1 **Original article**

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3 **Manuscript Title: Transient induction of a subset of ethylene biosynthesis genes is**
4 **potentially involved in regulation of grapevine bud dormancy release**

5 **Running Title:** Regulation of ethylene biosynthesis in grape buds

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Manuscript Title: Transient induction of a subset of ethylene biosynthesis genes is potentially involved in regulation of grapevine bud dormancy release

Key message: Transient increases in ethylene biosynthesis, achieved by tight regulation of transcription of specific ACC oxidase and ACC synthase genes, play a role in activation of grapevine bud dormancy release.

Abstract

The molecular mechanisms regulating dormancy release in grapevine buds are as yet unclear. It has been hypothesized that its core involves perturbation of respiration which induces an interplay between ethylene and ABA metabolism that removes repression and allows regrowth. Roles for hypoxia and ABA metabolism in this process have been previously supported. The potential involvement of ethylene biosynthesis in regulation of dormancy release, which has received little attention so far, is now explored.

Our results indicate that (1) ethylene biosynthesis is induced by hydrogen cyanamide (HC) and azide (AZ), known artificial stimuli of dormancy release, (2) inhibitors of ethylene biosynthesis and signalling antagonize dormancy release by HC/AZ treatments, (3) ethylene application induces dormancy release, (4) there are two sets of bud-expressed ethylene biosynthesis genes which are differentially regulated, (5) only one set is transiently upregulated by HC/AZ and during the natural dormancy cycle, concomitant with changes in ethylene levels, and (6) levels of ACC oxidase transcripts and ethylene sharply decrease during natural dormancy release, whereas ACC accumulates.

Given these results, we propose that transient increases in ethylene biosynthesis prior to dormancy release, achieved primarily by regulation of transcription of specific ACC oxidase genes, play a role in activation of dormancy release.

Keywords: *Vitis Vinifera*, ACC synthase (ACS), ACC oxidase (ACO), Bud, Dormancy, Ethylene, Grapevine

55 **Introduction**

56 Our previous efforts to understand the molecular mechanisms regulating dormancy
57 release in grapevine buds resulted in a working model that outlines biochemical pathways
58 potentially involved in artificially-induced bud dormancy release (Ophir et al. 2009). In this
59 model, perturbation of mitochondrial cytochrome-pathway activity leads to respiratory
60 stress resulting in an energy crisis. Anaerobic respiration is upregulated, triggering ethylene
61 biosynthesis, which regulates abscisic acid (ABA) metabolism and termination of ABA-
62 driven endodormancy (Ophir et al. 2009). Our further results supported the predictive power
63 of the model with respect to involvement of respiratory stress, hypoxia and ABA in bud
64 endodormancy regulation (Ophir et al. 2009; Vergara et al. 2017; Zheng et al. 2015).
65 However, up until this time, we addressed the involvement of ethylene only in a preliminary
66 manner (Ophir et al. 2009), through identifying a temporary increase in ethylene levels in
67 response to treatment with HC (an artificial bud dormancy release stimulus), and an
68 enhancement of bud break in response to exogenous ethylene.

69 Ethylene synthesis starts with conversion of methionine to S-adenosyl methionine
70 (SAM) by SAM synthases. SAM is converted to ACC by ACC synthase (ACS), and ACC
71 is converted to ethylene by ACC oxidase (ACO). While regulation of ACS activity serves
72 as the recognized control point, ACO activity also serve as the rate limiting step, as seen
73 in tomato climacteric ripening and hypoxic roots (Harpaz-Saad et al. 2012; Liu et al. 2015;
74 Van de Poel and Van Der Straeten 2014; Yoon 2015). *ACS* and *ACO* genes occur as
75 multigene families (Dal Ri et al. 2009; Iwai et al. 2006; Vanderstraeten and Van Der
76 Straeten, 2017), and individual members may be differentially regulated during
77 development, and under different environmental conditions, and serve for specific roles (Liu
78 et al. 2015; Tsuchisaka and Theologis, 2004; Tsuchisaka et al. 2009).

79 The involvement of ethylene in seed germination has been intensively studied. A dose-
80 dependent stimulatory effect of exogenous ethylene on seed germination is observed in
81 various dormancy situations, and increased ethylene production after onset of imbibition
82 and during germination in various plants is also reported (Corbineau et al. 2014). In
83 agreement, triggers of seed dormancy release, such as nitric oxide (NO) and hydrogen
84 cyanide (HCN), increase ethylene levels. Treatments with inhibitors of ethylene
85 biosynthesis/action and use of mutants impaired in ethylene signalling also support an
86 involvement of endogenous ethylene in dormancy release. ACO activity appears to play a
87 fundamental role during germination, and is regulated at a transcriptional level. In both

88 *Arabidopsis* and cress seeds, *ACO1* and *ACO2* are the major *ACOs* involved in ethylene
89 synthesis (Corbineau et al. 2014). *ACO1* and *ACO2* expression is down regulated by ABA
90 in *Arabidopsis* seed, and in agreement, higher level of *ACO* transcripts was recorded in
91 ABA-insensitive mutants and in the ABA biosynthesis mutant *aba2* (Carrera et al. 2008;
92 Penfield et al. 2006).

93 A role for ethylene in terminal bud formation and transition to endodormancy has been
94 documented. Expression of the ethylene receptor gene At*ETR1* carrying the dominant
95 mutation in birches abolished formation of terminal buds, suggesting that ethylene facilitates
96 short day (SD)-induced formation of terminal buds (Ruonala et al. 2006). In poplar,
97 photoperiod, low sugar, ethylene, and ABA are proposed to act sequentially in SD-induced
98 terminal bud formation and dormancy acquisition (Ruttink et al. 2007). Since ethylene
99 biosynthesis and signalling are transiently activated two weeks after exposure to SD, before
100 internode elongation ceases, it was proposed that ethylene regulates terminal bud formation.
101 It was also proposed that transiently low hexose pools, rather than SD signal itself, might
102 trigger transient activation of ethylene signalling, which regulates terminal bud formation,
103 whereas ABA is involved in endodormancy induction (Ruttink et al. 2007). In potato tubers,
104 endogenous ethylene levels are critical for the development of adequate endodormancy,
105 their involvement being restricted to the initial phase of endodormancy induction (Suttle,
106 1998). Similarly, for *Chrysanthemum*, ethylene induces dormancy, but this fails for an
107 ethylene-insensitive transgenic line (Sumitomo et al. 2008). The transition of leafy spurge
108 crown buds from para- to endodormancy and the SD-induced dormancy of *Vitis riparia* buds
109 also is proposed to involve a role for ethylene (Doğramacı et al. 2013; Fennell et al. 2015;
110 Horvath et al. 2008).

111 While the role of ethylene during seed dormancy release (Corbineau et al. 2014) and
112 submergence-induced growth promotion (Fukao and Bailey-Serres 2008) may imply a
113 similar function in buds, its role during bud dormancy maintenance and release, and in
114 growth resumption remains unclear. The role for ethylene in potato tuber dormancy
115 maintenance and release remains quite ambiguous (Aksenova et al. 2013; Sonnewald and
116 Sonnewald, 2014): on the one hand, ethylene treatments can either shorten or delay the
117 dormancy period, depending on treatment duration and concentration (Prange et al. 1998;
118 Rylski et al. 1974), Bromoethane (BE), a dormancy release stimulus, temporarily increases
119 ethylene production (Alexopoulos et al. 2009; Suttle, 2009), and ethylene response
120 functions are downregulated during sprouting (Hartmann et al. 2011). On the other hand,

121 the effect of BE is not negatively influenced by inhibitors of ethylene signalling, and
122 ethylene synthesis inhibitors did not inhibit bud break. Additionally, ethylene and ACC
123 treatments do not break minituber dormancy (Suttle, 2009).

124 In poplar, a gene set associated with ethylene signalling, is upregulated from para- to
125 endodormancy and downregulated from endo- to ecodormancy, suggesting that it is
126 involved both in dormancy maintenance and release (Howe et al. 2015). A similar function
127 for ethylene signalling was recently proposed for underground adventitious buds of leafy
128 spurge (Chao et al. 2017). Interestingly, overexpression of an ethylene response factor
129 (ERF) gene (*EBB1*) induced early bud-flush in poplar, whereas down-regulation delayed
130 bud-break. *EBB1* was undetectable during the dormancy, but rapidly increased prior to bud-
131 break, and was at highest levels in active meristems, suggesting a role in meristem
132 reactivation after winter dormancy (Yordanov et al. 2014).

133 We proposed a role for ethylene in regulation of grape bud dormancy release in
134 response to HC, following detection of increased expression of hypoxia-related ERF genes
135 accompanied by increased ethylene levels. The observation of enhanced bud break in
136 response to exogenous ethylene supported its relevance to dormancy (Ophir et al. 2009).
137 Alteration of ethylene-related functions by HC has been also recorded in cherry buds, where
138 it induces expression of *ACS* and *ACO* (Ionescu et al. 2017), and in grape summer buds,
139 where ERF gene expression was altered (Sudawan et al. 2016). Upregulation of several
140 ethylene biosynthesis and hypoxia-induced ERFs was recently reported during grape bud
141 growth resumption (Meitha et al. 2018).

142 In light of the limited attention dedicated so far to studying the involvement of
143 ethylene biosynthesis in the regulation of grape bud dormancy release, we undertook the
144 current study. Our results imply that a transient increase of ethylene biosynthesis prior to
145 dormancy release is achieved, in response to both artificial and natural stimuli of dormancy
146 release, by tight regulation of transcription of a specific subset of ethylene biosynthesis
147 genes, and this increase has a role in activation of dormancy release.

148

Materials and methods

Plant material

The experiments were conducted using mature buds collected from cordon-trained grapevines (*Vitis vinifera* cv. Early sweet) in a commercial vineyard located at Gilgal, Jordan Valley, Israel (Zheng et al. 2018). Vines were pruned to three-node spurs, and the detached canes, each carrying nine buds (in positions 4-12), were cut into single-node cuttings on arrival from the vineyard, randomly mixed, and groups of 10 cuttings prepared.

Natural dormancy curve

To describe the seasonal changes in the dormancy status of the bud population in the vineyard across the natural dormancy cycle, canes were sampled weekly, and nine groups of 10 single-node cuttings were placed in open vases containing water under the forcing condition of 22 °C under a 14 h/10 h light/dark regime. The bud break percentages at 21 d were used to prepare a seasonal dormancy curve. For gene expression analyses, three groups of one hundred buds were sampled weekly from the pool of cuttings and stored at -80 °C.

Analyses of the effect of chemical and physical treatments on bud break

Analyses performed using an Open Vases (OV) experimental system (see summary in Table S1): To test the effect of HC [3% Dormex®, 490 gr HC/L (SKW, Trostberg, Germany), v/v] and AZ [2% NaN_3 (Sigma-Aldrich), w/v] on bud dormancy release, nine groups of 10 single-node cuttings per treatment were treated as described in Zheng et al. (2015). The treated cuttings were placed in open vases containing water, and bud break was monitored. Cuttings treated with 0.02% Triton X-100 solution served as controls. For gene expression analyses, identical treatments were carried out, and the buds were sampled at 12, 24, 48 and 96 h and stored at -80 °C.

To test the effect of cobalt chloride ($CoCl_2$), 15 groups of 10 cuttings per treatment were placed in vases containing 150 ml 3.6 mM $CoCl_2$ solution (Merck, Darmstadt, Germany). After incubation for 48 h, cuttings were sprayed with 3% HC and returned to fresh $CoCl_2$ solution for an additional 10 days (with solution replacement every 48 h). The cuttings were then transferred to water for the rest of the bud break monitoring period.

To test the effect of silver thiosulfate (STS), nine groups of 10 cuttings per treatment were placed in vases containing 150 ml 0.5% or 2% (v/v) STS (Aldrich, Steinheim, Germany). After incubation of 24 h, cuttings were sprayed with 3% HC and returned to

182 identical fresh STS solutions for the rest of the bud break monitoring period (with solution
183 replacement every 48 h).

184 To test the effect of ethephon [0.7% Ethrel®, 480gr ethephon/L (Bayer CropScience,
185 Monheim am Rhein, Germany), v/v] on bud dormancy release, nine groups of ten single-
186 node cuttings per treatment were treated as described above for HC.

187 To test the effect of Ethrel on dormancy release of whole vines under vineyard
188 conditions, cv. Early sweet vines in a commercial vineyard, located in Argaman, Jordan
189 Valley, were pruned to three-node spurs in mid-January, and the total number of buds was
190 determined. The pruned vines were sprayed with 0.02% Triton X-100 without (control) or with
191 0.8% Ethrel to runoff (1 L/vine), using a 15 L knapsack sprayer (SOLO®, VA, USA). Each
192 treatment consisted of four blocks of three vines, in a randomised complete block design. The
193 numbers of bursting buds were counted at 42 days after Ethrel application.

194

195 **Analyses performed using a Sealed Vase (SV) experimental system** (see summary
196 in Table S1)**:** To compare the effect of ethylene, NBD, and hypoxia on bud dormancy
197 release, an enclosed environment was necessary. Thus, treatments were carried out in sealed
198 2 L glass jars (3 jars per treatment) as described in Ophir et al. (2009). A group of 70 cuttings
199 was placed in each glass jar containing 150 ml of water.

200 To test the effect of exogenous ethylene, ethylene was injected to a final concentration
201 of 100 ppm and the jars were left sealed for 48 h. For the control treatment, cuttings were
202 sprayed with 0.02% (v/v) Triton X-100 solution, and incubated in the presence of a
203 perforated tube containing vermiculite saturated with 7.43 g/100 ml $KMnO_4$ solution
204 (Merck, Darmstadt, Germany) as described in Ophir et al. (2009). After 48 h, nine groups
205 of 10 cuttings were transferred to open vases containing water for bud break monitoring as
206 previously described.

207 To test the effect of NBD on the stimulatory effects of HC and AZ, we employed HC,
208 AZ, HC-NBD, and AZ-NBD treatments. HC and AZ treatments were applied as described
209 above, and the cuttings placed in jars which were sealed for 48 h. For the combined
210 treatments, cuttings were initially sprayed with HC or AZ, and then placed in jars sealed for
211 48 h in the presence of 5 ml NBD (Aldrich, Steinheim, Germany) placed in an open 15 ml
212 tube. After 48 h, groups of 10 cuttings from each treatment were transferred to open vases
213 with water for bud break monitoring (3 groups of 10 cutting for HC and HC-NBD
214 experiment, 9 groups of 7 cuttings for AZ and AZ-NBD experiment).

215 Hypoxia treatments involved jars flushed with N_2 to reduce the O_2 level to 1% as
216 described in Zheng et al. (2015) and sealed for 48 h. Cuttings were then transferred to open
217 vases with water for bud break monitoring. Other details are as previously described.
218 For gene expression analyses, buds were sampled from jars sealed separately for 24 h,
219 and stored at -80 °C.
220
221 **Extraction and analysis of ACC and ethylene measurements**
222 Extraction and analysis of ACC were conducted as previously described (Müller and
223 Munné-Bosch 2011), using 0.1 g of homogenized powder prepared from five frozen buds
224 for each of three biological replicates.
225 LC-MS analyses were conducted using UPLC-Triple Quadrupole-MS (Waters Xevo
226 TQ MS). Chromatographic separation was performed on a Waters Acquity UPLC BEH C18
227 1.7 µm 2.1 x100 mm column with a VanGuard precolumn (BEH C18 1.7 µm 2.1 x 5 mm)
228 with isocratic elution water: acetonitrile (95:5) containing 0.1 % formic acid.
229 Ethylene assays were conducted as formerly described (Ophir et al. 2009).
230
231 **RNA extraction and quantitative real-time PCR (qRT-PCR) analyses**
232 Total RNA was extracted and cDNA synthesized as previously described
233 (Acheampong et al. 2017). The transcript levels were quantified by qRT-PCR as previously
234 described (Zheng et al. 2015). Gene-specific primers for transcription assay are in
235 Supplementary Table S2.
236
237 **Transcription factor binding site (TFBS) analysis**
238 Nucleotide sequence between positions -2000 to -600 bp upstream from the
239 transcription start site of the selected genes were downloaded from the 12X V1 grapevine
240 genomic database (CRIBI, http://genomes.cribi.unipd.it/grape/). TFBS analyses were
241 carried out using the Genomatix Genome Analyzer (GGA) MatInspector program, and
242 common TFBS was searched using the Common TFs program online
243 (http://www.genomatix.de) (Dekel et al. 2015). The parameters used were Matrix Family
244 Library version 11.0 (MatInspector Release professional 8.4.1, September 2017), core
245 similarity (0.85), and matrix similarity (0.80).
246
247

Statistical Analyses

Statistical analyses were performed on a JMP 13.1.0 (SAS Institute, Cary, NC, USA) by one-way ANOVA with Student's t-test (*, $p < 0.05$), or with Tukey's HSD with a *p*-value <0.05.

253 **Results**

254 **Ethylene participates in regulation of bud endodormancy release**

255 Our working model proposed that ethylene biosynthesis is triggered by artificial
256 stimuli of grapevine bud break, and affects a cascade of biochemical events that leads to
257 endodormancy release. In agreement, our results (Fig 1a) indicate that HC and AZ
258 treatments both induce significant increases in ethylene levels within dormant grapevine
259 buds at 6, 24 and 48 h from its application.

260 We then went on to examine the potential involvement of ethylene on bud
261 endodormancy release, through testing the effect of treatments with ethylene-biosynthesis
262 and ethylene-signalling inhibitors. Application of $CoCl_2$, a known inhibitor of ethylene
263 biosynthesis (Merritt et al. 2001), led to a complete inhibition of endodormancy release of
264 HC-treated buds and untreated buds (Fig. 1b). Incubation in silver thiosulfate solution (STS,
265 2%), which inhibits ethylene signalling (Beyer, 1976), significantly inhibited bud break of
266 HC-treated buds. Incubation in 0.5% STS solution resulted in a milder inhibiting effect on
267 the extent of induction following treatment with HC (about 1.9 and 1.1-fold at 14-18 d), and
268 both STS (0.5%)-HC and HC-treated buds displayed enhancements of bud break at 14-18
269 d, as compared to the control. Untreated buds were similarly inhibited by treatment with
270 STS (Fig. 1c).

271 We formerly reported that treatment with the ethylene signalling inhibitor 2, 5-
272 norbornadiene (NBD) inhibited bud break, as compared to untreated buds, despite
273 significant increases in ethylene production induced by this treatment (Ophir et al. 2009).
274 Here we show that combined treatment with NBD and HC (NBD-HC, Fig. 1d) or AZ (NBD-
275 AZ, Fig. 1e), significantly reduced the effects of HC and AZ on advancing bud
276 endodormancy release over the 28 d monitoring period (Fig. 1d and Fig. 1e). Finally, a
277 significant enhancement of bud break was seen in response to application of gaseous
278 ethylene (100 ppm), as compared to its control (10, 5, 2, and 1.3-fold at 12, 14, 18 and 21 d
279 from application; Fig. 1f). In agreement, our results indicate that application of Ethrel
280 (containing ethephon, which upon metabolism by the plant is chemically converted to
281 ethylene), resulted in significantly increased levels of bud break in the OV system (Fig 1g,
282 2.4-fold at 24 d from application) and in the vineyard (Fig 1h, 1.3-fold at 42d from
283 application), as compared to the appropriate control.

284

285

286 **Ethylene biosynthesis is regulated by HC and AZ at the level of transcription**

287 Given these results, we next examined whether the observed increases in ethylene 288 levels were driven by regulation of expression of members of the *ACS* and *ACO* gene 289 families. Previous analyses (Dal Ri et al. 2009; Muñoz-Robredo et al. 2013; Xu and Wang, 290 2012) identified nine putative grapevine homologs for *ACS* (*VvACS*; Fig. S1), and four for 291 *ACO* (*VvACO*; Fig. S2). Our preliminary expression analyses revealed that five members of 292 the *VvACS* family (*VvACS1*, *VvACS2*, *VvACS4, VvACS6*, *VvACS9)* and three members of 293 the *VvACO* family (*VvACO1, VvACO2, VvACO4*) are expressed in mature grapevine buds*,* 294 while transcripts of the others were below the level of detection across the natural cycle and 295 in response to HC (data not shown). Therefore, only these nine genes were used for the 296 detailed expression analyses described below.

297 Analyses of the effects of HC and AZ on the transcript levels of the bud-expressed 298 members of the *VvACS* and *VvACO* gene families were done using qRT-PCR. In agreement 299 with the observation of increased ethylene levels following HC and AZ treatments, both 300 treatments significantly upregulated the expression of *VvACS1*, *VvACS6*, *VvACO2* and 301 *VvACO4* (Fig. 2a, 2c, 2g and 2h; Table S3). Surprisingly, both HC and AZ treatments also 302 led to a significantly decreased expression of two bud-expressed members of *VvACS* gene 303 family (Fig 2b and 2d; Table S3). The expression of *VvACS4* was not affected, apart from a 304 significant decrease at 12 h in response to AZ (Fig 2e; Table S3).

305

306 **Ethylene and hypoxia upregulate expression of genes that are downregulated by HC**

307 To further study the differential response of bud-expressed members of the *ACO* and 308 *ACS* families to dormancy release stimuli, we examined the effects of ethylene and hypoxia 309 treatments on the expression of the gene family members listed above. Since hypoxia and 310 direct ethylene treatments necessitate an enclosed environment, their effects were compared 311 to those of HC and control treatments in the SV experimental system.

312 In agreement with effects noted in the OV experimental system (Fig. 2), *VvACS1* and 313 *VvACS6* expression levels are upregulated by 9 and 103-fold at 24 h following the HC 314 treatment in the SV system, whereas *VvACS2* and *VvACS9* expression levels were both 2- 315 fold down regulated by the same treatment (although differences were not statistically 316 significant). These data support the suitability of this system for further analyses (Fig. 3).

317 Interestingly, the segregating transcriptional responses of the two *ACS* subgroups was 318 also reflected in their responses to treatment with ethylene and hypoxia [in which the

319 ethylene level is significantly lower (Fig. S3), as compared to the SV control lacking
320 $KMnO_4$]. The genes whose expression was significantly induced by HC (*VvACS1* and
321 *VvACS6*) were not affected by hypoxia and ethylene (Fig. 3a and 3c). On the other hand, the
322 genes whose transcription was inhibited by HC (*VvACS2* and *VvACS9*), were upregulated
323 by hypoxia (Fig. 3b and 3d, 2.5 and 2.2-fold). These two genes were also upregulated by
324 ethylene (1.6 and 1.5-fold) although upregulation of *VvACS2* was not significant. The level
325 of *VvACS4* was upregulated only by hypoxia (2.8-fold; Fig. 3e).
326 Similar analyses were done to explore the responses of the bud-expressed *VvACO*
327 genes. The response of *VvACO2* was, in principal, similar to that of *VvACS1* and *VvACS6*,
328 as its transcript level increased in response to HC (9 -fold), and did not significantly change
329 in response to ethylene and hypoxia treatments (Fig. 3g). Expression of the other two genes,
330 *VvACO1* (Fig. 3f) and *VvACO4* (Fig. 3h), was upregulated by treatments with HC (2- and
331 20-fold) and ethylene (4- and 9-fold). *VvACO4* was highly responsive to hypoxia (34-fold).
332
333 **Seasonal changes in ethylene biosynthetic gene expression**
334 The next set of experiments examined whether a similar pattern of segregating
335 behavior of the bud-expressed members of the *VvACS* gene family occurs across the natural
336 endodormancy cycle. Transcript levels were recorded during endodormancy induction,
337 maintenance, and release (Fig. 4). It is important to note that while analyses of
338 endodormancy status under forcing conditions in growth chambers indicate that
339 endodormancy release is taking place from mid-December onwards, the bud population
340 sampled in the vineyard for transcript analysis is still ecodormant during January. Hence,
341 changes that are related to actual activation of meristem growth may not be recorded in this
342 analysis. The transcription profiles of *VvACS2* and *VvACS9* indicate that they are expressed
343 at relatively low levels during endodormancy induction and maintenance, and are induced
344 only during endodormancy release (Fig. 4b, 4d). The transcription profiles of *VvACS1* and
345 *VvACS6* suggest that they are significantly but only temporarily induced during the
346 transition from endodormancy induction to endodormancy maintenance, and subsequently
347 decrease to their lowest levels during endodormancy release (Fig. 4a, 4c). The transcription
348 profile of *VvACS4* did not display significant regulation over the analyzed period (Fig. 4e).
349 A similar analysis revealed that *VvACO1* transcripts sharply decrease when the
350 endodormancy cycle starts, and further decrease during endodormancy release (Fig. 4f).
351 *VvACO4* presents a high and stable transcript level over the period of endodormancy

induction, which sharply decreases during endodormancy maintenance, with a further decrease evident during endodormancy release (Fig. 4h). The transcriptional profile for *VvACO2* is similar to that of *VvACS1* and *VvACS6*, with increased transcription during endodormancy induction, reaching a maximum during endodormancy maintenance, followed by a gradual decrease during endodormancy release (Fig. 4g). Collectively, the levels of all three bud-expressed *VvACO* transcripts are lowest during endodormancy release (from Dec 18th to Jan 08th).

Identification of putative transcription factors binding sites in the promoters of the bud-expressed *VvACS* and *VvACO* genes

Based on the dichotomous transcriptional behavior presented above, the bud-expressed *ACO* and *ACS* genes were divided into two subgroups: subgroup I includes *VvACS1*, *VvACS6*, *VvACO2* and *VvACO4*, and subgroup II includes *VvACS2*, *VvACS9* and *VvACO1.* Towards understanding the source of this dichotomous behavior, their promoter sequences were subjected to *in silico* analysis of TFBSs, searching for *cis*-acting regulatory elements differentially enriched within the promoters of the two subgroups. We identified 103 unique overrepresented sequence motifs within a 1.4 kbp region upstream of the coding sequences (Supplementary Data File1). Of these, 16 TFBSs exist in the promoters of all eight genes (Supplementary Data File1), 7 TFBSs are enriched selectively within the promoters of subgroup I and 2 TFBSs are enriched selectively in the promoters of subgroup II (Table 1, Fig. S4). It should be noted that while *VvACS4* may be more related to subgroup II, one of the TFBSs that may characterize subgroup I is seen in its promotor. Among the TFBSs that are enriched within the promoters of the subgroup I genes are (1) Dehydration responsive element binding factors (DREB, Upadhyay et al. 2017), (2) Sucrose box, required for sugar responsive gene expression (Ibraheem et al. 2010; Tsukaya et al. 1991), and (3) Auxin response factor 3 (ARF3, Cheng et al. 2013; Ulmasov et al. 1997).

Among the TFBSs that are differentially enriched in the promoters of the subgroup II genes are: (1) APETALA2-like transcription factors (AP2L, Yant et al. 2010), and (2) Plant nitrate-responsive *cis*-elements (PNRE, Konishi et al. 2010).

384 **Seasonal changes in ethylene biosynthesis capacity and response to inhibition of**
385 **ethylene signalling**

386 To further examine changes in ethylene biosynthesis capacity across the natural
387 endodormancy cycle, ethylene production was recorded weekly in single node cuttings in
388 the SV system. The data (Fig. 5), which are based on analyses conducted in parallel in two
389 different vineyards, suggest that the ethylene biosynthesis capacity increases at the transition
390 point between endodormancy induction and endodormancy maintenance, but is significantly
391 decreased later.

392 We found that ACC levels in the bud were slightly reduced (approximately 1.2-fold)
393 in parallel with reductions in *VvACS1* and *VvACS6* expression at maximal dormancy depth
394 and were markedly increased (approximately 1.8-fold) during dormancy release (Fig. 6), in
395 parallel with increased expression of *VvACS2* and *VvACS9* and decreased expression of all
396 three *VvACOs* (Fig. 4).

397 Interestingly, our data suggests that the inhibiting effect of NBD on HC-induced bud
398 break is also timing-dependent. According to our data, there is a gradual reduction in the
399 degree of inhibition exerted by NBD, when the bud break percentage of NBD-HC-treated
400 bud populations is compared with that of HC-treated bud population (Fig. 7). It should be
401 noted that this decrease is in parallel with the decreased expression of all three *VvACO*s and
402 the reduced ethylene biosynthesis capacity.

403

404 **Seasonal changes in expression of thiosulfate sulfurtransferase**

405 Biosynthesis of ethylene from ACC is accompanied by the production of a cyanide
406 moiety, which is then converted to thiocyanate by thiosulfate sulfurtransferase (TST; Höfler
407 et al. 2016). Since upregulation of TST expression implies an accompanying increase in
408 cyanide levels and, indirectly, an increase in ethylene production, and since cyanide and
409 thiocyanate are inducers of grapevine bud endodormancy release (Sudawan et al. 2016), we
410 recorded the transcript levels of a grape homolog of the TST gene across the dormancy cycle
411 (Fig. 8). Levels gradually increased during endodormancy induction, peaked during
412 dormancy at maximal dormancy depth, and decreased during endodormancy release, similar
413 to the seasonal transcript profiles recorded for *VvACS1* (Fig. 4a), *VvACS6* (Fig. 4c), and
414 *VvACO2* (Fig. 4g), and to the profile of ethylene production (Fig. 5). Treatments with HC
415 and AZ resulted in significant upregulation of TST expression. The TST transcription
416 profiles serve as additional support for the concept of a temporary induction of ethylene

417 production, and also raise the possibility that byproducts of ethylene synthesis may also
418 contribute to dormancy release.
419

420 **Discussion**

421 Our current and previous results indicate that (1) ethylene biosynthesis is induced by 422 HC and AZ, known stimuli of grapevine bud dormancy release, (2) biosynthesis and 423 signaling of ethylene is essential for dormancy release, and (3) ethylene itself can serve as a 424 stimulus of dormancy release (Fig. 1; Ophir et al. 2009; Zheng et al. 2015). The results of 425 the current study further show that HC and AZ induce ethylene biosynthesis by upregulation 426 of the expression of genes encoding ACO and ACS, the enzymes that catalyze biosynthesis 427 of ethylene.

428

429 **Two sets of ethylene biosynthesis genes are expressed in the woody bud**

430 Interestingly, a dichotomy is apparent between the various bud-expressed members of 431 the ACS gene family in terms of their responses to HC and AZ. Whereas transcript levels 432 for *VvACS1* and *VvACS6* dramatically increased in response to HC and AZ, transcript levels 433 for *VvACS2* and *VvACS9* decreased in response to these same treatments. Furthermore, 434 whereas genes from the first group did not respond significantly to treatment with exogenous 435 ethylene and hypoxia (which are also dormancy release stimuli), transcript levels of genes 436 from the second group was significantly elevated by these treatments. Additionally, *VvACS1* 437 and *VvACS6* are expressed at relatively lower levels in naturally dormant buds, based on the 438 analysis of microarray data (Fasoli et al. 2012), and exhibit higher fold changes in transcript 439 levels in response to HC and AZ (Fig. 2). Excitingly, similar segregation patterns are seen 440 in the expression profiles of these genes across the natural endodormancy cycle. Whereas 441 *VvACS1* and *VvACS6* were induced during endodormancy induction and reduced during 442 endodormancy maintenance, *VvACS2* and *VvACS9* were induced only during endodormancy 443 release. This conservation of the pattern of segregation accompanying natural dormancy 444 supports its relevance to the biological phenomena under study. Based on its response to 445 hypoxia, *VvACS4* may belong to the second group, but based on the very limited regulation 446 induced by all other artificial or natural stimuli, it may be irrelevant to the dormancy cycle. 447 Its classification appears difficult also due to the presence of a sugar box in its promoter 448 sequence, which characterize the first subgroup (Table 1).

449 In examining the bud-expressed *VvACO* genes, only partial similarity to the above 450 pattern of segregating expression is evident. The response of *VvACO2* was very similar to 451 those of *VvACS1* and *VvACS6*, with significant induction of expression by AZ and HC, no 452 significant response to ethylene and hypoxia, increased expression during endodormancy

453 induction, and reduction in transcript level towards endodormancy release. *VvACO1* and
454 *VvACO4* shared significant induction by ethylene, as well as decreased expression
455 throughout the endodormancy cycle occurring in two characteristic steps. However, their
456 expression response patterns differed with respect to HC and AZ treatments, the response to
457 hypoxia, and in the timing of initial decrease during the endodormancy cycle. It should be
458 noted that *VvACO2* is expressed at relatively higher levels in naturally dormant buds, as
459 compared with *VvACO4,* and *VvACO1* transcript is barely detectable on microarray data
460 (Fasoli et al. 2012). Based on their transcriptional responses, we divided the bud-expressed
461 *ACO* and *ACS* genes into two subgroups, as previously listed. For reasons that will be further
462 discussed, we assigned *VvACO4* to Subgroup I.
463 Differential transcriptional responses of different members of the *ACS* and *ACO* gene
464 families in other species have been widely recorded. They include (1) differential expression
465 of the family members within a tissue, (2) differential expression of a given member across
466 tissues or developmental stages, and (3) differential responses of a given gene to abiotic
467 stresses (Harpaz-Saad et al. 2012; Van de Poel and Van Der Straeten 2014). The classic
468 example for differential behaviour of members of the *ACS* and *ACO* gene families has
469 been described in tomato, with a clear connection to the regulation of different stages of
470 fruit development. In the immature fruit, ethylene biosynthesis relies on transcription of
471 *SlACS1A* and *SlACS6* which is down-regulated by ethylene and thus declines when ripening
472 is initiated. Up-regulation of *SlACS2* and *SlACS4* transcript levels through positive feedback
473 by ethylene is responsible for the activation of an alternative ethylene biosynthesis system,
474 which operates during ripening and is known to be ethylene auto catalytic (Liu et al. 2015**)**.
475 Another relevant example is the differential behavior of members of the *Arabidopsis ACS*
476 gene family, documented in shoots and roots under hypoxia, with both positive and negative
477 effects of ethylene on expression of four of the 12 *ACS* genes induced under these conditions
478 (Peng et al. 2005).
479
480 ***VvACO2* may serve as the primary regulator of ethylene biosynthesis during the bud**
481 **dormancy cycle**
482 The presence of ACS transcript, and its product ACC, across the entire cycle (Fig. 4)
483 raises questions concerning the potential function of ACS activity as a primary regulator of
484 ethylene synthesis during the dormancy cycle, particularly in light of the significantly
485 decreased biosynthesis capacity towards the end of this cycle. This decrease, accompanied

486 by accumulation of ACC (Fig. 5 and 6), suggests that ethylene biosynthesis during the
487 dormancy cycle may be primarily regulated by the ACC oxidation capacity. The observation
488 of a negative correlation between the gradual changes in the transcript profile of *VvACO2*
489 and the bud dormancy status (Fig. 4), and a positive correlation with the ethylene
490 biosynthesis capacity (Fig. 5), raises the possibility that this gene serves as the primary
491 regulator of ethylene biosynthesis within the bud during dormancy, being upregulated by
492 environmental cues during dormancy induction and downregulated during endodormancy
493 release. The similarity of behavior between *VvACS1* and *VvACS6* implies that they may be
494 part of the same regulon.

495 A role for ACO as a primary regulator of developmental transitions has been
496 formerly documented for tomato ripening and seed germination (Corbineau et al. 2014; Liu
497 et al. 2015; Van de Poel and Van Der Straeten 2014). In tomato, the central relevance of
498 *SlACO1* in ripening is strongly supported by its regulation by several TFs that control tomato
499 ripening (Liu et al. 2015). In seeds, strong support for regulation of *ACO* expression by ABA
500 during germination indicates a central role for ACC oxidation in this process (Cheng et al.
501 2009; Linkies et al. 2009). Recent support for the potential importance of *VvACO2* in
502 grapevine bud dormancy is reflected by transcriptomic analyses documenting a significant
503 increase of this specific member of the *VvACO* family during SD-induced endodormancy of
504 *Vitis riparia* buds (Fennell et al. 2015), as well as in *Vitis vinifera* cv. Shine muscate buds
505 during endodormancy, as compared to paradormancy (Khalil-Ur-Rehman et al. 2017).

506 In light of the profile of *VvACO4* across the natural dormancy cycle and its response
507 to HC and AZ, we speculate that it acts in parallel with *VvACO2* and is affected by the same
508 regulon. Interestingly, expression of both *VvACO2* and *VvACO4* genes is induced during
509 berry ripening (Dal Ri et al. 2009; Fasoli et al. 2012).

510 *VvACO1*, which is sharply downregulated at the early stage of dormancy induction
511 (Fig. 4f), is probably irrelevant for ethylene biosynthesis during the dormancy cycle, since
512 it is regulated in an opposite direction (Fig. 5). However, this member of the VvACO family
513 may play a role in growing tissues, since increased transcript levels of *VvACO1* at bud burst
514 and phenological stages beyond are evident in the *Vitis vinifera* expression atlas based on
515 transcriptomic analyses (Fasoli et al. 2012). A role for *VvACO1* in growing tissues may be
516 also supported by its relatively high level of expression in the young berry, followed by a
517 decrease during ripening (Dal Ri et al. 2009; Fasoli et al. 2012).

518

519 **Ethylene production may be downregulated during dormancy release**

520 The increase in ethylene biosynthetic capacity during transition to dormancy 521 maintenance may define the window of ethylene function during the natural dormancy cycle. 522 The fact that the inhibitory effect of NBD on dormancy release is timing-dependent, and is 523 decreased when NBD is applied during late stages of endodormancy release, may support 524 the proposed window of ethylene influence. Of special interest is the limited ACO activity 525 observed during the period of actual endodormancy release, reflected by the inhibition of 526 expression of all the three bud-expressed *ACO* genes, the decreased ethylene biosynthesis 527 despite of the induction of *VvACS2* and *VvACS9*, and the accumulation of ACC during this 528 period. Together, the data implies that ethylene is not required at this stage.

529 Similar situations have been identified where ACS upregulation is accompanied by 530 ACO downregulation: in *Arabidopsis*, seed germination is associated with a reduced 531 expression of *ACO*, but accompanied by a transient induction of *ACS* (Linkies and Leubner-532 Metzger, 2012; Narsai et al. 2011). In apple buds, *ACS* transcripts are upregulated and *ACO* 533 transcripts downregulated during bud dormancy release (Kumar et al. 2017).

534 In light of the above, the relevance of *VvACS2* and *VvACS9* in the regulation of 535 ethylene biosynthesis during the dormancy cycle is questionable. Indeed, potential 536 upregulation of its activity during dormancy release may be speculated by significant 537 increase of expression only at a late stage of endodormancy release and by the 538 accompanying increase in the level of ACC. However, this same accumulation of ACC 539 suggests that ethylene production is inhibited, as supported by the parallel decrease in 540 availability of all three *VvACOs* and a decrease in ethylene biosynthesis capacity. One 541 potential scenario is that this accumulation of ACC may later serve for ethylene 542 production by *VvACO1* in order to regulate active shoot growth.

543 Based on the integration of the data and literature described so far, we speculate that 544 (1) the primary regulated step in ethylene biosynthesis during the dormancy cycle is ACC 545 oxidation by *VvACO2*, (2) an increased capacity of ACC oxidation during transition to 546 dormancy maintenance leads to increased endogenous ethylene biosynthesis over this 547 period, (3) the increased rate of ethylene synthesis further induces expression of *VvACO2*, 548 *VvACS1* and *VvACS6*, (4) at the time of transition to dormancy release, the levels of all the 549 bud-expressed *VvACO* transcripts are significantly decreased, and in agreement ethylene 550 production at this phase is significantly downregulated, and (5) a second set of ACS genes

551 (*VvACS2* and *VvACS9*) is induced. The induction of this set, in the absence of ACO activity,
552 may result in ACC accumulation.
553

554 **Potential sources for the behavior of subgroup I, and its reflection on a potential role**
555 **for ethylene in the regulatory network leading to dormancy release**

556 While it is clear that regulated biosynthesis of ethylene occurs during the natural
557 dormancy cycle, and in response to various artificial stimuli of dormancy release, the basis
558 for the differential response of the members of the *VvACS* and *VvACO* gene families is yet
559 to be determined, and the role of the increased biosynthesis capacity in the regulation of the
560 dormancy cycle is as yet unknown. Interestingly, the results of the *in silico* analysis of
561 transcription factor binding sites (Table 1, Fig. S4), which support the classification to two
562 subgroups based on different motifs shared between the members of each subgroup, revealed
563 that DREB, Sucrose Box and ABRE motifs are uniquely shared by subgroup I promoters.
564 These results raise the hypothesis that abiotic stress, ABA and sucrose may be potentially
565 involved in the regulation of group I genes during dormancy, as formerly suggested
566 (Tarancón et al. 2017 and references therein). Attractively, these motifs appear in
567 particularly high copy numbers in the promoter of the *VvACO2*, which is consistent with its
568 proposed central role in that regulon. In that context, attention should be drawn to the fact
569 that *VvACO2* expression peaks concomitantly with the sharp reduction in expression of *9-*
570 *CIS-EPOXYCAROTENOID DIOXYGENASE* (NCED), a key regulator in the biosynthesis
571 of ABA during dormancy induction, and with induction in the expression of *VvA8H-*
572 *CYP707A4*, responsible for ABA degradation in the woody bud (Zheng et al. 2015, 2018).
573 Examples of antagonistic effects between ethylene and ABA that trigger growth have been
574 previously documented. In submerged tissues, ethylene induces a decrease in endogenous
575 ABA levels, which leads to growth enhancement (Hoffmann-Benning and Kende 1992;
576 Saika et al. 2007). Similar antagonistic effects have been seen in the regulation of seed
577 germination: (1) ethylene overcomes the inhibitory action of ABA on germination, whereas
578 ABA also increases the ethylene requirement to release dormancy in various species, (2)
579 inhibition of germination by ABA is correlated with decreased *ACO* transcript level and
580 ethylene production, and (3) seeds of *Arabidopsis* mutant impaired in ethylene signalling
581 (*etr1*), have lower levels of *CYP707A2* and higher levels of *NCED* and ABA (Cheng et al.
582 2009; Corbinaue et al. 2014; Linkies et al. 2009).

583 In support for such potential antagonistic interactions in our current work (1) grape
584 buds exposed to ethylene signalling inhibitors, which inhibited bud break, also displayed
585 lower levels of *VvA8H-CYP707A4* and higher levels of *VvNCED1* (Zheng et al. 2015), (2)
586 maximal ethylene synthesis (Fig. 5) is synchronized with the sharp induction of ABA
587 degradation (Zheng et al. 2015), (3) antagonistic effects of application of ethylene and ABA
588 on bud break have been detected (Fig.1, Zheng et al. 2015). The hypothesis currently
589 proposed, in light of the data we accumulated so far and the current literature, is that at close
590 to deepest dormancy, induction of ethylene synthesis triggers a biochemical cascade that
591 antagonizes the inhibitory effect of ABA and mediates resumption of bud growth. This
592 hypothesis, which is also supported by recent reports related to bud and seed dormancy
593 (Tarancón et al. 2017; Xia et al. 2018) naturally calls for further study.
594
595 **Supplementary data**
596 **Table S1.** Schematic details of all the treatments in Fig 1.
597 **Table S2.** Primers used for qRT-PCR.
598 **Table S3.** Fold change values in Fig. 2.
599 **Figure S1.** Phylogeny of *ACS* homologues.
600 **Figure S2.** Phylogeny of *ACO* homologues.
601 **Figure S3.** Ethylene production in buds during hypoxia.
602 **Figure S4.** Position of 9 TFBSs in the promoter region of *VvACS* and *VvACO* genes.
603 **File S1.** Potential TFBSs identified in the promoter region of *VvACS* and *VvACO* genes.
604

605 **Acknowledgements**

606 This research was supported by the United States-Israel Binational Agricultural Research
607 and Development Fund (BARD grant no. IS-4639- 13 to EO, DG, and RO). We thank Felix
608 Shaya from the metabolomics unit of ARO for ACC quantitation. We also thank the China
609 Scholarship Council for joint PhD fellowships provided to Zhaowan Shi.

610

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Table 1. Transcription factor binding site (TFBS) analysis of the promoter sequences of the *VvACS* and *VvACO* genes. 9 TFBSs are listed that are enriched in the promoter region (0.6 - 2 kbp sequences upstream of the coding sequence) of *VvACS* and *VvACO* genes from subgroup I or subgroup II. The copy number of each motif within each promoter is indicated. Further details, including motif sequences and location are presented in Supplementary Figure S4 and Supplementary Data File1**.**

Matrix Family	Detailed Family Information	*p*-value	*VvACS1*	*VvACS6*	*VvACO2*	*VvACO4*	*VvACS2*	*VvACS9*	*VvACO1*	*VvACS4*
DREB	Dehydration responsive element binding factors	0.82	**1**	**6**	**3**	**1**	0	**1**	0	0
SUCB	Sucrose box	0.42	**2**	**4**	**3**	**2**	**1**	0	0	**5**
ARF3	Auxin Response Factor 3	0.83	0	**1**	**1**	**1**	0	0	0	0
PNRE	Plant nitrate-responsive cis-elements	0.25	0	0	0	**1**	**1**	**2**	**2**	0
AP2L	APETALA2-like transcription factors	0.91	0	**1**	0	0	**2**	**2**	**1**	0
ABRE	ABA response elements	0.97	**2**	0	**1**	0	0	0	0	0
BRRE	Brassinosteroid (BR) response element	0.81	**1**	**1**	0	0	0	0	0	0
CGCG	Calmodulin binding / CGCG box binding proteins	0.99	**1**	**1**	0	0	0	0	0	0
NCS2	Nodulin consensus sequence 2	0.45	**1**	**1**	0	0	0	0	0	0

Figure legends

Fig 1. Ethylene biosynthesis is induced by dormancy release stimuli and regulate bud break. Canes collected during endodormancy from a cv. Early sweet vineyard located in Jordan Valley were used to prepare single bud cuttings. (a) Ethylene levels were determined in air samples from 0.75-L jars each containing 30 cuttings treated with HC (3% Dormex®) or AZ (1% sodium azide). Jars containing Triton X-100 (0.02%)-treated buds served as the control. Three jars per treatment were sealed 2 h before air sampling at 6, 24 and 48 h after treatment. The sealed jars experimental system was termed SV system. Values are averages of three replications ± SE. (b) Bud break percentage was monitored for cuttings exposed to HC or combined $CoCl_2$-HC (3.6 mM $CoCl_2$) treatments. Triton X-100 -treated buds served as control. Treated cuttings were placed in water-filled open vases, exposed to forcing conditions at 22 °C under a 14/10 h light/dark regime. The open vases experimental system was termed OV system. Values are averages of fifteen groups, consisting of 10 cuttings each ± SE. (c) Bud break percentage was monitored for cuttings exposed to HC or combined STS-HC (0.2% or 2% STS) treatments, using the OV system. Values are averages of nine groups of 10 cuttings each ± SE. (d) Bud break percentages were monitored for cuttings sprayed with HC and placed for 48 h in sealed jars, in the absence (HC) or presence of NBD (HC-NBD; 5 ml NBD/jar). Values are averages of three groups of 10 cuttings each ± SE. (e) Bud break percentage was monitored for cuttings exposed to AZ or combined AZ-NBD treatments as detailed in D. Values are averages of nine groups of 7 cuttings. (f) Bud break percentage was monitored for cuttings exposed to HC, ethylene (100 ppm), or $KMnO_4$-control treatments, using the SV system. Values are averages of nine groups of 10 cuttings. (g) Bud break percentages were monitored at 24d after exposure to Ethrel (0.7%) treatment, using cuttings and the OV system. The values are averages of nine groups of ten cuttings each ± SE. For further details see (b). (h) Vines were pruned to three-node spurs and sprayed with Ethrel (0.8%) as detailed in Methods. Bars represent average bud break (at 42d after treatment) of the twelve grapevines in the four blocks for each treatment ± SE. (a, g, h) Statistical tests indicate differences between treatments and all time points. (b, c, f) Statistical tests indicate difference between treatments in each time point. Data points with different letters indicate significantly different values (*P* < 0.05) according to Tukey's HSD test. Asterisks between treatments indicate significant differences according to Student's t-test (*, *P* < 0.05). For additional details see Materials and Methods and Table S1.

Fig 2. The effect of HC and AZ on expression profile of the bud-expressed members of the VvACS and VvACO gene families. Total RNA was extracted from control, and from HC- and AZ-treated buds sampled at 12, 24, 48 and 96 h after treatments. Relative transcript levels were determined for *VvACS1* (a), *VvACS2* (b), *VvACS6* (c), *VvACS9* (d), *VvACS4* (e), *VvACO1* (f), *VvACO2* (g) and *VvACO4* (h), using qRT-PCR as described in Materials and Methods and normalized against *VvActin* and *VvGAPDH*. Values of qRT-PCR represent the mean ± SE of three biological replications, each with two technical repeats. Data points with different letters indicate significantly different values ($P < 0.05$) according to Tukey's HSD test.

Fig 3. The effect of exogenous ethylene and hypoxia on expression of the bud-expressed members of the VvACS and VvACO gene families. Total RNA was extracted from $KMnO_4$-control, HC, exogenous ethylene and hypoxia (1% O_2)-treated buds sampled at 24h after treatment. All the treatments were carried out in sealed jars. Transcript levels were determined for *VvACS1* (a), *VvACS2* (b), *VvACS6* (c), *VvACS9* (d), *VvACS4* (e), *VvACO1* (f), *VvACO2* (g) and *VvACO4* (h). For additional details see Fig. 1 and Fig. 2.

Fig 4. Expression profile of the bud-expressed members of the VvACS and VvACO gene families throughout the dormancy cycle. Canes were sampled weekly throughout the dormancy cycle. Single-node cuttings were prepared and randomly mixed. Nine groups of 10 cuttings were used for bud break monitoring as described in Fig. 1. The bud-break percentages at 21 d are shown (as line) in each panel. Values are averages of nine groups, consisting of 10 buds each ± SE. Total RNA was extracted from buds sampled weekly, upon arrival from the vineyard, and frozen. Relative transcript levels were determined for *VvACS1* (a), *VvACS2* (b), *VvACS6* (c), *VvACS9* (d), *VvACS4* (e), *VvACO1* (f), *VvACO2* (g) and *VvACO4* (h). For additional details see Fig. 1 and Fig. 2.

Fig 5. Changes in ethylene production throughout the dormancy cycle. Canes were collected separately from two cv. Early sweet vineyards located in Jordan Valley and analyses were conducted in parallel as describe in Fig. 1. The bud-break percentages at 21 d are shown (as line). At each collection point, and for each vineyard, three groups of 70 cuttings were placed in 2 L jars. For each vineyard, three jars per treatment were sealed 2 h before air sampling at 24 h after treatment. Ethylene level recorded is presented. Values are

the averages of six replications ± SE. All the other details are as in Fig. 1. Data points with different letters indicate significantly different values ($P < 0.05$) according to Tukey's HSD test.

Fig 6. Changes in ACC content in the buds throughout the dormancy cycle. Canes were sampled and bud break was monitored as described in Fig. 4. The bud-break percentages at 21 d is presented as line. Values are averages of nine groups, consisting of 10 buds each ± SE. ACC content was analyzed in bud extracts using a UPLC-Triple Quadrupole-MS. Values are averages of three biological replications ± SE. Data points with different letters indicate significantly different values ($P < 0.05$) according to Tukey's HSD test.

Fig 7. Differential effects of NBD treatment on bud dormancy release during the natural dormancy cycle. Single-node cuttings were prepared from canes that were harvested at several sampling dates throughout the dormancy cycle as described in Fig.4. The experiment was carried out as detailed in Fig. 1d. Values are the averages of three replications ± SE. In parallel with the actual bud-break data, calculated values are presented as the difference in bud-break percentages between HC and NBD-HC treatments. These values represent the mean of differences for seven monitoring time points (7, 10, 14, 18, 21, 24, and 28 d) for each sampling date.

Fig 8. The effect of natural and artificial dormancy release stimuli on the expression profile of bud-expressed homolog of the thiosulfate sulfurtransferase (TST) gene. The relative transcript levels were determined for *VvTST* (VIT_04s0023g03600) in response to HC and AZ treatment (a) and throughout the dormancy cycle (b). All the details are as in Fig. 1, 2 and Fig. 4.