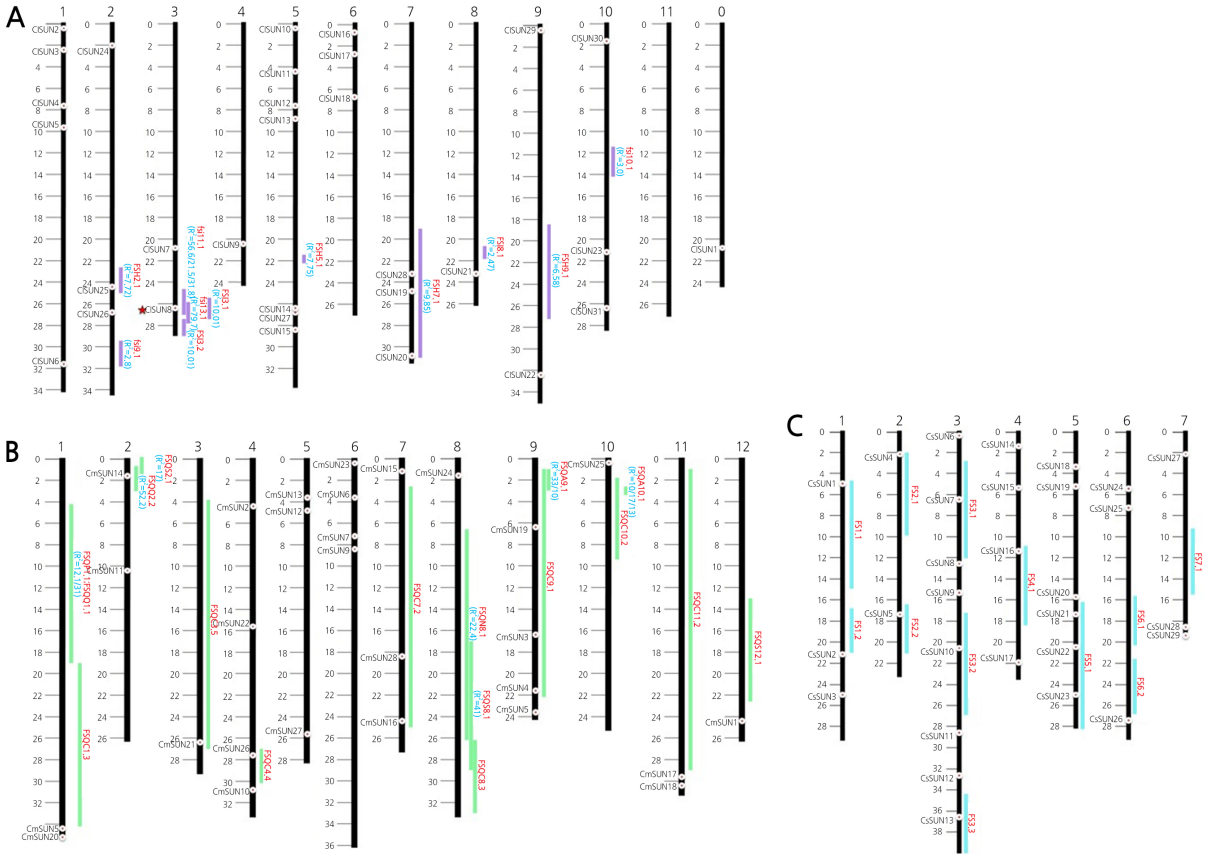

Fig. 2. Schematic diagrams of chromosomes showing co-localization of fruit-shape QTLs and IQD genes (SUN homologs) in watermelon (A), melon (B), and cucumber (C). Physical distances on the chromosomes are marked by 1 Mb intervals. The names of the SUN homologs (black letters and dots on the chromosomes) are shown on the left side of the chromosomes, while fruit-shape QTLs (red letters) are shown on the right side with their R^2(%) values and genomic regions (flanking marker intervals).

et al., 2015) (Table 2). In watermelon, a total of 11 QTLs have been reported for fruit shape index, including the major QTLs *fsi11.1* (Sandlin et al., 2012) and *fsi3.1* (Kim et al., 2015) that were detected on Chr. 3. The IQD gene *Cla011257* (named *ClSUN8* in this study) (Kim et al., 2015) was physically located in the vicinity of both QTLs. In melon, a total of 16 QTLs have been reported for fruit shape index, including the major QTLs *FSQQ2.2* (Périn et al., 2002) on Chr. 2 and *FSQS8.1* on Chr. 8 (Díaz et al., 2014), and we found that the melon IQD homolog *MELO3C015418* was located in the vicinity of *FSQQ2.2*. In cucumber, the length and diameter of ovaries and fruits were measured at anthesis (OvL and OvD), immature (FL and FD), and mature (MFL and MFD) stages and 22 QTLs were detected for the six traits (Weng et al., 2015). These fruit-shape (FS) QTLs were further integrated into 12 QTLs based on their location on the same chromosomal blocks (Weng et al., 2015). We confirmed that one of the cucumber IQD homologs, *Csa1G575000* (*CsSUN2*), was located in the vicinity of *FS1.2*.

Co-localization of these major QTLs with IQD genes showed that *Cla011257* was tightly linked to *fsi11.1* and *fsi3.1* in watermelon, *MELO3C015418* to *FSQQ2.2* in melon, and *Csa1G575000* to *FS1.2* in cucumber. In our phylogenetic dendrogram, these three IQD genes were clustered in the same subgroup, with a genetic similarity of 0.97 (Fig. 1). These results implied that IQD genes are not only associated with the fruit shape of cucurbit crops, but also show close orthologous relationships in these plant species.

Table 2. Fruit-shape QTL information that was used for colocalization of IQD gene homologs in cucurbits

Species	QTL	Chr.	R^2 (%)	Population	QTL franking marker		Reference
					Left	Right	
Watermelon	*FSH2.1*	2	7.72	garden female parent x LSW-177	WCI02-7	WCI02-31	Cheng et al. 2016
	fsi9.1	2	2.8	KBS x NHM	NW0248574	NW0249974	Sandlin et al. 2012
	fsi11.1	3	56.6/21.5	KBS x NHM	NW0248107	NW0250956	Sandlin et al. 2012
	fsi11.1	3	31.8	ZWRM x Citroides	NW0248107	NW0250956	Ren et al. 2014
	fsi3.1	3	79.7	Arka Manik x TS34	wsbin3-9	wsb3-24	Kim et al. 2015
	FSI3.1	3	10.01	LSW-177 x COS	WII03E02-87	WII03E09-92	Liu et al. 2016
	FSI3.2	3	38.22	LSW-177 x COS	WII03E09-92	WII03EXhoI-1	Liu et al. 2016
	FSH5.1	5	7.75	garden female parent x LSW-177	WCI05-13	WCI05-14	Cheng et al. 2016
	FSH7.1	7	9.85	garden female parent x LSW-177	WCI07-13	SSRW1-86	Cheng et al. 2016
	FSI8.1	8	2.47	LSW-177 x COS	WII08EKpnI-5	WII08EXhoI-5	Liu et al. 2016
	FSH9.1	9	6.58	garden female parent x LSW-177	WCI09-8	WCI09-13	Cheng et al. 2016
	fsi10.1	10	3.0	KBS x NHM	NW0250299	NW0249853	Sandlin et al. 2012
Melon	*FSQP1.1*	1	12.1	Ved x PI161 RIL	CMCTN86	CMCCA145	Périn et al. 2002
	FSQQ1.1	1	31	Ved x PI414723 RIL	CMCTN86	CMCCA145	Périn et al. 2002
	FSQC1.3	1		Piel de Sapo x PI161375 NIL	CMCCA145	CMCTN4	Eduardo et al. 2007
	FSQS2.1	2	17	Piel de Sapo x PI124112 NIL	CMPSNP431	AluICAPS	Díaz et al. 2014
	FSQQ2.2	2	52.2	Ved x PI414723 RIL	CMGA36	CmSUS1	Périn et al. 2002
	FSQC3.5	3		Piel de Sapo x PI161375 NIL	CSWCT10	TJ10	Eduardo et al. 2007
	FSQC4.4	4		Piel de Sapo x PI161375 NIL	CMAGN73	CMTC168	Eduardo et al. 2007
	FSQC7.2	7		Piel de Sapo x PI161375 NIL	CMAGN75	CMGA15	Eduardo et al. 2007
	FSQN8.1	8	22.4	PI414723 x Dulce RIL	CMCTTN232	CMAT141	Harel-Beja et al. 2010
	FSQS8.1	8	41	Piel de Sapo x PI124112 NIL	GCM241	PSI_25-H03	Díaz et al. 2014
	FSQC8.3	8		Piel de Sapo x PI161375 NIL	CMAT141	CMTCN56	Eduardo et al. 2007
	FSQA9.1	9	33/10	Piel de Sapo x PI161375 DHL	CMTC47	CM98	Monforte et al. 2004
	FSQC9.1	9		Piel de Sapo x PI161375 NIL	CMTC47	CMCTN7	Eduardo et al. 2007
	FSQA10.1	10	10/17/13	Piel de Sapo x PI161375 DHL	CMGA172	CMTCN67	Monforte et al. 2004
	FSQC10.2	10		Piel de Sapo x PI161375 NIL	CMCTN19	CMTCN8	Eduardo et al. 2007
	FSQC11.2	11		Piel de Sapo x PI161375 NIL	CMCT160a	CMGA104	Eduardo et al. 2007
	FSQS12.1	12		Piel de Sapo x PI124112 NIL	AI_35-A08	CMPSNP361	Díaz et al. 2014
Cucumber	*FS1.1*	1		Gy14 x9930	SNP.3689	SNP.118881	Weng et al. 2015
	FS1.2	1		Gy14 x9930	SNP.11145	SNP.12981	Weng et al. 2015
	FS2.1	2		Gy14 x9930	SNP.134265	SNP.121897	Weng et al. 2015
	FS2.2	2		Gy14 x9930	SNP.149645	SNP.107609	Weng et al. 2015
	FS3.1	3		Gy14 x9930	SNP.122373	SNP.35965	Weng et al. 2015
	FS3.2	3		Gy14 x9930	SNP.37745	SNP.45557	Weng et al. 2015
	FS3.3	3		Gy14 x9930	SNP.22341	SNP.51329	Weng et al. 2015
	FS4.1	4		Gy14 x9930	SNP.58133	SNP.61745	Weng et al. 2015
	FS5.1	5		Gy14 x9930	SNP.70369	SNP.77021	Weng et al. 2015
	FS6.1	6		Gy14 x9930	SNP.143873	SNP.91177	Weng et al. 2015
	FS6.2	6		Gy14 x9930	SNP.168061	SNP.94685	Weng et al. 2015
	FS7.1	7		Gy14 x9930	SNP.166749	SNP.106789	Weng et al. 2015

Detection of Allelic Variations for Fruit Shape-related IQ Genes

In our previous study (Kim et al., 2015), the genomic DNA sequence of ClSUN8 (Cla011257) revealed two SNPs (T to C and A to G) between lines TS (elongated fruit) and AM (round fruit), and a dCAPS marker was developed from one of these SNPs (A to G). In the present study using RACE PCR, we sequenced the full-length cDNA of Cla011257 to identify any mutations at the transcription or post-transcription levels that may relate to the function of this gene (Fig. 3, SM. 1). Sequence alignment of the cDNA sequences from the TS and AM lines revealed a 24-bp insertion/deletion (InDel) at the 3′-UTR and two SNPs at the 995th (T to C) and 1,179th (A to G) nucleotides in the third exon of the open reading frame (ORF). The SNP at the 995th nucleotide was synonymous (Ser203), whereas the SNP at the 1,179th nucleotide was non-synonymous, switching Lys (basic) acid in the TS line (Lys265) to Glu (acidic) in the AM line (Glu265) (Fig. 3, SM. 1), as reported by Kim et al. (2015). No significant changes at the transcriptional level, such as alternative splicing, were observed.

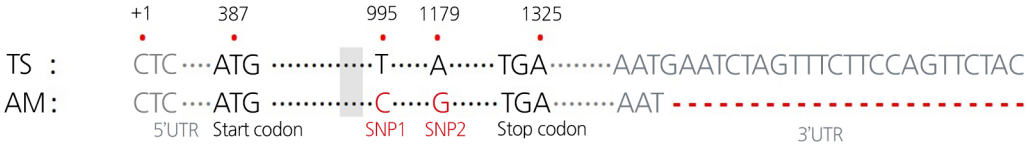

Fig. 3. The full-length cDNA sequences of ClSUN8 (Cla011257), a candidate IQD gene for fruit shape in watermelon accessions 'TS34' (TS, elongated) and 'Aarka Marnik' (AM, round). The 5'UTR and 3'UTR are shown in gray text. The nucleotide positions in the cDNA are marked by red asterisks. Mutation sites in the AM line [two SNPs in the coding sequence (CDS) and a 25-bp deletion in the 3'UTR] are shown in red text. A deletion region observed in several watermelon accessions, which resulted in failed PCR amplification of the Cla011257-dCAPS marker, is shaded in a gray box. The information for the complete sequence of the full-length cDNA sequences of ClSUN8 (Cla011257) is provided in Supplementary material 1.

In our previous report (Jin et al., 2017), allelic variations for ClSUN8 (Cla011257) that may exist in the watermelon population were evaluated by genotyping 85 watermelon accessions using the dCAPS marker developed for the non-synonymous SNP (A to G). We found that 81% of the accessions showed a marker-phenotype match, whereas failed PCR amplification was observed for 10 accessions. Those results indicated that there are allelic variations in Cla011257. Therefore, in the present study, DNA sequences of the PCR primer region for the dCAPS marker were verified by partially cloning Cla011257 from the four accessions. The new primer set [ClSUN8 (SCAR)] (Table 1) flanking the primers of the dCAPS marker amplified DNA fragments of expected size from all four accessions, and sequencing of the PCR fragments indicated that there was a 159-bp deletion that included the forward primer site of the dCAPS marker (SM. 1). Because the accessions showed a deletion mutation in Cla011257 regardless of fruit shape, fruit shape of these accessions may be controlled by another locus (or other loci), that is (are) possibly related to another IQD gene member (s) on different genomic region (s). This supposition may also apply to other watermelon accessions that showed mismatches between the dCAPS marker genotype and fruit shape (Kim et al., 2015).

In this study, we also cloned the genomic sequences of *MELO3C015418* from the melon inbred lines CM-P01 (oval) and MM-P02 (round) to identify allelic mutations. However, sequence alignment revealed no polymorphisms (data not shown). Although we cannot rule out the possibility that there may be mutations in the untranslated regions (UTR) and / or the promoter region of *MELO3C015418*, this sequencing result indicateed that a direct allelic comparison of the *MELO3 C015418* gene between the parents (Ved × PI414723) that were used for QTL mapping (Périn et al., 2002) must be examined

in order to elucidate the relationship between the IQD gene and fruit shape in melon.

The diversity of fruit shapes in cucurbits reflects genetic complexity and involvement of multiple genes in determining the trait. In addition, the loci conferring fruit shape may be different depending on the genetic background of the specific cultivar, as pointed out by Rodríguez et al. (2011). For example, different major QTLs were found in different mapping populations in melon [*CMFSI2.2* on Chr. 2 from 'Ved ×PI414723' (Périn et al., 2002) and *CMFSI8.2* on Chr. 8 from 'Piel de Sapo × PI124112' (Díaz et al., 2014)].

Morphological Assay and Expression Analysis of *ClSUN8*

The immature fruits (ovaries) at -4, -2, 0, 2, and 4 DAF were obtained from four watermelon inbred lines, SGR (highly elongated), TS (elongated), SBA (round), and AM (round), and their longitudinal length and diameter were measured (Fig. 4). At -4 DAF, the fruit shape index (FSI, length to diameter ratio) were SGR (FSI = 2.40), TS (1.89), AM (1.45), and SBA (1.37). All fruits rapidly expanded at 2 and 4 DAF (Fig. 4A). The FSI of each accession did not change significantly depending on the number of DAF, remaining constant from -4 to 4 DAF (Fig. 4B), implying that fruit shape had already been determined at the early stage of ovary formation.

Three different regions (proximal, middle, and distal) of the dissected fruit were observed (Fig. 5A). The size of the cells in these regions gradually expanded from -4 to -2 DAF (Fig. 5B). However, the cell sizes at any stage were very similar, indicating that the elongated fruit shape of SGR and TS was mainly due to a higher number of cells with increased cell division in the longitudinal direction (Fig. 5B). Extensive longitudinal growth during the early fruit developmental stages resulted in an elongated fruit shape of a mature tomato (Wu et al., 2011). In tomato, overexpression of the *SUN* gene led to the appearance of extremely long fruit and an increased FSI. A morphological analysis of *SUN*-overexpressing transformants

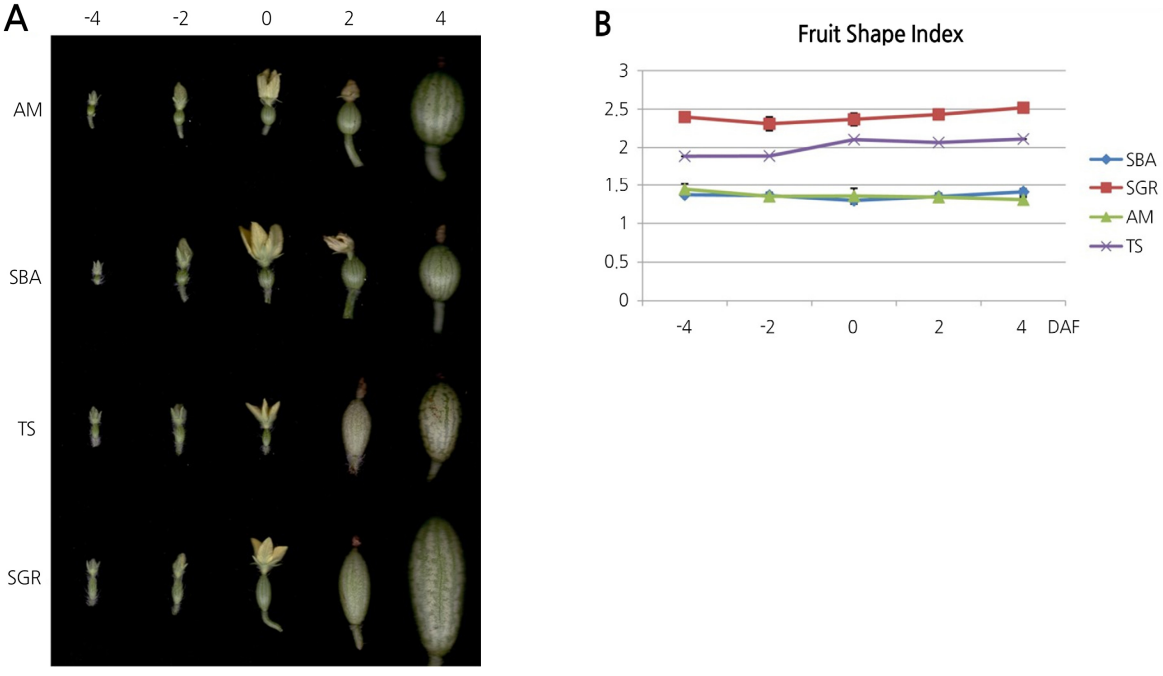

Fig. 4. Scanned images (A) and differences in the fruit shape index (B) of immature fruits harvested at -4, -2, 0, 2, and 4 days after fertilization (DAF) in four watermelon accessions (AM, TS, SBA, and SGR).

showed that accelerated cell division in the longitudinal direction and decreased cell division in the transverse direction was responsible for the elongated tomato fruits (Wu et al., 2011).

To investigate a possible association between the IQD gene expression level and fruit shape in watermelon, the mRNA transcript levels of *ClSUN8* were examined at different periods of fruit development (-4, -2, 0, 2, 4, and 6 DAF) for the SGR, TS, SBA, and AM lines (Fig. 6). The expression level of *ClSUN8* gradually decreased in all accessions, indicating that the

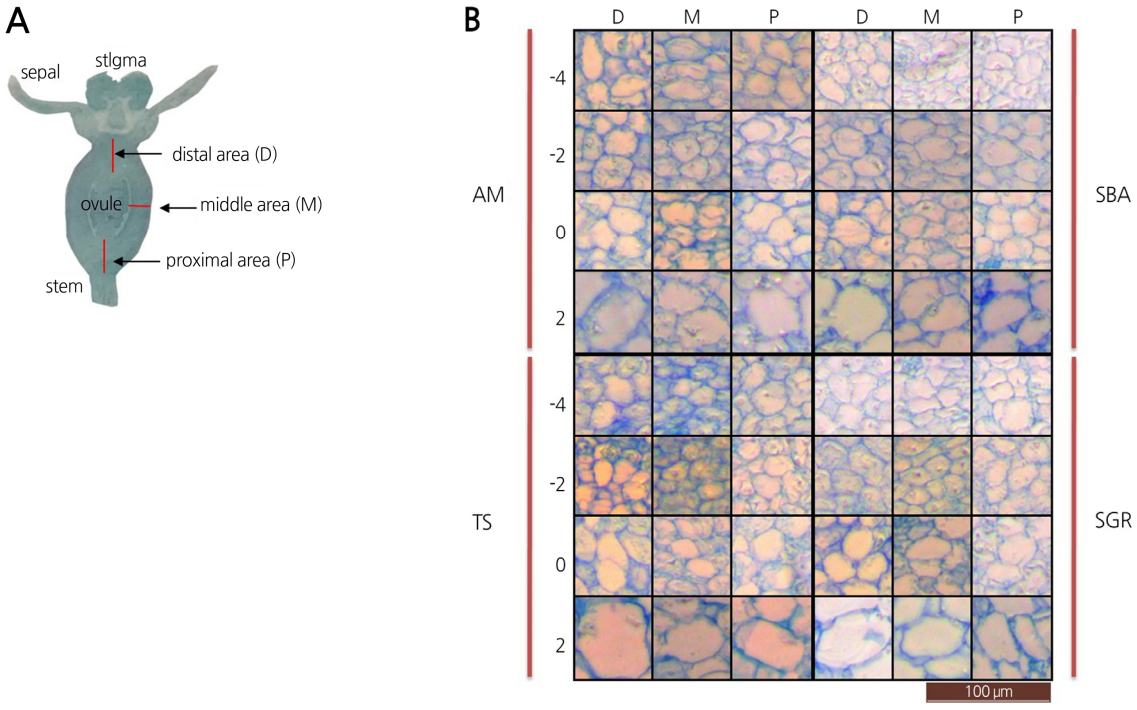

Fig. 5. Longitudinal section diagram of immature watermelon fruit (A) and histological analysis of flesh cells at three different areas [distal (D), middle (M), and proximal (P)] of immature fruits harvested at -4, -2, 0, 2, and 4 days after fertilization (DAF) in four watermelon accessions (AM, TS, SBA, and SGR).

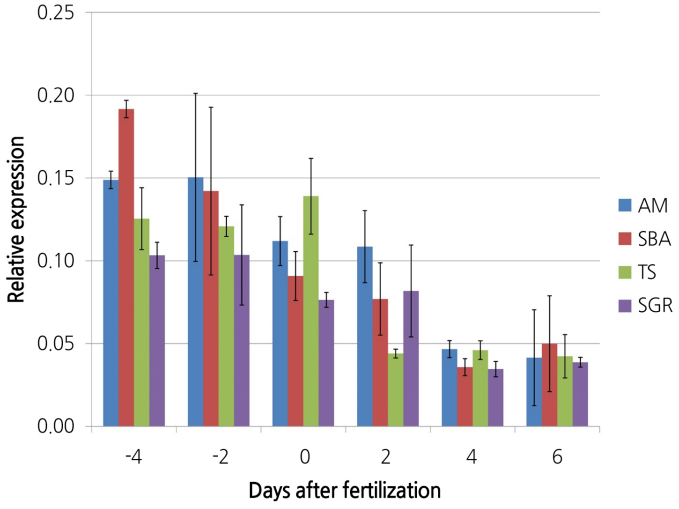

Fig. 6. Gene expression profiles of a candidate IQD gene for fruit shape, *Cla011257* (*ClSUN8*) at -4, -2, 0, 2, and 4 days after fertilization (DAF) in four watermelon accessions (AM, TS, SBA, and SGR).

regulation of gene expression for fruit shape determination occurs in the early developmental stages of ovary formation. In addition, there was no direct relationship between the gene expression level and fruit shape (Fig. 6). If *ClSUN8* is the gene responsible for fruit shape, our results imply that the elongated fruit shape of the TS line and the round shape of the AM line is not due to variations in the regulation of gene expression, but to the non-synonymous SNP and its effect on the molecular function of the IQD gene. To verify this hypothesis, a transformation confirmation test for functional characterization of *ClSUN8* will be necessary.

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