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Towards the identification of retrotransposon-based and ISSR molecular markers associated with populations resistant to ZYMV in melon

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ABSTRACT

Melon (*Cucumis melo* L.) is one of the most important commercial vegetable crops grown in Iran. Very few studies on the genetic structure and diversity of melon landraces of Iran are available in the literature. In addition, it is susceptible to a number of diseases causing reduction in both productivity and quality of the crop. In this study we applied inter retrotransposon amplified polymorphism (IRAP), retrotransposon-microsatellite amplified polymorphism (REMAP) and inter simple sequence repeat (ISSR) molecular markers to investigate genetic diversity and its association with populations resistant to ZYMV in 11 Iranian melon populations and 12 commercial lines. Significant differences ($P \leq 0.01$) were detected among populations for reaction to ZYMV infection. No symptoms observed on inbred line PI414723, while variety Charentais T showed the highest value of virus infection (1.87). Based on five IRAP, 15 REMAP and 20 ISSR primers, a total of 656 reproducible and scorable loci were generated, among them 75.41% were polymorphic. Population structure analysis using STRUCTURE software identified $K = 10$ as the reliable value for the number of clusters. Associations between molecular markers and populations resistant to ZYMV were tested using mixed linear model (MLM) approach that accounts for population structure. Highly significant associations were found for seven REMAP and four ISSR markers affecting ZYMV resistance ($P \leq 0.01$). This study provides evidence that using natural germplasms, the diversity can be enhanced for marker-trait associations that can be validated in segregating populations and exploited through marker-assisted selection.

1. Introduction

The family *Cucurbitaceae* contains 118 genus and 825 species with worldwide spreading especially in tropical and sub-tropical regions. The most economically important crops in this family are watermelon, cucumber, zucchini, squash, pumpkin and melon (Kirkbride, 1995). Melon (*Cucumis melo* L.) is an outcrossing diploid species ($2n = 2x = 24$). The crop is of interest for its specific biological properties and for its economic importance. The genome size of melon is 450 Mbp, approximately equal with that of rice (Garcia-Mas et al., 2012). The consensus genetic map of melon spans 1150 cM across the 12 melon linkage groups; with an average marker density of 0.72 cM/marker (Diaz et al., 2011). Melons belong to the two subspecies *melo* and *agrestis* (Robinson et al., 1997). Recently, Pitrat (2008) classified subsp. *melo* into 15 groups (*cantaloupensis*, *reticulatus*, *adana*, *chandalak*, *ameri*, *inodorous*, *chate*, *flexuosus*, *dudaim* and *tibish*) and subsp. *agrestis* into *momordica*, *conomon*, *chinensis*, *makuwa*, and *acidulous*. Three groups *Cantaloupensis*, *Inodorous*, and *Flexuosus* are the most popular groups of melons in Iran (Raghami et al., 2014). The origin of melon is under controversy, and based on the dispersion of wild melons in Kirkbride monograph, it was assumed that Africa is the primary center of melon diversity. Recently, using DNA sequences from plastid and nuclear markers of 100 *Cucumis* accessions from Africa, Australia, and Asia, Sebastian et al. (2010) concluded Australian sister species of melon, *Cucumis picrocarpus*, as the closest relative of melon and the divergence of *C. melo* from this species occurred approximately 3 Ma (Sebastian et al., 2010). Ancient documents have shown that melons, especially muskmelon, domesticated for the first time 3000 B.C. in Iran and Egypt (Staub et al., 1997).

Iran is the second producer of melons, generating over 1.7 million kilograms in 2010 (FAO, 2012) and more than 50% of total vegetable production in Iran is related to cucurbits. Nevertheless, there are a number of diseases that reduce both the production and quality of the crop. Members of *Cucurbitaceae* family are very sensitive to viral diseases and more than 35 viruses have been isolated from different *Cucurbitaceous* members. Almost all economical varieties of melons are sensitive to viral pathogens. Potyviruses include devastating viruses

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such as watermelon mosaic virus (WMV), zucchini yellow mosaic virus (ZYMV), papaya ring spot virus (PRSV) and melon necrotic spot virus (MNSV). They are the greatest viral group with severe damages on melon production through the world (Ekbic et al., 2010). ZYMV has special importance due to its high yield losses to cucurbits. ZYMV symptoms on cultivated crops are often very severe and induce significant yield reduction. In addition, fruits produced on infected plants exhibit severe deformation and color alterations, which render them unmarketable. The presence of ZYMV in Iran was first reported in 1988 from squash and muskmelon. Since then, ZYMV has spread to all vegetable-producing regions in Iran, making it the limiting factor in cucurbit crop production during summer and autumn, causing up to 100% yield loss (Benanej et al., 2008).

Different practices such as control of vector population, using virus-sterilized seeds, proper culture techniques and resistant varieties are commonly used to control and manage viral pathogens (Dasgupta et al., 2003). Among the mentioned strategies, application of resistant varieties is the most impressive way to control the viral diseases. Linkage mapping has proved its usefulness in detecting chromosomal regions and loci controlling important qualitative and quantitative traits such as resistance to viruses in crops (Frary et al., 2000). Danin-Poleg et al. (2002) identified one simple sequence repeat (SSR) marker tightly linked to a ZYMV resistance gene, designated Zym-1 in melon. Ling et al. (2008) developed a cleaved amplified polymorphic sequence (CAPS) marker based on a single nucleotide polymorphism (SNP) in the intron II region of the eIF4E gene sequence at a linkage distance of 7 cM in watermelon. Harris et al. (2009) identified a ZYMV-resistant polymorphic (ZYRP) marker linked to the ZYMV-FL resistance gene (at a genetic distance of 8 cM) in watermelon. One of the limiting factors in linkage mapping strategies conducted from a cross between two parents in many plant species, including melons, is that the effects of QTL is limited to a specific genetic background. Consequently the success in applying the results has been limited. A viable solution to this problem is association analysis, whereby genes and QTLs are detected in a random set of genotypes from a mixed genetic background (Flint-Garcia et al., 2005; Gupta et al., 2005). More importantly, due to the absence of tight linkage, only few tens of markers have been validated in a subset of melon genotypes (Blanca et al., 2011, 2012). To overcome this limitation, and as an alternative to planned populations, less expensive marker types without prior knowledge of the target DNA sequence have been identified through association analysis for traits of interest using germplasm collections (Ruan et al., 2009). Such markers have been adopted for association studies involving the use of germplasm collections for the identification of molecular markers in many plants (Chatterjee and Mohandas, 2003; Ganopoulos et al., 2011; Jugran et al., 2013). In addition, this approach is very effective for discovering new QTLs if the problem of spurious associations resulting from population structure can be resolved accurately (Zhu et al., 2008). A unified mixed linear model (MLM) approach for association studies in combination with Q (population structure matrix) and K (kinship matrix) provides a robust tool for identification of QTLs (Zhao et al., 2007).

To our knowledge, no association analysis has been used to identify molecular markers associated with populations resistant to ZYMV in Iranian melon germplasm. Hence, the aims of the current study were: i) to assess the genetic diversity of a set of Iranian melon populations and commercial lines and ii) to evaluate the current germplasm for the potential breeding for ZYMV resistance, and for preliminary evidence of genetic associations between molecular markers and populations resistant to ZYMV.

2. Materials and methods

2.1. Plant material and virus assessment

Seeds of 11 populations (eight individuals from each population) collected in Iran and 12 commercial lines were chosen in this study (Table 1). For commercial lines (hybrids and inbred lines); only one plant from each was assayed by molecular markers because, as expected, it was assumed that the hybrids and inbred lines are genetically uniform and pure. Virus assessment carried out based on double-antibody sandwich ELISA (DAS-ELISA) method (Clark and Adams, 1977) using polyclonal ZYMV antibody and alkaline phosphatase conjugated antibody (conjugate). All plants were grown under greenhouse condition in a completely randomized design with three replications. The temperature was adjusted to 27 ± 2 °C. At the first true leaf stage, the cotyledons were mechanically inoculated by rubbing Carborundum-dusted with extracts from 1 g of infected plants prepared in 0.02 M phosphate buffer and containing 0.1% 2-mercaptoethanol (pH = 7) and activated carbon (Hull, 2009). The seedlings were then washed using tap water (Ekbic et al., 2010). Presence or absence of virus symptoms was scored for each genotype in each replicate 3 weeks after inoculation. Virus symptoms were not considered a reliable way to assess virus concentration. The individual genotypes were also tested using DAS-ELISA for the detection of ZYMV. ELISA plates were read using ELISA reader (model *Anthos 2020*, Switzerland) in 405 nm wavelength. Optical density at this wavelenght is equal to E (Extinction Coefficient) × C (Concentration); hence OD and C have direct relationship but since standard curve with the purified virus was not available, thus it was not possible to relate optical density (OD) reading to virus quantity.

Table 1
Description of the melon germplasm assessed for genetic variation and ZYMV resistance.

Accession	Code	Abbreviation	Collection site/Breeding company	Horticultural group
Khagani M	1–8	KhM	Iran-Khorasan	*Inodorous*
Dargazi Tashkandi	9–16	DTa	Iran-Khorasan	*Inodorous*
Zivari Shahroud	17–24	ZSh	Iran-Semnan	*Inodorous*
Chahpaliz	25–32	Cha	Iran-Khorasan	*Inodorous*
Tashkandi M	33–40	TaM	Iran-Khorasan	*Inodorous*
Shadegani M	41–48	ShM	Iran-Khuzestan	*Inodorous*
Minoo	49–56	Min	Iran-Khorasan	*Inodorous*
Shadegani E	57–64	ShE	Iran-Khuzestan	*Inodorous*
Tal Shahroud	65–72	TSh	Iran-Semnan	*Cantaloupensis*
Jalali	73–80	Jal	Iran-Semnan	*Inodorous*
Khatooni Farimani	81–88	KhF	Iran-Khorasan	*Inodorous*
637	89	[a]Hyb1	Biotek-Turkey	*Inodorous*
Meanhno 9	90	Hyb2	Ergon-USA	–
Summit	91	Hyb3	Peto seed-USA	*Cantaloupensis*
Durango	92	Hyb4	Peto seed-USA	*Cantaloupensis*
Omega	93	Hyb5	–	*Cantaloupensis*
Ananas	94	Hyb6	Enza Zaden-Netherlands	–
Super soykan	95	Hyb7	Biotek-Turkey	*Inodorous*
Behta N397	96	Hyb8	Behta-Iran	*Inodorous*
Goldtropy	99	Hyb9	Canyon-USA	–
Dublone	97	[b]Lin1	INRA, Avignon-France	*Cantaloupensis*
PI414723	98	Lin2	INRA, Avignon-France	*Momordica*
Charentais T	100	ChT	INRA, Avignon-France	*Cantaloupensis*

Hyb: hybrid, Lin: Inbred line. The breeding company of cultivar Omega was not available.

2.2. DNA extraction and ISSR, IRAP and REMAP genotyping

Genomic DNA of the individuals was extracted using the method described by Doyle and Doyle (1990) with minor modifications. The concentration and quality of genomic DNA was determined by a spectrophotometer and by 0.8% agarose gel electrophoresis. Five inter-retrotransposon amplified polymorphism (IRAP), 15 retrotransposon-microsatellite amplified polymorphism (REMAP) and 20 inter simple sequence repeat (ISSR) primers, generating a clear, scorable and discernible banding pattern were selected for genetic analysis of entire germplasm (Table S1). For ISSR primers, PCR amplification was performed in 20 μl reactions containing 30 ng DNA, 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH = 8.3), 1.5 mM $MgCl_2$, 0.2 μM dNTP,