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Genetic relationships of some *Citrus genotypes* based on the candidate iron chlorosis genes

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Abstract: Iron is one of the most important elements in plant mineral nutrition. Fe deficiency is a critical abiotic stress factor for Mediterranean citriculture; the development of marker-assisted selection for this trait would greatly enhance rootstock breeding. In this study, DNA sequencing and single-stranded conformation polymorphism (SSCP) analyses were performed to determine the allelic diversity of genes associated with tolerance to iron chlorosis in citrus. Two candidate iron chlorosis tolerance genes were selected from existing *Citrus* EST databases and *Arabidopsis thaliana* genome databases. Ferritin-3 chloroplast precursor and putative membrane transporter candidate gene sequences were used to define primers in conserved regions. Six citrus genotypes from the basic taxon of *Citrus* were used to identify polymorphic regions in the genes. Direct sequencing of the amplified DNA fragments from the candidate genes was performed, and single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) were identified after sequence alignment. Based on the DNA sequencing analysis, a total of 6840 nucleotides of DNA were sequenced to identify SNPs and indels. In total, 263 SNPs and 15 indels were identified for both genes. We detected 38.45 SNPs and 2.19 indels for each 1000 b on average from the DNA sequencing results. New primers were designed in conserved areas flanking polymorphic ones for SSCP analysis. SSCP-PCR analysis was performed with 25 citrus genotypes. The neighbor-joining method was used for cluster analysis. Trifoliate genotypes and their hybrids (known to be sensitive to iron chlorosis) clustered together, whereas genotypes tolerant to iron chlorosis were more spread out on the dendrogram. Mandarins also showed high diversity for both genes according to SSCP results. Differences were found among sour orange genotypes known to have differential tolerance behavior to iron chlorosis.

Key words: Single-stranded conformation polymorphism, DNA sequencing, polymorphism, DNA

1. Introduction

Citrus is one of the most important and widely grown fruit crops, with total global production reported to be 117.7 × 10^6 t in 2010 (http://www.fao.org). Citrus fruit is produced throughout the tropical and subtropical regions of the world, where temperatures are warm enough for tree survival but cool enough for adequate chilling, and where water and suitable soils are sufficient to support tree growth and fruit production. The most significant production areas are found in the Americas (led by Brazil, the United States, Mexico, and Argentina), the Mediterranean basin (led by Spain, Italy, Egypt, and Turkey), and the South and East Asian regions (led by China, India, and Japan) (Talon and Gmitter, 2008).

Iron deficiency is one of the major abiotic stress factors for fruit trees in the Mediterranean area of southern Europe. The most important cause of this nutritional deficiency is the low availability of Fe in the calcareous soils (rich in lime) that are common in this semiarid area (Pestana et al., 2012). The most prevalent cause of iron chlorosis in the Mediterranean area is the bicarbonate ion, which occurs at high levels in calcareous soils. It is estimated that 20% to 50% of fruit trees in the Mediterranean basin suffer from iron chlorosis (Pestana et al., 2003). Low iron availability induces a typical leaf yellowing known as iron chlorosis, a general reduction of leaf chlorophyll, and a strong reduction in new vegetative flushes.

Citrus production in Mediterranean countries is increasing. More and more orchards are being planted on marginal lands, which increases plant abiotic stress. Most of these stresses can be individually managed by rootstock selection. However, due to the spread of the citrus tristeza virus (CTV) and its vectors all over the Mediterranean basin, the use of traditional sour orange rootstock (which has adequate tolerance to salinity and alkalinity) will soon be prohibited. In Spain, the risk of a socioeconomic

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disaster caused by CTV was so high that the government set up very severe rules to reduce losses produced by the virus. The use of sour orange was prohibited in new plantings and propagation of plants at citrus nurseries was strictly regulated, leading to a drastic reduction in its use (Navarro, 2012). Therefore, there is an urgent need to breed new citrus rootstocks. The required traits are present in the citrus germplasm, but the complexity of citrus biology makes it difficult to combine them through traditional breeding. Therefore, the implementation of marker-assisted selection (MAS) methods would greatly aid this research (Şimşek, 2009).

Plant ferritins and *pmt4* genes are candidates for iron chlorosis tolerance. Plant ferritins are important iron storage proteins located in plastids and they play a general role in Fe stress response in plants (Briat, 1996). Furthermore, it has recently been reported that legume and animal ferritins have a common eukaryotic origin due to their sequence homology; it is important to note that the sequence of eukaryotic ferritin diverges completely from the *Escherichia coli* bacterioferritin sequence (Ragland et al., 1990; Lescure et al., 1991; Spence et al., 1991; Lobreaux et al., 1992). However, an additional sequence is found in the NH_2-terminal of the plant protein. The first part of this extension is a transit peptide responsible for plastid targeting (Ragland et al., 1990; Lescure et al., 1991; Spence et al., 1991). The second part is a mature ferritin subunit and is known to be the site of free-radical cleavage, which occurs in vitro during iron exchange (Laulhere et al., 1989) and in vivo during germination (Lobreaux and Briat, 1991). Additionally, ferritins are not uniformly distributed in the different organs of a plant throughout its life cycle.

In addition to Fe storage, transporter genes such as *pmt4* may also play a role in Fe tolerance in plants. The *pmt4* gene belongs to a gene family of membrane transporters and is known to be important in Fe transport in plants. In cellular biology, the term "membrane transport" refers to the collection of mechanisms that regulate the passage of solutes, such as ions and small molecules, through biological membranes. The regulation of passage through the membrane is due to selective membrane permeability, a characteristic of biological membranes that allows for the separation of substances based on their distinct chemical nature. In other words, the membrane can be permeable to certain substances but not to others. In this paper, the *fer3* and *pmt4* genes were chosen from *Citrus* expressed sequence tag (EST) databases to investigate genetic diversity. İncesu (2011) reported molecular responses of *Citrus* genotypes that are sensitive and tolerant to iron chlorosis by microarray analyses. The *fer3* and *pmt4* genes were differentially expressed in *Citrus* genotypes under iron deficiency.

Single-stranded conformation polymorphism (SSCP) analysis is based on the sequence-specific differential migration of single-stranded DNA through a nondenaturing gel matrix. Partially denatured double-stranded DNA migrates as 2 single-stranded DNA bands in nondenaturing polyacrylamide gel electrophoresis. The migration of the 2 strands depends on their conformation under the electrophoresis conditions chosen, and therefore on the nucleotide sequence. Under appropriate conditions, it has been demonstrated that single-stranded nucleic acids form unique secondary structures depending on their sequence composition, and that small modifications such as single nucleotide polymorphisms (SNPs) can alter their secondary structure and thus their electrophoretic profile (Palacio and Duran-Vila, 1999; Xu et al., 2009). SSCP analysis is a powerful method for detecting the allelic diversity of genes. In this study, we performed SSCP analysis to discover the allelic diversity of candidate genes responsible for iron chlorosis in the *Citrus* germplasm. Our objective was to analyze potential associations between the allelic diversity of the candidate genes and the variability of tolerance to iron chlorosis. This knowledge could then be used to select for this trait in a citrus rootstock breeding program.

2. Materials and methods

2.1. Plant materials and DNA extraction

Six genotypes (Table 1), which were either sensitive or tolerant to iron chlorosis, were selected as plant material to search for polymorphisms between the *Citrus* and *Poncirus* basic taxa (*C. reticulata*, *C. maxima*, *C. medica*, *C. micrantha*, and *P. trifoliata*). Clementine was included as a reference, since its entire genome sequence has been released. In addition, 19 genotypes, representing a wide array of the citrus genome, were also analyzed with SSCPs (Table 2). Young leaves were collected from a single tree for each accession, immediately frozen in liquid nitrogen, and stored at –80 °C. High-molecular-weight genomic DNA was extracted from the leaf samples following the protocol for minipreps using CTAB (Edwards et al., 1991). The DNA concentration was measured using a NanoDrop ND 100 spectrophotometer (NanoDrop Technologies) and gel electrophoresis.

2.2. Candidate genes

Two candidate genes for iron chlorosis tolerance were selected from existing *Citrus* EST databases and *Arabidopsis thaliana* genome databases. The ferritin-3 chloroplast precursor (*fer3*, C20003E08) and putative membrane-transporter (*pmt4*, FC905918.1) genes were used. It should be noted that variability of the quantitative expression of the same genes was observed under stress and control conditions by microarrays (unpublished data). These genes were also checked in the *Arabidopsis thaliana*

Table 1. Genotypes selected for the search for polymorphisms in *Citrus* and *Poncirus*.

Genotype	Latin name (Tanaka classification)	Response to iron chlorosis*
Cleopatra Mandarin	*Citrus* × *reshni* hort. ex Tanaka	+++
Corsican Citron	*Citrus medica* L.	NI
Micrantha	*Citrus micrantha* Wester	NI
Nules Clementine	*Citrus clementina* hort. ex Tanaka	NI
Pink Pomelo	*Citrus maxima* (Burm.) Merr	-
Pomeroy Trifoliate	*Poncirus trifoliata* (L.) Raf.	-

*: No information, NI; very sensitive, - -; sensitive, -; acceptable, +; tolerant, ++; very tolerant, +++.

Table 2. Additional genotypes used for SSCP analysis.

Genotype	Latin name (Tanaka classification)	Response to iron chlorosis*
C35 Citrange	*Citrus sinensis* Osb. 'Ruby' × *Poncirus trifoliata* (L.) Raf.	+
Carrizo Citrange	*Citrus sinensis* Osb. 'Washington Navel' × *Poncirus trifoliata* (L.) Raf.	++
Changsha Mandarin	*Citrus reticulata* Blanco	++
Citrumelo 4475 (MN)	*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.	-
Duncan Grapefruit	*Citrus paradisi* Macfad.	+++
Eureka Lemon	*Citrus limon* (L.) Burm. f.	-
Fuzhu Mandarin	*Citrus erythrosa* hort. ex Tanaka	-
Gou Tou Sour Orange	*Citrus aurantium* L.	+++
Marumi Kumquat	*Citrus japonica* Thunb.	++
Mexican Lime	*Citrus aurantifolia* (Chistm.) Swingle	-
Nasnaran Mandarin	*Citrus x amblycarpa* (Hassk.) Ochse	++
Poncirus Flying Dragon	*Poncirus trifoliata* (L.) Raf.	--
Rangpur Lime	*Citrus* × *limonia* Osb.	+
Red Rough Lemon	*Citrus jambhiri* Lush.	++
Rubidoux Trifoliate	*Poncirus trifoliata* (L.) Raf.	-
Shekwasha Mandarin	*Citrus depressa* Hayata	++
Sunki Mandarin	*Citrus sunki* (Hayata) hort. ex Tanaka	+++
Tuzcu 3131 Sour Orange	*Citrus aurantium* L.	+++
Volkameriana	*Citrus* × *volkameriana* Tan. & Pasq.	++

*: Very sensitive, - -; sensitive, -; acceptable, +; tolerant, ++; very tolerant, +++.

genome databases to be sure they were related to iron chlorosis.

2.3. Direct sequencing

Direct DNA sequencing was performed in order to detect SNPs and insertions/deletions (indels) in candidate genes. The primers used in sequencing were designed from existing *Citrus* ESTs of these genes. PCR amplification reactions were performed in 26 µL of reaction mixture that contained 20 ng of citrus DNA, 0.2 mM primers, 200 mM dNTPs, 1 U of Taq DNA polymerase (Fermentas EP0402), and 1X reaction buffer. Amplification was carried out in a Master Gradient thermal cycler (Eppendorf) using the following program: initial denaturation for 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 45 s at 57 °C, and 2 min at 72 °C; and a final 10 min of elongation at 72 °C. PCR product purification was carried out either immediately or after cutting single bands out of the agarose gel, using the QIAquick PCR Purification Kit and the QIAquick Gel Extraction Kit (QIAGEN), respectively. Sequencing was ordered and performed with Sanger technology using an ABI310 genetic analyzer (Applied Biosystems). DNA strands were sequenced for each gene in all 6 genotypes by using forward primers with 2 replications. Sequence alignment and SNP and indel searches were carried out with BIOEDIT software (version 7.2.1, Ibis Biosciences) (Hall, 1999).

2.4. Design of SSCP primers and SSCP analysis

From the results of direct sequencing on the basic taxa, we designed new primers (Table 3) for further diversity and routine genotyping using the SSCP method. Primers were defined in conserved regions in the DNA sequences of 6 different *Citrus* species. Using Primer 3 (version 0.4.0) software (Rozen and Skaletsky, 2000), 2 pairs of primers were designed for the *fer3* gene and 3 pairs were designed for *pmt4*. SSCP-PCR analysis was performed with 25 citrus genotypes. PCR amplification reactions were performed in 26 µL of reaction mixture, which contained 20 ng of citrus DNA, 0.2 mM primers, 200 mM dNTPs, 1 U of Taq DNA polymerase (Fermentas EP0402), and 1X reaction buffer. The amplification was carried out in a Master Gradient thermal cycler (Eppendorf) using the following program: initial denaturation of 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 45 s at 57 °C, and 2 min at 72 °C; and a final 10 min of elongation at 72 °C as indicated above. PCR products were denatured at 95 °C for 15 min using the thermal cycler and were then transferred to ice immediately. PCR products were subjected to electrophoresis in a nondenaturing 12% polyacrylamide gel at 4 °C. The gels were stained with silver nitrate using Silver Sequence Staining Reagents (Promega), and the SSCP data were recorded as 1 for the presence of a band and 0 for its absence. Only reproducible bands were scored for all the genotypes tested. For cluster analyses, the neighbor-joining method was used from Jaccard dissimilarities with 1000 bootstraps in Darwin software (http://darwin.cirad.fr/darwin).

3. Results

In this study, the allelic diversity of 2 candidate genes for tolerance to iron chlorosis was analyzed in a set of genotypes representative of the citrus rootstocks. Six genotypes, including both those sensitive and tolerant to iron chlorosis, were selected as plant material to search for polymorphisms between the *Citrus* and *Poncirus* taxa by DNA sequencing. Based on the DNA sequencing analysis, a total of 6840 nucleotides of DNA were sequenced to identify SNPs and indels. A total of 48 SNPs and 6 indels were identified for the *fer3* gene, and 215 SNPs and 9 indels were found for the *pmt4* gene (Table 4). New primers were

Table 3. SSCP primers for the *fer3* and *pmt4* genes.

Primer name	Sequence (5' à 3')	Length (b)
Fer3-1 Forward	GCAATAGCATGCCTCTGACA	20
Fer3-1 Reverse	CTGACTTGATCTGCTCGTTGA	21
Fer3-2 Forward	GCGACGTTGCTCGTGATATT	20
Fer3-2 Reverse	GGCATTGGACACGTATGAGA	20
PMT4-1 Forward	CATCCACTCCAATCCTCCAT	20
PMT4-1 Reverse	CAAAGAAAGGCAGCAAGGTT	20
PMT4-2 Forward	GACAATTACCTTCTTTTTGTTGTTTTT	27
PMT4-2 Reverse	GAATAGCCGGAAGTAACTCCA	21
PMT4-3 Forward	TCCTTATCAATATACCCTTTGCTACTC	27
PMT4-3 Reverse	AGCCCCTTTTTCAAGCATTT	20

Table 4. Indels and SNPs obtained from DNA sequencing of candidate genes.

Gene	Size (b)	SNP number	Indel number
Ferritin-3 chloroplast precursor (*fer3*)	2520	48	6
Putative membrane transporter (*pmt4*)	4320	215	9
Total	6840	263	15
SNPs or indels/1000 nucleotides	-	38.45	2.19

developed from the results of direct sequencing for further SSCP analyses. SSCP primers designed to investigate the allelic diversity of 2 different candidate genes for tolerance to iron chlorosis were successfully amplified in the 25 citrus genotypes analyzed. The neighbor-joining dendrograms are presented in Figure 1 for the *fer3* gene and in Figure 2 for the *pmt4* gene.

4. Discussion

In our study, we detected an average of 38.45 SNPs and 2.19 indels for each 1000 b. The results show that *Citrus* species have a high mutation frequency. In the present study, *fer3* and *pmt4* genes responsible for iron chlorosis were selected and studied; in a previous study, root iron transporter 1 (*irt1*) and iron-sulfur assembly protein

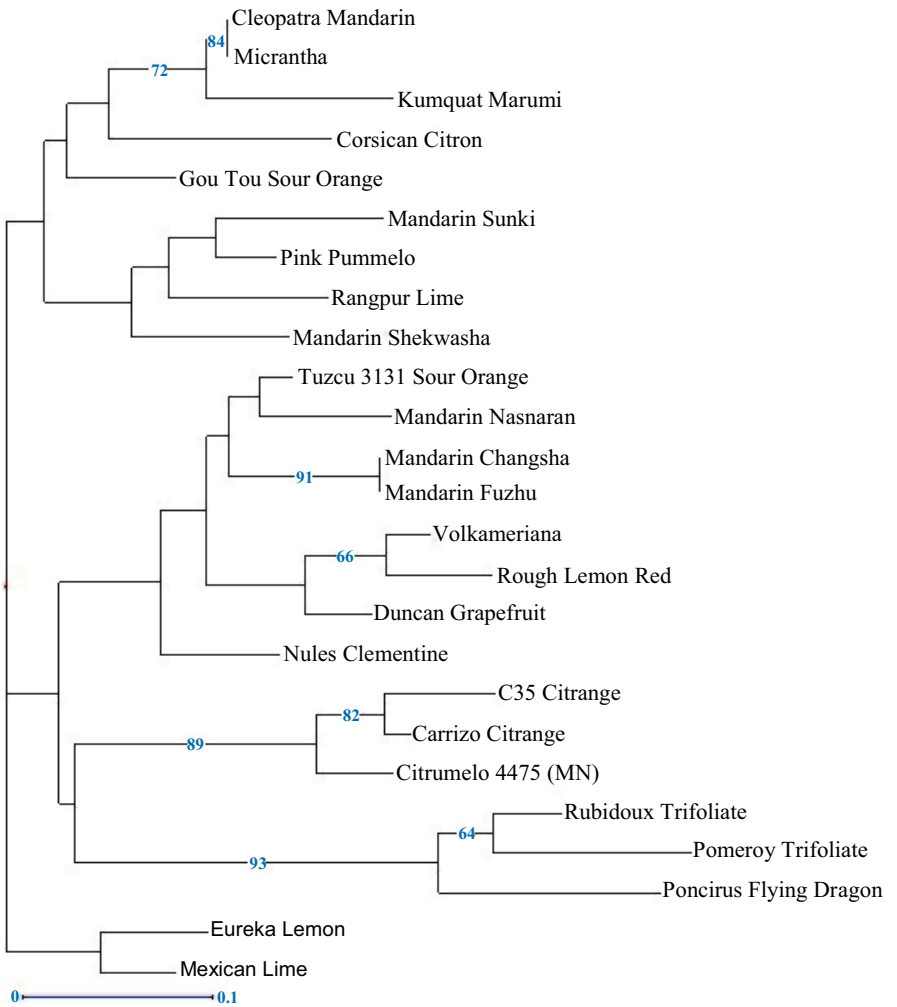

Figure 1. Dendrogram for the *fer3* candidate gene (1000 bootstraps performed). Branch support values of over 50% are shown.

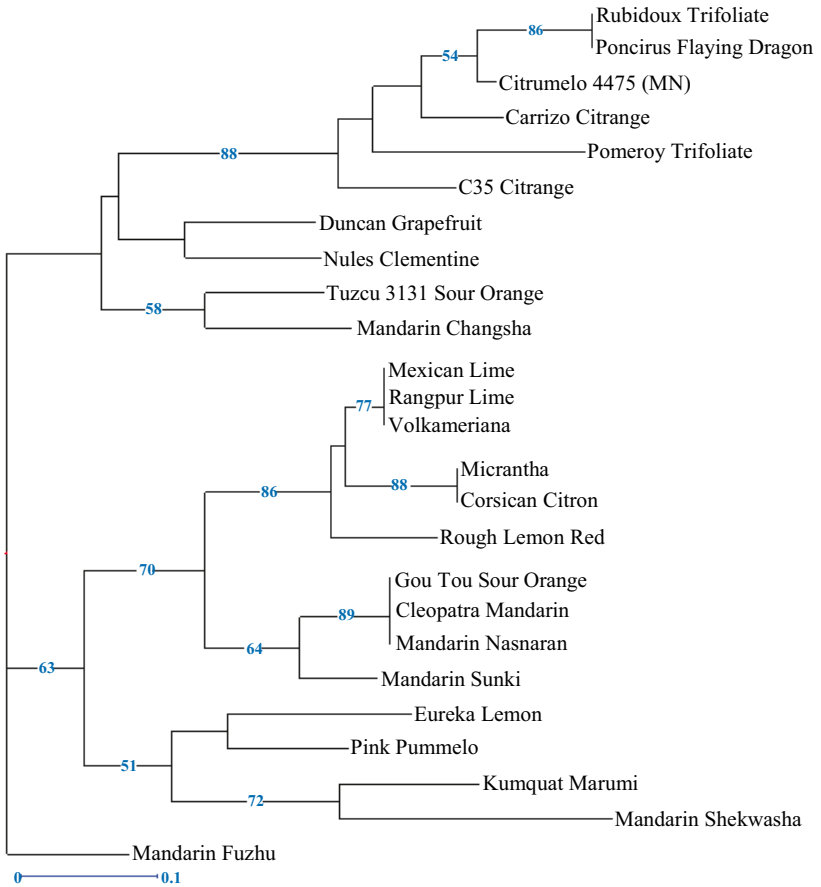

Figure 2. Dendrogram for the *pmt4* candidate gene (1000 bootstraps performed). Branch support values of over 50% are shown.

(*Fe-S*) genes associated with iron chlorosis were used in *Citrus* to investigate allelic diversity by SSCP and DNA sequencing. The *Fe-S* gene function in plants is not clearly known, but in animal cells it is thought to be responsible for the transport of iron. The *irt1* gene is responsible for the transport of iron in plants. The researchers found a mean of 25.28 SNPs and 0.90 indels for each 1000 b using the same genotypes (Table 1) used in this study (Simsek et al., 2011). Based on the results of DNA sequencing, it is clear that the *fer3* and *pmt4* genes have a higher polymorphism frequency than the *irt1* and *Fe-S* genes.

Trifoliate genotypes and their hybrids, known to be sensitive to iron chlorosis, clustered together for both genes. This low polymorphism between *Poncirus* and their differentiation with *Citrus* species is consistent with previous reports. Fang et al. (1997) showed 10% polymorphism among trifoliate genotypes in a study to determine variations among trifoliate genotypes by using RFLP and ISSR markers. Pang et al. (2007) reported high similarity among 3 *P. trifoliata* genotypes and a large differentiation from *Citrus* using AFLP markers. Simsek et al. (2011) worked with 2 different iron chlorosis tolerance genes to investigate the allelic diversity of some citrus rootstocks by SSCP markers and DNA sequencing. The researchers reported that trifoliate genotypes and their hybrids have the same allelic positions and are clustered together for both genes. Mandarin appears as the most polymorphic species for both genes in this study. Similarly, Luro et al. (1995) found high polymorphism among mandarin genotypes using microsatellite (SSR) markers. Additionally, mandarins contained a large number of genetic polymorphisms in many studies that were performed to clarify the diversity and taxonomy of *Citrus* (Nicolosi et al., 2000; Luro et al., 2001; Barkley et al., 2006; Ollitrault et al., 2012; Garcia-Lor et al., 2013). Simsek et al. (2011) reported that mandarins have high allelic diversity in iron chlorosis tolerance genes (Fe-S assembly protein and *irt1*).

In this study, differences in the *fer3* gene were found among lemon and lime genotypes, which are generally known to be tolerant to iron chlorosis. For this gene, the Eureka lemon, Mexican lime, Volkameriana, and

Red Rough lemon clustered in the same branches of the dendrogram; however, the Rangpur lime was located on a different branch. For the *pmt4* gene, many of the lemon and lime genotypes were in the same cluster of the dendrogram, except the Eureka lemon and Red Rough lemon. The *fer3* gene showed a higher allelic diversity than the *pmt4* gene in the lemon and lime genotypes. Aka-Kacar et al. (2005) showed, using RAPD markers, that lemon genotypes are genetically different. Gülşen and Roose (2000) worked with cpRFLP, ISSR, and isozyme markers to discover the origin of the Interdonato lemon. Based on the cpDNA markers, there were no differences between the Interdonato lemon and other lemons. However, from the results of the nucleus DNA markers, genetic differences were detected among lemon genotypes. Moore (2001) reported that limes are complex hybrids like lemons, but that limes have more citron genes than lemons. Molecular data (Nicolosi et al., 2000; Ollitrault et al., 2012; Garcia-Lor et al., 2013) have shown that common lemons (Lisbon, Eureka, etc.) resulted from a direct hybridization between sour orange and citron. Our study of the *pmt4* gene supported this result; the Corsican citron was clustered together with lemon and lime genotypes.

Polymorphisms were detected among sour orange genotypes known to be tolerant to iron chlorosis. The classical sour orange genotype (Tuzcu 31-31) did not cluster with the sour orange Gou Tou genotype. The Gou Tou sour orange was already identified as a hybrid of sour orange in a previous study.

However, trifoliate genotypes have been known to be sensitive to iron chlorosis and they are important plant breeding material because of their CTV tolerance. Sour oranges are significant rootstocks for citrus; on the other hand, sour oranges are known to be sensitive to CTV but generally tolerant to iron chlorosis. To have the desired rootstocks for citrus, breeding studies have been continued all around the world and it is useful to know the molecular structure of the desired feature.

The difficulty of allelic interpretation using the SSCP profile is an important limitation of such technology. However, SSCP is quite powerful, and thus can be used as a preliminary tool to identify polymorphisms and as a routine tool for segregating population analysis when a clear link can be made between the SSCP profiles and the different genotypes present in the population (Simsek et al., 2011). MAS is an important tool used in plant breeding. To develop molecular markers for selection, the polymorphism information is vitally important. Molecular markers can be developed and used for early selection using the polymorphic SSCP profiles in segregating populations.

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