The spatial dynamics and molecular mechanism of oxygen-dependent signaling during bud burst in grapevine

This thesis is presented for the degree of Doctor of Philosophy at the University of Western Australia

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Life can give everything to whoever tries to understand and is willing to receive new knowledge.

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Abstract

Deciduous perennial plants bear the majority of global fruit and nut production. Bud burst is the first stage in the annual growth cycle and the timing of this event is a key determinant of productivity. Hypoxia and oxidative stress have been proposed to create the optimum conditions for meristem growth and development during bud burst, evident from the upregulation of genes involved in hypoxia responses (i.e. antioxidant defence, alternative metabolism and respiratory pathways, stress signalling, and hormonal balance). The overall aim of this PhD study was to extend the knowledge of physiology and biomolecular processes of the grapevine bud, and how the developmental transition from quiescence to bud burst is achieved, with the dynamics of internal oxygen partial pressure ($pO_2$) as the main hub of seasonal perception. Measurements of internal $pO_2$ in quiescent buds showed a hypoxic core and spatiotemporal oxygenation within the bud from the early stages of the transition, prior to bud burst. The patterning of reactive oxygen species (ROS) was also regulated spatially, indicating that $pO_2$ and ROS were tightly regulated during the early stages prior to bud burst. The presence of light accelerated the spatial development of $pO_2$ and ROS during bud burst and transcriptomic and metabolic data suggested that light signalling is critical to the orderly development of bud burst, even before the leaves emergence from within the bud scales. Recalling that hypoxia is more widely considered to be a stress condition, the role of oxygen sensing in a developmental context is not fully understood. The role of group VII ethylene response factors (ERFVII) in direct oxygen sensing by N-end targeted proteolysis was recently demonstrated in plants. Via this pathway, ERFVIIIs are targeted for proteasomal degradation in an oxygen-dependent manner, whereby ERFVII proteins are stabilised under hypoxic conditions, enabling their function as transcription factors to activate hypoxia-responsive gene pathways. An in vitro translation assay was used to study the role of the grapevine homologues of ERFVII. At least two of the three VvERFVIIIs were shown to be substrates for the N-end rule pathway. Furthermore, in silico analysis of transcriptome data identified putative
gene targets in transcripts regulated during bud burst, which bear conserved hypoxia-
responsive promoter elements. Together these data are consistent with a role for the
VvERFVIIIs in oxygen-dependent molecular signalling in the internal tissues of the bud during
the transition in $pO_2$. In conclusion, this study suggests the importance of oxygen-dependent
signalling during bud burst in grapevine, and the study has implications for other hypoxic
organs such as fruit or in conditions of stress-induced hypoxia.
Thesis declaration

I hereby declare that this thesis contains no experimental material that has been, is being, or to be submitted for any degree at University of Western Australia (UWA) or any other institution. This thesis presents the conducted research during my enrolment for a PhD degree at School of Biological Sciences, UWA, and its original contribution to the field of plant oxidative biology. The raised hypotheses were results of discussions with my supervisors and so were the experimental designs. I have conducted the experiments, analysed and interpreted the data, prepared drafts for the papers, and finally composed this thesis. All of the mentioned pursuits would be impossible to achieve without the guidance from my supervisors as well as the collaborations with co-authors of the arising publications from this thesis. For all the un- and published papers, I have co-authors’ permission to include the works in this thesis. The contributions of me and co-authors are described in the preface of each experimental chapter.
This thesis contains three experimental chapters, which are preceded by a chapter of general introduction and mini literature review, and concluded by a general discussion chapter. Experimental chapter 2 has been published in a peer reviewed journal, hence it is presented in the format of the publication. Experimental chapters 3 and 4 have been prepared as the manuscript of journal publications, but are not formatted in any journal style as they have not been published. The use of “we” as the subject represents the authors involved in the experiments, which are listed in ‘Papers to be submitted’ section of Statement of original contribution. The appendix is a submitted methods chapter for book publication describing the techniques used in experimental chapters 2 and 3.
Statement of original contribution

The research presented in this thesis is an original contribution to the field of plant oxidative signalling. The hypotheses and framework are my original ideas, which were constructed through discussions with my supervisors. I designed and conducted all of the experiments, except RNA sequencing, and I did the data analysis and prepare the corresponding section of each manuscript (with feedback and editing from my supervisors). My supervisors and all the contributors who I share the authorship of the manuscripts have greatly helped me during the research. Their contributions are acknowledged in each preface of the experimental chapters and in the section of author contributions below. For the published paper contained in this thesis, I have permission of all co-authors to include this work in my thesis.

Publications arising from this thesis:

Published paper and accepted book chapter


Papers to be submitted:

- Chapter 3 of this thesis:

Author contributions:

KM optimised and performed the physiological analyses, drafted related methods and results including Figures 3.1-3 and Supplemental Figure S3.1 and contributed to the introduction and discussion. PAR performed the RNAseq, bioinformatic analysis, drafted related methods and results including Figure 3.4, Supplemental Figure S3.2 and Supplemental Tables S3.1-3. SS contributed to metabolic analysis and drafted related results including Figures 3.5-6. JAC supervised histology and data analysis for Figure 3.2. CHF co-conceived the study with MJC and contributed to the discussion and conclusions. MJC drafted Supplemental File S3.1, Supplemental Tables S3.4-6 and compiled the manuscript with contributions from co-authors described above. All authors approved the submitted manuscript.

• Chapter 4 of this thesis:


Author contributions:

KM performed the molecular biology and in vitro biochemistry, and drafted the manuscript with guidance by MC. KM and DG conceived the promoter analysis. PAR did the RNA sequencing, supervised differential and computational analysis of promoter motifs, and provided advice on the manuscript. SS and DH performed some replicates of the in vitro analysis and provided advice on the manuscript. DJG provided vectors and positive controls, and advice on the in vitro analysis. CF provided advice on the scope of the study. MC conceived the in vitro analysis, performed Genevestigator analysis and contributed to the manuscript.
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1 General introduction and mini literature review

1.1 General introduction

The formation of a winter bud is an important survival strategy for deciduous perennial plants. The bud enables survival through seasonal environmental conditions generally limiting for vegetative and reproductive growth. The perennial bud develops and matures under quiescence, whereby metabolism and cell division are slowed (Lavee and May, 1997). Transition from quiescence to bud burst is rapid, similar to seed germination, and is thus expected to require a considerable re-organisation of cellular metabolism, enabling the transition to a photosynthetic shoot (Fleming, 2006). Oxygen is a principal requirement of aerobic life; all energy metabolism involves a change in the reduction/oxidation (redox) state, and depends on redox cycling. It is expected however, that bud internal structures reside in a hypoxic condition, given that buds are a dense organ, and that an oxidized niche is crucial to cell identity and fate, as it is in the root meristem (De Tullio et al., 2010; Jiang, 2003). In contrast with redox regulation during seed germination which is widely studied (Hourmant and Pradet, 1981; Liu et al., 2010; Müller et al., 2009; Oracz et al., 2009), knowledge of the oxygen and redox state of perennial buds is scarce and almost entirely reliant on abstract assumptions of gene transcription patterns. Hence, there remain several key questions concerning the physiology of the bud, and how the transition from quiescence to bud burst is achieved. As the majority of global fruit and nut production derives from perennial plants with a seasonal habit, this area of research has considerable importance for the viability and security of present and future food production.
The transition from quiescence to bud burst is naturally driven by the seasonal environmental cues of temperate and Mediterranean-type climates. Here, I refer to quiescence in the generic form, as the experimental chapters in thesis does not address dormancy per se. Dormancy is a unique, internally repressed state of quiescence of the meristem, which requires entrainment to enable the resumption of growth in favourable conditions (Baskin and Baskin, 2004; Considine and Considine, 2016; Lang et al., 1987). In nature, it may be an advantage to have a prolonged bud burst, hedging against the risk of frost. In production systems however, chemical and physical management are typically employed to enforce uniform bud burst. Outside temperate or Mediterranean-type climates, or during aberrant seasons, production of grape fruit is problematic (Shulman et al., 1983). A delayed and erratic bud burst results in decreased shoot and cluster counts per vine and poor uniformity of fruit development (Dokoozlian et al., 1995). Chemical agents such as hydrogen cyanamide (HC) are thus a mainstay in perennial fruit industries. However, chemicals such as HC are highly toxic or even lethal to the meristem when application is mistimed or applied at too high concentration (George and Nissen, 1990; Or et al., 1999; Pires et al., 1995; Shulman et al., 1983). As there are no visible markers of the developmental and metabolic state of the mature bud, this risk is considerable. Furthermore, forecast increases in global temperatures and frequency of extreme weather events will exacerbate this bud burst management problem (Pagter and Arora, 2013). For example it is estimated that the increase in total heat accumulation and frequency of extreme hot days (>35°C) in the growing season will significantly reduce production of grapes in many current regions of cultivation by the late 21st century (White et al., 2006). For these reasons, a better understanding of the physiology of bud burst is also economically important. Improved knowledge will enable more accurate management intervention and the development of more environmentally friendly and efficient techniques to manage grapevine bud burst.
Knowledge from seed and other plant organs, as well as from animals, indicates an important role for temporal and spatial regulation of a low internal oxygen ($pO_2$, partial pressure of oxygen) in curating cell identity, function and fate (Kelliher and Walbot, 2012; van Tuyl et al., 2005). However, when $pO_2$ regulation fails to meet the physiological requirements of the growing and developing tissues, cell functions may be negatively affected (Colmer and Greenway, 2011; Geigenberger et al., 2000; McMinn et al., 2005). Regulation of internal $pO_2$ during the transition time of dormancy release and bud burst is highly likely to be co-dependent on respiration, the fundamental driver of cellular metabolism. Rolletschek et al. (2003) demonstrated a significant decrease in the respiration rate of broad bean embryos when cultivated in an oxygen atmosphere of 10% of the normal atmospheric oxygen concentration. The internal $pO_2$ of seed of several species was shown to be dynamically regulated during seed development and maturation, and highly dependent on the photosynthetic capacity of the seed, indicating a dynamic relationship between respiration and photosynthesis during development and quiescence, although no data is yet available during the transition to germination (Borisjuk and Rolletschek, 2009). In the bud of kiwifruit and poplar, both deciduous perennials, an increase in the respiration rate was detectable even prior to any visible growth in early spring (Bachelard and Wightman, 1973; McPherson et al., 1997). To date no studies have investigated the internal $pO_2$ in perennial buds. However, based on the available knowledge in seeds and other plant and animal organs, it is hypothesised that the regulation and maintenance of $pO_2$ is central to facilitate the transition of bud into shoot, in order to ensure the successful transition to phototrophic growth.

Reactive oxygen species (ROS) are by-products of key metabolism processes, and in recent years their role in cell signalling has been acknowledged. Typically, an increase in aerobic respiration results in an elevated production of superoxide ($O_2^-$) due to the increase in reduction of the mitochondrial ubiquinone pool and subsequent partial reduction of molecular oxygen by respiratory complexes of the mitochondrial electron transfer chain (Considine and
O$_2^-$ has a short half-life and is rapidly converted into hydrogen peroxide (H$_2$O$_2$) and subsequently to water through the action of metabolic and enzymatic antioxidants, such as superoxide dismutase, catalase and the ascorbate/glutathione cycle (Foyer and Noctor, 2011). The roles of O$_2^-$ and H$_2$O$_2$ are increasingly well-studied in the context of cell signalling in plants. For example, AtrobohB (NADPH-oxidase) produces the majority of O$_2^-$ in the embryo of Arabidopsis germinating seeds, and removal of AtrobohB gene leads to reduction of protein carbonylation (Muller et al., 2009). An immediate and transient accumulation of O$_2^-$ and H$_2$O$_2$ accompanies seed imbibition of Pisum sativum, although only O$_2^-$ coincides with radicle elongation (Kranner et al., 2010). Presently, the roles of O$_2^-$ and H$_2$O$_2$ during quiescence and the onset of bud burst are only implied by observations of changes in the expression of genes antioxidant scavenging, such as catalase, glutathione reductase and glutathione S-transferase, superoxide dismutase and glutathione peroxidase (Ophir et al., 2009; Or et al., 2002; Pacey-Miller et al., 2003; Perez and Lira, 2005).

In addition to hypoxia and ROS-dependent signalling, knowledge from rapid phase transitions in other plant and animal systems, highlight the need for direct sensing of the cellular energy status. In animals, the SNF- and Target of Rapamycin (TOR)-signalling pathways play important roles in sensing and transducing conditions of starvation or energy surplus (Fingar and Blenis, 2004; Lefebvre et al., 2001). Plant homologues of these pathways have been identified, for example the Arabidopsis KIN10 and KIN11 are functional homologues of the animal sucrose-non-fermenting1-related-kinase1 (SnRK1) (Baena-González et al., 2007). Although less solid evidence is available for the role of plant homologues of the TOR pathway, an additional regulator, trehalose-6-phosphate (T6P), has been identified as an important cellular sensor of energy status (Avonce et al., 2004). Not surprisingly, both SnRK1 (Baena-González et al., 2007) and T6P (Kretzschmar et al., 2015) are also implicated in conditions of hypoxia and oxidative stress in plants, with both pathways have been identified to be important in seed germination under various conditions (Tsai and Gazzarrini, 2014). However, presently there is little
knowledge on the direct relationship between these pathways and hypoxia in developmental transitions, and evidence for a role in perennial bud burst is only made by reference to gene expression (Or et al., 2002).

To summarise, contemporary evidence from developmental systems in plant and animal biology implicate oxygen and redox signalling as fundamental positional cues for determining cell identity and fate. In addition, the balance of sugars, particularly sucrose, hexoses and trehalose metabolites are primary indicators of cell energy status, which affect the appropriate coordination of molecular signalling and metabolism during developmental transitions. To date, there is only indirect evidence of a role for these signalling networks in perennial bud development, quiescence and burst, primarily via gene expression. Although some physiological studies and the results of treatments, such as HC or heat treatment are consistent with there is a considerable need to integrate spatial regulation into the developmental changes in the bud.

The transition from quiescence to bud burst is a useful system for which to begin to study these signalling networks. This transition is quite easily managed and predictable in controlled conditions, using single node cuttings (Mullins, 1966). An improved understanding of how perennial buds utilise these pathways to sense and respond environmental cues under controlled conditions will allow us to build on this platform to study wider environmental challenges in controlled, natural and field-managed conditions. Ultimately, these studies will guide the development of more accurate and environmentally safe management strategies. These studies will focus on grapevine (Vitis vinifera L.), as it is the most economically important fruit crop worldwide and is grown commercially in nearly 100 countries (FAOSTAT, 2014). Further, grapevine has become the scientific model perennial plant, with the genome sequence published in 2007 (Jaillon et al., 2007; Velasco et al., 2007).
1.2 Thesis aims

- Identify the gaps in knowledge of oxygen-, ROS- and energy-dependent signalling during perennial bud burst, with a particular focus on practical implications on the regulation of grapevine bud burst (Chapter 1 – mini literature review).

- Define the spatio-temporal dynamics of \( pO_2 \) and ROS, and relationship with respiration during the transition from quiescence to bud burst in grapevine (Chapter 2).

- Determine the role of light in coordinating the effective integration of \( pO_2 \), ROS, energy metabolism and transcriptional changes during the transition from quiescence to bud burst in grapevine. In particular to identify whether the absence of light results in a metabolic and transcriptional state of heightened hypoxia and starvation (Chapter 3).

- Identify the capacity of grapevine homologues of the group VII ethylene response factors (ERF VII) to function as substrates for the oxygen-dependent N-end rule of proteolysis. This study will involve in silico and in vitro experimentation, and identify putative targets of the ERF VII in the grapevine genome (Chapter 4).

- Synthesise these data into a guiding thesis on the roles and potential crosstalk of oxygen-ROS- and energy-dependent signalling in coordinating perennial bud burst in a general discussion (Chapter 5).

1.3 Mini literature review

1.3.1 Bud dormancy – a survival strategy

Latent buds in perennial plants bear important structures that determine the next season of growth. The perennial bud begins development during the early stages of shoot growth in spring. In woody perennial, such as grapevine and several other species develop the perennial bud in the leaf axils, while in other species such as poplar and apple the apical and more distal buds perenniate (Anderson et al., 2010). In the leaf axil of a grapevine shoot, two principal
types of buds form: the prompt bud, which is does not perenniate and typically develops into a summer lateral shoot, and; the perennial bud, which is actually a compound of three buds, the primary, secondary and tertiary (Pratt, 1974). It is this compound bud which later matures and becomes dormant during autumn and winter, before bursting into a new shoot in the following spring. Typically, the primary bud develops 12-15 nodes during the first season including 2-3 inflorescences meristems (Mullins et al., 1992; Pratt, 1979). The secondary and tertiary buds may develop inflorescence meristems, depending on cultivar and climate. If healthy, only the primary bud will burst in the spring, and is hence the primary determinant of productivity.

Dormancy, in general, is the failure to grow in conducive conditions. Dormancy is a state of meristematic or embryonic organs, and hence the two most notable organs capable of dormancy are seeds and perennial buds. It should be noted at this point that not all plant meristems can or do enter dormancy per se, for example meristems of annual species such as Arabidopsis are not capable of dormancy, only quiescence (Considine and Considine, 2016). Similarly, not all angiosperms develop dormant seed, although extending the Arabidopsis example, they do, to varying extend depending on ecotype (Alonso-Blanco et al., 2003; Bentsink et al., 2010). To some extent their signature physiological behaviour and dependence on environmental cues are comparable (Considine and Considine, 2016; Doramaci et al., 2011; Fennell et al., 2015). However, the similarities and differences in their physiology are also arguable and so is the classification of dormancy in both structures (Baskin and Baskin, 2004). Lang et al. (1987) is one of the earliest studies that distinguished plant dormancy types, primarily based on the origin of signals that induce such conditions. The proposed three types of dormancy are: (1) endodormancy, when the inhibition to resume growth originates from the dormant structure itself; (2) paradormancy, when the growth inhibition is due to repression from other parts of the plant; and (3) ecodormany, where environmental factors provoke the inhibition. In this classification, endodormancy is equivalent to primary or
physiological dormancy of seeds, while paradormancy and ecodormancy can be considered as more opportunistic modes of quiescence, where the bud will regrow as soon as the external repression is removed (Considine and Considine, 2016). Henceforth, I will refer to dormancy and quiescence, where the experimental chapters of this thesis are solely concerned with quiescence and regrowth, or ecodormancy according to Lang et al. (1987).

Bud dormancy, in perennial plants, is an evolutionary strategy to maximise survival in seasonal conditions, and with which to endure the unfavourable condition and ensure the survival of the shoot apical meristem (Vegis, 1964; Vitasse et al., 2014). The seasonal climate governs the annual life cycle of a bud with photoperiod and temperature as the two most predictable cues (Arora et al., 2003; Olsen, 2010; Rohde and Bhalerao, 2007; Wareing, 1956). The declining day length and temperature in autumn are widely considered to provide the environmental cue to commence acclimation and dormancy programs (Rohde and Bhalerao, 2007). The bud will continue to harden until mid-winter or until their maximum cold hardiness is reached, following this the cells will be physiologically ready to endure the very cold temperatures (Dokoozlian et al., 1995), down to -70°C in some cold-adapted species (Korner, 2012; Vitasse et al., 2014). The accumulation of exposure to cold conditions is widely considered a prerequisite for the release of bud dormancy, although there is a considerable genetic effect, and no chilling models developed to date applies to all varieties or climate conditions (Lavee and May, 1997; Mullins et al., 1992). Several authors, particularly in the seed literature have questioned whether acclimation and dormancy are two separate processes which can be uncoupled (D'Angeli et al., 2016; Ruttink et al., 2007). Current evidence suggests a high degree of correlation between cold-acclimation and dormancy, for example in the patterns of gene expressions, ROS signalling, water and soluble sugars concentrations, and hormonal balance (Arora and Rowland, 2011; Considine and Considine, 2016; Dhuli et al., 2014; Fennell et al., 2015).
1.3.2 The transitions between bud dormancy, quiescence, bud burst and shoot growth

The term ‘bud dormancy release’ generally refers to the conclusion of endodormancy or commencement of ecodormancy, when the bud is in a quiescent state and still tightly wrapped by the lignified scales but will rapidly grow when transferred to a favourable condition. There are no visible signs of this transition, it can only be measured through ‘forcing’ studies, where the bud is placed in conditions conducive to growth (Mullins, 1966). Following dormancy release, the bud enters a resting or quiescent condition, anticipating the arrival of a suitable condition to resume rapid and visible growth, or bud burst. Thus the release of dormancy is commonly referred to as the competence to germinate or burst. The early progression to bud burst has been classified into four stages by Eichhorn and Lorenz (1977) with modification by (Coombe, 2004): EL-1, winter bud; EL-2, bud scales opening; EL-3, woolly bud with more or less green tissue showing; and EL-4, bud burst with the first visible leaf tip (Figure 1.1).

Once temperature and day length increase (Lavee and May, 1997), the bud is begins to rehydrate and swell, transitioning from EL-1 to the subsequent stages. Early growth entirely relies on heterotrophic metabolism, relying on stored reserves in the trunk and roots (Lebon et al., 2008). The development of photosynthetic capacity is believed to commence during or soon after bud burst (EL-4), as the leaves emerge green. However, net autotrophic capacity does not develop until several leaves have emerged, EL-12 - EL-15 (Fleming, 2006; Lebon et al., 2008). In seeds, the counterplay between mitochondrial respiration and photosynthesis during the early stages of germination and etiolation are reasonably well defined and considered to involve a so-called photo-heterotrophic metabolism, where mitochondrial respiration and photosynthesis are co-dependent (Borisjuk et al., 2004). Presently however, there is no knowledge on the balance of respiration and photosynthesis in the early stages of bud burst.
Progression of bud dormancy to burst in grapevine (*Vitis vinifera* L.). Winter bud (EL 1) is distinguished by the tightly protected shoot apical meristem under layers of lignified scales. In this morphological state, the bud could be at the endodormancy (induced by short day and low temperature), dormancy release, or ecodormancy stage. Increasing day length and temperature direct the buds from dormancy release and onwards stages of EL2 – EL4 (own photographs and Figure compiled following the system in (Coombe, 2004)).

### 1.3.3 A possible role of hypoxia in bud dormancy release, and the proposed molecular oxygen sensing mechanism

It is not fully understood how deciduous perennial plants orchestrate the metabolic, physiological and molecular responses towards the change in environmental cues to terminate bud dormancy and resume growth. Gene expression studies suggest that oxidative stress and potentially hypoxia is one of the prerequisite conditions during bud dormancy release and burst (Halaly *et al.*, 2008; Ophir *et al.*, 2009; Or *et al.*, 2000; Perez and Lira, 2005; Vergara *et al.*, 2004).
Moreover, the placement of bud under anaerobic condition and inhibition of oxygen uptake by sodium azide, an inhibitor of mitochondrial respiration, or hydrogen cyanamide (HC) causes dormancy to be broken (Lavee and May, 1997; Pérez et al., 2009; Pouget, 1963). Although the precise mode of action of HC is not known, similarities in the effect on gene expression have been observed with other treatments such as sodium azide and heat shock, which do inhibit mitochondrial respiration (Halaly et al., 2008; Or et al., 2002). However, to date no study has documented the internal $pO_2$ profile in buds or indeed investigated the effects of various treatments on the $pO_2$ during dormancy, quiescence or bud burst.

The condition of hypoxia during seed germination is well documented in legumes with a minimum of 0.4% $pO_2$ at the earliest stage of embryo development (Rolletschek et al., 2002; Rolletschek et al., 2003), which becomes detrimental when maintained in the subsequent phase following the globular and heart-shape stage (Kuang et al., 1998). More widely, the significance of hypoxia in several key stress and developmental processes in plant and animal biology has become acknowledged. Low levels of $pO_2$ in Zea mays germ line cells results in precocious activation of meiotic genes (Kelliher and Walbot, 2014), which further increases germ cell numbers and stimulates germ cell formation (Kelliher and Walbot, 2012). Direct measurement of tissue $pO_2$ in developing mammalian embryo, lung, kidney and heart revealed that these tissues also harbour low oxygen tension (Loughna et al., 1998; Mitchell and Yochim, 1968; van Tuyl et al., 2005; Yue and Tomanek, 1999). Subsequently, a core set of 49 hypoxia-responsive genes, which are induced in oxygen-limiting conditions across the domains of life was identified (Mustroph et al., 2009).

The survival of Arabidopsis (Gibbs et al., 2011; Licausi et al., 2011b) and Barley (Mendiondo et al., 2016) during the low oxygen condition is mediated by the N-end rule protein degradation pathway. The N-end rule pathway of proteolysis degrades proteins based on their N-terminus sequence with the three studies identifying members of the group ERF VII as the substrates
for the specific branch of the pathway, the Cys/Arg-branch. Group ERFVII of Arabidopsis, rice, barley, and potato share a consensus of initiating motif methionine-cysteine (MC) at the N-terminus, MCGGAI/L, which is crucial for the destabilisation processes (Gibbs et al., 2011; Licausi et al., 2011a; Mendiondo et al., 2016). In normoxic conditions, removal of N-terminal methionine by a methionine amino peptidase (MAP) exposes the cysteine to oxidation by a plant cysteine oxidase (PCO) (Gibbs et al., 2011; Weits et al., 2014). The oxidised cysteine creates a secondary destabilising residue, allowing the addition of arginine (R) by arginine transferase (ATE), creating a primary destabilising residue at the N-terminus. Subsequently, proteasome will target the arginylated protein for proteosomal degradation via proteolysis 6 (PRT6), a specific E3 ligases (Gibbs et al., 2011; Mendiondo et al., 2016).

Licausi et al. (2011b) demonstrated that the cellular localisation of one of the functional Arabidopsis ERF VII homologues (RAP2.12), is determined by the oxygen tension. The RAP2.12 protein initially associates with membrane-bound acyl-CoA-binding proteins, and when the oxygen tension declines, RAP2.12 dissociates from this complex and shuttles into the nucleus to induce expression of hypoxia-responsive genes. Upon re-oxygenation, the RAP2.12 moves back into cytosol and goes through the N-end rule degradation pathway. Recent research in Arabidopsis revealed that the functional ERF VIIIs bind to an evolutionarily conserved cis-motif within the hypoxia-responsive genes to regulate their expression (Gasch et al., 2016). To date, the functionality of these interactions in perennial plants have not been defined.

Thus, given that the expression of a range of hypoxia-responsive genes is up-regulated during bud burst in grapevine, it is reasonable to investigate whether the quiescent buds are hypoxic and how the $pO_2$ is dynamically regulated throughout bud burst in conditions that affect the course or coordination of the resumption of growth. Furthermore, with the recent advances in our understanding of direct oxygen signalling in plants, at least in stress conditions, it is reasonable to investigate whether such signalling is present and functional during bud burst.
1.3.4 ROS interacts with phytohormone regulations in dormancy release signalling

The roles of ROS in plant signal transduction have been widely acknowledged, engaging in a diverse range of crucial processes from induction of cell mitosis to senescence (Foyer and Noctor, 2005). The involvement of ROS-dependent signalling in the release of dormancy and onset of bud burst in perennial plants, including in grapevine, has been suggested by a transient rise in H$_2$O$_2$ prior to bud burst, and through the profiles of several core antioxidant genes (Perez and Lira, 2005). Or et al. (2002) found increasing activity of catalase enzyme, an H$_2$O$_2$ scavenger, during dormancy, and decreasing when commencing release or after application of HC, which may corroborate the increase in H$_2$O$_2$ through a decline in the capacity to scavenge it. Additionally, exogenous application of H$_2$O$_2$ advances bud burst, although not as efficiently as HC (Perez et al., 2008). Further, H$_2$O$_2$ during oxidative stress in grapevine buds is mainly produced by disruption of the mitochondrial electron transport chain (Vergara et al., 2012a). Up-regulation of antioxidant, fermentation, stress related kinase, hypoxia related and alternative respiratory pathway genes has further suggested the interconnection between hypoxia, oxidative stress and ROS signalling during dormancy release (Table 1.1).

Elevated ROS concentrations are shown to mediate responses of phytohormones during seed dormancy release and germination. H$_2$O$_2$–imbibed Arabidopsis seeds have elevated expression of genes encoding the catabolism of abscisic acid (ABA) and biosynthesis of gibberellic acid (GA), resulting in a shift in the balance of these primary hormones that favours the release dormancy and promotes germination (Liu et al., 2010). When growth in Arabidopsis seedlings was stimulated by low-dose gamma irradiation, ROS also mediated the ABA signalling pathway (Qi et al., 2015). Oracz et al. (2008) proposed that cyanide, an inhibitor of the mitochondrial cytochrome oxidase, as well as Cu/Zn superoxide dismutase and catalase, is able to interact with ethylene receptors to further promote the release of dormancy in sunflower seeds.
Moreover, phytohormones are able to generate \(\text{H}_2\text{O}_2\) production via the activation of NADPH oxidases during signal transduction (Bartoli et al., 2013), considered as amplifying oxidative signalling (Considine and Foyer, 2014).

**Table 1.1** Regulated genes during bud dormancy release and commencing burst in grapevine.

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene expression in grapevine bud dormancy release or commencing burst</th>
<th>Reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fermentation related</td>
<td>Pyruvate decarboxylase</td>
<td>Or et al. (2000); Vergara et al. (2012b)</td>
</tr>
<tr>
<td></td>
<td>Alcohol dehydrogenase</td>
<td>Or et al. (2000); Vergara et al. (2012a)</td>
</tr>
<tr>
<td></td>
<td>Sucrose synthase</td>
<td>Halaly et al. (2008); Vergara et al. (2012b)</td>
</tr>
<tr>
<td>Sensors of stress signal</td>
<td>GDBRPK, an SNF-like protein kinase</td>
<td>Or et al. (2000)</td>
</tr>
<tr>
<td>Alternative respiratory pathway</td>
<td>Alternative oxidases</td>
<td>Vergara et al. (2012a)</td>
</tr>
<tr>
<td>Hypoxia responsive Dormancy release related</td>
<td>Non-symbiotic haemoglobin</td>
<td>Vergara et al. (2012b)</td>
</tr>
<tr>
<td></td>
<td>Flowering locus T</td>
<td>Vergara et al. (2012b)</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>(\alpha)-amylase genes</td>
<td>Rubio et al. (2014)</td>
</tr>
<tr>
<td>Antioxidant defence</td>
<td>NADH-dehydrogenases</td>
<td>Vergara et al. (2012a)</td>
</tr>
<tr>
<td></td>
<td>Glutathione reductase</td>
<td>Halaly et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Ascorbate peroxidase</td>
<td>Halaly et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Thioredoxin</td>
<td>Halaly et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Glutathione S-transferase</td>
<td>Halaly et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Superoxide dismutase</td>
<td>Vergara et al. (2012a)</td>
</tr>
<tr>
<td>Down-regulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antioxidant defence</td>
<td>Catalase</td>
<td>Or et al. (2000)</td>
</tr>
</tbody>
</table>

*The cited studies were performed by forcing bud break using HC (Halaly et al., 2008; Or et al., 2000; Rubio et al., 2014; Vergara et al., 2012b), hypoxia (Rubio et al., 2014; Vergara et al., 2012a; Vergara et al., 2012b), heat stress (Halaly et al., 2008), \(\text{H}_2\text{O}_2\), ethylene (Vergara et al., 2012a), or chilling (Rubio et al., 2014).

### 1.3.5 Bud respiration activities during hypoxia and as oxygen supply increases

While a condition of low oxygen tension is predicted to promote the release of bud dormancy and enable bud burst, hypoxia can also limit mitochondrial energy production. When the
oxygen tension is less than 5% in potato tubers, adenylate energy status declines and metabolic activities are compromised, creating a starvation response (Geigenberger et al., 2000). Legume embryos develop in a hypoxic environment (e.g. 0.4% oxygen), in which the early growth may be energy-limited, that is, respiration does not cover energy demand (Rolletschek et al., 2002). Grapevine buds stimulate fermentative and alternative respiratory pathways when respiration is inhibited by exposure to hypoxia or HC (Halaly et al., 2008; Or et al., 2000; Vergara et al., 2012a; Vergara et al., 2012b). A responsive respiration trend in pea roots towards decreasing oxygen availability - an initial linear decline of respiration which is followed by a nonlinear inhibition - indicates energy-dependent signals are an important component of optimising metabolism during hypoxia (Zabalza et al., 2009). An alternative perspective of this study is conveyed by Armstrong and Beckett (2011) by using a mathematical model, noting the multicylindrical diffusive and respiratory characteristics of roots and the kinetics of the scavenging process. The results showed that the initial decrease of respiration is due to the spread of severe hypoxia from the root centre, rather than energy status sensing and a down-regulation of respiration to ‘conserve’ oxygen.

During the transition of cells from the quiescent to the metabolically active state, regulation of respiration under and during the release from hypoxia as a result of bud burst, is central to the oxidative regulation (Considine and Foyer, 2014). However, exposure of endodormant grapevine buds to high winter temperature (maximum temperatures ≥10°C) causes an increase in mitochondrial respiratory capacity, and inevitably, its level of H₂O₂. This often leads to an erratic bud burst in areas lacking normal temperate winter-spring transition (Pérez et al., 2007).

A pattern of declining respiration rates during autumn and increasing again by the end of winter - or after dormancy release - is unique to buds from deciduous trees (Ross, 1985; Westwood, 1993). By using differential scanning calorimetry (an indirect measure of respiration), Gardea et al. (1994) detected steadily increasing respiration rates in grapevine
buds by the late winter (Oregon, USA) and continuing as the buds progressed to burst. Poplar buds during winter-autumn transition also display a comparable trend with a more pronounced increase in respiration after bud burst (Bachelard and Wightman, 1973). The study by Gardea et al. (1994) also identified that the highest metabolic efficient phase is during ecodormant, quantified as heat (kJ) released per CO$_2$ produced. These studies emphasise that an increase in mitochondrial energy production precedes bud burst.

1.3.6 Temporal accumulation of carbohydrates during bud dormancy and release, and their suggested functions in stress signalling

Earlier studies on the nexus of hypoxia and respiration in coordinating bud burst have also implicated a role of sugars in signalling the cellular energy status and in providing protective functions against cold- or oxidative stress. For example, Rubio et al. (2014) suggested that hypoxia induced the expression of α-amylase genes in grapevine buds, providing a potential relationship between hypoxia and the remobilisation of starch energy reserves into soluble sugars during dormancy release. A transient increase in starch hydrolysis, leading to abrupt accumulation of sugars, particularly sucrose, has also been shown when buds were treated with near-lethal heat stress (ben Mohamed et al., 2014). Typically, hypoxic conditions limit the production of ATP, whereby oxygen is the terminal electron acceptor of oxidative phosphorylation, which generates the maximal yield of ATP synthesis. In these conditions, the end-products of glycolysis are instead oxidised by fermentation pathways, which serves to recycle electron carriers (NAD$^+$), so that glycolysis can continue. These conditions in fact lead to an increase in glycolysis, in order to generate at least some ATP. This can have profound effects on the sugar status and in particular the ratio of different sugars in the cell.

A comparison of trends of sugars and starch concentrations in the buds of poplar (Populus balsamifera), several grape genotypes, and common ash (Fraxinus excelsior) prior to, during, or after endodormancy is presented in Table 1.2. The modulation in soluble sugars and starch
concentrations are also related to bud dormancy stages in deciduous species. The considerable changes in metabolic control during bud development, dormancy and bud burst, notably the activity of photosynthesis (Dhuli et al., 2014) and connection to the mother plant (Rinne et al., 2011) require ingenious and variable energy storages. Fennell et al. (2015) suggested the potential role of trehalose, raffinose and resveratrol in bud dormancy commitment in grapevine, with raffinose accumulation suggested as an early step in the cold acclimation process (Grant and Dami, 2015). Bachelard and Wightman (1973) suggests that the increase is continuous and when the buds are getting ready to burst, elevated monosaccharide concentrations appear to be due to the movement of sugars into the buds as a result of sap flow. However, the precise timing of vascular reconnection of bud to the plant body, during bud dormancy release and burst, is not clearly defined. In poplar bud, chilling up-regulates the expression of group 2 GLUCAN HYDROLASE FAMILY 17 genes (GH17s) via the enhanced synthesis of GA (Rinne et al., 2011). Group 2 GH17s are known to hydrolyse callose and re-open connections through sieve plate pores and plasmodesmata in both shoot apical and rib meristem. A role for specific sugars in stress and developmental signalling is also becoming widely accepted. In particular, evidence points to trehalose-6-phosphate (T6P) in playing a key role in signalling changes in the cellular energy status in both stress and developmental contexts, and particularly through its relationship with the conserved hub of starvation responses, the sucrose-non-fermenting1-related kinase1 (SnRK1) (Tsai and Gazzarrini, 2014). Trehalose is a non-reducing sugar that presents in a very low concentration in plant tissues (Lunn et al., 2006). T6P is an intermediate product in a two-step trehalose biosynthetic pathway. In the first reaction, T6P synthetase (TPS) synthesizes T6P from UDP-glucose and glucose-6-phosphate, followed by dephosphorylation to trehalose by T6P phosphatase (TPP). Trehalase then hydrolyses trehalose to two molecules of glucose (Elbein et al., 2003). Interestingly, regulation of TPS and TPP coincides with coordination of plant response towards
prevailing carbon supply (Paul, 2008). For example, elevated expression of a rice \textit{TPP} (\textit{OsTPP7}) during germination results in an increase in the balance of trehalose to T6P, which enhances

Table 1.2 Generalised trends of soluble sugars and starch concentrations at various stage of bud dormancy and burst in poplar \textit{(Populus balsamifera)}, several grapevine \textit{(V. vinifera)} genotypes and common ash \textit{(Fraxinus excelsior \textit{L.})}. – indicates not-measured.

<table>
<thead>
<tr>
<th>Time/period</th>
<th>Disaccharide</th>
<th>Raffinose family oligosaccharide (RFO)</th>
<th>Monosaccharide/fructans family</th>
<th>Polysaccharide</th>
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<tr>
<td>Late winter</td>
<td>High</td>
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<td>Mid summer</td>
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<td>Early and mid autumn</td>
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<td>Moderate</td>
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<td>Mid summer</td>
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<td>Early and mid winter</td>
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<td>Late spring</td>
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starch mobilisation and promote embryo growth and coleoptile elongation under anaerobiosis (Kretzschmar \textit{et al.}, 2015). This pattern of metabolic regulation is consistent with a starvation syndrome. The molecular mechanism of the starvation response has been shown to directly relate to T6P, rather than trehalose, whereby T6P is a negative regulator of SnRK1 (Tsai and Gazzarrini, 2014). For example in \textit{Arabidopsis} leaves, treatment with T6P results in decreased expression of SnRK1 transcripts, which then allows the upregulation of genes which are
normally repressed by SnRK1, such as genes coding for protein and nucleotide synthesis, or those involved in the mitochondrial tricarboxylic acid cycle and electron transport chain (Zhang et al., 2009). Under conditions of hypoxia or extended darkness, the expression of SnRK1-target genes increase. This includes ca. 300 genes that are widely activated under a number of starvation-related conditions, and conserved across species (Baena-González et al., 2007; Baena-Gonzalez and Sheen, 2008). Importantly, the SnRK1-mediated responses can be partially rescued by treatment with sugars such as glucose. Further, in flooded rice seeds, SnRK1 induces production of a key transcription factor for α-AMYLASE genes, MYBS1, to trigger the cascade reaction of starch hydrolysis down to germination (Lee et al., 2014). Given the implied importance of energy status and oxidative metabolism and signalling during bud burst, the regulation of sugars and in particular T6P may also be important in this developmental transition.

1.3.7 Conclusions

The conceptual conclusions of this mini literature review are presented in Figure 1.2, highlighting the predicted cascades during bud burst in grapevine with the presumed hypoxia as the start point. Bud burst is a transition of considerable metabolic complexity, likely to see considerable changes in source/sink relations as well as the early stages of photosynthetic development. Thus the timing and metabolic coordination of the transition is crucial, and failure to achieve an orderly bud burst in a production system often leads to laborious and expensive management methods and low productivity. Existing literature shows that hypoxia responsive genes are up-regulated during dormancy release and when commencing bud burst, suggesting that hypoxia within the bud is mechanistically involved in this transition. The involvement ROS-signalling in bud burst has also been suggested by gene expression patterns. However, direct measurement of the internal oxygen tension and in situ detection of ROS are currently absent from the literature. Recent advances in knowledge of direct oxygen perception and signalling in plants has also yet to be applied to bud burst. Sugar status is also
likely to be important in signalling changes in energy state. Hence, together with the internal oxygen tension, respiration and ROS localisation data, analysis of carbohydrate composition could reveal a deeper understanding of the underlying physiological processes during bud burst. By coupling these physiological and metabolic analyses with transcriptome analysis, this thesis will also make an important contribution to bridge the divide between gene expression patterns and physiology in bud quiescence and burst.

Figure 1.2 Diagram of the conceptual conclusions presented in this mini literature review. Hypoxia is predicted as a prerequisite for bud burst initiation in grapevine, due to the increased expression of hypoxia responsive genes during this crucial transition. Some of the highly expressed genes are responsible for antioxidant defence, which lead us to hypothesise that ROS accumulation is also important in stimulating the growth and development. The rapid change of growth and development of the buds into shoots will inevitably require immediate energy sensing mechanism to readjust metabolism responding to the priorities. The proposed sequences of induction and feedback are presented in this diagram. ROS, reactive oxygen species; CHO, energy source in the form of carbohydrate, lipid, or protein. Solid arrow represents the predicted causal relationship and dashes arrow indicates feedback.
1.4 References


2 Spatio-temporal relief from hypoxia and production of reactive oxygen species during bud burst in grapevine (*Vitis vinifera*)

Preface

This chapter has been published in the journal Annals of Botany, vol. 116, pp. 703-711. The paper was the first to report *in situ* measurements of internal partial pressure of O$_2$ ($p$O$_2$) in perennial buds. This tested a fundamental assumption of earlier gene expression studies which suggested hypoxia and a transient oxidative burst were key signals driving bud burst. Thus, the experiments in this chapter were design to examine the tissue $p$O$_2$ and reactive oxygen species (ROS) spatial patterns, and respiratory measurements during bud burst. The experimental work involved a four months research visit to Prof Foyer’s laboratory at Leeds University, to develop the histochemical techniques to assess accumulation of ROS during bud burst. I contributed to all of the experimental data including optimising the experimental conditions. The profiling of internal $p$O$_2$ using O$_2$ microelectrodes was performed in collaboration with Dr Konnerup. I made a valuable contribution to drafting the manuscript. Dr Considine and Prof Colmer guided me through the discussions to generate the ideas and framework, as well as providing continuous feedback during the experiments.
PART OF A SPECIAL ISSUE ON REACTIVE OXYGEN SPECIES AND NITRIC OXIDE

Spatio-temporal relief from hypoxia and production of reactive oxygen species during bud burst in grapevine (Vitis vinifera)

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• Background and Aims Plants regulate cellular oxygen partial pressures (pO2), together with reduction/oxidation (redox) state in order to manage rapid developmental transitions such as bud burst after a period of quiescence. However, our understanding of pO2 regulation in complex meristematic organs such as buds is incomplete and, in particular, lacks spatial resolution.

• Methods The gradients in pO2 from the outer scales to the primary meristem complex were measured in grapevine (Vitis vinifera) buds, together with respiratory CO2 production rates and the accumulation of superoxide and hydrogen peroxide, from ecodormancy through the first 72h preceding bud burst, triggered by the transition from low to ambient temperatures.

• Key Results Steep internal pO2 gradients were measured in dormant buds with values as low as 2.5 kPa found in the core of the bud prior to bud burst. Respiratory CO2 production rates increased soon after the transition from low to ambient temperatures and the bud tissues gradually became oxygenated in a patterned process. Within 3h of the transition to ambient temperatures, superoxide accumulation was observed in the cambial meristem, co-localizing with lignified cellulosic associated with pro-vascular tissues. Thereafter, superoxide accumulated in other areas subtending the apical meristem complex, in the absence of significant hydrogen peroxide accumulation, except in the cambial meristem. By 72h, the internal pO2 gradient showed a biphasic profile, where the minimum pO2 was external to the core of the bud complex.

• Conclusions Spatial and temporal control of the tissue oxygen environment occurs within quiescent buds, and the transition from quiescence to bud burst is accompanied by a regulated relaxation of the hypoxic state and accumulation of reactive oxygen species within the developing cambium and vascular tissues of the heterotrophic grapevine buds.

Key words: Bud burst, Vitis vinifera, grapevine, reactive oxygen species, ROS, superoxide, hypoxia, oxygen partial pressure, meristem, development, respiration, ecodormancy, quiescence.

INTRODUCTION

The buds of perennial trees and vines comprise one or more embryonic shoots with multiple meristems of diverse organogenic states, encased in a protective shell of dense scales. Similar to germinating seeds, the transition from quiescence to metabolically active occurring during bud burst is rapid, and requires the re-structuring of intercellular communication, respiratory and biosynthetic metabolism, and cell division and expansion. The identity, pluripotency and fate of cells in the meristem is determined by spatial organization (Esau, 1977; van den Berg et al., 1995), which is compounded in the embryonic shoot. Hence, this transition requires intricate spatial and temporal coordination of intercellular signalling networks within and between the functional domains of each meristem.

Oxygen is an essential substrate and signal in all aerobic organisms. Plants regulate the availability of oxygen and its metabolism during key transitions, including the regulation of quiescence (Considine and Foyer, 2014). Within this context the cellular reduction/oxidation (redox) hub plays a key role (Gapper and Dolan, 2006; Considine and Foyer, 2014), and we suggest the partial pressure of oxygen (pO2) also plays an important role, as known in animals and other aerobic organisms (Brahimi-Horn et al., 2007). The complex roles of redox processes in seed germination (Diaz-Vivancos et al., 2013, and references therein) and the control of pO2 are far from understood (Bradford et al., 2008; Borsjak and Rolletschek, 2009).

Similarly, our current knowledge of redox and pO2 sensing and signalling during bud burst is limited, particularly in terms of the spatial resolution of oxygen dynamics. Animal stem cell models consider that the redox environment, together with hypoxia (low pO2), are central regulators of the stem cell niche, which are key to cell identity and the maintenance of quiescence and pluripotency (Mohyeddin et al., 2010; Wang et al., 2013). The quiescent centre of the root meristem resides in an oxidized niche (Jiang et al., 2013; Jiang and Feldman, 2005). It is probable that the organizing centre and stem cells of the...
In plants, as in animals, intracellular redox signals govern the cell cycle (Coluci et al., 2002; Jiang et al., 2003; Rothstein and Lucchesi, 2005; Díaz-Vivancos et al., 2010). The local perception of pO2 in animals enables acclimation during developmental transitions, as well as mediating responses to various stress conditions and pathologies (Brahimi-Horn et al., 2007). Recent studies have increased understanding of the sensing and signalling of pO2 in plant oxygen-stress responses (Gibbs et al., 2011; Licausi et al., 2011). However, this type of regulation has scarcely been studied in developing systems other than seeds.

Regulation of respiration is central to the transition from quiescence to the metabolically active state. During seed germination or bud burst, respiration increases because of the requirement for oxidative phosphorylation and reducing power (Morohashi and Shimokoriyama, 1975; Hourmant and Pradet, 1981; Bewley, 1997). Studies on seeds have demonstrated a regulatory role of redox signalling during germination and clear spatial gradients that illustrate the function of reactive oxygen species (ROS) and low-molecular-weight antioxidants in cell division and expansion (Gidrol et al., 1994; Schopfer et al., 2001; Oracz et al., 2009; Kramer et al., 2010; Rewers and Siwinska, 2014).

The transition to bud burst can be accelerated by numerous sub-lethal stresses, including transient inhibition of respiration, heat shock or hypoxia (Esashi and Nagao, 1973; Erezz et al., 1980; Erezz, 1987), as is also the case with seed germination (Roberts, 1962; Siegel et al., 1962, 1964; Chen, 1970; Al-Ani et al., 1985). ROS are proposed to be key signalling agents induced by respiratory inhibition, as they function both directly on the cell cycle and by modulating activities of plant growth regulators such as ethylene, abscisic acid and auxin (Ophir et al., 2009). This fits with earlier suggestions that repressed catalase activity (Shlakman et al., 1983; Nir et al., 1986) and increased production of hydrogen peroxide stimulate bud burst in grapevine (Perez and Lira, 2005; Vergara et al., 2012a). Indirect evidence that dormant buds reside in an hypoxic state comes from analyses of gene expression. Transcripts encoding proteins involved in oxidative phosphorylation and the tricarboxylic acid (TCA) cycle are repressed in dormant buds while those encoding components involved in glycolysis, pyruvate metabolism, fermentation and redox networks are increased (Halalay et al., 2008; Ophir et al., 2009; Vergara et al., 2012b). Much of these data come from buds under stress conditions.

The scales of buds have low oxygen permeability and so the enclosed tissues are likely to be hypoxic, similar to the situation in dry seeds (Borisjuk and Rolletschek, 2009). In the seeds of some species, the suberized cell layers beneath the seed coat act as a barrier to oxygen diffusion, and their removal accelerates germination (Collis-George and Melville, 1974; Rolletschek et al., 2007). To date, no studies in the literature report data on pO2 values in buds. The following studies were therefore performed to resolve this issue, and to examine the cellular redox poise and pO2 status during bud burst. Furthermore, we aimed to resolve the spatio-temporal changes in these parameters that accompany the transition to bud burst, in a simplified developmental system that may provide a platform for further studies in a range of conditions and quiescent states (Considine and Foyer, 2014). The following experiments were performed on grapevine (Vitis vinifera), which is one of the most economically important woody perennial crop species, and has become a model species for research on perennial woody plants. Due to the anatomical complexity of the grapevine bud relative to other meristematic organs, it is useful to describe grapevine bud structure (Pratt, 1974; May, 2004). The mature bud complex, or N+2 according to May (2004), comprises a hierarchy of three buds – primary, secondary and tertiary – each resembling primordial shoots (Fig. 1). The primary bud is the most developed and by maturity bears 12–15 nodes, including inflorescence, tendril and leaf primordia, enclosed by layers of bracts and hairs. During maturation prior to winter, outer bracts lignify and harden to physically protect the bud over winter. Concurrent with this is a gradual cessation of meristematic activity and the acquisition of tolerance to desiccation and chilling (Schrader et al., 2004; Rohde et al., 2007; Ruttkink et al., 2007). The cessation of growth involves the acquisition of dormancy, defined as the failure of an intact, viable bud to burst in otherwise conducive conditions, until repressive factors are overcome through entrainment to seasonal signals such as chilling and photoperiod (Bewley, 1997), otherwise known as endodormancy (Lang et al., 1987). Once endodormancy is overcome, the bud is said to be ecodormant, i.e. quiescent but awaiting conducive conditions for growth. In this study, we refer to the mature bud complex as a whole, although pO2 measurements were directed at the primary bud, and the secondary and tertiary buds were often lost during histological processing. The data presented here show that ecodormant buds undergo a regulated transition from hypoxia to the oxygenated state during bud burst. These findings provide a platform to further explore and dissect the roles of these signalling agents in mediating transitions in bud dormancy governed by environmental and developmental inputs.

**MATERIALS AND METHODS**

**Plant material**

Grapevine *Vitis vinifera* L. ‘Grimson Seedless’ canes with mature dormant buds were harvested mid-winter from a vineyard in Yallingup, Sidings, Western Australia (33-694'S, 115-102'E). Canes with buds intact were stored at 4 °C in the dark until they had received at least 5500 chilling hours (approx. 7 months). The low degree of quiescence of the buds after cold-storage was confirmed by growing single-node cuttings of nodes 5–7 (explants, numbered acropetally) at 23 °C in vermiculite in darkness, with water maintained at field capacity (see Fig. 1 for developmental progression). Nodes 5–7 were chosen due to positional effects noted previously (Antcliff and May, 1961). The cumulative rate of bud burst was scored similarly to that described by Antcliff and May (1961) and according to the modified Eichorn–Lorenz scale (EL; Coombe, 2004), showing that 50 % of buds had reached EL–4 after 96 h at 23 °C and 80 % bud burst by 240 h (data not shown). On this basis we chose to study a time series over 72 h from transfer to 23 °C, in continuous darkness to minimize complexity. One or more single nodes were considered a biological replicate, as described for each assay.
Internal O$_2$ partial pressure

The internal pO$_2$ of buds were measured after 3, 24 and 72 h at 23°C, using a Clark-type oxygen microelectrode with tip diameter of 25 μm (OX-25; Unisense A/S, Aarhus, Denmark). Internal pO$_2$ was also measured in buds with the outer scales removed by scalpel 10 min earlier, after 3 h at 23°C. Microelectrodes were calibrated at atmospheric pO$_2$ (20.87 kPa) and at zero O$_2$, then mechanically guided into the buds, from the outer scale surface to the core of the primary bud, in 25-μm steps to a depth of 2000 μm using a motorized micro-manipulator (MC-232; Unisense). The microelectrode recording was allowed to stabilize for 20 s after each step with measurements taken over the subsequent 10 s. Means and 95% confidence intervals of individual buds (n = 5) were calculated using R (R Development Core Team, 2014) and graphics were compiled using the latticeExtra package and functions within (Sarkar and Andrews, 2013).

Bud respiratory CO$_2$ production

Four buds per biological replicate were excised from the cane by transverse sectioning at the base of the bud, weighed and placed onto thin agar plates, cut-side down, so that O$_2$ entry and CO$_2$ exit would occur across the bud scales rather than via the cut base. The rate of CO$_2$ production of each biological replicate was measured in the dark, in an insect respiration chamber (6400-89; Li-COR, Lincoln, NE, USA) attached to an Li-6400XT portable gas exchange system. Measurements were performed at 23°C in CO$_2$-controlled air (380 μmol CO$_2$ mol$^{-1}$ air) with 100 μmol m$^{-2}$ s$^{-1}$ air flow, at 55–75% relative humidity. The system was allowed to stabilize for 10 min before recording and until the "stable" value was equal to 1, i.e. the condition of humidity, CO$_2$ and air flow were in equilibrium and stable. Means and 95% confidence intervals were determined by fitting the time-series of CO$_2$ evolution to a quadratic equation of the form, $y = a + bx + cx^2$, using the linear model function within R (R Development Core Team, 2014) and plotted using ggplot2 (Wickham, 2009).

Histology

Chemicals for histology were supplied by Sigma (St Louis, MO, USA) unless otherwise stated. To confirm the path of the pO$_2$ microelectrode, buds were fixed for sectioning immediately after measurement. Before excision and fixation, a vector was cut in a sagittal plane from each side of the bud complex, adjacent to the primary bud and parallel to the path of the microelectrode to aid penetration of the fixative. Buds were then excised from the cane by transverse sectioning at the base of the bud, then fixed in 10% (v/v) formaldehyde (Chem-Supply, Adelaide, Australia) with 5% (v/v) propionic acid (Ajax Chemicals, Sydney, Australia) overnight at 4°C, and subsequently dehydrated in serial ethanol solutions (15, 20, 25, 30, 50, 75, 90 and 100%, v/v) for 30 min each, with gentle agitation at 4°C. Buds were then embedded in paraffin wax. Sagittal sections (5 μm) of the bud were made on a microtome (RM2255; Leica Biosystems, Nussloch, Germany), transferred to slides, de-waxed and stained with 0.05% (w/v) toluidine blue O in 0.1 M phosphate buffer, pH 4.8. The sections were then scanned at 20× magnification using an Aperio Scanscope LX (Leica Biosystems).

Histological detection of hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$) were performed on bud sections from explants grown for 0, 3, 23 or 72 h at 23°C. The methods of Groten et al. (2005) were followed with minor change: nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) were each dissolved in 10 mM phosphate buffer, pH 7.8, without dimethylsulfoxide. Buds were excised from the cane as described to visualize the path of the microelectrode, and stained under light vacuum for 8 h at room temperature in darkness. Stained buds were fixed in 4% (v/v) formaldehyde (Chem-Supply) in a buffer of 5 mM MgSO$_4$, 5 mM EGTA and 50 mM PIPES, pH 6.9, vacuum infiltrated for 1 h, incubated overnight at 4°C, dehydrated in serial ethanol solutions (15, 20, 25, 30, 50, 75, 90 and 100%, v/v), for 30 min each, with gentle agitation at 4°C. The buds were then transferred to 1:1 (v/v) ethanol/Steedman's wax solution (Norenborg and Barrett, 1987) and incubated overnight at room temperature prior to embedding. Serial sagittal sections of the bud were made at 20-μm intervals using a microtome (RM2255; Leica Biosystems), transferred to slides and de-waxed in 100% followed by 50% (v/v), 5 min each solution. The sections were then scanned at 20× magnification using an Aperio Scanscope LX (Leica Biosystems).

To visualize lignin, NBT-stained buds were counter-stained with 0.05% (w/v) Auramine-O (Ajax Chemicals) in deionized water. A drop of stain solution was placed on each section and...
left to absorb for 1 min before washing the slides with sprayed water. The stained sections were then visualized using a Carl Zeiss microscope (D-708 Z; Oberkochen, Germany) with blue light at 450–490 nm.

RESULTS

Respiratory CO₂ production and internal pO₂

Respiratory CO₂ production rates increased from approx. 4.0 to 5.2 nmol CO₂ g f d⁻¹ s⁻¹ in ecodormant buds maintained at 23 °C over the first 72 h following the transition from low to ambient temperatures. Subsequently, respiration rates fell to approx. 4.0 nmol CO₂ g f d⁻¹ s⁻¹ by 144 h (Fig. 2), showing that metabolic activity was increased upon transition to conducive growth conditions for bud burst.

We determined the internal pO₂ profile from the outer scale towards the core of the primary bud complex: at 3 h after transfer to 23 °C, which was the earliest stage of measurement, the internal pO₂ was hypoxic immediately within the scale (approx. 10 kPa air = 20–6 kPa), declined towards 5 kPa within the outer 500 μm and declined steadily to approx. 2.5 kPa through to the core of the bud complex (Fig. 3A). Some replicate data showed undetectable O₂ (severe hypoxia/potential anoxia) at the core. Removal of the outer layer of scales at this time point resulted in oxygenation of the outer 15–1800 μm of the tissue profile, relative to the intact bud, although the core remained near 2.5 kPa (Fig. 3B). Despite this effect, de-scaling buds had no significant effect on the rate or completion of bud burst to stage EL-4, relative to intact buds (data not shown; see Materials and Methods). We then determined the pO₂ profiles of intact buds at 24 and 72 h after transfer to 23 °C to determine whether removal of the scale at 3 h simply expedited the normal progression of oxygenation within the bud. By 24 h, only the pO₂ of the outer 500 μm of the bud had increased, up to approx. 15 kPa pO₂ immediately within the scale, while the remaining pathway towards the core remained near levels seen in intact buds at 3 h (Fig. 3C). By 72 h, the pO₂ profile of the outer 1400 μm of tissue resembled that of the de-scaled buds at 3 h, although the pO₂ of the inner 500 μm had increased, resulting in a biphasic profile such that the minimum pO₂ along the electrode’s transect was approx. 7 kPa at 1400 μm depth from the scale, while at 2000 μm depth, the pO₂ was >10 kPa (Fig. 3D). Figure 3E shows the path of the microelectrode in a representative section.

Histological detection of superoxide and hydrogen peroxide

Using replicate buds of the same developmental series and treatment conditions as used for pO₂ microelectrode measurements, we stained for the local accumulation of superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), detected as the products of reactions with NBT or DAB, respectively. Immediately upon removal from 4 °C (0 h) and after 3 h at 23 °C, O₂⁻ accumulation was observed in the cambial meristem tissues. For the first 3 h no H₂O₂ accumulation was detected in tissues around the apical meristem but low levels were observed in the cambial meristem tissue (Fig. 4E, F). After 24 h, O₂⁻ levels were
increased in a wider zone of tissues of the apical meristem complex and retained in the cambial meristem tissues, while $\text{H}_2\text{O}_2$ was not accumulated in the tissues with the exception of the cambial meristem (Fig. 4C, G). At this time point the $p\text{O}_2$ at the core of the bud complex remained low. A more distinct pattern of $\text{O}_2^{-}$ localization emerged at 72 h, which suggested association with the developing pro-vascular tissues (Fig. 4D). At 72 h, no $\text{H}_2\text{O}_2$ accumulation was observed in the bud tissues (Fig. 4H). By this stage, the $p\text{O}_2$ at the core of the bud complex had increased, suggesting a possible association between the patterns.

To investigate the cell types associated with the distinct $\text{O}_2^{-}$ pattern seen at 72 h, we counter-stained sections to visualize lignin. Figure 5 shows a clear co-localization of $\text{O}_2^{-}$ with lignified cellulose as early as 3 h from transfer to 23°C, but not earlier, providing further evidence that these are developing pro-vascular tissues. At 0 h, $\text{O}_2^{-}$ accumulation was localized in the meristematic tissues but very little lignin was associated with this pattern (Fig. 5C and D show magnified images of the boxed areas of Fig. 5A and B). By contrast, at 3 h the co-localization of $\text{O}_2^{-}$ and lignin was observed (Fig. 5D-F shows the individual and superimposed images). Close inspection of Fig. 5E reveals the typical ladder-like perforation plates of xylem vessel elements.

**DISCUSSION**

The experimental system presented here mitigated the potentially confounding effects of endodormancy and the influence of light. Endodormancy in grapevine, as in many perennial trees and vines, is primarily overcome by an accumulated exposure to chilling. Adequately chilled buds are termed ecodormant, a qualitative condition that is repressed by the unfavourable growth environment (i.e., cold) and therefore more comparable to quiescence in other organs and forms of life. Bud burst per se does not require the presence of light (Pouget, 1963), although several studies have demonstrated influences of light intensity and photoperiod on organogenesis at other stages of development (Buttrose, 1970; Srinivasan and Mullins, 1981).

There is no knowledge of whether photosynthesis may initiate in the bud prior to bud burst. Drawing analogy to seeds, where in several species photosynthesis influences the internal $p\text{O}_2$, even during development or when mature and imbibed prior to germination (Boriskin and Rolletschek, 2009), we may expect this to be the case in buds. Hence, overcoming endodormancy and excluding light allowed us to accurately and precisely study heterotrophic metabolism during the acute phase of bud burst.

Cells in a quiescent state are defined by very low metabolic rates, with minimal respiration until environmental or metabolic triggers prime the metabolic systems to resume growth. While several authors have described conserved responses to hypoxia or other oxidative stress across species and life forms (Hochachka, 1986; Jones et al., 2000; Mustroph et al., 2010), it is not possible to construct a generalized description of the metabolic state of quiescent cells or the changes that occur upon the transition to the metabolically active state or subsequent proliferation (Valecourt et al., 2012; Teslia and Teitell, 2015). The findings of the present study provide new insights into the management of hypoxia when dormancy is broken in quiescent grapevine buds by exposure to chilling and the subsequent transition to ambient temperatures. While respiration rates are rapidly increased and superoxide accumulation is observed in and around the developing lignified zone of the cambium following the transition to ambient temperatures, the release from the hypoxic state is gradual and occurs in specific regions of the bud as the developmental transition progresses.

Rapid acceleration of respiratory CO$_2$ production was observed in the buds following the transition from low to ambient temperatures, demonstrating alleviation of the constraints maintaining the quiescent state. This process, which was observed
over the 72 h of bud burst measured at 23°C, resembles the pattern observed during seed imbibition (Bewley, 1997) and in other studies on perennial buds (Holins and Tepper, 1971; Shulman et al., 1983; Gardea et al., 1994; McPherson et al., 1997; Perez et al., 2008). Measurements of respiratory CO₂ production do not allow discrimination between TCA cycle activity, fermentation, the pentose phosphate pathway or other pathways. Evidence suggests that fermentation occurs during bud burst under stress conditions and that the imposition of stress accelerates bud burst. For example, acetaldehyde and ethanol accumulate in ecodormant grapevines treated with sodium azide, hydrogen cyanamide or heat shock (Ophir et al., 2009). Hydrogen cyanamide, heat shock and hypoxia increase the levels of transcripts that are orthologues of ALCOHOL DEHYDROGENASE, PYRUVATE DECARBOXYLASE and SUCROSE SYNTHASE in ecodormant grapevine buds (Or et al., 2000; Ophir et al., 2009; Vergara et al., 2012b).

However, in each case, untreated controls showed a slower or weaker transcriptional response with negligible fermentation activities observed during bud burst. These observations suggest that stress-induced changes in transcript profiles do not reflect the transcriptome signatures of developmental regulation of bud burst. Some evidence of pentose phosphate pathway activity was seen throughout seasonal development in pear buds (Zimmerman and Faust, 1969), and during chilling of potato tubers (Dwelle and Stallknecht, 1978) or peony buds (Gai et al., 2013). However, these studies represent quite different physiological states compared with bud burst.

Many plant tissues and organs, including dry seeds, have permeability barriers that reduce oxygen diffusion. In the case of seeds, the hypoxic state may contribute to maintaining quiescence (see Introduction). The data presented here show that the scales of the dormant bud are a significant barrier to oxygen. Crucially, however, the meristematic core of the bud tissues remained in a hypoxic state even when the outer scales were removed. While Iwasaki and Weaver (1977) suggested some acceleration of bud burst in de-scaled ecodormant grapevine buds, removal of the outer scales did not affect the rate of bud burst in our study (data not shown). Schneider (1968) also showed that removal of scales attenuated quiescence of Rhododendron floral buds. However, in these earlier studies there was very limited replication of experiments. Nevertheless, it is conceivable that the buds used in our study were near to 100% labile and hence very little effect of scale removal would be seen.

The data reported here demonstrate that the pO₂ at the meristematic core of the bud complex was in an hypoxic state for up to 24 h after the environmental trigger to resume growth had caused an increased in respiration. Respiratory CO₂ production rates had increased by 15% in 24 h and superoxide accumulation was observed in the cambial tissues underlying the meristematic core of the bud complex. By 72 h, however, the oxygen profile was biphasic, the oxygen levels within the bud core had increased and superoxide accumulation was pronounced within the pro-vascular tissues. The present data are insufficient to explain the biphasic profile of oxygenation. In the heterotrophic conditions presented, even once the resistance to diffusion of the outer scales and compacted tissues was relaxed, the increased respiratory rates would contribute to substantial declines in pO₂ with distance into the tissue. Further investigation of the vascular flow and metabolic activities at the core of the bud complex are required. Our group is currently exploring
these features, and also the developmental processes and controls that preserve in the presence of light, where photosynthesis may contribute to oxygenation even prior to bud burst, as is the case during germination of some seeds (Borisjuk and Rolletschek, 2009).

Vascular development and re-activation of intercellular communication are proposed to be essential early features of the transitions to and from quiescence in plant organs, including grapevine buds (Essau, 1948; Rinne et al., 2001; Paul et al., 2014). Cell expansion, cell-wall thickening and the conductivity of plasmodesmata in vascular tissues are all dependent on, or influenced by, ROS accumulation (Gapper and Dolan, 2006; Benitez-Alonso et al., 2011). Ogawa et al. (1997) showed a strong co-localization of lignin and superoxide (NBT) in vascular tissue of spinach hypocotyls. Moreover, these authors demonstrated that inhibition of Cu/Zn SUPEROXIDE DISMUTASE (Cu/ZnSOD) or NAD(P)H OXIDASE reduced vascular lignin biosynthesis. More recently, ectopic expression of Cu/ZnSOD and/or ASCORBATE PEROXIDASE (APX) in Arabidopsis resulted in enhanced vascular lignin synthesis (Shafi et al., 2015). SOD, APX and catalase were found in cell membranes that had been partially purified from lignin-producing tissues of Norway spruce (Karkonen et al., 2014). Together, these data suggest that vascular lignin synthesis is dependent on superoxide and/or hydrogen peroxide production. Note that hydrogen peroxide did not accumulate in vascular tissues of the buds studied here.

Taken together, the data presented here add to the growing body of evidence showing that regulation of redox and oxygen metabolism is critical to organ development (Considine and Feyrer, 2014). The present study demonstrates that during bud burst, the complex network of enclosed shoot meristems undergoes a controlled transition from hypoxia to increasing \( \text{pO}_2 \). This transition is accompanied by a highly localized accumulation of ROS in and around the developing cambium and vascular tissues. These data clearly demonstrate the spatial and temporal nature of the control of the oxygen and redox environments within the bud that occurs during the transition from quiescence to burst in heterotrophic grapevine buds.

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LITERATURE CITED


3 Light enhanced oxygen and energy metabolism suggests a dominant role for chloroplast biogenesis during bud burst

Preface

This chapter is currently under consideration to publish in the journal New Phytologist, submitted on 30/7/2016 (NPH-MS-2016-22526). The key findings of Chapter 2 on buds in dark conditions encouraged further analyses of ROS and $pO_2$ in buds in the presence and absence light in order to manipulate energy and oxygen metabolism in physiologically relevant conditions. Energy metabolism was examined by contrasting the composition of metabolites in buds grown in continuous darkness with those in a dark/light photoperiod cycle. The experimental design was generated through discussions with Dr Considine and Prof Foyer, and particularly to include the investigation of transcriptomic data. I planned and undertook all of the physiological experiments and metabolite assays, data analyses, and drafted the related methods and results, and contributed to the introduction and discussion of the submitted manuscript. Dr Agudelo-Romero performed the RNA sequencing and transcriptome analysis, and Dr Signorelli assisted with interpretation of differentially regulated transcripts relation to energy and ROS functions. These contributions are reflected in the authorship statement and acknowledgements of the submitted manuscript.
Abstract

The importance of tissue oxygen tension in regulating developmental transitions in plants has only recently emerged. This prompted a revisit of suggestions that stress-dependent signalling associated with hypoxia and mitochondrial impairment were mechanistic in triggering bud burst in perennials. Here we investigated relationships between tissue oxygen tension, localisation of reactive oxygen species (ROS) and transcriptional regulation during bud burst in grapevine (*Vitis vinifera* L.). We found that the presence of light temporally and spatially augmented tissue oxygenation and *in situ* patterning of ROS. Transcriptome data suggested a dominant role for chloroplast biogenesis in activating energy metabolism during the developmental transition to bud burst. Regulated hypoxia is a key feature of bud burst, explaining the absence of oxidative stress markers. The expression of chloroplast/photosynthetic genes highlights the importance of activating autotrophic growth early during this transition. Moreover, trehalose-6-phosphate plays a key role in the sucrose-dependent orchestration of bud burst. Together these data help to bridge the divide between tissue oxygen tension and molecular regulation of developmental phase transitions in plants.
3.1 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 status, which both function as powerful cues influencing cell function and fate. The role of light in calibrating development is well understood, particularly in the skoto- to photomorphogenic transition (Kami et al., 2010). Recent research has shown that oxygen tension, or more particularly hypoxia, is an important positional cue guiding biochemical adaptations that enable successful photomorphogenesis (Abbas et al., 2015). More broadly however, our understanding of the signalling roles of oxygen- and reactive oxygen species (ROS) during developmental transitions is only beginning to emerge, with considerably more attention in the past given to stress conditions (Considine & Foyer, 2014; Considine et al., submitted).

Seed germination and spring bud burst are the two principal events in the higher plant life cycle that see the initiation of autotrophic programming. During quiescence or dormancy of seeds, a relatively high proportion of cells reside in the G0/G1 stage of the cell cycle, the cellular matrix is relatively desiccated and hypoxic and the principal energy organelles exist in prototypical forms (Borisjuk & Rolletschek, 2009; Considine & Considine, 2016). Here we refer to quiescence as the more generic term for reversible latency, while dormancy is a specialised condition of quiescence, entrained in the organism’s life history (Considine & Considine, 2016). Germination commences with imbibition and release of hydrolases and proteases to enable catabolism of starch, proteins and lipids from storage tissues, which support respiration and synthetic metabolism (Bewley, 1997). Respiration thus increases early in germination, enabled by the activation of glycolysis, the pentose phosphate pathway and oxidative phosphorylation (Bewley, 1997), indicating that mitochondrial biogenesis resumes rapidly (Howell et al., 2006).
Our understanding of the roles of oxygen tension, ROS and reactive nitrogen species (RNS) in coordinating essential molecular events during germination has also increased rapidly in recent years. In particular, ROS and RNS have become known as primary spatial cues, modulating hormone- and kinase-dependent signalling and curation of transcription networks (Diaz-Vivancos et al., 2013). Synthesis and accumulation of ROS, principally superoxide and hydrogen peroxide, occurs within the embryonic axis and peripheral tissues, driven by apoplastic peroxidases and NADPH oxidases (Kranner et al., 2010; Ishibashi et al., 2015). Here, ROS appear to function in cell wall elasticity and cross-linking to enable extension growth of the radicle (Kärkönen & Kuchitsu, 2015), as well as carbonylation of specific proteins (Oracz et al., 2007).

Light is also an important positive cue for germination, whereby phytochrome signalling moderates the balance of gibberellins and abscisic acid, which determines germination competence (Seo et al., 2008). The active conformation of phytochromes is promoted by red light and this conformational change is accompanied by import into the nucleus, where it binds to several interacting proteins that regulate germination and other developmental transitions. Light also promotes plastid differentiation to actively photosynthetic chloroplasts (Pogson & Albrecht, 2011). Plastid and mitochondrial biogenesis during the early stages of germination may be co-dependent, in a state of so-called photoheterotrophic growth (Borisjuk et al., 2004). Germination in prolonged darkness however, promotes a state of starvation (Hong et al., 2012; Avin-Wittenberg et al., 2015). The transcription of numerous genes are highly responsive to prolonged darkness or starvation (Osuna et al., 2007), leading to a reorganisation of metabolism towards the use of alternative substrates and pathways for energy provision, including autophagy (Avin-Wittenberg et al., 2015). The roles of primary signalling hubs for energy status, particularly sucrose nonfermenting1-related kinase1 (SnRK1) and trehalose-6-phosphate and hexokinase and their interaction and crosstalk with other signalling nodes
during germination have begun to emerge (Baena-González & Sheen, 2008; Hong et al., 2012; Avin-Wittenberg et al., 2015).

In contrast to germination, our knowledge of the processes that govern the early stages of bud burst remains poor. The spatial organisation of tissues in buds is considerably more complex than that of seeds; depending on the species, the bud comprises one or more embryonic shoots, often with both vegetative and reproductive meristems (van der Schoot et al., 2013). Gene expression data from several previous investigations have suggested that hypoxia and mitochondrial respiratory stress are key events triggering the transition from quiescence to bud burst in grapevine. Accordingly, hypoxia was proposed as a physiological trigger for an increase in mitochondria-derived ROS, impaired oxidative phosphorylation and a reconfiguration of energy metabolism towards substrate-level phosphorylation and fermentation. However, these insights were drawn from buds treated with a chemical agent known to induce a stress response, potentially compromising the relevance to development under more physiologically normal conditions. Nevertheless, we recently demonstrated that the quiescent bud is indeed hypoxic, whereby the oxygen tension (partial pressure of oxygen, $pO_2$) is $<3$ kPa at the bud core (Meitha et al., 2015). During the early stages of bud burst, the respiration rate increases and yet there is a gradual, spatial relaxation of hypoxia, in part mediated by a weakening of the outer barriers to oxygen diffusion (Meitha et al., 2015). We also provided spatial resolution of ROS patterning, whereby superoxide appeared to initially localise to the meristematic tissues and gradually shifted towards the provascular tissues at the interface between the bud and branch (Meitha et al., 2015), perhaps promoting vascular development and augmenting the re-establishment of source-sink relations and intercellular communication (Benitez-Alfonso et al., 2011). Although these data improved our physiological understanding of the transition to bud burst, they were drawn from an acute experiment in the absence of light. Given the importance of light as a cue for promoting germination and particularly in photosynthetic oxygen synthesis, the present study investigated the role of light.
in augmenting oxygen, redox and energy metabolism during the developmental transition of bud burst in grapevine. Although bud burst in grapevine does not require light (Pouget, 1963), as in some species such as *Rosa* spp. (Girault *et al.*, 2008), the contrast of a dark/ light regime against continuous darkness was expected to provide useful insight to the developmental regulation of oxygen and energy metabolism in physiologically relevant conditions. It was hypothesised that the presence of light would accelerate internal oxygenation in the bud and by corollary that persistence of hypoxia under extended darkness would manifest in a starvation response, reflected in transcriptional and metabolic responses. A profound and somewhat surprising finding was the prominent role of chloroplast biogenesis during bud burst, as previous studies had implicated the mitochondria as the hub of energy metabolism during the transition to bud burst.

### 3.2 Materials and methods

#### 3.2.1 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 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. Prior to each 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) and deionised watered to field capacity (*i.e.* substrate water-holding with drainage), for a period of 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 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 (Supplemental Figure S3.1). Both time points were classified at the bud-swell stage of EL2/3 (modified Eichorn-Lorenz (EL) scale; Coombe, 2004), i.e. prior to 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).

Bud respiration (4 biological replicates of 4 buds each), internal partial pressure of oxygen (pO₂; 3 replicates of one bud each) and histology (3 replicates of one bud each) were performed as described previously (Meitha et al., 2015). Moisture content was measured on the same buds used for respiration (4 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. For statistical comparison of respiration, moisture content and sugar concentration, ANOVA was performed using a Tukey multiple comparison of means with 95% family-wise confidence at P≤0.05 (n = 4 replicates of four buds per replicate). 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. The buds for metabolite analyses were the same as those used for respiration and hydration (4 biological replicates of 4 buds each), while 3 replicates (3 buds each) were used for RNA-seq analysis.
3.2.2 Sugar and starch analysis

Metabolite extraction was performed as previously described (Gomez et al., 2002) with minor modifications and in 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 x g 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.
3.2.3 RNA extraction, Illumina 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-seq 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).

3.2.4 Data processing analysis

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; Supplemental Table S3.1) and the log$_2$ Transcripts-Per-Million (logTPM) was obtained. A linear model (limma) was then applied to do a differential expression analysis (Supplemental Table S3.2). $P$-values were corrected for multiple-testing using the Benjamini-Hochberg's method (FDR $P\leq0.05$) (Benjamini & Hochberg, 1995). Data were then filtered considering a fold change (FC|2|).
3.2.5 Clustering and functional enrichment analysis

Genes differentially expressed were grouped in 2×3 self-organising maps (SOMs) (Kohonen, 1990) using Acuity 4.0 software (Axon Molecular Devices, Sunnyvale, USA) with Euclidean squared similarity metrics. Gene lists were analysed further with FatiGO (Al-Shahrour et al., 2004) to identify significant functional enrichment in Babelomics 5 (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 & Hochberg, 1995).

3.3 Results

3.3.1 Physiology and metabolite analysis

3.3.1.1 Respiration, water and sugar content show minimal effect of light during bud burst

To investigate the physiology of pre-burst buds in grapevine, we assessed respiratory CO₂ production and moisture content of buds grown in continuous darkness (D) or a dark/ light photoperiod (DL; 12/ 12 h), over 72 and 144 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; Supplemental Figure S3.1). Moisture content increased through time, but was unaffected by treatment condition (Figure 3.1.A). Bud respiration increased in both conditions from time zero (see Fig. 2 in...
Meitha *et al.*, 2015), but only continued to increase between 72-144 h in the DL condition ($P<0.05$; **Figure 3.1.B**). Differences in respiration between the D and DL conditions at 144 h were evident but not significant ($P>0.05$; **Figure 3.1.B**). In order to gain further insight to respiration, we then compared trends in respiratory substrates in each condition. Significant decreases in sucrose, glucose and fructose but not in starch were observed in the DL condition between 72 and 144 h (**Figure 3.1.C-F**). In contrast, changes in substrates in the D condition over this time were not significant, however direct comparisons between the D and DL conditions were also not significant. Together these data suggest that the presence of light in the DL condition temporally augments the activation of reserve mobilisation and respiration in buds after bud burst has commenced.

### 3.3.1.2 Internal tissue oxygenation is accelerated in the presence of light during bud burst

We then investigated the internal oxygen partial pressure ($pO_2$), as this provides a spatial resolution of oxygen metabolism in the contrasting conditions, which our respiratory approach cannot. We have previously reported that oxygenation during bud burst commences from the outer bud layers, followed by a net increase in $pO_2$ from within the bud core by 72 h (see Fig. 3 in Meitha *et al.*, 2015), revealing that the $pO_2$ minimum was peripheral to the bud core. In the previous report, buds were kept in continuous darkness. Here we demonstrate that internal oxygenation was further augmented in the presence of light (**Figure 3.2**), despite no detectable difference in net respiration at this time (**Figure 3.1.B**). In the DL condition, the $pO_2$ minimum at 72 h was apparently greater (less hypoxic) than in D and the $pO_2$ at the core of the bud (2000 µm) was greater, although these data were not amenable to rigorous statistical analysis, as we have used spline fits. Hence, increases in the internal $pO_2$ appeared to be spatially and temporally augmented by light. Analysis of internal $pO_2$ was not reliable at 144 h in our hands and thus not presented.
Figure 3.1. Overview of water content (A), respiration rates (B) and metabolite profiles (C-F) of bursting single bud grapevine explants after removal from 4 °C storage and growth for 72 or 144 h in continuous darkness/D or a 12/12 h photoperiod/DL. Respiration rates were measured as CO₂ production in darkness (B), after which excised buds were freeze-dried and weighed for moisture content (A). Subsequently, sucrose (C), glucose (D) and fructose (E) were quantified by gas chromatography and starch (F) by an enzymatic assay. All data presented on a dry weight (DW) basis except for water content, on fresh weight (FW) basis. Means with different letters for treatment or time point indicate significant difference according to a Tukey multiple comparison of means with a 95 % family-wise confidence level at $P \leq 0.05$ (n = 4 replicates of four buds per replicate).
Superoxide and hydrogen peroxide patterning is accelerated in the presence of light during bud burst

The bud complex of grapevine comprises a primary, secondary and tertiary bud, each a primordial shoot (Figure 3.3.A). We focussed our ROS histology on the primary bud as the primary determinant of production; the other two typically remain suppressed and are not fruitful. Superoxide localisation was visualised as formazan, resulting from the oxidation of NBT in the presence of superoxide (Figure 3.3.B-D). We have previously shown that after 72 h in the D condition, superoxide shifts from the meristematic tissues to the pro-vascular tissues of the bud complex, i.e. the junction with the shoot (see Figure 4D in Meitha et al., 2015). This was supported by detection of lignin by co-localisation (see Fig. 5 in Meitha et al., 2015). Here
we show that this pattern persists to 144 h in the D condition (Figure 3.3.D). By comparison, in the DL condition superoxide became more localised towards differentiating shoot organs by 72 h (Figure 3.3.B) and appeared more diffuse and less abundant by 144 h (Figure 3.3.C). Thus the spatial dynamics of superoxide of buds also appeared to be temporally augmented in the presence of light.

Figure 3.3 In situ localisation of reactive oxygen species of grapevine buds during the transition to bud burst. An unstained bud at 0 h after removal from 4 °C storage (A). In situ localisation of superoxide (B, C) by nitrobluetetrazolium (NBT) and hydrogen peroxide (E-G) by 3,3'diaminobenzidine (DAB). Prior to staining, the buds were removed from the 4 °C storage and grown as single bud explants for 72 (B, E) and 144 h (C, F) in a 12/12 h photoperiod, or for 144 h in continuous darkness (D, G). Refer to Meitha et al., (2015) for 72 h in continuous darkness. Scale bar = 1 mm. Figures are representative of three independent replicates of individual buds.
Hydrogen peroxide (H₂O₂) was visualised by oxidation of DAB to a brown precipitate. The previous report showed diffuse patterns of H₂O₂ detection in meristematic and differentiating organs in the first 72 h of the D condition (see Fig. 4E-H in Meitha et al., 2015). Here, this pattern apparently persisted to 144 h in the D condition (Figure 3.3.G) and to 72 h in the DL condition (Figure 3.3.E), while H₂O₂ had apparently dissipated and was undetectable by 144 h in the DL condition (Figure 3.3.F). Taken together with the dynamics of superoxide, these data suggest a more rapid development of the vascular system and differentiation of meristematic organs in the presence of light. However we acknowledge that our approach could not determine whether the ROS was causal or consequential of differentiation.

3.3.2 Transcriptome analysis

3.3.2.1 Transcriptional analysis and comparison with public data indicate a strong influence of light signalling in augmenting oxygen and energy metabolism

A full description of the transcriptome comparisons, annotation and enrichment are provided in Supplemental File S3.1, Supplemental Figure S3.2, Figure 3.4, Supplemental Table S3.3). We performed Self Organising Map analysis with the differentially expressed genes (DEG) that met our criteria for false discovery rate and fold-change (Figure 3.4, refer to Supplemental File S3.1). In order to interrogate these data in the context the wider public data, we queried transcriptional profiles of Arabidopsis homologues using Genevestigator (Hruz et al., 2008; date accessed 04/07/2016). We selected all genes that met DEG criteria in the DL/ D comparison at 144 h, as this may provide insight to the genes induced in the dark condition and whether this was consistent with our hypothesis concerning the impact of light augmenting oxygen and energy metabolism during the transition to bud burst. Comparisons with all available perturbation data highlighted similarities with post-germination photomorphogenesis, particularly studies investigating light- and carbon-dependent signalling
(Supplemental Table S3.4.A-B, refer to Supplemental File S3.1). For example, the role of plastid biogenesis in mediating light-dependent signalling (Ruckle et al., 2012) and a study identifying CARBON AND LIGHT INSENSITIVE mutants (Thum et al., 2008). Refining the query to studies of seeds suggested similarities of the DL condition with developmentally advanced seeds (Narsai et al., 2011; Supplemental Table S3.4.C, refer to Supplemental File S3.1). Finally we constrained the query to conditions of oxygen- or oxidative stress, whereby we sought conditions most different to the DL/ D comparison, as we expected these signatures to be more pronounced in the D condition. These data suggested similarities of the D condition with transient hypoxia, rather than oxidative stress (Supplemental Table S3.4.D, refer to Supplemental File S3.1).

Hence, these data provide considerable validation of our approach and evidence of the influence of light and sugar signalling in particular. With this confidence, we explored the key transcriptional changes in our DL/ D data according to the functional enrichment. We have used the terms ‘upregulated’ or ‘downregulated’ for simplicity in describing these changes, where we refer to the DL condition relative to D, unless otherwise indicated.
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**Figure 3.4** Self-organising maps (SOMs) of transcripts differentially expressed during grapevine bud burst in the presence (DL) or absence (D) of light. Differentially expressed genes (DL/ D) that met our criteria for false discovery rate and fold change criteria (FDR $P \leq 0.05$, FC$|2|$) at 72 and 144 h against 0h were organised into SOMs, labeled as 1 to 6. The number of Unigenes belonging to each SOM category is indicated in the bottom left hand of each SOM. Numbers in brackets refer to genes identified after functional enrichment using FatiGO (Al-Shahrou et al., 2004). The tables display the summary of the functional categories along with their adjusted $P$ values, which cluster 4 does not present any of them. Significant enrichment according to Bonferroni-Hochberg adjusted $P$ value < 0.05 in a Fisher’s exact test. Green represents downregulated expression (DL/ D), magenta represents upregulation and black represents no change. Colour intensity reflects magnitude of differential expression, where greater intensity corresponds to a greater differential. A full description is available in Supplemental File S3.1, refer also to Supplemental Figure S3.2.

### 3.3.2.2 Photosystem assembly and chlorophyll biosynthesis implicate chloroplast biogenesis early during bud burst in the presence of light

Early markers of the perception of light or prolonged darkness in the respective conditions included a homologue of *EARLY LIGHT-INDUCIBLE PROTEIN* (VIT_05s0020g04110), which was progressively upregulated in the DL condition (SOM1) and a homologue of *DARK-INDUCED6* (*DIN6*, VIT_06s0004g06830, also known as *ASPARAGINE SYNTHETASE1, ASN1*), which was progressively downregulated in DL relative to D (upregulated in D, SOM6). The upregulation of *DIN6* is a hallmark of various stress conditions including extended darkness and hypoxia, which limit photosynthesis and respiration, while being repressed by more energetic conditions or treatment with sucrose or glucose (Baena-Gonzalez et al., 2007). In particular, *DIN6* was induced by specifically induced by the *Arabidopsis* homologues of the catalytic subunits of SnRK1 (KIN10, KIN11), a conserved hub of starvation signalling (Baena-Gonzalez et al., 2007).

Transcripts involved in protein synthesis and transport, particularly related to chloroplast and mitochondrial biogenesis, were among those most greatly upregulated in the DL condition relative to D. The upregulation of numerous genes coding for components of photosystems I and II, the light-harvesting complexes and associated functions are illustrated in Figure 3.5.
Several of these were homologous to genes encoded in the plastid genome. Other homologues of organelle-encoded genes included a plastid-encoded RNA polymerase (VIT_06s0004g05580) and ribosomal protein (VIT_09s0070g00920), as well as a mitochondrial-encoded subunit of cytochrome oxidase (VIT_03s0110g00190). Nuclear-encoded import components of the mitochondria (VIT_03s0063g01900) and plastid (VIT_04s0008g07020) were also upregulated in the DL condition, in addition to two ankyrin domain-containing proteins (VIT_07s0151g00180, VIT_19s0014g05330), which may be involved in successful insertion of LHC components from the stroma to the thylakoid membrane. Furthermore, several genes coding for primary active transporters or transporters driven by light or electrochemical potential were upregulated in the DL condition (Figure 3.5).

Several genes coding for steps in the chlorophyll synthetic pathway were upregulated by 144 h in the DL condition, including the primary rate-limiting GLUTAMYL-tRNA REDUCTASE (Figure 3.5). Interestingly, a negative regulator of chlorophyll synthesis, FLUORESCENT IN BLUE LIGHT and the catabolic Mg-CHLOROPHYLLASE 1, were also upregulated in the DL condition, suggesting that chlorophyll turnover was already active. Following these data, we investigated chlorophyll synthesis, assayed as pheophytin and found significantly more total pheophytin at 72 h in the DL condition than D (Tukey’s test, $P \leq 0.05$, data not shown). Differences were apparent at 144 h but not significant. Several steps of the Calvin cycle were upregulated, as further described in the subsequent section (Figures 3.5, 6).

Together, these data provide a strong indication that chloroplast biogenesis plays an important role during the early stages prior to bud burst and the minimal light requirement to augment this. Evidence of mitochondrial biogenesis was less pronounced or less light-dependent.
Figure 3.5 Upregulation of genes in grapevine buds coding for photosynthetic and chlorophyll metabolic functions at 144 h in the presence of light (DL/ D). Purple colour indicates upregulation at 144 h of DL respect to D. ALA, Aminolevulinic acid; CAO, CHLOROPHYLL A OXYGENASE; CHL, Mg-CHLOROPHYLLASE 1; CHLH, Mg-CHELATASE subunit; CRD, Mg-PROTOPORPHYRIN IX MONOMETHYLESTER CYCLASE; Cytb6/F, CYTOCHROME b6-F COMPLEX IRON-SULFUR subunit (PETC); Fd, FERREDOXIN; FLU, FLUORESCENT IN BLUE LIGHT; FNR, Fd NADP+ OXIDOREDUCTASE; GUN4, GENOMES UNCOUPLED4; HCF136, PSII STABILITY/ASSEMBLY FACTOR; HEMA, GLUTAMYL-tRNA REDUCTASE; LHC, LIGHT-HARVESTING COMPLEX; POR, NAPDH-PROTOCHLOROPHYLIDE OXIDOREDUCTASE; PSI, PHOTOSYSTEM I; PSI1, PHOTOSYSTEM II; PsaD, PSI REACTION CENTRE (RC) subunit II, chloroplast precursor; PsaE B, PSI RC subunit IV B; PsaG, PSI RC subunit V; PsaH, PSI RC subunit VI; PsaK, PSI subunit X; PsaL, PSI subunit XI; PsaN, PSI RC subunit N; PsaO, PSI subunit O; PsbS, PSI 22 kDa protein; PsbW, PSI RC W; PsbX, PHOTOSYSTEM II subunit X; PsbY, PSI CORE COMPLEX PROTEIN (chloroplast precursor); psbZ, PSI core complex proteins; RuBisCO, RIBULOSE BISPHOSPHATE CARBOXYLASE; RuBP, Ribulose 1,5-bisphosphate.
Figure 3.6 Differential expression of genes during grapevine bud burst coding for carbon- and energy-related functions at 144 h in the presence (DL) or absence (D) of light. Processes and reactions in purple and green reflect up- and downregulation respectively at 144 h. α1,4G, α-1,4-GLUCOSIDASE; BCAAs, branched-chain-amino acids; BCAT, BRANCHED-CHAIN-AMINO-ACID AMINOTRANSFERASE; Epi, ALDOSE 1-EPIMERASE; FBP, FRUCTOSE 1,6-BISPHOSPHATASE; FBPA, FRUCTOSE 6-PHOSPHATE ALDOLASE; G3PDH, GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE B; ME, NADP-DEPENDENT MALIC ENZYME; PEPC, PHOSPHOENOLPYRUVATE CARBOXYKINASE; RuBisCO, RIBULOSE BISPHOSPHATE CARBOXYLASE; SUS, SUCROSE SYNTHASE; TP, Trehalose-phosphatase; TPP, Trehalose-6-phosphate phosphatase; TPS, Trehalose-6-phosphate synthase.

3.3.2.3 Transcripts involved in starch and sucrose metabolism, glycolysis and the Calvin cycle indicate a predominant metabolic role of the chloroplast in the presence of light and the mitochondria and nucleus in its absence.

Given that whole organ starch, sucrose and hexose levels were not significantly different between buds in D and DL treatment conditions at either time point, nor was respiration (Figure 3.1), it is interesting that several transcripts related to starch, sucrose and hexose
metabolism exhibited strong temporal regulation and equally strong contrasts between the treatment conditions. Caution must be exercised in interpreting these changes, as many enzymes contribute to more than one metabolic pathway and some catalyse reversible reactions. Notwithstanding this, transcripts particularly upregulated at 72 h in the DL condition included homologues of STARCH PHOSPHORYLASE (VIT_04s0008g03590), BETA-1,3-GLUCANASE (VIT_09s0002g06880) and two SUCROSE SYNTHASE (SUS; VIT_04s0079g00230, VIT_17s0053g00700; (Supplemental Table S3.3). The activation of starch and sucrose hydrolysis was largely attenuated at 144 h, although STARCH PHOSPHORYLASE remained upregulated at 144 h, while a homologue of CALLOSE SYNTHASE was downregulated in the DL condition at 144 h (Supplemental Table S3.3).

Taking a cue from DIN6 expression, which suggested a mild starvation syndrome in the D condition at 144 h and to be consistent with Figure 3.5, we illustrated the differential regulation of genes coding for carbon metabolic pathways at 144 h in Figure 3.6. The clear upregulation of plastid carbon metabolism in the DL condition was evident through several components of the Calvin cycle, in addition to genes coding for up- and downstream catabolic functions. The upregulation of a homologue of the plastid-localised NADP\(^+\)-dependent MALIC ENZYME in provides further evidence of active photosynthesis in the DL condition, as the enzyme encoded by this gene contributes NADP\(^+\)/NADPH homeostasis and provision of reducing power for the Calvin cycle (Wheeler et al., 2005). In contrast, the D condition was characterised by the upregulation of genes coding for the catabolism of branched-chain amino acids in the plastid, as well as the PHOSPHOENOLPYRUVATE CARBOXYKINASE in the cytosol (Figure 3.6). These suggest the requirement for alternative substrates to fuel the mitochondrial tricarboxylic acid pathway (Araújo et al., 2010; Avin-Wittenberg et al., 2015). Trehalose-6-phosphate (T6P) is a primary sensor of cellular energy status. Two TREHALOSE-6-PHOSPHATE PHOSPHATASE homologues (TPP) were upregulated in the DL condition at 144 h, while a further TPP and a TREHALOSE-6-PHOSPHATE SYNTHASE (TPS) were downregulated
(upregulated in the D condition, Figure 3.6). These transcriptional differences suggest reduced levels of T6P or alternatively increased turnover in the DL condition. Following these insights and in addition to the identification of the the DIN6 homologue, we contrasted all of the DEGs identified in the DL/ D comparison at 144 h, to the core 600 putative targets of the Arabidopsis KIN10 (Baena-Gonzalez et al., 2007). Indeed, this contrast corroborated our identification of the catabolism branched-chain amino acids, plus the upregulation of TREHALOSE-6-PHOSPHATE SYNTHASE in the D condition (repressed in DL/ D; Supplemental Table S3.5). Of further interest from this contrast were a DORMANCY/AUXIN ASSOCIATED1 (DRM1; VIT_10s0003g00090), two genes coding for thioredoxins (VIT_00s0532g00030, VIT_08s0007g06700) and two members of the NBS-LRR leucine-rich repeat superfamily (VIT_11s0118g00480, VIT_09s0002g08270), each implicated in sugar starvation responses (Baena-Gonzalez et al., 2007).

Together, these data provide evidence of a prominent role for chloroplast processes in carbon and oxygen (energy) metabolism during bud burst and the requirement for light to orchestrate chloroplast biogenesis. Alternative pathways for catabolism became evident under continuous darkness, suggesting catabolism of branched-chain amino acids to fuel the mitochondrial tricarboxylic acid cycle, consistent with a mild starvation syndrome.

3.3.2.4 Gibberellic acid and auxin signalling were upregulated, ethylene and cytokinin signalling were downregulated in the presence of light

Investigation of hormone-related transcriptional changes indicated a strong and transient upregulation of transcripts assigned to gibberellic acid (GA) and auxin biosynthesis or signalling and downregulation of GA catabolism (Supplemental Table S3.6). There was also a strong downregulation of ethylene biosynthesis or signalling in the DL condition, or by corollary upregulation in the D condition. Notable were two homologues of the ETHYLENE RESPONSE
FACTOR family (VIT_18s0001g10150, VIT_05s0077g01860), which includes a homologue of the *Arabidopsis RELATED TO AP2.3*, a known hypoxia-responsive gene (Gibbs et al., 2011). Of particular interest however, genes coding for functions in cytokinin signalling and transport were downregulated in the light, which is contrary to the condition recently shown in bud outgrowth in *Rosa* sp. (Roman et al., 2016). These included two genes coding for PURINE PERMEASE1 (VIT_18s0001g06910, VIT_18s0001g06940) and a ARR1-type1 protein (VIT_06s0004g05120), homologues of which were shown upregulated by cytokinin or light during bud outgrowth in *Rosa* sp. (Roman et al., 2016). However, physiologically the *Rosa* buds studied were young sylleptic buds, suppressed by apical dominance, which had yet to undergo maturation or dormancy sensu stricto (Considine & Considine, 2016).

3.3.2.5 Protein kinase signalling was differentially regulated, implicating a starvation in the absence of light

Following the identification of key homologues of *Arabidopsis* genes previously implicated in starvation or hypoxia, we looked to the transcripts assigned to signalling pathways other than hormone components, *e.g.* protein kinase-dependent signalling, which may provide evidence of the dominant signalling networks governing cellular communication during bud burst. Indeed, a homologue of the *RAPTOR1* (VIT_08s0058g00910) component of the target of rapamycin pathway (Baena-González & Sheen, 2008) was upregulated in the DL condition. The target of rapamycin pathway promotes growth and differentiation in conditions of energy sufficiency (Xiong et al., 2013; Xiong & Sheen, 2014). Perhaps consistent with this, an *AKIN GAMMA* (VIT_17s0000g01460), homologue of the *Arabidopsis* family of *SnRK1*-like genes was downregulated in the DL condition. *SnRK1*-like protein kinases function in stress and developmental conditions, notably mediating cellular responses to starvation (Baena-Gonzalez et al., 2007; Baena-González & Sheen, 2008).
3.4 Discussion

The impact of hypoxia imposed by stress conditions such as flooding has been widely studied (Voesenek & Bailey-Serres, 2015; Loreti et al., 2016). The role of select group VII ethylene response factors (ERF VII) in the direct perception and signal transduction of changes in oxygen tension was recently defined in this context (Gibbs et al., 2011; Licausi et al., 2011), providing a mechanistic explanation for genetic variation in resilience of crops to submergence. For example, the success of the Swarna-Sub1 rice (Ismail et al., 2013), which involves expression of a constitutive ERF VII that is not susceptible to degradation under normoxia (Gibbs et al., 2011). Earlier studies of the transition from quiescence to bud burst in perennials also implicated hypoxia and oxidative stress in triggering the developmental re-activation of metabolism and growth (Or et al., 2000; Ophir et al., 2009; Vergara et al., 2012). However, physiological data were limited and buds in the latter studies were subject to chemical stress, which questions the relevance of these insights in a developmental context. Very recently, mechanistic approaches have established a role for oxygen-dependent signalling in developmental transitions (Considine et al., submitted), notably during seed germination (Gibbs et al., 2014) and the skoto-photomorphogenic transition in seedlings (Abbas et al., 2015). Our previous studies have provided some insights into the physiological role of oxygen tension in vivo ($pO_2$) during the developmental transition of bud burst (Meitha et al., 2015). Controlled hypoxia in the quiescent buds and regulated spatiotemporal oxygenation during bud burst provided some support for the hypothesis that hypoxia was a physiological cue for bud burst (Ophir et al., 2009). The data presented here increase our knowledge of the physiological roles of $pO_2$ and reactive oxygen species (ROS) in orchestrating bud burst in grapevine under physiologically relevant conditions, notably in the absence of chemical treatment. By contrasting the presence and absence of light during bud burst, we show that light accelerates oxygenation and the spatial patterning of ROS in vivo (Figures 3.2, 3), well before the emergence of leaves, which defines bud burst sensu stricto (Coombe, 2004).
Transcriptome analysis allowed us to explore the implications for cellular signalling and metabolism further, particularly during the transition to autotrophic metabolism.

The most prominent signature of the transcriptome data was chloroplast biogenesis and establishment of photosynthetic functions of light harvesting and carbon fixation (Fig. 5). This dominant feature represented an essential preparation for the onset of autotrophic metabolism. A role for chloroplast biogenesis has been well-established during seed germination (Pogson & Albrecht, 2011), a model which bears much resemblance to bud burst. Interestingly, a recent report suggested that chloroplast biogenesis was secondary to cytokinin signalling during light-dependent bud burst (Roman et al., 2016). Profiles of cytokinin-related genes in our data were contrary to those presented therein. However, Roman et al. (2016) studied suppressed syleptic buds, which had yet to undergo maturation or dormancy sensu stricto (Considine & Considine, 2016). Hence this signalling may resemble the condition of syleptic buds of annual plants which bear no dormancy and where auxin, strigolactone, cytokinin and sugar demand play key roles (Domagalska & Leyser, 2011; Mason et al., 2014).

The physiological distinction between suppressed syleptic buds and dormant or post-dormant buds appears to be fundamental in determining the targets of light signalling and in particular the relative roles of plastid- and cytokinin-dependent signalling and metabolism.

The regulation of metabolic and bioenergetic functions of the mitochondria were not as prominent as had been suggested in the literature (Ophir et al., 2009; Vergara et al., 2012). Therein, it was proposed that dormancy-releasing stimuli which accelerate bud burst, such as hydrogen cyanamide, heat shock, hypoxia or sodium azide, act by impairing mitochondrial oxidative phosphorylation. In turn, this triggers a ROS-mediated oxidative stress and activates a hypoxic/ starvation syndrome of enhanced glycolysis and fermentation, leading to ethylene-dependent signalling. The data presented here show conclusively that hypoxia persisted under continuous darkness and the transcriptome data suggested a mild starvation syndrome in this
condition, with dark-induced expression of putative targets of the low-energy signalling hub sucrose nonfermenting1-related kinase1 (SnRK1; Baena-Gonzalez et al., 2007; Baena-González & Sheen, 2008), as well as catabolism of branched-chain amino acids and ethylene synthesis and signalling. These patterns are similar to those proposed to be central to the relief of dormancy and acceleration of bud burst (Ophir et al., 2009; Vergara et al., 2012), however bud burst was not accelerated under continuous darkness and there was little evidence of mitochondrial or oxidative stress in our data. The accelerated transcriptional activation of chloroplast biogenesis and carbon metabolism in the presence of light add further question to the physiological relevance of hypoxia or oxidative stress as triggers for the transition to bud burst and highlight the need for further research into organelle biogenesis in perennial buds. A previous study of metabolic control in isolated mitochondria of grapevine buds was largely unrevealing (Perez et al., 2008), as mitochondria were not intact. There are no reports of intact chloroplasts isolated from mature or post-dormant buds in the current literature. Research into organelle ultrastructure, histology and biogenesis during bud development and burst is ongoing in our laboratory.

Sucrose, glucose and fructose levels decreased between 72 and 144 h in the the presence of light but not in continuous darkness (Figure 3.1.C-F). Importantly in relation to sucrose metabolism, a gene coding for a TREHALOSE-6-PHOSPHATE (T6P) SYNTHASE was upregulated in the condition of continuous darkness, while two genes coding for T6P PHOSPHATASE were higher in the presence of light (Fig. 6). Taken together, these observations suggest that T6P synthesis was favoured in darkness and that it was decreased at the end of the bud burst profile in the presence of light. T6P is a determinant of sink strength and an important metabolic signal conveying information of cellular carbon status. The cell autonomous expression of trehalose pathway genes allowing fine tuning of specific developmental responses to sucrose availability (Griffiths et al., 2016). T6P exerts its effects through SnRK1, homologues of which were downregulated in the presence of light in the present study. The
data presented here show that similar to the situation in seeds, sucrose and trehalose metabolism are key to ensuring appropriate resource allocation to drive growth and development of the bud.

The N-end rule of proteolysis, which directly perceives and transduces changes in oxygen tension, was also recently shown to be involved in overcoming dormancy in seed and enabling germination, through both oxygen- and NO-dependent signalling (Gibbs et al., 2014). Furthermore, hypoxia was shown to be an important positional cue mediating post-germinative photomorphogenesis, in a manner dependent on the N-end rule of proteolysis (Abbas et al., 2015). The oxygen-dependent N-end rule of proteolysis is mediated by select group VII ethylene response factors (ERF VII), which are targeted for proteasomal degradation under normoxia, but stabilised under hypoxia, whereby they activate conserved hypoxia-responsive processes (Gibbs et al., 2011; Licausi et al., 2011). One of the two ethylene response factors which were differentially regulated in our data was homologous to the *Arabidopsis RELATED TO AP2.3 (RAP2.3)*, previously shown to be a substrate for N-end rule (Gibbs et al., 2011). In our data, this ERF VII was upregulated in the condition of continuous darkness. It is tempting to suggest that this upregulation was mechanistic in the transcriptional and metabolic response in these conditions and further evidence of the physiological persistence of hypoxia in the buds. However, the ability of the grapevine ERF VII s to function as substrates for the oxygen-dependent N-end rule of proteolysis is ongoing work in our laboratory. The role of nitric oxide in seed germination, in relation to the N-end rule of proteolysis and abscisic acid-dependent signalling in particular, also suggests functions yet to be explored in perennial buds.

The accelerated spatial patterning of ROS localisation in the presence of light is interesting in the context of vascular development. By counterstaining for lignin, we have previously shown that superoxide localises to the vascular tissues soon after the transition to bud burst
commences, where it may function in cell wall elasticity and cross-linking required for vascular development (Meitha et al., 2015). This suggests accelerated vascular development in the light, which may be provide an internal pathway for oxygen diffusion by reconnecting the bud to the mother plant at a time when xylem pressure is high. The oxygen profiles clearly show at the later stages of bud burst that the oxygen minimum is peripheral to the core, indicating that oxygenation is not merely a passive process of diffusion through the outer scales. Two explanations for this are plausible; either photosynthesis becomes established at the core of the bud, or oxygen diffusion via vascular connections becomes established. These explanations are not mutually exclusive. An additional role for ROS in this context may related to the re-establishment of intercellular communication. In poplar buds, the plasmodesmata become occluded by callose deposition during the onset of bud dormancy and degraded with dormancy release and the onset of bud burst (Rinne et al., 2001). Chilling and gibberellic acid accelerate the callose degradation via β-1,3-glucanases restoring intercellular communication and conductance, notably for sucrose and phloem-mobile elements such as the FLOWERING LOCUS T (Rinne et al., 2001). However, according to the model proposed by Benitez-Alfonso and Jackson (2009), ROS would augment callose deposition. Nevertheless, a gene coding for callose synthase was repressed in the presence of light and hence further research is required to establish whether ROS may participate in re-establishing intercellular conductance.

3.5 Conclusions

The quiescent bud of perennials such as grapevine is a complex organ of one or more embryonic shoots of differing organogenic states. Data presented here bridge divide between spatial and temporal regulation of physiological oxygen tension and transcriptional activation of organelle biogenesis and energy metabolism in the developmental transition towards bud burst. These data demonstrate a predominant role for chloroplast biogenesis and increased capacity for light harvesting and carbon fixation in the orderly transition to bud burst. These
data also suggest that light-dependent hormone signalling in mature proleptic buds may be quite distinct to the behaviour of suppressed sylleptic buds.

3.6 References


3.7 Supplemental information

File S3.1 (E-copy). Transcriptome overview, annotation and enrichment and query of perturbations available on Genevestigator. Full description of the transcriptome analysis and comparisons of data in the presence (DL) or absence (D) of light. Includes description of data in Supp. Fig. S3.2, Fig. 3.4 and Supp. Tables S3.3, 4.

Table S3.1 (E-copy). Log$_2$ of transcripts per million (logTPM) dataset normalised by the Trimmed Mean of M-values (TMM) method. Unique_ID refers to the Vitis vinifera annotation by Grimplet et al. (2012). D and DL refer to the conditions of continuous darkness or the dark/ light photoperiod. 72 and 144 refer to the corresponding time of sampling in hours. Three biological replicates are indicated for each condition.

Table S3.2 (E-copy). Limma analysis statistics. Limma analysis was performed using log transcript per million (logTPM) values in the presence/DL or absence/D of light at 72 h (A) and 144 h (B). Differentially expressed genes (DEGs) were filtered using a fold change (logFC|1|) and multiple test (FDR≤0.05, adj. P.value) criteria. (A) Comparison of DL/ D at 72 h. (B) Comparison of DL/ D at 144 h.

Table S3.3 (E-copy). Differentially expressed genes (DEG) which met fold change and false discovery rate criteria (FC≥|2|, FDR $P$≥0.05) in the comparison of the dark/ light condition (DL) against continuous darkness (D) at 72 and 144 h and after functional enrichment (Al-Shahrour et al., 2004). Annotation and Arabidopsis homologues represent the annotation by Grimplet et al. (2012).

Table S3.4 (E-copy). Comparison of differentially expressed gene expression, after functional enrichment with public Arabidopsis microarray data available at Genevestigator. Refer to Supp. File S1 for a full description. Arabidopsis homologues, according to the Vitis vinifera reference genome (Grimplet et al., 2012) were identified for genes meeting the criteria for differential expression (false-discovery rate $P$≤0.05, fold-change |2|) in the 144 h DL/ D comparison (Supp. Table S3). The resulting genes were trimmed of duplicates (A) and submitted to the Signature tool of Genevestigator (Hruz et al., 2008; date accessed 04/07/2016). Comparisons against all 3020 Perturbations including all genetic backgrounds (B), or constrained lists of 136 germination studies (C) or 100 hypoxia-related studies (D) were made. (A) The submitted list of genes from Supp. Table S3. (B) The exported data from Genevestigator perturbations unconstrained, listing Arabidopsis gene accession (row 1), ATH1 probe ID (row 2) and top 50 most similar transcriptome profiles to our data. (C) The exported data from Genevestigator perturbations constrained to germination, listing Arabidopsis gene accession (row 1), ATH1 probe ID (row 2) and top 50 most similar transcriptome profiles to our data. (D) The exported data from Genevestigator perturbations constrained to studies involving hypoxia or oxidative stress, listing
Arabidopsis gene accession (row 1), ATH1 probe ID (row 2) and top 50 most different transcriptome profiles to our data.

Table S3.5 (E-copy). Comparison of differentially expressed gene expression, after functional enrichment with the 600 putative targets of the Arabidopsis KIN10 homologue of the catalytic subunit of SnRK1 from Baena-Gonzalez et al. (2007; Suppl. Table S3.4 therein). Arabidopsis homologues, according to the Vitis vinifera reference genome (Grimplet et al., 2012) were identified for genes meeting the criteria for differential expression (false-discovery rate $P \leq 0.05$, fold-change $|2|$) in the 144 h DL/ D comparison (Supp. Table S3). Differential expression of the resulting genes were contrasted with the 600 differentially expressed putative targets of KIN10, which were differentially regulated by starvation conditions. Because our data were DL/ D, this analysis sought opposite direction of change, i.e. reflecting the D/ DL condition.

Table S3.6 (E-copy). Subset of differentially expressed genes (DEG) from Supp. Table S3.3 related to hormone metabolism, signalling and transport. Fold-change (FC) data for the comparison of the dark/light (DL) conditions against continuous darkness (D) at 72 and 144 h are presented, together with the corresponding Arabidopsis homologue and functional annotation according to Grimplet et al. (2012). Additional details presented in Supp. Table S3.3.

Figure S3.1. Morphometric development of single bud explants of grapevine grown in the presence (DL) or absence (D) of light for 72 or 144 h, showing no visible differences in development. Representative images shown.
Figure S3.2. Transcriptional regulation of grapevine bud burst in the presence (DL) or absence (D) of light. (A) Percentage of variances from a principal component analysis (PCA). (B) PCA plot of dependent-light samples according to their transcripts per million (TPM) values normalised by Trimmed Mean of M-values (TMM) method. (C) Differentially expressed genes (DEG) which met false discovery rate and fold change criteria (FDR P≤0.05, FC≥2) in the comparison of the dark/light condition (DL) against continuous darkness (D). (D) Venn diagram of DEGs from the DL/D comparison according their stage of development (72 h and 144 h). (E) Distribution of functional categories the core set of the 870 modulated DEGs. (F) Further detail on the metabolism functional category corresponding to 238 DEGs.
Preface

The results of Chapters 2 and 3 suggested that hypoxia was mechanistically involved in curating the transcriptome during quiescence and the transition to bud burst. Hence, the experiments were looking to the possible mechanistic regulation of hypoxia responsive genes during bud burst. The ideas and framework of this Chapter were constructed through discussions with Dr Considine, Prof Foyer, Dr Gibbs (University of Birmingham) and Prof Colmer. I planned and conducted all of the molecular experiments including cloning, subcloning and site-directed mutagenesis, in vitro protein translation, and performed the bioinformatic analysis. Dr Santiago Signorelli and Ms Dina Hermawaty performed some replicates of the in vitro analysis and provided advice on chapter. Dr Agudelo-Romero performed the RNA sequencing and supervised the bioinformatic analysis of the RNA sequencing data. I wrote the chapter with constructive comments on the text from all supervisors and collaborators.
Abstract

Recent insights have drawn attention to a developmental role for tissue oxygen tension during phase transitions such as germination and photomorphogenesis. Regulated hypoxia was also recently demonstrated to be a key feature of bud burst in grapevine (*Vitis vinifera* L.). In the present study we investigated the capacity of the group VII ETHYLENE RESPONSIVE FACTORS (ERFVII) of grapevine to function in the direct perception of molecular oxygen through the N-end rule pathway of targeted proteolysis. According to the arginine/N-end rule pathway in plants, the stability of ERFVII proteins is dependent on the N-terminal motif (N-degron), which under normoxia is modified to enable proteasomal degradation, but under hypoxia/anoxia is stable, enabling import to the nucleus to function in transcriptional activation of target genes. The grapevine genome possesses three *VvERFVIIs*, and deduced amino acid sequences show each possess the conserved N-degron motif. *In vitro* translation of two *VvERFVIIs* in rabbit reticulocytes showed that the wild-type proteins were degraded under normoxic conditions but stabilised under anoxia or in the presence of a proteasome inhibitor. Mutagenesis of the N-terminal cysteine to alanine also prevented proteasomal targeting, verifying that the N-terminal cysteine is the primary destabilising residue. Transcriptome profiles of bursting grapevine buds, which differed in tissue oxygen tension identified the presence of several differentially regulated genes which possessed the hypoxia responsive promoter element (HPRE), a motif that is directly targeted by ERFVIIs in *Arabidopsis*. We discuss these data in the context of the role of oxygen signalling during the transition from a hypoxic, quiescent condition to active biosynthetic metabolism.
4.1 Introduction

Hypoxia is a common abiotic stress confronted by plant cells which is caused by a sudden increase in respiratory demand, reduced oxygen levels in the environment, or as a consequence of residing in a dense organ (Banks, 1993; Biais et al., 2009; Magness, 1920). The low oxygen condition has considerable consequences for cellular metabolism and long term exposure can induce cell death (Biemelt et al., 1999; Colmer and Greenway, 2011; Geigenberger et al., 2000). Hypoxia has become acknowledged as a key condition to trigger cell differentiation in plants and animals under non-stress conditions (Abbas et al., 2015; Kelliher and Walbot, 2012; Mitchell and Yochim, 1968; van Tuyl et al., 2005). For example, hypoxia is a key positional cue guiding male germ line fate (Kelliher and Walbot, 2012), germination (Gibbs et al., 2011) and the skoto-photomorphic transition in seedlings (Abbas et al., 2015). A regulated transition in tissue oxygen tension is also documented to occur during bud burst, a defining event in the phenology of deciduous perennials (Meitha et al., 2015). The quiescent bud of grapevine (Vitis vinifera L.) is a complex dense organ that bears the shoot apical meristem. The lignified scales of buds protect shoot apical meristem through conditions unfavourable for growth (Mullins et al., 1992; Pratt, 1974, 1979), and were shown to be a barrier to oxygen diffusion (Meitha et al., 2015). The mean internal oxygen partial pressure ($pO_2$) of quiescent buds is ~2.5 kPa and increases during bud burst (Meitha et al., 2015). Gene expression studies during bud dormancy release and burst in grapevine suggest the upregulation of putative hypoxia-responsive genes (HRG), which include functions in antioxidant defence, fermentation and hormonal regulation (Halaly et al., 2008; Or et al., 2000b; Vergara et al., 2012a; Vergara et al., 2012b). Our previous study (Meitha et al., 2015) showed that oxygenation is spatio-temporally regulated during bud burst, which indicates a possible interaction between $pO_2$ dynamics and regulation of HRG expression during this pivotal transition from quiescence to active growth.
The direct perception of molecular oxygen in plants occurs via an N-end rule of targeted proteolysis (Gibbs et al., 2011; Licausi et al., 2011; Sasidharan and Mustroph, 2011). This pathway degrades substrate proteins via the recognition of amino acids sequence at the amino (N)-terminal, known as N-degron (Bachmair et al., 1986; Lee et al., 2005; Marino et al., 2015; Varshavsky, 1996). Proteins of group VII ethylene response factor (ERFVII) in Arabidopsis (Gibbs et al., 2011) and barley (Mendiondo et al., 2016) have been identified as functional substrates for the arginylated branch of the N-end rule pathway (Arg/N-end), whereby hypoxia attenuates proteolysis, enabling signal transduction to activate hypoxia-responsive genes and acclimation (Figure 4.1). Recent detection of an ERFVII in hypoxic apple fruit is further evidence that this function of the ERFVII transcription factors is widely conserved (Cukrov et al., 2016). Indeed, the exception proves the rule, where the waterlogging-resistant SUB1A locus of rice encodes an ERFVII which does not conform to N-end rule proteolysis, and is thus constitutively abundant and able to function in rapid acclimation to hypoxic stress (Ismail et al., 2013). The consensus motif of N-degron in ERFVII, MCGGAI/L, is crucial for oxygen sensing and commitment for the subsequent steps of proteolysis (Gibbs et al., 2011; Licausi et al., 2011; Sasidharan and Mustroph, 2011). However, not all ERFVII proteins possessing the motif are susceptible to the Arg/N-end (e.g. SUB1A of rice), indicating that 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 degradation process is initiated by methionine removal from the N-terminus by METHIONINE AMINOPEPTIDASE (MetAP), exposing cysteine for oxidation by PLANT CYSTEINE OXIDASE 1 or 2 (PCO1/2), a step requires both oxygen and nitric oxide (Gibbs et al., 2014; Weits et al., 2014). Oxidised cysteine is then arginylated by an ARGINYL tRNA TRANSFERASE (ATE), which triggers interaction with E3 ligases (N-recogin) for ubiquitination, and inevitably the targeted protein will undergo proteasomal degradation (Tasaki et al., 2012).
Figure 4.1 Schematic diagram of the Arg/N-End rule pathway under normo- and hypoxia. The degradation of group ERFVII transcription factors requires molecular oxygen and nitric oxide. Under normoxia, cysteine is oxidised following the constitutive cleavage of methionine. The oxidised residue will then be arginylated, attracting E3 ligase to bind as the proteolysis signal for proteasome. Amino acids are indicated by single letter; MetAp, methionine amino peptidase; PCO, plant cysteine oxidase; NO, nitric oxide; C*, oxidised cysteine; ATE, arginine transferase; RC*, arginylated cysteine; PRT6, E3 ligase.

The stabilisation of RELATED TO APETALA 2.12 (RAP2.12), one of five ERFVIIIs in Arabidopsis, promotes transcription of 8 HRG (Kosmacz et al., 2015). The RAP2.12 transcription factor directly binds to a specific motif in HRG promoters, consisting of a 12-bp cis-regulatory sequence (5’-AAACCA[G/C][G/C][G/C]GC-3’), known as the hypoxic-responsive promoter element (HRPE, Gasch et al., 2016). The motif presents 1-3 times in the promoter region of 39 out of 49 core HRG (Mustroph et al., 2009), and is adequate for the activation of the core HRG AtPCO1 and LATERAL ORGAN BOUNDARIES PROTEIN 41 (AtLBD41) by two redundant ERFVIIIs (RAP2.2 and 2.12) in Arabidopsis (Gasch et al., 2016). Earlier studies of HRG identified the anaerobic responsive element (ARE) in promoters of ALCOHOL DEHYDROGENASE 1 (ADH1) genes of Arabidopsis and maize (Dolferus et al., 1995; Hoeren et al., 1998; Walker et al., 1987). The ARE of ZmADH1 is a bipartite element with two copies of GT-rich (5’-[T/C]GGTTT-3’) and two GC-rich (5’-GCC[G/C]C-3’) components (Hoeren et al., 1998; Walker et al., 1987), and AtADH1 comprises a somewhat similar motif that is necessary for promoter activity in hypoxia-stressed plants (Dolferus et al., 1995). Similarities exist between HRPE and ARE; both function
in forward and reverse complement orientation, their activity multiplies when present in multiple copies, and they are sufficient for reporter gene induction under hypoxia (Gasch et al., 2016; Olive et al., 1990; Walker et al., 1987).

Here, we report on the behaviour of two VvERFVIIIs under normoxia (~21 kPa $pO_2$) and anoxia (~0 kPa $pO_2$) using an in vitro translation system. RNA sequencing data confirmed the presence of the conserved N-degron motif in the VvERFVII coding sequence. Differential expression analysis of quiescent versus bursting buds differing in $pO_2$ was performed to determine the differentially regulated transcripts during this phase transition. The expression patterns of homologues of the 49 HRG of Arabidopsis (Mustroph et al., 2009) are described and the presence of HRPE motifs in the 5’ untranslated region (5’UTR) was examined, along with GT- and GC-rich components of ARE. Data were consistent with a role for the VvERFVII in oxygen perception and the transcriptional regulation of key enzymes involved in glycolysis and fermentation during the transition from quiescence to bud burst.

4.2 Materials and methods

4.2.1 Plant materials and growth conditions

Grapevine (Vitis vinifera var. Crimson Seedless) canes with buds intact were sampled from a vineyard in Western Australia (33.694°S, 115.102°E) during mid-year, and stored at 4°C until required for experiments, as described previously (Meitha et al., 2015). Prior to each experiment, the stored canes were removed from 4°C, and buds from node 5-7 were cut into individual cuttings with cane intact (explant). The explants were then planted in vermiculite and maintained in growth conditions with a constant temperature of 23°C, in darkness and watered to field capacity, for a period of 3 and 72 hours (h). The buds were excised from cane and plunged into liquid nitrogen immediately after the growth period, with three biological replicates from each treatment consisting of 3 buds each. Internal oxygen tension of these buds represented the following range: for 3 h, 10 kPa immediately within the scale to 2.5 kPa
at the bud core, with much of the transect <5 kPa, and; for 72 h, 20 to 7.5 kPa, noting that the profile was biphasic (Meitha et al., 2015).

4.2.2 RNA extraction, Illumina library construction and sequencing

Frozen buds were ground into fine powder with liquid nitrogen treatment. Total RNA extraction was performed by using the Spectrum Plant Total RNA kit with an on-column DNase treatment according to the supplier’s instructions (Sigma-Aldrich, Castle Hill, Australia), followed by an isopropanol/acetate precipitation. Electrophoresis and spectrophotometry analysis (NanoDrop 100, Thermoscientific, Scoresby, Australia) were conducted to confirm the quality and quantity of extracted RNA. Only RNA with an OD 260/280 nm ratio greater than 1.95 was chosen for the subsequent analyses. RNA-seq 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 100bp single-end runs.

4.2.3 Data processing analysis

Transcripts data 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 carried out using edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015) Bioconductor packages. The counts matrix obtained from Kallisto was examined in edgeR and the quality of the replicates was checked using Pearson's correlation, the values ranged between 0.86 and 0.97. Then raw data was normalised using trimmed mean M-value (TMM) method (Robinson and Oshlack, 2010) (Supplemental Figure S4.1) and log2 Transcript-Per-Million (log2TPM) was obtained. A linear model (limma) was applied to do a differential expression analysis (Supplemental Table S4.1). P-values were corrected for multiple-testing using the method of Benjamini-Hochberg (FDRs0.05) (Benjamini and Hochberg, 1995). Functional annotation was constructed following Grimplet et al. (2012), group ERFVII transcripts were compared against
those previously reported (Licausi et al., 2010) and MotifSearch (Kanehisa and Susumu, 2000) was used to locate APETALA2/ERF domain in the genes.

4.2.4 Plasmid construction, in vitro protein translation and immunodetection

The cDNA synthesis was made using Tetro cDNA Synthesis Kit according to the manufacturer’s instruction (Bioline, Sydney, Australia). Specific primers were used to amplify group ERFVII genes of grapevine (VvERFVII) 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 haemaglutinin (HA) tag for western blot detection. Site-directed mutations to alter the penultimate codon of the N-degron sequence of VvERFVII proteins were performed according to manufacturer’s instructions (QuickChange II, Agilent Technologies, Mulgrave, Australia), replacing the N-terminal Cys with alanine (Ala) (Gibbs et al., 2011). Sequence validation was performed by sequencing and list of primers is presented in Supplemental Table S4.2.

Translations were performed by using TNT-T7 Coupled Reticulocyte Lysate System (Promega, Alexandria, Australia) in normal and zero oxygen conditions. The $pO_2$ was quantified at the start and end of incubation by inserting the micro-respiration sensors (Unisense, Aarhus, Denmark) into the anoxia chamber. Normal oxygen level (normoxia) was the air-equilibrated oxygen concentration, $pO_2 \sim 21$ kPa, $[O_2] \sim 260 \mu$M, $\sim 20.95\%$ (v/v) of atmospheric air. The $pO_2$ of the anoxia treatment remained below detection at the two time points tested (0 kPa). For all translation experiments, the reactions were firstly incubated at 30°C in normoxia for 30 minutes to produce the initial amount of proteins. After the addition of 0.1 mM cyclohexamide to inhibit protein synthesis, the reaction was sampled immediately as the first time point. As required, MG132 was also added at this time to inhibit the proteasome inhibition. The reaction tubes were then subjected to their committed oxygen treatments at 30°C. The anoxic condition was made by putting the tubes in a custom-built respiration chamber, flushed with
100% N\textsubscript{2} gas, and then submerged in a water-bath with the N\textsubscript{2} gas continuously flowing during the incubation. The incubation of reaction under anoxia was terminated after 90 minutes and the reaction products were sampled. Protein immunoblotting was performed as described in Gibbs \textit{et al.} (2011) with the modification of primary antibody anti-HA titre of 1:20,000, and chemiluminescence detection was made using a ChemiDoc\textsuperscript{TM} MP system (Biorad, Gladesville, Australia). Equal loading was confirmed by chemiluminescent detection of the proteins after resolving in TGX Stain-Free\textsuperscript{TM} (Biorad) precast gels and equal transfer was checked after the transfer onto PVDF membrane (Supplemental Figure S4.2).

4.2.5 Promoter analyses

Transcripts were filtered considering a log fold change of 1 (log\textsubscript{FC} \geq |1|) in 3 h versus 72 h buds to generate a list of putative hypoxia regulated transcripts. Internal pO\textsubscript{2} condition of 3 h buds is potentially hypoxic when compared to 72 h, see Figure 3A and C in Meitha \textit{et al.} (2015). Differentially expressed genes were then queried against homologues of the HRG (Mustroph \textit{et al.}, 2009), which were identified following a grapevine-specific functional classification of 12X V1 predicted transcripts (Grimplet \textit{et al.}, 2012), cross-referenced with Plaza3.0 database (Proost \textit{et al.}, 2015) and by BLAST method (Altschul \textit{et al.}, 1990). Following this, hypoxia regulated transcripts were refined based on the list of HRG with HRPE motif (Gasch \textit{et al.}, 2016).

The 5’UTR (-3000 bp from ATG) of matched transcripts were retrieved from Ensembl Genomes (Kersey \textit{et al.}, 2016). The position-specific scoring matrices (PSSM) of HRPE motif was obtained from Gasch \textit{et al.} (2016), and then RSA-tool matrix scan (Turatsinze \textit{et al.}, 2008) was used to predict the occurrences of HRPE in putative promoter sequences, applying a cut-off \textit{P}-value of 1.10\textsuperscript{-4}. Consensus sequences of HRPE, GT- and GC-rich components of ARE (Hoeren \textit{et al.}, 1998; Walker \textit{et al.}, 1987) were also submitted to DMINDA-motif scanning analysis (Ma \textit{et al.}, 2014) to detect motifs occurrences in the promoters, with unchanged parameters.
4.3 Results

4.3.1 The grapevine genome contains 3 ERFVII, each possessing the N-degron motif

Analysis of transcript data identified minor genotype variations in the coding sequence of the three grapevine ERFVII previously identified (Licausi et al., 2010). The three ERFVII 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), based on protein similarity as described below (Table 4.1). The APETALA2/ERF domain and N-degron motif were found in the deduced amino acid sequence of each VvERFVII (Figure 4.2A). The APETALA2/ERF is a unique domain important for DNA binding and its presence confirms that these proteins are members of ERF family (Nakano et al., 2006), whilst the N-degron motif is a key motif that dictates proteolysis via the Arg/N-end rule pathway (Gibbs et al., 2011; Licausi et al., 2011). The VvERF058.1, VvERF057.1 and VvERF059.1 genes were expressed in 3 h buds but not differentially regulated in the treatment comparison (3 vs 72 h), logFCs ≤ |1| (Supplemental Table S4.1), noting that not all plant ERFVII are differentially regulated by oxygen at the transcript level (Mustroph et al., 2009).

Hierarchical cluster analysis (Figure 4.2C) of group ERFVII proteins from the present study and Licausi et al. (2010) VvERF057, VvERF058, and VvERF059, and ERFVII of Arabidopsis HRE1 and HRE2, RAP2.3, RAP2.3 and RAP2.12, showed that VvERF058.1 and VvERF058 were identical and showed least similarity to the other ERFVII of grapevine or Arabidopsis. 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. Relative to VvERF057, VvERF057.1 lacked 57 residues (AA 158-179 and 189-223, position respect to start codon), whereas VvERF059.1 possessed an additional 48
residues (AA 228-242, 250-259 and 325-347) and missing 15 (AA 84-98) when compared with VvERF059 (Supplemental Table S4.3). These variations were confirmed by direct sequencing of the subcloned coding sequence, and are likely to be genotype-specific alleles, as our data represented the variety Crimson Seedless, whereas Licausi et al. (2010) reported on the Pinot Noir (PN40024) genome (Jaillon et al., 2007).

Table 4.1 Details of grape ERFVII with their functional annotations according to Grimplet et al. (2012) and their homologous genes in Arabidopsis. Presented logFCs are a comparison of average log2TPM (n=3) in 3 h versus 72 h buds.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Unique ID in 12X V1</th>
<th>LogFC</th>
<th>Location</th>
<th>Functional annotation (Grimplet et al., 2012)</th>
<th>Best Arabidopsis match (Plaza 3.0 annotation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VvERF058.1</td>
<td>VIT_05s0077g01860</td>
<td>-0.545</td>
<td>chr5: 1463190-1464371 (+)</td>
<td>ERF (ethylene response factor) sub B-2</td>
<td>AT3G16770 (Related to AP2 3)</td>
</tr>
<tr>
<td>VvERF057.1</td>
<td>VIT_07s0005g00820</td>
<td>0.443</td>
<td>chr7: 3447132-3448343 (+)</td>
<td>AP2-like ethylene-responsive transcription factor</td>
<td>AT2G47520 (Hypoxia responsive ERF 2)</td>
</tr>
<tr>
<td>VvERF059.1</td>
<td>VIT_09s0002g00470</td>
<td>-0.239</td>
<td>chr9: 355010-357884 (+)</td>
<td>Ethylene response factor</td>
<td>AT1G53910 (Related to AP2 12)</td>
</tr>
</tbody>
</table>
Figure 4.2 Structure and hierarchical clustering of group ERFVII transcription factors in grapevine. (A) Structure of the three members of group ERFVII in grapevine. All possess APETALA2/ERF domain and N-degron motif of MCGGAI from the consensus MCGGAI/L. (B) Dendogram of group ERFVII in grapevine against *Arabidopsis*. The tree was built by using Jukes-Cantor genetic distance model in Neighbor-Joining method, with Bootstrap resampling values are shown in the nodes. It indicates that VvERF057.1 and VvERF057, 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 possibly related to AtRAP2.12 and AtRAP2.2.
4.3.2 The VvERF057.1 and VvERF059.1 proteins are in vitro substrates for the N-end rule pathway

The stability of VvERF058.1, VvERF057.1 and VvERF059.1 proteins were examined in normoxic and anoxic conditions. We were not able to express the VvERF058.1. In each experiment, the translation reaction was terminated after 30 minutes by the addition of cycloheximide, thus protein abundance at this point was considered as the basal amount prior to degradation. The degradation of wild type MC-VvERF057.1 and MC-VvERF059.1 was detected when the translated proteins were continuously exposed to normoxia (atmosphere-equilibrated O\textsubscript{2}, ~21 kPa pO\textsubscript{2}, ~260 μM [O\textsubscript{2}]), while the mutant versions (MA-VvERF057.1 and MA-VvERF059.1) were found to be stable throughout the experiment (Figure 4.3.A). The addition of proteasome inhibitor attenuated proteolysis of MC-VvERF057.1 and MC-VvERF059.1 (Figure 4.3.B). In the anoxic condition, MC-VvERF057.1 and MC-VvERF059.1 proteins were stable even after 90 minutes of incubation (Figure 4.3.B), indicating that, similarly to ERFVII of Arabidopsis, oxygen is required to stimulate destruction via the N-terminal Cys. Collectively these data indicate that grapevine ERFVIIs are targeted for degradation via the proteasome in an oxygen-dependent manner, and that the Cys2 residue is critical for their instability, identifying them as targets of the Arg/N-end rule pathway.

4.3.3 Investigation of hypoxia-regulated transcripts in quiescent buds of grapevine

Having established that at least two of the grapevine ERFVIIs are in vitro substrates for N-end rule proteolysis, we sought to establish whether changes in expression of putative target genes were consistent with a role for the ERFVIIs in oxygen-dependent transcriptional changes during bud burst. For this, we selected a comparison of buds at 3 h against 72 h during bud burst, which we previously demonstrated represented a considerable difference in in vivo oxygen tension, from a hypoxic state to one more oxygenated (see Figure 3 in Meitha et al., 2015).
Figure 4.3 In vitro translation of *VvERF057.1* and *VvERF059.1* genes. (A) Degradation of the wild type (MC) version of the proteins were detected under normoxia, but abolished in the mutant (MA). (B) The addition of proteasome inhibitor (MG132) aided the stabilisation of MC-*VvERF057.1* and MC-*VvERF059.1* in the presence of oxygen, and under anoxia abolished protein degradation. Presented figures are representatives of at least two independent replicates. Min indicates the sampling points of reaction after the specified minutes of incubation following the addition of cycloheximide to terminate protein synthesis.

Following RNA sequencing, we found 1947 differentially regulated transcripts in 3 h buds against 72 h, 913 up- and 1034 downregulated (Supplemental Table S4.4). According to the annotation of Grimplet *et al.* (2012), the major functional class represented among differentially expressed genes was metabolism, with 87% belonging to the subclass of primary metabolism (Supplemental Figure S4.3). We queried the presence of homologues of the 49 core HRG of *Arabidopsis* (Mustroph *et al.*, 2009), and identified 12 up- and 6 downregulated transcripts (Table 4.2). The dominant feature of this refined list of HRG was greater carbon flux through glycolysis and fermentation, as well as ethylene synthesis and catabolism of abscisic acid in the 3 h against 72 h comparison (Table 4.2). The downregulation of 6 of the HRG homologues suggests signals other than the oxygen tension are driving their transcription during bud burst. This is plausible, as bud burst is accompanied by considerable
developmentally-driven transcription (See Chapter 3). It is also worth noting that the 49 core HRG (Mustroph et al., 2009) were defined in the context of abiotic stress, rather than developmental conditions. Nevertheless, we interrogated public datasets through Genevestigator (Hruz et al., 2008) to gain further understanding of the expression patterns of these genes, particularly where downregulated. For example, the *PHOSPHATE-INDUCED PROTEIN 1* (AT1G35140) is downregulated in conditions of increased salicylic acid (ArrayExpress accession E-TABM-518), implicating a negative association with pathogen responses. A similar pattern was revealed in the *LOB DOMAIN-CONTAINING PROTEIN 41* (AT3G02550), when *Arabidopsis* were treated with a bacterial elongation factor EF-Tu (GEO accession GSE40354; Tintor et al., 2013). It is possible that the buds were infected with pathogens, as they were directly derived from a commercial vineyard and had no antibiotic treatment.

4.3.4 Analysis of the HRPE motif, and GC- and GT-rich components of ARE in grapevine HRG promoter regions

We then queried whether the differentially expressed HRG possessed the hypoxic-responsive promoter element (HRPE), as identified in *Arabidopsis* (Gasch et al., 2016). Differences were found between the two methods, RSAT and DMINDA (Supplemental Table S4.5). Only transcripts implicated by both methods are discussed further. The HRPE was found in two of the upregulated transcripts, *ALCOHOL DEHYDROGENASE 6* (*VvADH6*) and *PHOSPHOFRUCTOKINASE* (*VvPFK*) genes. Unexpectedly, the HRPE was also found in two downregulated transcripts, *PHOSPHATE-INDUCED PROTEIN 1* (*Vv-PHI1.3a*) and *LATERAL ORGAN BOUNDARIES PROTEIN 38* (*VvLOB38*) (Table 4.3). GT- and GC- components of ARE were also detected in the promoter sequences of these transcripts in dispersed locations (Supplemental Table S4.5).
Table 4.2 Differentially regulated transcripts of 18 core hypoxia-responsive genes homologue in grapevine (*Vitis vinifera*) buds during mild hypoxia, ~4.2 kPa. Bold fonts indicate best *Arabidopsis* match were obtained from matching against Grimplet et al. (2012) annotation and BLAST, while normal fonts only by BLAST. Presented logFCs are a comparison of log2TPM average (n=3) in 3 h versus 72 h buds.

<table>
<thead>
<tr>
<th>Unique ID</th>
<th>LogFC</th>
<th>Functional annotation (Grimplet et al., 2012)</th>
<th>Best <em>Arabidopsis</em> match (Plaza 3.0 annotation)</th>
<th>BLAST e-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIT_02s0087g00710</td>
<td>3.15</td>
<td>ABA 8′-hydroxylase/CYP707A1</td>
<td>AT5G45340 (Abscisic acid 8-hydroxylase 3/CYP707A3)</td>
<td>0.0,</td>
</tr>
<tr>
<td>VIT_04s0023g03600</td>
<td>2.93</td>
<td>SEN1 (dark inducible 1)</td>
<td>AT2G17850 (Rhodanese/cell cycle control phosphatase superfamily protein)</td>
<td>2e-37,</td>
</tr>
<tr>
<td>VIT_04s0044g01110</td>
<td>1.77</td>
<td>Alcohol dehydrogenase 6</td>
<td>AT1G77120 (Alcohol dehydrogenase 1)</td>
<td>NA</td>
</tr>
<tr>
<td>VIT_06s0004g06900</td>
<td>1.52</td>
<td>Pyruvate decarboxylase isozyme 1</td>
<td>AT4G33070 (Pyruvate decarboxylase isozyme 1)</td>
<td>0.0,</td>
</tr>
<tr>
<td>VIT_08s0217g00100</td>
<td>3.38</td>
<td>Pyruvate decarboxylase isozyme 2</td>
<td>AT5G54960 (Pyruvate decarboxylase isozyme 2)</td>
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</tr>
<tr>
<td>VIT_11s0016g02850</td>
<td>1.49</td>
<td>Phosphofructokinase</td>
<td>AT4G32840 (ATP-dependent 6-phosphofructokinase 6)</td>
<td>0.0,</td>
</tr>
<tr>
<td>VIT_12s0059g01380</td>
<td>2.37</td>
<td>1-aminocyclopropane-1-carboxylate oxidase 1</td>
<td>AT2G19590 (1-aminocyclopropane-1-carboxylate oxidase 1)</td>
<td>8e-87,</td>
</tr>
<tr>
<td>VIT_14s0171g00320</td>
<td>2.94</td>
<td>Kelch repeat-containing protein</td>
<td>AT3G27220 (Galactose oxidase/kelch repeat-containing protein/HUP6)</td>
<td>1e-168,</td>
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<tr>
<td>VIT_18s0001g06020</td>
<td>3.10</td>
<td>Phosphate-induced protein 1</td>
<td>AT1G35140 (Phosphate-induced protein 1/exordium like 1)</td>
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<td>AT1G35140 (Phosphate-induced protein 1/exordium like 1)</td>
<td>9e-117,</td>
</tr>
<tr>
<td>Unique ID</td>
<td>LogFC</td>
<td>Functional annotation</td>
<td>Best <em>Arabidopsis</em> match</td>
<td>BLAST e-value</td>
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<tr>
<td>------------------</td>
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<td>-----------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>VIT_01s0011g03540</td>
<td>-2.20</td>
<td>Lateral organ boundaries protein 41</td>
<td>AT3G02550 (LOB domain-containing protein 41)</td>
<td>8e-67,</td>
</tr>
<tr>
<td>VIT_01s0026g01460</td>
<td>-1.79</td>
<td>Thioredoxin H-type 2 (Trx-H-2)</td>
<td>AT5G61440 (Atypical cys his rich thioredoxin 5)</td>
<td>7e-06,</td>
</tr>
<tr>
<td>VIT_02s0025g01610</td>
<td>-1.17</td>
<td>Phosphatidylinositol 4-kinase type-II</td>
<td>AT1G26270 (Phosphatidylinositol 3- and 4-kinase family protein)</td>
<td>3e-55,</td>
</tr>
<tr>
<td>VIT_03s0017g01210</td>
<td>-2.18</td>
<td>Phosphate-induced protein 1</td>
<td>AT1G35140 (Phosphate-induced protein 1/exordium like 1)</td>
<td>1e-92,</td>
</tr>
<tr>
<td>VIT_03s0038g03860</td>
<td>-1.35</td>
<td>Phosphate-induced protein 1</td>
<td>AT1G35140 (Phosphate-induced protein 1/exordium like 1)</td>
<td>6e-41,</td>
</tr>
<tr>
<td>VIT_18s0001g09250</td>
<td>-2.80</td>
<td>Lateral organ boundaries protein 38</td>
<td>AT3G02550 (LOB domain-containing protein 41)</td>
<td>1e-38,</td>
</tr>
</tbody>
</table>
Table 4.3 Locations and sequences of detected hypoxic-responsive promoter element (HRPE) in the 5’ untranslated region of 4 regulated transcripts in quiescent grapevine buds. HRPE consensus sequence is 5’-AAACCA(G/C)(G/C)(G/C)GC-3’ and occurrences in -3 kb regulated transcripts were detected by RSAT and DMINDA. Occurrences of HRPE in grapevine genes is consistent with a role for the ERFVII transcription factors in transcriptional regulation during hypoxia.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location (relative to start codon)</th>
<th>Sequence</th>
<th>Recognised by</th>
<th>Gene</th>
<th>Location (relative to start codon)</th>
<th>Sequence</th>
<th>Recognised by</th>
</tr>
</thead>
<tbody>
<tr>
<td>VvADH6</td>
<td>-207 to -195</td>
<td>AAAACCAGGGGT</td>
<td>DMINDA and RSAT</td>
<td>VvPHI1.3a</td>
<td>-1406 to -1395</td>
<td>AAAACAACGGGC</td>
<td>DMINDA and RSAT</td>
</tr>
<tr>
<td>VvPFK</td>
<td>-1500 to -1489</td>
<td>GAAACCTGTGAC</td>
<td>DMINDA and RSAT</td>
<td>VvLOB38</td>
<td>-2282 to -2270</td>
<td>GAAACCACGAGC</td>
<td>DMINDA and RSAT</td>
</tr>
<tr>
<td></td>
<td>-908 to -897</td>
<td>AAAACCGTAGAC</td>
<td>RSAT</td>
<td></td>
<td>-817 to 807</td>
<td>AAACCACCCAC</td>
<td>DMINDA</td>
</tr>
<tr>
<td></td>
<td>-192 to 181</td>
<td>GAAACCTGTGAC</td>
<td>RSAT</td>
<td></td>
<td>-271 to -260</td>
<td>AAAACACGGAGC</td>
<td>RSAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-261 to -250</td>
<td>GGCACGAGCGGC</td>
<td>RSAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-49 to -38</td>
<td>AAATCCGGGGGC</td>
<td>RSAT</td>
</tr>
</tbody>
</table>
4.4 Discussion

Earlier studies which indicated a role for hypoxia in transcriptional regulation during bud burst (Or et al., 2002; Ophir et al., 2009) pre-dated the resolution of the direct oxygen signalling mechanism in plants (Gibbs et al., 2011; Licausi et al., 2011a). Nevertheless our recent study confirmed hypoxia in quiescent buds and that bud burst was accompanied by a regulated oxygenation (Meitha et al., 2015). The questions we sought to address here was whether the grapevine homologues of the group VII ETHYLENE RESPONSE FACTORs (ERFVII) were also capable of functioning in direct oxygen perception, and whether gene expression patterns during bud burst were consistent with a role in transcriptional activation. These studies are an important contribution to understanding how deciduous perennials perceive changes in the environment and commit to resume growth following an extended period of quiescence and dormancy.

The three VvERFVIIIs each possessed the N-degron motif required for oxygen-dependent proteolysis by the N-end rule (Gibbs et al., 2011). To establish whether this was sufficient to target the proteins for proteolysis, we developed an in vitro translation system based on those used to investigate N-end rule proteolysis in other species (Gibbs et al., 2011; Lee et al., 2005). Both the VvERF057.1 and VvERF059.1 proteins were degraded in an oxygen-dependent manner. We were unable to establish a translation system for VvERF058.1. Conditions of low oxygen stabilise ERFVII proteins by reducing the likelihood of oxidation of the N-terminal Cys, which under normoxia would occur after the constitutive removal of methionine by methionine amino peptidases (MetAP) (Frottin et al., 2006; Gibbs et al., 2011; Ross et al., 2005). Site-directed mutation of the penultimate cysteine to alanine uncoupled the VvERFs from oxygen dependent degradation, demonstrating that the cysteine was the primary destabilising residue. This supports similar findings of oxygen perception mechanisms across life forms; RGS4, RGS5 and RGS16 proteins in mammalian (Hu et al., 2005; Lee et al., 2005), and group ERFVII in plants (Gibbs et al., 2014; Gibbs et al., 2011; Mendiondo et al., 2016; Weits
et al., 2014). Treatment with a proteasome inhibitor MG132 has greatly enhanced stability of MC-VvERF057.1 and MC-VvERF059.1 under normoxia, demonstrating that both of the proteins are substrates of the Arg/N-end rule pathway.

Each of the VvERFs were transcribed in buds but not differentially regulated in the conditions we compared, which differed in tissue oxygen tension. This was not remarkable, as transcriptional regulation by oxygen conditions has been demonstrated for some but not all ERFVIIIs in plants. For example the Arabidopsis HRE1 and HRE2 are upregulated under hypoxia but not the remaining three Arabidopsis ERFVIIIs, despite the demonstrated functions of the encoded proteins by the oxygen-dependent N-end rule (Licausi et al., 2010c; Papdi et al., 2015). Nevertheless, we made an assumption that the transcribed RNA was translated in vivo, in order to investigate whether the VvERFVIIIs may function as oxygen-dependent transcription factors. A conserved set of 49 hypoxia responsive genes (HRG), which are induced by hypoxia, was previously identified (Mustroph et al., 2009). We queried whether homologues of these were differentially expressed in hypoxic quiescent buds by comparison with buds later during bud burst. The tissue oxygen tension of the quiescent buds (3 h) was <5 kPa pO2 for all but the outer 500 μm, while the bursting buds (72 h) were >10 kPa pO2 for all but a portion of the profile peripheral to the bud core (see Figure 3A and D in Meitha et al. (2015)). A total of 18 transcripts representing homologues of 14 of the conserved 49 HRG were differentially regulated in the 3/72 h comparison (Table 4.2). The fact that six of these were downregulated in the more hypoxic condition here most likely indicates cues other than oxygen are signalling transcription, notably developmental regulation. This is supported by the patterns of two genes involved in hormone regulation during bud burst; ABSCISIC ACID (ABA) 8′-HYDROXYLASE (VvABA8′OH), which encodes the controlling step of ABA catabolism and ACC OXIDASE (VvACO) in ethylene synthesis. This is consistent with the suggested links between hypoxia and ABA-ethylene interplay after the application of dormancy-releasing stimuli (hydrogen cyanamide and heat shock) to grapevine buds (Ophir et al., 2009). In quiescent buds prior to bud burst,
VvACO expression increases, and exogenous ethylene application leads to enhanced bud burst (Ophir et al., 2009), whereas ABA levels decline (Or et al., 2000a), along with increasing expressions of the catabolic VvABA8'OH (Zheng et al., 2015) and decrease in the ABA biosynthetic gene 9-CIS-EPOXYCAROTENOID DIOXYGENASE (VvNCED) (Ophir et al., 2009; Zheng et al., 2015). The results also suggested an increase in glycolysis through the upregulation of PHOPHOFRUCTOKINASE transcripts, and fermentation pathway through the upregulation of ALCOHOL DEHYDROGENASE 6, and PYRUVATE DECARBOXYLASE ISOZYME 1 (VvPDC1) and VvPDC2. In plants, hypoxia compromises mitochondrial respiration and leads to reconfiguration of central carbon metabolism to overcome limited ATP production and regeneration of NAD⁺ by enhancing glycolysis and fermentative pathway (Bailey-Serres and Chang, 2005; Bailey-Serres et al., 2012). Hypoxia treatments, 41% and 25% of normoxia (8.6 and 5.2 kPa), to dormant buds increased the rate of bud burst and expression of VvADH2, VvPDC and SUCROSE SYNTHASE (VvSUSY) (Vergara et al., 2012b). Moreover, upregulation of VvADH (Keilin et al., 2007; Or et al., 2000b), VvPDC (Or et al., 2000b) and VvSUSY (Halaly et al., 2008; Keilin et al., 2007) were also documented in quiescent buds when treated with the forcing chemical hydrogen cyanamide. Hence, the transcriptional condition of quiescent buds in this study is consistent with a response to hypoxia. Additionally, several non-enzymatic coding transcripts of the 49 core HRG (Mustroph et al., 2009) were also upregulated in hypoxic buds, although their associations with bud dormancy release and burst have not been clearly defined.

To further investigate the possible mechanism of gene regulation in quiescent buds, the presence of hypoxia associated promoter motifs in the 5’UTR of the candidate HRG was examined. The 33 bp hypoxic-responsive promoter element (HRPE) was recently identified in Arabidopsis, as being sufficient to drive activity of a luciferase reporter under anoxic conditions in vivo or when the AtRAP2.2 or AtRAP2.12 transcription factors were transiently expressed in vitro (Gasch et al., 2016). Regulatory function of the anaerobic responsive element (ARE) in
ADH1 of maize (Walker et al., 1987) and Arabidopsis (Hoeren et al., 1998) were examined in conditions of ~5 kPa $pO_2$, and the results suggested that GT- and GC-rich components are key regions for transcriptional regulation. Here we queried the presence of HRPE and/or GT- and GC-rich components of the ARE in the differentially expressed HRG of grapevine using two bioinformatic tools. The results obtained by both methods provided strong evidence of the presence of the HRPE in the promoters of upregulated ($VvADH6$ and $VvPFK$) and downregulated ($VvLOB38$ and $VvPHI.3a$) transcripts (Table. 4.3). Considering the possible stabilisation of $VvERF057.1$ and $VvERF059.1$ in the hypoxic quiescent buds, the upregulation of $VvADH6$ and $VvPFK$ is consistent with a regulatory interaction between these transcription factors and the HRPE in the 5'UTR. By contrast, the downregulation of $VvLOB38$ and $VvPHI.3a$ suggest additional signals may prevail on their transcription. Considering the HRG and HRPE and ARE motifs were defined in the context of abiotic stress, it is reasonable to consider different behaviours in a developmental context. Comparison with public transcriptome data also suggested the quiescent buds may have been infected, and that this was overcome during bud burst. Nevertheless, the 5'UTR of $VvADH6$ and $VvPFK$, $VvLOB38$ and $VvPHI.3a$ also contained GT- and GC-rich components of the ARE, which were dispersed in the 3 kb region. In the 5'UTR of grapevine, the distance between detected GT- and GC-rich components of ARE was relatively large, exceeding the acceptable range of its homologue in maize (Olive et al., 1990), and similarly when compared with the $AtADH1$ promoter. However, the fact that both $At$- and $VvADH$ transcripts were induced under mild hypoxia tempts us to consider that the increased spacing of GT- and GC-components of ARE is a consequence of genetic evolution, suggesting that further functional analysis of the promoter region is required.

Several key assumptions were made in the progress of this study, notably that the in vivo regulation of the VvERFVIIIs resembled in vitro behaviour, and that the transcriptome of the bud was spatially homogenous. Both assumptions require further examination, for example by heterologous complementation of mutants of the Arabidopsis ERFVII or by overexpressing an
MA- construct of a VvERFVII, and establishing tolerance to hypoxic conditions. The reverse-genetic path to establishing function in a perennial bud is challenging, particularly due to the generation time. RNA in situ hybridisation or PCR may provide a suitable compromise for investigating the spatial expression patterns of the VvADH6 or VvPFK under conditions of hypoxia versus normoxia, although this alone will not resolve the function of the VvERFVIIs. The functional interactions between VvERFVII and HRPE may best be established by further in vitro analysis, similar to the yeast-two hybrid assays reported by Gasch et al. (2016).

4.5 Conclusions

The transition from a quiescent bud to an emergent shoot requires considerable metabolic reorganisation, and must cope with a large change in tissue oxygen tension, in addition to light and hydration. Here we have established that two of the grapevine group VII ETHYLENE RESPONSE FACTORS (VvERFVII) function in oxygen-dependent proteolysis via the Arg/N-end rule in vitro, and that transcriptional regulation of key metabolic functions is consistent with stable expression of one or more VvERFVIIs in the early stages of bud burst. Promoter motif analysis identified putative binding sites for the VvERFVIIs in the 5’UTR of ALCOHOL DEHYDROGENASE and PHOSPHOFRACTOKINASE homologues, which were upregulated in the hypoxic quiescent buds. Transcriptional regulation of these functions at the earliest stage of bud burst enables rapid substrate-level phosphorylation independent of mitochondrial respiration, which requires oxygen. As the tissue becomes oxygenated during bud burst and mitochondrial respiration becomes more favourable, the VvERFVII more rapidly degrade via the N-end rule, attenuating their function in transcriptional activation. Additionally at this stage, the influence of light-dependent chloroplast biogenesis may support synthetic metabolism. Hence, we suggest that VvERF057.1 and VvERF059.1 transcription factors play a role in spatially mediating the interaction between oxygen and molecular responses in the hypoxic quiescent buds.
4.6 References


Similar mechanisms might be triggered by alternative external stimuli that induce dormancy release in grape buds. *Planta* **228**, 79-88.


The contribution of submergence-tolerant (Sub1) rice varieties to food security in flood-prone rainfed lowland areas in Asia. *Field Crops Research* **152**, 83-93.


4.7 Supplemental information

**Table S4.1 (E-copy)**. Limma analysis statistics. Limma analysis was performed using log2 transcript per million (log2 TPM) values in 3 and 72 h treated buds. Differentially expression genes were filtered using a fold change (logFC|1|) and multiple test (FDR≤0.05, adj. P. value) criteria, comparing 3 vs 72 h transcript data.

**Table S4.2.** List of primers used in this study. MC- primers were used to amplify the coding sequence of respective genes from grape RNA extract. The obtained fragments were then mutated by using the MA-primers (site directed mutagenesis).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-ERF5_fw</td>
<td>GGCCGAATTCATGTGGAGGTGC</td>
</tr>
<tr>
<td>MC-ERF5_rev</td>
<td>TCCATCTAGAAAAACGCAGCG</td>
</tr>
<tr>
<td>MC-ERF7_fw</td>
<td>GGCAGAATTCATGGCTGGAGGTGTATCA</td>
</tr>
<tr>
<td>MC-ERF7_rev</td>
<td>TTCATCTAGATAGACGCTAGGTGGG</td>
</tr>
<tr>
<td>MC-ERF9_fw</td>
<td>TGGTGAATTCATGGCTGGGTGTCGCA</td>
</tr>
<tr>
<td>MC-ERF9_rev</td>
<td>TATATCTAGAGAAACTCCCCCACA</td>
</tr>
<tr>
<td>MA-ERF5_fw</td>
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</tr>
<tr>
<td>MA-ERF5_rev</td>
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</tr>
<tr>
<td>MA-ERF7_fw</td>
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<tr>
<td>MA-ERF7_rev</td>
<td>GAGATGATAGACACCTCCAGCCATGAATTCTCGAGTGCAAAA</td>
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<td>MA-ERF9_fw</td>
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</tr>
<tr>
<td>MA-ERF9_rev</td>
<td>AGATGATGAGCCACCACCAGCCCATGAATTCTCGAGTGCAAAA</td>
</tr>
</tbody>
</table>

**Table S4.3 (E-copy)**. Group ERFVII transcription factors in grapevine from cultivar Crimson Seedless (this study) and Pinot Noir (Licausi et al., 2010).

**Table S4.4 (E-copy)**. Differentially expressed genes (DEG) which met fold change and false discovery rate criteria (FC≥|1|, FDR P≤0.05) in the comparison of 3 h against 72 h treated buds and after functional enrichment (Al-Shahrour et al., 2004). Annotation and Arabidopsis homologues represent the annotation by Grimplet et al. (2012). (A) Upregulated and (B) Downregulated.
Table S4.5 (E-copy). List of HRPE scanning results by RSAT and DMINDA tools, and GT- and GC-rich components of ARE by DMINDA in 3 kb 5' untranslated region of regulated transcripts, in up- (A) and (B) downregulated transcripts of HRG homologues.

Figure S4.1. Mean M-values of the resulting transcript reads from 3 and 73 h buds, (A) before and (B) after normalization with trimmed mean M-values (TMM) method.
Figure S4.2. Equal loading assessment. (A) After resolving in TGX Stain-Free™ gels, and (B) after transferring the proteins onto PVDF membrane. Lane 1-4 are MC-VvERF057.1 and 5-8 are MC-VvERF059.1 translation products in TNT-T7 Coupled Reticulocyte Lysate System (Promega), respectively sampled after 30, 60, 90, and 120 minutes of reaction. Translation reactions were terminated after 30 minutes of incubation by adding cycloheximide, and proteasome inhibitor MG132 was included as a treatment.
Figure S4.3. (A) Distribution of functional categories the core set of the 1034 upregulated DEGs. (B) Further detail on the metabolism functional category corresponding to 130 DEGs.
5 General discussion

The overall aim of the series of experiments described in this thesis was to extend the knowledge of physiology and biomolecular processes of the grapevine bud, and how the transition from quiescence to bud burst is achieved. The predicted sequence of molecular and physiological changes in this transition stage of buds was presented in Figure 1.2. The key findings of chapters 2-4 have developed the understanding of how hypoxia, generally $pO_2$ regulation, interacts with environmental cues in inducing bud burst and the following processes (Figure 5.1).

**Figure 5.1** Diagram of involved molecular and physiological changes during grapevine bud burst. The rapid transition of bud burst in grapevine involves growth and development processes from EL1; quiescent, into EL4; bud burst (Coombe, 2004). The internal oxygen pressure ($pO_2$) of EL1 buds is hypoxic, ~4.2 kPa, and transcript regulation is considered to be mechanistically regulated by group ERFVII. These include the transcriptional regulation of genes involved in fermentation, glycolysis and reactive oxygen species (ROS) signalling or metabolism. ROS regulation also interacts with cell expansion, which mainly is the consequence of hydration. Moreover, fermentation and glycolysis networks overlap with ROS regulations, and chloroplast biogenesis under the treatment of light. The balance between sugar metabolism and buds chloroplast triggers sugar signalling to indicate source and to initiate photosynthesis immediately after burst when light presence.
Hypoxia, through numerous molecular studies (Ophir et al., 2009; Or et al., 2000a; Pérez et al., 2008; Vergara et al., 2012a; Vergara et al., 2012b), has been proposed as a prerequisite condition to induce the resumption of growth in winter buds of grapevine. The key physiological assumption underlying this hypothesis was tested in Chapter 2 of this thesis, presenting the first data of tissue oxygen tension (partial pressure or oxygen, $pO_2$) in a perennial bud. Therein I established that the quiescent bud was hypoxic, and that a spatially regulated oxygenation accompanies the transition to bud burst. The results from this experiment raised further questions about whether and how metabolic functions are regulated by hypoxia, a condition that to date has largely been studied in the context of abiotic stress, rather than development. Measurements of respiration and the in situ localisation of reactive oxygen species (ROS) were carried out to provide context for further investigations seeking to relate the prevailing signals to metabolic change and growth resumption (discussed below in section 1.1).

Similarly to seed germination, bud burst requires rapid adjustment of cellular processes to enable the hetero- to photo-autotrophic transformation. The ability to sense light and to rapidly establish photosynthetic capacity is crucial, given that the buds contain a finite amount of carbon reserves. Dormant buds are an independent organ with a disconnected vascular system from the mother plant, attributed to callose plugs in sieve plate pores and plasmodesmata (Rinne et al., 2011). Although the precise timing of vascular reconnection is unclear, data presented in Chapter 2 strongly pointed to a role for ROS-dependent vascular development in the very early stages of bud burst. Nevertheless, Chapter 2 was carried out in the absence of light, in order to establish a simplified system, free from the confounding cues of light and particularly oxygenic functions of the chloroplast. Chapter 3 elaborates on that system by experimentally contrasting the physiological and molecular changes in the presence or absence of light.
The spatially and temporally dynamic changes in tissue $pO_2$ during bud burst demonstrated in Chapters 2 and 3, provided context to explore the mechanisms of direct oxygen perception, and particularly their role in recalibrating cellular priorities for survival and rapid growth. The role of group VII ethylene responsive factor (ERFVII) transcription factors in mediating plant oxygen sensing was recently discovered, involving proteasomal degradation under normal oxygen conditions (normoxia) through the N-end rule pathway, but stability in conditions of low oxygen (hypoxia) (Gibbs et al., 2011; Licausi et al., 2011). The identification of three ERFVII in grapevine (Licausi et al., 2010; Zhuang et al., 2009) preceded a mechanistic understanding of role of ERFVII in oxygen signalling. In Chapter 4, interactions between $pO_2$ regulation and stabilisation of grapevine ERFVII (VvERFVII) were investigated. Moreover, the incorporation of transcriptomic data analysis enabled the prediction of the transcriptional targets of VvERFVII in the hypoxic buds.

5.1 Key findings

5.1.1 $pO_2$ regulation, light incidence, and physiological changes

Hypoxia has been widely studied in the context of acute or chronic abiotic stress in plants, such as flooding or waterlogging, whereby prolonged exposure compromises mitochondrial ATP synthesis and can lead to cell death. Studies in animal systems and more recently in plants have begun to demonstrate a developmental role for hypoxia, and oxygen regulation (Considine et al., submitted; Rajatapiti et al., 2010; Rolletschek et al., 2002; van Tuyl et al., 2005). This thesis investigated the regulation of the internal $pO_2$ (kPa) of grapevine buds in controlled conditions of bud burst, in the presence or absence of light (Chapters 2, 3). The tissue oxygen profiles revealed an internal hypoxic condition of buds at the earliest stage of burst (quiescent bud, 3 h), with the $pO_2 < 5$ kPa in all but the outermost 500 µm within the bud scales. The question of whether the scales presented a mechanistic barrier to oxygen diffusion was tested, demonstrating that although the $pO_2$ of quiescent buds increased when scales
were removed, the bud core remained hypoxic. This profile contrasted with that observed after 72 h of controlled bud burst with scales intact, where the profile displayed a biphasic pattern due to the elevation of $pO_2$ in the bud core. Introducing light to the controlled system accelerated the spatial oxygenation pattern. These data demonstrate that internal oxygenation during bud burst is spatially and temporally regulated and partially light dependent. While the outer scales provided a physical barrier, the pattern of oxygenation could not be explained purely by physical diffusion constraints.

The importance of hypoxia in setting a conducive environment for cell growth and development in plants and animals is correlated with the increase in ROS production, as the messenger in oxidative signalling (Clanton, 2007; Considine and Foyer, 2014). Although the mechanisms and intracellular source of ROS during hypoxia remains in question (Pucciariello et al., 2012), the biosynthesis of antioxidant enzymes (Blokhina et al., 2003; Vergara et al., 2012a) and ROS-mediated growth are evident in hypoxia treated plants (Steffens et al., 2013). In deep-water rice, superoxide promotes aerenchyma formation, mediated by ethylene-dependent signalling (Steffens et al., 2011). ROS is also involved in phytohormone signaling pathways during seed germination, evidence by the failing endosperm rupture when apoplastic-ROS accumulation is inhibited by ABA (Oracz and Karpinski, 2016). The involvement of ROS-dependent signalling in the release of dormancy and onset of bud burst in grapevine has been suggested by a transient rise in $H_2O_2$ prior to bud burst and through the profiles of several core antioxidant genes (Halaly et al., 2008; Or et al., 2000b).

Histochemical analysis of ROS during bud burst demonstrated the co-localisation of $O_2^{-}$ and lignin within 3 h of treatment in growth conditions when the internal $pO_2$ was still hypoxic (Chapter 2). This suggested an association between $O_2^{-}$ and lignification of the developing vascular tissue in hypoxic buds, as previously shown in spinach hypocotyls (Ogawa et al., 1997). Further, ROS accumulation in the vicinity of cell wall has been correlated to loosening during the elongation growth in seedlings (Schopfer, 2001) and cross-linking of the building
components in maize coleoptile and sunflower hypocotyls (Liszkay et al., 2003; Passardi et al., 2004). The spatial patterning of H$_2$O$_2$ and O$_2^-$ localisation is concurrent with developmental progressions, pO$_2$ regulation and light incidence (Chapters 2 and 3). Although the roles of pO$_2$ and ROS towards plant cell development is increasingly acknowledged, existing literature on light-induced ROS is largely focused on the stress response of high light (Carmody et al., 2016; Li et al., 2009; Miller et al., 2009). Taken together, the results indicate that the spatio-temporally and partially light-dependent regulations of pO$_2$ and ROS play a role in bud burst in grapevine.

The internal oxygenation during bud burst was concurrent with an increasing trend of respiration rates (Chapters 2 and 3). Further, the incidence of light augmented both respiration and pO$_2$, clearly indicating that the change in pO$_2$ was not a result of declining oxygen consumption by the mitochondrial electron transport chain. The declining concentration of soluble sugars during bud burst was only evident in the presence of light, indicating that glycolysis and other metabolic functions upstream of respiration were induced in the presence of light. These insights prompted further query of the role of the chloroplast during bud burst, which I investigated with colleagues in Chapter 3 through transcriptional data.

5.1.2 pO$_2$ regulation, light incidence, and the transition of metabolic state

Seed germination and bud burst represent a considerable metabolic reorganisation heterotrophic to photoautotrophic growth. The analysis of transcriptomic data revealed the upregulation of glycolysis and fermentation related genes at the earlier stage of burst (3 h) when the internal pO$_2$ condition is hypoxic (Chapters 2, 4). In seeds, cell metabolic state is dependent on tissue oxygen concentration. Studies of internal oxygen in various seeds have demonstrated a steep decrease of oxygen immediately below the seed coat, but that the incidence of light can immediately increase oxygen concentration in green tissues (Borisjuk and Rolletschek, 2009). This internal oxygenation could be attributed to seed photosynthesis,
such as in barley, which the green layer of seed pericarp does not suffer from hypoxia (Borisjuk and Rolletschek, 2009) and is photosynthetically active within 4-8 days following fertilisation (Wobus et al., 2005). Soybean seeds display a decreasing photosynthesis trend during maturation in parallel to the increasing lipid content (Borisjuk et al., 2005). Hence, germination in hypoxic seeds could be supported by glycolysis and fermentation, providing substrate level phosphorylation, as long as the carbon source is available (Strommer and Garabagi, 2009). The production of acetaldehyde and alcohol has been documented to occur in seeds of a variety species (Zhang et al., 1997; Zhang et al., 1995). Furthermore, hypoxic response in Arabidopsis seedlings involves the upregulation of genes associated with glycolysis and fermentation (Liu et al., 2005). Hypoxia-tolerance in seeds is also often associated with the elevated expression of fermentation genes, such as ALCOHOL DEHYDROGENASE (ADH), PYRUVATE DECARBOXYLASE (PDH) (Estioko et al., 2014) and ALDEHYDE DEHYDROGENASE (ALDH) (Fukao et al., 2003). In a similar way, the observed upregulation of fermentative genes at the onset of bud burst in grapevine may represent a strategy to survive hypoxia and supply energy for growth. Although buds possess green tissues, access to light is apparently limited by the scales. Moreover, the comparison of the 144 h transcriptome with public datasets indicates that hypoxia-associated conditions are still apparent in the dark treated buds, as suggested by the activation of the branched-chain amino acids catabolism (e.g. 144 h dark/light vs dark). Thus, fermentation and amino acid catabolism are sought as an alternative metabolism pathway during hypoxia and dark conditions.

Clear evidence of a metabolic transition into photoautotrophic growth was seen in buds grown in the presence of light for 144 h. Transcriptomic data suggested that chloroplast biogenesis takes place long before the emergence of leaves from the bud. This was evidenced by the upregulation of genes involved in the Calvin Cycle, the light phase of photosynthesis and the chlorophylls synthesis and turnover (Chapter 3).
Taken together, transcriptome data suggest the transition of metabolic state of buds from heterotrophic into photoautotrophic. The hypoxic condition and the metabolic requirements during the early stage of bud burst are considered the prevailing features driving substrate-level phosphorylation and fermentation, rather than oxidative phosphorylation at this very early stage. The incidence of light upon the buds was shown to induce the chloroplast biogenesis to enable photosynthesis as soon as the first leaf emerges.

5.1.3 $pO_2$ and transcriptional regulations

The ability of plants to endure and survive hypoxic conditions is highly dependent on their transcriptional response, as demonstrated in the hypoxia-tolerant seeds (Estioko et al., 2014; Fukao et al., 2003). However, perception and activation of the transcriptional response is dependent on the modification of nascent proteins, specifically the conditional proteolysis of group VII ETHYLENE RESPONSE FACTORS (ERFVII) via the N-end rule pathway (Gibbs et al., 2011; Licausi et al., 2011). Three members of group ERFVII were previously identified in the grapevine genome and in Chapter 4 I demonstrated that each possess the N-degron motif, which primarily determines the ability to function via the N-end rule. These genes were transcribed in hypoxic quiescent buds, but not differentially regulated during bud burst, which is consistent with three of the five ERFVII homologues of Arabidopsis (Licausi et al., 2010b; Papdi et al., 2015). An in vitro translation study demonstrated that two VvERFVII were substrates for oxygen-dependent N-end rule proteolysis, exhibiting stable abundance under anoxia. Moreover, site-directed mutagenesis of the amino terminal cysteine-to-alanine confirmed the importance of this cysteine as the primary destabilising residue, again consistent with substrate ERFVII homologues in Arabidopsis and barley (Gibbs et al., 2011, Gibbs et al., 2014).

The localisation of ERFVII to the nucleus in Arabidopsis is detectable under a mild hypoxic condition of 10.5 kPa and more pronounced when $pO_2$ is further decreased (Kosmacz et al., 2015). Considering the $pO_2$ in quiescent buds is lower (3 h, ~4.2 kPa) than the threshold of
ERFVII stabilisation in *Arabidopsis*, further experiments tested whether gene expression patterns were consistent with the stable expression of the VvERFVII proteins *in vivo*. Previous studies had identified a set of 49 conserved hypoxia responsive genes (HRG) (Mustroph *et al.*, 2009) and identified a hypoxic response promoter element (HRPE) in the 5′ untranslated region of several of these genes, which directly interacts with the ERFVII protein (Gasch *et al.*, 2016). Among these were genes coding for key glycolytic and fermentation enzymes. I then queried the expression of the grapevine homologues of the 49 HRG in a comparison of 3 h against 72 h bud during bud burst, which I’d previously shown to represent considerable differences in internal $pO_2$ (Chapter 2). The key glycolytic and fermentative HRG were significantly upregulated at 3 h against 72 h. The presence of the HRPE in the 5′ untranslated regions of these genes was then confirmed by bioinformatic tools. Together, these data are strong evidence that the hypoxia in quiescent buds is physiologically relevant and transcription is curated by oxygen-dependent signalling via the VvERFVII. This is the first report to make a direct relation between tissue oxygen tension and functionality of the N-end rule in a developmental process.

### 5.2 Future research

The recognition of $pO_2$ regulation in the developmental processes of plants is emerging, and my studies have greatly contributed to this understanding in the context of bud burst. The results from this study have traversed several research areas, related to $pO_2$ dynamics, which will broaden the knowledge of the physiological and molecular regulation underlying bud dormancy and burst. I propose three main areas to be elucidated further:

1. The assessment of internal $pO_2$, respiration rates (as $CO_2$ production and $O_2$ consumption), ROS accumulation and metabolites composition of buds throughout the whole course of endo and eco-dormancy will help us to determine the reason of hypoxic-core in buds at the early stages of burst, as suggested by the results of
Chapter 2. The investigation of vascular connection in buds prior to, during and post dormancy will also help to confirm the source of internal oxygenation of the core, whether it is attributed to bud photosynthesis or delivered from other parts of the plants.

2. The differences in oxygen availability under bud burst and differential expression of fermentative genes suggest that a deeper analysis on the metabolites will provide further insight. For example, the determination of ATP/ADP and NAD\(^+\)/NADH.H\(^+\) ratios may help to understand if these conditions impact the tissue/cell energy status. In addition to this, the role of soluble sugars in mediating developmental transitions is also achieving wider acknowledgement, notably those of trehalose, glucose and sucrose (Couee et al., 2006). To analyse the concentrations of all sugars in the tissues during the whole course of dormancy would help to understand the interactions between oxidative signalling and sugars modulations. Additionally the feeding of sugars or ROS quenchers to the replicate of buds will provide a comprehensive understanding.

3. To the best of my knowledge there is no information about photosynthetic activity during bud development. Transcriptome data of dark/light treated buds at 144 h in this thesis suggest that at the later stages of bud burst, the organ is ready to photosynthesise. This clearly opens a new area of research in bud development study. The investigation of photosynthesis in buds could be challenging if using classical non-invasive methods, such as by using a chlorophyll fluorometer (e.g. the quantum yield of photosystem II, the non-photochemical quenching, etc.) or by using the IRGA (e.g. internal CO\(_2\), net photosynthesis, etc.), due to the enormous modification of the tools to measure in buds intact to the main plant. However, as a first approach, the dissection of the buds to evaluate chlorophyll fluorescence derived parameters in endo- and ecodormant buds would be helpful to provide some insights. The use of
$^{13}$CO$_2$ would be a very valuable tool to detect whether the bud carbohydrate is already accumulated prior to dormancy or if there is any production during the dormant stage.

4. This thesis also presented results of group ERFVII post-translational regulation which suggested the role of $pO_2$ dynamics in transcriptional control. To analyse genes expression and protein contents in buds exposed to different hypoxia degrees and the in vivo stabilisation of ERFVII proteins would be beneficial to confirm the regulating role of oxygen. Analysis of ERFVII stability in in vivo system could be achieved by transiently transforming grapevine buds, using Agrobacterium mediated or biolistics method, to integrate a fusion of ALCOHOL DEHYDROGENASE 6 or PHOSPHOFRUCTOKINASE promoter with reporter gene into its genome. This knowledge could be useful to optimise the storage condition of canes with buds, in terms of oxygen concentration, for commercial propagation purposes.

5.3 Conclusions

Bud burst in grapevine is a phase of rapid transformation that establishes the founding steps to transition from a metabolic state of heterotrophy into photoautotrophy. The perception of favourable temperature conditions signals the onset of bud burst, and the progression is accelerated by light incidence. The fact that quiescent bud meristem resides in a hypoxic condition has corroborated the emerging role of hypoxia in developmental processes. This is mediated by ROS regulation, especially during the stage of tissue differentiation. The implication of internal $pO_2$ dynamics is also evident in the respiration rates and composition of metabolites during bud burst. In conclusion, this study has contributed to the knowledge of physiology and molecular processes during the developmental transition of grapevine bud, pointing to the dynamics of internal $pO_2$ as the hub of seasonal perception during bud burst.
5.4 References


Appendix: Mitochondrial respiration and oxygen tension

Preface

This appendix is the submitted manuscript for a chapter in the Methods in Molecular Biology series, Plant Respiration and Internal Oxygen. Daniel Shaw and I contributed equally in the manuscript preparation with comments and revisions by Dr Considine and Prof Foyer. Section 3 of this book chapter is the detailed description of methods used in Chapters 2 and 3 of this Thesis; the measurement of respiration rates and internal oxygen partial pressure ($pO_2$).
Mitochondrial respiration and oxygen tension

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Summary

Measurements of respiration and oxygen tension in plant organs allow a precise understanding of mitochondrial capacity and function within the context of cellular oxygen metabolism. Here we describe methods that can be routinely used for the isolation of intact mitochondria, and the determination of respiratory electron transport, together with techniques for \textit{in vivo} determination of oxygen tension and the measurement of respiration by both CO\(_2\) production and O\(_2\) consumption that enables calculation of the respiratory quotient [CO\(_2\)]/[O\(_2\)].

**Key words.** Respiration, oxidative phosphorylation, \textit{in vivo}, \textit{in vitro}, mitochondria, oxygen tension, respiratory quotient (RQ), respiratory control ratio (RCR), gradient centrifugation.
Introduction

Respiration is one of the most intensively studied and well characterised physiological processes in plants. Sophisticated and accurate commercial instruments for the measurements of CO₂ and O₂ exchange have been available since the middle of the last century (1, 2). However, it was only when relatively simple methods became available for the determination of oxygen tension (partial pressure of oxygen, \( pO_2 \); ca. 21kPa at standard atmosphere and pressure) within plant organs that it was realised than many plant dense organs have hypoxic regions (3). The spatial regulation of oxygen tensions and hence oxygen metabolism can be complex in tissues such as the root (4), seed (5) or perennial bud (6). Readers interested in more high-throughput analyses, such as with the Seahorse XF (Agilent Technologies, Santa Clara USA) or Q2 technologies (Astec Global, Sheridan USA) can find relevant information in references by Sew, Millar and colleagues (7, 8).

Intact plant mitochondria can be successfully isolated from actively growing plant tissues and organs such as potato tubers, pea shoots and soybean cotyledons and roots (9-11) where mitochondria are relatively abundant and tissues are relatively free of lignins, polysaccharides and other cellular components. Here we describe a method for isolating intact mitochondria from Arabidopsis leaves, followed by purification by density gradient centrifugation using Percoll and polyvinylpyrrolidone (PVP). The Percoll/PVP gradient serves to separate broken chloroplasts and thylakoids from the intact mitochondria (9). Methods to assess the physical and functional integrity of the isolated mitochondria are described here, as well as an assay to determine respiration function and methods for the determination of respiration rates in intact organs, such as the grapevine buds. We refer the reader to other literature for commonly used assays to determine respiration function, which vary according to the research question (12, 13). These methods can be applied to measure O₂ consumption, CO₂ production and profile internal \( pO_2 \) from the same material within a relatively short time, <20 minutes.
This is useful because the physiological state of plant organs change with time, particularly once they are detached from the plant.

The methods described to measure O₂ consumption utilise a Clark-type oxygen electrode (2, 14), which operates in a closed system. Although measurement in a closed chamber have some limitations, the electrodes are highly specific for O₂. In addition, commercially available O₂ detection equipment that operates in an open system, a differential fuel cell system, is still considerably expensive. The oxygen electrode method assumes linearity and hence prior testing should be used to establish the conditions where changes in oxygen levels are linear, and to ensure that oxygen depletion does not alter the rate of respiration (See Note 1).

Several methods are available for the sensitive internal pO₂ detection, which predominantly involve sophisticated and expensive equipment, such as the optical oxygen sensor system (chemiluminescent or fluorescent) or electron paramagnetic resonance (EPR) oximetry. In a comparison of sensitivity in measuring oxygen consumption by tumour cells, Clark-type oxygen electrodes were shown to be more sensitive than MitoXpress fluorescent assays but inferior to the EPR method (15).

The measurement of internal pO₂ is often challenging in plant dense organs, such as buds, roots and seeds. The method described here uses a Clark-type electrode that allows penetration into an aimed depth. The electrode is housed in a conical thin glass with a tip diameter of 25 µm to minimize disruption, and in which the tip size also allows extensive measurement. In our experience, the 25 µm electrode is sufficiently robust to penetrate layers of highly lignified scales of a grapevine bud (6). For more fleshy tissues, electrodes as thin as 10 µm, or considerably wider for more hardened tissues, are available (see e.g. www.unisense.com/o2). A stable signal can be obtained using these electrodes within 1 second of the start of the measuring period. Experiments measuring the internal pO₂ of seeds have been conducted by using O₂-sensitive optical glass-sensors, which are more time efficient because they do not require repetitious calibration or a pre-polarization period (16).
An additional consideration when using Clark-type electrodes is the consumption of oxygen by redox reactions (15). It is important to ensure that this is negligible relative to the sensitivity of the electrode. When measuring the respiration rate, this problem can be considerably reduced by using a very small electrode and by continuous stirring in a micro-respiration chamber to avoid a gradient of O₂. In spite of this limitation, Clark-type O₂ electrodes are highly reliable tools to determine and quantify internal pO₂ and to determine O₂ consumption rate in dense and small plant tissues.

The measurement of respiration by both CO₂ production and O₂ consumption enables calculation of the respiratory quotient [CO₂]/[O₂], which provides important information about respiratory control and substrate use. Hence we also present a method to detect CO₂ evolution in an open system by using an infra-red gas analyser (IRGA). This high-resolution, rapid response method requires rigorous environmental control of gas system exchange (2). Two important considerations common to the measurement of CO₂ production by an IRGA and O₂ by a micro-respiration system are:

1. To seal any wound that could occur from detaching the tissue or organ from the rest of the plant as this may become an unwanted source of gas exchange activity. This can be simply achieved by mounting the sample on a block of agar.

2. To use a measuring chamber with a minimal volume, as this will greatly aid fast detection of the minute O₂ depletion or CO₂ evolution.
Extraction of intact mitochondria

Materials

Density gradient

1. Light gradient fraction: 28% Percoll (GE Healthcare, Little Chalfont, UK), 0.3 M sucrose, 10 mM 2-(2-[hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethane sulfonic acid (TES), 0.1% (w/v) bovine serum albumin (BSA). Adjust pH to 7.5 with NaOH.

2. Heavy gradient fraction: 28% Percoll, 4.4% PVP-40, 0.3 M sucrose, 10 mM TES, 0.1% (w/v) BSA. Adjust pH to 7.5 with NaOH.

3. Gradient former (e.g. Model 485 Gradient Former from Bio-Rad Laboratories Ltd, Hemel Hempstead, UK; optional).

4. Peristaltic pump, pump-head and silicone tubing (e.g. #WZ-07522-30; #WZ-77200-62 and #WZ-95802-02, respectively, from Cole-Parmer Instrument Co. Ltd, London, UK; optional).

Isolation of mitochondria

1. Powder-free gloves.

2. Miracloth (Merck Biosciences, Nottingham, UK). Ensure they are completely clean and free from detergent (see Note 2).


4. Large mortar and pestle.

5. Cell extraction medium: 0.3 M sucrose, 25 mM sodium pyrophosphate (Na$_4$P$_2$O$_7$), 1% (w/v) BSA, 1% (w/v) PVP-40, 10 mM KH$_2$PO$_4$, 2 mM EGTA. Adjust pH to 7.5 with
hydrochloric acid (HCl). If prepared before day of use, store at 4°C. Add Sodium L-
ascorbate to 18 mM and cysteine to 20 mM on day of use.

6. 50 mL Centrifuge tubes.

7. Centrifuge with fixed-angle rotor (Beckman JA-20 or equivalent).

8. Mitochondria wash medium: 0.3 M sucrose, 10 mM TES, 0.1% BSA. Adjust pH to 7.5
with NaOH. If prepared before day of use, store at 4°C.


10. 28% Percoll, 0–4.4% PVP-40 gradient (see Subheadings 2.1.1 and 2.2.1).

11. 2.5 ml or 5 ml plastic pipette OR autopipette with a cut tip – this is essential, as
conventional pipette tips will shear mitochondria.

**Determination of mitochondrial integrity**

1. Mitochondria isolated according to Subheading 2.2.2.

2. Clark-type oxygen electrode, such as those manufactured by Hansatech (Kings Lynn,
UK) or Rank Brothers (Cambridge, UK).

3. Mitochondrial reaction medium: 0.3 M mannitol, 10 mM TES-KOH pH 7.5, 3 mM
MgSO₄, 10 mM NaCl, 5 mM KH₂PO₄, 0.1% (w/v) BSA.

4. Stock solutions for cytochrome-c oxidase (COX) assay: 0.5 M Na-ascorbate; 5 mM
cytochrome-c; 10% (v/v) Triton X-100.
Measuring respiratory control, non-phosphorylating leak state and maximum uncoupled flux in Mitochondria isolated according to Subheading 6.2.2.2

1. Stock solutions of respiratory substrates: 1 M succinate, 500 mM pyruvate, 100 mM nicotinamide adenine dinucleotide (NADH), 50 mM adenosine 5’-diphosphate (ADP), and 50 mM malate. All to be made up in 500 mM TES-KOH, pH 7.5.

2. 10 mM ADP made up in 500 mM TES-KOH pH 7.5.

3. 5 mg/ml oligomycin made up in 500 mM TES-KOH pH 7.5.

4. 10 mM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) made up in 500 mM TES-KOH pH 7.5.

Methodology

The isolation of intact mitochondria from plant tissues using density gradient centrifugation has been a standard procedure over a decade (17). The main ideas are the disruption of the plant cells so as to release the contents of the cell from within the cell wall; maintaining the integrity of the organelles by protecting them from compounds released from other subcellular compartments; and separating, by use of density gradient centrifugation, the mitochondria from other organelles such as the thylakoids. Here we describe a method to isolate mitochondria from Arabidopsis tissue and assess the organelle integrity as well as respiratory function. Mitochondria from green Arabidopsis leaves were isolated using a protocol based on Day et al. (1985) (9).

Preparation of density gradients

1. The formation of this gradient involves a gradient maker which in its simplest form consists of two cylindrical chambers connected by a small pipe at their base, a tap to meter outflow from these chambers and an outflow pipe from one chamber (see
Figure 1). Alternatively one can prepare the gradient manually using pipettes which is not described here. Two solutions are prepared: a heavy gradient fraction consisting of 28% (v/v) Percoll and 4.4% (v/v) PVP-40 from a 20% (w/v) stock made up in buffer, and a light gradient fraction made up of 28% v/v Percoll in buffer (See Section 2.1.1).

2. Pour the light gradient solution (17.5 ml) into the chamber with no outflow and pour the heavy gradient solution (17.5 ml) into the chamber with the outflow.

3. Place a magnetic stir bar in the chamber with the outflow, and rapidly stir the solution. The outflow pipe should be secured against the inside of a 50 ml centrifuge tube held at a 45° and on ice.

4. Use a peristaltic pump to pull the solution through the pipes and into the 50ml centrifuge tubes. Allow heavy gradient solution to run until half dispensed. Open connection valve between chambers, allowing solutions to mix. Allow both heavy gradient and light gradient solutions to dispense until the chambers are empty (see Note 1).

Isolation of mitochondria

1. Where possible conduct all steps, but especially steps 4-6, in a 4°C cold room. This greatly aids the fraction of intact mitochondria.

2. Ensure that all plasticware and glassware is free from detergent (see Note 2). Perform all procedures at 4°C. Solutions, plasticware and glassware should be pre-chilled. Centrifugation should be performed using a refrigerated centrifuge at 4°C.

3. Harvest seedlings (~50g), place in a Buchner funnel, and wash with sterile distilled water.

4. Place washed seedlings in a clean mortar, cover with 300ml cell extraction medium (see Note 3), and homogenize tissue with a pestle.
5. Filter extract through two layers of Miracloth to remove starch, cell debris, and unbroken cells. Lightly wring the Miracloth to aid filtration.

6. Transfer filtrate to several 50ml centrifuge tubes and centrifuge at 2450 x g for 5 minutes. Transfer supernatant into fresh centrifuge tubes, taking care not to transfer any of the pellet.

7. Centrifuge the supernatant from step 5 at 17500 x g for 20 minutes. Use slow braking. Aspirate and discard the supernatant.

8. Add 1ml mitochondria wash medium to the pellets and gently resuspend using a small paintbrush. Transfer the resuspended pellets to two 50 ml centrifuge tubes and add mitochondria wash medium to each to 40 ml.

9. Repeat steps 5 and 6.

10. Resuspend the final pellets in 1ml mitochondria wash medium using a paintbrush as before. You now have an organelle suspension.

11. Carefully pipet the two organelle suspensions onto the surface of two 28% Percoll, 0–4.4% PVP-40 gradients (see Subheading 2.2.1). Centrifuge at 40000 x g for 40 minutes. Disengage the centrifuge brake at the end of the run as rapid deceleration can disturb the contents of the gradient.

12. Towards the bottom of the gradient, mitochondria will form a white/pale brown band and the thylakoids will form a dark green band in the upper fraction of the gradient (Figure 2). Aspirate and discard the upper fraction of the gradient. Carefully transfer the mitochondria band to a fresh 50 ml centrifuge tubes.
13. Add mitochondria wash medium to the mitochondria to a total volume of 40ml and centrifuge at 31000 x g for 15 minutes. Aspirate and discard most of the supernatant, leaving ~3 ml in the bottom of the tube and ensuring that the pellet is not disturbed.

14. Repeat step 13 and aspirate the resultant supernatant. Finally, add 0.5 ml mitochondria wash medium and resuspend gently with a small paintbrush. This solution contains your isolated mitochondria.

**Determination of mitochondrial integrity**

1. Fill the chamber of a Clark-type oxygen electrode with 2 ml Mitochondrial Reaction Buffer and warm to 25°C.

2. Calibrate the oxygen electrode by aerating the buffer and setting this value as 100%, and by adding Sodium hydrosulphite and setting this value as 0%.

3. Once calibrated, drain the chamber, flush with water and fill the chamber once more with 2ml Mitochondrial Reaction Buffer.

4. Determine mitochondrial protein, according to Lowry (18)

5. Add 500 µg of mitochondria (in a volume of 10-40 µl) to the chamber and close it.

6. Respiration rates are calculated by measuring oxygen saturation of buffer and dividing by time.

7. Once a base respiration rate is established add 40 µl of 0.5 M ascorbate using a 50 µl Hamilton syringe to reduce endogenous cytochrome c (rate a).

8. Two minutes later 20 µl 5 mM cytochrome c is added (rate b).

9. Two minutes later add 10 µl 10% Triton X-100 to solubilise mitochondrial membranes (rate c).
10. The following equation was used to calculate COX activity:

\[ \text{rate } c - \text{rate } a \]

11. The following equation was used to calculate the proportion of intact mitochondria:

\[ 100 - \frac{\text{rate } b - \text{rate } a}{\text{rate } c - \text{rate } a} \times 100 \]

See Note 4 & Figure 3

**Measuring respiratory control, non-phosphorylating proton leak state and maximum uncoupled flux**

1. Fill the chamber of the oxygen electrode with 2 ml Mitochondrial Reaction Buffer and warm to 25°C.

2. Add 500 µg of mitochondria (in a volume of 10-40 µl) to the chamber and close it.

3. Determine the background rate of oxygen consumption (rate a).

4. Add 10 µL of each of the respiratory substrates listed in Subheading 2.1.4 and determine the rate of oxygen consumption (rate b).

5. Add 10 µl 10 mM ADP. The rate of oxygen consumption should increase.

6. After a few minutes, the ADP will be depleted, and the rate of oxygen consumption will be reduced to (rate c). The respiratory control ratio is given by:

\[ \frac{\text{rate } c - \text{rate } a}{\text{rate } b - \text{rate } a} \]

7. Add Oligomycin to a concentration of µg.ml⁻¹. The rate of oxygen consumption should reduce (rate d). Max Leak is given by:

\[ \text{rate } c - \text{rate } d \]
8. Add titrations of 10 mM FCCP in steps of 0.1 μl corresponding to a step increase in the final concentration of 0.5 μM FCCP every two minutes. Maximum uncoupled flux (capacity of the electron transfer system, ETS; rate e) once the rate of oxygen consumption no longer increases with titrations of FCCP. Max ETS is given by:

\[
\frac{rate_d}{rate_e}
\]

2.3 Notes

1. Prevent bubbles from forming in the gradient by stopping the peristaltic pump once air bubbles are visible towards the end of the outlet tube.

2. Ensure that all equipment is detergent-free, as detergent will damage the mitochondrial membranes. It is recommended that a plasticware and glassware is cleaned using hot water only and is dedicated solely to mitochondrial isolations.

3. A common cause of a low extraction rate of intact mitochondria is the insufficient use of mitochondria extraction medium in relation to the amount of tissue harvested. *Arabidopsis thaliana* cells are highly vacuolated, and upon rupture, the cell volume is able to substantially dilute the osmoticum. If an insufficient volume of extraction medium is used, then the dilution of the osmoticum can lead to the rupture of mitochondria.

4. Cytochrome c cannot traverse an intact outer mitochondrial membrane and so if the outer membrane is intact, COX will be 100% latent. Thus, by comparing COX activity in the absence and presence of Triton X-100, an estimation of outer mitochondrial membrane integrity can be obtained.
**In vivo respiration and internal oxygen tension**

**Materials**

**General materials**

1. Plant material: the methods described here work best to determine O$_2$ consumption, CO$_2$ production rates, and internal pO$_2$ profile in fresh plant material (*see Note 1*).

2. Agarose 0.8%

3. Calibration chamber for micro-respiration sensors and O$_2$ microsensors.

4. Scalpel blades and handle.

5. Balance.

6. Pycnometer to measure sample volume.

7. 100% nitrogen (N) gas.

8. Air pump.

**Measuring O$_2$ consumption**

1. Instruments of the micro-respiration system (*See Notes 2 and 5*): micro-respiration sensors and chambers, rack for the sensors and chambers, solid glass cylinder which fits the micro-respiration chambers, glass ring, nylon mesh, glass coated stirring magnet, stirrer controller, signal amplifier, acquisition software for data logging and calculations.

2. Vaseline or industrial grease.
**Measuring CO₂ production**

1. Instruments of the IRGA system (See Note 3): IRGA console and hand piece, respiration chamber, relevant cables and tubings.

2. Small soda charger (8 g).


4. Desiccant (Drierite, Ohio, USA).

**Measuring internal pO₂ profile**

1. Instruments of micro-profiling system (see Note 3 and 5): O₂ microsensor, signal amplifier, motorized micromanipulator, calibration chamber and acquisition software for data logging.

2. Stereoscopic microscope.

3. Solid blocks for plant material stage.

**Methodology**

All the methods described below are to be performed in a controlled temperature room, especially for the ones involving O₂ as the gas is very sensitive to temperature fluctuation. If using the same materials for the three measurements, then it is best to conclude with pO₂ profiling since it is destructive. It is important to remove all possible light sources during measurement to avoid photosynthesis reaction, which will confound the results.

**Measurement of O₂ consumption rate**

1. Connect and assemble the instruments of micro-respiration system as directed in the manufacturer manuals (See Note 6).
2. Calibrate the micro-respiration sensors by setting zero oxygen reading in fully flushed water by 100% N gas, and atmospheric O\textsubscript{2} reading in continuously bubbled water by ambient air. This step is performed in the calibration chamber.

3. Assemble a stirring magnet, glass ring, nylon mesh and solid glass cylinder (if necessary for very small samples) inside the micro-respiration chamber (See Figure 4).

4. Proceed to setting up CO\textsubscript{2} and \textit{p}O\textsubscript{2} measurement systems.

5. When all measuring systems are ready, start detaching the plant samples and immediately record the weight and volume.

6. Place the samples onto a thin layer of 0.8% agarose, then cut-side to fit and seal the wounding site (See Note 6).

7. Place the sample-on-agar inside the prepared micro-respiration chamber, put on the rack and pull down the sensor.

8. Remove all possible source of light.

9. Record O\textsubscript{2} depletion in approximately 5-6 minutes (caution, see Note 5), the software will ask to input sample and chamber volumes prior to this.

10. Transfer the sample-on-agar into the CO\textsubscript{2} production rates measurement chamber.

11. Micro-respiration data output could be saved as an excel sheet to aid further calculations if required.

**Measurement of CO\textsubscript{2} production rate**

1. Connect and assemble the instruments of IRGA system as directed in the manufacturer manuals (See Note 7).
2. Calibrate and inspect that all elements in the IRGA console and hand piece are working well, detailed instructions are available in the manuals. Calibration involves the using of soda lime and desiccant to set zero CO₂ and H₂O reading subsequently.

3. Warm up the system and set the desired experimental conditions, for example: air flow at 100 µmol.m⁻².s⁻¹, CO₂ concentration at 380 µmol.mol⁻¹ air and 55 – 75% relative humidity. Allow the system to stabilize in this condition for 10 minutes.

4. Place the sample-on-agar into the CO₂ production rates measurement chamber and log in sample weight.

5. Remove all possible light sources that directly irradiate the CO₂ chamber.

6. On the display of IRGA console, pay attention to the “stable” value and record the respiration rate when this value equals to 1. This suggests that the condition of humidity, CO₂ and air flow are in equilibrium and stable. Keep the fresh weight (FW in grams) data handy to easily log them into the console when prompted prior to recording the respiration rates.

7. Remove the sample-on-agar from the CO₂ chamber and immediately place on the sample stage of internal pO₂ profiling system.

8. Data output is also available as a spreadsheet and is readily transferred from the IRGA console into computer.

**Measurement of pO₂ micro-profile**

1. Connect and assemble the instruments of internal pO₂ micro-profiling system as directed in the manufacturer manuals *(See Note 2).*

2. Prepare a stage to place and hold the sample during internal pO₂ profiling.
3. Position O₂ microsensor above the sample stage that there is enough space for securely swapping the material between profiling (See Note 8).

4. Establish the microscope to be able to visualize clearly O₂ microsensor location relative to the sample (see Figure 5). This will greatly aid setting up the start point of each measurement (e.g. 0 µm).

5. Prior to the measurement, calibrate O₂ microsensor similarly as the calibration for micro-respiration sensors.

6. Continue setting up the pO₂ micro-profiling system and input all required information according to the conditions of experiment or instrument being used.

7. Determine the depth of measurement to conduct based on the material size and purpose of experiment. Use a calliper to precisely quantify the dimensions (length, width, height or diameter) of the material. For example, to measure internal O₂ concentration in a grapevine bud up to the meristem area (6), diameter of the bud was firstly determined to determine how deep the O₂ microsensor was inserted from the surface.

8. Adjust all parameters to suit the experiment in the profile settings page of the acquisition software. For instance, to measure internal O₂ in a grapevine bud with 4 mm diameter the settings would be: start (0 µm), end measurement (2,000 µm), step size (25 µm), safe (-1,000 µm), sensor angle (0°), wait before measure (3 s), measure period (1 s), replicates (3), delay between (0 s), number of cycles (1). See Note 9 for the explanation of key parameters.

9. Place and secure the sample on stage and position O₂ microsensor at step one of measurement or e.g. 0 µm depth then start measurement. The outer surface of the measured material is commonly considered as the step one.
10. When the final step of measurement is done, save the data in an excel format from the software and carefully remove profiled sample from the stage.

11. Store the plant material in a microtube and keep at 4°C for a couple of days to visualize O₂ microsensor trace inside the sample. The browning of damaged tissue due to O₂ microsensor insertion will be visible against the lighter colour of healthy/undamaged surrounding tissues. However, for tissues with natural dark colour or a combination of dark and light colour, such as the grapevine bud, this could be achieved by preparing microscope slides of 5 µm sections (See Reference (6)).

**Calculation of respiration rate and interpretation of respiratory quotient**

1. The respiratory quotient is a dimensionless expression of respiratory control, *i.e.* molar consumption CO₂/ molar consumption of O₂ (19). Therefore the units of expression of respiratory data must be equal.

2. The CO₂ production data are acquired as μg(CO₂).g(FW)⁻¹.min⁻¹, which is readily converted to a dry weight (DW) basis is preferred. For RQ, convert these data to mol(CO₂).g(FW)⁻¹.min⁻¹.

3. The O₂ consumption data are acquired as μmol.L⁻¹ of O₂ concentration inside the micro-respiration chamber at the given times (ms). Firstly, assess all data acquired to determine the linear range of oxygen consumption (See Note 1). Set the ‘initial’ time point as the beginning of the linear range and the ‘final’ time point as the end of the linear range. Determine total O₂ consumed by subtracting the O₂ concentration at the final point of recording from the initial. Then, divide total O₂ consumed by the total time between two concentration points, here units are μmol.L⁻¹.s⁻¹. Convert these data to mol(O₂).g(FW)⁻¹.min⁻¹.
4. RQ can then be calculated as the ratio between the number of moles CO$_2$ produced and O$_2$ consumed (19). This ratio is an indicator of substrates types used in respiration and the following energy usage to support biosynthesis. For example an RQ of 1.0 suggests the use of glucose as the sole substrate of respiration and is fully oxidised to CO$_2$ and H$_2$O, in the absence of biosynthesis. An RQ lower than 1 may indicate the use of substrates more reduced than glucose (e.g. lipid or protein), RQ>1 may indicate use of more oxidised substrates (e.g. organic acids). However, these can be simplistic assumptions, as the requirement of reducing power for cellular processes other than primary metabolism may alter the relationship. However, typical relationships of RQ to substrate and metabolic control have been demonstrated, e.g. for seed metabolising predominantly lipids vs carbohydrates during germination (20).

**Calculating pO$_2$ data and statistical treatment**

Output data files present O$_2$ concentration (µmol.L$^{-1}$) at all given measurement depth. The data are usually ready to display after measurements of soft organs, such as submerged root or germinating seeds. However, when measuring organs with alternate layers of hard and soft tissues, e.g. the alternate layers of bracts and hairs in grapevine buds, some false values or “noise” are sometimes recorded too. These noises are caused by the tension to the microsensor when penetrating hard tissues after the softer ones. The generated vibration briefly influences electrical field inside the microsensor, and is recognised by the single presence of abruptly peaked reading. In this case, statistical analysis to visualise trends without removing the genuine reading is preferable. Treating the data as multivariate is suggested, in which trends visualisation as graphs could be easily generated. For example, by using latticeExtra package (21) in R statistics software (22).
Notes

1. Measurement of the rate of oxygen consumption in a closed system assumes linearity between the initial and final time points used for the calculations above. Thus the optimal range should be determined prior to experimentation for each treatment condition or tissue.

2. For the O₂ and CO₂ gas exchange measurements, it is a must to use fresh material and to be performed as soon as the sample is detached as the physiological state of the tissues will change gradually once they are separated from the rest of the plant. The use of dry plant material for \( pO_2 \) is possible but not ideal. Dehydration often causes brittleness or constriction of the tissue that could limit accessibility of the O₂ microsensor. When the tissue is too hard to penetrate, it is not impossible to break the O₂ microsensor. However, this \( pO_2 \) profiling method is not limited to use only in biological samples, measurement could be performed in any penetrable material such as in agar medium of plant tissue or bacterial culture, etc.

3. We use the micro-respiration and \( pO_2 \) micro-profiling systems from Unisense (Aarhus, Denmark). For the \( pO_2 \) micro-profiling, ensure to choose the best diameter size of O₂ microsensor outside tip that suits the dimensions of the sample. The use of motorized micromanipulator is recommended for measurement with fine step sizes of < 100 µm.

4. We use an insect respiration chamber attached to a portable IRGA (gas exchange) system from Li-COR (Lincoln, NB, USA).

5. The signal of the micro-respiration sensors and O₂ microsensors are very small (10⁻¹³ to 10⁻¹⁰ ampere) and electrical fields may interfere. It is recommended to switch off any unnecessary electrical/mechanical equipment and avoid touching the sensor or wires during measurements. Once connected to the electricity current, the sensors signal (pA) usually takes a few moments to stabilize for pre-polarization. Ensure to turn on the
system at least 2 hours prior to calibration, this is crucial to remove all built up oxygen in the electrolyte when the sensor was not in use. Check and repeat calibration frequently to ensure that all measurements are calibrated to correct concentrations.

6. In addition to block O₂ entry and CO₂ exit through the cutting site, the agar also helps to retain sample moisture content during measurement.

7. Pay extra attention to the fine cables around the hand piece when removing swapping leaf to insect chamber. The cables are easily trapped in between metal parts that may break them.

8. Make sure that there is enough room between the stage and the tip of O₂ microsensor, for the plant material and operator hand to move freely. This is crucial as often the O₂ microsensor tip was broken when operator accidentally touching the sensor when swapping the material in between profiling.

9. For the full explanation of all parameters please refer to the manual of SensorTrace Profiling software. Here are some key parameters of internal pO₂ profiling in dense plant tissue:

A. Start (µm): the first depth in which the measurement is to be taken.

B. End (µm): the final depth in which measurement is to be taken. Both A and B are to be decided based on the experiment purposes and dimensions.

C. Step size (µm): the vertical step depths by which O₂ microsensor is moved from start to end position.

D. Wait before measure (s): after the O₂ microsensor is moved to a certain depth, it will wait for a period of seconds before it starts measuring. This period is important to get the sensor signal stabilized after the movement. Adjust
according to the character of the sample, harder sample may require longer waiting period.

E. Measure period (s): duration of the measurement in each position, with each measurement is an average value over this period of time. When measuring in a noisy environment, measuring in a longer period is recommended.

References


Figures

**Figure 1.** Diagrammatic representation of the density gradient maker used to prepare gradients for purification of isolated organelle fractions.
Figure 2. Example of a Percoll gradient after centrifugation showing the position of the intact mitochondria.
Figure 3. Example of trace showing the changes in oxygen uptake after the addition of ascorbate and cytochrome to intact mitochondria, and then the detergent, Triton to disrupt the membranes, allowing access of the substrate to cytochrome C oxidase with subsequent detection of enzyme activity by oxygen uptake.
Figure 4. Diagrammatic representation of the micro-respiration chamber
Figure 5. Diagrammatic representation of the use of a microscope to visualize the location of the O₂ microsensor within the tissue under analysis.