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QTL identification and candidate gene identification for monoterpene content in grape (*Vitis vinifera* L.) berries

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Summary

Great progress has been made during the last decade in clarifying the molecular details of aroma accumulation in grape berries. However, the multigene complex controlling monoterpene accumulation in grape is not well understood. To shed light on this issue, the grapes of 149 F1 progenies from the cross 87-1 (*Vitis vinifera* L.) × 9-22 (*Vitis vinifera* L.) were characterized at the mature stage for three representative free monoterpenes during five growing seasons. A total of 202, 184 and 255 polymorphic SSR (simple sequence repeat) markers were contracted on the maternal 87-1, paternal 9-22 and consensus genetic maps, respectively. On the consensus map, we confirmed a major QTL (quantitative trait locus) for free linalool, nerol and α-terpineol content on linkage group (LG) 5, and a stable QTL for free linalool and α-terpineol was detected on LG 10. In addition, two new stable QTLs for free monoterpene (linalool, nerol and α-terpineol) contents were identified on LG 11 and LG 18 that explained up to 42.5 % of the total variance. Eleven promising candidate genes related to pentatricopeptide repeat (PPR)-containing proteins, seed maturation protein, RING finger protein, and AP2/ERF transcription factors might be potentially involved in monoterpene accumulation. The stable QTLs and candidate genes identified in this study provide new insights into free monoterpene accumulation in grape.

K e y w o r d s : grape; monoterpene; linkage map; QTL; candidate gene.

Introduction

Grape (*Vitis vinifera* L.) is an important fruit species worldwide due to its potential economic value as fresh or dry fruit, wine and liquor. Volatile aroma components in grapes and wines are among the most crucial characteristics targeted in grape cultivar breeding programs. These aroma components vary among different grape cultivars, and the corresponding genetic architecture has been widely studied in recent years (Doligez *et al.* 2006b, Battilana *et al.* 2009, Wu *et al.* 2016, Costantini *et al.* 2017, Yang *et al.* 2017). It is well known that volatile aroma compounds' inheritability and metabolism are typical quantitative traits and are controlled by polygenes in grape berries (Battilana *et al.* 2009, Duchêne *et al.* 2009, Wu *et al.* 2013, Liu *et al.* 2016). Previous studies have shown that volatile aroma compounds in grape berries include mainly C6 volatile compounds, aldehydes, esters and terpenes (Kalua *et al.* 2009, Liu *et al.* 2016, Wu *et al.* 2016). The characteristic volatile aroma compounds in Muscat grape cultivars are monoterpenes (Gunata *et al.* 1985, Mateo and Jiménez 2000, D'Onofrio *et al.* 2016, Wu *et al.* 2016). Free monoterpenes are volatile compounds directly involved in aroma flavor, such as linalool, nerol, α-terpineol, citronellol and geraniol. They play a major role in Muscat grapes and wines, due to free monoterpenes having low odor detection thresholds (Selli *et al.* 2006, Ugliano *et al.* 2008, Bordiga *et al.* 2013) and total concentrations in Muscat-flavored grape varieties can be as high as 6 $mg \cdot L^{-1}$ (Mateo and Jiménez 2000). In contrast, bound glycoside monoterpenes are non-volatile compounds with no direct contribution to the aroma of grapes, but they can constitute a large source of potential volatile molecules after hydrolysis or fermentation (Hjelmeland and Ebeler 2015).

Monoterpenes (C10) are representative terpenoid compounds. Lichtenthaler (1999) proposed that monoterpene synthesis in plants utilizes two five-carbon (C5) compounds, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), from two independent pathways, including the cytoplasmic mevalonic acid (MVA) and the plastidial 2-methyl-D-erythritol-4-phosphate (MEP) pathways. It has been suggested that the MEP pathway supplies precursors for many volatile compounds in plants (Gershenzon and Dudareva 2007). 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) has traditionally been considered a key rate-limiting enzyme of the MEP biosynthetic pathway in plants (Estévez *et al.* 2001). The MEP pathway is the dominant route for monoterpene biosynthesis in grape (Luan and Wüst 2002), and monoterpene linalool, nerol, and geraniol contents have been suggested to colocalize with *VvDXS* in berries (Battilana *et al.* 2009). Early published studies found quantitative trait loci (QTLs) for monoterpenes (linalool, nerol, α-terpineol, citronellol and geraniol) on linkage groups (LGs) 1, 2, 5, 6, 7, 10, 12, 13, 15 and 16. The most likely candidate gene for monoterpene QTLs is on LG 5 and is associated with the *VvDXS* gene (Doligez *et al.* 2006b, Battilana *et al.* 2009, Duchêne *et al.* 2009). Despite this

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progress, the MEP pathway rate-limiting enzymes 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and 1-hydroxy-2-methyl-2- butenyl 4-diphosphate reductase (HDR) have been suggested to play key roles in several plant species (MAHMOUD and CROTEAU *et al.* 2001, BOTELLA-PAVÍA *et al.* 2004). Particularly, a large number of grapevine terpene synthase (*VvTPS*) genes have been reported to generate linalool, geraniol and α-terpineol (MARTIN *et al.* 2010). HDR and GPPS (geraniol diphosphate synthase) have been suggested to regulate monoterpene accumulation during grape development (MARTIN *et al.* 2012). These previous studies have indicated that monoterpene accumulation in grape involves complex multigene control beyond the important *VvDXS* gene. In addition, some transcription factors (TFs), such as NAC, AP2/ERF and MYB families, have recently been proposed to be involved in the transcriptional regulation of terpene synthesis genes in grape (WEN *et al.* 2015, CRAMER *et al.* 2014, COSTANTINI *et al.* 2017). However, QTLs for monoterpene accumulation related to TFs have not been reported in grape. In the present study, we aim at identifying new candidate genes, in addition to *VvDXS*, for monoterpene accumulation in Muscat-flavored varieties. main free monoterpene (linalool, nerol and α-terpineol)-related QTL detection was performed from an intraspecific cross between 87-1 and 9-22 grapes (*Vitis vinifera* L.) over 5 successive years, and some probable candidate genes were identified according to the 12X V2 of the grape genome at CRIBI (http://genomes.cribi.unipd.it/grape/, VITULO *et al.* 2014).

Material and Methods

Plant material: An F1 population derived from the cross 87-1 (*Vitis vinifera* L.) × 9-22 (*Vitis vinifera* L.) consisted of 149 plants. The female parent 87-1 with typical Muscat flavor and the male parent 9-22 without aroma flavor were described by GUO *et al.* (2014). The parents and progeny were grafted onto 'Beta' (*Vitis riparia* × *Vitis labrusca*) rootstock to resist chilling and were grown at Shenyang Agricultural University (Liaoning, China, 41° 50' N, 123° 24' E, 55 masl). Winter pruning and spring thinning were conducted as in commercial orchards. The young grape leaves collected from each plant were used for genomic DNA (gDNA) extraction. From 2011 to 2015, three clusters were collected from each progeny as replicates at maturation stage, which the total soluble solid (TSS) content was approximately 18 °Brix in berries, and stored at -80 °C until use.

Free monoterpenes analysis: The free monoterpenes were extracted with headspace (HS) solid-phase microextraction (SPME) and analyzed with gas chromatography-mass spectrometry (GC-MS) (7890A-5795C, Agilent Technologies, Santa Clara, USA) according to a previous report (CANUTI *et al.* 2009) with some minor modifications. The berries were defrosted at 4 °C before volatile isolation. After removing the seed, 50 g berries and 3 g NaCl were homogenized with commercial blender, and then 10 g puree was sealed in 20 mL capped solid-phase microextraction vial with 100 µl 5 $mg \cdot L^{-1}$ cyclohexanol as an internal standard. A SPME fiber (PDMS/DVB 65 µm, Supelco, Bellefonte, USA) coated with polydimethylsiloxane-divinylbenzene was used for volatile extraction *via* dynamic HS-SPME at 37 °C for 30 min. After extraction, the fiber was directly desorbed into the injection port of the GC at 250 °C for 5 min in splitless mode, with a nitrogen gas linear flow rate of 1 $mL \cdot min^{-1}$. Volatile compounds were separated on a VF-Waxms column (30 m × 0.25 mm id × 0.25 µm film thickness, Agilent, Santa Clara, USA) by applying the following temperature program: 70°C for 5 min, 70-120 °C at 1 $°C \cdot min^{-1}$, 120 °C for 2 min, 120-210 °C at 3 $°C \cdot min^{-1}$ and 210 °C maintained for 3 min. The transfer line temperature was 240 °C. MS operated in electron impact (EI) mode at 70 eV, and data were collected at a scanning rate of 2.88 $scans \cdot s^{-1}$ within a mass scanning range of m/z 30-500 amu. Mass spectra were retrieved using the NIST11 spectral library.

Including the parents, 10 intensely Muscat-flavor individuals and 10 No-flavor individuals were selected to generate free monoterpene concentration 'Highest' group and 'Lowest' group, and used for free monoterpenes GC-MS qualitative analysis in 2011. The result ensured that the free linalool, nerol and α-terpineol were the main free monoterpenes in the 87-1 × 9-22 population, then chosen for further QTL identification. A model grape puree (5 $g \cdot L^{-1}$ tartaric acid dissolved in Milli-Q water, pH adjusted to 4.5 with NaOH) with added chemical aroma standards linalool, nerol and α-terpineol (Tokyo Chemical Industry, Tokyo, Japan) for calibration curve preparation was produced. Standard concentrations ranged from 0.01 $\mu g \cdot L^{-1}$ to 1 $mg \cdot L^{-1}$.

Phenotypic evaluation of the free linalool, nerol and α-terpineol: The normality of the free linalool, nerol and α-terpineol distribution was evaluated by the Shapiro-Wilk test. Because most data distributions significantly deviate from normality, phenotypic correlations between traits within years were determined using the nonparametric Spearman correlation coefficient. H^2 was calculated as previously described by DUCHÊNE *et al.* (2009). Statistical analyses were performed with the *R* 3.5.1 package (R DEVELOPMENT CORE TEAM 2014).

SSR marker selection and polymorphic marker identification: gDNA was extracted from young leaves of the progeny and parents by the CTAB method (HANANIA *et al.* 2004). 454 SSR primer sequences were obtained from previous studies, including VMC (*Vitis* Microsatellite Consortium, managed through AGROGENE, Moissy Cramayel, France), VVS (THOMAS and SCOTT 1993), VVMD (BOWERS *et al.* 1996, 1999), VrZAG (SEFC *et al.* 1999), UDV (DI GASPERO *et al.* 2005), VVI (MERDINOGLU *et al.* 2005) and Chr (BLASI *et al.* 2011). In addition, based on the locations of microsatellite repeat regions on the grape chromosome genome (JAILLON *et al.* 2007, http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/), a total of 567 new SSR primers (VLG- and Y-series, suppl. Tab. 1) were designed by Primer 3.0 for use in this study. PCR amplification and polyacrylamide gel electrophoresis were carried out according to a previous study (GUO *et al.* 2014).

Linkage map construction and QTL analysis: The genetic maps of 87-1 and 9-22 and the consensus map were constructed based on SSR marker segregation in 149 F1 individuals. Polymorphic markers

(ab × cd, ef × eg, hk × hk, lm × ll, and nn × np) were scored as previously described by Guo *et al.* (2014), and the segregation of each marker was tested for goodness-of-fit to the appropriate expected ratio by χ^2-tests before construction of linkage maps. The maps were created using JoinMap 3.0 (Van Ooijen and Voorrips 2001) under the following parameters: "CP mapping function", LOD ≥ 3.0, maximum recombination rate = 0.4. A total of 314 and 306 polymorphic SSR markers were used for the maternal 87-1 and paternal 9-22 construction, respectively. The consensus map was generated by merging 432 segregating SSR markers. The LGs were numbered according to grape reference genetic maps (Doligez *et al.* 2006a) and VLG- and Y-series SSR marker position. The final parental and consensus maps were drawn with Mapchart 2.2 (Voorrips 2002).

QTL analysis was performed on a consensus map according to each trait evaluated in each year. QTL identification was carried out using the R/qtl package (Broman *et al.* 2003) with 4-way cross and importing geno, pheno and map files in MapQTL format. Multiple QTL regression was carried out with the "stepwiseqtl" function according to a previous report (Huang *et al.* 2012) with some minor modifications. The linkage map was scanned at 1-cM steps for the presence of a significant QTL. The maximum QTL number was set to 5 (max.qtl = 5) for backward/forward selection. A 5 % LOD significance threshold was generated for each QTL analysis using 1000 permutation tests at the chromosome-wide level, and the "bayesint" function (prob = 0.95, expandtomarkers = TRUE) was used to derive the 95 % QTL location confidence interval. A QTL was considered to be stable if it was detected reproducibly in at least 2 of the 5 successive years (Ban *et al.* 2016). The QTL regions on the linkage maps were illustrated in MapChart 2.2 (Voorrips 2002).

Identification of candidate genes: To identify genes potentially implicated in grape monoterpenoid metabolism, candidate gene identification was followed the method of Doligez *et al.* (2013) with some modifications as described below. The sequence of the SSR markers delimiting the QTL confidence interval was blasted against the 'Pinot Noir' genomic sequence (Jaillon *et al.* 2007), and the physical interval of the confidence QTL intervals was anchored to the grape genome. The prediction genes in focused QTL intervals were extracted from 12X V2 of the grape genome at CRIBI (http://genomes.cribi.unipd.it/grape/), and their putative function was checked. In addition, as a complementary approach, consulting the available literature or GO term in CRIBI database (http://genomes.cribi.unipd.it/grape/), the extracted prediction genes that were possibly involved in monoterpenoid metabolism (e.g. IPP biosynthetic process, MEP or MVA biosynthesis) were analysed.

Results

Phenotypic data: The free linalool, nerol and α-terpineol contents exhibited continuous variation in the 87-1 × 9-22 population, most progenies showed low free linalool, nerol and α-terpineol contents but wide transgressive segregation (Fig. 1). Phenotypic correlations between traits (free linalool, nerol and α-terpineol) averaged over 5 years varied from 0.52 to 0.79 and were always highly significantly positively correlated ($P > 0.001$). The correlation between free linalool and α-terpineol was highest (0.79) among the three traits (Fig. 2). In this study, the broad sense heritability (H^2) for all traits was rather large and varied between 0.94 and 0.99 (suppl. Tab. 2).

Genetic mapping: In the 87-1 map, 202 polymorphic simple sequence repeat (SSR) markers were assigned to 19 LGs with a total length of 1509.4 cM. The average length of a LG was 79.4 cM. The largest group was LG 14, with a genetic distance of 130.9 cM (Tab. 1, suppl.

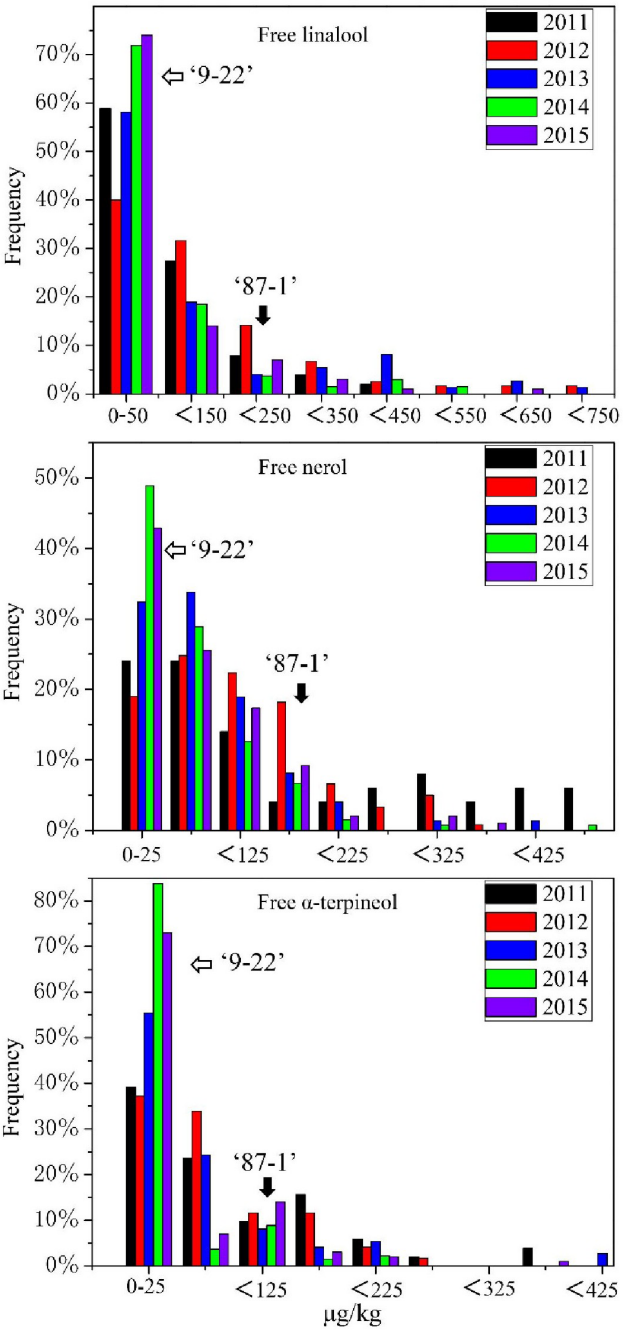

Fig. 1: Distribution of the free linalool, nerol and α-terpineol contents in the 87-1 × 9-22 population. Histograms show data variations from 2011 to 2015. Arrows indicate the mean parental values.

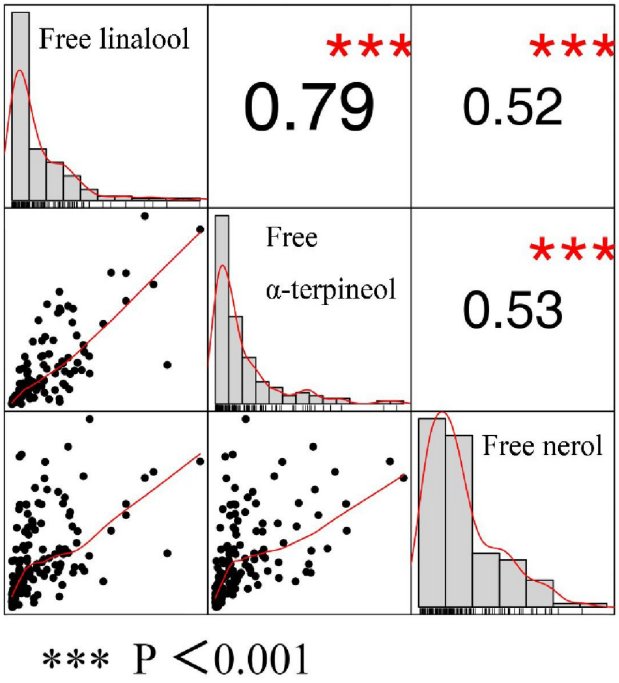

Fig. 2: Phenotypic correlations between the free linalool, nerol and α-terpineol averaged over 5 years.

Figure). Similarly, in the 9-22 map, 19 LGs covered a total genetic distance of 1554.1 cM with 184 polymorphic SSR markers. The average length of a LG was 81.8 cM, and the largest group was LG 14, with a genetic distance of 139.5 cM. In the consensus map, a total genetic distance of 1787.3 cM was covered by 19 LGs with 255 polymorphic SSR markers. The average length of a LG was 94.1 cM. The largest group was LG 14, with a genetic distance of 185.4 cM, which consisted of 24 SSR markers. The smallest LG was LG 13 (42.2 cM), consisting of 6 SSR markers (Tab. 1 and suppl. Figure). Compared with the reference map, almost all the SSR markers in the 19 LGs had the same collinearity except for some minor inversions. In particular, four markers (VLG10-V-1, VLG10-O-1, VVIP09 and VLG10-P-1) were expected to be located in LG 10 but mapped to LG 14 or LG 19 in the consensus map, and LG 8, 9, 14 and 18 were divided into two parts in the consensus map (suppl. Figure).

QTL analysis: Because the consensus map contained the polymorphic SSR markers derived from 87-1 and 9-22, we focused on the stable QTLs that were detected in the consensus map for at least two years in this study. Stable QTLs detected in four genomic regions on LGs 5, 10, 11, and 18 individually explained up to 42.5 % of the total phenotypic variance (Tab. 2, Fig. 3).

The results confirmed a major QTL for free linalool content previously found on LG 5 in 2011 and 2013 that explained 11.2-15.8 % of the total phenotypic variation. This locus also explained 10.2-19.3 % of the total variance in free α-terpineol content during 2011 and 2012 and 6.8-33.1 % of the total variance in free nerol content during 2011, 2012 and 2014 (Tab. 2). A stable QTL for the contents of free linalool and α-terpineol was detected on LG 10 during 2011 and 2014 that explained 12.4-20.5 % of the total variance in free linalool content and 12.8-29.9 % of the total variance

Table 1

Main characteristics of linkage groups in the maternal 87-1, paternal 9-22 and consensus maps

LGs	87-1		Consensus		9-22	
	No. of SSR makers	Length (cM)	No. of SSR makers	Length (cM)	No. of SSR makers	Length (cM)
1	9	56.7	11	57.3	11	57.3
2	10	70.2	11	72.6	8	59.9
3	10	73.8	10	73.8	9	83.7
4	11	98.1	15	98.6	12	63.6
5	15	126.5	16	117.1	9	91.1
6	7	65.8	8	86	8	86
7	12	94.4	13	103.3	7	76.9
8	8	87.9	13	106.3	11	109.7
9	7	71.3	12	103.5	9	69.3
10	7	54	13	76.3	10	110.4
11	7	58.6	12	106.7	8	96.2
12	5	59.2	6	68.3	6	68.3
13	3	24	6	42.2	6	42.2
14	20	130.9	24	185.4	16	139.5
15	8	79.6	11	101.3	9	98.6
16	11	58.3	11	58.3	9	36.5
17	15	91.4	19	85.9	10	78.6
18	21	128.2	24	152.1	9	90.9
19	16	80.5	20	92.3	17	95.4
Total	202	1509.4	255	1787.3	184	1554.1
Average	10.6	79.4	13.4	94.1	9.7	81.8

Table 2

Summary table of the QTLs for free linalool, nerol and α-terpineol in the consensus map

Trait	Year	QTL	LG	Threshold of LOD[#]	LOD score	LOD peak (cM)	CI extremes (cM)	Flanking markers	R^2
Free linalool	2011	*LIN_2011_1*	5	3.4	7.0	13.7	8.0-34.0	VLG5-G-1, VrZAG79	15.8
		LIN_2011_2	10	2.93	8.5	31.0	22.0-38.0	VLG10-A-1, UDV073	20.5
		LIN_2011_3	11	3.28	9.1	62.0	59.0-64.0	VLG11-F-1, Y-11-10	22.0
	2013	*LIN_2013_1*	5	3.42	6.4	0.0	0.0-19.0	VLG5-G-1, VRZAG47	11.2
		LIN_2013_2	11	3.14	5.9	55.0	43.0-58.0	Y-11-4A, VLG11-F-1	10.3
		LIN_2013_3	18	3.22	4.1	77.0	76.0-83.0	Y-18-28B, Y-18-29B	6.7
	2014	*LIN_2014_1*	10	2.7	3.8	4.0	0.0-14.0	VLG10-F-1, VLG10-A-1	12.4
Free α-terpineol	2011	*TER_2011_1*	5	3.36	7.9	27.8	10.0-32.0	VLG5-G-1, VrZAG79	19.3
		TER_2011_2	10	2.82	10.6	27.0	22.0-31.0	VLG10-A-1, UDV073	29.9
	2012	*TER_2012_1*	5	3.17	4.2	12.0	7.0-16.0	VLG5-G-1, VRZAG47	10.2
	2014	*TER_2014_1*	10	2.71	4.7	7.0	0.0-17.0	VLG10-F-1, VLG10-A-1	12.8
	2015	*TER_2015_1*	11	3.21	4.8	55.0	45.0-49.0	Y-11-4B, Y-11-10	10.8
Free nerol	2011	*NER_2011_1*	5	3.25	17.3	8.0	5.0-13.0	VLG5-G-1, VLG5-F-1	33.1
		NER_2011_2	18	3.33	15.5	76.0	76.0-76.0	Y-18-28B, Y-18-27A	26.8
	2012	*NER_2012_1*	5	3.17	8.5	14.0	12.0-17.0	VLG5-G-1, VRZAG47	19.3
	2013	*NER_2013_1*	18	3.03	13.4	77.0	77.0-78.3	Y-18-28B, Y-18-27A	42.5
	2014	*NER_2014_1*	5	3.17	3.4	14.0	0.0-19.0	VLG5-G-1, VRZAG47	6.8
		NER_2014_2	11	3.07	3.3	36.0	31.0-42.0	VLG11-A-1, Y-11-4B	6.7

[#] 5 % LOD significance threshold at the chromosome-wide level

in free α-terpineol content, respectively (Tab. 2). In addition, two new stable QTLs for free monoterpenes (linalool, nerol and α-terpineol) were identified on LG 11 and LG 18 (Tab. 2, Fig. 3). The QTL for free monoterpenes (linalool, nerol and α-terpineol) on LG 11 had a stable effect over 4 years and explained 6.7-22 % of the total variance during 2011, 2013, 2014, and 2015. The QTL peaks on LG 18 were always located between the new SSR markers Y-18-28B and Y-18-27A, and this LG explained up to 42.5 % of the total phenotypic variance in free nerol during 2013.

Identification of potential candidate genes: In total, several hundred candidate genes were identified in the four stable QTL confidence intervals (suppl. Tab. 3). However, 11 promising candidate genes in the focused QTL intervals were closely investigated because they could potentially regulate monoterpene accumulation or the progress of MEP or MVA biosynthesis. The annotations or putative gene functions of these genes include pentatricopeptide repeat (PPR)-containing proteins, seed maturation protein, RING finger protein, and AP2/ERF TFs (Tab. 3).

Discussion

Genetic maps: Based on the double pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994), we constructed a genetic map of grape (*Vitis vinifera* L.) using SSR markers. Except for the very few SSR markers mapped to different places, most of the SSR markers positioned on the parental and consensus maps were consistent with the reference map (suppl. Figure). The differences might be due to the significant differences in recombination rate between common loci simultaneously scored in different crosses (Doligez *et al.* 2006a). Another possible reason is that the occurrence of chromosomal rearrangements in the cross cannot be excluded, which also leads to marker order discrepancies with the previous map. Most of the VLG- and Y-series SSR markers designed in this study were precisely mapped to the 19 grape LGs, except for the three markers VLG10-V-1, VLG10-O-1 and VLG10-P-1, and all the SSR markers formed a high-quality consensus map. Therefore, these maps can serve as an effective tool for detecting QTL and marker-assisted selection (MAS) breeding. In addition, four LGs (8, 9, 14,18) were divided into two sublinkage groups in consensus map. A similar phenomenon has been observed in some published grape linkage maps (e.g. Lowe and Walker 2006, Moreira *et al.* 2011). This fact might hint at the possibility that the linkage strength between linked markers in subregions of LG 8, 9, 14, 18 were not tight enough. This deficiency may be further improved by modern sequencing technology, such as genotyping-by-sequencing (GBS) or single nucleotide polymorphisms (SNP) methods, which are able to provide a sufficient number of markers for mapping.

Heritability of monoterpene contents in grape: The typical Muscat aromas are primarily attributed to the abundant monoterpene components in grape berries (Gunata *et al.* 1985, Mateo and Jiménez 2000, Wu *et al.* 2013, D'Onofrio *et al.* 2016, Liu *et al.* 2016, Wu *et al.* 2016). In the present work, the contents of the three free monoterpenes linalool, nerol and α-terpineol showed continuous variation in progenies, which is typical of quantitative traits and consistent with previous studies (Battilana *et al.* 2009, Duchêne *et al.* 2009). Various studies have shown that the free monoterpenes in the progenies were skewed towards the low content area (Doligez *et al.*

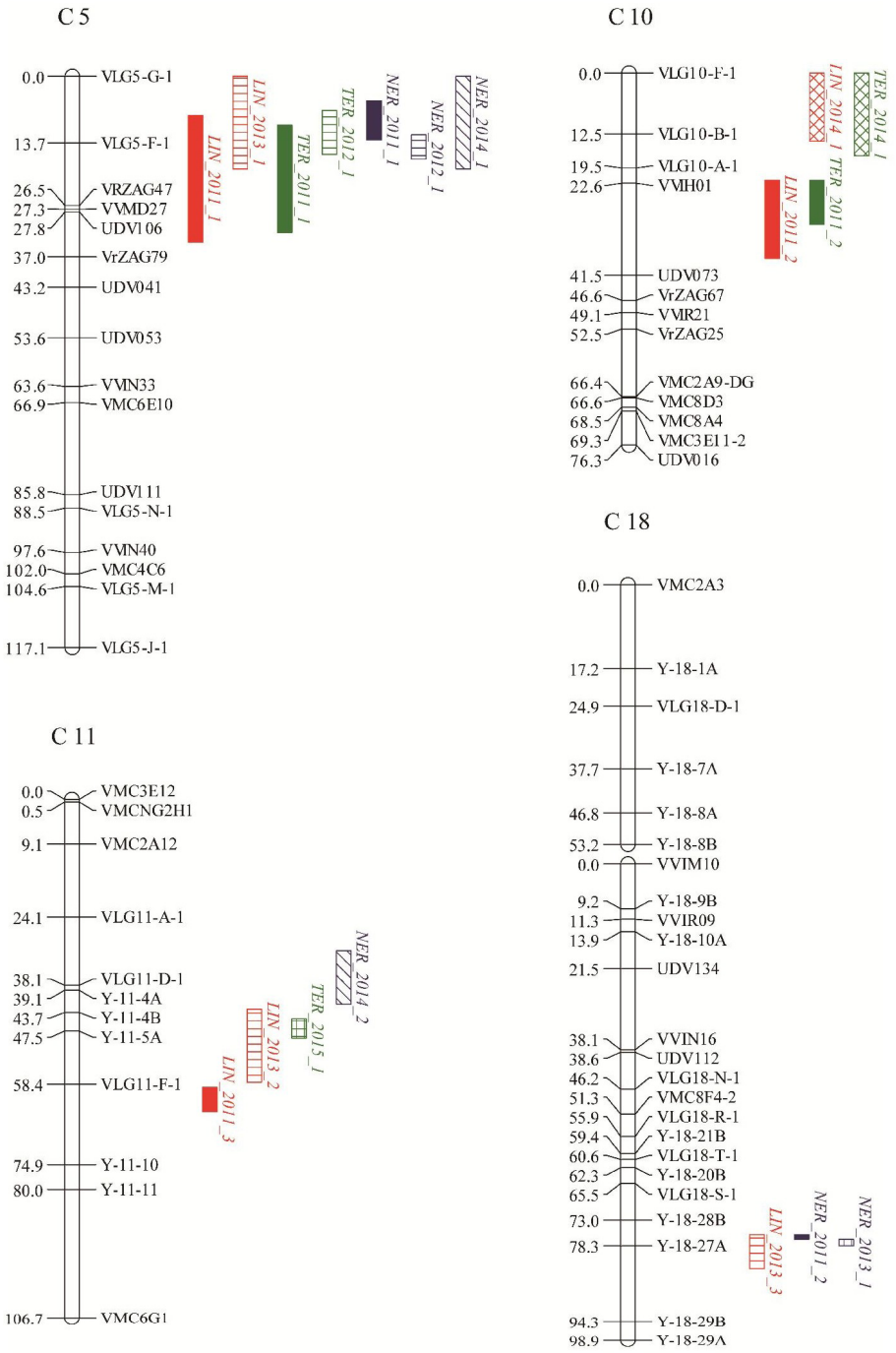

Fig. 3: Stable QTLs for free linalool, nerol and α-terpineol on the consensus map. Linkage group distances are in cM Kosambi. QTLs are shown on the right side and are named starting with the abbreviation of the trait (LIN for linalool, NER for nerol and TER for α-terpineol), followed by the year (2011 to 2015) and the order of the identified QTL per season.

2006b, BATTILANA *et al.* 2009, DUCHÊNE *et al.* 2009), and the skewness was also observed in total monoterpene content (LIU *et al.* 2 016). A similar trend in monoterpene content distribution was also observed in this study. Moreover, there was widely transgressive segregation in the progeny, which is consistent with a previous study (BATTILANA *et al.* 2009). AGRAMA *et al.* (1999), suggesting that increased genetic variance will increase the efficiency of QTL detection for the trait, and high H^2 may reflect the absence of interaction between genetic and environmental variance. In the present study, the monoterpene contents showed high H^2 (0.94). This is in agreement with the high values of H^2 (0.904) for monoterpene contents found by DUCHÊNE *et al.* (2009). These results indicate that variations in monoterpene contents are the main genotypic effect and that the QTL detection for monoterpene content is stable.

Stable QTLs for free linalool, nerol and α-terpineol: Previous studies have done QTL identification for different types of free monoterpenes (e.g. linalool, nerol, α-terpineol and geraniol) in different mapping populations (DOLIGEZ *et al.* 2006b, BATTILANA *et al.* 2009, DUCHÊNE *et al.* 2009). Possibly due to the difference of mapping population and growing environment, we found that linalool, nerol and α-terpineol were the main free monoterpenes

Table 3

Published functional candidate genes potentially involved in free monoterpene components in grape colocalized with QTLs identified

Grapevine 12X gene ID	LG	Position in 12X	Annotation or putative gene function	Reference
VIT_205s0020g03490	5	5264210-5264834	Ferredoxin, involved in IPP biosynthetic process and MEP pathway	Seemann *et al.* 2006; Vitulo *et al.* 2014
VIT_205s0020g03530	5	5290176-5291777	Seed maturation protein, involved in regulation of terpenoid biosynthetic process	Vitulo *et al.* 2014
VIT_205s0020g03570	5	5302968-5305900	Lovastatin insensitive 1 (LOI1), a pentatricopeptide repeat protein, involved in IPP biosynthetic process, MEP pathway and MVA pathway	Kobayashi *et al.* 2007; Vitulo *et al.* 2014
VIT_210s0116g01910	10	1165545-1176649	UPF0051 protein ABCI8, involved in IPP biosynthetic process and MEP pathway	Vitulo *et al.* 2014
VIT_210s0003g00110	10	1407224-1411356	Zinc finger CCCH domain-containing protein	Costantini *et al.* 2017
VIT_210s0003g00140	10	1442862-1444120	Ethylene response factor 3, AP2/ERF domain	Cramer e*t al.* 2014; Wen *et al.* 2015; Costantini *et al.* 2017
VIT_211s0016g04250	11	3539829-3550442	Protein mrp homolog, involved in IPP biosynthetic process and MEP pathway	Vitulo *et al.* 2014
VIT_211s0016g04450	11	3757752-3762574	E3 ubiquitin-protein ligase, zinc finger (C3HC4-type RING finger)	Costantini *et al.* 2017; Spyropoulou *et al.* 2014
VIT_211s0016g04730	11	4024220-4034033	E3 ubiquitin-protein ligase, zinc finger (C3HC4-type RING finger)	Costantini *et al*. 2017; Spyropoulou *et al.* 2014
VIT_218s0041g00290	18	24707099-24716985	C3HC4-type RING finger protein	Costantini *et al.* 2017; Spyropoulou *et al.* 2014
VIT_218s0041g01110	18	25669617-25672961	Pentatricopeptide repeat-containing protein	Kobayashi *et al.* 2007

in 87-1 × 9-22 population, so we did not quantify geraniol, which is also one of the most important free monoterpenes involved in Muscat flavor. In the present work, we identified four stable free monoterpene (linalool, nerol and α-terpineol) content-related QTLs on the consensus map. Notably, all four stable QTLs have an effect on two or three monoterpene compounds (Fig. 3). Previous studies have reported that one QTL is related to one or more traits (Doligez *et al.* 2006b, Mejía *et al.* 2007, Battilana *et al.* 2009, Doligez *et al.* 2013). The results in this study are consistent with the genetic variability of Muscat aroma possibly being due to a few genes with pleiotropic effects (Doligez *et al.* 2006b, Battilana *et al.* 2009). These genetic correlations are also supported by the phenotypic correlations, which are highly significantly positive (0.52-0.79) among free linalool, nerol and α-terpineol (Fig. 2).

In grape, a major QTL for monoterpene content was detected on LG 5 in several previous studies. Doligez *et al.* (2006b) detected a QTL for linalool, nerol and geraniol content on LG 5 between the markers VrZAG79 and VVC6. Battilana *et al.* (2009) found that the QTL for monoterpene (linalool, nerol and geraniol) contents on LG 5 was significantly associated with the marker DXS in V. *riparia* and VrZAG47 in 'Moscato Bianco' (*V. vinifera*). In addition, a major QTL for monoterpene (linalool, geraniol, nerol, citronellol and α-terpineol) content reported by Duchêne *et al.* (2009) on LG 5 collocated with a *DXS* gene. Yang *et al.* (2017) detected a QTL for linalool, nerol and α-terpineol content near the marker VLG5-F-1 by association study. The grape genomic sequence of *V. vinifera* (Jaillon *et al.* 2007) made it possible to identify positional candidate genes underlying QTL confidence intervals. We searched the ESTs of SSR markers from the grape genome. The physical interval between VVC6 and VrZAG79 on chromosome 5 as described by Doligez *et al*. (2006b) is 441,633 - 5,692,717. The relevant consequence is that the *DXS* gene (3,851,155 - 3,856,278) is within the QTL confidence interval, and in accordance with that the *DXS* gene plays an important role in monoterpene accumulation in grape (Battilana *et al.* 2009, Duchêne *et al.* 2009, Emanuelli *et al.* 2010, Battilana *et al.* 2011, Dalla Costa *et al.* 2018). In this study, the QTL for free linalool on LG 5 was located between markers VLG5-G-1 (5,464,833 - 5,464,959) and VrZAG79 (5,692,460 - 5,692,717), and the confidence interval overlapped with the QTL for free nerol and α-terpineol on LG 5. Although the interval between VLG5-G-1 and VrZAG79 is 1.6 - 1.9 Mb distant from the *DXS* gene on chromosome 5, four markers between the interval of VLG5-G-1 and VrZAG79 (including UDV106 (4,373,195-4,373,390), VLG5-F-1 (4,423,604 - 4,423,792), VRZAG47 (4,472,037 - 4,472,195) and VVM27 (4,472,022 - 4,472,201) are only 0.5 - 0.6 Mb distant from the *DXS* gene, and there was no marker mapped to the end of chromosome 5 closer than markers VLG5-G-1 or UDV106. This might be indi-

cating that *DXS* was also the key gene for free monoterpene accumulation in 87-1 × 9-22 population, which is consistent with previous studies (DOLIGEZ *et al.* 2006b, BATTILANA *et al.* 2009, DUCHÊNE *et al.* 2009).

A previous study has shown that a positive correlation between *VvDXS1* expression profile and monoterpenoid accumulation in 'Moscato Bianco', and a G/T SNP at gene position 1822 was found to be significantly associated to aroma based on association analysis in two germplasm collections (EMANUELLI *et al.* 2010). BATTILANA *et al.* (2011) found that the *VvDXS* N284 encodes an enzyme with greater catalytic efficiency than that encoded by *VvDXS* K284. The increased accumulation of monoterpenes in tobacco leaves and in microvine berries was transformed with distinct *VvDXS1* alleles (DALLA COSTA *et al.* 2018). Moreover, EMANUELLI *et al.* (2014) developed user-friendly functional molecular markers for *VvDXS* for accurate selection of muscat flavor at early stages of grape breeding programs (EMANUELLI *et al.* 2014). Therefore, it will be interesting to check the *DXS* genotype of the two parents (in the position of the SNP1822) and to map the *DXS* gene in the 87-1 × 9-22 consensus map based on its segregation in the progeny.

For free linalool content, we detected three stable QTLs on LGs 5, 10 and 11. On LG 10, BATTILANA *et al.* (2009) has detected a QTL for linalool near markers VrZAG67 and cnd41, and DUCHÊNE *et al.* (2009) has detected a QTL for linalool near marker VrZAG64. In this study, the QTL for free linalool on LG 10 was located between markers VLG10-F-1 and UDV037. Due to the sequences of VLG10-F-1 and VLG10-A-1 having been re-located to "chromosome unknown" in current genome, we checked the physical position of markers VLG10-B-1 (1,339,894 - 1,340,142), VVIH01 (1,181,256 - 1,181,498) and UDV073 (1,292,945 - 1,293,119), and found that the physical position of marker VLG10-B-1 on chromosome 10 was a 108 Kb distant from the marker VrZAG67 (1,447,733 - 1,447,886) and located at the same position with marker VrZAG64 (1,339,932 - 1,340,094). These results suggest that the QTL on LG 10 is important for free monoterpene accumulation, and it is necessary to analyze the function of genes in the interval of markers VVIH01 to VrZAG67.

WAGNER (1967) suggested that the terpinol content might be determined by up to six loci, and a large number of *VvTPS* genes have been reported to be associated with terpinol content (MARTIN *et al.* 2010). In addition, several previous studies found a QTL for linalool content on LG 2 (DOLIGEZ *et al.* 2006a, BATTILANA *et al.* 2009, DUCHÊNE *et al.* 2009). In this study, we did not detect a stable QTL for linalool content on LG 2, but we found two new QTLs for free linalool content on LGs 11 (VLG11-A-1 to Y-11-10 with interval 25,492 - 9,669,078) and 18 (Y-18-28B to Y-18-29B with interval 24,403,001 - 28,125,303) that overlap the QTL for free nerol or α-terpineol content. These results suggest that the difference in Muscat aroma components might be due to a few genes with pleiotropic effects, and it is also possible that there are linked genes influencing the content of different aromatic compounds (DOLIGEZ *et al.* 2006a, BATTILANA *et al.* 2009). As the physical interval between markers VLG11-A-1 and Y-11-10 is too wide and contains too many genes, we focused on QTL peak area between markers VLG11-D-1 and VLG11-F-1 (319794 - 4856106) when doing candidate gene identification for LG 11. The new stable QTLs on LGs 11 and 18 might interact with the QTLs on LGs 5 and 10 for the free linalool, nerol and α-terpineol contents in grape. In particular, the new SSR markers in the stable QTL confidence intervals provide new information for Muscat aroma grape breeding.

Candidate genes: Although the MEP pathway has been suggested as the main route for monoterpene biosynthesis in grape (LUAN and WÜST 2002), previous studies have reported that there is exciting metabolic flow between MVA and MEP metabolism in plants (NAGATA *et al.* 2002, HEMMERLIN *et al.* 2003). Within the positional information provided through QTLs, evidence obtained from the GO term in CRIBI database or previous literature suggested that the candidate genes were potentially associated with monoterpene contents (Tab. 3).

PPR-containing proteins are thought to regulate the expression of genes encoded in organelle genomes by post-transcriptional regulation in mitochondria or plastids (SMALL and PEETERS 2000). The Lovastatin insensitive (*LOI1*) gene encodes a novel PPR protein that is predicted to localize in mitochondria and has the ability to bind single-stranded nucleic acids. The posttranscriptional regulation of mitochondrial RNA may be involved in isoprenoid biosynthesis in both the MVA and MEP pathways (KOBAYASHI *et al.* 2007). In this study, we found two *LOI1* genes (VIT_205s0020g03570 and VIT_218s0041g01110) in the stable QTL confidence intervals on LGs 5 and 18. This observation suggests that the *LOI1* gene is involved in monoterpene accumulation.

The TF of AP2/ERF (APETALA 2/ethylene-responsive element binding factor) has been reported to be involved in the transcriptional regulation of terpene syntheses in plants (YU *et al.* 2012). According to transcriptomic analysis, the expression of AP2/ERF has been shown to be involved in terpene accumulation in grape (CRAMER *et al.* 2014, WEN *et al.* 2015). Furthermore, COSTANTINI *et al.* 2017 suggested that the RING finger (C3HC4-type and CCCH-type) protein is involved in monoterpene accumulation during grape berry ripening. Considering these data, we propose that the genes annotated as AP2/ERF (VIT_210s0003g00140), and RING finger protein (VIT_210s0003g00110, VIT_211s0016g04450, VIT_211s0016g04730 and VIT_218s0041g00290) might be involved in monoterpene accumulation.

Conclusions

In summary, a QTL mapping approach was used to identify QTLs for free monoterpene (linalool, nerol and α-terpineol) contents in grape during five consecutive seasons. We constructed a high-quality genetic map of grape (*Vitis vinifera* L.) that covered 19 LGs. We confirmed two important free monoterpene (linalool, nerol and α-terpineol) content-related QTLs on LGs 5 and 10 and identified two new stable QTLs on LGs 11 and 18 for the contents of free linalool, nerol and α-terpineol. Eleven potential candidate genes within the confidence interval of these QTLs were functionally relevant and could be used for improving Muscat grape cultivar breeding via molecular design in the future.

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