Detection of polymorphism in ancient Tempranillo clones (*Vitis vinifera* L.) using microsatellite and retrotransposon markers

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Abstract
Tempranillo is one of the most widely cultivated grapevine varieties in Spain. After several years of clone selection, some highly recommended old clones have been identified in terms of both their qualitative and production characteristics. This study was designed to discriminate among 28 ancient clones of the cultivar Tempranillo (*Vitis vinifera*). DNA samples from clones were analysed using two different molecular markers; microsatellites or simple sequence repeats (SSR) and retrotransposons. The results of this study indicate that one variant genotype was expressed as three alleles. Further analysis revealed the presence of a chimera, in which the third allele was present in the leaf but not root or wood tissue, indicating a functionally double-layered apical meristem. The present research also showed that one of the retrotranposon marker was able to discriminate one grapevine clone (VP1) from the remaining clones.
Keywords: *Vitis vinifera* L.; Intravarietal variability; microsatellites; retrotransposon

INTRODUCTION

The origin of the grape cultivar Tempranillo -the most widely cultivated in the Rioja region of Spain- is unknown. Tempranillo grapes have been cultivated for many generations to create a vineyard variety grown across the Rioja region that is known today worldwide. The Tempranillo cultivar is propagated by cuttings and the resulting clones are genetically identical to the mother plant. The long-term vegetative propagation in a grapevine variety can be composed of a range of clones differing in minor genetic and phenotypic characteristics. Clone selection has been the only technique used to improve the quality and production of elite varieties. Clonal diversity is the product of two main factors: the environmental and the appearance of genetic mutations (Hartman *et al*., 1997). Intracultivar variability can also result from epigenetic modifications in response to the environment (Schellenbaum *et al*., 2008; Kaeppler *et al*., 2000) The molecular basis of genetic mutations in grapevine is poorly understood. A very important clonal polymorphism in grape is the skin color. This mutation has arisen as a result of retrotransposn activity (Kobayashi *et al*., 2004).

Old Tempranillo clones preserved by growers are in some cases 100 years old and these clones have interesting phenotypic characters. These traditional clones are ideal for improving a given trait, since they give rise to a more diverse crop in terms of yield, quality and morphologically distinct phenotypes. Unfortunately, however, in most cases the selected clones cannot be identified according to their morphological characters. This prevents distinguishing the clones available and hinders their certification and registration, with consequent repercussions on all sectors of the wine industry.

To date, several molecular markers have been used to characterize genetic diversity at the DNA level in the genus *Vitis*, and microsatellites have been identified as useful molecular markers for fingerprinting grapevine varieties. However, the use of microsatellite

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markers for clone discrimination has rendered contradictory results (Hocquigny *et al*., 2004; Ibáñez *et al*., 2003; Imazio *et al*., 2002). Polymorphisms identified by microsatellite markers have shown the presence of trialellic loci, referred in grapevines as chimeras (Hocquigny *et al*., 2004; Riaz *et al*., 2002) produced by mutations in cells of the meristem layers L1 and L2 (Thomson and Olmo, 1963). Although the chimeric state of a few grapevine clones has been previously demonstrated (Franks *et al*., 2002), the importance of this phenomenon is unknown. Compared to other methods, marker systems based on transposable elements are able to identify substantial genome changes. Retrotransposon systems detect insertion elements, hundreds to thousands of nucleotides long. The long terminal repeats (LTRs) that bind to a complete retrotransposon contain ends that are highly conserved in a given family of elements and thus a junction is formed between these conserved LTR ends and the anonymous flanking genomic DNA. These LTR sequences allow for experimental procedures such as retroelement-microsatellite amplified polymorphisms (REMAP) and inter-retroelement amplified-polymorphism (IRAP). Effectively, both these tools have been of exceptional value for the development of molecular markers in plants (Branco *et al*., 2007). However, when these retrotransposon markers have been used in grape, the two techniques have revealed polymorphisms among different cultivars but not among clones (Pereira *et al*., 2005).

The aim of the present study was to assess the possibility of using these molecular markers to detect intravarietal variation between ancient clones of the cultivar Tempranillo.

MATERIALS AND METHODS

DNA extraction: Twenty eight plant tissue samples were taken from ancient clones of the grapevine cv. Tempranillo growing in different vineyards and showing phenotypic differences. Once collected, unexpanded young leaves were frozen in liquid nitrogen and stored at -80ºC until further use. Frozen tissue was grounded in liquid nitrogen and DNA was extracted following the instructions provided in the DNeasy Plant Mini-kit (Quiagen, Hilden, Germany).

Microsatellites analysis: The following 43 microsatellite markers were used: 2 UCH markers developed by Lefort *at al*. (2003); 6 VrZAG markers developed by Sefc *et al*. (1999); 8 VVMD markers developed by Bowers *et al*. (1999); 14 VVI markers developed by Merdinoglu *et al*. (2005) and 12 VMC markers developed by the Vitis Microsatellite Consortium (Agrogene, France).

PCR amplifications were performed in a reaction mixture volume of 10 µl containing 10 ng of template DNA, 0.25-0.5 µM primers labelled with 6-FAM, HEX or NED fluorophores, 0.5 µM non-labelled primers, 150 µM of each dNTP (Boehringer Mannheim, Germany), 2.5 mM $MgCl_2$, 1X Ampli*Taq* buffer, and 0.2 U of Ampli*Taq* polymerase (PE Applied Biosystems, Foster City, California). The PCR cycle was conducted in a thermocycler (GeneAmp PCR System 9700, PE Applied Biosystems). The cycling programme consisted of the following steps: 10 min at 94ºC, followed by 35 cycles of 45 s at 92ºC, 1 min at 57ºC, 1 min and 30 s at 72ºC, and a final extension of 5 min at 72ºC. The amplification products were separated by capillary electrophoresis in an automated 310 ABI PRISM DNA sequencer (PE Applied Biosystems, Foster City, California) using the fluorophore HD400-ROX as an internal size standard. PCR fragments were detected using the GENESCAN analysis software (version 3.1) (PE Applied Biosystems) and alleles were scored using the GENOTYPER DNA fragment analysis software (version 2.5.2) (PE Applied Biosystems).

At each locus, a genotype displaying one allele was considered homozygous, and a genotype displaying two alleles as heterozygous. To confirm each variant genotype, DNA from the same extract was analysed twice.

IRAP and REMAP markers: We used the REMAP and IRAP primers described by Pereira *et al*. (2005) on the retrotransposon *Gret1* and compared their DNA profiles among Tempranillo clones. The IRAP and REMAP PCR were performed in a 20 µl reaction mixture containing 20 µg of DNA. All the five primer combinations were used: (*Gret1*LTR-reverse/Microsat-GA, *Gret1*LTR-reverse/Microsat-CT, *Gret1*LTR-forward/Microsat GA, *Gret1*LTR-forward/Microsat-CT for REMAP and *Gret1*-reverse/*Gret1*-forward for IRAP. PCR products were separated by 1.5% (w/v) agarose gel electrophoresis and detected by ethidium bromide staining.

RESULTS

Plant Material: Although the methodology for clonal selection is variable between countries, the two main

objectives of this research were elimination of major viral infections and improvement in yield related characteristics. During the first phase of the selection process vineyards with more than 80 years-old were growing in the Rioja Alta and Rioja Alavesa region and were surveyed during 3 years. In this phase were preselected 28 clones designated (VP) based in this parameters; yield, cluster weight, soluble solids, pH total, acidity, anthocianyn content, alcoholic degree and sugar content.

Molecular analysis of Tempranillo clones using microsatellite markers: The amplification products of the 43 SSRs for the 28 DNAs clones showed no differences among Tempranillo clones, except for the locus VrZAG79. Three alleles were detected in leaves of Tempranillo clones (VP3); 244 bp and 248 bp alleles and an additional 250 bp variant allele (Fig. 1, Table 1). The former alleles (244:248bp) have been well defined by other authors (Ibañez *et al*., 2003) as the most frequently detected alleles in different clones of the same cultivar. In DNA extracted from wood tissue of this clone, only the two alleles with sizes of 244 bp and 248 bp were detected at the locus VrZAG79. The appearance of three alleles at the same locus could be the result of a chimeric structure in which the genotypes of layers L1 and L2 bear different alleles. To investigate whether chimerism could explain the detected polymorphism, the genotypes in the wood and root tissues were examined because these are composed of L2 cells only, while the leaves comprise L1 and L2 cell layers (Table 2). Results of this study indicate that the alleles of the VrZAG79 locus (244 bp and 248 bp) are present in the L2 cells of Tempranillo. These findings also reveal that L1 cells feature the 244 bp and 250 bp alleles.

Table 1. Genotypes of 43 microsatellites loci in Tempranillo clones.

SSR	Genotype	SSR	Genotype
UCH11	238/258	VVIO55	392/394
UCH29	209/209	VVIP02	265/271
VMC1A2	105/115	VVIP31	177/179
VMC1E11	195/197	VVIP60	320/324
VMC1E12	240/244	VVIQ52	80/80
VMC2H4	204/206	VVIV67	363/365
VMC3D12	200/216	VVMD2	238/252
VMC4f3.1	178/182	VVMD7	236/250
VMC5A1	163/169	VVMD21	245/253
VMC5g7	186/212	VVMD24	205/211
VMC8A7	152/170	VVMD27	180/180
VMC8d11	132/138	VVMD28	50/54
VMC8F10	208/232	VVMD31	207/209
VMC9b5	238/238	VVMD32	232/248
VVIH01	167/169	VVS2	142/144
VVIH54	163/165	ZAG62	194/198
VVIM25	165/189	ZAG47	158/158
VVIB01	289/293	ZAG79	244/248/250
VVIN16	150/152	ZAG21	203/205
VVIN31	166/168	ZAG25	235/243
VVIN83	237/237	ZAG93	195/197
VVIO01	240/242		

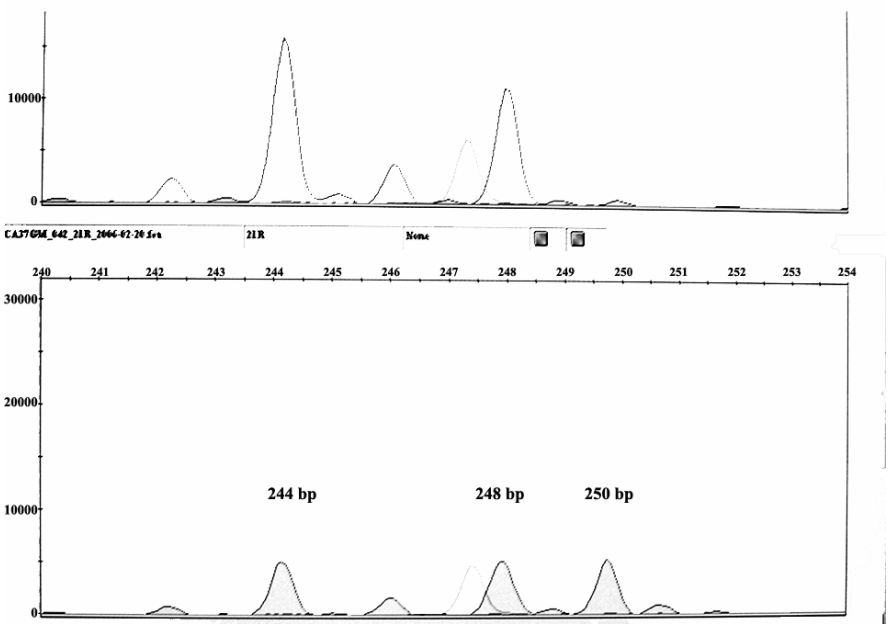

Figure 1. Electrophenogram of the three alleles detected in the Tempranillo clone VP13 at the ZAG79 locus.

Table 2. Genotype of different plant origin from the Tempranillo clone VP3 at the ZAG79 locus.

DNA origin	Locus allele (bp)	Cell layer
Leaves	244:248:250	L1+L2
Wood	244:248	L2

Molecular analysis of *V. vinifera* using inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellites polymorphism (REMAP) markers: Retrotransposons can potentially insert in the genome in any direction and members of the retrotransposon family can exist in various orientations, such as head-to-head, head-to-tail and tail-to-tail. To increase the probability of finding bands, primers for the 5´, 3´ and LTR ends can be combined or LTR primers can be combined with SSR primers. The REMAP primers were able to amplify genome regions in which the *Gret1* LTRs were flanked by microsatellites separated by a distance of between 100 bp to 2 Kb. The IRAP primers amplified genome regions between two LTRs occurring 400 bp to 2 Kb apart. Profile complexity ranged from one to 10 bands. All IRAP and REMAP reactions amplified bands of varying intensity in the 28 clones used in this study. No differences in the IRAP amplified bands were detected among the Tempranillo clones. In contrast, nine clones showed different profiles of one REMAP combination (*Gret1*LTR-F/MicrosatCT) (Table 3). Clones VP2, VP13, VP14, VP15, VP17, VP18, VP21 and VP23 showed no amplification products. When attempts were made to amplify the same DNA in these clones using different primers, it was only the REMAP primer combination that failed. This suggests a point mutation in one of the annealing sequences. Only one clone, VP1, showed a small deletion polymorphism (Table 3), which was checked and repeated six times. Indeed, this is the first report of the use of IRAP and REMAP markers to distinguish clones.

Table 3. Fingerprinting of *Gret1* LTR forward-Microsat CT marker in Tempranillo clones (VP). The (-) marker indicate the presence of the amplification band.

	LTR-F/CT			
Clone	Nº fragments	(size of fragments bp)		
		1310	1600	1745
VP1	2	-	-	
VP2	0			
VP3	3	-	-	-
VP4	3	-	-	-
VP5	3	-	-	-
VP6	3	-	-	-
VP7	3	-	-	-
VP8	3	-	-	-
VP9	3	-	-	-
VP10	3	-	-	-
VP11	3	-	-	-
VP12	3	-	-	-
VP13	0			
VP14	0			
VP15	0			
VP16	3	-	-	-
VP17	0			
VP18	0			
VP19	3	-	-	-
VP20	3	-	-	-
VP21	0			
VP22	3	-	-	-
VP23	0			
VP24	3	-	-	-
VP25	3	-	-	-
VP26	3	-	-	-
VP27	3	-	-	-
VP28	3	-	-	-
Contro	3	-	-	-

DISCUSSION

This study described the use of a different type of molecular marker to distinguish among grapevine clones. Currently, microsatellite markers represent the most widely used DNA markers to identify cultivars. However, these markers fail to discriminate among clones of the "Muscat group" (Crespan and Milani, 2001), Traminer (Imazio *et al*., 2002), Garnacha (Ibañez *et al*., 2003) and some table grape cultivars (Dangl *et al*., 2001). In contrast, microsatellites have served to detect polymorphisms at the clone level in Riesling (Regner *et al*., 2000), the Pinot family (Hocquiniy *et al*., 2004), Chardonnay (Berts *et al*., 2005), Tannat (González-Techera *et al*., 2004) and other cultivars (Crespan, 2003). The present results indicate that the Tempranillo clones analyzed are genetically very uniform and the ampelographic differences observed in different clones probably reflect epigenetic differences. However, it was nevertheless possible to distinguish between clones differing in the alleles shown for the SSR marker VrZAG79. Standard allele sizes of 244 and 248 pb have been reported previously for the Tempranillo cultivar (Ibañez *et al*., 2003), yet three alleles (244:248:250) were detected in clone VP3. The apical meristem of the grapevine is

composed of two or more cell layers forming the tunica as an addition to the corpus (Franks *et al*., 2002). In the leaf tissue of Tempranillo, which is derived from the L1 layer and inner cell layer L2, the microsatellite marker ZAG79 revealed the two standard alleles plus a variant allele while wood tissue samples showed the two standard alleles. The presence of a third allele in the leaf tissue suggests a periclinal chimera in which a mutant allele only exists in the L1 cell layer, as described by Riaz *et al*. (2002). This mutant allele is likely to have replaced one of the standard alleles in the wood and root tissue, whereas in the leaves, the mutant allele is found along with the standard allele. The results of this research, therefore suggest that a 2 bp insertion in the VrZAG79 248 bp allele giving rise to the 250 bp allele, only occurs in the L2 cell layer.

Retrotransposon elements such as *Tvv1* are novel markers that have proved useful for analyzing genetic diversity and relatedness in the genus *Vitis*, but these elements are conserved between vegetatively propagated clones (Pelsy *et al*., 2007). However, since most plant retrotransposon elements are activated in somatic cells by several biotic or abiotic stress factors, the propagation of grapevines using cuttings might increase the likelihood of a retrotransposon to transpose and multiply. Thus, retrotransposons could be a major force driving the creation of additional genetic variability in the grapevine. In effect, the skin color mutation of Tempranillo (from red to white) has been attributed to a retrotransposon *Gret1* insertion in the promoter of a *Myb*-related gene that regulates anthocyanin biosynthesis (Kobayashi *et al*., 2004). The gypsy-type retrotransposon *Gret1* is the first complete retrotransposon sequence identified in *V. vinifera* (Kobayashi *et al*., 2004). Given the reported *in situ* results indicating that *Gret1* is clustered in the *Vitis* genome (Pereira *et al*., 2005), REMAP and IRAP markers would be expected to generate a complex band profile. There are a number of possible explanations for the relatively few bands obtained here using this technique. One is that the repeated insertion sites represent genomic regions where *Gret1* is inserted as a non-evenly distributed tandem. In addition, the distance between the transposon and the microsatellite loci used in this study may have been excessive for conventional PCR amplification. From a molecular perspective, our PCR results indicate that are several clones with differences probably related to a point mutation in the primer sequence of the *Gret1* retrotransposon. This is the first time this technique has been successfully used to distinguish among grapevine clones, although similar techniques using universal retrotransposon based sequences have been used successfully (Wegscheider *et al*., 2009).

In conclusion, our findings indicate that SSR and retrotransposon markers could be useful tools for identifying ancient Tempranillo clones. The recent publication of the complete grapevine genome has paved the way for the detailed analysis of its transposon content. Recently, Benjak *et al*. (2008) reported the transduplication of these elements and the consequent amplification of cell sequences, some of which have been domesticated and probably fulfil cellular functions. These observations provide further evidence that the mobility of these elements has contributed to the genetic variability of this species.

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