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Characterization of the *Lsi1* Homologs in *Cucurbita moschata* and *C. ficifolia* for Breeding of Stock Cultivars Used for Bloomless Cucumber Production

Jaemin Jung[1†], Joonyup Kim[3†], Bingkui Jin[1], Youngmi Choi[1], Chang Oh Hong[2], Hyun Ho Lee[2], Youngwhan Choi[1], Jumsoon Kang[1], and Younghoon Park[1,3*]

[1]Department of Horticultural Bioscience, Pusan National University, Miryang 50463, Korea
[2]Department of Life Science and Environmental Biochemistry, Pusan National University, Miryang 50463, Korea
[3]Life and Industry Convergence Research Institute, Pusan National University, Miryang 50463, Korea

*Corresponding author: ypark@pusan.ac.kr

†The authors contributed equally to this manuscript.

Abstract

Bloomless cucumber fruits are commercially produced by grafting onto the pumpkin stocks (*Cucurbita moschata*) to restricted silicon (SiO_2) absorption. Inhibition of silicon absorption in bloomless stocks is conferred by a mutant allele of the *CmLsi1* homologous to *Lsi1* in rice. In this study, we characterized the *Lsi1* homologs in pumpkin (*C. moschata*) and its cold-tolerant wild relative *C. ficifolia* ('Heukjong') in order to develop a DNA marker for selecting a bloomless trait and to establish the molecular basis for breeding bloomless stock cultivars of *C. ficifolia*. A Cleaved amplified polymorphic sequence (CAPS) marker (CM1_CAPS) was designed based on a non-sysnonymous single nucleotide polymorphism (SNP, C>T) of the *CmLsi1* mutant-type allele, and its applicability for Marker-assisted selection (MAS) was confirmed by evaluating three bloom and five bloomless pumpkin stock cultivars. Quantitative RT-PCR of the *CmLsi1* for these stock cultivers implied that expression level of the *CmLsi1* gene does not appear to be associated with the bloom/bloomless trait and may differ depending on plant species and tissues. A full length cDNA of the *Lsi1* homolog [named *CfLsi1*(B^+)] of 'Heukjong' (*C. ficifolia*), was cloned and sequence comparison between *CmLsi1*(B^+) and *CfLsi1*(B^+) revealed that there exists total 24 SNPs, of which three were non-synonymous. Phylogenetic analysis of *CfLsi1*(B^+) and *Lsi1* homologs further revealed that *CfLsi1*(B^+) is closely related to Nodulin 26-like intrinsic proteins (NIPs) and most similar to *CpNIP1* of *C. pepo* than *C. moschata*.

Additional key words: cucurbitaceae, *Lsi2*, *Low silicon rice 1*, marker-assisted breeding, transporters, RACE PCR

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Introduction

Cucumbers (*Cucumis sativas* L.), the Cucurbitaceae, are fruits and vegetables with a total annual production of 75 million tons (FAO, 2014). Total production of domestic cucumber in Korea is 271,040

tons per year (KOSIS, 2015), of which 28.4 tons is exported to Japan (UNI-PASS, 2015). For marketability in Japan, cucumber fruit without bloom (bloomless cucumber) is inevitable because it displays a clean and shiny appearance on the surface of the fruit (Choi et al., 2013; Mitani et al., 2011). In major Asian countries including Japan and Korea, oriental pumpkin (*C. moschata* Duch.) is used as a stock in grafting of cucumber for prevention of Fursarium wilt disease, maintenance of vegetative growth, and improvement of cold tolerance. For producing cucumbers exported to Japan, therefore, a specific type of pumpkin stock (bloomless stock) that prevents the cucumber fruit from blooming must be used (Seo et al., 2004; Choi et al., 2013). The seeds for bloomless stock are imported mainly from Japan, while no domestic stock cultivars are commercially available to date.

For production of winter greenhouse cucumber, a wild type pumpkin cultivar 'Heukjong', (*C. ficifolia* Bouche.) is widely used as a stock due to its nature of cold-tolerance over other non-tolerant pumpkin stocks (Tachibana, 1988; Seo et al., 2004). However, 'Heukjong' produces bloom in grafting of the cucumber and is inadequate for cultivating bloomless cucumbers in the winter season. Furthermore, despite the necessity for cold-tolerant bloomless stock cultivars, introgression of the bloomless trait into *C. ficifolia* from *C. moschata* is not straightforward due to the infeasibility of cross-fertilization between these two species (Robinson and Decker-Walters, 1997).

The bloom of cucumber fruit is generated when absorbed silicon (SiO_2) is exuded with water onto the skin and then dried (Yamamoto et al., 1989). A gene that facilitates the uptake of silicon, *Low silicon rice 1* (*Lsi1*), was first identified in rice (Ma et al., 2006), and its homologous genes were also identified in many other plants including maize and barley (Chiba et al., 2009; Mitani et al., 2009). There are two types of transporters that mediate silicon absorption, a channel-type transporter (or an influx transporter) and an efflux transporter (Ma and Yamaji, 2015). In rice, the *Lsi1* gene encodes a channel-type transporter that facilitates passive transport of silicon across the plasma membrane between apoplast and the plant cell (Yamaji et al., 2008), while an efflux transport is mediated by the *Lsi2* gene that belongs to an uncharacterized anion transporter family (Ma and Yamaji, 2015). In response to silicon, expression of *Lsi1* was down-regulated in rice and cucumber (Ma et al., 2006; Sun et al 2017), but was unaltered for its homologous genes in maize and barley (Chiba et al., 2009; Mitani et al., 2009). Recently, it was reported that pumpkin homologs, *CmLsi1* and *CmLsi2*, are associated with the transport of silicon (Mitani et al., 2011; Mitani-Uneo et al., 2011). Furthermore, a bloomless pumpkin cultivar 'Super-unryu' possesses a missense mutation (P>L) at position 242 amino acid that is linked to the transport activity of silicon (Mitani et al., 2011).

DNA markers have been employed to identify DNA polymorphisms and analyze genotypes and genetic linkage between genes and traits (Lee and Chung, 2011; Kwon and Choi, 2013). In modern breeding strategies, Marker-assisted selection (MAS) is the most popular application of the DNA marker. MAS shortens the time required for breeding, is not influenced by environmental factors, and can greatly increase reliability and precision in comparison to phenotype-based selections (Tanksley, 1983; Collard and Mackill, 2008; Jonah et al. 2011). Although it has been reported for mutational effect of *CmLsi1* related to the bloomless trait (Mitani et al., 2011), a DNA marker that can be used to analyze allelic or mutational variations of the *CmLsi1* gene is not publicly available.

Here, we developed a molecular marker for selection of a bloomless trait based on nucleotide polymorphisms of *CmLsi1*. We cloned the homologous gene of *Lsi1* in *C. ficifolia* to establish the molecular basis for breeding of cold-tolerant bloomless stock cultivars of *C. ficifolia*..

Materials and Methods

Plant Materials

Three cultivars of bloom pumpkin stocks ['Heukjong' (Kyung Nong Seed, Busan, Korea), 'Odaetojwa' (Kyung Nong Seed, Busan, Korea), and 'Arirang' (Koregon, Anseong, Korea)] and five cultivars of bloomless pumpkin stocks [('Ohmai Stock' (Kyung Nong Seed, Busan, Korea), 'Ohmai Summer Stock' (Kyung Nong Seed, Busan, Korea), 'Union' (Koregon, Anseong, Korea), 'Nunbusyeo' (Koregon, Anseong, Korea), 'Newtype' (Koregon, Anseong, Korea)] were used for the determination of silicon uptake and gene analysis.

Gene-Based DNA Marker

***Extraction of DNA*:** Genomic DNA was extracted from young leaves of the two-true leaf stage of the plant. Collected leaf tissues were placed into a 1.5 mL micro-centrifuge tube with beads and 600 μL of DNA extraction buffer and subjected to Tissuelyser (TissueLyser II, QIAGEN, Venlo, Netherlands) for homogenization. Samples were lysed in a 65°C waterbath for 45 min and further incubated by adding 200 μL of 7.5 M ammonium acetate on ice for 15 - 20 min. Incubated samples were separated by centrifugation for 10 min at 14,240 x g. The supernatant of centrifuged samples was placed in a 1.5 mL tube containing 5 μL (5 mg · mL^{-1}) of glycogen solution and 600 μL of isopropanol in a 1.5 mL tube and centrifuged for 10 min at 14,240 x g. After decanting the supernatant, remaining pellet was washed using 300 μL of 70% EtOH. The washed pellet was resuspended in 200 μL of 0.1 M Tris. Concentration of DNA was measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and 20 ng · μL^{-1} was used for PCR reactions.

***Development of CAPS Marker*:** Coding DNA Sequences (CDS) of wild-type allele *CmLsi1*(B^+) (AB551949) and mutant-type allele *CmLsi1* (B^-) (AB551950) of the *C. moschata Lsi1* were obtained from NCBI and aligned for comparison using CLUSTALW (www.genome.jp/tools/clustalw) and confirmed for the SNP of the mutant allele (Fig. 1). Identified SNPs were searched for restriction sites (NEBcutter V 2.0, NEB®, Ipswich, MA, USA) to generate a Cleaved Amplified Polymorphic Sequence (CAPS) marker using Primer premier 5 (PREMIER Biosoft, Palo Alto, CA, USA) (Fig. 1). PCR conditions for genotyping consisted of 1 μL of template DNA (20 ng · μL^{-1}), 0.5 μL of forward and reverse primer (10 pmol), 0.1 μL of Taq polymerase (5 U · μL^{-1}, eTaq Solg ™, SolGent, Daejeon, Korea), 0.2 μL of dNTPs (Solg ™, SolGent, Daejeon, Korea), 1 μL of 10X buffer (Solg ™, SolGent, Daejeon, Korea) and 6.7 μL of ddH_2O in a total volume of 10 μL. Reactions were incubated at 95°C for 2 min, and cycled 35 times as follows: denaturation at 94°C for 15 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min using a PCR cycler (T100 ™, BIO-RAD, Hercules, CA, USA). After the last cycle, reactions were incubated at 72°C for 3 min. Restriction digestion of PCR products was performed by adding 0.3 μL of *Hae* III (10,000 U · mL^{-1}, Time-Saver ™, NEB®, Ipswich, USA), 1.5 μL of 10X buffer (CutSmart ™, NEB®, Ipswich, Massachusetts, USA), 3.2 μL of ddH_2O to the PCR product and incubated at 37°C for 1 h. Digested products were run on a 2% agarose gel at 70V for 40 min and stained with EtBr, and checked for the results using Gel Image Analysis System (CoreBio i-MAXTM, Davinch-K, Seoul, Korea).

Analysis of CmLsi1 Gene Expression

***RNA Extraction and cDNA Synthesis*:** Young leaves and roots from at least three plants per cultivar were collected and

immediately frozen in liquid nitrogen. Total RNA was extracted using a Seed/Fruit Kit (Ribospin™, GeneAll®, Seoul, Korea) according to the manufacturer's instructions. Quantitated RNA using Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA) was diluted to 10 ng · μL^{-1} and used for cDNA synthesis with an RT Premixture kit (HyperScript ™, GeneAll®, Seoul, Korea) following the manufacturer's instructions.

***Quantitative PCR (qPCR)*:** Gene-specific primers (Q_F and R) for *CmLsi1* (B^+) (AB551949) were designed using Primer premier 5 (Table 1). Quantitative real-time RT-PCR (qPCR) was performed in a Real-time PCR cycler (LightCycler® 480, Roche, Basel, Switzerland) for 'Heukjong', 'Odaetojwa', 'Ohmai Stock', and 'Ohmai Summer Stock'. Reactions were performed by adding 1 μL of cDNA (10 ng · μL^{-1}), 0.5 μL of each forward and reverse primer (10 pmol), 5 μL of 2X SYBR Green I Master (Roche, Basel, Switzerland), and 3 μL of ddH2O in a total volume of 10 μL. *CmACTIN* was used as a reference gene as described by Obrero et al. (2011) (Table 1). All reactions were incubated at 95℃ for 5 min, and cycled for 45 times as follows: 95℃ for 10 sec, 55℃ for 20 sec, 72℃ for 1 min. Relative quantitation of the *Lsi1* transcript to *CmACTIN* gene was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2011).

Table 1. PCR primers used in this study.

	Primer name	Primer Sequence (5'- 3')	Tm (℃)	Enzyme
CAPS	CM1_CAPS_F	GACATTAGGACCTGCAATGG	58.2	*Hae*III
	CM1_CAPS_R	CTTTCACCTTCACCAACGTC	58.1	
RACE PCR	Cm1_3'RACE	GATTACGCCAAGCTTGGCGGCATTGAACGGGAGCGATGTG	76.4	
	Cm1_5'RACE	GATTACGCCAAGCTTACCGATACCAAAGCCGTGGGAGAGCTG	75.7	
qPCR	Q_F	GTTGGTTCTGCCGTGTGTAT	55	
	Q_R	TTTGAGTGAAAATGAGTGAGGAG	55	

Cloning of *Lsi1* Homolog in *C. ficifolia*

***5'and 3'RACE*:** The full-length cDNA sequence of *CmLsi1* homologous gene from *C. ficifolia* was obtained using RACE 5'/3' Kit (SMARTer®, Takara, Kusatsu, Japan). Total RNA was extracted as outlined above and RACE reactions were performed according to the manufacturer's instructions. Gene-specific primers were designed from *CmLsi1* (B^+) CDS (AB551949) with primer lengths of 23-28 bp, GC contents of 50-70%, T_m>70°C and 15 bp 5'-end primer overlap sequence (GATTACGCCAAGCTT).

***Cloning and Sequencing of CfLsi1*:** RACE reactions were checked on an agarose gel and purified the product using a gel extraction kit (ExpinTM, GeneAll®, Seoul, Korea). Purified RACE product was cloned into T-Easy Vector System I (pGEM, Progma, Madison, WI, USA) and verified the sequence by the dye terminator method (Genotech, Daejeon, Korea).

Sequence Alignment and Phylogenetic Analysis

The nucleotide sequence of *CfLsi1* was used as a query sequence to conduct BLAST searches to identify homologs of the *CfLsi1* from NCBI (www.ncbi.nlm.nih.gov). To determine the relationship between searched sequences, a phylogenetic tree (dendrogram) of homologs was generated with MEGA 5 (Tamura et al., 2011) using the Neighbor-Joining (NJ) algorithm and Tamura-Nei parameter model with 1,000 bootstrap replicates.

Measurement of Silicon Uptake

Seeds of eight commercial cultivars (see above) were sown in Baroker potting soil (Seoul Bio, Eumseong, Korea) in a 50 - holed tray. After 25 days of sowing, seedlings were transplanted in a plastic pot (15 cm × 13 cm × 19 cm) and grown for 30 days in a greenhouse at Pusan National University (Miryang, Korea). While transplanting, the soil collected from a paddy field of the Agriculture Research Station at Pusan National University was air-dried and sifted, and 2.4 kg of the soil was filled in the plastic pot. Three plants of eight cultivars were grown in a completely randomized design with three biological replicates. Bulk density of soil was 1.37 g · cm^{-1}, and 576 mL of distilled water was added so that the water content in soil was 60% of the pore volume with a particle density of 2.65 g · cm^{-1}. After transplanting, the silicate fertilizer (Nonepong Eco, Nousbo, Suwon, Korea) was dissolved in 100 mL of tap water and added at the rate of 0 (untreated control), 100 and 200 mg Si · kg^{-1} in each pot. Throughout the whole growth, nitrogen (12.8 mg · kg^{-1}), phosphoric acid (8.75 mg · kg^{-1}) and potassium (12.7 mg · kg^{-1}) and compost (1 g · kg^{-1}) were supplemented with the same amount, and water was added to compensate the decreased amount by weighing the pot once every four days. After 30 days of transplanting, whole plants were collected from soil and dried in a dry oven at 70℃ for 72 h and then pulverized. One gram of the pulverized sample was lysed in the lysis buffer (H_2SO_4:HClO:H_2O = 5:9:1, Samchun, Pohang, Korea) and then the content of silicon was analyzed with ICP-AES (GBC model X-100, GBC, Melbourne, Australia).

Results and Discussion

Development of *CmLsi1* Marker

Coding DNA Sequences (CDS) of wild-type allele (B^+) and mutant-type allele (B^-) of the *CmLsi1* were used for alignment to confirm the non-synonymous SNP (C>T) missense mutation (P>L). To design a CAPS marker for the identified SNPs, restriction enzyme sites within the sequence were searched and recognition sites of *Hae* III was selected for the study. A PCR primer set CM1_CAPS (Table 1) was designed spanning the *Hae* III restriction site to cut the amplified product (201 bp) into 140 bp and 61 bp in the mutant-type allele (Fig. 1). Genotyping analysis of the eight stock cultivars using the CM1_CAPS marker revealed that bloom and bloomless traits were correlated with each genotype (Fig. 2). For a bloom F_1 cultivar 'Arirang', marker analysis indicated that it is heterozygous genotype for *CmLsi1* (Yamaji and Ma, 2007), which suggests that one of the parental lines of 'Arirang' was a bloomless cultivar homozygous for the mutant allele of *CmLsi1*. From this, we confirmed use of the CM1_CAPS marker for MAS-assisted breeding of bloomless trait.

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---------- CAACCGATACCAAAGCCGTGGGAGAGCTGGCAGGTTTGGCAGTTGGTTCTGCCGTGTGTATCACATC  617
CATCTTGGCTGGACCCGTATCAGGTGGGTCGATGAACCCTGTGAGGACATTAGGACCTGCAATGGCAAGTGATAATT  694
ATAAAGGACTTTGGGTGTACTTTGTTGGGCC/TGGTTACAGGAACCCTATTAGGGGCATGGTCATATAAGTTCATAC  769
GTGCCAGTGATAAACCTGTGCACTTAATTTCTCCTCACTCATTTTCACTCAAACTTCGAAGAATGTCAAGATCTGAC  846
GTTGGTGAAGGTGAAAGATGA  857
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Fig. 1. Coding DNA sequence (867 bp) of the *CmLsi1* (B^+) (AB551949). PCR primers for CM1_CAPS marker (yellow) and quantitative real time RT-PCR (red) are color-coded. Restriction site used in this study for *Hae*III is boxed and the SNP (C/T) to *CmLsi1* (B^-) (AB551950) is red-colored.

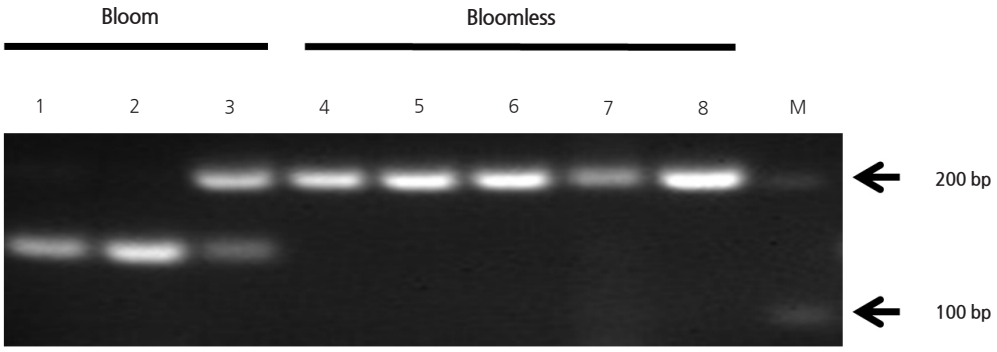

Fig. 2. Agarose gel (2%) image showing the result of CM1_CAPS genotyping for the *CmLsi1* gene in eight stock cultivars. Lane 1, 'Heukjong'; 2, 'Odaetojwa'; 3, 'Arirang'; 4, 'Nunbusyeo'; 5, 'Ohmai Stock'; 6, 'Ohmai Summer Stock'; 7, 'Union'; and 8, 'Newtype'.

Transcripts of *CmLsi1* in Bloom and Bloomless Stock Cultivars

Expression levels of endogenous *CmLsi1* gene were examined in leaf and root tissues from bloom ('Heukjong' and 'Odaetojwa') and bloomless ('Ohmai Stock' and 'Ohmai Summer Stock') cultivars using Quantitative real time RT-PCR (qRT-PCR) (Fig. 3). From the tissues tested for representative cultivars, relative expression of *CmLsi1* was higher in root than leaf of these cultivars (Fig. 3). The expression pattern of *Lsi1* was consistent with the result from rice (*Oryza sativa*) and cucumber (*Cucumis sativus*), which showed higher expression of *Lsi1* in root than leaf (Ma et al., 2006; Sun et al., 2017). Among cultivars of pumpkins (*C. moschata*), it has been shown that expression of *Lsi1* gene in a bloomless cultivar 'Super-unryu' was higher than a bloom cultivar 'Sintosa' (Mitani et al., 2011). Curiously, it was also reported that expression of *CmLsi1* in shoot apex was

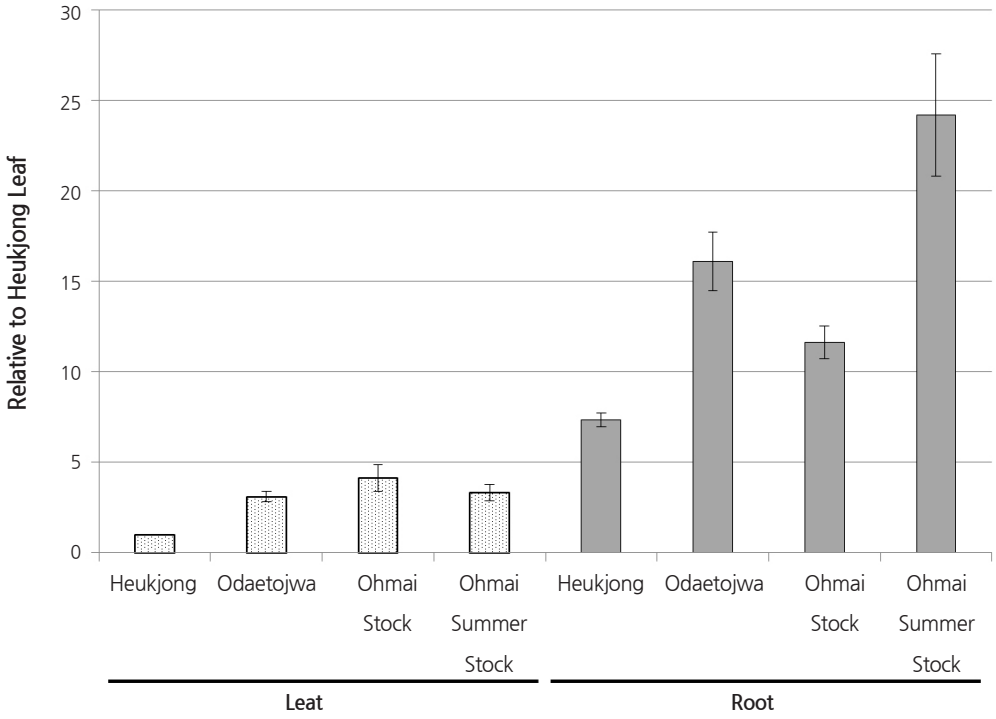

Fig. 3. Tissue-specific expression of *CmLsi1* in four stock cultivars using a gene-specific primer. Relative values from leaf and root tissues to *CmACTIN* are normalized to that in leaf of 'Heukjong'.

higher than that in root of pumpkin (Mitani et al., 2011). The findings suggest that expression of *Lsi1* is species- and tissue-specific, and the relation of *Lsi1* genotypes with the bloom / bloomless trait may not involve the transcriptional regulation of *Lsi1* genes. However, exact mechanisms underlying these transcriptional regulations and their possible relation with functions in silicon uptake remain to be determined.

Taken together, expression pattern of the *Lsi1* gene does not appear to be associated with variations of alleles in pumpkin (this study), and the expression level may differ depending on the species, cultivars and tissues examined. A link between bloomless trait of pumpkin resulting from the missense mutation caused by a non-synonymous SNP in the protein and suppression of *Lsi1* expression in a bloomless cultivar needs further examination.

Cloning of *CfLsi1*

We identified a full-length cDNA of *CmLsi1*(B^+) homologous gene in *C. ficifolia* through 5' and 3' RACE and named *CfLsi1*(B^+) (Fig. 4). A sequence comparison between *CmLsi1*(B^+) (accession AB551949) and *CfLsi1*(B^+) revealed that there exists total 24 SNPs, although the length of CDSs was identical. Of these, only three SNPs appeared to substitute (non-synonymous) amino acids at positions 14 (S>A), 75 (V>A), and 162 (L>F) (Fig. 5). Considering the ability of silicon exerted likely by *Lsi1*, these missense mutations do not appear to affect the function of the *CfLsi1* gene.

It seems plausible to introgress a bloomless allele from *C. moschata* to 'Heukjong' (*C. ficifolia* Bouche.) by hybridization to breed a cold-tolerant bloomless stock cultivar for cucumber. However, given the crossing barriers between these two species, other approaches like gene silencing technologies (RNAi) specifically targeting *CfLsi1*(B^+) expression in 'Heukjong' cultivar

ACATGGGGATAAGCGCAGTTTTTATAATATTATCAGCGTTTACGTTGAAACAGAGCGAGAAGACAAGGCTTCT
TCCTCCTTCTCCTCCAAAAATGAGTTCTTCCCAGGATCCTCAGCTTGTTCAACAACAAGCTGTTGTGGATGTT
GAAGAATTTGTCTCCGTTGAAAACCCAGATTCCAAACGCTCCCAGTTTGGATCCTTGTTCAAAAACCATTACC
CTCCCGGCTTTTCCCGAAAGCTTGTAGCAGAGGTGATAGCGACGTATTTGCTAGTGTTTGTAACGTGTGGGGC
GGCGGCATTGAACGGGAGCGATGCACAAAGAGTGTCGCAGCTTGGTGCCTCGGTCGCCGGTGGCCTTATTGTG
ACGGTGATGATTTACGCCGTCGGACACATTTCCGGCGCCCATATGAACCCGGCTGTCACCACGGCTTTTGCTG
CAACACGACACTTTCCATGGAAACAGGTTCCATTGTATGCAGCAGCCCAATTGAGTGGGGCAACATGTGCAGC
CTTTACACTGCGCCTATTATTACATCCCATCAAACACTTGGGCACTACAACGCCATCGGGATCAGATTTTCAA
GCACTCGTCATGGAGATTGTTGTTACCTTCTCAATGATGTTTGTCACATGTGCTGTTGCAACTGATACCAAAG
CAGTGGGAGAGCTGGCAGGTTTGGCAGTTGGTTCTGCCGTGTGTATCACATCCATCTTGGCTGGACCCGTATC
AGGTGGGTCGATGAACCCTGTGAGGACATTAGGACCTGCAATGGCAAGTGATAATTATAAAGGACTTTGGGTG
TACTTTGTTGGGCCAGTTACAGGAACCCTATTAGGGGCATGGTCATATAAGTTCATACGTGCTAGTGATAAAC
CTGTGCACTTAATTTCTCCCCATCATTTTCACTCAAACTTCGGAGAATGTCAAGATCTGACGTTGGTGAAGG
TGAAAGATGA**GCACTAACTCAACATCTTCCTCTACGACCTTTTAAGTATTGGAAAGAATGTGAACTAGTATGA**
GTGTTTAATGAGCATAGTGATCTCATGTACTATGTTACAATTAAGTTTACTTCTTACCATCTAGCAGCGTATG
AAAGCTTACTTTCTATTAGTTTAGCACTATGTAAGAAGTAGCCTTTGTAATTTAGCATCTTCTATGAATGAAA
TTTTCTACTTTTGTA

Fig. 4. The full-length cDNA sequence of the *CfLsi1* (B^+) gene. 5' and 3' untranscribed sequence (UTR) at both ends are bolded, and 24 SNPs to the *CmLsi1* (B^+) gene were red-colored.

Fig. 5. Comparison of the amino acid sequences for *Lsi1* gene homologs in *C. ficifolia* Bouche. [*CfLsi1* (B^+)] and C. moschata Duch. [*CmLsi1* (B^+)]. Of 24 SNPs, three SNPs were predicted to substitute amino acids in non-synonymous way. Three nonsynonymous changes are highlighted as indicated.

that may alter the fuction of *CfLsi1* protein might be useful as an alternative. Nonetheless, we consider that a full-length cDNA sequence of *CfLsi1*(B^+) identified in this study would provide valuable molecular platform that can be used in future molecular breeding.

Phylogenetic Analysis of CfLsi1 and Lsi Homologs

Nucleotide sequences that showed high sequence similarity to *CfLsi1*(B^+) of *C. ficifolia* from different plant species were used to generate a phylogenetic tree (Fig. 6). As expected, the retrieved sequences with high similarity revealed that *CfLsi1*(B^+) is closely related to Nodulin 26-like intrinsic proteins (NIPs) that belong to a larger family of Major intrinsic proteins (MIPs) (Wallace et al., 2006). It has been shown that among channel-type silicon transporters (or influx transporters) all *Lsi1* belong to the NIP III group of aquaporin proteins (Ma and Yamaji, 2015).

Sequence comparisons using *CfLsi1*(B^+) showed that *CfLsi1* is most similar to *CpNIP1* of *C. pepo* than *C. moschata*. Also, *CfLsi1* was closely related to homologs in cucumber and melon. Cucumber, melon (*Cucumis melo*), and watermelon (*Citrullus vulgaris*) are in the same Benincaseae tribe whereas *C. ficifolia*, *C. moschata* and *C. pepo* belong to the Cucurbiteae tribe (Schaefer et al., 2009). It is worth noting that phylogenetic analysis of *CfLsi1*(B^+) and its select homologs (the current study) is also supported by the previous finding that within Cucurbiteae tribe *C. ficifolia* Bouche. was closer to *C. pepo* than either to oriental and western pumpkin based on analysis of relationship using SSR markers (Kim et al., 2015).

Measurement of Silicon Content

The accumulation of silicon for eight stock cultivars was measured as described above. Overall, the silicon content measured in all the pumpkin stock cultivars ranged from 0.2% to 0.5% of the total dry mass. The most significant difference between

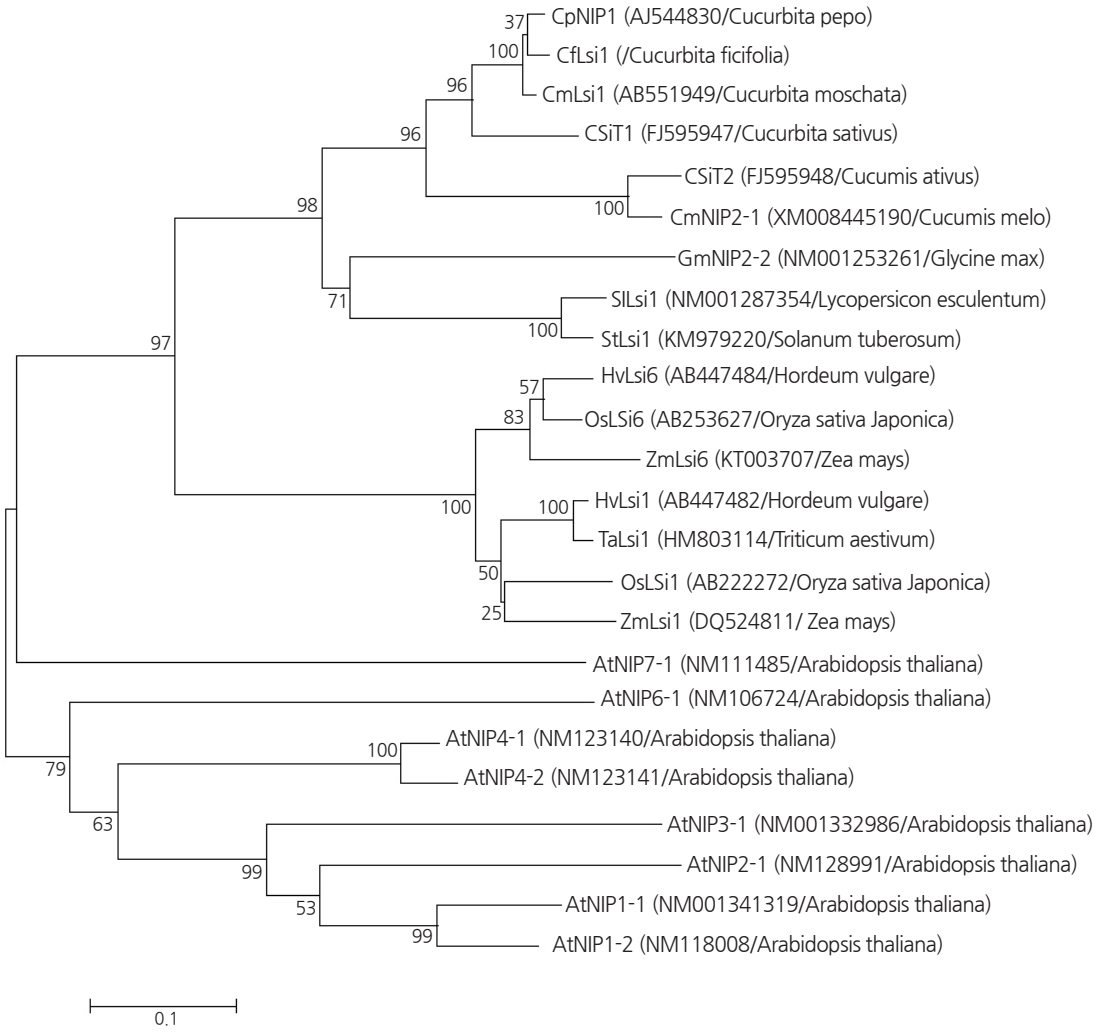

Fig. 6. A phylogenetic tree based on the nucleotide sequences of the *Lsi1* gene homologs in different plant species. The phylogenetic tree was constructed using the neighbor-joining algorithm by MEGA 5.05 software after ClustalW alignment with 1000 bootstrap trials.

bloom and bloomless cultivars was found to be the ones supplied with 0.1 g·kg^{-1} and 0.2 g·kg^{-1} of silicon (Table 2). The content was higher in all the treated bloom cultivars ('Heukjong', 'Odaetojwa', and 'Arirang') than most of the bloomless cultivars ('Ohmai Stock', 'Ohmai Summer Stock', 'Union', 'Nunbusyeo' and 'Newtype'). Interestingly, the highest amount of silicon content was found in the bloom cultivars at 0.2 g·kg^{-1} of silicon treatment particularly in 'Heukjong' cultivar (0.5% of total dry mass) (Table 2). Above findings agree with the previous results that the silicon content in shoots of bloom stock in pumpkin was higher than that of bloomless stock cultivar in pumpkin (Mitani et al., 2011), and the higher content of silicon in the cucumber grafted to the bloom stock than that grafted to the bloomless stock (Choi et al., 2013).

It was reported that the shoot of bloom cultivar in pumpkin contained 1% of silicon whereas the shoot of bloomless cultivar had 0.1% (Mitani et al 2011). In our study, however, bloom cultivars of pumpkin stocks contained approximately 0.3 - 0.5% of silicon which was less than expected, which may be attributed to the different experimental settings. In addition, a silicon content in plants may be affected by various factors other than genetic or allelic variations. For instance, silicon could be fixed to

Table 2. Silicon concentration in eight stock cultivars used for cucumber fruit production. Each treatment was replicated three times.

Cultivars	Trait[z]	Marker genotype[y]	Silicon treatment ($g \cdot kg^{-1}$)		
			0	0.1	0.2
Heukjong	B	*Lsi1/Lsi1*	4.69 d[x]	4.52 c	5.15 f
Odaetojwa	B	*Lsi1/Lsi1*	3.71 c	4.71 d	3.89 e
Arirang	B	*Lsi1/lsi1*	3.31 bc	3.24 b	3.86 e
Nunbusyeo	BL	*lsi1/lsi1*	3.16 b	2.91 b	2.87 c
Ohmai Stock	BL	*lsi1/lsi1*	2.36 a	2.26 a	2.16 ab
Ohmai Summer Stock	BL	*lsi1/lsi1*	2.51 a	2.54 a	1.95 a
Union	BL	*lsi1/lsi1*	2.61 a	2.25 a	2.43 b
Newtype	BL	*lsi1/lsi1*	3.19 bc	2.24 a	3.47 d

[z]B, bloom; BL, bloomless.
[y]*Lsi1/Lsi1*, homozygous marker genotype for wild-type allele; *lsi1/lsi1*, homozygous marker genotype for mutant type allele.
[x]Least significant difference, $p < 0.05$

soil surface and changed into unavailable forms due to various environments such as pH, redox potential, temperature, and electrical conductivity in soil (Patrick Jr et al., 1987; Szulc et al., 2015; Carmo et al., 2016). Therefore, the difference in growth conditions and organs measured as well as experimental settings may have contributed to the differential contents of silicon. Furthermore, it has not been determined for general amount of silicon uptake in pumpkin, which makes it difficult to compare the amount of silicon uptake in the current study with others.

It is not clear for the observed silicon contents bloomless stock cultivars. It may have been affected by other genetic factors other than *Lsi1*, such as *Lsi6* that might otherwise have affected the uptake. The presence and function of *Lsi6* homologs has not been determined in pumpkin. The *Lsi6* gene in rice is presumed to play a role in transferring silicon from xylem to leaf, but exact mechanisms of action are still unclear. According to Misson et al. (2004), it was reported that the mutation in *Pht1;4-1* gene caused about 40% of reduction in total absorption of Pi under low inorganic phosphate (PI) conditions, but not complete inhibition. Therefore, it is thought that the missense mutation (P > L) of the *CmLsi1* reduces the silicon absorption rate rather than complete inhibition in bloomless pumpkin stocks. Although we provide molecular basis for breeding of bloomless stock cultivars, functional relevance of the *CmLsi1* gene that can inhibit silicon uptake and its bloomless effect on the cucumber silicon still await further investigation.

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