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Developmental control of hypoxia during bud burst in grapevine

Running title: Developmental hypoxia in grapevine buds

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**Brief summary statement:**  
Hypoxia has recently emerged as an important developmental cue. Tissue oxygen levels are dynamic and highly regulated during bud burst in grapevine. Here we show that the timing and coordination of bud burst in grapevine displays hallmarks of oxygen-dependent signalling, including the oxygen-dependent N-end rule proteolysis of Group VII ETHYLENE RESPONSE FACTORS.

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Abstract

Dormant or quiescent buds of woody perennials are often dense, and in the case of grapevine (Vitis vinifera L.) have a low tissue oxygen status. The precise timing of the decision to resume growth is difficult to predict, but once committed the increase in tissue oxygen status is rapid and developmentally regulated. Here we show that more than a third of the grapevine homologues of widely conserved hypoxia-responsive genes, and nearly a fifth of all grapevine genes possessing a plant hypoxia-responsive promoter element were differentially regulated during bud burst, in apparent harmony with resumption of meristem identity and cell-cycle gene regulation. We then investigated the molecular and biochemical properties of the grapevine ERF-VII homologues, which in other species are oxygen labile and function in transcriptional regulation of hypoxia-responsive genes. Each of the three VvERF-VIIIs were substrates for oxygen-dependent proteolysis in vitro, as a function of the N-terminal cysteine. Collectively these data support an important developmental function of oxygen-dependent signalling in determining the timing and effective coordination bud burst in grapevine. In addition, novel regulators, including GASA-, TCP-, MYB3R-, PLT- and WUS-like transcription factors, were identified as hallmarks of the orderly and functional resumption of growth following quiescence in buds.

Keywords: Bud burst; hypoxia; redox; quiescence; woody perennial plant; transcriptome; meristem; cell cycle; ETHYLENE RESPONSE FACTOR; N-end rule proteolysis.
INTRODUCTION

The transition from heterotrophic to autotrophic metabolism is a defining feature of vegetative organogenesis in higher plants. The initial events during this transition are rapid and accompanied by considerable changes in the cellular and extracellular environment, notably light and oxygen-dependent cues, which function as powerful signals influencing cell function and fate. Bud burst in woody perennial species is an important ecological and agricultural context of this transition. Much of the global supply of fruits and nuts are generated from perennials and reliant on the successful timing and orchestration of bud burst, as it helps to synchronise flowering, fruit set and harvest. Several studies have modelled ecological requirements for bud burst (e.g. Chuine and Cour, 1999; Chuine, 2000; Linkosalo et al., 2006; Nendel, 2010), monitored the effect of particular chemical stresses (Halaly et al., 2008; Lavee and May, 1997; Ophir et al., 2009) or hormones (Aloni et al., 1991; Aloni and Peterson, 1997; Lavee and May, 1997; Rinne et al., 2011) on the degree of latency, or carried out detailed molecular analysis of changes during the preceding stages of dormancy onset and release (Mazzitelli et al., 2008; Rohde et al., 2007, Ruttink et al., 2007). However, few studies have investigated the physiology in relation to transcriptional change during the course of bud burst. In particular, few studies have investigated the physiology of tissue oxygen status, and accompanying oxygen-dependent metabolism and signalling. Tissue oxygen status has emerged as an important context for the regulation of developmental transitions (Considine et al., 2017) and acclimation to stress (Voesenek and Bailey-Serres, 2015), as both an essential substrate for aerobic metabolism as well as important medium of post-translational modification and signal transduction.
Grapevine is among the most-well studied woody perennials. The mature proleptic bud of grapevine is a complex organ comprising multiple meristems of differing organogenic states. Metabolism reaches a minimum during dormancy and begins to increase with hydration prior to bud burst, then accelerates rapidly with the onset of bud burst (Gardea et al., 1994; Meitha et al., 2015). It is commonly accepted that a chilling requirement to satisfy dormancy (endodormancy) precedes the rise in metabolism and onset of bud burst (Lavee and May, 1997). However, bud burst can be induced by a number of chemical and physical stresses, and indeed commercial practice commonly exploits one or other of these. Oxidative stress is a common theme among responses to these stresses, for example inhibition of mitochondrial respiration by azide, application of hydrogen peroxide, heat shock or hypoxia (see Or, 2009).

Treatment with heat shock or cyanamide (commonly used in production) resulted in similar induction of antioxidant, glycolytic and fermentation enzymes, metabolism and gene expression (Halaly et al., 2008; Ophir et al., 2009; Or et al., 2000). Hypoxia and other inhibitors of mitochondrial respiration also elevated the levels of hydrogen peroxide (Vergara et al., 2012a), while hypoxia or cyanamide induced the expression of a number of conserved hypoxia-responsive genes as well as a homologue of FLOWERING LOCUS T (Vergara et al., 2012b) and \( \alpha \)-AMYLASE (Rubio et al., 2014). More recently, cyanamide was shown to increase expression of a number of homologues of cyclins and cyclin-dependent kinases (CDK; Vergara et al., 2016), reduce levels of abscisic acid (Vergara et al., 2017) and promote the level of nitric oxide in the bud prior to bud burst (Sudawan et al., 2016). Although most of these studies were conducted in stress conditions, the weight of evidence and practicality to industry suggest an important role for oxygen-dependent metabolism and signalling in the natural process of bud burst.
A signalling role and mechanism for cellular hypoxia was recently established in plants (Gibbs et al., 2011; 2014; Licausi et al., 2011). Despite the prevailing focus on stress-induced hypoxia in the literature, regulated hypoxia has emerged as an important developmental cue, essential for meiosis (Kelliher and Walbot, 2012), seed germination (Gibbs et al., 2014) and photomorphogenesis (Abbas et al., 2015), and likely to be important in preserving genome integrity through maintaining quiescence within the stem cell niche of meristems (Considine et al., 2017). The bud is enclosed by lignified bracts, a structure resembling a seed coat. We have previously shown that the core of the quiescent bud (post-dormant) of grapevine has a low tissue oxygen status (internal partial pressure of oxygen, $pO_2 < 3$ kPa = 40 μM [O$_2$]; Meitha et al., 2015). During the early stages of bud burst, $pO_2$ increased in a regulated spatial manner and there was a spatial and temporal shift in the localisation of superoxide, from meristematic tissues to provascular tissues (Meitha et al., 2015). Although the outer scales are a barrier to oxygen diffusion, the $pO_2$ was clearly regulated by a balance of primary metabolism, as the $pO_2$ minimum was peripheral to the bud core. However, physiological hypoxia cannot be defined by an objective measure of $pO_2$ alone (Sasidharan et al., 2017). A number of conserved metabolic and transcriptional responses have been observed among species in response to physiologically hypoxic conditions. These include restriction of oxidative phosphorylation and increased starch and sucrose catabolism, substrate-level phosphorylation (glycolysis) and fermentation, and activation of the γ-aminobutyric acid (GABA) shunt (Voesenek and Bailey-Serres, 2015).

Many of the conserved responses to hypoxia are transcriptionally regulated, in particular by Group VII ETHYLENE RESPONSE FACTORS (ERF-VII; Gibbs et al., 2011; Licausi et al., 2011; Gibbs et al., 2014). Oxygen-sensitive ERF-VIIIs are targeted for proteolysis in the presence of oxygen.
and nitric oxide, as a function of their N-terminal motif (N-degron), a process termed N-end rule proteolysis. The consensus motif of the N-degron in ERF-VIIs, MCGGAI/L, is crucial for O$_2$/NO-dependent proteolysis. The degradation process is initiated by the cleavage of methionine from the N-terminus by a METHIONINE AMINOPEPTIDASE (MetAP), exposing cysteine for oxidation by the PLANT CYSTEINE OXIDASE1 or 2 (PCO1/2), a step which requires both oxygen and nitric oxide (Gibbs et al., 2014, Weits et al., 2014; White et al., 2017). Oxidised cysteine is then arginylated by an ARGINYL tRNA TRANSFERASE (ATE), which triggers interaction with E3 ligases (N-recognition) for ubiquitination and proteasomal degradation. In conditions of low oxygen and nitric oxide, the ERF-VIIs are stabilised and effect transcriptional regulation. For example, the stabilisation of RELATED TO APETALA2.12 (RAP2.12), one of five Arabidopsis thaliana (arabidopsis) ERF-VIIs, promotes transcription of several hypoxia responsive genes (Kosmacz et al., 2015). The RAP2.12 transcription factor directly binds to a specific motif in the promoter region of hypoxia responsive genes, consisting of a 12-bp cis-regulatory sequence (5'-AAACCA[G/C][G/C][G/C]GC-3'), known as the hypoxia-responsive promoter element (HRPE; Gasch et al., 2016). The motif presents 1-3 times in the promoter region of 39 out 49 core hypoxia responsive genes of arabidopsis (Mustroph et al., 2009), and is adequate for the activation of the AtPCO1 and LATERAL ORGAN BOUNDARIES PROTEIN41 (AtLBD41) RAP2.2 and RAP2.12 in arabidopsis (Gasch et al., 2016). Nevertheless, not all plant ERF-VIIs are substrates for N-end rule proteolysis, as the position of a downstream lysine and a tertiary structure that exposes the N-terminus also determines ability to function via the N-end rule (Gibbs et al., 2011).

The aim of the present study was to investigate the role of oxygen-dependent signalling in coordinating the effective resumption of transcription and metabolism during bud burst in
grapevine. We demonstrate that the rapid development of a physiologically hypoxic state is reflected in genome-wide transcriptional regulation during the first 24 h of the resumption of growth in the post-dormant buds. This was evident in the enrichment of numerous conserved hypoxia-responsive gene homologues, as well as genes with a hypoxia-responsive promoter element (HRPE), within 24 h, during which time tissue oxygen status remained low, as respiration, sugar consumption and starch hydrolysis increased. Further, we demonstrate that the Vitis ERF-VII transcription factors are substrates for oxygen-dependent N-end rule proteolysis in vitro, providing strong evidence for a developmental role of hypoxia signalling during bud burst in grapevine. Moreover, the transcriptional and physiological patterns were in harmony with the orderly resumption of an autotrophic capacity for growth and metabolism, even in the absence of light. This study provides a new benchmark for understanding the developmental regulation of bud burst in perennial plants. Moreover, these data implicate a facultative light requirement for bud burst in grapevine, which we explore in an accompanying manuscript (Signorelli et al., 2017).

MATERIALS AND METHODS

Plant material and physiological assays

Unless otherwise stated, water used throughout the study was Milli-Q® water (MQW, Merck-Millipore, Bayswater, Australia) and chemicals were analytical grade from Sigma-Aldrich (Castle Hill, Australia). Grapevine (Vitis vinifera var. Crimson Seedless) canes with quiescent buds intact were sampled from a vineyard in Western Australia (33.694 °S, 115.102 °E) in mid-winter and stored at 4 °C in the dark until required (4 months). This period of chilling was similar to that used to study dormancy release in raspberry (Mazzitelli et al., 2008) and enables the experimental approximation of a temperate-climate winter. Prior to the
experiment, the stored canes were removed from 4 °C and buds from node 5-7 were cut into intact single-node cuttings (explants), with ca. 10 mm cane above the node and 40 mm cane below. The explants were then planted in moist vermiculite and maintained in growth conditions with a constant temperature of 23 °C, in constant darkness or dark/ light treatment (12/ 12 h dark/ light photoperiod, 150 µmol quanta.m⁻².s⁻¹, LED tubes #108D18-V10, Everfine, Hangzhou, China) and deionised watered to field capacity (i.e. substrate water-holding with drainage), for a period of 3, 24, 72 or 144 h. The dark/ light regime commenced with 6 h dark, followed by alternating 12 h light and dark cycles, such that the 24, 72 and 144 h samples were taken in the middle of the light period. There were no visible differences in development between these time points, irrespective of light condition (Supporting Information Fig. S1). Both time points were classified at the bud-swell stage of EL2/ 3 (modified Eichorn-Lorenz (EL) scale; Coombe, 2004), i.e. prior to the emergence of leaf tips, which indicates bud burst sensu stricto (EL4). In these conditions, buds reached 50 % bud burst (EL4) within 7-10 days, with minimal difference between treatment conditions, however a trend towards acceleration of bud burst in the presence of light was evident (data not shown). For metabolite and transcript analysis, buds were excised from the cane immediately upon sampling, directly submerged in liquid nitrogen and stored at 80 °C until required.

Bud respiration (3 biological replicates of 4 buds each) were performed as described previously (Meitha et al., 2015). Internal partial pressure of oxygen (pO₂; 3 replicates of one bud each) was measured as described for buds without removing the outer scales in Meitha et al. (2015). It is worth mentioning that the degree of noise in the pO₂ data is due to structural heterogeneity of the bud. For this reason we presented the raw data as well as the spline fit and 95 % confidence intervals. Analysis of internal pO₂ was not reliable at 144 h in our hands.
and thus not presented. Moisture content was measured on the same buds used for respiration (3 replicates of 4 buds each), as follows: clean excised buds, without agar were weighed fresh (FW) then dipped in liquid nitrogen and stored at -80 °C or directly transferred into a freeze-dryer for >48 h before dry weight (DW) was recorded. Hydration was expressed as the percentage of water mass (g) per 100 g FW.

**Sugar, starch and chlorophyll analysis**

The buds for metabolite analyses were the same as those used for respiration and hydration (3 biological replicates of 4 buds each). For sugars and starch, extraction was performed as previously described (Gomez et al., 2002) with minor modifications and in a smaller scale. Freeze-dried buds were ground in an automated cryo-grinder (Geno/Grinder® 2010, SPEX SamplePrep, New Jersey, USA), with three 2.8 mm ceramic beads (Precellys, Scoresby, Australia) added to each replicate tube of buds. The extraction solution (MQW:methanol:chloroform, 5:5:3), containing 10 µM ribitol, was added into 25 mg of bud powder, vortexed rigorously and immediately dipped in liquid nitrogen. Ribitol was dissolved in MQW prior to preparing the extraction solution and included in the analysis as an internal standard during the gas chromatography (GC) of soluble sugars. The samples were then gently agitated at 4 °C for 20 min to disperse. After 10 min of sonication at 60 °C, the tubes were allowed to cool down on ice for 2 min. A two-liquid phase separation was obtained after 3 min of 13,000 xg centrifugation at 4 °C for 3 min. The polar supernatant was used for the subsequent processes of soluble sugars assay, while the pellet was washed in 100 % (v/v) methanol, dried in a speed-vac and retained for starch quantification as previously described (Gomez et al., 2003). For GC, bud extracts were derivatised by mixing with 20 mg.mL⁻¹ methoxyamine hydrochloride in pyridine at 65 °C for 120 min. The derivatives were injected
in split mode (10:1) into a GC (Agilent Technologies, 7890 GC System, Mulgrave, Australia) with a 30 µm capillary injection column (VP5-MS, 0.25 mm diameter and 0.25 µm film). Injection temperature was 280 °C and oven ramp was 325 °C, held for 3.5 min, 6 °C.min⁻¹ ramp to 215 °C, held for 1 min, 40 °C.min⁻¹ ramp to 320 °C, held for 22 min. Helium, the carrier gas, was at a constant flow rate of 0.9 mL.min⁻¹. Glucose, sucrose and fructose were identified and peaks quantified (Chemstation Quantitation Process Program; Agilent Technologies and Agilent MassHunter Workstation Software for Quantitative Analysis) by comparison with authentic standards and the internal standard.

For chlorophyll analysis, snap-frozen buds were ground to a powder in a 1.5 mL tube with a micropestle. Chlorophyll was extracted as pheophytin by addition of 0.2 N hydrochloric acid (Sigma-Aldrich), in a mass per volume ratio of 10 mg FW/100 µL at room temperature. After centrifugation at 14,000 x g for 8 min at 4 °C, the supernatant was separated for pheophytin quantification as described previously (Queval and Noctor, 2007). The pellet was kept to determine the dry weight. Chlorophyll was expressed as µg pheophytin per g DW.

For statistical comparison of respiration, moisture content, sugar, starch and chlorophyll, data were analysed by ANCOVA using a 2nd order polynomial to fit the time-response relationship (R-Core Team). Where the treatment factor was statistically significant, the residual standard error was used to estimate the 95 % confidence limits for the predicted means.

RNA extraction, library construction and sequencing

Samples were ground in liquid nitrogen to a fine powder. Total RNA extraction was performed using the Spectrum Plant Total RNA kit with an on-column DNase treatment according to the
manufacturer’s instructions (Sigma-Aldrich), followed by an isopropanol/acetate precipitation. The quality and integrity of the isolated RNA was tested using a NanoDrop 100 spectrophotometer (Thermo-Scientific, Scoresby, Australia) and agarose gel electrophoresis. Only RNA with an A_{260}/A_{280} ratio ≥1.95 was retained for analysis. RNA libraries were prepared with the TruSeq Stranded Total RNA with Ribo-Zero Plant kit according to manufacturer’s instructions (Illumina, Scoresby, Australia). Sequencing was performed on an Illumina HiSeq1500 instrument as 100 bp single-end runs. Raw data files have been deposited in NCBI BioProject (PRJNA327467; http://www.ncbi.nlm.nih.gov/bioproject/327467).

**Transcript data processing analysis**

Summary statistics of read length and read mapping are provided in Supporting Information Fig. S2. Quality control of raw reads was assessed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and adapter contamination and quality trimming was performed with Trimmomatic (Bolger et al., 2014). BBMAP was used to remove PCR duplication (http://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/).

Resulting reads were aligned to the whole 12X V1 *Vitis vinifera* PN40024 reference genome (Jaillon et al., 2007) using Kallisto (Bray et al., 2016). Gene expression profiling was performed using edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015) Bioconductor packages.

The counts matrix obtained from Kallisto was read in edgeR and the quality of the replicates was checked using Pearson’s correlation (0.90-0.98). Raw data was then normalised using the Trimmed Mean of M-values method (TMM; Supporting Information Table S1) and the log_{2} Transcripts-Per-Million (logTPM) was obtained. A linear model (limma) was then applied to do a differential expression analysis (Supporting Information Table S2). *P*-values were
corrected for multiple-testing using the Benjamini-Hochberg’s method (FDR P≤0.01) (Benjamini and Hochberg, 1995). Data were then filtered considering a fold change (FC|2.5|).

**Functional enrichment analysis and principal component analysis (PCA)**

Three lists of genes differentially expressed from each comparison, up- and down-regulated genes and both (up- plus down-regulated genes) were used to perform a functional enrichment analysis using FatiGO (Al-Shahrour et al., 2004), in order to identify significant functional categories in Babelomics 5 ([http://babelomics.bioinfo](http://babelomics.bioinfo)), following a grapevine-specific functional classification of 12X V1 predicted transcripts (Grimplet et al., 2012), which was adapted from the MIPS FunCatDB database (Ruepp et al., 2004), with modifications according to the GO database (Ashburner et al., 2000). Fisher’s exact test was carried out in FatiGO to compare each study list with the list of total non-redundant transcripts housed in the grapevine 12X V1 gene predictions (Grimplet et al., 2012). Significant enrichment was considered for P-values (P≤0.01) after Benjamini and Hochberg correction for multiple testing (Benjamini and Hochberg, 1995). Finally, the covariance matrix of the PCA was built using the prcomp() function of R. Eigenvalues and eigenvectors were calculated using the factoextra library of R.

**Cis-regulatory motif analysis and BLAST**

Grapevine promoter sequences (2.5 kb upstream of the coding sequence) of differentially expressed genes (DEG) for the first two temporal comparisons in continuous darkness (3/0 h, 24/3 h) were retrieved from Regulatory Sequence Analysis Tools (RSAT, [http://floresta.eead.csic.es/rsat/](http://floresta.eead.csic.es/rsat/)); to prevent the overlap with neighbouring genes, the noorf option was selected. Enrichment of cis-regulatory elements and the Hypoxia-Responsive
Promoter Element (HRPE; Gasch et al., 2016) were performed using Homer (v4.9; Heinz et al., 2010). The evolutionarily conserved set of hypoxia-responsive genes from arabidopsis (Mustroph et al., 2009) was used to identify homologous sequences in the grapevine genome 12X V1. BLAST tool from CRIBI was used (blastn: http://genomes.ccrib.unipd.it/grape/), where all the sequences with an e-value ≤ 35 were selected to further analysis.

Plasmid construction, in vitro protein translation and immunodetection

In vitro assays to determine the capacity of VvERF-VII proteins to act as substrates for N-end rule proteolysis was performed as previously described, with modifications (Gibbs et al., 2011). The cDNA synthesis was made using Tetro cDNA Synthesis Kit according to the manufacturer’s instructions (Bioline, Sydney, Australia). Specific primers were used to amplify ERF-VII genes of grapevine (VvERF-VII) with addition of EcoRI restriction site in the 5’ and XbaI in 3’ terminal, to aid construction into pTNT-3xHA vector (Gibbs et al., 2011). The coding sequence of each gene was fused with 3x haemagglutinin (HA) tag for Western blot detection. Site-directed mutations to alter the penultimate codon of the N-degron sequence of VvERF-VII proteins were performed according to manufacturer’s instructions (QuickChange II, Agilent Technologies, Mulgrave, Australia), replacing the N-terminal cysteine (Cys2) with alanine, which attenuates Cys/Arg-N-end rule proteolysis (Gibbs et al., 2011). Sequence validation was performed by Sanger sequencing of cloned constructs. All unique primers used for this experiment are listed in Supporting Information Table S3.

Translations were performed by using TNT-T7 Coupled Reticulocyte Lysate System (Promega, Alexandria, Australia) in ambient and zero oxygen conditions in a customised oxygen chamber. The normoxic condition was air-equilibrated (pO₂ ca. 21 kPa, ca. 21 %

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and the anoxic condition was established by flushing with 100 % N₂ gas. The pO₂ was quantified at the start and end of incubation using micro-respiration sensors (Unisense, Aarhus, Denmark), verifying that pO₂ remained unchanged within detection limits. After establishing oxygen conditions, reaction tubes were placed in the chamber, and the whole chamber was sealed and submerged in a water bath at 30 °C. For all translation experiments, the reactions were first incubated at 30 °C in normoxia for 30 min to produce the initial amount of protein. After the addition of 0.1 mM cycloheximide to inhibit protein synthesis, a T0 sample was taken immediately. The reaction tubes were then subject to oxygen treatments at 30 °C. At 30 min and 60 min of incubation subsamples were taken and the reaction terminated by placing the tubes on ice with SDS buffer. SDS-PAGE was performed according to the method of Laemmli (1970) in 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad, Gladesville, Australia). Each well was loaded with 12.5 μL of the in vitro reaction product, plus Laemmli sample buffer. Equal loading was confirmed by chemiluminescent detection of the proteins after resolving in precast gels and equal transfer was checked after the transfer onto PVDF membrane (Supporting Information Fig. S3). Protein immunoblotting was performed as described in Gibbs et al. (2011) with the modification of primary antibody anti-HA titre of 1:20,000 (Sigma, H9658, monoclonal Anti-HA antibody, mouse) and chemiluminescence detection was made using a ChemiDoc™ MP system (BioRad). Experimental conditions were validated with positive and negative controls from arabidopsis (Gibbs et al., 2011) and the use of the proteasome inhibitor MG132 (data not shown).
RESULTS AND DISCUSSION

PHYSIOLOGY AND METABOLITE ANALYSIS

Resumption of primary metabolism and tissue oxygenation is largely independent of light

Temperate woody perennials such as grapevine are commercially propagated from cuttings of bud-wood bearing dormant proleptic buds, which comprise an embryonic shoot enclosed in layers of bracts and scales. The bud-wood is typically stored in the dark for several weeks or months at ca. 4 °C to overcome dormancy and encourage a more uniform resumption of growth. We studied the resumption of growth in such buds using single-node explants, which have been widely used as a model system to study the physiology of early shoot and inflorescence development in grapevine (Antolín et al., 2010; Geny et al., 1998; Lebon et al., 2005; Mullins, 1966; Mullins and Rajasekaran, 1981; Ollat et al., 1998). To investigate the physiological changes during the resumption of bud growth, we assessed respiratory CO₂ production and moisture content of buds during the first 144 h of growth following removal from 4 °C. To manipulate growth, particularly primary metabolism, in non- or low-stress conditions, we contrasted the effects of the presence and absence of light on bud burst. Light incidence serves signalling and substrate functions that are crucial to meristem activity, enabling or augmenting a number of important plant developmental transitions via photoreceptor-mediated signalling and the photosynthetic generation of oxygen and sucrose (Considine, 2018; Signorelli et al., 2018). Light incidence is required for bud outgrowth in many species (Leduc et al., 2014). While grapevine buds apparently do not require light to burst, light incidence does have a strong influence on inflorescence initiation and fruitfulness within buds (Buttrose, 1970). In this study we were primarily interested in the role of light as a substrate that promotes photosynthetic oxygen synthesis. The incidence of light has a strong influence on primary metabolism and tissue oxygen tension in photosynthetic seeds.
(Borisjuk and Rolletschek, 2009), and as hypoxia was a key focus of this study, we hypothesised that the degree of hypoxia (low bud oxygen tension) would be directly influenced by light. As such, explants were grown in continuous darkness (D) or a dark/ light photoperiod (DL; 12/ 12 h). There were no visible phenotypic differences between these two time points, irrespective of light condition; all experimental buds were at the bud-swell stage (EL2/3), prior to bud burst (EL4; Coombe, 2004; Supporting Information Fig. S1). The increase in moisture content and respiration followed a similar pattern, with significant developmental effects but no significant impact of light (P≤0.05; Fig. 1a). Changes in chlorophyll content were not statistically significant, although there appeared to be an interaction between treatment (+/- light) and time, such that the net chlorophyll content was only maintained in the presence of light. Temporal changes in sugar and starch contents were significant but the effect of light was only significant in interaction with time and generally minor. Glucose and fructose concentration appeared to peak at 72 h, followed by a decline, which was also reflected in the decline in starch content (Fig. 1a).

Tissue oxygen status (pO₂) provides a spatial resolution of oxygen metabolism. The bud is relatively hypoxic at the earliest stages of bud burst (3 h), followed by a considerable oxygenation within 24 h (Meitha et al., 2015). In the previous report, however, buds were grown in continuous darkness only. Here we show there was negligible difference in the pO₂ minimum of the profile, or the pO₂ at the core of the bud at 72 h in the DL cf. D conditions (Fig. 1b).

Taken together, these data show that the resumption of primary metabolism is rapid during bud burst, with an increase in respiration and starch hydrolysis within 72-144 h. We earlier
hypothesised that contrasting the presence and absence of light would significantly affect oxygen-dependent physiology and metabolism. Of the measures we assayed, only chlorophyll content appeared to be affected and this was not significant, and thus we conclude that the resumption of metabolism during bud burst in grapevine was unaffected by the presence of light.

TRANSCRIPTOME ANALYSIS

We performed short-read RNA sequencing in order to further investigate the dynamics of transcriptional regulation during bud burst over six days, which precedes leaf emergence (0, 3, 24, 72, 144 h). A dendrogram and PCA of the normalised TPM data demonstrated that replicates of each condition clustered together and that the first and second components discriminated conditions by time and treatment, accounting for ca. 40% of the variance collectively (Supporting Information Fig. S4a, b). The transcriptomes at each time point were contrasted against the preceding time for each treatment, allowing interpretation of developmental transitions and the impact of light. The number of differentially expressed genes (DEGs, FDR≤0.01) was considerably greater in the first 3 and 24 h (3/ 0 h, 24/ 3 h) than between subsequent stages, despite the greater lapse in time in later stages (Supporting Information Fig. S4c, d). The only remarkable difference in number and magnitude of DEGs in the presence cf. absence of light occurred in the final stage (144/ 72 h), when a greater number of genes were differentially regulated in the DL condition than D, particularly at ≥10-fold (Supporting Information Fig. S4c, d). For further analysis we constrained the data to FC≥2.5, resulting in a core set of 4022 DEGs represented in at least one comparison (Supporting Information Fig. S4d). Functional annotations were assigned to the core set of DEGs against the Vitis vinifera reference genome (Grimplet et al., 2012). Of the total core set,
14.9 % DEGs (846) were matched to an ‘unknown’ branch category function (Supporting Information Fig. S4e). Of the twelve branch categories represented in the remaining 3176 DEGs, primary metabolism (28.9 %), transport overview (12.6 %) and signalling (10.2 %) comprised over 50 % of the assigned primary function of all genes. For transcripts that belonged to more than one functional category, each was taken into account.

Further analysis and functional enrichment of transcript data was performed in the condition of continuous darkness. Dynamic transcriptional regulation is evident in the first two temporal comparisons (3/0 h, 24/3 h), including organelle and cell biogenesis, cell division, metabolism and trafficking. By comparison, only primary metabolism was enriched in the 72/24 h comparison and no categories were enriched at 144/72 h (Fig. 2a-d). The strong regulation of genes linked to photosystems in the first two stages supports a conclusion that quiescent buds are primed to develop autotrophic metabolism even in the absence of light, and that temperature rather than light is the primary trigger for bud burst in grapevine.

Expression profiles and unique identifiers of all transcripts subsequently discussed are shown in Supporting Information Fig. S5.

**Stage-specific transcriptional regulation of bud burst**

A Venn diagram of stage-specific DEGs shows very few transcripts were commonly regulated between successive stages (Fig. 2a). Only 13 of the 996 transcripts upregulated in the 3/0 h transition were further upregulated at 24/3 h. In fact, this number remained 13 even when the FC criteria was relaxed to consider genes not significantly downregulated at 24/3 h (i.e. FC≥-2.5; Supporting Information Table S4). Among these were homologues of a SERINE CARBOXYPEPTIDASE, HISTONE H4, and a GASA-like protein (GIBBERELLC ACID-STIMULATED
ARABIDOPSIS; Supporting Information Fig. S5). These transcripts were representative of the major regulated functional categories in the 3/0 h transition (Fig. 2a), and particularly the strong signature of protein synthesis, folding and trafficking, as well as regulation of the cell cycle, glycolysis and fermentation, calcium, and gibberellic acid (GA) signalling. Only one transcript was commonly repressed between the first two stages, a \( \text{GA3-BETA-HYDROXYLASE} \), encoding an enzyme which catalyses the synthesis of GA4 and which is repressed by feedback inhibition by GA (Middleton et al., 2012). Looking to the transcripts specifically upregulated in the 3/0 h transition, resumption of the cell cycle was evident. In particular, homologues of a \( \text{HISTONE H3, CYCLIN-D (CYCD), CELL DIVISION CYCLE20 (CDC20), CDC7 and PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA)} \). The CYCD and PCNA are acutely involved in replication licensing and have been shown to functionally interact in pea and arabidopsis (Shimizu and Mori, 1998; Strzalka et al., 2015). Meristem functions were also implicated by the strong induction of two MADS Box \( AGL24 \) homologues, as well as \( \text{VERNALIZATION INSENSITIVE3 (VIN3)} \), described further below. Several auxin-responsive transcripts were also uniquely upregulated at 3 h, including two homologues of \( \text{SMALL AUXIN UP RNA (SAUR)} \), an \( \text{AUXIN BINDING PROTEIN (ABP)} \) and \( \text{AUXIN RESPONSE FACTOR (ARF)} \). Genes specifically repressed at 3 h included several transcripts associated with cell cycle or growth arrest, such as two KIP-related \( \text{CYCLIN-DEPENDENT KINASE INHIBITOR} \) homologues, \( \text{DOG1 (DELAY OF GERMINATION1)} \) and \( \text{BAK1 (BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE1)} \). Transcripts that were specifically induced at 3 h but repressed at 24 h included a \( \text{CDC6} \), and a \( \text{CDT1A} \) cyclin dependent protein kinase, and two additional \( \text{GASA} \) homologues. The arabidopsis \( \text{CDC6} \) and \( \text{CDT1A} \) are reported targets of the E2F transcriptional activator of G1/S phase genes, and directly involved in licensing DNA replication (Castellano et al., 2004). \( \text{GASA} \) proteins are widely reported targets of GA signalling, serving developmental and
meristem-related functions, and several have been shown to be redox-regulated (Sun et al., 2013; Trapalis et al., 2017). The prevalent signature of GA signalling immediately upon the induction of bud burst, and in the absence of light, is notable given that GA is central to the photocontrol of bud outgrowth in Rosa sp. (Choubane et al., 2012). A HYPOXIA RESPONSIVE2 (HRE2, also named ERF071) and two ALCOHOL DEHYDROGENASE (ADH) homologues were also specifically regulated in this way, as described in further detail below.

A number of common pathways were implicated in the subsequent transition of 24/3 h, including representing the GA and auxin-related pathways. Regulation of the cell cycle was also enriched among upregulated transcripts, however here it was mitotic genes such as those encoding A- and B-type cyclins and CDK that were particularly expressed (transcripts of three CYCB, three CDKB, three CYCA, one CDC20, a WEE1 protein kinase and a DIMERIZATION PARTER- (DP)-like DEL1 were elevated). Other uniquely upregulated genes at this transition included transcripts related to meristem functions, for example a MADS Box SHORT VEGETATIVE PHASE (SVP) and a BRUSHY1 (BRU1), a CONSTANS-like homologue (COL16) as well as numerous genes encoding components of photosystems, light-harvesting complexes and other regulatory proteins involved in chloroplast biogenesis, discussed further below.

Genes differentially regulated between the final two transitions included some common pathways with earlier stages, for example auxin- and GA-responsive transcripts and a histone, upregulated at the 72/24 h transition, while uniquely regulated transcripts were largely associated with primary metabolism, discussed below.
Glycolysis, fermentation and hypoxia responsive transcripts are elevated early in bud burst

Looking more specifically to transcriptional control of pathways implicated in oxygen-dependent signalling and metabolism, transcripts associated with substrate-level phosphorylation and fermentation were upregulated at 3 h (e.g. ALPHA AMYLASE, three SUCROSE SYNTHASE, a VACUOLAR INVERTASE, three PHOSPHOFRACTOKINASE, two GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE, a PYRUVATE KINASE, three ADH and two PYRUVATE DECARBOXYLASE transcripts). Many of these have been identified as hallmarks of hypoxia-induced metabolism (Voeseke and Bailey-Serres, 2015) as well as stress-induced metabolism during the release of bud dormancy or quiescence (Or, 2009; Considine and Considine, 2016). By 24 h, glycolytic transcripts remained at higher expression levels, however transcripts involved in fermentation were largely reduced relative to 3 h. Perhaps surprisingly, a GLUTAMATE DECARBOXYLASE, encoding a central enzyme of the GABA shunt was repressed at 24 h, following no change at 3 h. As the GABA shunt is a common feature of hypoxia-induced metabolism and release of bud dormancy; it may be that this pathway was already engaged at 0 h, and hence unchanged at 3 h before repression at 24 h. Nevertheless, a need for additional substrates to fuel metabolism at 24 h is suggested in the elevated expression of components of gluconeogenesis (e.g. FRUCTOSE 1,6 BISPHOSPHATASE and NADP-DEPENDENT GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE). Although there was no clear direction of transcription regulation of metabolism in the last two transitions, there was evidence that transcripts coding for glycolytic and fermentation functions were repressed.

The differential regulation of HRE2 described above is particularly relevant to the aims of this study. The arabidopsis HRE2 is one of the ERF-VII substrates for the O₂/·NO-dependent N-end rule of proteolysis (Gibbs et al., 2011; 2014; Licausi et al., 2011), and one of only two that are
known to respond transcriptionally to hypoxia. In addition, there was strong upregulation of an HYPOXIA-RESPONSIVE homologue and two HYPOXIA UPREGULATED1 HSP70, specifically at 3 h, further indicating the development of a hypoxic state early during bud burst. VIN3, described above has also been shown to be required for acclimation to hypoxia, as it is for the vernalisation response in winter annuals (Bond et al., 2009). Strikingly, by 24 h, expression of two homologues of the SUCROSE NONFERMENTING1-RELATED KINASE1 (SnRK1) catalytic subunit AKIN gamma were elevated, which is consistent with the earlier identification of a SnRK1-homologue during dormancy release in grapevine buds (Or et al., 2000). SnRK1 is a major hub of energy homeostasis (Baena-Gonzalez et al., 2007) and shown to be induced in hypoxic conditions, acting as a positive regulator of sugar response elements in the promoter region of α-AMYLASE genes, enabling substrate supply to glycolysis and fermentation (Lee et al., 2014). In addition, in our data four homologues of CIPK (CBL-INTERACTING PROTEIN KINASE) genes, which positively regulate SnRK1-mediated starvation responses in plants were induced at 3 h and/or 24 h. This is also compatible with the strong evidence for GA signalling, which can also promote amylase expression. In contrast, there was no clear direction of the regulation of trehalose-6-phosphate abundance, which is a major counterpart to SnRK1 in energy homeostasis and signalling, as genes coding for the synthesis and catabolism of trehalose-6-phosphate were differentially regulated over this period.

The plant hormone ethylene has been widely implicated in acclimation to hypoxia (Voesenek and Bailey-Serres, 2015). A number of homologues of ethylene synthetic and signalling genes were differentially regulated in the first 24 h, including an ACC SYNTHASE, five ACC OXIDASE transcripts and two homologues of the ETHYLENE OVERPRODUCER1 (ETO1), encoding a negative regulator of ACC SYNTHASE (Supporting Information Table S4). Additionally, a
number of AP2/EREBP (APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN) homologues were regulated, described further below. Although the ACC SYNTHASE levels were reduced at 3 h, so too was an ETO1, while three of the ACC OXIDASE transcripts were elevated. These transcripts were unchanged at 24 h, indicating that expression levels were maintained, and hence suggesting that ethylene synthesis was elevated in the first 24 h of bud burst. Together, these data provide strong evidence for the transcriptional activation of a state of metabolic control that is typical of hypoxia-induced responses.

Perturbed redox metabolism and signalling suggests increased redox activity early during bud burst

Redox metabolism and signalling have also been implicated in cross-talk or direct regulation of hypoxia, metabolic, hormone and cell-cycle regulating pathways in plant development (Considine and Foyer, 2014; Considine et al., 2017). Several studies highlighted in the introduction have demonstrated induction of hydrogen peroxide as well as numerous classical antioxidant enzymes during the stress-induced release of bud dormancy or quiescence (Or, 2009; Considine and Considine, 2016). In our data, an increase in cellular oxidation was suggested in the first 3 h and further at 24 h, by the elevated expression of two ALDEHYDE DEHYDROGENASE, a number of PEROXIDASE, including three ASCORBATE PEROXIDASE, and a number of THIOREDOXIN-like homologues (Supporting Information Table S4; Fig. S5). Nevertheless, a homologue of THIOREDOXIN h, previously shown to be induced by cyanamide in grapevine buds (Halaly et al., 2008), as well as three GLUTAREDOXIN homologues were repressed, relative to 0 h. Transcripts encoding CATALASE, SUPEROXIDE DISMUTASE, GLUTATHIONE REDUCTASE or other enzymes of the ascorbate-glutathione cycle and related soluble antioxidants, some of which have previously been shown to respond
transcriptionally to stress-induced release of bud dormancy or quiescence (Or, 2009), were not differentially regulated. Taken together these data suggest that redox homeostasis and signalling is a key developmental feature of bud burst in grapevine, however some particular redox enzymes, such as CATALASE, THIOREDOXIN h, may be more responsive to particular stress conditions such as cyanamide, where previous studies have consistently shown their transcriptional induction. In contrast to the activation of glycolysis, fermentation and other hypoxia-related metabolic and signalling processes, transcriptional regulation of redox homeostasis became more pronounced following the first 3 h of bud burst, suggesting that hypoxia-induced signalling preceded the substantial development of redox signalling.

Transcription factors and cis-regulatory elements indicate response to stress and re-establishment of meristem identity precede mitosis during bud burst

Several transcription factor families were enriched in the first 24 h, including AP2/EREBP, TCP (TEOSINTE BRANCHED1/ CYCOIDEA/ PCF1) and GASA family transcription factors at 3 h, and MYB and B-type ARR (ARABIDOPSIS RESPONSE REGULATOR) transcription factors at 24 h (Fig. 2, Supporting Information Table S4, Fig. S5). To gain further insight to the prevailing transcriptional control during bud burst we carried out enrichment for known cis-regulatory motifs in the 2.5 kb upstream region of the DEGs. We retained focus on the first 24 h (3/0 h, 24/3 h) in darkness. Several motifs were commonly enriched throughout the first two transitions such as the E-box and three bZIP motifs (bZIP16, bZIP68, AREB; Fig. 3a; Supporting Information Table S5). The prevalence of these binding motifs is understandable: E-box motifs are most-well understood in the establishment of a circadian rhythm (Seitz et al., 2010); AREB motifs are known to function in dehydration stress (Urano et al., 2017); while the bZIP16 and bZIP68 binding motifs are involved in the redox-dependent regulation of gene expression.
during photomorphogenesis (Shaikhali et al., 2012). Also of interest, although less significantly enriched, were binding motifs for ELONGATED HYPOCOTYL (HY5) and PHYTOCHROME-INTERACTING FACTORS (PIF), again highlighting the constitutive activation of autotrophic development (Supporting Information Table S5).

Particular binding motifs were enriched in a stage-specific manner (Fig. 3b). At 3 h, BCP1 (BASIC PENTACYSTEINE1), FAR1 (FAR-RED IMPAIRED RESPONSIVE) and CAMTA1 (CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR1) binding motifs were highly enriched, together with several MYB and AP2/EREBP motifs. Both BCP1 and FAR1-like transcription factors have been implicated in a number of meristem and developmental functions, converging on SHOOT MERISTEMLESS, an important regulator of meristem identity and cell cycle genes in the shoot apical meristem (Simonini and Kater, 2014; Aguilar-Martínez et al., 2015). The enrichment of CAMTA1 motifs may be a product of the temperature transition at this stage, although calcium signalling and responses were among the highly regulated transcripts at 3 h (Supporting Information Table S4), and have previously been implicated in the release of bud dormancy in grapevine (Pang et al., 2007).

At 24 h, fewer binding motifs were uniquely enriched, approximately half the number found at 3 h, however three MYB3R motifs were considerably enriched. Plants possess only a small number of three-Myb-repeat proteins (MYB3R), and many of those in arabidopsis have been shown to directly regulate canonical mitotic proteins (Haga et al., 2011). Knockout and biochemical studies showed that the arabidopsis MYBR1 and MYB3R4 act as transcriptional activators of G2/M phase genes, while MYBR3 and MYB3R5 act as repressors, possibly functioning to promote the endocycle and differentiation (Haga et al., 2011; Kobayashi et al., 2011).
2015). In our data, binding motifs for the MYB3R1, MYB3R4, MYB3R5 were enriched. This may correlate well with the elevated expression of mitotic A- and B-type cyclins and CDKs at 24 h, as indicated earlier. Additional motifs of interest that were particularly enriched at 24 h included the WUSCHEL1 motif. WUSCHEL1 represents a conserved family of homeotic transcription factors that are expressed in meristems, and particularly important for constraining the stem cell niche, together with other positional cues, including PLETHORA1 (PLT1) in the root apical meristem. Together these data suggest that predominantly, the transcriptional response to stress/ stimuli and re-establishment of meristem identity precede the resumption of mitosis during bud burst. Although expression of genes coding for DNA replication licensing were elevated at 3 h, the considerable number of A- and B-type cyclins and CDKs that were elevated at 24 h supports this conclusion, particularly relating to mitosis.

**Conserved hypoxia-responsive genes and representation of a hypoxia-responsive promoter element (HRPE) suggest oxygen-dependent signalling is important during bud burst**

Previous studies have identified a set of hypoxia-responsive genes conserved across phyla, which corresponds to 49 in arabidopsis (Mustroph et al., 2009). In addition, a unique plant HRPE was recently identified and shown to function in oxygen-dependent signalling (Gasch et al., 2016). We used BLAST to identify a set of 122 *Vitis* homologues of the 49 arabidopsis hypoxia-responsive genes and queried the expression profiles of our transcript data, using the existing FC and FDR criteria. 44 of the 122 *Vitis* homologues (36 %) were differentially expressed in the first 24 h (Fig. 4 upper panel). More than half of these were elevated at 3 h, and many were unchanged or only moderately repressed at 24 h (relative to 3 h). Many of the elevated transcripts at 3 h have already been described, particularly with regard to glycolysis, fermentation and hypoxia, including HRE2, but notable also were four homologues
of *PLANT CYSTEINE OXIDASE*, which function upstream of the ERF-VII in O$_2$/NO-dependent N-end rule proteolysis (Weits et al., 2014). The HRPE is a specific and recently defined plant *cis*-regulatory motif that is not present in the databases used by HOMER. We identified a total of 252 genes in the *Vitis* genome with one or more HRPE in the upstream promoter region. Of these, 55 were present in the total core set of 4022 DEGs, and 47 of these were differentially expressed in the first 24 h, i.e. nearly 20 % of the whole HRPE-containing transcriptome (Fig. 4). This included 9 of the 44 *Vitis* homologues of conserved hypoxia-responsive genes (not duplicated in the lower panel of Fig. 4). Furthermore, many of the HRPE-containing genes that were differentially regulated at 3 h were not further changed at 24 h. Although cautious interpretation is warranted, these data suggest oxygen-dependent signalling is most important within 3 h of the resumption of growth during bud burst, however there is no striking repression of these, suggesting that hypoxia-dependent gene expression may be maintained throughout the first 24 h. This is consistent with the persistence of low oxygen status during the first 24 h in the bud, followed by oxygenation (Meitha et al., 2015). The presence of an HRPE in one of the induced GASA-like transcripts is particularly interesting given the prominence of these transcription factors in data already presented, as well as the redox-regulation of several members of this family in *arabidopsis* (Sun et al., 2013; Trapalis et al., 2017). Hence GASA-like transcription factors may provide an important point of convergence between GA-, oxygen- and redox-dependent signalling.

**IN SILICO AND IN VITRO PROPERTIES OF THE GRAPEVINE GROUP VII ERFs (VvERF-VII)**

Given the elevated expression of a *Vitis* homologue of *HRE2* and the enrichment of the HRPE motif in promoter regions of DEGs, we sought to investigate the potential of the *VvERF-VII* homologues to function as substrates for O$_2$/NO-dependent N-end rule proteolysis. This
would potentially implicate them as regulators of the developmental hypoxia observed during bud burst. Analysis of transcript data identified minor genotype variations in the coding sequence of the three VvERF-VII previously identified (Licausi et al., 2010). The three VvERF-VII transcripts in this study were designated VvERF058.1 (VIT_05s0077g01860), VvERF057.1 (VIT_07s0005g00820) and VvERF059.1 (VIT_09s0002g00470) according to the nomenclature of Licausi et al. (2010). The APETALA2/ERF domain and N-degron motif were found in the deduced amino acid sequence of each VvERF-VII (Fig. 5a). Although only VvERF057.1 was differentially expressed in data presented here, not all plant ERF-VIIs are transcriptionally regulated by oxygen levels (Mustroph et al., 2009). In arabidopsis for example, only the HRE1 and HRE2 are transcriptionally induced under hypoxia.

Hierarchical cluster analysis (Fig. 5b) of group ERF-VII proteins from the present study and Licausi et al. (2010), and the ERF-VII proteins of arabidopsis, rice and maize show that VvERF058.1 and VvERF058 were identical and possessed least similarity to the other ERF-VII of grapevine or other species presented. High similarity was found between VvERF057.1 and VvERF057, and VvERF059.1 and VvERF059, and the latter clustered with RAP2.2 and RAP2.12. Minor sequence variation was identified in the comparison of the deduced VvERF057.1 and VvERF059.1 amino acid sequences against those previously reported, reflecting genotypic differences between the studies.

**VvERF-VIIs are substrates for oxygen-dependent N-end rule proteolysis in vitro**

The N-terminal cysteine (Cys2) is a tertiary destabilising residue in ERF-VIIs which are subject to N-end rule proteolysis (Gibbs et al., 2011; 2014). Nevertheless, presence of Cys2 does not adequately predict N-end rule proteolysis; the rice ERF-VII SUB1A was previously shown to
evade this regulation, despite bearing Cys2, for example (Gibbs et al., 2011). We thus examined the stability of the VvERF-VIIs using an in vitro translation system, using full-length wild type (MC-) constructs and with those where the Cys2 was mutated to alanine (MA-), which attenuates N-end rule proteolysis. In addition, we examined the stability in conditions of normoxia or anoxia. Each of the wild type MC-VvERF-VIIs were rapidly degraded in normoxic conditions, while the mutated MA- constructs were stable, as were the MC-constructs in anoxic conditions (Fig. 5c). Hence, the VvERF-VIIs are substrates for the oxygen-dependent N-end rule proteolysis in vitro via the and the Cys2 residue, as in arabidopsis (Gibbs et al., 2011), which together with the transcriptome and physiological data presented suggest an important developmental regulatory function for the VvERF-VIIs in coordinating the effective resumption of growth during bud burst.

CONCLUSIONS

The main focus of hypoxia-related acclimation and development has centred on conserved transcriptional responses, largely regulated in plants by a small family of oxygen- and nitric oxide-labile transcription factors, the ERF-VIIs (Considine et al., 2017). The data presented here provide strong evidence in support of an important function for oxygen-dependent signalling via the grapevine ERF-VIIs in the transition from quiescence to bud burst in grapevine. The acute transition of tissue oxygen status and primary metabolism during bud burst was accompanied by the transcriptional hallmarks of hypoxia, however this was in harmony with the resumption of meristem identity and cell proliferation genes. Approximately 20 % of genes in the grapevine genome possessing a HRPE promoter motif were differentially regulated in the first 24 h of bud burst. The HRPE motif is required for transcriptional regulation of several conserved hypoxia-responsive genes by ERF-VII
transcription factors. Each of the three ERF-VII proteins coded in the grapevine genome are substrates for oxygen-dependent N-end rule proteolysis in vitro, identifying these transcription factors as strong candidates for the orderly transcriptional regulation of growth and metabolic functions during the transition from quiescence to bud burst in grapevine. In addition, hypoxia-induced transcription preceded a substantial accumulation of transcripts involved in redox homeostasis, which is consistent with our previous observations of a spatial shift in superoxide during bud burst (Meitha et al., 2015). The extensive transcriptional approach also enabled the identification of novel candidate regulators of the orderly and functional resumption of growth following quiescence in buds, including GASA-, TCP-, MYB3R-, PLT- and WUS-like transcription factors. Functions of each of these in meristem and cell cycle regulation have been demonstrated in other plant systems, but with the exception of the TCP family, which includes BRANCHED1, no evidence has been provided of their function in bud outgrowth or branching. We suggest that the TCP and GASA proteins are particularly interesting, as several members of each have been shown to be redox-regulated, and at least one of the differentially regulated GASA genes identified here possesses an HRPE promoter motif.

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AUTHOR CONTRIBUTIONS

KM carried out biological experiments, performed all physiological and biochemical analysis and prepared material for metabolite assays. PAR carried out all bioinformatic analysis and prepared all figures. SS supervised and assisted with physiological, metabolic and biochemical analysis and interpretation of transcript data. DJG provided vectors, protocols and guidance of the in vitro studies. JAC provided advice on experimental conditions, conducted statistical analysis of $pO_2$ data and advised statistical analysis of metabolite data. CHF contributed to the conception of the study and drafting the results. MJC conceived and
designed the experiments and wrote the manuscript. All authors approved of the final version of the manuscript.

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Figure 1. Overview of the carbohydrate metabolites and physiological traits during bud burst of grapevine (*Vitis vinifera* var. Crimson Seedless) at 23 °C following chilling, under conditions of continuous darkness (D, pink line) or a 12/12 h dark/light (DL, blue line) photoperiod regime. (a) Moisture content (H$_2$O), respiration rates (CO$_2$), and the concentration of chlorophyll, glucose, sucrose, fructose and starch were measured throughout bud development at 3, 24, 72 and 144 h, reported against fresh or dry weight (FW, DW, n = 3 replicates of four buds per replicate). ANCOVA was performed using a 2$^{nd}$ order polynomial to fit the time-response relationship, and the residual standard error was used to estimate the 95 % confidence limits for the predicted means (error bars), where the model was statistically significant (n = 3 replicates of four buds per replicate, raw data over-plotted, open circles). (b) Internal oxygen partial pressure (pO$_2$) profiles at 72 and 144 h under DL (blue) and D (pink) conditions. Data represent scatterplots of raw data (n = 3 individual buds), with a regression curve applied and 95 % confidence intervals shown as grey shading. pO$_2$ data for the D condition are represented from Meitha et al., 2015, licensed under CC BY.
Figure 2. Venn diagrams and functional enrichment analysis of differentially expressed genes (DEG) from buds grown in continuous darkness (D) in the developmental comparisons 3/0 h, 24/3 h, 72/24 h and 144/72 h. (a) Venn diagrams showing the distribution of DEGs (false discovery rate (FDR) $P \leq 0.01$) classified as up- (fold change (FC) $\geq 2.5$, purple) and down-regulated (FC $\leq 2.5$, green). (b, c and d) Bar plots summarise the functional categories significantly enriched (FDR $P \leq 0.01$ in a Fisher’s exact test) in the developmental comparisons 3/0 h (b), 24/3 h (c), and 72/24 h (d); no categories were enriched in the 144/72 h data.

Three different functional enrichment analysis were performed for each comparison, where ‘All’ (up- plus down-regulated genes, grey), ‘Up’ (up-regulated genes, purple) and ‘Down’ (down-regulated genes, green) make reference to the list of genes assessed.
Figure 3. Enrichment analysis of known cis-regulatory binding motifs identified in promoter regions of DEGs (FDR P≤0.01, FC |2.5|) in the 3/0 h and 24/3 h developmental comparisons under continuous darkness (D). (a) Bar plots showing the ten most significantly enriched motifs (lowest P-value) for the comparisons, 3/0 h (upper left corner) and 24/3 h (lower left corner). (b) Venn diagram discriminating the common and unique motifs in the 3/0 h and 24/3 h comparisons. A scatter plot lists the unique motifs found in each comparison.
Figure 4. Differential expression of putative *Vitis* hypoxia-responsive genes among the DEGs (FDR $P \leq 0.01$, FC $\geq 2.5$) during the first 24 h of bud burst in continuous darkness (D). Homologues of conserved hypoxia-responsive genes of *arabidopsis* (Mustroph et al., 2009) are shown in the upper panel of the heatmap (adjacent the yellow column). DEGs containing one or more Hypoxia-Responsive Promoter Element (HRPE; Gasch et al., 2016) in the 2.5 kb upstream promoter region (refer methods) are shown in the lower panel of the heatmap (adjacent the green column). There was a total of 197 genes containing one or more HRPE in the *Vitis* reference genome (Grimplet et al., 2012). Genes marked with an asterisk are among the homologues of *arabidopsis* hypoxia-responsive genes which contain one or more HRPE motif. The gene marked with a black box (■) is a homologue of the *arabidopsis* Group VII ETHYLENE RESPONSE FACTOR (ERF-VII) *HRE2* (also named *ERF071*).
Figure 5. Structure, hierarchical clustering and \textit{in vitro} translation of grapevine ERF-VII genes. (a) Structure of the three members of group ERF-VII in grapevine. These three transcription factors possess APETALA2/ERF domain and N-degron motif. (b) Dendrogram of group ERF-VII amino acid sequences in the grapevine genome against 	extit{arabidopsis} (\textit{Arabidopsis thaliana} - At), rice (\textit{Oryza sativa} - Os) and barley (\textit{Hordeum vulgare} - Hv). The tree was built using a Jukes-Cantor genetic distance model in Neighbor-Joining method from protein sequences, with Bootstrap resampling values are shown in the nodes. These data indicate that VvERF057.1 and VvERF058, and VvERF059.1 and VvERF059 are similar, while VvERF058.1 and VvERF058 are identical. These two identical proteins are also identified as outliers of the group, whereas VvERF059.1 and VvERF059 are more closely related to AtRAP2.12 and AtRAP2.2. Sequence analysis in (a) and (b) were made in Geneious (ver.7.1.9 - Kearse et al., 2012). (c) Degradation of the VvERF-VII proteins (MC-VvERF057.1, MC-ERF058.1 and MC-VvERF059.1) in an \textit{in vitro} rabbit reticulocyte system was dependent on the N-terminal cysteine (MC-), as site-directed mutation of the Cys2 to alanine attenuated degradation. Additionally, the wild-type MC-constructs were stabilised in the absence of oxygen, confirming oxygen-dependent lability of the wild type VvERF-VIIs. Presented figures are representatives of at least three independent replicates. 0, 30 and 60 indicates the time (min) of sampling points following addition of cycloheximide to terminate further protein synthesis \textit{in vitro}. 

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