Tracing Heterozygosity in the *Vvlexp1* Locus in Grapevine by Sequencing and High-resolution Melt Analysis

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ABSTRACT. Multiple loci in a continuously asexually reproducing genome such as vegetatively propagated grapevine (*Vitis vinifera*) can be heterozygote. The methodology to analyze heterozygous loci is manifold ranging from traditional breeding and studying segregating offspring, codominant marker analyses to whole sequence analysis. Results of heterozygosity studies on challenging loci need to be carefully confirmed to ensure accuracy and avoid misinterpretation. One of these methods is high-resolution melt (HRM) analysis in combination with sequencing and segregation analysis. We present first the adoption of HRM analyses for grapevine and its potential to confirm heterozygotic markers with low or no sequence size differences.

Multiple allelism is part of the evolutionary diversity in the animal and plant world. Gregor Mendel was the first to discover multiple allelic traits and allelic series in plants. This was the major and essential step to marker-assisted selection and molecular breeding. Multiple allelism is not an exception of the rule but the rule. If a trait is connected to a heterozygote allele, there is a possibility the heterozygote loci might even give the plant the required fitness to tolerate environmental changes quicker than plants carrying only homozygote alleles (Hansson and Westerberg, 2002). Many loci in a continuously asexually reproducing genome such as the vegetatively propagated cultivated grapevine display a high level of heterozygosity. The methodology to analyze heterozygote alleles is manifold ranging from traditional breeding and studying segregating offspring, codominant marker analyses and association mapping to whole sequence analysis.

Various studies have concentrated on analyzing heterozygosity between grapevine cultivar clones (Anhalt et al., 2011; Blauch et al., 2007; Fanizza et al., 2005; Konradi et al., 2007; Wegscheider et al., 2009), but heterozygosity is as well the basis of every breeding study. Segregating alleles allow finding loci linked to traits of interest. Heterozygosity arising from the crossing of two parents can result in a population with a high level of segregation (Anhalt et al., 2008). Mapping and marker-assisted selection (MAS) of these loci are highly

achievable, e.g., in potato [*Solanum tuberosum* (Danan et al., 2011; Kloosterman et al., 2010)], tomato [*Solanum lycopersicum* (Davis et al., 2009; Hutton et al., 2010)], rape seed [*Brassica napus* (Chen et al., 2011; Smooker et al., 2011)], or in grapevine (e.g., Costantini et al., 2008; Doligez et al., 2002, 2006; Duchene et al., 2012). Codominant markers, like microsatellites (simple sequence repeat), single nucleotide polymorphisms (SNP), or restriction fragment length polymorphism markers, are efficient for MAS, because they can display allelic variations. Especially SNPs are of high interest because of their possibility to trace single base changes in the genome in coding regions in contrast to microsatellite markers (Salmaso et al., 2004), which mainly occur in non-coding regions.

Genome sequencing is a challenge in heterozygote plants as a result of the assembling of the heterozygote sequences into separate contigs of the two haplotypes and can only be solved by physical mapping (Scalabrin et al., 2010) or deep sequencing. In addition to these challenges, technical pitfalls can occur during amplification through polymerase chain reaction (PCR) and cloning. In the PCR approach two heterozygote alleles can be amplified with different proportions during the amplification step. Therefore, poorly represented alleles can get missed in the subsequent cloning and the sequencing step. Additionally, transformation artifacts can be produced by cloning (Zhang and Hewitt, 2003), which can biased cloning and sequencing results. We exemplify misinterpretation through cloning pitfalls and showing a study searching for a marker linked to cluster architecture. The sequence locus *Vvlexp1* was analyzed by molecular cloning, marker analysis, and sequencing (Hoffmann et al., 2009; Vaclavicek, 2004) with the ‘Pinot Noir’ clones from the Geisenheim Research Center (Geisenheim, Germany) with compact clusters (clone ‘1-84Gm’)

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vs. loose clusters (clone '18Gm'). What was not recognized was the fact that both clones were heterozygous and both had one allele with a deletion and a SNP and one allele without. Cloning and the close size similarity of the marker bands were misleading to the conclusion that '1-84Gm' and '18Gm' had different homozygous loci. Because cloning catches only one of the alleles in each bacteria colony, it is necessary to analyze several clone colonies to catch heterozygote loci. In the unfortunate case repeating only one same allele was analyzed and thus homozygosity was concluded. This was the case in the stated studies and this was the reason to look for an alternative way of analyzing this locus. In the presented study, the locus was re-analyzed with grapevine cultivars, clones, and on individuals of two selfing populations of '18Gm' and '1-84Gm' with sequencing of PCR fragments and the HRM approach.

The aim of the presented study was to find stable methods to efficiently analyze such challenging loci like *Vvlexp1*. We present the HRM approach and its potential to confirm heterozygotic markers with low or no sequence size differences.

Materials and Methods

PLANT MATERIAL. Two 'Pinot Noir' clones from Geisenheim Research Center, '18Gm', '1-84Gm', were used as reference

plant material. Additionally, two grapevine species, a collection of 41 different grapevine cultivars, nine clones of 'Pinot Noir', 10 clones of 'Plavac mali', 15 clones of 'Škrlet', and 27 individuals of two selfing populations (Table 1) one of the 'Pinot Noir' clone '18Gm' and one of '1-84Gm' were analyzed. The plant leaf material was achieved by collecting young leaves, which were snap-frozen and stored at -20°C in 50-mL tubes until the DNA extraction. Genomic DNA was extracted using the E.Z.N.A. SP Plant DNA Miniprep Kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions with one modification, prolonging the incubation step to 30 min. The cultivar collection was mainly obtained as extracted DNA from the Geisenheim Research Centre, University of Zagreb (Zagreb, Croatia) or from the University of California, Davis.

LOCI ANALYSIS. Locus *Vvlexp1* was analyzed with a molecular marker (F:GGGCATATTTCAGCCAGAGCC, R:ACAAT TGTGTCCCTGCTCAC) designed with the online primer design program Primer 3 (Rozen and Skaletsky, 2000) using the sequence (AM482589) to analyze differences among loci of the cultivars and clones. The following protocol was used for PCR amplification with TAQ DNA polymerase (5 U/ μL ; Fermentas, St. Leon-Rot, Germany): 3-min initial denaturation at 95°C , 35 cycles of 45 s for denaturation at 95°C , 45 s for

Table 1. Grapevine sequences of locus *Vvlexp1* of different cultivars, clones, and individuals of the selfing population from Geisenheim Research Centre (Geisenheim, Germany) '18Gm' (A1-A27) and '1-84Gm' (B2 to B16) are shown.

Locus	Allele codes	Sequence of alleles	Individuals
Consensus		AAAAAAAGCCXCCXXXXTXXCTTTTXXCCCAA bp 1- - - - -1213- -1617181920-2223- - - -29- - -33- - -38	
<i>Vvlexp1-1</i>	a	AAAAAAAGCCCTTCCTCCCTTTTCTTTTACCCAA	'18Gm', '1-84Gm', Selfing populations: A1, A3, A5, A6, A7, A8, A9, A11, A15, B3, B4, B6, B2, B3, B14, B15, '105 S-Frank', '120-13 Gm', '1-44 Gm', 'Pinot Noir ENTAV 115', 'Pinot Noir ENTAV 777', 'Pinot Noir 777', 'Pinot Noir 943', 'Blauer Trollinger', 'Cardinal', 'Chardonnay', 'Chasselas Fr.38-95', 'Chenin, Findling', 'Flora', 'Freiburger', 'Gamay', 'Gauburgunder', 'Malingre Precoce', 'Melon', 'Rakatsiteli', 'Sauvignon Blanc', 'Schwarzriesling', 'St. Laurent', 'Weisser Burgunder'
	b	AAAAAAAGCCCTTCCT- - -TTTCTTTTACCCAA	
<i>Vvlexp1-2</i>	a	AAAAAAAGCCCTTCCTCCCTTTTCTTTTACCCAA	Selfing populations: A10, A14, A27, B2, B5, B6, 'Annabella', 'Balgär', 'Catarratto', 'Cipar', 'Garant', 'Marinkovica Grozdje', 'Mueller Thurgau', 'Riesling', 'Saba Kiralynoje', 'Trebiano Di Soave'
	a	AAAAAAAGCCCTTCCTCCCTTTTCTTTTACCCAA	
<i>Vvlexp1-3</i>	b	AAAAAAAGCCCTTCCT- - -TTTCTTTTACCCAA	Selfing populations: A19, A22, B10, B11, B16
	b	AAAAAAAGCCCTTCCT- - -TTTCTTTTACCCAA	
<i>Vvlexp1-4</i>	a	AAAAAAAGCCCTTCCTCCCTTTTCTTTTACCCAA	'Cabernet Franc', 'Detskij Rannuej', 'Trebiano Di Toscana', 'Plavac mali' clones, 'Škrlet' clones
	d	AAAAAAAGCCCTTCCTCCCTTTTCTTTTACCCAA	
<i>Vvlexp1-5</i>	c	AAAAAAAGCCCTTCCTCCCTTTTCTTTT- TTTACCCAA	'Angelina', 'Blauer Portugieser', 'Kraljevina', 'Krisimis Luscsisztij', 'Merlot', 'Muscat A Petits Grains', 'Muscat D'Alexandrie', 'Pošip bijeli', 'Regent', 'Semillon', 'Siegerrebe', Vitis cinerea
	c	AAAAAAAGCCCTTCCTCCCTTTTCTTTT- TTTACCCAA	
<i>Vvlexp1-6</i>	e	AAAAAAAGCCCG-CC- -CTT- -CTTTT-TTT-CCCAA	Vitis rotundifolia
	e	AAAAAAAGCCCG-CC- -CTT- -CTTTT-TTT-CCCAA	

annealing at 53 °C, 1 min for elongation at 72 °C, and 10 min for a final elongation at 72 °C. The PCR products were sequenced at AGOWA (Berlin, Germany).

HRM analysis for homo-/heterozygosity of alleles was performed with a Type-it HRM kit (Qiagen, Hilden, Germany) following the manufacturer’s manual. The Type-it HRM PCR Kit contains the dsDNA-binding fluorescent dye EvaGreen. The HRM primer (F: 5’-AAGGCCACATTGACAAATGCAA-3, R: 5’-TCTTGCCCTTTTCTTGGGTA-3) was designed with the program Primer 3. PCR conditions were chosen as follows: 5-min initial denaturation at 95 °C, 40 cycles of 10 s for denaturation at 9 °C, and 30 s for strand annealing at 55.5 °C. HRM was conducted from 65 to 95 °C with a temperature rise of 0.1 °C every 4 s. Samples were analyzed on a Rotor-Gene Q (Qiagen) in three technical and three biological replicates. Results were visually evaluated with the help of the normalized fluorescence curves and the melting curves of the Rotor-Gene Q Series Software Version 2.0.2.

Results

SEQUENCING AND CODOMINANT MARKER STUDY. The amplification products were sequenced and presented in Table 2. The data confirm that ‘18Gm’ and ‘1-84Gm’ share the same heterozygote locus: allele “b” with a deletion of 4 bp and a SNP and allele “a” with no deletion and no SNP. Individuals of both populations showed either both alleles or are homozygote for either the allele “a” or “b.” The cultivars consisted of the mentioned alleles “a,” “b,” or alleles (“c,” “d,” “e”). All together six different allele combinations (*Vvlexp1-1* to *Vvlexp1-6*; Table 1) could be found with the sequencing approach.

After amplifying and sequencing, the alleles of individuals of both selfing populations were identified (Fig. 1). Heterozygote loci showed clearly the two alleles in the sequence graph as an overlay of the two allele sequences (Fig. 1). However, still no clear homozygosity could be certified because facing the problem that visually homozygote displayed alleles could be as

Table 2. Grapevine sequences of the two ‘Pinot Noir’ clones (‘18Gm’ and ‘1-84Gm’) and of the individuals of the selfing population, ‘18Gm’ (A1 to A27) and ‘1-84Gm’ (B2 to B16), from Geisenheim Research Centre (Geisenheim, Germany) are given including their allele codes.

Clones	Allele code	Sequence of allele
‘1-84Gm’, ‘18Gm’, A1, A3, A5, A6, A7, A8, A9, A11, A15, B3, B4, B6, B2, B3, B14, B15	a	AAAAAAAAGCCCTTCCTCCCTTTTCTTTTCTTTACCCAA
	b	AAAAAAAAGCCCTTCCT- - -TTTCTTTTTTTTACCCAA
A10, A14, A27, B2, B5, B6	a	AAAAAAAAGCCCTTCCTCCCTTTTCTTTTCTTTACCCAA
	a	AAAAAAAAGCCCTTCCTCCCTTTTCTTTTCTTTACCCAA
A19, A22, B10, B11, B16	b	AAAAAAAAGCCCTTCCT- - -TTTCTTTTTTTTACCCAA
	b	AAAAAAAAGCCCTTCCT- - -TTTCTTTTTTTTACCCAA

Fig. 1. The two grapevine sequences of the heterozygous ‘Pinot Noir’ clones ‘1-84Gm’, ‘18Gm’ (alleles “ab”), and two of the homozygous genotypes of the selfing population of ‘18Gm’ (A10, A19) (alleles “aa” and “bb”) from Geisenheim Research Centre (Geisenheim, Germany) are displayed. Alleles “ab” in ‘Pinot Noir’ clones overlaying each other. The sequence shift in allele “b” is the result of a deletion of 4 bp. Star = position of the deletion; box = single nucleotide polymorphism in allele “a” and “b”.

well heterozygote as a result of one poorly represented allele through PCR (Zhang and Hewitt, 2003).

HIGH-RESOLUTION MELT. HRM analysis confirmed the homo- and heterozygosity of the genotypes. The two homozygous genotypes can be distinguished from the identical sequences of the clones '1-84Gm' and '18Gm' (Fig. 2A). The melting temperature of the homozygote loci of the individuals of the selfing populations showed sequence melting at 74.3 °C (e.g., A19) and at 75 °C (e.g., A10). The heterozygote loci had their melting point at 74.6 °C. The melting curves (Fig. 2B) show the single peak for the melting of the homozygote loci and the double peak for the heterozygote loci of the '18Gm' and '1-84Gm' clones. As a result of the different melting temperatures of the two heterozygote alleles, a double peak occurs. These results confirmed that both genotypes carry the alleles without and with deletion and SNP.

The sequence loci of the different cultivars could be as well identified through single or double peaks as homo- or heterozygote genotypes.

Discussion

Clearly defining the heterozygosity/homozygosity state of grapevine genotypes is challenging. Especially if alleles show similar sizes and differ in few SNPs, correct identification may be impossible only with traditional methods. The possibility of a fully sequenced genome of *V. vinifera* cultivar Pinot Noir (Jaillon et al., 2007; Velasco et al., 2007) opens doors for in silico searches, which may be hampered because the two databases (one a homozygote and one a heterozygote genome

of 'Pinot Noir'), may show differences in regions (Scalabrín et al., 2010).

The results of the presented locus study challenge the results in the previous studies (Hoffmann et al., 2009; Vaclavíček, 2004) in which the heterozygote loci of '18Gm' and '1-84Gm' were difficult to identify in the first place. The sequencing results visualized first an overlay of two sequences (Fig. 1) and alleles could not be clearly defined as a result of the overlays. The study was repeated several times to exclude technical pitfalls and provide adequate DNA quality and excellent technical conditions for both cloning and sequencing, resulting in the same outcome. As a control, individuals of the selfing populations were analyzed and showed segregation in the different allele combinations indicating the need to imply a more sensitive method for identifying allelism in similar alleles. Another study in grapevine faced the same challenge and solved it by studying the hetero- and homozygote genotypes through single-strand conformational polymorphisms and heteroduplex analysis (Salmaso et al., 2004). Other approaches to catch single alleles is allele-specific sequencing, but it requires the polymorphic restriction site information of the alleles and is restricted because of the limitation to determine all haplotypes (Zhang and Hewitt, 2003) or to resolve haplotypes and mutations by denaturing high-performance liquid chromatography (DHPLC). DHPLC can detect heteroduplexes in PCR products and is therefore suitable to analyze single base mutations (O'Donovan et al., 1998) as shown to detect juice ingredients from six fruit species, one of them in grapevine, and to targeted different regions of the chloroplast genomes and traumatin-like proteins (Han et al., 2012).

Fig. 2. Upper and lower curves show the two homozygous loci sequences of the selfing grapevine populations '1-84Gm' (individual A10) and '18Gm' (individual A19) from Geisenheim Research Center (Geisenheim, Germany) and in the middle the heterozygous loci of the 'Pinot Noir' clones '1-84Gm', '18Gm' analyzed with the high-resolution melt approach in grapevine. (A) Normalized fluorescence curves. (B) Melting curves; homozygous loci give one peak and heterozygous loci two peaks; DF/dT = derivative of fluorescence vs. temperature.

HRM has been previously applied in grapevine studies as an identification method of rootstocks and cultivars (Mackay et al., 2008). It is also well suited for mutation studies and for breeding studies such as “blind” mapping (Studer et al., 2009). In the presented study we show that HRM can be helpful to analyze homo- and heterozygote sequences of interest and show small sequence changes, which can be easily overlooked. In combination with sequencing, it is a stable, sensitive, and fast method to confirm challenging loci and confirm their allelism.

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