Abiotic stresses differentially affect the expression of *O-methyltransferase* genes related to methoxypyrazine biosynthesis in seeded and parthenocarpic fruits of *Vitis vinifera* (L.).

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Running title: Abiotic stresses affect methoxypyrazine biosynthesis.

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Abstract

MPs (3-alkyl-2-methoxypyrazines) are grape-derived aroma compounds that are associated with detrimental herbaceous flavours in some wines. It is well known that several viticultural and environmental parameters can modulate MP concentrations in grapes, although comprehensive molecular studies have not been conducted in this field. Although the biosynthesis pathway of MPs has not been fully elucidated, four *Vitis vinifera O-methyltransferase* genes (*VvOMT1-4*) have been demonstrated to be involved in MP biosynthesis. We assessed whether sunlight restriction and abiotic stress induction have an impact on MP levels in grapes and wines from seeded and parthenocarpic fruits. Our results show that *VvOMT1* and *VvOMT3* expression correlates with 3-isobutyl-2-methoxypyrazine (IBMP) levels in seeded fruits during both abiotic stresses, whereas no correlation was found in parthenocarpic fruits. These results are discussed in the context of how different viticultural practices can modulate *VvOMT* gene expression, which has a direct impact on IBMP levels in wines.

Keywords: *Vitis vinifera*, abiotic stress, parthenocapy, methoxypyrazine, wine flavor, methyltransferase, *VvOMT.*

1. Introduction

Vitis vinifera (L.) cultivar (cv.) Carmenere was believed to be extinct after a grape phylloxera (*Dactylosphaera vitifoliae*) attack in European vineyards in the 19th century but was rediscovered in Chile during the 1990s. This cultivar was widely cultivated in the Médoc region due to its exceptional wine quality, including a particular bouquet (Mondaca & Hinrichsen, 2007) and has the opportunity to be the emblematic grapevine cultivar of the Chilean wine industry. However, Carmenere wines have generated significant criticism associated with problems related to a strong vegetative character associated with high methoxypyrazine concentrations (Belancic & Agosin, 2007). Carmenere is a cultivar of medium to high vigour, with an abundant production of lateral shoots, and exhibits a low basal bud fertility that should be cane pruned. This cultivar has medium productivity and often shows parthenocarpic and fruit abscission problems (Moreno & Vallarino, 2011). It generally requires extra leaf removal in summer (Belancic & Agosin, 2007) and deficit irrigation management to decrease herbal aromas in the wine.

Some grapevine cultivars, such as Merlot, Malbec and Carmenere, exhibit two main reproductive problems: i) the loss of berries by abscission during the earliest stages in the season, which mainly impacts production, and ii) the presence of small parthenocarpic berries mixed in with normal-size seeded berries in the same clusters (commonly called “millerandage”), which impacts grape cluster quality. This outcome could be the result of the presence of parthenocarpic fruits caused by incomplete fertilisation. Moreover, the

73 percentage of underdeveloped berries can affect wine quality, but the causes
74 for this are poorly understood.
75
76 MPs (3-alkyl-2-methoxypyrazines) are striking flavourants present at very low
77 concentrations in foods and are associated with herbaceous aromas (Murray
78 & Whitfield, 1975) among some *V. vinifera* red cultivars, such as Cabernet
79 Sauvignon, Merlot (Hashizume & Umeda, 1996), Cabernet Franc (Roujou de
80 Boubee, Van Leeuwen & Dubourdieu, 2000), and Carmenere (Belancic &
81 Agosin, 2007) and white cultivars such as Sauvignon blanc, Chardonnay,
82 Semillon and Riesling (Allen, Lacey, Harris & Brown, 1991; Hashizume &
83 Samuta, 1999). In red wine, the sensory detection threshold of MPs is as low
84 as 15 pg/mL (Roujou de Boubée et al., 2000). In grapes and wine, the MPs
85 most often studied are 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-
86 methoxypyrazine (IPMP), and 3-*sec*-butyl-2-methoxypyrazine (sBMP).
87 Quantitatively, IBMP is predominant and is normally present at
88 concentrations an order of magnitude higher than those of IPMP and sBMP
89 (Alberts, Stander, Paul & Villiers, 2009). IBMP masks fruity aromas, and a
90 relationship between “green” flavours, wine quality and IBMP has been
91 reported (Belancic & Agosin, 2007; Dunlevy et al., 2010; Roujou de Boubee
92 et al., 2000; Vallarino, Lopez-Cortes, Dunlevy, Boss, Gonzalez-Nilo &
93 Moreno, 2011). Different grapevine cultivars contain diverse quantities of
94 MPs, suggesting that these compounds can contribute to their varietal
95 distinction (Belancic & Agosin, 2007; Koch, Doyle, Matthews, Williams &
96 Ebeler, 2010; Ryona, Leclerc & Sacks, 2010). This observation also suggests
97 that MPs or their precursors originate in the berry, which depends on grape

genotypes, implying that MPs do not translocate to the fruit from the shoots (Koch et al., 2010).

It is well known that several viticultural and environmental parameters can modulate IBMP concentrations in grapes and wines (Ryona, Pan, Intrigliolo, Lakso & Sacks, 2008; Sala, Busto, Guasch & Zamora, 2005; Sala, Busto, Guasch & Zamora, 2004; Scheiner et al., 2010). In this respect, it is known that conditions that stimulate vine vigour, such as high water availability and low bud numbers, are associated with high methoxypyrazine concentrations (Chapman, Thorngate, Matthews, Guinard & Ebeler, 2004; Sala et al., 2005). Additionally, light and temperature play a role in the accumulation of IBMP. Higher temperatures during ripening periods are thought to enhance methoxypyrazine degradation, leading to lower concentrations at harvest (Lacey, Allen, Harris & Brown, 1991). Several groups have reported that exposed clusters accumulate less IBMP than shaded clusters, and proportional differences persist until harvest (Ryona et al., 2008). Furthermore, complete shading of clusters reportedly increases the concentration of IBMP by more than 100% (Koch, Ebeler, Williams & Matthews, 2012). Consequently, management practices that improve cluster exposure, such as leaf removal, can reduce IBMP when imposed at the pre-veraison stage (Scheiner et al., 2010).

There is very little information about MP biosynthesis in plants. It has been suggested that the pathway for MPs begins with the condensation of NH_3 with an amino acid, such as valine or leucine, and glyoxal to form 3-alkyl-2-(1*H*)-pyrazin-2-one and 3-alkyl-2-hydroxypyrazine (HP) (Cheng, Reineccius,

123 Bjorklund & Leete, 1991; Gallois, Kergomard & Adda, 1988; Jones, 1949;
124 Murray & Whitfield, 1975). Subsequently, HPs are *O*-methylated to form MPs
125 (Murray & Whitfield, 1975; Rizzi, 1990).

126 Four *V. vinifera* O-methyltransferase (OMT) genes, *VvOMT1*, *VvOMT2,*
127 *VVOMT3,* and *VvOMT4*, have been isolated and were shown to be able to
128 methylate HPs to form MPs (Dunlevy et al., 2010; Dunlevy, Dennis, Soole,
129 Perkins, Davies & Boss 2013; Guillaumie et al., 2013; Vallarino et al., 2011;).
130 The timing of *VvOMT1* expression in the skin and flesh of developing
131 Cabernet Sauvignon grape berries is associated with the period of MP
132 accumulation in these tissues (Dunlevy et al., 2010). Additionally, a more
133 recent study showed that *VvOMT3* appears to be a key gene in the
134 biosynthesis of these compounds in grapevines (Dunlevy et al., 2013;
135 Guillaumie et al., 2013). In this study, we describe how different viticultural
136 practices can affect the expression of these *O-methyltransferase* genes and
137 the production of methoxypyrazines in cv. Carmenere, which is reported to
138 produce a “greener” red wine with the highest level of MPs reported thus far
139 in the literature (Belancic & Agosin, 2007).
140
141
142 **2. Materials and methods**
143
144 **2.1. Experimental design**
145 A commercial vineyard located in Colchagua Valley, Chile (34°30' S,
146 longitude 70°53' W) was used in this study. The cv. Cabernet Sauvignon was
147 used as a control to contrast with previous gene expression pattern reports

148 (Dunlevy et al., 2010). The experiments were performed on *V. vinifera* cv.
149 Carmenere during the 2008/09 and 2009/10 seasons. Vine spacing was 1.5
150 m between vines and 2.2 m between rows, and the vines were cane pruned
151 for a vertical shoot-positioning trellis system. The experimental design was a
152 randomised complete block with 4 replications. The experimental plot at each
153 site consisted of 4 rows, and each experimental unit consisted of 50 vines
154 per row.
155 Treatments consisted of (1) a control (normal growth conditions; Figure S1),
156 (2) clusters with sunlight restriction (Figure S2) or (3) stress induced by a
157 combination of lateral shoot removal and water deficit (Figure S3). For
158 treatment 1 (control), standard practices for irrigation, fertilisation and pest
159 control were utilised. For treatment 2, clusters were covered at stage 27
160 (setting) in the modified Eichhorn and Lorenz system (Coombe, 1995) with a
161 filter paper bag that filters out 95% of photosynthetically active radiation
162 (PAR). The bags were left open at the bottom to prevent temperature
163 increases outside the range of the control treatment (Sala et al., 2004). For
164 treatment 3, we combined (i) lateral shoot thinning and (ii) cut-off of irrigation
165 to the vines from setting until harvest.
166
167 **2.2. Grape sampling**
168 Cluster samples were selected at random from throughout a vineyard block
169 according to their developmental stage and phenotypic condition (seeded
170 and parthenocarpic fruits), frozen in liquid nitrogen and stored at -80ºC until
171 use. Samples were taken at weekly intervals from setting until mature stages.
172 The phenologically analysed stages and their corresponding numbers

according to the modified E-L system (Coombe, 1995) were as follows: setting, 27; pre-veraison 1, 31; pre-veraison 2, 33; veraison, 35; post-veraison, 36-37; and mature, 38.

2.3. Wines

Clusters from different treatments were used to make microvinifications. Wines were made from each treatment group according to standard winemaking practices (Centro Tecnológico de la Vid y el Vino, Universidad de Talca) with 4 biological and 3 technical replicates. Each replicate consisted of 50 ± 1 kg of hand-harvested fruit collected from the field treatments. Grapes were hand-destemmed, and the berries were inoculated with yeast (*Saccharomyces bayanus*, EC-1118, Lallemand, Montreal, Canada) at a rate of 20 g hL^{-1} in stainless steel fermenters. The fermentation temperature was maintained between 22ºC and 23ºC to a density of 990 g/L, and malolactic fermentation was not allowed. After alcoholic fermentation, the wines were pressed, and $K_2S_2O_5$ was added to 30 ppm of SO_2 in the wine.

2.4. Methoxypyrazine analysis

MPs were quantified in wine samples using a stable isotope dilution assay as described in Ryona, Pan & Sacks (2009).

For grape samples, the extraction was performed as described by Dunlevy et al. (2010). Multiple frozen berries were ground and homogenised in liquid nitrogen, and 1 g was added to 10 mL of citrate/phosphate buffer (0.1 M $NaHPO_4$, 50 mM citric acid, pH 5). A volume of 80 µL of 5 µg L^{-1} internal standard d_3-IBMP was added prior to solid phase microextraction (SPME).

198 IBMP and IPMP were analysed by SPME-GC-MS and quantified using
199 external standards.
200
201 **2.5. Analysis of *VvOMT1*, *VvOMT2, VvOMT3,* and *VvOMT4* mRNA**
202 **expression by qRT-PCR**
203 Total RNA was extracted from berry samples according to Reid, Olsson,
204 Schosser, Peng & Lund (2006) with minor modifications. The integrity of the
205 extracted RNA was confirmed by electrophoresis under denaturing conditions
206 after treating the RNA with RNase-free DNase I (Ambion). First strand cDNA
207 synthesis of total RNA (1 µg per sample) was performed using oligo (dT)
208 primer according to the manufacturer's instructions (Revertaid First Strand
209 cDNA Synthesis K1622 Kit, Fermentas).
210 Expression of the *VvOMT1, VvOMT2, VvOMT3, and VvOMT4* genes was
211 analysed by quantitative polymerase chain reaction (qPCR) using the Brilliant
212 SYBR Green Master Mix (Stratagene, USA) as described previously (Gainza-
213 Cortes et al, 2012). Relative quantification of the target was performed using
214 the comparative cycle threshold method. The following primers were used:
215 for analysis of *VvOMT1* transcript levels (GenBank accession no.
216 GQ357167), forward, 5′-GGTTGGTGTTCGATTTACTGATGA-3´, reverse 5´-
217 GGTAAAGTGGAGATCGAAATCTCCAG-3´; for *VvOMT2* (GenBank
218 accession no. GQ357168), forward 5´-GTACGGCTAAGATCTCCATCAA-3´,
219 reverse 5´- GATACGCCTTCACCACCTCC-3´; for *VvOMT3* (GenBank
220 accession no. KC243500), forward 5´-ATGATGGCTCATACTACTAC-3´,
221 reverse 5´-CCTAATTTCGTGTCCTAATG-3´ (Dunlevy et al., 2013); and for
222 *VvOMT4* (GenBank accession no. KC243503), forward, 5´-

223 GATGGCACATACTACTACA-3´, reverse 5´-GGGATTTACCTTGCGATA-3´
224 (Dunlevy et al., 2013).
225 To normalise gene expression for differences in the efficiency of cDNA
226 synthesis, transcript levels of the constitutively expressed *VvGAPDH* gene
227 (GenBank accession no. CN938023) and ubiquitin gene from *V. vinifera*
228 (GenBank accession no. TC32075) were measured using the following
229 primers: for *VvGAPDH*, forward, 5′-TTCCGTGTTCCTACTGTTG-3′, reverse,
230 5′-CCTCTGACTCCTCCTTGAT-3′; and for *VvUBQ,* forward, 5′-GTGGTATTA
231 TTGAGCCATCCTT-3′, reverse 5′-AACCTCCAATCCAGTCATCTAC-3′.

232

3. Results

3.1. Influence of different stress treatments on methoxypyrazine content in wines

MP concentrations in Carmenere wines resulting from the treatments described in the experimental design section were determined for two consecutive years (2009 and 2010). Compared with the control (C), an increase in the levels of IBMP was observed in the T-2 (sunlight restriction treatment) and T-3 (stress by lateral shoot removal plus water deficit) groups (Figure 1A) when wines were made from clusters containing both seeded and parthenocarpic berries.

Different fruit sizes were observed between parthenocarpic and seeded berries from the same cluster in all treatments **(**Figure S4**)**. Therefore, different ratios between skin and fresh weight were produced by parthenocarpic and seeded berries. In grapes, MPs are primarily located in berry skins (Dunlevy et al., 2010; Roujou de Boubee et al., 2000). Therefore, we sought to determine whether parthenocarpic and seeded berries differentially contribute to total MP content in wines. Consequently, both IBMP and IPMP contents were measured in Carmenere wines made with parthenocarpic or seeded fruits from the control, T-2 or T-3 groups.

Interestingly, no differences in IBMP content were observed in the T-2 and T-3 groups compared with the control group in wines made with parthenocarpic grape berries (Figure 1B). However, IBMP levels were higher in both treatment groups (T-2 and T-3) than in the control group when only seeded

257 grape berries were used for winemaking (Figure 1B) as was observed in wine
258 from a mixture of parthenocarpic and seeded berries (Figure 1A). IPMP
259 levels were below the limit of detection in all samples (data not shown).
260

261 **3.2. Influence of different stress treatments on methoxypyrazine**
262 **content in grapes**

263 Given these changes in IBMP content in wine and that a previous study
264 reported that the concentration of methoxypyrazines in wines correlates with
265 concentrations in the grapes used for winemaking (Ryona et al., 2009), IBMP
266 and IPMP levels during development and ripening in both parthenocarpic and
267 seeded berries in all treatment groups were evaluated (Figure 2). Similar to
268 previous studies, IBMP concentrations increased early in the season,
269 reaching a maximum at the pre-veraison 2 stage, followed by a steady
270 decrease until mature seeded berries (Figure 2 A, B, C). Interestingly, in
271 seeded berries, the IBMP concentration was consistently higher in the T-2
272 and T-3 treatment groups, which had a peak of 546 ± 78 ng kg^{-1} and 480 ±80
273 ng kg^{-1}, respectively, at pre-veraison 2 compared with a maximum of 323 ±
274 48 ng kg^{-1} measured in control seeded berries at the same stage of
275 development (Figure 2 A, B, C). Surprisingly, the highest level of IBMP was
276 found in parthenocarpic berries at the veraison stage, which decreased
277 dramatically in the T-2 and T-3 groups (Figure 2 B, C), whereas in the control
278 group, IBMP levels decreased at the post-veraison stage (Figure 2 A).

279 In the control group, a 6-fold higher maximum IBMP peak in seeded berries
280 compared with parthenocarpic berries was observed (Figure 2A) despite the

281 smaller size of parthenocarpic berries (Figure S4). The same result was
282 obtained when comparing seeded and parthenocarpic berries in the T-2 and
283 T-3 treatment groups (Figure 2 B, C). This observation was surprising
284 because MP accumulation is mainly associated with skins (Dunlevy et al.,
285 2010; Roujou de Boubee et al., 2000).

286 In the control seeded berries, the decrease in IBMP began between the pre-
287 veraison 2 and veraison stages (Figure 2A) as is generally described in the
288 literature (Dunlevy et al., 2010; Koch et al., 2012; Roujou de Boubee et al.,
289 2000; Ryona et al. 2008). However, in seeded berries from the T-2 and T-3
290 treatment groups, no significant decrease in IBMP was observed until several
291 days after veraison (Figure 2 B, C). IBMP levels were 2-fold higher at the
292 mature stage in both the T-2 and T-3 treatment groups than in the control
293 group (67 ± 10, 59 ± 9 and 33 ± 8 ng kg^{-1} FW at the mature stage in the T-2,
294 T-3 and control groups, respectively). In agreement with a previous report
295 (Ryona et al., 2008), a correlation was observed between IBMP levels in the
296 pre-veraison and mature stages. Additionally, IPMP levels remained
297 relatively low in all samples (between 9 ± 4 ng kg^{-1} FW and 67 ± 15 ng kg^{-1}
298 FW) compared with IBMP concentrations as previously described (Allen &
299 Lacey, 1993; Dunlevy et al., 2010; Ryona et al., 2010) (Figure 2).

300

301 **3.3. Analysis of *VvOMT* gene expression during fruit development and**
302 **ripening in Carmenere and Cabernet Sauvignon varieties**

303 The *V. vinifera* genome is known to contain four genes encoding functional
304 *S*-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferase (OMT)

isoforms, *VvOMT1, VvOMT2, VvOMT3,* and *VvOMT4,* which have been cloned and shown to be involved in methoxypyrazine biosynthesis (Dunlevy et al., 2010; Dunlevy et al., 2013; Guillaumie et al., 2013; Vallarino et al., 2011). The relative expression of *VvOMT1*, *VvOMT2, VvOMT3,* and *VvOMT4* in cvs. Carmenere and Cabernet Sauvignon grown in the Chilean mesoclimate was analysed at six developmental and ripening stages (setting, pre-veraison 1, pre-veraison 2, veraison, post-veraison, and mature) to compare with previously published data from cvs. Carmenere and Cabernet Sauvignon grown in France and Australia, respectively (Dunlevy et al., 2010; Dunlevy et al., 2013; Guillaumie et al., 2013). Importantly, similar expression patterns were found between Cabernet Sauvignon and Carmenere cultivars for all genes. qRT-PCR evaluation of the *VvOMT1* and *VvOMT2* genes demonstrated an expression peak at the pre-veraison 1 stage, which decreased during fruit ripening (Figure 3 A, B). Similarly, the expression of *VvOMT3* and *VvOMT4* were compared throughout the development and ripening of Cabernet Sauvignon and Carmenere berries. In Carmenere, *VvOMT3* and *VvOMT4* expression increased markedly after setting, peaking at pre-veraison 2, and then decreased rapidly to low, nearly undetectable levels at mature stages (Figure 3 C, D). In Cabernet Sauvignon, a similar expression profile for *VvOMT3* and *VvOMT4* was obtained (Figure 3 C, D). Here, *VvOMT3* and *VvOMT4* expression increased from the setting stage, reached a peak in expression at the pre-veraison 1 stage and was low from pre-veraison 2 to mature stages (Figure 3 C, D).

3.4. Different stress treatments produced differences in *VvOMT* expression during fruit development and ripening

Given the changes in IBMP and IPMP content among the different fruit stages in the different treatment groups, which may indicate an alteration in the rate of synthesis or degradation of methoxypyrazines, the levels of the four known methoxypyrazine-related genes, *VvOMT1*, *VvOMT2, VvOMT3,* and *VvOMT4,* were measured by qRT-PCR during fruit development and ripening in the T-2 and T-3 treatment groups in comparison with the control group.

During the development and ripening stages in the control group, *VvOMT1* showed the highest expression at the pre-veraison 1 stage in seeded berries, whereas in parthenocarpic fruit, the peak was three weeks later at the veraison stage (Figure 4 A). *VvOMT1* expression gradually decreased thereafter (Figure 4 A). Interestingly, when both sunlight restriction (T-2) and stress by lateral shoot removal plus water deficit (T-3) were applied, a similar *VvOMT1* expression pattern was observed as with both treatments applied separately to parthenocarpic and seeded berries (Figure 5 A). With both treatments, *VvOMT1* expression increased markedly at the pre-veraison 1 stage as in the control group, but expression was not reduced until the post-veraison stage in seeded fruits (Figure 5 A). Similarly, in parthenocarpic berries, *VvOMT1* exhibited the same behaviour as in seeded fruits in the T-3 treatment group, whereas in the T-2 treatment group, *VvOMT1* was reduced at an earlier stage (veraison stage; Figure 5 A).

In the control group, the expression of *VvOMT2* peaked at the pre-veraison 1 stage and then rapidly decreased in both parthenocarpic and seeded berries (Figure 4 B). Moreover, across development and ripening stages, this pattern was repeated in the sunlight-restricted treatment group (T-2; Figure 5B). However, in the lateral shoot removal plus water deficit group (T-3), *VvOMT2* expression reached a maximum 2-3 weeks after pre-veraison 1 at the pre-veraison 2 stage after which transcript levels steadily declined (Figure 5 B).

In seeded fruits, the expression of *VvOMT3* in the sunlight-restricted (T-2) and lateral shoot removal plus water deficit groups (T-3) were similar (Figure 5 C). Interestingly, in seeded fruit, *VvOMT3* expression increased from the pre-veraison 1 stage, reached a peak in expression at pre-veraison 2 in the control group and was low from the veraison to mature stages (Figure 4 C). In contrast, *VvOMT3* expression was slightly altered after reaching a peak, remaining high until the veraison stage in the T-2 and T-3 treatment groups (Figure 5 C). In parthenocarpic fruits, *VvOMT3* increased after the pre-veraison 1 stage, reaching a peak in the pre-veraison 2 and veraison stages and then decreased in the control (Figure 4 C) and T-2 and T-3 (Figure 5 C) groups.

The maximum expression of *VvOMT4* in seeded fruits occurred at the pre-veraison 2 stage in the control (Figure 4 D) and in both the T-2 and T-3 groups (Figure 5 D). In parthenocarpic fruits, *VvOMT4* reached a peak at the pre-veraison 2 stage in the control group (Figure 4 D), whereas the peak was at veraison in the T-2 treatment group (Figure 5 D) after which transcript levels declined (Figure 4 D and 5 D). Surprisingly, *VvOMT4* expression in

parthenocarpic fruit in the T-3 group was maintained at high levels from pre-veraison 1 to the post-veraison stages (Figure 5 D).

4. Discussion

MPs are grape-derived wine aroma compounds that have been associated with vegetative, green bell pepper aromas among some red grape cultivars, including Carmenere. This odour is generally considered detrimental to quality in red wines. Moreover, the levels of MPs in wine are known to directly correlate with the content of these compounds in grape berries at harvest (Ryona et al., 2009).

It is known that climactic parameters, such as cluster light environment, temperature, and water availability, influence MP levels in grapes during ripening (Koch et al., 2012; Lacey et al., 1991). However, the mechanisms involved are still unknown. In this study, the levels of MPs in Carmenere grapes were compared during development and ripening under two stress-inducing growth conditions, 1) clusters under sunlight restriction (T-2) and 2) the combination of lateral shoot removal plus water deficit (T-3), compared with normal growth conditions. As has been described previously, IBMP levels were dominant and present at concentrations an order of magnitude higher than IPMP levels (Alberts et al., 2009). In general, IBMP concentrations in Carmenere followed the expected pattern over the growing season, with pre-veraison accumulation followed by mature degradation (Ryona et al., 2008) in all treatment groups. Additionally, we correlated smaller berry size with parthenocarpic fruit at the mature stage in the same

400 cluster across all treatments. Given that MPs are primarily localised to berry
401 skins (Dunlevy et al., 2010; Roujou de Boubee et al., 2000) and also that the
402 highest ratio of skin:total fresh weight was observed in parthenocarpic fruits,
403 MPs were quantified in parthenocarpic and seeded berries during
404 development and ripening across all treatments individually. Interestingly, we
405 observed that seeded berries exhibited the highest IBMP levels per fresh
406 weight in all samples analysed.

407 Moreover, when comparing the two treatments (T-2 and T-3) and the control
408 in seeded fruits, similar IBMP behaviour during development and ripening
409 were observed. The concentration of IBMP increased several fold, reaching a
410 peak at the pre-veraison stage and then decreasing throughout the rest of
411 the season (Ryona et al., 2008). However, altering the light environment
412 continuously from setting to harvest as well as the induction of stress by the
413 combination of lateral shoot removal and water deficit produced an
414 approximately 2-fold increase in IBMP from pre-veraison to the mature stage
415 compared with the control group. These data suggest that both stress-
416 inducing treatments can change MP biosynthesis behaviour and that
417 traditional agronomic practices can modulate IBMP concentrations in seeded
418 fruit at the mature stage. Furthermore, as has been described previously, a
419 correlation was observed between IBMP concentrations at the pre-veraison
420 and mature stages (Ryona et al., 2009). However, a different behaviour was
421 observed in parthenocarpic fruits throughout development and ripening.
422 Interestingly, compared with seeded fruit, IBMP levels in parthenocarpic fruit
423 were generally reduced throughout development and ripening. In
424 parthenocarpic fruit in the control group, IBMP levels reached a peak at the

425 veraison and post-veraison stages, whereas this peak was shifted to earlier
426 developmental stages by either reducing light conditions (T-2) or reducing
427 plant vigour plus water deficit (T-3). In contrast with a previous report (Ryona
428 et al., 2008), no correlation was observed between IBMP levels in the pre-
429 veraison 2 and mature stages in parthenocarpic fruits. Moreover, higher
430 IBMP levels during development and ripening of parthenocarpic fruits were
431 observed in both stress-inducing treatment groups compared with the control
432 group. However, non-significant differences in IBMP content at the mature
433 stage were observed between treatments. The data presented here indicate
434 that both sunlight restriction and removal of lateral shoots plus water deficit
435 activate different MP synthesis and/or degradation mechanisms in seeded
436 and parthenocarpic fruits. These observations are in agreement with higher
437 IBMP levels in wine made from seeded berries.

438 It is not fully understood why MP accumulation is linked to temperature, plant
439 vigour or water availability. It was recently reported that complete shading of
440 clusters increases IBMP concentrations more than 100% compared with
441 unshaded controls (Koch et al., 2012). In this study, the authors also
442 indicated that both light and temperature significantly affect IBMP levels in
443 mature fruit (Koch et al., 2012). Another metabolic study in grapes reported
444 that metabolic processes such as photosynthesis increase with increasing
445 temperature (Keller, 2010). Recently, four *O*-methyltransferase genes
446 (*VvOMT1, VvOMT2, VvOMT3,* and *VvOMT4*) involved in MP biosynthesis
447 were identified (Dunlevy et al., 2010; Dunlevy et al., 2013; Guillaumie et al.,
448 2013; Vallarino et al., 2011). All genes were shown to be responsible for the
449 methylation of HP intermediates described as precursors of MPs (Dunlevy et

al., 2010; Dunlevy et al., 2013; Guillaumie et al., 2013; Hashizume, Tozawa, Hiraga, & Aramaki, 2001a; Hashizume, Tozawa, Endo, & Aramaki, 2001b; Harris, Ryona & Sacks, 2012; Vallarino et al., 2011). Although Carmenere ripens more slowly than Cabernet Sauvignon, in both cultivars, *VvOMT expression* followed the expected pattern as has been described previously (Dunlevy et al., 2010; Dunlevy et al., 2013; Guillaumie et al., 2013).

To our knowledge, the light-, plant vigour- and water availability-dependence of *VvOMT1*, *VvOMT2, VvOMT3,* and *VvOMT4* gene expression has not been characterised. When the expression of *VvOMT1* and *VvOMT2* was analysed in seeded fruits in the control group, the peak expression of both genes preceded the maximum peak of IBMP levels as previously described (Dunlevy et al., 2010). Expression of both genes exhibited a peak at the pre-veraison 1 stage, whereas the IBMP peak was observed at the pre-veraison 2 stage. In contrast, the levels of *VvOMT3* and *VvOMT4* closely mirrored IBMP accumulation patterns in seeded fruits. The maximum peak of expression of both genes at pre-veraison 2 matched the peak of IBMP levels in the fruit. These correlations between gene expression and IBMP levels have been described in total Carmenere berries (Guillaumie et al., 2013). Interestingly, in parthenocarpic fruit, peak *VvOMT1* expression was shifted to the veraison stage in accordance with the delay in colour change of these berries (data not shown), whereas *VvOMT2, VvOMT3,* and *VvOMT4* transcripts exhibited the same maximum expression peak in seeded berries.

In seeded fruits from shaded clusters as well as in samples from the lateral shoot removal plus water deficit group, *VvOMT1* and *VvOMT3* transcripts began to accumulate at the same stage as in the control group: at the pre-

475 veraison 1 and pre-veraison 2 stages, respectively. In contrast to the control
476 group, maximal *VvOMT1* and *VvOMT3* mRNA accumulation was maintained
477 until the post-veraison stage when IBMP levels also decreased. Interestingly,
478 no changes in the expression pattern of *VvOMT4* were observed when
479 comparing the control group and the stress induction treatment groups (T-2
480 and T-3). However, differential regulation of *VvOMT2* was observed when
481 altering plant vigour and water availability. In the T-3 treatment group, the
482 *VvOMT2* expression peak was shifted to later stages compared with the
483 control group, whereas no differences in the *VvOMT2* expression profile were
484 observed in the shaded clusters. Taken together, these expression data are
485 consistent with roles for *VvOMT1* and *VvOMT3* as key genes in IBMP
486 biosynthesis in seeded grape fruit under sunlight restriction or lateral shoot
487 removal plus water deficit.

488 In parthenocarpic fruits, the *VvOMT1* expression profile throughout
489 development and ripening in both stress induction treatments was similar to
490 seeded fruits. Similar to the control group, *VvOMT1* and *VvOMT3* expression
491 correlated with IBMP accumulation in the pre-veraison and veraison stages in
492 both treatments as well as in seeded fruit, although parthenocarpic fruits
493 contain 3- to 7-fold less IBMP. In the control group, the degradation of IBMP
494 seems to be delayed by two weeks relative to the stress treatments. By
495 contrast, no correlation was found between *VvOMT2* and *VvOMT4*
496 expression and IBMP or IPMP levels in accordance with studies by
497 Guillaumie et al. (2013) and Vallarino et al. (2011).

498 In summary, our results show that different agronomical management
499 practices, such as restriction of light exposure and removal of lateral shoots

500 plus water availability, differentially influence MP concentrations in seeded
501 and parthenocarpic fruits. In both stress induction treatments in which the
502 vine balance was changed, higher IBMP levels at the pre-veraison and
503 veraison stages than in the control group were observed, which led to higher
504 IBMP concentrations at mature stages in seeded fruits. In contrast, although
505 different IBMP levels at the veraison and post-veraison stages were exhibited
506 in both stress induction treatment groups from the control, no differences
507 were observed at the mature stage among all treatments in parthenocarpic
508 fruits. Therefore, we suggest that different MP synthesis and/or degradation
509 mechanisms operate in seeded and parthenocarpic fruits. In this regard, we
510 present here compelling evidence of the importance of *VvOMT1* and
511 *VvOMT3* in the control of IBMP levels in seeded fruits in agreement with
512 previous molecular modelling data (Guillaumie et al., 2013; Vallarino et al,
513 2011). We observed a correlation between higher *VvOMT1* and *VvOMT3*
514 expression and higher IBMP content at the mature stage in both stress
515 induction treatment groups in seeded fruits. Moreover, we also found a
516 correlation between IBMP levels in wine with IBMP content in grapes at the
517 mature stage (Ryona et al., 2009). Therefore, the results presented here
518 indicate that different agronomic interventions that affect vine balance can
519 modulate *VvOMT1* and *VvOMT3* expression, which has a direct impact on
520 IBMP concentrations in grapes and wines. It is important to improve our
521 understanding of MP metabolism and unravel the underlying network of
522 highly flexible regulatory mechanisms to gain insight into MP regulation. This
523 knowledge will assist in the development of new strategies for breeding new

524 varieties and new agronomic practices for obtaining grape fruit with
525 appropriate levels of MPs.

526

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533

534 **Appendix A. Supplementary data**

535 **Figure S1**. Photo of *Vitis vinifera* cv. Carmenere plants grown under normal
536 conditions (control treatment).

537 **Figure S2**. Photo of *Vitis vinifera* cv. Carmenere plant clusters that were
538 covered with bags that filter out 95% of photosynthetically active radiation
539 (PAR) (sunlight restriction treatment; T-2).

540 **Figure S3**. Photo of *Vitis vinifera* cv. Carmenere plants under stress
541 generated by a combination of lateral shoot removal and water deficit (T-3
542 treatment).

543 **Figure S4**. Size of parthenocarpic (A) and seeded fruits (B).

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Figure legends

Figure 1. Influence of sunlight restriction (T-2) and removal of lateral shoots plus water deficit (T-3) on methoxypyrazine content in Carmenere wines.

A, IBMP concentration in Carmenere wines made with grapes from the control group (C), sunlight restriction group (T-2) and lateral shoot removal plus water deficit group (T-3). **B**, IBMP concentration in Carmenere wines made from grapes from the control group (C), T-2 and T-3 groups, considering seeded and parthenocarpic berries separately. IPMP was not detectable. Four biological replicates were analysed. Different letters indicate significant differences using ANOVA and the Tukey HSD test adjusted to a 95% significance level.

Figure 2. Methoxypyrazine concentrations in seeded and parthenocarpic berries throughout development and ripening under sunlight restriction (T-2) and removal of lateral shoots plus water deficit (T-3).

IBMP and IPMP concentrations, expressed as ng kg^{-1} fresh weight, in seeded and parthenocarpic berries during development and ripening from the control (A), T-2 (B), and T-3 (C) groups. Four biological replicates were analysed. Different letters indicate significant differences using ANOVA and the Tukey HSD test adjusted to a 95% significance level (uppercase for IBMP levels and lowercase for IPMP levels).

Figure 3. *VvOMT* gene expression in whole fruit clusters (cvs. Carmenere and Cabernet Sauvignon) throughout development and ripening.

Relative expression of four *Vitis vinifera* *O*-methyltransferase genes (*VvOMT1* (A)*, VvOMT2* (B)*, VvOMT3* (C), and *VvOMT4* (D)) in whole fruit clusters throughout development and ripening in cvs. Carmenere and Cabernet Sauvignon were determined by qRT-PCR. Four biological replicates were analysed. Different letters indicate significant differences using ANOVA and the Tukey HSD test adjusted to a 95% significance level.

Figure 4. *VvOMT* gene expression in seeded and parthenocarpic Carmenere berries throughout development and ripening.

Relative expression of *VvOMT1* (A), *VvOMT2* (B), *VvOMT3* (C), and *VvOMT4* (D) in seeded and parthenocarpic berries from the control group as determined by qRT-PCR. Four biological replicates were analysed. Different letters indicate significant differences using ANOVA and the Tukey HSD test adjusted to a 95% significance level.

Figure 5. *VvOMT* gene expression in seeded and parthenocarpic Carmenere berries throughout development and ripening under sunlight restriction (T-2) and lateral shoot removal plus water deficit (T-3).

752 Relative expression of *VvOMT1* (A), *VvOMT2* (B), *VvOMT3* (C), and
753 *VvOMT4* (D) in seeded and parthenocarpic berries from the sunlight
754 restriction treatment group (T-2) and lateral shoot removal plus water deficit
755 treatment group (T-3) as determined by qRT-PCR. Four biological replicates
756 were analysed. Different letters indicate significant differences using ANOVA
757 and the Tukey HSD test adjusted to a 95% significance level.