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Functional characterization of watermelon (*Citrullus lanatus* L.) EST–SSR by gel electrophoresis and high resolution melting analysis

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ABSTRACT

The present work was conducted to characterize the functionality of 257 watermelon EST–SSR primer pairs for their PCR amplification and polymorphisms. EST–SSR markers were tested on DNA sample panels of six watermelon cultigens and two related species of *Citrullus lanatus* var. *citroides* and *Citrullus colocynthis* based on agarose gel electrophoresis and high resolution melting (HRM) analysis. Successful PCRs were shown for 240 primer pairs (79%), and 173 primer pairs (67%) were polymorphic in a watermelon DNA sample panel on agarose gel electrophoresis. In addition, HRM analysis of 24 EST–SSR markers that were monomorphic on agarose gel separation identified an additional 19 polymorphic markers, indicating that HRM is an efficient tool for the rapid screening of sequence variations and allele discrimination. In the assessment of genetic relationships, six watermelon cultivars were closely related together (0.91–0.97) and demonstrated a narrow genetic base in the watermelon genetic pool. A high level of genetic dissimilarity (0.36–0.97) was shown between watermelon species and other related species. Marker transferability to melon species (*Cucumis melo* L.) was examined by cross-species PCR amplification and genetic diversity assessment in eight melon cultigens. Of the 257 EST–SSR primer pairs, 79 (32.9%) showed successful PCR amplification from melon DNA samples. A dendrogram of the genetic relationship based on 22 EST–SSR markers showed a clear classification of melon genotypes in accordance to fruit characteristics. The EST–SSR markers characterized in this study will contribute to diverse genomic investigations and breeding efforts, including comparative genome mapping, marker-assisted selection, and DNA fingerprinting for genetic diversity and cultivar identification in many cucurbit crops.

1. Introduction

Watermelon (*Citrullus lanatus* L.) is a fruit crop of the family *Cucurbitaceae*, along with other cucurbit crops including the melon, cucumber, and zucchini. Watermelon thrives in the temperate regions of Africa, central Asia, and the Mediterranean (Jeffrey, 1975; Whitaker and Bemis, 1976). World-wide production of watermelon fruits has increased steadily during the last century. Today, watermelon accounts for 6.8% of the world area devoted to vegetable production. In 2008, net production value of the top 20 countries cultivating watermelons was $ 8.6 billion and the amount of fruit production was 89 million tons; South Korea was the eighth largest watermelon producing country following China, India, and the United States (FAO, 2009). Watermelon is a major vegetable crop in South Korea, accounts for a farm production value of $ 930 billion with a cultivation area of 20,750 ha in 2009.

Commercial watermelon breeding in South Korea is focusing on the production of F1 hybrid cultivars. Breeding goals have been narrowly concentrated on improving just a few traits like sugar content, disease resistance, and fruit shape. This has led to the formation of a narrow genetic base among domestic breeding sources and, consequently, to limitations in developing new cultivars with diverse characteristics that are required for the changing global seed market. To expand genetic diversity, breeding strategies that incorporate exotic gene pools from wild-type germplasm or foreign elite cultivars are necessary.

Molecular markers are an useful tool for assessment of genetic diversity, cultivar identification, molecular tagging of important genes by genetic map, and incorporating target traits by marker-assisted selection (MAS). Furthermore, markers of known

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Table 1
List of watermelon cultivars and related species used for the DNA sample panel.

EN	Cultigen	Species	Origin	Fruit weight (kg)	Fruit shape	Flesh color	Rind color	Seed size	Seed color
1	52192	*C. lanatus* var. *lanatus*	Korea	7.0	Round	Red	Black	NS	Black
2	Ts34	*C. lanatus* var. *lanatus*	Korea	7.0	Elongated	Red	Jubilee stripe	TS	Black
3	Drdfrm	*C. lanatus* var. *lanatus*	Korea	7.0	Oblong	Deep red	Dark green	SS	Black
4	JXM	*C. lanatus* var. *lanatus*	China	6.0	Round	Red	Jubilee stripe	NS	Black
5	Arka manik	*C. lanatus* var. *lanatus*	India	6.0	Round	Red	Crimson stripe	NS	Dotted brown
6	All sweet	*C. lanatus* var. *lanatus*	USA	12.0	Elongated	Red	Crimson stripe	NS	Dotted brown
7	PI 494817	*C. lanatus* var. *citroides*	Zambia	3.0	Round	White	Green	GS	Black
8	PI 386024	*C. colocynthis*	Iran	0.1	Round	White	Dotted green	SS	Grey

GS, giant size; wild-type accessions panels and related species integration DNA, respectively. 2011 selfed that have difficulties in allele disc; NS, normal size; SS, small size; TS, tomato seed size.

chromosomal locations are useful for selecting the genomic background of recurrent parents in advanced backcross breeding. A key for successful application of these techniques is to identify a number of polymorphic markers that can easily discriminate genomic variations existing among the plant individuals. In the watermelon, germplasm characterization and genetic map constructions have been conducted using different marker types such as amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and simple sequence repeat (SSR) (Lee et al., 1996; Levi et al., 2001; Che et al., 2003; Kwon et al., 2010).

SSRs, or microsatellite markers, are powerful PCR-based tools applicable in plant breeding and genome study due to their reproducibility, multi-allelic nature, and co-dominant inheritance. SSRs are highly abundant and distributed with wide genome coverage. SSR markers can be designed from genomic DNA (gSSRs) (Panaud et al., 1995) or expressed sequence tags sequences (ESTs). Advantages of EST–SSRs include their locus-specificity to the expressed genes and usability as functional markers in the study of the association of candidate genes with phenotypic variations and comparative studies for genetically related different species (Ritschel et al., 2004; Park et al., 2010). EST–SSRs have been developed in many horticultural plant species including eggplant (Nunome et al., 2009), peppers (Yi et al., 2006), melons (Kong et al., 2007), tomato (D'Agostino et al., 2007), and roses (Park et al., 2010). In cucurbit crops, melon EST–SSR markers have been used in the molecular mapping of important traits and DNA fingerprinting studies (Levi et al., 2006a,b, 2008; Kong et al., 2007). However, only a few studies on EST–SSR application in the watermelon have been reported (Levi et al., 2008; Verma and Arya, 2008).

High resolution melting (HRM) analysis allows genotyping by the discrimination of DNA sequence variant such as single point mutations and small insertion and deletions (In/Del) based on the shape of melting transitions (*Tm*) of the PCR products (Wittwer et al., 2003; Zhou et al., 2005; Wittwer, 2009). The methods generally involve the gradual denaturation (melting) of the PCR amplicons and real-time detection of the subtle changes in fluorescent signal over temperature by double-strand DNA-binding fluorescent dyes present in the amplification reaction (Erali and Wittwer, 2010). Most single base variants or single nucleotide polymorphisms (SNPs) can be genotyped by HRM because many homozygotes differ in *Tm*. Genotyping by HRM analysis is beneficial to investigators primarily due to its cost-effectiveness. This technique is also faster and simpler than alternative approaches requiring post-PCR processing enzyme restriction and electrophoresis, or labeled probes for SNP detection. HRM can be applied not only for allele discrimination by targeting well-characterized SNPs, but for screening for the existence of unknown sequence variations without a sequencing process. The use of HRM in genotyping and gene scanning have been reported in many crops including rice (Li et al., 2011), tomato (Bae et al., 2010), pepper (Jeong et al., 2010), melon (An et al., 2010) and almond (Wu et al., 2008).

The International Cucurbit Genomics Initiative (ICuGI, http://www.icugi.org/) database provides information for genomic tools to improve fruit quality in melon and related cucurbit crops. The ICuGI contains over 4700 EST unigenes representing a wide array of genes expressed in watermelon fruits and leaves (Levi et al., 2006a,b). These unigenes were analyzed for SSR-containing ESTs, and at least 257 EST–SSR primer pairs were designed for public uses. From this new set of watermelon EST–SSR primer pairs, 40 were characterized and a relatively high polymorphism rate among heirloom watermelon cultivars in the United States was reported (Levi et al., 2008). However, there are many remaining EST–SSR primer pairs that have not been verified concerning their marker functionality.

In the present study, we performed a functional evaluation of 257 watermelon EST–SSR markers deposited in the ICuGI database involving PCR amplification and polymorphism determination using watermelon and melon germplasm collections. With the EST–SSR primer pairs successfully amplified by PCR, we assessed (1) EST–SSR allele variations within a set of watermelon cultivars and other related species based on regular agarose gel electrophoresis and HRM analysis, (2) cross-species amplification in diverse melon cultigens for marker transferability, and (3) genetic relationship among cultigens in each watermelon and melon sample panel.

2. Materials and methods

2.1. Plant material

A set of DNA sample panel comprised of eight watermelon accessions (Table 1) was used for functional evaluation of 257 EST–SSR primer sets. This DNA sample panel included six domestic and foreign watermelon cultigens (*C. lanatanus* var. *lanatanus*) and two related wild-type subspecies and species (*C. lanatus* var. *citroides* and *Citrullus colocynthis*). For study of marker transferability, eight melon accessions (*Cucumis melo* L.) (Table 2) representing diverse subspecies and fruit characteristics were used for PCR with watermelon EST–SSR markers. Seed samples for watermelon and melon were obtained from a private breeding company and the Agricultural Research and Extension Service (ARES) in Jinju, South Korea, respectively. These samples were also used for the assessment of their genetic relationship.

2.2. DNA isolation

Seeds were germinated in a greenhouse, and young cotyledons and true leaves were collected for DNA extraction. Leaf tissues were ground in 1.5 ml microcentrifuge tubes with steel beads and 600 μl DNA extraction buffer using a TissueLyser (Qiagene, Venlo, Netherlands). The tubes were incubated at 65 °C for approximately 45 min, 200 μl of 7.5 M ammonium acetate was added, and the tubes were placed in ice for 15–20 min. The lysates were centrifuged for 10 min at 14,240 × *g*, and each supernatant was