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Evaluation of Chloroplast Genotypes of Korean Cucumber Cultivars (*Cucumis sativus* L.) Using sdCAPS Markers Related to Chilling Tolerance

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Abstract. DNA markers can determine the genotype of many species. Single nucleotide polymorphism (SNP) detection is difficult without sequencing but it becomes easier with sdCAPS method. Here an experiment was performed for developing molecular markers using two SNPs, CSatpB-SNP and CSycf1-SNP, of chloroplast in cucumber plants. Properly designed primers with nucleotide sequences for restriction enzymes proved success of PCR and efficacy of digestion by the restriction enzymes. Then these markers were used to study the genotyping of cucumber breeding lines and cultivars obtained from various sources in respect of their chilling stress response. We confirmed that a U.S. cucumber line, 'NC76' known to possess a nuclear factor for the chilling tolerance showed the chloroplast genotypes related to chilling tolerance. However all Korean cucumber cultivars tested in this study showed the chloroplast genotypes related to chilling susceptibility. In conclusion, to develop chilling tolerant cucumber, both maternal and a nuclear factors related to chilling tolerance should be transferred from 'NC76' when 'NC76' is used as a female source and other elite lines as recurrent parents.

***Additional key words*:** chilling injury, dCAPS, molecular markers, SNP

Introduction

Cucumber (*Cucumis sativus* L.) is one of the most important vegetable plant in the world (FAO, 1993). Breeding of cucumber for horticulturally important traits has received more attention recently. For example, low temperature (0°C to 12°C, chilling temperature) can cause severe damage on leaves of cucumber. Therefore, genetics of chilling tolerant trait in cucumber has been studied (Chung et al., 2003, 2007; Kozik and Wehner, 2008; Smeet and Wehner, 1997). The previous study showed that chilling tolerant trait is maternally transmitted and is likely associated with chloroplast DNA genotypes (Chung et al., 2003). Chung et al. (2007) showed that cucumber chloroplast genome has a very narrow genetic variation and only three chloroplast DNA regions (1: between TrnK and Rps16, 2: AtpB, and 3: Ycf1) are polymorphic between 'GY14' (chilling susceptible) and 'Chipper' (chilling tolerant) cucumber plants. Among these three single nucleotide polymorphisms (SNPs) of chloroplast DNA (cpDNA), two SNPs of AtpB and Ycf1 are located in the middle of AtpB and Ycf1 genes, respectively. AtpB gene encodes a beta subunit of ATP synthase (Cozens et al., 1986). Drought and salt stresses may change the gene expression level of atpB in plants (Ji et al., 2012; Kamal et al., 2012). In yeast, ycf1 gene produces a protein involved in cellular resistance to cadmium (Li et al., 1996) and it is also involved in attenuating oxidative stress response (Paumi et al., 2012). In cucumber, the genotypes of ycf1 and atpB genes are completely correlated with chilling response in 4 chilling tolerant and 4 chilling susceptible varieties (Chung et al., 2007). This result indicates that nucleotide positions of the two SNPs (SNP-atpB and SNP-ycf1) related to chilling response might be useful as markers for development of low temperature tolerant cucumber cultivar.

DNA marker is a technique to determine the genotypes of many crop species. Recently, several types of molecular markers such as RAPD (Fazio et al., 2003), AFLP (Park et al., 2000), and simple sequence repeat (SSR) markers (Fazio et al., 2003; Ren et al., 2009) have been developed and used for breeding of cucumber. These studies have shown that molecular markers linked to specific traits may be useful for marker assisted selection (MAS) in cucumber breeding.

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Single nucleotide polymorphism (SNP) is also a powerful marker type to detect polymorphism among species which possess a narrow genetic background. However, detection of SNP is difficult and requires high price equipment such as sequencer or analyzer for high resolution melting curve detection. In order to provide easy and economical SNP detection system for small scale laboratory, derived cleaved amplified polymorphic sequence (dCAPS) method, a way to convert SNP to a PCR-based marker type (Neff et al., 1998) is available to geneticist and breeders.

The main purpose of this work was to provide cucumber breeders with an easy detection method to determine chloroplast genotype that could be related to chilling tolerance in cucumber plants. We used sdCAPS (simply derived cleaved amplified polymorphic sequence) method (Jeong et al., 2011) to convert SNP information to PCR-based markers. The developed markers were then used to determine unknown chloroplast genotypes of Korean cucumber cultivars and U.S cultivar, 'NC76' which is known as a new chilling tolerant cucumber (Kozik and Wehner, 2008).

Materials and Methods

Plant Materials

Chloroplast sequences from chilling tolerant cucumber line, 'Chipper' and susceptible line, 'GY14' were used for sdCAPS marker development (Fig. 1). Cucumber cultivars of Korea, Japan, and China were purchased from different seed companies (Table 1). Chilling susceptible cucumber line, 'GY14' and Chilling tolerant cucumber lines, 'NC76' and 'Chipper' were obtained from United State Department of Agriculture (USDA), Agriculture Research Service (ARS) Madison, WI.

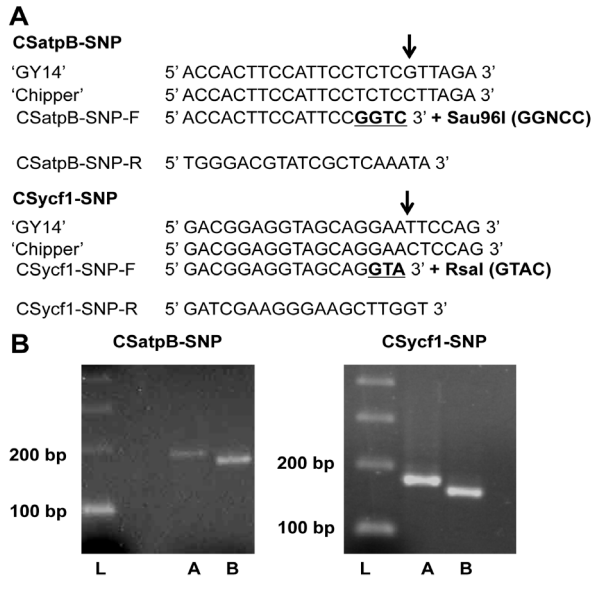

Fig. 1. Graphic depiction of analysis of simply derived cleaved amplified polymorphic sequence (sdCAPS) sites. (A) Arrows indicate the SNP sites and Restriction enzymes (RE)s for targeted SNP genotypes were selected according to sdCAPS method (Jeong et al., 2011), Bold letters in sdCAPS primer designations indicate mismatches between the sdCAPS primer and target DNA sequences. (B) Agarose gel images show the differences in length variation after restriction enzyme treatment for genotyping of both CSatpB-SNP and CSycf1-SNP, where A is for 'GY14', B for 'Chipper', and L for size ladder.

sdCAPS Marker Development

Chloroplast DNA (cpDNA) sequences of cucumber lines, 'GY14' (GenBank number: DQ865975.1) and 'Chipper' (GenBank number: DQ865976.1) were used for sequence alignment. Two SNPs of AtpB (CSatpB-SNP) and Ycf1 (CSycf1-SNP) genes of chloroplast DNA were aligned and compared (Fig. 1). According to sdCAPS method (Jeong et al., 2011), the forward primers were designed and restriction enzymes (REs), *Sau*96I and *Rsa*I were selected for CSatpB-SNP and CSycf1-SNP, respectively. Reverse primers were selected from the sequences to produce approximately 200 base pair nucleotides through PCR amplifications (Fig. 1).

DNA Extraction, PCR, and Restriction Enzyme Analysis

Seedling leaf tissue (second to third leaf stage) was collected from cucumber plants and stored at -80°C until DNA extraction. The frozen tissues were lyophilized followed by grinding and then mixing with 1X lysis buffer (50 mM Tris (pH 8.0), 50 mM EDTA, 5% sodium dodecyl sulfate) after that the mixtures were incubated for 1 h at 65°C. Proteins were precipitated and removed by adding one-third volume of 5 M ammonium acetate. DNAs were then precipitated by adding 100% ethanol and subsequently washed twice with 70% ethanol. DNA was dissolved with 10 mM Tris buffer (pH 8.0).

All PCR reagents were purchased from the Solgent Corporation (Seoul, South Korea) and all REs used for experimentation were purchased from NEB (Ipswich, MA, USA). Each 15 μL reaction volume contained 4.0 mM of $MgCl_2$, 0.3 mM of dNTPs, 15 ng of DNA, 0.45 μM of primers, polymerase buffer and 0.2 units of Taq DNA polymerase. All amplifications were conducted using the following cycling profile: 94°C for 5 min; 40 cycles of 94°C for 30 s, 50°C for 60 s, 72°C for 60 s; 72°C for 6 min, and then followed by an indefinite soak at 4°C. REs were applied to PCR products according to manufacturer's protocol.

To confirm PCR amplification, the amplified and digested samples were electrophoresed in 1.6% agarose gels in 0.5X TBE buffer (0.045 M Tris-borate and 1.0 mM EDTA pH 8.0) for 3 h at approximately 170 V, and then gels were

Table 1. List of cucumber cultivars tested for chloroplast genotypes.

No.	Name	Source	Country	Chilling Tolerance[z]
1	Heungnongbaegdadagi	Seminis Korea	Korea	weak
2	Saeronbanbaeg	Danong, Inc.	Korea	N/A[y]
3	Asiachungjang	Asia seeds Co.	Korea	strong
4	Saeronchungjang	Danong, Inc.	Korea	weak
5	Eunsungbaegdadagi	Seminis Korea	Korea	weak
6	Glorysamchug	Asia seeds Co.	Korea	N/A
7	Baegrogdadagi	Syngenta seeds, Inc.	Korea	weak
8	NC76	USDA	USA	strong
9	Chipper	USDA	USA	strong
10	Suiseifushinari 2-go	Kurume Vegetable breeding	Japan	N/A
11	Joeunbaegdadagi	Seminis Korea	Korea	strong
12	Hangangmatbaegdadagi	Syngenta seeds, Inc.	Korea	strong
13	Sinjungpum	Dongbu Hannong Co.	Korea	weak
14	White	Dongbu Hannong Co.	Korea	weak
15	Donggwan	Tianjin, Inc.	China	strong

[z]Information of chilling tolerance is based on seed description from company or source.
[y]N/A: no information is available.

stained with ethidium bromide (0.5 $mg \cdot mL^{-1}$ in TBE) and banding patterns were captured with a digital camera (WGD-30, Daihan Scientific, Seoul, South Korea).

Results and Discussion

For detection of SNPs among two cucumber lines 'Chipper' and 'GY14' sequences were aligned. Alignment of sequences clearly shows the CSatpB-SNP [i.e. guanine (G) in 'GY14' and cytosine (C) in 'Chipper'] and for CSycf1-SNP [i.e. thymine (T) in 'GY14' and cytocine (C) in 'Chipper'] (Fig. 1). By using sdCAPS method (Jeong et al., 2011), a dCAPS forward primer was designed having restriction enzyme sites i.e. *Sau*96I (GGNCC) for the CSatpB-SNP. For CSycf1-SNP, a dCAPS forward primer was designed for the restriction enzyme *Rsa*I (GTAC). The reason for designing these primers with the said restriction enzyme sites is that they cut PCR amplified products only from 'Chipper' genotype but not from 'GY14' genotype (Fig. 1).

Considering both SNPs [CSatpB-SNP and CSycf1-SNP] (Fig. 1), the reverse primers were designed to amplify the target regions of DNA with size approximately 200 bps. With this size, when forward primer region of PCR product from the genotype of 'Chipper' is removed, the difference in size between cut and uncut PCR products would be certainly appeared in electrophoresis using the agarose gel. In case of CSatpB-SNP, the forward primer carrying *Sau*96I restriction enzyme site and a reverse primer produced PCR amplification with both 'Chipper' and 'GY14' DNAs even though the forward primer possess two mismatch sequences with the target DNA sequences (Fig. 1). According to sdCAPS strategy (Jeong et al., 2011), maximum two mismatches can be generated between sdCAPS primer sequences and target DNA sequences. This case clearly demonstrated that two mismatches did not affect the PCR amplification.

The expected size of the PCR products for CSatpB-SNP was 217 bps according to the sequences from GeneBank database. Since the PCR amplified product of 'GY14' was not supposed to be cut by *Sau*96I enzyme in the forward primer region, its size should be around 217 bps. However, after employment of the restriction enzyme, *Sau*96I, the sizes of the PCR product with 'GY14' and 'Chipper' were around 190 bps and 175 bps, respectively. Although the results showed that both PCR products from 'GY14' and 'Chipper' were digested by the restriction enzyme. However, developed sdCAPS marker for CSatpB-SNP detected the polymorphism (i.e. 190 bps and 175 bps) for 'GY14' and 'Chipper' respectively, detected in 1.6% agarose gel (Fig. 1). The reason that the sizes of cut PCR products were different was explained when the *Sau*96I recognition site was found in both targeted nucleotide sequences of 'GY14' and 'Chipper'. After PCR amplification using the sdCAPS primer, two *Sau*96I recognition sites including the forward primer region were found from 'Chipper'. In the 'GY14' sequence, there is only one *Sau* 96I recognition site after the PCR amplification. Because of this, the size of final product of 'Chipper' is 15 bps less compared to the final product of 'GY14' after digestion with the restriction enzyme

treatment (Fig. 1).

In case of CSycf1-SNP, the forward primer was designed with *Rsa*I restriction site. The expected size of 'GY14' was 198 bp but actual size of cut PCR product was 177 bp due to an extra *Rsa*I restriction site in the targeted sequence region. It was located near reverse primer region of the sequences. When 'Chipper' was digested with *Rsa*I it resulted in three fragments 16 bp (left side), 21 bp (right side) and the large fragment 157 bp. Nevertheless, this CSycf1-SNP sdCAPS marker still detected the polymorphism in size of the final products between 'Chipper' and 'GY14'. Therefore both (CSatpB-SNP and CSycf1-SNP) sdCAPS markers showed the polymorphic bands between chilling tolerant chloroplast (cp) genotypes and chilling susceptible cp genotypes in cucumber (Fig. 1).

In order to exam the efficacy of these markers and cpDNA genotypes of Korean cucumber cultivars, different cucumber varieties numbering from 1 to 15 obtained from different sources (Table 1) were employed for genotyping of the markers. Targeted cpDNA regions were amplified with respective sdCAPS markers developed in this study. Among Korean cucumbers, 'Asiachungjang', 'Joeunbaegdadagi', and 'Hangangmatbaegdadagi' are chilling tolerant and rest of all are chilling susceptible according to the descriptions of seed companies (Table 1). Two chilling tolerant cucumber lines 'Chipper' and 'NC76' are from United States, Suiseifushinari 2-go is from Japan, and Donggwan is from China. The results of genotyping for CSatpB-SNP and CSycf1-SNP showed differences in sizes of the PCR products. 'NC76' and 'Chipper' from USA possess the same chloroplast genotypes while all other cucumber cultivars from Korea, Japan, and China did show same chloroplast genotypes (Fig. 2).

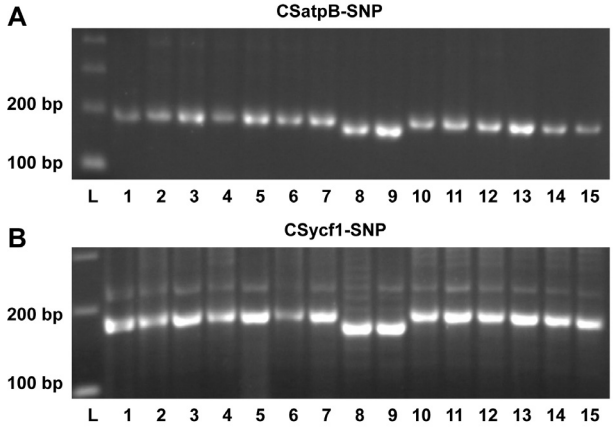

Fig. 2. Chloroplast DNA (cpDNA) genotyping of lines of cucumber 1 to 15 (see Table 1) shows differences in length variation. Line number 8 ('NC76') and 9 ('Chipper') show cpDNA genotypes related to chilling tolerance in both CSatpB-SNP (A) and CSycf1-SNP (B). L is size ladder.

'NC76' was originated from PI 246930, collected from Afghanistan (Wehner and Shetty, 1997). Although PI 246930 has small fruits and it is known as susceptible to downy mildew, 'NC76' was selected for high chilling tolerance (Kozik and Wehner, 2008) from PI 246930. A single dominant nuclear gene controls chilling tolerance in 'NC76' according to Kozik and Wehner (2008). 'NC76' has been also used for reciprocal crosses to determine maternal inheritance of chilling injury (Gordon and Staub, 2011). Although cpDNA genotypes were not verified in their study, inheritance of chilling response with 'NC76' indicated maternal effects of chilling injury exist in the crosses with 'NC76'.

The result of genotyping presented in this study shows that the cpDNA genotypes of 'NC76' are same as 'Chipper' which contains the maternally inherited source of chilling tolerance (Chung et al., 2007). Therefore, chilling tolerant nature of 'NC76' is speculated with the combination of some chloroplast DNA and nuclear DNA. 'Donggwan' cultivar is also known as chilling tolerant cucumber (from personal communication) but here it possesses cpDNA genotypes related to chilling susceptible. According to seed companies, various chilling responses exist among the cucumber lines tested in this study (Table 1). In spite of that all Korean cucumber cultivars along with 'Suiseifushinari 2-go' from Japan showed cpDNA genotypes related to chilling susceptible (Fig. 2). This fact indicates that all cucumber varieties from Korea, Japan, and China tested in this study did not have the chloroplast genotypes related to chilling tolerance. Therefore, their nature of mechanisms for chilling tolerance in chilling tolerant cucumber varieties such as 'Asiachungjang', 'Joeunbaegdadagi', 'Hangangmatbaegdadagi' and 'Donggwan' may be different than maternal factors of 'Chipper' or 'NC76'. This result suggested that their phenotypes of chilling response and the genetics of chilling tolerant factor(s) should be verified to understand the mechanism.

In conclusion we confirmed that the sdCAPS method is useful to develop PCR based SNP analysis for breeders and genome researchers. To exam the genotypes related to chilling tolerance in cucumber cultivars, the sdCAPS markers of (CSatpB-SNP and CSycf1-SNP) clearly demonstrate the cpDNA genotypes of the cucumber germplasm. Therefore, when new germplasm are chosen to breed cucumber cultivars for chilling tolerance, these markers could be useful to exam the chloroplast genotypes of the new germplasm. In addition, because 'NC76' has both nuclear and maternal genetic factors for chilling injury, it might be possible to develop chilling tolerant cucumber cultivars by transferring the maternal factor(s) and nuclear factor for chilling tolerance from

'NC76'. This breeding scheme can be conducted by making crosses with 'NC76' as a female source and other elite lines as recurrent parents. In addition, the single dominant nuclear factor of 'NC76' for chilling tolerance should also be genetically mapped and closely linked molecular markers to chilling tolerance can be identified. Using a strategy of marker assisted selection, this would accelerate breeding of chilling tolerant cucumber cultivars.

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