A reverse-genetics approach to low-oxygen tolerance in *Arabidopsis*

Michelle N. K. Demers

Department of Biological Sciences,

Faculty of Science and Engineering

Macquarie University, Sydney NSW Australia

A thesis submitted for the degree of Masters of Research

Submission date: 23 December 2016

DECLARATION

I certify that the material of this thesis has not been previously submitted as part of the requirements for a higher degree to any other university or institution.

This thesis contains no material previously published or written by any other person. I certify that all information sources and literature used are indicated in the thesis.

I wish to acknowledge the following assistance with the research detailed in this report:

- Dr. Brian Jones for assistance with methods for growing Arabidopsis
- Dr. Andrew Allen for assistance with the statistical analysis of my anaerobic data
- Dr. Juan Pablo Matte Risopatron for assistance with analysis of qRT-PCR data
- Dr. Roberto Gaxiola for generously providing us with plant material
- Dr. Brian Atwell, my supervisor for assistance with experimental design and manuscript preparation

All other research described in this report is my own original work.

Michelle Demers

23 December 2016

NOTE TO EXAMINERS

This thesis is written in the form of a single journal article for Annals of Botany. The majority of the author guidelines have been followed, except for minor deviations detailed here and where the guidelines clash with Macquarie University thesis formatting requirements. All figures and tables have been presented at the appropriate places in the text to enhance readability.

ACKNOWLEDGEMENTS

This project would not have been completed without the generosity of Dr. Brian Jones, who graciously allowed me the use of his lab, growth facilities, and expertise in dealing with *Arabidopsis*. I would also like to thank the community of staff and graduate students working in the Biomedical Building Environment at the University of Sydney for their help and support for this project.

Special acknowledgements in Faculty of Agriculture and Environment at the University of Sydney include Nathan Danckert for giving me a helping hand to get started with my lab work and lending me supplies whenever I ran out. Dr. Neil Wilson for lending me his wealth of knowledge whenever I needed advice, and for lending me his key whenever I got locked out. Peri Tobias for always having a listening ear and lastly, Mathilde Lagarde for being a wonderful lab partner.

A variety of people provided me with assistance on various analyses which allowed this project to progress smoothly. I would like to thank Dr. Andrew Allen from Macquarie University for working with me to figure out which stats analyses I needed and how to interpret them, and to Juan Pablo Matte Risopatron from Pontificia Universidad Catolica de Chile for aiding in my understanding of qPCR and helping me to make sense of my data, even from a world away.

I would like to thank Dr. Robeto Gaxiola for his generous donation of AVP1-1 seed for use in these experiments. If not for that, this project would have taken a very different direction and not have been as much of a success as it turned out to be.

I would like to thank my supervisor, Dr. Brian Atwell, for his continued support and guidance throughout this project in overcoming multiple nearly impossible obstacles, helping me gather my thoughts, improving my writing and lugging certain supplies across the city for me whenever it was needful.

I would like to thank my friends and family for their moral support, listening to my problems, helping me with editing and in general for their love and support for me while completing this project.

Lastly and most sincerely, I want to thank Thomas Gretzinger for listening to all of the ups and downs of my project, always providing honest feedback, helping me find solutions to all of my problems and, most importantly, providing support when I needed it most. Know that I would not have been able to complete this without you.

TABLE OF CONTENTS

ABBREVIATIONS

AMP	Adenosine monophosphate
ANP	Anaerobic proteins
ARE	Anaerobic response element
ATP	Adenosine 5'-Triphosphate
AVP1	*Arabidopsis thaliana* vacuolar pyrophosphatase 1
AVP1-1	*Arabidopsis* transgenics overexpressing *AVP1*
AVP2/AVPL2	*Arabidopsis thaliana* vacuolar pyrophosphatase 2
CTAB	Cetyl trimethylammonium bromide
EF1α	Elongation Factor 1-α
ERF	Ethylene response factor
GMP	Guanosine monophosphate
NAD^+	Nicotinamide adenine dinucleotide
NMP	Nucleoside Monophosphate
NTP	Nucleoside triphosphate
OVP3	*Oryza sativa* vacuolar pyrophosphatase 3
PCR	Polymerase Chain Reaction
PPase	Pyrophosphatase
PPi	Pyrophosphate
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse-Transcriptase Polymerase Chain Reaction
SE	Standard Error
TCA	Tri-carboxylic acid
UDP	Uridine monophosphate
UTP	Uridine 5'-triphosphate
V-ATPase	Vacuolar adenosine triphosphatase
V-PPase	Vacuolar pyrophosphatase
WT	Wild-Type *Arabidopsis*

ABSTRACT

Anoxia causes an energy crisis in plants because oxygen is required to generate ATP. In anoxia-sensitive species, cell death occurs after sustained anoxia through loss of membrane integrity because insufficient energy is available for proton pumping. *Arabidopsis* vacuolar H^+- pyrophosphatase proton pumps (*AVP1*) reputedly improve survival in anoxia because pyrophosphate substitutes for ATP to maintain proton pumping. Plants overexpressing *AVP1* were grown on medium containing 1% or 0.1% sucrose then in anoxia for up to 2 days. RNA was extracted for qPCR during anoxia or plants were given two weeks in normoxia to recover. Survival and dry weight of roots and shoots after two weeks, and resumption of shoot and root growth, were measured over this period. Survival rates post-anoxia were higher on high-sucrose medium but overexpression of *AVP1* in roots increased survival, regardless of sucrose supply. However, biomass was only enhanced by *AVP1* overexpression in normoxia. *AVP1* expression was strongly induced in roots by anoxia, especially on 0.1% sucrose plates, while exogenous sucrose probably inhibited *AVP1* induction. *AVP1* is a critical component of acclimation to anaerobiosis as its induction in anoxic roots enhances shoot function during an energy crisis. This is an ideal gene target for improved flood tolerance.

1. INTRODUCTION

1.1 General Introduction

In order to produce enough food to feed nine billion people by 2050, our agricultural productivity will need to double from current levels (Bailey-Serres *et al.* 2012; Kole 2013). This would be a formidable task if conditions were to remain as they are now but becomes even more challenging as extreme weather events become increasingly common due to climate fluctuations. Periodic and sustained waterlogging events are detrimental to crops and have increased markedly over the last 10 years in all continents except Antarctica (Bailey-Serres *et al.* 2012). In the United States, economic losses due to excess water are second only to drought (Setter and Waters 2003) and in Australia, waterlogging results in yield losses of approximately $AUD 300 million per year (Setter and Waters 2003). In south and southeast Asia, 20 million hectares are flooded annually with an associated cost of approximately $US 1 billion per year (Kole 2013). Crop losses due to excess water can range anywhere between 10 and 100% depending on the severity and duration of waterlogging and crop growth stage (Kole 2013). Waterlogging can result from heavy rainfall, rising ocean levels and natural disasters such as tropical storm surges, all of which are expected to increase in future years due to human-influenced climate change (Kole 2013). Alarmingly, none of the world's major economic crops are resilient to this stress save rice, and even this tolerance is limited because of its acute sensitivity to salinity (Yeo and Flowers 1986).

Rice, the most flood-tolerant of all crops, accounts for roughly 20% of global caloric intake and is the staple food for nearly half of the global population (Normile 2008; Kole 2013). Nearly 144 million hectares worldwide are dedicated to rice production, and approximately 35% of this is in flood-prone areas (Kole 2013). Rice consumption has risen by 50 million tonnes from 2004 to 2011, making continued research and development of new varieties of utmost importance to global food security (Mohanty 2013).

At germination, many paddy rice varieties are extraordinarily flood-tolerant; these varieties can germinate in the complete absence of O_2 and coleoptiles can elongate by many centimetres while completely submerged (Atwell *et al.* 1982). Adult rice plants exhibit a much wider range of tolerances to submergence; some low-yielding landraces of the *indica* sub-species are able to tolerate fluctuating levels of standing water throughout an entire growing season, whereas many modern hybrids suffer huge yield losses unless soil conditions are ideal (Kole 2013). These low-yielding landraces are commonly the types grown by the most impoverished farmers in rain fed areas due to the unpredictable nature of transient floods; this situation could be greatly improved with the development of high-yielding varieties

that are more flood-tolerant in their mature growth stages (Kole 2013). Understanding how tolerance to flash floods and longer term flooding has evolved, and how to incorporate these mechanisms into crops, could ameliorate the impacts of short and long-term floods on food production.

1.2 Waterlogging creates an energy crisis

Waterlogging (the submergence of roots) and flooding (complete submersion) compromise oxygen supply to respiring cells. This can happen in a variety of circumstances in both temperate and tropical cropping systems – during transient floods, sustained floods after heavy rainfall and following excessive irrigation (Drew 1997). The effects are exacerbated when soils have poor drainage, allowing standing water to persist for days, or even weeks (Drew 1997; Setter and Waters 2003). When the amount of water flooding the air-filled fraction in soil exceeds a threshold, loss of continuity in the gas phase and the low solubility of oxygen in water (oxygen diffuses 10,000 times more slowly in water than in air) restrict transfer of oxygen to the roots (Armstrong 1980). As temperatures rise, plant respiration rates increase and so organs will become hypoxic (deficient in oxygen) more quickly, eventually becoming anoxic (the complete absence of oxygen), which results in an energy crisis (Drew 1997) .

Oxygen plays a vital role in energy production through oxidative phosphorylation, generating ATP in the electron transport chain in the mitochondria (Gibbs and Greenway 2003). When oxygen availability is restricted in either hypoxia or anoxia, oxidative phosphorylation is progressively restricted and energy generation becomes reliant instead on substrate-level phosphorylation (Dennis *et al.* 2000; Gibbs and Greenway 2003; Bailey-Serres and Chang 2005; Bailey-Serres and Voesenek 2008). Substrate-level phosphorylation regenerates NAD^+ through the fermentation of pyruvate to a combination of ethanol, lactate and alanine, yielding small amounts of ATP via glycolysis (Dennis *et al.* 2000; Gibbs and Greenway 2003; Bailey-Serres and Chang 2005; Bailey-Serres and Voesenek 2010) (Fig. 1.1). Where oxidative phosphorylation yields 30 – 36 mol ATP per mole of hexose, substrate-level phosphorylation only yields 2 – 4 mol ATP per mol of hexose. Glycolysis can accelerate in response to low oxygen (Gibbs and Greenway 2003; Huang *et al.* 2003), and pyrophosphate metabolism, which utilizes pyrophosphate (PPi) to obtain energy in place of ATP, can also mitigate the impact of low ATP regeneration (Atwell *et al.* 2015). Even so, this extreme drop in energy production compromises the function of plants markedly (Bailey-Serres and Voesenek 2010). After the onset of anoxia and an energy crisis, proton transport is inhibited and given time, pH in the cytosol decreases and both the plasma membrane and tonoplast become depolarised. Eventually, compartmentation breaks down and solutes such as potassium are lost from the cytosol (Atwell *et al.* 2015). If O_2 levels do not recover, cell death is inevitable. However, plants

such as rice and other wetland species have evolved a suite of adaptive structural and metabolic responses that allow them to survive excessively wet environments.

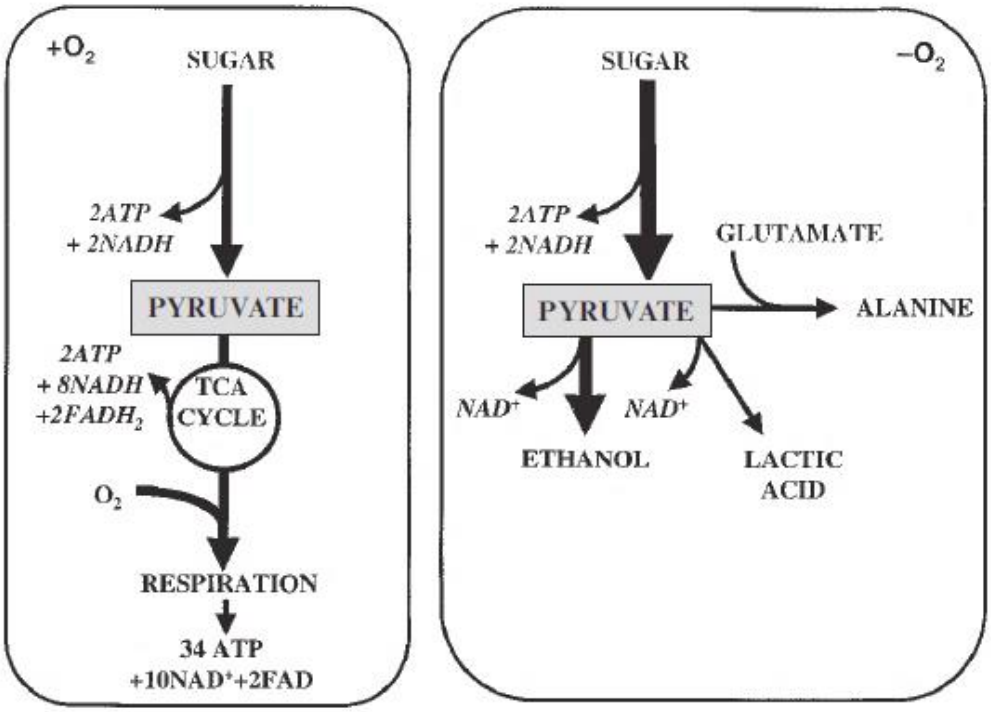

Fig. 1.1. Shift in metabolism occurring in plants treated by low oxygen conditions (Dennis et al. 2000).

1.3 Adaptations to oxygen deficits

Adaptations to low oxygen in plants are very complex. Strategies plants typically engage to survive oxygen deficits are escape or quiescence (Setter and Waters 2003). The development of internal air channels (aerenchyma) is a major response to flooding associated at times with both these survival strategies. Each strategy has apparently evolved in response to a particular duration and frequency of waterlogging. The 'escape' strategy involves rapid elongation of shoot tissues so that they can outpace rising water to reach the atmosphere, thus acting like a snorkel (Bailey-Serres and Voesenek 2010; Hattori *et al.* 2011; Muto *et al.* 2011). This shoot organ can then facilitate the transfer of oxygen to submerged tissues through development of aerenchyma that connect leaves to roots and facilitate gas exchange between aerated sections of the plant and those that remain submerged (Drew 1997; Setter and Waters 2003; Bailey-Serres and Voesenek 2010). This adaptation is most advantageous when waterlogging is frequent and flood levels are relatively shallow (Setter and Waters 2003). Significantly, the 'escape' strategy is metabolically expensive and depletes carbohydrate reserves rapidly; should

shoots fail to reach the surface before carbohydrate reserves run out, plant death is inevitable (Bailey-Serres and Voesenek 2010; Vashisht *et al.* 2011).

In deep or transient floods of up to a few weeks' duration, a 'quiescence' strategy is the most advantageous. In this circumstance, plants alter their metabolism to reduce energy requirements, begin fermenting hexoses and remain dormant until floods subside, where they then become re-aerated and resume growth (Bailey-Serres *et al.* 2012; Atwell *et al.* 2015). In order to do this effectively, not only must growth be halted but what little ATP is made must be preferentially allocated to key processes for survival. These includes maintenance of membrane gradients (Atwell *et al.* 2015), maintaining ion and solute transport (Atwell *et al.* 2015), synthesizing anaerobic proteins (ANPs) (Greenway and Gibbs 2003), maintaining a pHstat (Felle 2005) and using pyrophosphate (PPi) as an energy source (Greenway and Gibbs 2003; Atwell *et al.* 2015) Quiescence is considered by some to be a true tolerance mechanism (Bailey-Serres and Voesenek 2008). In order for plants to trigger this physiological response, the expression of a great many genes must change.

1.4 Genetic responses to anoxia

1.4.1 Low oxygen sensing and signalling

In order for plants to undergo quiescence, they must sense oxygen levels and trigger extensive downstream modifications in gene expression. There are a variety of putative mechanisms for direct and indirect sensing of cell oxygen status. These modifications serve to elicit a physiological response which puts the plant in a quiescent state.

The most widely accepted theory of direct oxygen sensing in plants came with the discovery of the N-end rule pathway of targeted proteolysis. This is where the stability of the N-terminal region of a 'sensor' protein determines expression of multiple transcription factors, and the stability of the N-terminus is determined in turn by the sequence of amino acids present (Gibbs *et al.* 2011; Licausi *et al.* 2011). This in particular applies to the type VII ethylene response factors (ERFs), which are responsible for eliciting the core metabolic response and have been demonstrated to be targeted to the nucleus in hypoxia (Gibbs *et al.* 2011; Bailey-Serres *et al.* 2012). In plants, N-end rule pathway substrates contain an N-terminal region consisting of NH_2-Met_1-Cys_2; the methionine residue is cleaved by a protein called Met amino peptidase leaving only the cysteine residue, which easily becomes spontaneously oxidised. Once oxidised, the amino acid is then targeted for 26S proteasome-mediated degradation (Bailey-Serres *et al.* 2012). In hypoxic and anoxic conditions, these residues are able to avoid degradation and so these critical

transcription factors can act on key O_2-senstive genes (Gibbs *et al.* 2011; Licausi *et al.* 2011; Bailey-Serres *et al.* 2012). Gibbs *et al.* (2011) and Licausi *et al.* (2011) both demonstrated that when the N-terminal region was mutated, type VII ERF proteins remained stable regardless of oxygen status. When Gibbs *et al.* (2011) mutated the N-terminal region of two type VII ERFs, HRE1 and HRE2, this resulted in plants better able to withstand hypoxic stress, presumably due to a constitutive anaerobic response. Interestingly, Licausi *et al.* (2011) noticed increased mortality in plants containing a similar N-terminal mutation of another type VII ERF, RAP2.12, presumably due to its inability to properly trigger acclimation responses.

Unsurprisingly, plants have evolved multiple responses to oxygen deficits as well as suspected mechanisms for directly and indirectly sensing oxygen status. Cytosolic [Ca^{2+}] levels increase rapidly in anoxia (Drew 1997) and are known to be involved in low-oxygen signalling pathways by inducing signal transduction cascades (Liu *et al.* 2005). A drop in pH levels following anoxia may be an indirect signal of anoxia because of resultant de-energised ATP-driven proton pumps, which will also cause cytosolic [Ca^{2+}] to rise (Drew 1997; Gibbs and Greenway 2003; Bailey-Serres and Chang 2005; Gibbs *et al.* 2011). Ethylene is also involved in the anaerobic response through its induction of ERFs (Bailey-Serres *et al.* 2012). Additionally, rising levels of reactive oxygen species and rising levels of nitric oxide may be involved (Drew 1997; Bailey-Serres and Chang 2005).

There are also many other families of transcription factors known to play a role in low-oxygen signalling besides type VII ERFs, including heat shock factors, MADS-box proteins, bZIP proteins, Ethylene-response binding proteins, AP2-domains, leucine zippers, basic helix-loop-helix, zinc fingers, NAC and WRKY factors; all have been demonstrated to significantly increase under anoxia (Klok *et al.* 2002; Loreti *et al.* 2003; Branco-Price *et al.* 2005; Liu *et al.* 2009; Narsai *et al.* 2009, 2015).

1.4.2 Microarray studies

Microarray studies (Klok *et al.* 2002; Branco-Price *et al.* 2005; Gonzali *et al.* 2005; Liu *et al.* 2005; Lasanthi-Kudahettige *et al.* 2007; Narsai *et al.* 2009, 2015) have been useful in studying gene expression responses to anoxia. Microarrays aid in understanding not only which genes are differentially regulated in anoxia, but also the extent to which transcripts are altered, the functional groups of genes affected, the timing in which they are changed and even the identity of new genes that may play a role in the pathway of interest. A microarray study done by Liu (2005) looking at *Arabidopsis* found 1,266 genes whose expression is differentially altered during low oxygen conditions, whereas in coleoptiles of rice, which are so much more tolerant to low oxygen, Lasanthi-Kudahettige (2007) found 3,138 probe sets with

differentially-altered expression in anoxia. In microarray studies of both species, gene expression exhibited differential timing when they were upregulated, indicating that different elements of the anaerobic response may be advantageous at different stages after the onset of anoxia (Klok *et al.* 2002; Liu *et al.* 2005; Lasanthi-Kudahettige *et al.* 2007). Microarray studies have also demonstrated low oxygen responses to be tissue-specific. A microarray study by Narsai *et al.* (2015) looked at gene expression differences between rice coleoptile bases and tips and found many differentially expressed genes in response to hypoxia. Using the response to anoxia as an example, coleoptile tip and base tissues upregulated 944 and downregulated 1971 of the same genes in response to anoxia, tips upregulated and downregulated an additional 451 and 299 genes respectively not observed in bases, and bases upregulated and downregulated an additional 245 and 170 genes respectively that were not observed in tips (Narsai *et al.* 2015)

In order to determine which transcripts were induced purely by aerobic and anaerobic environments and exclude any gene responses that could be a result of previous growth conditions or secondary responses, Narsai (2009) studied gene expression using microarrays of transcripts from young rice coleoptiles from three distinct treatments. Their criterion for a core anaerobic transcript was one that was significantly upregulated in both anoxic germination and when plants were switched from an aerobic to an anaerobic environment, and significantly downregulated when plants were switched from an anaerobic environment to an aerobic one. They found 730 anaerobic transcripts, which included a significant overrepresentation of transcripts involved in carbohydrate and lipid metabolism, and a significant underrepresentation of genes involved in the TCA cycle, which supports the fact that this pathway is largely superfluous[1] without oxygen to drive oxidation of NAD^+ (Narsai *et al.* 2009). Many other upregulated genes were involved in glycolytic and fermentative pathways, but they found transcripts involved in most major metabolic pathways, a finding which has been confirmed in *Arabidopsis* (Dolferus *et al.* 2003; Branco-Price *et al.* 2005; Gonzali *et al.* 2005; Liu *et al.* 2005; Lasanthi-Kudahettige *et al.* 2007). Therefore, the anaerobic response is more complex than previously considered.

1.4.3 Induction of anaerobic proteins

Classical studies show that upon the onset of anoxia, a core set of ANPs are induced, as was well described in maize by Sachs (1980). These mainly consist of enzymes involved in glycolysis and

[1] Assuming that the haemoglobin cycle is not active to any significant degree (see paper in the Atwell et al Tansley review or search on 'Igamberdiev' and 'hemoglobin')

fermentation, such as alcohol dehydrogenase, sucrose synthase, pyruvate decarboxylase and lactate dehydrogenase, all of which are constitutively induced while in anoxia for maize, rice and *Arabidopsis* (Sachs *et al.* 1980; Drew 1997; Branco-Price *et al.* 2005; Liu *et al.* 2010) (Fig. 1.1). These genes are characterised by having common elements in their promotor regions specifically activated by low oxygen, referred to as an anaerobic response element (ARE). AREs consist of GC motifs (GCCCATTG), GT anaerobic response element motifs (TGGTTT and GCAAAACC), G-box and TATA box elements (Matsumura *et al.* 1998; Klok *et al.* 2002; Liu *et al.* 2010). More recently, vacuolar pyrophosphatases may also be considered as ANPs, as they have been shown to contain multiple AREs in rice, and have strong upregulation and translation during anoxia in rice and *Arabidopsis* (Carystinos *et al.* 1995; Huang *et al.* 2005; Liu *et al.* 2010). This demonstrates that during anoxia, metabolism shifts away from oxidative phosphorylation in favour of glycolysis and fermentation, and towards using pyrophosphate (PPi) as an energy source to maintain a strong transmembrane potential.

1.5 Hypoxia acclimates cells to low oxygen conditions

A period of hypoxia preceding anoxia is well known to increase tolerance to anoxia in plants; this is demonstrated with maize (Greenway *et al.* 1992; Xia and Roberts 1996), *Arabidopsis* (Ellis *et al.* 1999; Hunt *et al.* 2002), and wheat (Albrecht *et al.* 2004). In hypoxia, plants will rapidly undergo a transition where they begin to reduce levels of oxidative phosphorylation and other metabolic processes that are highly energy-consuming and begin to increase fermentation rates (Albrecht *et al.* 2004; Bailey-Serres and Voesenek 2010). While providing signals to plants that anoxic conditions may be imminent, hypoxia also triggers synthesis of proteins necessary for anaerobic survival, a metabolically-expensive process, while they are still able to source energy from oxidative phosphorylation for protein synthesis (Bailey-Serres and Voesenek 2010). This allows plants to properly acclimate to this stress.

When soils become waterlogged, oxygen gradually becomes inaccessible depending on factors such as temperature, rates of root respiration, aerobic microbial activity in the soil, and the diffusion rates of O_2 through the soil; this can occur within hours to a few days (Setter and Waters 2003). The same situation applies when water subsides; roots are often not completely aerated again until days after water levels have drained (Setter and Waters 2003). This gradual reduction and increase in soil water and therefore oxygen levels prior-to and post waterlogging gives plants time to acclimate pre-empting anoxic conditions and the effects of re-oxygenation (Gibbs and Greenway 2003; Setter and Waters 2003). Therefore, moving plants directly from normoxia into anoxia and *vice versa* in an experimental setting imposes either an anoxic or an oxidative shock, causing injuries from other effects such as an excessive

drop in pH or the formation of free radicals (Gibbs and Greenway 2003; Felle 2005). Observing plant responses after an anoxic or aerobic shock probably yields results different from those seen in plants that have first undergone a hypoxic pre- or post-treatment and so may not necessarily be indicative of responses to hypoxia in a more natural setting (Ellis *et al.* 1999; Hunt *et al.* 2002; Setter and Waters 2003; Albrecht *et al.* 2004; Felle 2005). However, experiments observing plants directly exposed to anoxia do have their merits: they demonstrate how the core anaerobic response performs without the complication of an acclimation period, and could reveal mechanisms to cope with secondary effects associated with the anoxic shock.

1.6 Anaerobic metabolism

1.6.1 Energy budgets in anoxia

Anoxia represents an energy crisis for cells. Even if glycolysis were accelerated by feedback regulation through changing cell energetics, ATP production could never exceed more than a third of that observed in normoxia. This accelerated carbohydrate breakdown is the result of the Pasteur effect (Gibbs and Greenway 2003). This does not always occur in practice, as demonstrated by a recent study of rice coleoptiles which found that anoxic cells yielded only approximately 12% of the energy they can produce in normoxia (Edwards *et al.* 2012). Regardless of the percentage reduction, for cells to survive prolonged periods of anoxia, they must greatly reduce their energy expenditure to preferentially sustain three main functions: maintaining intracellular pH in compartments via a biochemical pHstat, synthesise new proteins required for anaerobic metabolism and maintain transmembrane gradients and ion transport (Greenway and Gibbs 2003; Atwell *et al.* 2015). A recent paper by Edwards *et al.* (2012) demonstrated this by devising an energy budget for cells in anoxic conditions by examining such processes as protein synthesis, DNA synthesis, biosynthesis of sucrose and rates of ion uptake. They found an 8-fold decrease in available ATP in anoxia, and all rates of metabolic processes mentioned above were decreased in anoxia compared with normoxia. Of the ATP available, the greatest amount was allocated to protein synthesis (52% of available ATP in anoxia compared with 19% in normoxia), and lipid synthesis rates were only 25% lower in anoxia than normoxia, emphasizing how important these processes are to survival in anoxia (Edwards *et al.* 2012). Rates of DNA synthesis in anoxia were approximately half of those seen in normoxia, and rates of net uptake of K^+ in anoxia were reduced by approximately one-third (Edwards *et al.* 2012). All available ATP could be accounted for by just a few key metabolic functions in anoxia while those same processes only account for half of available ATP in normoxia (Edwards *et*

al. 2012). The proportion of decreases in different metabolic processes are clearly not uniform, demonstrating the preferential allocation of energy in anoxia.

1.6.2 pH changes in anoxia

At the onset to anoxic conditions, plants undergo a distinct pH drop from the normal set point of 7.5 to between 6.8 - 7.2, (Greenway and Gibbs 2003; Felle 2005; Kulichikhin *et al.* 2009). This shift favours enzymes involved in anaerobic metabolism that function better at a pH slightly below 7, and a new pH 'set point' will be maintained at this level (Felle 2005). It also serves to reduce the energy cost of transmembrane gradient regulation through H^+ pumping (Atwell *et al.* 2015). If anoxic conditions persist, membranes will continuously leak K^+ into the apoplast and cells will eventually succumb to acidosis after a variable period of stability, depending on the plant's level of tolerance (Felle 2005). This occurs not because of compromised acid-base equilibria but rather due to a lack of energy to maintain proton pumps, therefore reducing transmembrane gradients and causing membrane potentials to break down. The vacuole eventually releases its contents into the rest of the cell resulting in death (Felle 2005). Provided that the injuries sustained during anoxia are not terminal, pH may be fully restored once plants are restored to normoxic conditions (Kulichikhin *et al.* 2009). This was observed for excised rice coleoptiles which restored their cytoplasmic pH in air after undergoing 92 hours in anoxia at an external pH of 6.5 (Kulichikhin *et al.* 2009).

1.6.3 Ion transport and transmembrane gradients

In normal conditions, ion transport is very metabolically expensive and may account for as much as half of all available ATP (Atwell *et al.* 2015). Therefore, in anoxia, ion transport must be reduced only to that which is necessary for survival, or 'maintenance' levels (Greenway and Gibbs 2003). This is well demonstrated in anoxic rice coleoptiles, which showed a 17-fold decrease in the permeability of membranes to K^+ and Cl^- compared to fully aerated controls (Colmer *et al.* 2001).

Maintenance of membrane integrity and transmembrane gradients during an energy crisis is also believed to be a crucial requirement for survival (Felle 2005; Atwell *et al.* 2015). Maintaining transmembrane gradients ensures that cellular functions remain compartmentalised and able to maintain solute transport, which also contributes to the maintenance of the biochemical pHstat (Atwell *et al.* 2015). This important function is consistent with preferential use of energy for lipid synthesis in rice coleoptiles during anoxia (Edwards *et al.* 2012), and enzymes involved in lipid metabolism were commonly reported as being

upregulated in microarray experiments (Dolferus *et al.* 2003; Liu *et al.* 2005; Lasanthi-Kudahettige *et al.* 2007).

1.7 Vacuolar Pyrophosphatase

1.7.1 Transmembrane proton pumps

Transmembrane gradients in anoxia, particularly in the vacuole, will function so long as proton pumps are available to maintain the proton motive force across them (Felle 2005). It is well known that two families of electrogenic transmembrane proton pumps work simultaneously in the vacuolar membrane: vacuolar ATPases (V-ATPases) and vacuolar pyrophosphatases (V-PPases) (Sarafian *et al.* 1992; Rea and Poole 1993; Kim *et al.* 1994; Carystinos *et al.* 1995; Maeshima 2000; Li *et al.* 2005; Atwell *et al.* 2015). Both V-ATPase and V-PPase are abundant throughout the vacuolar membrane and substantially contribute to the maintenance of proton transport across membranes and electrochemical potential gradients (Sarafian *et al.* 1992; Kim *et al.* 1994). This electrochemical gradient is the result of electrically-coupled transport processes, which function to maintain membrane compartmentation (Sarafian *et al.* 1992; Kim *et al.* 1994). The V-ATPase consumes relatively large amounts of energy because of the free energy of ATP hydrolysis, and its rates are highly dependent upon cytoplasmic ATP availability; when ATP levels drop during an energy crisis, this pump is deactivated, rendering V-PPase the sole functioning proton pump on the vacuolar membrane (Felle 2005; Atwell *et al.* 2015).

1.7.2 Vacuolar pyrophosphatases utilise PPi

Vacuolar pyrophosphatases produce energy by hydrolysing PPi as a substrate instead of ATP, allowing proton transport to be energised even during an energy crisis (Rea and Poole 1993; Carystinos *et al.* 1995; Maeshima 2000; Atwell *et al.* 2015). This allows for prolonged maintenance of the vacuolar transmembrane gradient, limited ionic transport and compartmentalization under severe stress conditions. The substrate for V-PPase is PPi, which contains a high-energy phosphoanhydride bond (Maeshima 2000). Pyrophosphate is generated as a by-product of many reactions, such as protein synthesis, RNA synthesis, starch and cellulose synthesis, DNA and RNA polymerization, and β-oxidation of fatty acids, and there is a constant pool of PPi available in the cytosol (Maeshima 2000; Atwell *et al.* 2015) (Fig. 1.2). This PPi pool is involved in plant growth, as PPi must be hydrolysed in order to facilitate macromolecule biosynthesis, or high levels may risk inhibiting metabolism (Ferjani *et al.* 2011). Levels of free PPi have been shown not to change in response to changes in light or dark, during changes in respiration or even in anoxia, all conditions which would elicit drastic changes in

available ATP (Rea and Poole 1993). As well, the resultant drop in pH raises the free energy of hydrolysis of PPi, while simultaneously lowering the free energy of ATP (Carystinos *et al.* 1995; Drew 1997). The stability and increased free energy of PPi hydrolysis makes it an ideal energy source, particularly under stress conditions when plants are often energetically compromised, reducing the demand on ATP. Plants tolerant to anoxia appear to have conserved the activity of various pyrophosphatases including V-PPase. For example, in rice coleoptiles, transcripts coding for PPi-dependent enzymes involved in glycolysis are preferentially increased in anoxia, including V-PPase (Atwell *et al.* 2015).

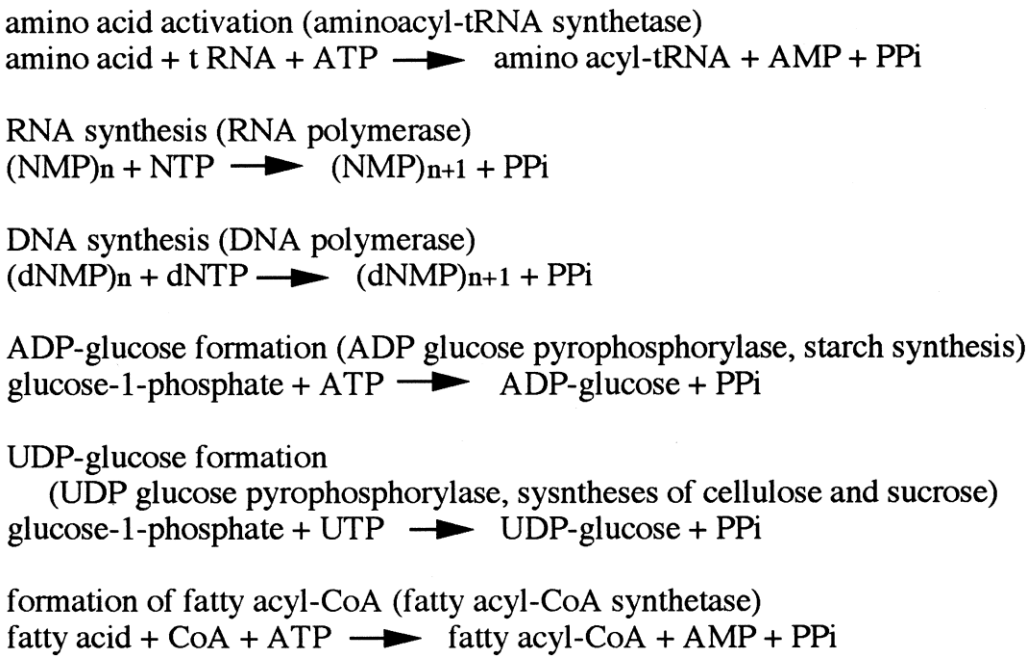

FIG. 1.2. Pathways of PPi formation in cells. ATP: Adenosine 5'-triphosphate; AMP: Adenosine monophosphate; NMP: Nucleoside monophosphate (any of AMP, UMP and GMP), NTP: Nucleoside triphosphate (any of ATP, UTP and GTP); UTP: Uridine 5'-triphosphate; UDP (Maeshima 2000).

1.7.3 Regulation of vacuolar pyrophosphatase proton pumps

There are three classes of pyrophosphatases (PPases) in many organisms: membrane PPases, soluble PPases and V-PPases. V-PPases are the only type of PPase able to transport H^+ across membranes (Maeshima 2000). Where V-ATPase is found in all eukaryotes, V-PPase is limited to plants, phototrophic bacteria, algae, protozoa and archaebacteria (Maeshima 2001). Vacuolar pyrophosphatases are encoded by a single gene, unlike V-ATPase which may be encoded by up to 26 genes, (Sarafian *et al.* 1992; Gaxiola *et al.* 1999; Li *et al.* 2005). Within higher plants, V-PPase homologues have been documented in many plants including barley, wheat, tobacco, beetroot, rice and *Arabidopsis*, with three main

conserved regions: CS1, CS2 and CS3 (Maeshima 2000; Liu *et al.* 2010; Lin *et al.* 2012; Asaoka *et al.* 2014) (Fig. 1.3). The V-PPases in plants and other organisms all contain a highly conserved catalytic domain in CS1 for substrate hydrolysis with the amino acid sequence DVGADLVGKVE (Rea and Poole 1993; Maeshima 2000). In rice, a plant very tolerant to anoxia, as many as six genes encoding for V-PPases have been found and one of these isoforms, *OVP3*, has been shown to be significantly upregulated by anoxia (Liu *et al.* 2010). There is evidence that V-PPases in rice are involved with other stresses aside from anoxia, as other rice V-PPase isoforms have also been found to respond to chilling (Carystinos *et al.* 1995). In *Arabidopsis*, a single V-PPase protein has been found localised to the tonoplast, *AVP1*, and has shown to be dependent on cytosolic K^+ for its activity and inhibited by Ca^{2+} (Li *et al.* 2005; Igamberdiev and Kleczkowski 2011). A second V-PPase gene named AVP2/AVPL2 was identified, but subsequently found to be localised to the Golgi apparatus (Drozdowicz *et al.* 2000; Mitsuda *et al.* 2001). *AVP1* overexpressers in *Arabidopsis* have demonstrated increased tolerance to salt and drought (Gaxiola *et al.* 2001), and have also been shown to have increased tolerance to conditions of reduced phosphates (Yang *et al.* 2007). *AVP1* has been successfully transformed into other plant species such as alfalfa and tomato, also yielding beneficial results of increased tolerance to salt and drought stresses (Bao *et al.* 2009; Gaxiola *et al.* 2011; Wang *et al.* 2016).

Although *AVP1* overexpression in *Arabidopsis* has been demonstrated to confer tolerance to various abiotic stresses, it has not yet been properly investigated with respect to anoxia. As *AVP1* in *Arabidopsis* is analogous to *OVP3* in rice, the question remains as to whether *AVP1* overexpression will also increase tolerance to anoxia by means of providing increased membrane integrity during an energy crisis. A pilot study by Liu *et al.* (2009) has yielded promising results in this respect, but requires further investigation.

1.7.4 Vacuolar pyrophosphatase transgenics in Arabidopsis

Arabidopsis plants overexpressing *AVP1* are overall very vigorous (Gaxiola *et al.* 2001). These overexpressers have larger leaves due to increased cell numbers, a greater number of leaves in their rosettes, and larger root systems (Li *et al.* 2005). *AVP1* overexpressers also retain more water and solutes than wild type plants, likely relating to their increased ability to withstand salinity and drought (Gaxiola *et al.* 2001). It appears that *AVP1* is also involved in a number of functions for plant growth and development, as knockout mutants with a deactivated *AVP1* gene showed altered morphological characteristics. The majority of cotyledons were heart-shaped, leaves were smaller due to reduced cell counts and root tips were deformed with unusually small cells (Li *et al.* 2005). Approximately one-third of these loss-of-function mutants initiated flowering, and of the flowers that formed none developed

normally (Li *et al.* 2005). *AVP1* is clearly important for healthy plant growth as well as a general tolerance strategy for a variety of stressors. However, the degree of tolerance that *Arabidopsis* overexpressing *AVP1* may have in low-oxygen conditions has yet to be determined.

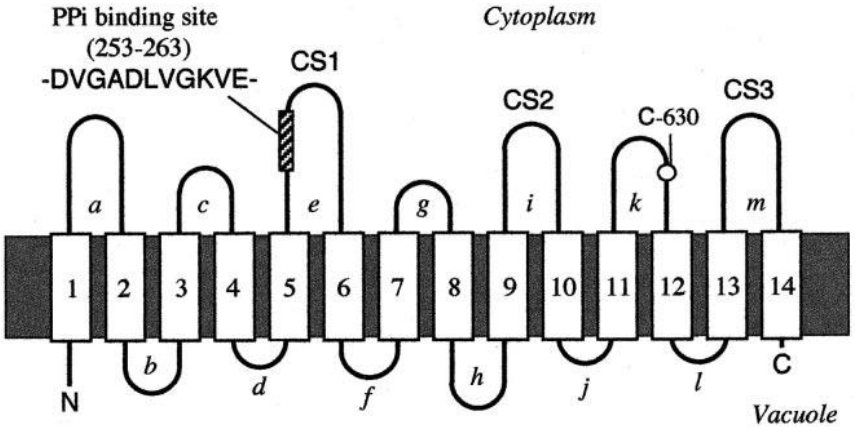

FIG. 1.3. Topological model of V-PPase from mung bean. Fourteen transmembrane domains are predicted, with the putative substrate- binding site located in loop e. Three conserved segments, CS1, CS2 and CS3 are labelled (Maeshima 2000).

1.8 Summary

Waterlogging and flooding of crops is a serious issue worldwide, as it results in a deficit of oxygen which inhibits oxidative phosphorylation, resulting in an energy crisis. In order to sustain their energy status, plants can sense low oxygen situations and alter their metabolism by expressing genes involved in anaerobic pathways such as fermentation, and switching off other non-vital processes. Plants must also preferentially allocate energy to three main processes: 1) synthesis of anaerobic proteins, 2) maintaining a pHstat and 3) maintaining transmembrane gradients. Another strategy to cope with reduced ATP is to use PPi as an alternative energy source. V-PPases are energised by PPi and are able to maintain vacuolar transmembrane electrical potential, even in anoxia.

1.9 Aims of this thesis

I plan to study *Arabidopsis thaliana* accession Columbia overexpressing *AVP1*, a gene that codes for a V-PPase which might become especially significant in conditions of low oxygen supply. I will determine the degree of tolerance that overexpression of a V-PPase will confer in *Arabidopsis,* a plant which is normally intolerant to anoxia. I will test this by subjecting 6 – and 7-day-old plants to complete anoxia in light and darkness, and will determine plant survival over a following two-week recovery period. I hypothesise that plants overexpressing *AVP1* will not only be likely to survive longer periods

of low-oxygen conditions than wild-type plants, but will also have more vigorous growth following a recovery period due to their enhanced ability to maintain membrane integrity. As *Arabidopsis* is a model plant, this study will yield valuable insights into how all plants may more efficiently cope with the stress of low-oxygen conditions.

2. MATERIALS AND METHODS

2.1 Plant Materials

2.1.1 Plants

Seeds of *Arabidopsis thaliana* acc. Columbia were generously donated by Professor Roberto Gaxiola (Arizona State University). Comparative studies were performed using the transgenic line AVP1-1 in which a tonoplastic vacuolar pyrophosphatase proton translocase was over-expressed. These constitutive overexpressers were made as previously described (Gaxiola *et al.* 2001). In brief, AVP1-1 transgenics were selected by using Agrobacterium-mediated transformation with an insert containing the open reading frame of *AVP1,* a tandem repeat of the 35S promotor and the polyadenylation signal and selection of transformed plants was based on resistance to kanamycin (Gaxiola *et al.* 2001). Plants were determined to contain two copies of the 35S-*AVP1* transgene and were selected for two generations to ensure homozygosity (Gaxiola *et al.* 2001).

2.1.2 Chlorine gas sterilization

Seeds were dry-sterilised using a chlorine gas method. Dry seed was placed inside 1.5-mL microcentrifuge tubes to a maximum volume of 4mm. Tubes were placed inside of a fume hood. A solution of concentrated sodium hypochlorite (NaClO) was prepared by dissolving 11g of pool chlorine (Poolbrite, containing 650g/kg available chlorine) in 50mL of water in a 100-mL glass beaker. The beaker was placed inside a glass desiccator beside the open Eppendorf tubes and 3mL of 1 N HCl was added to the NaOCl to release 14% active chlorine gas into the closed desiccator. Seeds were left exposed to the chlorine gas for 3 - 4 h. Eppendorf tubes containing sterile seeds were transferred to a flow bench where the lids were left open for approximately 30 min to allow any excess chlorine gas to evaporate.

2.1.3 Ethanol sterilization

All steps were performed in a sterile flow bench at room temperature. Sterile Whatman No. 1 filter papers were immersed in 100% ethanol, placed at the back of a sterile lamina flow hood to dry. Seeds were placed in 1.5-mL microcentrifuge tubes to a maximum volume of 200 µl. A wash solution containing 70% (v/v) ethanol and 0.05% Triton X-100 was prepared, and 1 mL was added to each microcentrifuge tube. Tubes were occasionally vortexed over the following 10 min, after which the wash solution was removed via pipette. Seeds were rinsed with 1 mL of 100% (v/v) ethanol for 5 min with occasional vortexing. The ethanol was removed via pipette and the rinse step repeated. After 5 min, seeds were

taken up along with a final rinse of 100% ethanol using a 1-mL pipette tip and spread onto a sterile filter paper where they dried over the next 10 min. Seeds were either directly sown onto plates or placed in sterile microcentrifuge tubes and stored at 4°C.

To screen for possible contamination, a subset of 10-12 seeds from each sterilised tube were plated in a sterile environment onto ½ strength Murashige and Skoog media containing 1% sucrose and 0.4% Phytagel (Sigma). Plates were wrapped around the edges with micropore tape and placed directly into the growth room. Contaminating organisms were visible after 4 d and any seed rows not displaying contamination were considered sterile.

2.1.4 Media, plating and growth conditions

Seeds were sown on sterile plates with media consisting of ½ Murashige and Skoog medium, 10% 5mM MES buffer, either 1% or 0.1% sucrose and 0.4% Phytagel was added prior to autoclaving. This medium was made by mixing 2.2 g of Murashige and Skoog Basal Medium (Sigma), 10 g of sucrose, 100 mL of 5 mM MES buffer (pH 5.6) into reverse osmosis water and diluted to 1 L. The mixture was then raised to a pH of 5.7 using 1 M KOH and 4 g of Phytagel was added to the mixture immediately prior to autoclaving. The sterile medium was then poured into 15 cm diameter petri dishes (Jet Biofil) inside a lamina flow hood to maintain sterility and left to cool for 1 h until solidified. Plates were left at room temperature for 2 d to ensure sterility before being placed for storage at 4°C or sown with seed.

Sterile seeds were placed onto the medium using a toothpick and sterilised Whatman No. 1 filter paper in the laminar flow hood. A total of 12 seeds were planted on each plate at 1 cm spacing, with half of the plate (6 seeds) containing wild-type Columbia seed and the other half containing AVP1-1 transgenic seed (Fig. S-1). Plates with seed were stratified in the dark at 4°C for 3 d. Following stratification, plates were stood upright on racks in a growth chamber at a constant 22°C under fluorescent cool white light (Philips, ~ 120 µmol m^{-2} s^{-1}) with a 16 h light / 8 h dark photoperiod.

2.1.5 Seed bulking

Seeds were sown into soil in 6 cm x 6 cm x 9.5 cm pots containing 1.5 g L^{-1} of soil with a slow-release fertiliser (Osmocote Exact Mini, Everris). Seeds were placed in a growth cabinet at a constant temperature of 23°C under cool-white fluorescent light (Philips, ~ 120 µmol m^{-2} s^{-1}) with a 16 h light / 8 h dark photoperiod. Pots were watered by placing them in 2 cm of standing water, which was allowed to dry out during early growth stages but kept full during the formation of seed pods. Watering was stopped once half of the seed pods turned yellow, and seed was harvested by hand after the entire plant was dry.

Harvested seeds were dried by placing them in open tubes next to silica gel within a sealed container for 2 weeks, and then stored at 4°C for future use.

2.2 Anaerobic Treatments

2.2.1 The anaerobic glove box

We modified a sealed glove box designed for microbiological work for anaerobic studies in the experiments reported here (Fig. 2.1). The box was placed inside of a growth room to ensure a constant temperature throughout the experiments. The anaerobic box contained two small holes for gas inlet and outlet tubes. The gas inlet tube was connected directly to a flow meter, which was in turn attached to a regulator on a G-size cylinder of high purity nitrogen gas (BOC, Sydney, Australia). The same side of the box also contained an isolation chamber, from which two doors could be opened and closed separately, one from the outside and the other from the inside. The front of the box was sealed by a perspex pane in order to see inside while preventing gas leakage and the inner chamber of the box was accessible through a pair of rubber gloves. Gaseous oxygen levels were monitored using a KXL-803 Oxygen meter (U4W6, China) which had been initially calibrated with a Clarke oxygen electrode (Rank Bros, UK).

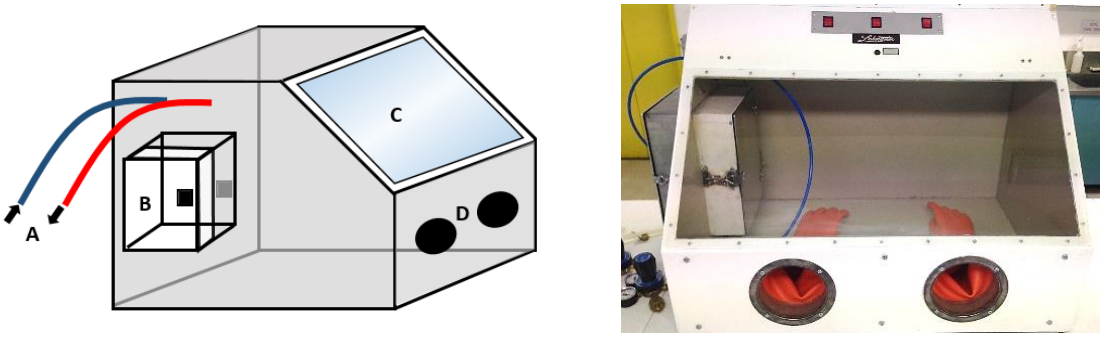

FIG. 2.1**.** Left: schematic diagram of the anaerobic glove box used for experiments. A) gas inlet tube (blue) and outlet tube (red); B) isolation chamber with separate sealing doors from the outside and inside; C) Perspex viewing pane; D) two arm-holes attached to rubber gloves on the inside of the box. Right: actual photo of the anaerobic glove box described.

2.2.2 Plant cultivation prior to treatment

Prior to undergoing an anaerobic treatment, plants grew to the two-leaf stage when roots were approximately 2 - 3 cm long; this enabled measurement of continuing root and leaf growth to be assessed.

Plants grown on 1% sucrose in the light were 7-d-old prior to treatment, but this was amended to 6 d for future treatments on this media because rapid root growth caused overcrowding after 7 d. Seven days' growth was appropriate for plants grown on 0.1% sucrose media due to slower growth rates (Fig. S-2).

2.2.3 Anaerobic treatments at 22°C

Prior to using the anaerobic box, it was flushed with high purity nitrogen (BOC Australia) for approximately 1 h at a flow rate >1 L min^{-1} until the oxygen meter registered complete anoxia. The flow rate was kept high while plates were introduced to the box, creating a positive pressure to ensure minimal oxygen contamination. Plates that were to enter the box had their micropore tape removed to facilitate gas exchange, and two small pieces of masking tape were placed on each side of the lid to ensure that the lid remained on top of the plate. This enabled oxygen inside the plates to diffuse out slowly as plants approached complete anaerobiosis. Plates were placed inside the isolation chamber and the door was sealed from the outside. The inner door of the isolation chamber was opened briefly to move plates into the main chamber, where they were placed upright in small wire racks and covered with a thick black cloth to prevent photosynthesis from occurring. Control treatment plates were wrapped with three layers of aluminium foil to maintain darkness and placed upright on the bench for the duration of the treatment. As some oxygen contamination inevitably occurred each time the inner isolation chamber door was opened, a half-hour grace period was given to plates just entering the inner chamber, thereafter they were considered to be fully anaerobic and the treatment time was started. Once the oxygen monitor read that the inner chamber was fully anaerobic (0.00% O_2), the nitrogen gas flow was reduced to 1 L min^{-1}. The oxygen monitor was checked periodically to ensure no oxygen contamination had occurred. Once an anaerobic treatment was complete, the nitrogen gas flow was increased >1 L min^{-1} to minimise oxygen contamination to plates that were to remain longer in the anaerobic box for subsequent sampling times. For a list of the number of plates sampled at each time point at a given sucrose concentration, refer to Table S-1. For anaerobic treatments in constant light, two red/blue bulbs were strung along the outside of the anaerobic box near the glass pane. Light intensity was measured around 250µmol m^{-1} s^{-1} using a light meter (LI-250A, LI-COR Inc., USA). For treatments requiring darkness, the red/blue bulbs were switched off, a thick black cloth was spread out over plates to prevent photosynthesis, and the front Perspex pane was covered with a layer of aluminium foil.

Upon completion of a treatment, the inner isolation chamber door was opened and completed treatment plates were placed inside. The inner door was then shut and the outer isolation chamber door opened to return plates to normoxia. Micropore tape was replaced around plate edges, and plates were placed back

upright onto the bench in the growth room. Aluminium foil was removed from control pates at this time and all plates were left to recover in normal growth conditions for two weeks. During this time, plates were observed every other day to collect data.

2.3 Survival, recovery rate and dry weight analyses

2.3.1 Survival and dry weight analyses

Upon return to normoxia, root tip positions of treatment and control plates were immediately scored on the backs of petri plates using a scalpel, and the number of leaves on each plant was recorded. Root survival was described as elongation of the primary root axis or the nearest lateral root beyond the length at return to normoxia and shoot survival was described as the ability to produce a new leaf beyond those present at the imposition of normoxia. Plates that became contaminated by fungus were excluded from the survival analyses if the contamination was determined to have an effect on their survival (i.e. reaching the plants before any new growth was evident). After two weeks of growth, roots and shoots were harvested for dry weight analysis as a measure of how well they recovered after anoxia. Contaminated plates were always excluded from this analysis, as well as any individual plants that may have begun growing only *after* the anaerobic treatment was complete. Roots and shoots were separated using a scalpel, and six plants of the same genotype on the same plate were bulked. Roots were briefly rinsed in water to remove any media, and roots and shoots were placed into labelled paper bags before being placed into a drying oven set at 60°C. Bags were left to dry for a minimum of 3 d before tissue was weighed.

2.3.2 Recovery rate analysis

Plates were recorded on alternate days for the elongation of roots or growth of new leaves, and the number of days required to produce the first new leaf or first root elongation past the initial root length was recorded as 'days to recovery'. When calculating the average days to recovery, plants which never recovered had a value of '0'. Average days to recovery of plates with low overall survival were therefore skewed to appear very low which was unrepresentative of what was observed, whereas removing all dead plants from the analysis resulted in an unbalanced design that no longer fit with the statistical model. Recovery was therefore expressed as a rate, calculated as 'days to recovery'$^{-1}$, which allowed plants that never recovered to be factored into this analysis and given an arbitrary value of 0.

2.4 PCR

2.4.1 DNA extraction

DNA was extracted using a CTAB method (Sanchez-Serrano and Salinas 2014). Tissue was snap-frozen in liquid nitrogen, and between 50 - 80 mg of tissue per sample was ground while frozen using a small pestle inside microcentrifuge tubes. A CTAB buffer was prepared by adding 100 mL Tris-HCl (pH 8.0), 280 mL of 5 M NaCl, 40 mL of 0.5 M EDTA (pH 8.0) and 20 g of cetyltrimethyl ammonium bromide (CTAB) into reverse osmosis water, increasing the final volume up to 1 L. A volume of 500 µL of CTAB buffer was added to each sample tube and left to incubate in a hot water bath set at 55°C for 1 h, mixing after 30 min. Following this, samples were removed from the water bath and into a fume hood, where 500 µL of isoamyl-chloroform was added to each tube and gently mixed by inverting 3 - 5 times. Tubes were centrifuged at 16 rcf for 7 min, after which the top aqueous phase was transferred via pipette to a new tube. To each volume of aqueous phase in the new tubes, cold 7.5 M ammonium acetate was added at 8% of this volume and cold isopropanol at 54% of the volume, both stored at 4°C.. Tubes were mixed thoroughly by inverting 20 - 30 times, and then placed in a -20°C freezer for 30 min. Tubes were then centrifuged at 16 rcf for 3 min and the supernatant was discarded, taking care not to dislodge the pellet. 700 µL of 70% (v/v) ethanol was added to each tube, mixed by inverting 5-10 times, centrifuged for 1 min at 16 rcf and then the supernatant was discarded. This last step was repeated once using 95% (v/v) ethanol. After removing the 95% ethanol supernatant, tubes were inverted onto a clean Kimwipe and left to dry for 15 min. Tubes were then placed upright with caps open, covered by a Kimwipe and left to dry for a further 45 min. DNA pellets were hydrated with 50 µL of nuclease-free water and left at room temperature to re-suspend overnight. Concentrations were analysed using a Nanodrop 2000c (Thermo Scientific). DNA samples were diluted 100 ng/µL working solutions for PCR and stored at -20°C for future use.

2.4.2 PCR conditions and primers

The presence or absence of the *AVP1* transgene was determined by PCR amplification using a primer pair spanning an intron specific to *AVP1*, denoted AVP1a, which amplified a band at 1031bp for genomic DNA and765bp for cDNA. All primer sequences and expected amplicon lengths can be viewed in Table 2.1. A set of primers specific to the 35S promotor were also used to confirm the presence of transgenes (Table 2.1). Reactions were run in volumes of 10 µL and the master mix consisted of 6.4 µL Millipore water, 2 µL MyTaq Red buffer (Bioline, Alexandria, New South Wales, Australia), 0.5 µL of 100 ng/µl DNA, 0.5 µl each of forward and reverse primer (10 µM) and 0.1 µL of MyTaq (Bioline, Alexandria,

New South Wales, Australia). The PCR conditions used were an initial denaturation phase of 95°C for 20 s, followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, elongation at 72°C for 30 s and a final elongation step of 72°C for 5 min. Gel electrophoresis using either a 1% or 2% (w/v) agarose gel with 5 µL/100mL of SYBR Safe (Invitrogen, Mt Waverley, Victoria, Australia) was used to visualise PCR products. Gels were visualised using a ChemiDoc MP Imaging System with Image Lab Software (Bio-Rad, Gladesville, NSW, Australia).

2.4.3 PCR product purification

PCR products used for standard curve dilutions were purified using a QIAquick® PCR purification kit (Qiagen Valencia, CA, USA) according to the manufacturer's instructions. PCR products destined for purification were run in a 25 µL PCR reaction, master mix consisted of 16 µL Millipore water, 5 µL MyTaq Red buffer (Bioline, Alexandria, New South Wales, Australia), 1.25 µL of 100 ng/µl DNA, 1.25 µL each of forward and reverse primer (10 µM) and 0.25 µL of MyTaq (Bioline, Alexandria, New South Wales, Australia)

2.5. Quantitative Reverse-Transcriptase PCR (qRT-PCR)

2.5.1 Tissue collection

A single sample tube was collected for root and shoot tissues of both genotypes at 0, 12, 24 and 36 h of anoxia in darkness with both 1% and 0.1% sucrose media. An additional three sample tubes were collected for tissues only at 24 h of anoxia in darkness grown in 0.1% sucrose media due to limited resources. Roots and shoots were separated inside the anaerobic box and tissues of a single genotype from a single plate were bulked together in 1.5 mL microcentrifuge tubes. Each tube was a pooled sample consisting of tissue from approximately 6 plants, which was sealed while inside the box and immediately dropped into liquid nitrogen upon entering normoxia. Tissues were stored in a -80°C freezer until further use.

Total RNA was extracted from shoots and roots using an Isolate II RNA Plant Kit (Bioline, Alexandria, New South Wales, Australia) according to the manufacturer's instructions. Tissues were disrupted while frozen using a Tissuelyser (Qiagen Valencia, CA, USA) and 300 µL of 1-mm diameter zirconia/silica beads (Daintree Scientific, Tasmania).

Tissuelyser parameters for shoot tissues were 20 s at a frequency of 25 s^{-1} and for root tissues were 30 s at a frequency of 30 s^{-1}. Changes made to the RNA extraction protocol include the following: the DNAseI

digest was left at room temperature for 25 min and for root tissues the final elution step was repeated using the same aliquot of water to improve RNA yield. RNA integrity was verified on a 1% (w/v) agarose gel containing 10 µL/100 mL SYBR Safe stain (Invitrogen, Mt Waverley, Victoria, Australia) (Fig. S-3). RNA was stored at -80 °C for further use. Concentrations of RNA were analysed using a Nanodrop 2000c (Thermo Scientific).

To test for DNA contamination, first the amount of RNA that would be used for each reverse-transcriptase reaction was calculated. The amount of RNA used was kept uniform across all samples as an additional measure of standardization, and this same concentration was used to test for DNA contamination in each sample. The largest amount of RNA that could be uniformly obtained from each sample was calculated by multiplying the smallest RNA concentration by the maximum volume of RNA that could be added to the reaction (12 µL). The required volume of RNA from all other samples were diluted with RNAse free water to achieve a final concentration of 264 pg in 12 µL of water to use with the reverse-transcriptase reaction. A PCR reaction was performed using diluted RNA in place of template DNA using the method described in section 2.4.2 using Elongation Factor 1-α (EF1α) primers. PCR product was run on a 1% (w/v) agarose gel to check for amplification. If no bands were visible, the RNA was considered free from DNA contamination.

TABLE 2.1. *PCR primers to confirm transformation of transgenics and qRT – PCR primers.*

Genes for PCR	**Primer Name**	**Sequence (5' – 3')**	**Amplicon Length (bp)**	**Source**
AVP1	AVP1a_F	AGA GTG TTG TCG CTA AGT G	1031 765*	Bao *et al.* (2009)
AVP1	AVP1a_R	CAG TGA AGT CGT GGT TGA T		Bao *et al.* (2009)
35S	35S_F	CCT ACA AAT GCC ATC ATT GCG	207	Wolf *et al.* (2000)
35S	35S_R	GGG TCT TGC GAA GGA TAG TG		Wolf *et al.* (2000)
Genes for qRT-- PCR	**Primer Name**	**Sequence (5' – 3')**	**Amplicon Length (bp)**	**Source**

Actin	Act_F	GGC GAT GAA GCT CAA TCC AAA CG	490 390*	Brini *et al.* (2007)
Actin	Act_R	GGT CAC GAC CAG CAA GAT CAA GAC G		Brini *et al.* (2007)
AVP1	AVP1b_F	GCC CTA GTC TCC TTG GCT CT	141	Undurraga *et al.* (2012)
AVP1	AVP1b_R	AGA GCT GCA CTT CCC ACA CT		Undurraga *et al.* (2012)
EF1α	EF1α_F	TCC AGC TAA GGG TGC C	264	Gutierrez *et al.* (2008)
EF1α	EF1α_R	GGT GGG TAC TCG GAG A		Gutierrez *et al.* (2008)

* *cDNA amplicon length*

2.5.2 cDNA synthesis

RNA was converted to DNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Mt Waverley, Victoria, Australia) according to the manufacturer's instructions. 264 pg of RNA from each sample was used in the reaction as described in section 2.5.1, which was incubated at 50°C for 45 min. Modifications to the protocol included the replacement of three components from the protocol: Oligo$(dT)_{23}$ primers at 50 µM were used instead of the Oligo$(dT)_{20}$ Primer (50 µM), dNTP Mix (Bioline, Alexandria, New South Wales, Australia) was used in place of the 10mM dNTP mix, and RNasin (Promega, Alexandria, Victoria, Australia) was used in place of RNase H. A negative RT reaction was conducted, whereby all reagents were used except the reverse transcriptase enzyme.

The quality of cDNA was determined using 1 µL of each cDNA sample in a PCR reaction as described above with Actin primers denoted Act (Table 2.1). These primers yield a 490 bp fragment from DNA and a 390 bp fragment from cDNA. The products were run on a 1% (w/v) agarose gel, and the presence of a single 390 bp band indicated that cDNA was being amplified. cDNA was stored at -20°C for further use (Fig. S-4).

2.5.3 Quantitative PCR analysis of transcript levels

Levels of *AVP1* mRNA were determined by quantitative PCR using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Gladesville, NSW, Australia). Standard curves were derived from a pooled cDNA sample consisting of cDNA from 8 different samples (4 from shoots and 4 from roots) which was used to make a dilution series covering effectively five orders of magnitude for *Ef1α* and four for *AVP1.* All primer sequences for qRT-PCR can be viewed in Table 2.1.

An optimal primer temperature was determined by performing a melt curve analysis by heating PCR products at the end of the amplification from 60 – 95 °C and monitoring fluorescence. In consideration with available time and resources, *EF1α* was used as a reference gene after two other common reference gene primer sets obtained from literature gave unreliable amplification. AVP1b primers were used to amplify the *AVP1* gene (Table 2.1). To measure transcript levels in tissues, each quantitative reaction was performed in triplicate 20 µL reaction volumes containing 10 µL Brilliant II SYBR© Green QPCR Master Mix (Qiagen, Valencia, CA, USA), 1 µL of each primer (10mM solution), 1 µL of undiluted cDNA and 8 µL of Nuclease-free water. The parameters for the qPCR were as follows: a denaturing phase of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and then 60°C for 30 s. Following qRT-PCR, amplified products were run on a 2% agarose gel to confirm the presence of an amplicon of the expected size (Fig. S-5).

2.5.4 Analysis of qRT-PCR Results

As a preliminary analysis of mRNA expression of *AVP1*, pooled samples were collected from *Arabidopsis thaliana* WT and a 35S::*AVP1* transgenic line overexpressing *AVP1* at 0, 12,, 24, and 36 h of anoxia in darkness on 1% and 0.1% medium sucrose as described above. A more detailed analysis was performed at 24 h for 0.1% sucrose medium, where 3 biological replicates were taken of pooled samples for each tissue at this time point.

Relative *AVP1* expression was analysed calibrated to *EF1α* as a reference gene. Raw data from qPCR was analysed using the C2Pe method using the C2Pe method (unpublished method, available in www.cienciapura.cl/qPCR/), and used as a reference a series of published methods that are part of the assumption free methods group (Pfaffl 2001; Liu and Saint 2002; Peirson *et al.* 2003; Ramakers *et al.* 2003; Rutledge 2004; Zhao and Fernald 2005; Guescini *et al.* 2008; Ruijter *et al.* 2009). Each PCR curve

was fitted into the model to calculate the Ct, taken into account the efficiency of the exponential phase and the reaction quality given by the linear phase**.**

To validate the use of the C2Pe method, the Relative Error (RE) was calculated from standard curve using the default Ct from the thermocycler and the Ct from the C2Pe method, and the RE values are shown in Table 2.2. C2Pe resulted in less error in the analysis, therefore this method was selected for the qPCR analysis.

$$RE = \frac{1}{n} \cdot \sum_{i=0}^{n} \left| \frac{O_i - E_i}{E_i} \right|$$

Where RE is the relative error, E is the expected Ct value using the known dilution concentration and O is the observed Ct value calculated from the standard curve method.

The C2Pe Ct value of each sample was used to calculate the relative concentration of initial mRNA using the standard curve method, then the relative concentration of *AVP*1 was normalised with *EF1a* as a reference gene for the AVP1-1 and WT plants.

2.6 Statistical analysis

Statistical analysis was conducted using R software (R, version 3.2.4) unless otherwise stated. The significance level for all analyses was 0.05 and all error bars represent the standard error of the mean. A Chi Squared test was used to analyse overall shoot survival on 1% sucrose media in the dark and for overall root and shoot survival on 0.1% sucrose media. A Fisher's Exact Test for Count Data was used for overall root analysis in 1% sucrose in darkness, and to compare data within treatments and within controls for all survival experiments. A two-way ANOVA was used for the recovery rate analysis, and a two-way ANOVA with a Tukey's HSD post hoc test was used for the dry weight analysis. A square root transformation was used for all dry weight data. A Student's t-test was used (Microsoft Excel, 2016) for percentage differences in dry weights between normoxic controls and for expression levels of roots and shoots at 24 h on 0.1% sucrose media.

TABLE 2.2. *Calculated relative error values for both default Ct and C2Pe for* AVP1 *and* EF1α *primers.*

	Default Ct	**C2Pe**
AVP1	0.906198315	0.644665513
EF1α	0.232486496	0.210626553
Total	0.60677084	0.451759308

3. RESULTS

3.1 Morphology

3.1.1 Effect of sucrose-free medium on growth of AVP1-1 and WT plants

To test the effect of sucrose-free medium on seedling development in normoxia, plants were initially grown on sucrose-free medium, repeating the conditions of the previous experiments of Ellis *et al.* (1999). Sucrose-free medium resulted in highly inconsistent growth in AVP1-1 and WT plants prior to anoxic treatment, with a small percentage of plants growing at a rapid rate while growth in the majority of seedlings arrested (Fig. 3.1A-D). When plants grew for longer periods, these differences became even more pronounced (Fig. 3.1E-F). This lead to an altered series of experiments new series of experiments using 0.1% and 1% sucrose (see Section 3.2.3 and 3.2.4).

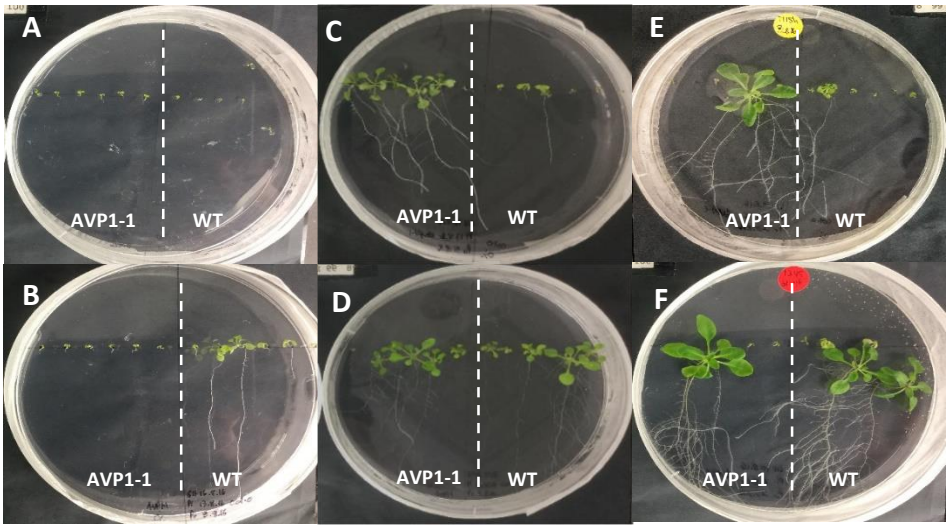

FIG. 3.1. *Arabidopsis* AVP1-1 transgenics and WT grown aerobically on plates with no exogenous sucrose, illustrating the variability of growth under normal conditions: (A-D) 14-d-old plants exhibiting a range of growth responses; (E, F) 20-d-old plants grown on sucrose-free medium.

3.1.2 Morphology in normoxia and following anoxia

When *Arabidopsis* was grown in soil under normoxic conditions, leaf area of AVP1-1 plants was substantially greater than that of WT plants (Fig. 3.2A). This was also observed for plants grown on plates with both 1% and 0.1% sucrose medium (Fig. 3.2B,C).

When plants of both genotypes were exposed to anoxia on phytagel plates, most existing root tips of both genotypes would cease to elongate. By contrast, lateral roots a few mm back from the primary root apex would often appear and continue elongating past the apex (Fig. 3.3A). These plants were considered to have survived anoxia: from a functional point of view, even though the primary root apex was dead, other lateral roots were initiated, allowing the plant to recover and resume normal growth and development. A slightly different response to anoxia was observed with shoots, particularly in low-sucrose medium. Leaf development was impaired in both genotypes; in place of a new leaf of normal shape and size, leaves that developed after anoxic treatment were smaller than their control counterparts, more numerous and sometimes became developmentally disorganised (Fig. 3.3B)

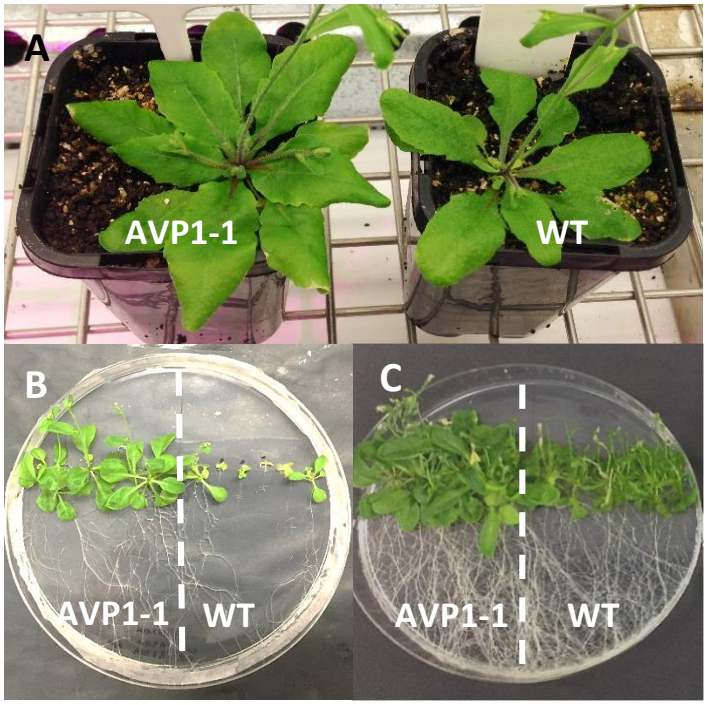

FIG. 3.2. Phenotype of AVP1-1 transgenics grown under normal conditions; (A) AVP1-1 (left) and WT (right) grown in soil displaying an increased leaf area for AVP1-1; (B) AVP1-1 and WT plants grown for 21 d on 0.1% sucrose media in normoxia; (C) AVP1-1 and WT plants grown for 32 days on 1% sucrose media. Larger leaves were evident on AVP1-1 plants under all conditions.

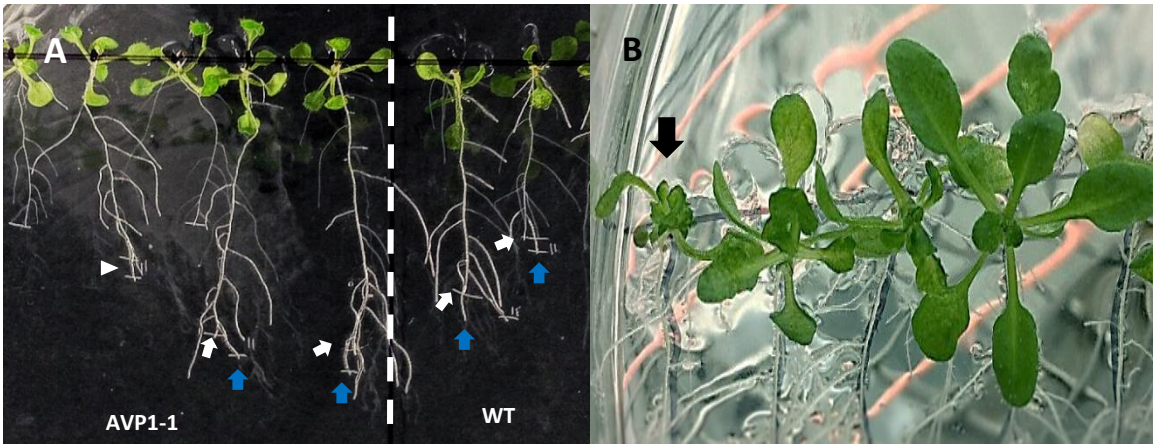

FIG. 3.3. Anoxically-treated roots and shoots displaying abnormal growth on plates containing 1% sucrose; (A) anoxically-treated roots, with white arrows indicating primary root apices which have failed to elongate following anoxia, blue arrows indicating the closest lateral roots which have elongated past the initial growth point, and a triangle indicating a primary root apex that has continued to elongate; (B) anoxically-treated shoots with the far left shoot, indicated by an arrow, displaying abnormal leaf growth.

3.2 Expression of the 35S::AVP1 insertion

3.2.1 Verification of the AVP1 *transgene insertion*

Both AVP1-1 and WT *Arabidopsis* were tested with PCR to confirm the presence or absence of the inserted 35S::*AVP1* construct. When tested with AVP1-specific primers (Table 2.1), AVP1-1 plants consistently showed a double band, the largest being between the 1000 and 1200 markers, and the smallest being slightly below the 800 marker (Fig. 3.1, lanes 1,2). These band sizes are consistent with expected sizes for genomic DNA and cDNA (1031 and 765 bp, respectively). When *AVP1* DNA was tested with primers specific to 35S, two band sizes appeared. The first was around 200 bp, consistent with the expected size of 207 bp and the second band was present around 600 bp, indicating that there may be a triple tandem repeat of the 35S promotor (Fig. 3.1 lanes 6,7). These data demonstrate that AVP1-1 plants are transgenic; they contain multiple copies of the 35S promotor, as well as both the genomic DNA and cDNA sequence for *AVP1*.

When cDNA from AVP1-1 plants was tested with AVP1a primers, only the predicted cDNA band was visible at 765 bp, and no band was visible when tested with 35S primers (Fig. 3.1 lanes 3,8). When testing WT DNA, a single large band consistent with the genomic DNA size appeared for primers specific to AVP1-1 (Fig. 3.1 lanes 4,5) and no bands appeared for 35S (Fig. 3.1 lanes 9,10). This demonstrates that

the WT did not contain any additional DNA for the *AVP1* gene, and that generation of cDNA from AVP1-1 plants was successful.

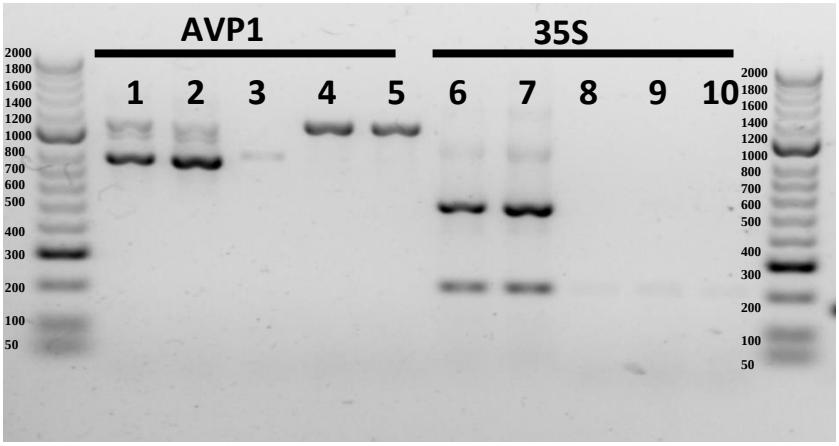

FIG. 3.1**.** Gel electrophoresis of PCR products from AVP1-1 and WT *Arabidopsis.* PCR products were amplified with primers specific to *AVP1* (lanes 1-5) and 35S (lanes 6-10) and run on a 1% (w/v) agarose gel with a 50bp Hyperladder (Bioline). DNA used with AVP1-specific primers includes AVP1-1 DNA (1, 2), AVP1-1 cDNA (3), and WT DNA (4, 5). DNA used with 35s-specific primers includes AVP1-1 DNA (6, 7), AVP1-1 cDNA (8) and WT DNA (9, 10). The primer pair used to amplify *AVP1* (AVP1a) spanned an intron, with an expected amplicon size of 1031 bp for genomic DNA and 765 bp for cDNA. The 35S primer set had an expected single amplicon size of 207 bp.

3.3 Quantitative expression of AVP1 in anoxia

3.3.1 Overview of expression in AVP1-1 transgenics

Transcript levels of *AVP1* mRNA in root and shoot tissues at varying times of anoxia were determined by qRT-PCR using primers specific to *AVP1*. Please note that all further results were normalised with a single reference gene, *EF1α,* therefore results may wish to be interpreted with a note of caution. Pooled samples consisting of either six shoots or six roots from a single plate were collected across 0, 12, 24 and 36 h of anoxia, both from 1% and 0.1% sucrose medium plates; although insufficient resources were available for biological replicates, three technical replicates were performed for each qPCR reaction. Coefficients of variation between the three technical replicates across all reported expression levels varied from 0.2 - 4.8 %. The degree of transgenic *AVP1* overexpression was analysed by comparing *AVP1* mRNA in AVP1-1 plants relative to *AVP1* mRNA in WT roots and shoots (Fig. 3.2). In normoxia (0 h), AVP1-1 roots in 1% and 0.1% sucrose medium had a 0.6- and 0.8-fold increase in expression relative to WT plants, respectively (Fig. 3.2A,C), whereas shoots in 0.1% sucrose medium had a 1.6-fold increase in *AVP1* expression relative to WT plants, respectively (Fig. 3.2D). This is similar to what

Gaxiola *et al.* (2001) found − a 1.6-fold increase in protein of AVP1-1 shoots was observed relative to WT shoots for plants grown in soil under normoxic conditions. With prolonged anoxia, *AVP1* expression in roots in 1% sucrose became approximately equivalent to that of WT plants, whereas in 0.1% sucrose, expression levels in AVP1-1 increased with longer times in anoxia, expressing as much as 3.6-fold more mRNA in 36 h than WT plants (Fig. 3.2C). Expression in shoots in 1% and 0.1% sucrose relative to WT behaved similarly, decreasing relative to WT shoots after 12 h anoxia but becoming near equivalent to WT expression levels by 36 h. Therefore, *AVP1* expression of the AVP1-1 transgenic is different to expression observed in WT plants, and both genotypes respond differently in different tissues and in different media sucrose levels.

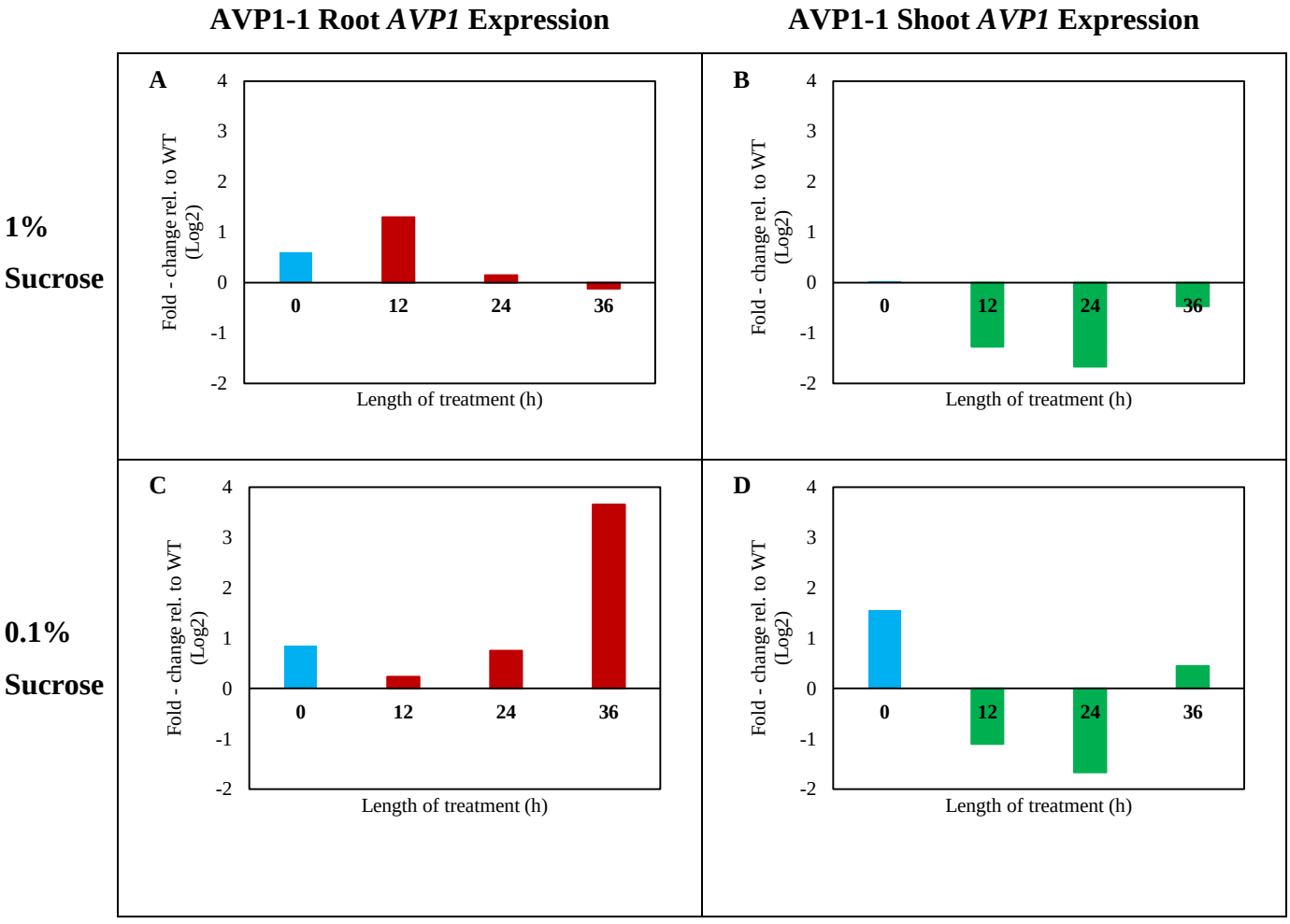

FIG. 3.2. Fold-change in expression of *AVP1* mRNA in AVP1-1 transgenics relative to WT plants in different tissues and medium, normalised to the reference gene EF1α. Figures show: 1% sucrose medium (A) roots and (B) shoots; 0.1% medium (C) roots and (D) shoots. Relative concentrations of *AVP1*

mRNA have undergone a log2 transformation to represent the fold change in expression. Blue bars represent relative expression in normoxia of AVP1-1 compared with WT plants.

3.3.2 Overview of AVP1 *expression across four times in anoxia*

Changes in *AVP1* expression over prolonged anoxia were examined for both AVP1-1 transgenics and WT plants relative to expression levels in normoxia (Fig. 3.3). Levels of expression in normoxia relative to the reference gene can be seen in Table S-2. Expression levels varied greatly between genotypes, times and particularly with sucrose supply. Both genotypes follow a similar trend for roots in 1% sucrose medium; by 12 h expression had fallen by 4- to 5.7-fold, then recovered by 2.6- to 4-fold for AVP1-1 and WT plants respectively by 36 h of anoxia (Fig. 3.3A,C - blue bars). Patterns of root expression of *AVP1* between both genotypes in 0.1% sucrose followed a similar trend to 1% sucrose, albeit with overall greater mRNA levels (Fig. 3.3A,C – red bars). *AVP1* expression of both genotypes increased rapidly on 0.1% sucrose over prolonged periods of anoxia, increasing between 12 and 36 h by 6.6- and 3.2-fold for AVP1-1 and WT, respectively.

Shoot *AVP1* expression was more strongly induced in normoxia than was root expression in 1% sucrose medium for both genotypes, with WT shoots exhibiting stronger overall expression in anoxia than AVP1-1 shoots (Fig. 3.3B,D – blue bars). However, shoot expression on 1% sucrose was not responsive to increasing anoxia to the degree that roots were, and decreased slightly over time (Fig. 3.3B,D – blue bars). Between normoxia and 12 h of anoxia, AVP1-1 shoot expression did not change whereas WT expression actually increased by 1.4-fold; however, from 12 – 36 h of anoxia, expression levels declined by 1.4- and 2.2- fold for AVP1-1 and WT plants, respectively (Fig. 3.3B,D – blue bars).

On 0.1% sucrose medium, both genotypes exhibited greater shoot expression over normoxic levels compared to 1% sucrose medium, and WT shoots on average had higher expression levels than WT roots (Fig. 3.3B,D – red bars). Shoot expression on 0.1% sucrose medium in anoxia was overall higher in WT plants compared with AVP1-1 transgenics, and had increased by 1.9-fold after 12h of anoxia compared with a decline of 0.79-fold during that same period observed for AVP1-1 transgenics (Fig. 3.3B,D – red bars). Expression changed by a total of 1- and 0.46- fold between 12 - 36 h of anoxia for AVP1-1 and WT plants, respectively, demonstrating that shoot expression overall on 0.1% sucrose was even less responsive to anoxia over time than on 1% sucrose medium (Fig. 3.3B,D – red bars).

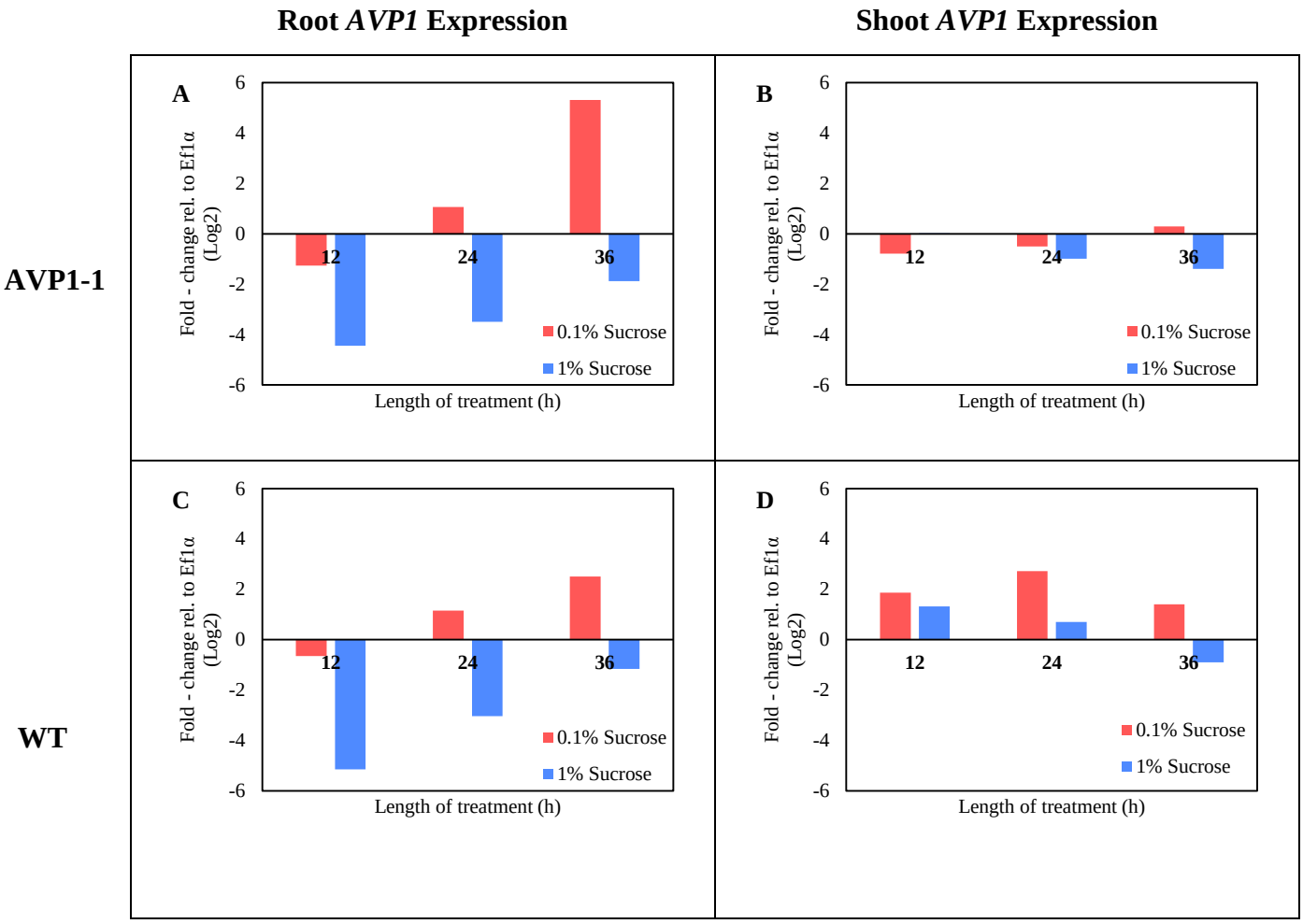

FIG. 3.3. Expression of *AVP1* mRNA relative to normoxia expression levels across times in anoxia in darkness for both AVP1-1 transgenics and WT plants, normalised to *EF1α*; (A) AVP1-1 roots (B) AVP1-1 shoots; (C) WT roots; (D) WT shoots. The log 2 - transformed value of expression levels in normoxia have been subtracted from the log 2 - transformed expression at each time point. Coefficients of variation across all expression levels varied between 0.2 – 4.8%.

3.3.3 Detailed expression at 24 h of anoxia on 0.1% sucrose medium

A detailed analysis of *AVP1* expression in pooled samples of roots and shoots after 24 h of anoxia was performed on three biological replicates growing on 0.1% sucrose medium (Fig. 3.4). When relative *AVP1* expression levels were compared between AVP1-1 transgenic and WT plants, no statistically significant differences were found in *AVP1* expression ($p = 0.25$). After 24 h anoxia, relative to *EF1α,* average root expression of *AVP1* was 33% higher than expression in WT plants, whereas shoot expression of *AVP1* was 26% higher than WT plants, respectively.

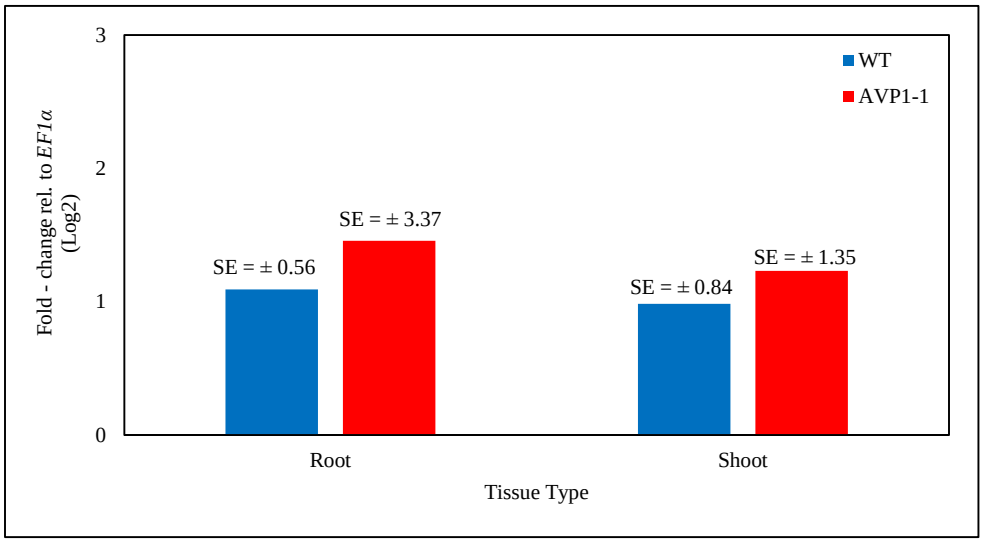

FIG. 3.4. Comparison of expression of *AVP1* relative to *EF1α* for WT and AVP1-1 plants at 24 h of anoxia grown on 0.1% sucrose medium. Three biological replicates were sampled for qPCR analysis, with three technical replicates repeated for each sample. SE values are displayed above each bar. Genotypic differences between relative *AVP1* expression were non-significant (t-test, $p = 0.25$).

3.4 Survival after anoxic shock

3.4.1 Justification for adoption of darkness for survival studies

A series of anaerobic treatments involving varying lengths of time in anoxia, light and medium sucrose concentrations were performed in order to test the tolerance of both WT and AVP1-1 overexpression plants to an energy crisis. An initial experiment was performed on 7-d-old plants grown on 1% sucrose medium with constant light while in anoxia. Most plants of both genotypes survived, with no significance difference between the survival roots ($p > 0.5$) and shoots ($p = 1$) for AVP1-1 and WT plants (Fig. 3.5A). To impose strict anoxia in further treatments, plants were kept in constant darkness during the anaerobic treatment to prevent the generation of gaseous oxygen from photosynthesis. Normoxic control plants were always grown on plates in constant darkness for the duration of the anoxic treatment.

3.4.2 Comparison of genotypic differences on root and shoot survival

In order to analyse genotypic-specific variations in response to anoxia, the percent increase in survival of AVP1-1 compared with WT plants was calculated as survival rates for (AVP1-1 - WT) / (WT) (Fig. 3.5). There was no significant difference ($p > 0.5$) between root and shoot responses on 1% sucrose medium in light (Fig. 3.5A). However, significant differences were evident for shoots on both 1% and

0.1% sucrose medium in darkness ($p = 0.006$ and 0.001, respectively) (Fig. 3.5B,C). On 1% sucrose medium in the dark between 12 – 27 h of anoxia, AVP1-1 shoots survived 30% better than WT plants, whereas on 0.1% sucrose medium AVP1-1 transgenics survival rate improved by 500% and 420% after 24 and 30 h of anoxia, respectively. Root survival was also significantly enhanced for AVP1-1 transgenics in anoxia treatments in darkness, particularly with 0.1% sucrose medium ($p = 0.001$), where root survival was 17 - 92% greater than in WT plants between 24 - 42 h anoxia. Overexpression of AVP1-1 displays a clear advantage in survival of both roots and shoots, and these gains more evident with increased severity of anoxia treatment conditions. Survival percentages of each genotype plotted over time can be seen in Figure S-6.

3.4.3 Root survival on 1% and 0.1% sucrose media after anoxic treatment

Having considered the effect of AVP1-1 on survival after anoxia in Fig. 3.5, it became apparent that the interaction between exogenous sucrose and duration of anoxia was significant and distinct for roots and shoots. Therefore, combined effects in both genotypes are shown in Fig. 3.5. Please refer to figure S-6 to see survival of both genotypes separately. For all treatments in darkness, normoxic controls displayed consistent survival rates of around 100% for both roots and shoots, which were significantly different from anoxically-treated roots and shoots in all instances ($p < 0.001$) (Table S-3). Therefore, normoxic controls are not shown in Fig. 3.5.

In the presence of light, 1% sucrose medium treatments up to 24 h resulted in root survival rates above 80%; this result was repeated with 1% sucrose medium in darkness for up to 24 h (Fig. 3.5A, C). Throughout anoxia in darkness on 1% sucrose medium, overall root survival remained high; the highest average mortality rate was 57% after 30 h of anoxia in darkness (Fig. 3.5C). Surprisingly, after a 33-h anoxic treatment, survival rates were > 80%, while a 36-h exposure to anoxia further reduced survival. Overall, roots appeared to be more resilient to anoxic stress in 1% than 0.1% sucrose medium, regardless of light or darkness; after 36 h of anoxia in darkness, survival was still greater than 60% (Fig. 3.5C).

To test the interaction of sucrose supply with the energy crisis caused by anoxia, plants were grown on medium containing 0.1% sucrose and kept in the dark during the anoxic treatment (Fig. 3.5C). Root survival in 0.1% sucrose medium showed a marked decrease in comparison with 1% sucrose medium, indicating that added sucrose prolonged survival during an energy crisis (Fig. 3.5C,E). This difference in survival is particularly clear between 18 - 24 h. Interestingly, a distinct peak in survival rates was observed at both sucrose levels, either at 33 h (1% sucrose) and later at 42 h (0.1% sucrose). Very few plants survived the 48-h anoxic treatment in 0.1% sucrose medium.

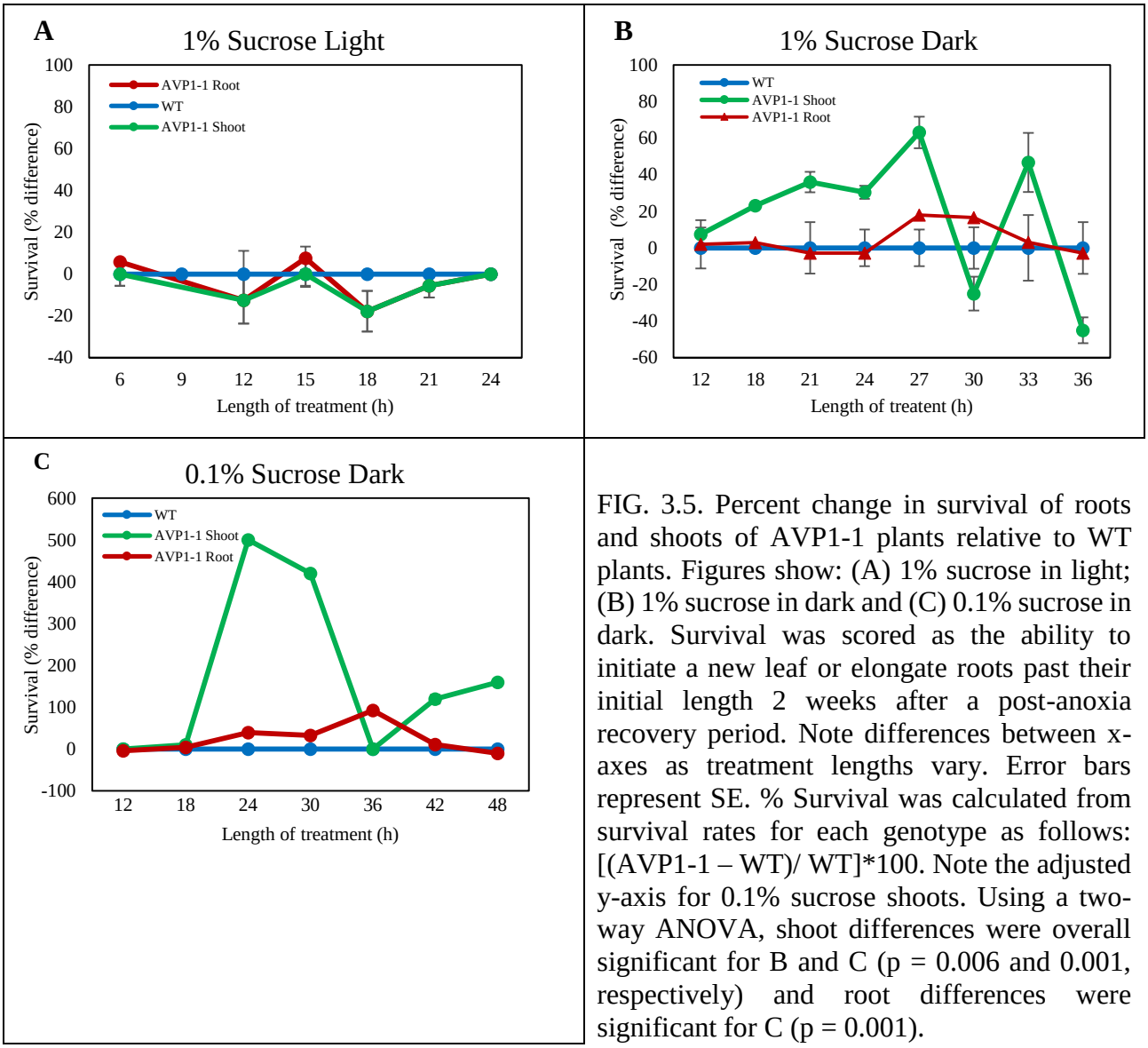

FIG. 3.5. Percent change in survival of roots and shoots of AVP1-1 plants relative to WT plants. Figures show: (A) 1% sucrose in light; (B) 1% sucrose in dark and (C) 0.1% sucrose in dark. Survival was scored as the ability to initiate a new leaf or elongate roots past their initial length 2 weeks after a post-anoxia recovery period. Note differences between x-axes as treatment lengths vary. Error bars represent SE. % Survival was calculated from survival rates for each genotype as follows: [(AVP1-1 – WT)/ WT]*100. Note the adjusted y-axis for 0.1% sucrose shoots. Using a two-way ANOVA, shoot differences were overall significant for B and C ($p = 0.006$ and 0.001, respectively) and root differences were significant for C ($p = 0.001$).

3.4.4 Survival of shoots in 1% and 0.1% medium

Overall, roots were more tolerant to anoxia than shoots in all treatments except 1% sucrose in light, where shoot survival appears equal to that of root survival (Fig. 3.5A, B). However, where equivalent survival in anoxia was observed in 1% sucrose medium for roots up to 24 h in both light and darkness, shoot survival in darkness had already begun to decline, indicating that darkness in anoxia had a more negative impact on shoots than on roots (Fig. 3.6B,D).

Shoots were very sensitive to the levels of sucrose in the media, as shoot survival suffered severely on 0.1% sucrose medium (Fig. 3.6F). After 12-h of anoxia, shoot survival in 1% sucrose medium was 75%, whereas on 0.1% sucrose media shoot survival had already fallen to approximately 50% (Fig. 3.6D,F).

At 24 h, the difference was even more severe, as survival of plants grown in 1% sucrose media was above 60%, whereas survival of plants in 0.1% sucrose media was less than 20% (Fig. 3.6D,F). An example of this can be seen in Figure S-7. As observed with roots, survival improved briefly by 42 h but dropped by 48 h (Fig. 3.6D,F).

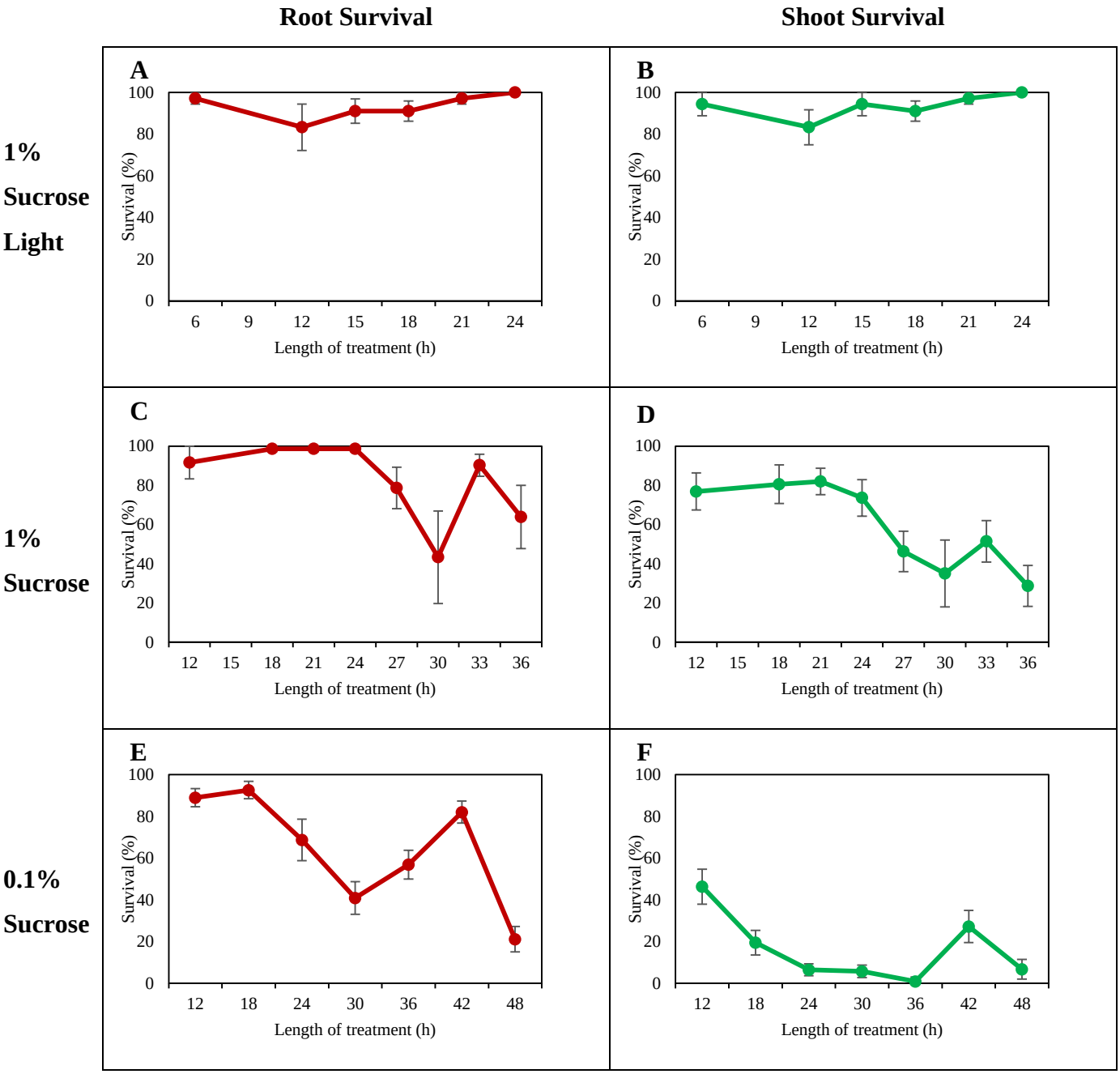

FIG 3.6. Survival percentages after various periods of treatment in anoxia for roots and shoots, averaged between AVP1-1 and WT. Figures show: 1% sucrose for plants treated in light for (A) roots and (B) shoots; 1% sucrose for plants treated in darkness for (C) roots and (D) shoots; and 0.1% sucrose for plants treated in darkness for (E) roots and (F) shoots. Survival was scored as the ability to initiate a new leaf or elongate roots past their initial length 2 weeks after the anoxic treatment. Note differences between x-axes as treatment lengths vary. Error bars represent SE averaged between both genotypes.

3.5 Biomass responses to anoxic treatment

3.5.1 Exogenous effects of sucrose on dry weight in normoxia

As a measure of how rapidly plants were able to recover and resume growth following anoxia, dry weights of root and shoot tissues were measured after a recovery period of two weeks. As a basic measure of the impact of a ten-fold reduction in sucrose medium on growth, the means and percent decrease between dry weights of normoxic controls on 1% and 0.1% sucrose medium were compared (Table 3.1). Mean dry weights of both roots and shoots grown on 0.1% sucrose media in normoxia were significantly smaller than mean dry weights of plants on grown on 1% sucrose in normoxia ($p < 0.001$ for all genotypes and tissues). Mean reductions in dry weight ranged from a minimum of 61% for AVP1-1 shoots up to 86% for WT roots (Table 3.1). There were no significant differences in responses of shoots compared with roots, nor were there any differences with respect to genotype. This indicated that a reduction in medium sucrose level effectively resulted in a reduction of biomass accumulation by more than 60% in all tissues. All means and percent differences discussed in this section can be viewed in Table S-2**.**

TABLE 3.1. *Mean dry weights of AVP1-1 and WT roots and shoots anoxically-treated on 1% sucrose and 0.1% sucrose. Dry weights for each genotype and media sucrose level were averaged across time points 12, 18, 21, 24, 30, and 36 h of anoxia, and rounded to two decimal places. Percentage decrease on 0.1% sucrose medium is relative to the corresponding tissue in 1% sucrose medium.*

Genotype	Medium sucrose	Root weight average (mg)	Shoot weight average (mg)	Root % decrease	Shoot % decrease
AVP1-1	1% / dark	2.35	6.65	-	-
WT	1% / dark	1.70	5.60	-	-
AVP1-1	0.1% / dark	0.54	2.60	77	61
WT	0.1% / dark	0.24	1.25	86	78

3.5.2 Dry weight of plants on 1% sucrose medium after a period of anoxia in light

When plants were exposed to anoxia on a 1% sucrose medium in the light, they were one day older than 1% sucrose treatments in the dark, and were left to recover for 15 d in a slightly warmer growth room than the other treatments (24°C). However, no significant differences in dry weight between WT and AVP1-1 were observed for either root or shoot tissues, with no other significant interactions present (Fig. 3.6A-B). This shows that these conditions were not stringent enough to elicit any effect of anoxia on genotype-specific responses in dry weight, presumably because of photosynthetic oxygen generation.

3.5.3 Dry weight of roots in 1% and 0.1% sucrose medium in darkness

When darkness was imposed during anoxia for plants grown on 1% and 0.1% sucrose, several levels of significance were observed for root dry weights (Fig. 3.6C,E). Root dry weights in both conditions were significantly depressed after anoxia compared with normoxia. Additionally, the duration of exposure to anoxia had a highly significant effect, with both these factors having a p-value of < 0.001 for each treatment. There was also a significant duration × anoxic treatment interaction ($p < 0.001$), demonstrating the increasing impact of anoxia on root growth over time. For anoxic treatments in 1% and 0.1% sucrose in darkness, there was a significant effect of genotype (both $p = 0.001$), and a significant genotype × anoxic treatment interaction ($p = 0.02$ and 0.001, respectively). This indicated that individual time points had to be examined to determine whether significance could be attributed to differences between genotypes in normoxic controls or anoxic treatments.

After ≤ 30 h in anoxia on 1% sucrose medium, p-values for the genotypic contrast in normoxia were as low as 0.05, whereas p-values for anoxic treatments always exceeded 0.38, indicating that genotype-specific significance arose from differences between normoxic controls from 12 – 30 h (Fig. 3.6C). In 0.1% sucrose medium, p-values were lower in normoxic controls compared with plants exposed to anoxia ($p = 0.07 – 0.5$ and $p = 0.86 – 1$, respectively), indicating again that genotype effects were present in the normoxic controls only (Fig. 3.6E).

3.5.4 Dry weight of shoots in 1% and 0.1% sucrose medium

Shoot dry weights for plants grown on 1% sucrose medium in darkness responded similarly to roots under the same conditions, with highly significant effects of being treated with anoxia *vs* normoxia, the length of time, and a significant duration × treatment interaction (all $p < 0.001$) (Fig 3.7). For shoots in 1% sucrose, however, there was a mildly significant effect of genotype ($p = 0.049$) and a genotype × treatment interaction that was borderline significant ($p = 0.055$) (Fig 3.7D). Both of these factors were highly significant for shoots in 0.1% sucrose (Fig 3.7F). Individual time points were examined for anoxic treatments *vs* normoxic controls to determine which treatment elicited significantly different genotypic responses.

At 1% sucrose, all genotypic effects in shoots were the result of the first 24 h of darkness. The range of p-values in normoxic controls was lower than in anoxic treatments up to 24 h ($p = 0.051$ - 0.12 and $p =$ 0.8 - 0.99, respectively), after which there were no significant genotype effects. This indicated that the significant effect of genotype over the entire 24-h period could best be ascribed to the plants in normoxia.

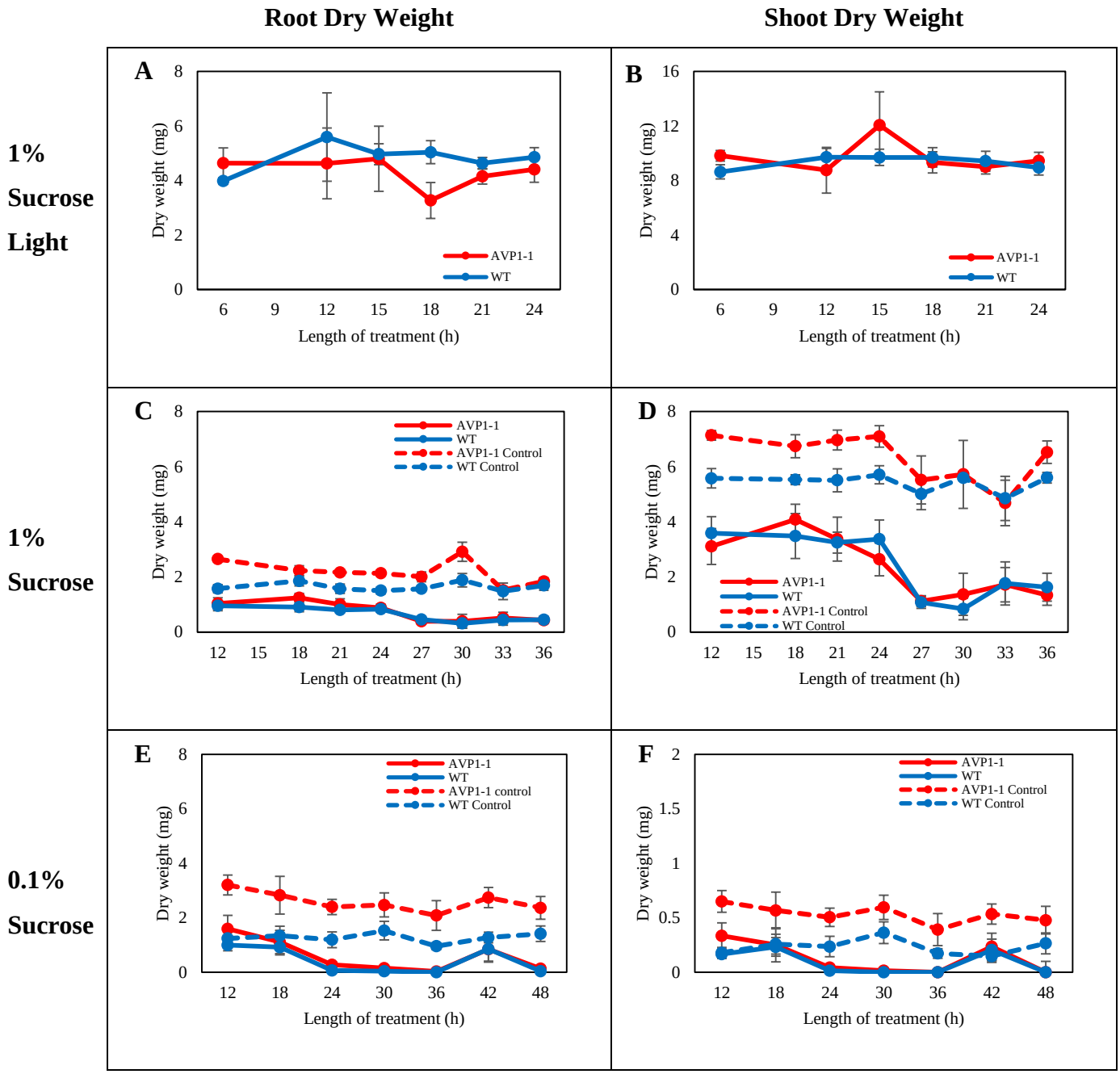

FIG. 3.7. Mean dry weight of roots and shoots over time for both AVP1-1 and WT. Figures show: 1% sucrose in light for (A) roots and (B) shoots; 1% sucrose in darkness for (C) roots and (D) shoots; and 0.1% sucrose in darkness for (E) roots and (F) shoots. Plants recovered in normoxia for 14 d (aside from 1% sucrose in light, which had a 15-d recovery). Once treatments were complete, dry weights of shoots and roots were averaged for all six plants per genotype from each plate. Notice differences between x-axes as treatment times differed, and note the y-axis range for 1% sucrose shoots in light (B) and 0.1% sucrose roots in darkness (F). Error bars represent SE. Using a two-way ANOVA with a Tukey's HSD, significant difference between genotypes (all $p < 0.05$) were observed in controls up to 30 h of darkness (C), up to 24 h of darkness (D), and across all time points (E, F).

Therefore, for roots and shoots under both media sucrose conditions in darkness, the AVP1-1 phenotype enhanced dry weight only in normoxia. In 0.1% sucrose medium across all times in anoxia (up to 48 h), p-values for genotypic differences within normoxic controls were lower than in anoxic treatments ($p = 0.014 - 0.27$ *vs* 0.2 - 0.99, respectively), again indicating that any genotypic differences observed were from the normoxic control group only.

3.6 Rate of Recovery

3.6.1 Recovery from darkness in normoxia

Recovery rates were represented as the inverse of days to recovery (units of d^{-1}; Allen, pers. comm.). This analysis was performed for treatments in darkness only. Plants in continuous normoxia recovered from a darkness period within 2 d, regardless of sucrose level. Therefore, normoxic control data were excluded from these figures. All average recovery rates across all times and treatments can be seen in Table S-3.

3.6.2 Effects of AVP1-1 overexpression on recovery

The highly significant factors that slowed the recovery rate for roots and shoots were: (i) exposure to anoxia ($p < 0.01$ for roots and $p < 0.001$ for shoots) and (ii) the length of the anoxic treatment ($p < 0.001$ for both tissues). There was also a highly significant interaction between time and treatment ($p < 0.001$ for both tissues). There were no significant differences on recovery rates for roots between AVP1-1 and WT plants for roots grown on both 1% and 0.1% sucrose (Fig. 3.8A,B respectively).

However, shoots of AVP1-1 grown on 1% and 0.1% sucrose medium recovered faster after anoxic treatments ($p = 0.001$ and 0.049 for plants on 1% and 0.1% sucrose medium, respectively), depicted in green and red in Figure 3.8. For example, in 1% sucrose medium in darkness, shoots of AVP1-1 recovered faster than WT plants (~36%) between 12 - 27 h of anoxia (Fig. 3.8A). In 0.1% sucrose medium at 24 h and 30 h of anoxia, AVP1-1 plants had 140% and 660% greater recovery rates compared with WT plants. These significant genotypic interactions were also significant for normoxic controls, where AVP1-1 shoots recovered marginally faster than WT plants (Fig. S-8B,D).

When recovery rates were plotted over time, it appears that anoxia had a greater negative impact on roots growing on 1% sucrose (Fig. S-8A,C); by 36 h, roots in 1% sucrose recovered at a rate of 0.2 d^{-1}, while plants grown on 0.1% sucrose media recovered at 0.35 d^{-1}.

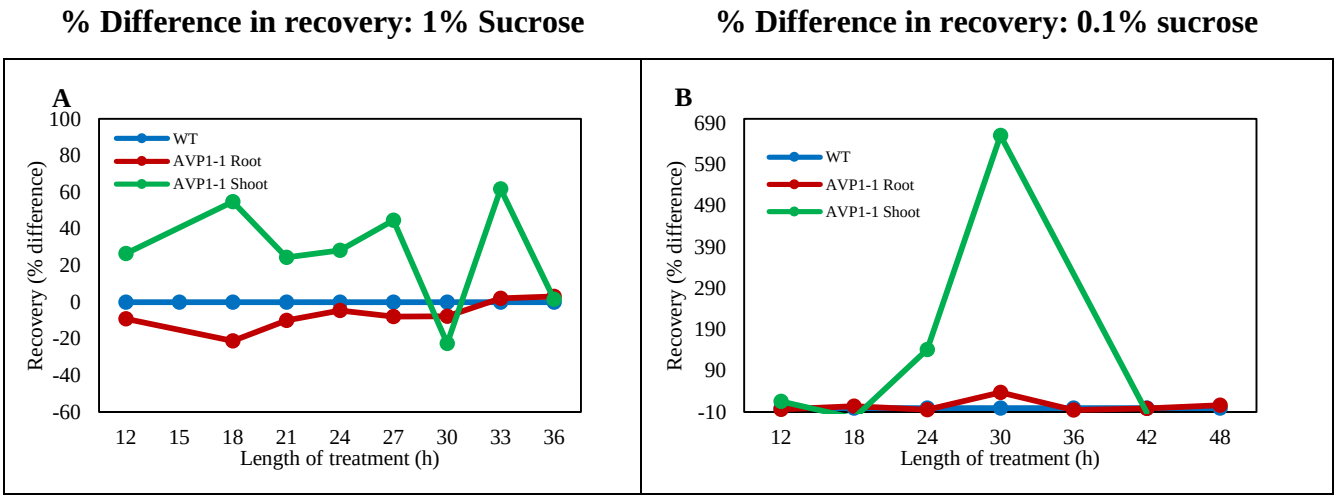

FIG. 3.8. Recovery rate of AVP1-1 tissues relative to WT in darkness. Figures show: (A) 1% sucrose and (B) 0.1% sucrose medium. Recovery rate was measured as the inverse of the first day that plants produced a new leaf and the first day that roots elongated past their initial length after anoxia. Note differences in x-axes as treatment lengths vary, and in y-axes. Error bars represent SE. % recovery was calculated from recovery rates for each tissue and genotype as follows: [(AVP1-1 – WT)/ WT]*100. Using a two-way ANOVA, significant differences were observed for shoots on 1% ($p = 0.001$) and 0.1% sucrose medium ($p = 0.049$).

4. DISCUSSION

4.1 Rationale behind the use of Arabidopsis and the survival analysis

Arabidopsis was chosen for this analysis for two major reasons: is a relatively flood-intolerant plant, and so overexpressing a gene that confers flood tolerance should show a distinct phenotype when tested under appropriate conditions, and its intolerance to flooding is shared with most of the world's important agricultural crops (Setter and Waters 2003; Vashisht *et al.* 2011; Kole 2013). This results in making any tolerance mechanisms of broad relevance. Moreover, *Arabidopsis* has a single isoform of the *AVP1* gene, which codes for a tonoplastic proton transporter energised by pyrophosphate so it is only necessary to manipulate a single gene to study its phenotype. The *AVP1* gene in *Arabidopsis* has already been proven to be effective in increasing tolerance to other abiotic stressors such as salinity and drought, but has not previously been formally linked to tolerance in anoxia (Gaxiola *et al.* 2001, 2012; Li *et al.* 2005). Therefore, an *Arabidopsis* transgenic that overexpresses *AVP1* (AVP1-1) was tested in a series of anaerobic treatments to study what effects this gene may have on survival and growth during an energy crisis.

The main cause of tissue death in anoxia is depolarisation of the vacuolar membranes. By translocating protons into the vacuole, *AVP1* allows membrane charge to be maintained using PPi as an alternative energy source to ATP. Hence, the focus of this study was to determine whether overexpression of *AVP1* would maintain membrane integrity after periods of anoxia up to 48 h. Consistent with the role of *AVP1*, survival was the main focus of this study. If *AVP1* were a critical gene in an energy crisis, survival, growth and recovery post-anoxia should have been influenced by its expression.

Responses of *AVP1*-overexpressing plants compared with WT plants were deliberately studied in a series of severe energy shocks, where plants were exposed to anoxia for various times up to two days then returned to air (normoxia). Because *Arabidopsis* becomes dormant in anoxia and is susceptible to tissue death, physiological observations at the end of each anoxic treatment do not reveal the extent of damage. Therefore, plants were subjected to a period of normoxia to assess the scale of damage in anoxia. In other words, survival is not evident in *Arabidopsis* immediately post-anoxia because plants become quiescent, conserving energy and only recovering over time if damage is non-lethal (Ellis *et al.* 1999; Van Dongen *et al.* 2009; Vashisht *et al.* 2011). Therefore, replicating the approach of Ellis *et al.* (1999) and Vashisht

et al. (2011), plants were given a two-week window in which to recover and resume growth following anoxia. Plants that failed to recover were scored as dead.

4.2 Survival of Arabidopsis plants after a period of anoxia

4.2.1 Comparison of root and shoot survival

Survival rates in roots and shoots diverged significantly. Root survival proved generally resilient to anoxia when compared with shoots, particularly on 1% sucrose medium. Surprisingly, mortality of a substantial proportion of roots (> 20%) was only evident after about 24 h of anoxia in either sucrose level. While root apices and the stele are especially vulnerable to low-oxygen conditions (Fig. 2.3A; Gibbs & Greenway, 2003), even in *Arabidopsis* root tissues generally appeared to be remarkably tolerant to O_2 deprivation. Shoots proved to be more susceptible to anoxia than roots. However, almost all shoots survived in the presence of light, proving that trace amounts of free O_2 generated from photosynthesis were sufficient to make the atmosphere hypoxic and ensure cell survival. When *Arabidopsis* plants were completely submerged, plants survived much longer when in light than in darkness (Vashisht *et al.* 2011). Once darkness was imposed under 24 h anoxia, shoots immediately suffered an increased mortality rate of 20% while roots were completely unaffected.

4.2.2 Comparison with survival rates reported in previous research

The most similar study on effects of anoxia in *Arabidopsis* were performed by Ellis *et al*. (1999), where three-week-old WT plants were transplanted into liquid sucrose-free medium then immediately exposed to anoxia in darkness. Under these shock conditions, survival rates in anoxia decreased rapidly from 100% at 6 h of anoxia to complete mortality by 24 h for roots and at 36 h for shoots (Ellis *et al.* 1999), which is much higher than what was observed in current experiments. The high mortality likely contrasts with the current experiments because of the methodology used. The discrepancy between these two related studies on WT *Arabidopsis* must be explained. It can be ascribed to four factors as follows:

(a) the definition of root survival - in our experiments, elongated lateral roots were counted as root survival whereas Ellis *et al.* (1999) examined only the primary root apex. Lateral roots were observed in their experiment to have survived 0.1% O_2 for up to 48 h, suggesting that the entire root system might not perish with the primary root apex. No data were reported for anoxic roots;

(b) different media – in Ellis *et al*. (1999), plants were grown for three weeks in a medium completely devoid of sucrose, whereas we established that a sucrose-free medium on plates led to very uneven growth (Fig. 3.1). Because we show that survival after anoxia was dependent on the level

of sucrose, we presume that the carbohydrate-starved plants used by Ellis *et al.* (1999) might have been especially vulnerable to anoxia;

(c) severity of anoxic shock - in the system we used, lids of the phytagel plates were left on to prevent contamination and desiccation while inside of the anaerobic box. Furthermore, the anaerobic box took a finite time (~ 20 min) to reach anoxia after nitrogen flushing. Because this slowed the decline from normoxia to anoxia around the plants (see Materials and Methods), it afforded an acclimation period of what we estimate to be 30 min. In Ellis *et al.* (1999), anoxia appears to have been imposed instantaneously by argon flushing;

(d) Lastly, but most importantly, plants in Ellis *et al.* (1999) had suffered from transplant shock immediately prior to treatment, which could have a large impact on mortality especially when combined with anoxic shock. Plants in our experiments were never transplanted; any damage occurring from treatments was purely from anoxia.

4.3. Effects of AVP1-1 on survival, growth and recovery after anoxia

4.3.1 Effects of AVP1-1 phenotype on survival of shoots and roots

The *AVP1* gene proved to be effective in prolonging survival after the anoxia treatment, especially when survival rates for WT plants dropped to the range of 50 - 70%. This meant that genotype differences in shoot survival for AVP1-1 overexpressers were already evident after 18 h of anoxia in 1% sucrose medium in darkness, but were not observed in roots until they had undergone more than 24 h of anoxia in 0.1% sucrose medium. By the time genotypic differences were evident in roots, shoot survival had already fallen below 20%. The AVP1-1 genotype enhanced survival most graphically after the anoxia treatment had induced a threshold level of damage.

Despite the fact that AVP1-1 overexpressers had enhanced root and shoot survival rates compared with WT plants after various periods of anoxia, WT shoots had paradoxically higher levels of expression of *AVP1* (green bars - Fig. 3.2); this indicates that *AVP1* expression in *shoots alone* is not sufficient to ensure survival after an anoxic treatment. Therefore, we postulate that root expression of *AVP1* is essential for plants to survive O_2 deprivation. Survival after anoxia and *AVP1* expression in roots are consistent with this hypothesis, particularly in 0.1% sucrose medium where the survival of AVP1-1 plants (Fig. 3.4C) coincided with high levels of expression of *AVP1* in roots (Fig. 3.1C). This leads to our claim that shoot survival after anoxia always requires high levels of *AVP1* expression in roots.

Further evidence for roots playing the dominant role in whether plants survive anoxia is the high induction of *AVP1* unique to roots (Figure 3.3A,C). This can be ascribed to regulatory elements upstream of *AVP1*, implying the presence of root-specific anaerobic promoters similar to those reported in rice (Liu *et al.* 2010).

4.3.2 Expression of AVP1 in roots also enhances recovery and biomass accumulation

When *AVP1* expression patterns in AVP1-1 and WT plants were compared, it was evident that expression of this gene was a strong predictor not only of survival (Section 4.3.1) but also growth and recovery after anoxia. As for survival, which is essentially a cell maintenance phenomenon, recovery of shoot and root growth after anoxia was also dependent on induction of *AVP1* expression in roots. In spite of higher *AVP1* expression in shoots of WT plants relative to AVP1-1 plants, recovery and growth were always superior in the overexpresser, leading to the conclusion that root expression of *AVP1* was responsible for both survival and recovery.

Enhanced biomass accumulation for AVP1-1 plants was observed only in normoxic roots and shoots regardless of sucrose supply. While it might appear surprising that the genotypic contrasts were absent in anoxically treated plants, enhanced biomass in normoxic plants has also been previously described by Li *et al*. (2005) for AVP1-1 transgenics. The consistently increased dry weight seen in AVP1-1 plants growing in air, while *AVP1* overexpression was localised to the roots, again implies that roots are responsible for the phenotype seen in AVP1-1 plants. There was a more significant difference in dry weights between genotypes on 0.1% than 1% sucrose medium. This might be the result of slightly increased expression of AVP1-1 relative to WT in normoxic roots at 0.1% sucrose (*cf.* 1% sucrose – blue bars). Moreover, the higher expression of *AVP1* in roots on 0.1% sucrose medium was mirrored in shoots, possibly contributing to the increased biomass of AVP1-1 overexpressing plants when grown in normoxia. This leaves the question of why it is that in anoxically treated plants, *AVP1* overexpression did not enhance dry weight. In anoxia, energy reserves are preferentially allocated towards protein synthesis, lipid synthesis, and in general maintenance of transmembrane gradients (Felle 2005; Edwards *et al.* 2012; Atwell *et al.* 2015). We speculate that protein turnover and maintenance in AVP1-1 plants were still taking precedence over growth because plants were still recovering after sustained anoxia. The priority of maintenance over growth is evidenced by the recovery of meristematic activity after anoxia in AVP1-1 plants (Fig. 3.8), while biomass accumulation was not changed (Fig. 3.7). It is likely that if left to grow for longer, recovery would be followed by faster growth in AVP1-1 (Fig. 4.1).

4.4 Effects of exogenous sucrose on cell energetics and AVP1 *induction*

4.4.1 Effects of sucrose on survival

The variability in seedling establishment seen on a sucrose-free medium led to the use of 1% sucrose, where the effects of anoxia on physiology were surprisingly modest. As a result, 0.1% sucrose was used in the phytagel medium, revealing a strong interaction between sugar supply and anoxic response. When sucrose in the medium was reduced to 0.1%, survival of roots and particularly shoots after an anoxic period was reduced (*cf.* 1% sucrose). The supply of exogenous sucrose to the medium (and thus directly to the roots) clearly ameliorates the energy deficit caused by a lack of oxygen. In anoxia, substrate-level phosphorylation is the main source of energy for plants, yielding small quantities of ATP through glycolysis (Webb and Armstrong 1983; Dennis *et al.* 2000; Bailey-Serres and Chang 2005; Huang *et al.* 2005; Bailey-Serres and Voesenek 2010), and glycolysis rates in anoxia can be accelerated by exogenous sugars (Gibbs and Greenway 2003; Kulichikhin *et al.* 2009). Furthermore, Huang *et al.* (2003) found that supplying excised rice coleoptile tips of an anoxia-intolerant variety (IR22) with exogenous sucrose, anoxia tolerance became equal to that of a known anoxia-tolerant cultivar (Amaroo). The exact mechanism by which this interaction is achieved is not entirely clear. Within the realm of energetics, possibilities include enhanced transport or catabolism of sugars at high exogenous sugar concentrations, thereby accelerating fermentation and generating ATP by substrate-level phosphorylation. These events are likely to be replicated in the shoots of anaerobic seedlings, particularly in the dark where a carbohydrate deficit will be severe. However, this thesis reports specifically on the interaction between overexpression of *AVP1* and sucrose supply on: (i) recovery after anoxia and subsequent growth in normoxia and (ii) survival after anoxia. This can be tentatively linked to a general switch to metabolism energised by pyrophosphate, with a consequent upregulation of PPi-dependent enzymes (Atwell *et al.* 2015). The catabolism of sucrose through the engagement of sucrose synthase, and pumping of protons into vacuoles by a highly expressed vacuolar pyrophosphatase (*AVP1*) are consistent with the sucrose-induced stimulation observed in these experiments. The divergent responses of growth and survival, which are stimulated by overexpression of *AVP1* in normoxia, and post-anoxia respectively, are addressed below (Fig. 4.1)

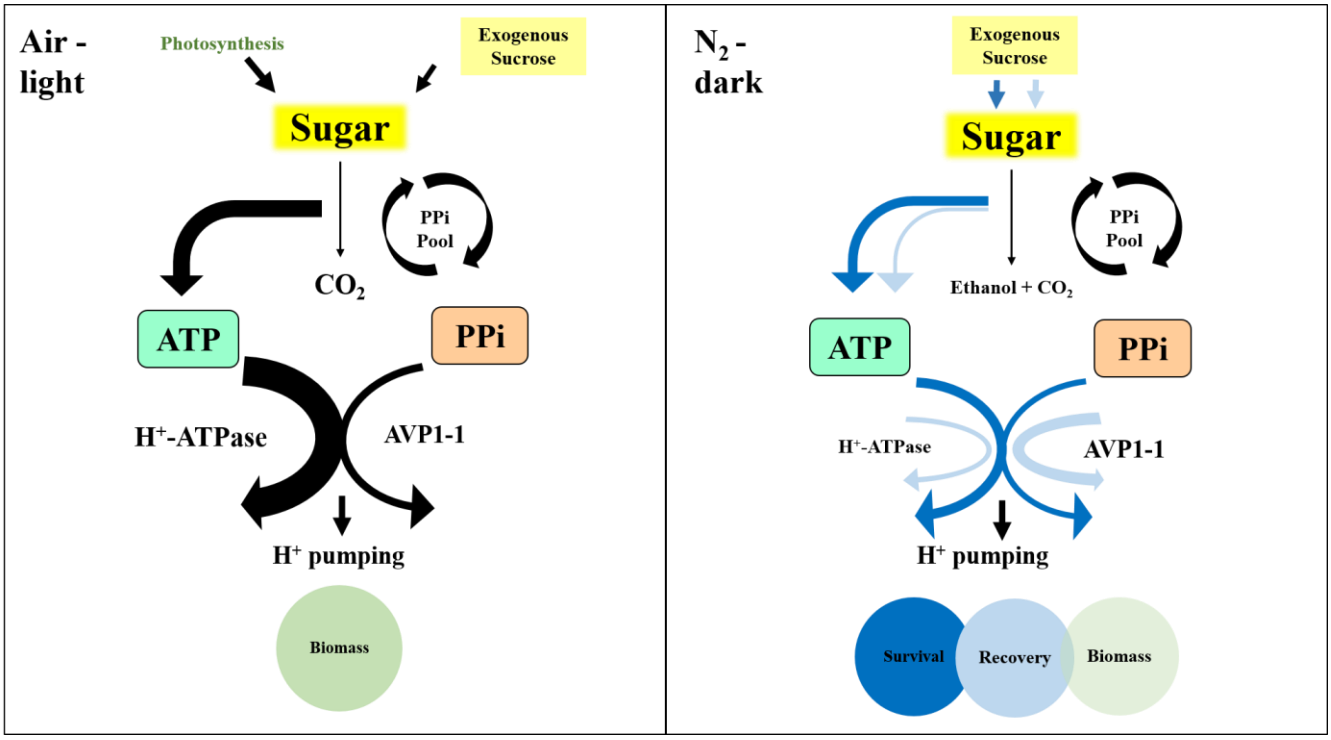

FIG 4.1 Conceptual model explaining the implications of proton pumping into the vacuole, capturing the effects of overexpression of the *Arabidopsis AVP1* gene (AVP1-1). The left-hand panel shows the pathways under optimal conditions (normoxic and illuminated), with ATP generation and proton pumping by ATPases dominant. A proportion of protons are pumped by *AVP1*, resulting in a measurable increase in final biomass of both roots and shoots. In anoxia and darkness, plants relied upon exogenous sucrose supplied at two levels: 1% sucrose (dark blue) and 0.1% sucrose (pale blue). High sucrose levels enhanced survival, presumably by accelerating fermentation and increasing ATP supply for H^+-ATPase (dark blue). By contrast, low sucrose levels had much more impact on shoot recovery after anoxia. In that, *AVP1* expression in roots was strongly upregulated in these low-sucrose plants (pale blue), the claim in made that increased proton pumping and resultant membrane integrity enhanced recovery after anoxia, partially compensating for the low rates of ATP generation by fermentation. Because biomass accumulation in these AVP1-1 plants was not greater than in the wild-type plants, it is hypothesized that the AVP1 gene contributes to cell maintenance in anoxia while contributing to cell growth in normoxia. Arrow thickness represent notional activity abundance.

4.4.2 Effects of sucrose on AVP1 *induction*

Exogenous sucrose directly affected expression levels of *AVP1* in normoxia (Fig. 3.2); *AVP1* expression increased in plants on 1% sucrose medium compared with 0.1% sucrose. However, from 0 - 12 h of anoxia, expression of *AVP1* in roots on 1% sucrose medium was strongly downregulated in both genotypes, decreasing ~4-fold from expression levels in normoxia. In contrast, roots of both genotypes on 0.1% sucrose medium exhibited a much smaller decrease in *AVP1* expression during this same period. As anoxia progressed, *AVP1* mRNA in roots was more strongly induced on 0.1% sucrose medium than

1% sucrose across both genotypes; this phenomenon was also observed in shoots, likely as a result of import of sugars via the phloem. This suggests that exogenous sucrose supply may be linked to downregulation of *AVP1* expression. Sucrose has been demonstrated to be a signalling molecule involved in a wide array of cellular process, including stress and energy signalling, as reviewed by Tognetti *et al.* (2013). Sucrose has been associated with the regulation of both WRKY and bZIP transcription factor families (Tognetti *et al.* 2013), which are known to be involved in low-oxygen signalling (Hunt *et al.* 2002; Loreti *et al.* 2003; Branco-Price *et al.* 2005; Narsai *et al.* 2015). The exact nature of the relationship between these anaerobically induced transcription factors and sucrose signalling is unclear; sucrose has been demonstrated to downregulate bZIP transcription factors while opposite effects of up- and down-regulation has been demonstrated within different members of the WRKY family (Tognetti *et al.* 2013). The relationship between sucrose, cell energy status and the transcription of *AVP1* requires deeper analysis. However, it is unlikely that increased sucrose supply strongly downregulates transcription of other anaerobic proteins because plants grown on 1% sucrose could still acclimate to anoxia (Fig. S-6).

Despite increased levels of *AVP1* mRNA in 0.1% sucrose medium, *survival rates* on 1% sucrose medium were still higher, implying that an adequate exogenous sucrose supply was more important than high levels of expression of *AVP1* in ensuring that plants survived anoxia. By contrast, *AVP1* plays a more critical role than sucrose in recovery once damage in anoxia has already taken place. This theory is supported by the fact that recovery rate in roots is actually impaired on 1% sucrose medium compared with 0.1% sucrose, reflecting the importance of *AVP1* for initiation of dormant meristems.

There are two theories as to why this could be possible, both relating to downregulated expression of *AVP1* seen with higher exogenous sucrose. The first theory is that increased expression of *AVP1*, as seen in plants grown on 0.1% sucrose medium, would sustain membrane function for longer under anoxia than the lower levels reported in plants on 1% sucrose medium, despite the energetic benefits that higher sucrose levels confer. Higher levels of depolarisation of the tonoplast membrane in plants in 1% sucrose could delay recover from anoxia. The second possibility is that anaerobically induced genes other than *AVP1* were downregulated in the presence of exogenous sucrose. Anaerobic proteins not only acclimate plants to anoxia, but also protect tissues from oxidative damage on return to normoxia (Sachs *et al.* 1980; Gibbs and Greenway 2003; Setter and Waters 2003). We postulate that the stimulus to glycolysis through sucrose catabolism helped counter downregulation of the expression of anaerobically induced genes. These 1%-sucrose plants could therefore be more vulnerable to oxidative stress on return to normoxia, explaining the reduced recovery rates relative to plants at 0.1% sucrose (Fig. S-8).

4.4.3 Anoxic shock and acclimation

The longer plants remained in anoxia, the more damage tissues sustained as a result of an energy crisis. Declining polymer synthesis began to affect cell function and eventually membrane integrity failed, causing loss of solutes and cell death. However, acclimation to anoxia occurs in many tissues, most notably in rice coleoptiles where energy is directed towards protein synthesis (Edwards *et al.* 2012) and the composition of the protein complement (Narsai *et al.* 2015). The classical study of Sachs *et al.* (1980) identified a set of anaerobically induced proteins in maize roots; even if insufficient energy is available to achieve new growth (biosynthesis), maintenance of protein turnover can enable cells to survive even in anoxia (Gibbs and Greenway 2003). This 'anaerobic response' is responsible for synthesis of proteins that enable acclimation to both anoxia and oxidative damage after re-oxygenation (Sachs *et al.* 1980; Gibbs and Greenway 2003; Setter and Waters 2003; Narsai *et al.* 2015). In *Arabidopsis*, Figure 3.5 reveals a higher survival rate for plants that had been exposed to anoxia for longer than 30 h than those returned to normoxia after exactly 30 h. This was repeated in roots and shoots across many replicates while anoxia for longer still (36 - 48 h) caused greater declines in survival (Fig. 3.5C-F). The paradox of a longer period in anoxia enhancing subsequent survival in normoxia suggests that some acclimation events occurred in the hours immediately after 30 h of anoxia. In that shoots of AVP1-1 plants in this acclimation period had a higher survival rate than shoots of WT plants after return to normoxia, we speculate that *AVP1* expression plays a role in the acclimation response and therefore should be classified as an ANP. This was found to be the case in previous studies with rice and *Arabidopsis* (Carystinos *et al.* 1995; Huang *et al.* 2005; Liu *et al.* 2010). Because the peak at > 30 h was also seen in WT roots and shoots, *AVP1* alone cannot be the only contributor to anoxic acclimation. Many other proteins with energetic, transport and protective functions are likely to enable plants to survive short-term anoxia.

5. CONCLUSION

Arabidopsis is a relatively flood-intolerant plant; it might not a priori not be considered an ideal choice for discovery of flood tolerance mechanisms if key genes have been eliminated from the genome by evolution. Its flood intolerance has actually been shown to result from low levels of transcription of key flood tolerance genes rather than their absence (Narsai *et al.* 2011). It is therefore an ideal genetic background for testing the impact of overexpressed genes for flood tolerance. Against this background, we tested the overexpression of a gene encoding a tonoplastic proton pump energised by pyrophosphate (*AVP1*).

Overexpression of *AVP1* improved survival of *Arabidopsis* seedlings up to 2 d of anoxia, provided that it was highly expressed in roots. *AVP1* expression in roots was induced in anoxia and improved survival of both roots and shoots after plants were returned to air. While survival in anoxia can be ascribed to many anaerobically induced proteins, this study shows that *AVP1* improves survival after an energy crisis and should be considered an adaptive protein for flood tolerance. Its expression in roots appears to improve root function and subsequently, resilience of shoots in complete anoxia (in darkness).

While *AVP1* overexpression in anoxia improved survival of plants after a substantial anoxic treatment, shoot or root biomass were no greater in these plants after a period of recovery in air. Curiously, *AVP1* overexpression did cause plants continuously growing in air to grow larger than controls, suggesting an *AVP1* phenotype that was independent of oxygen deprivation. This led to our hypothesis that the AVP transporter contributes to growth in optimal conditions as well as cell maintenance (survival) after an energy crisis.

Interactions between exogenous sucrose and plant performance after anoxia indicated a subtle role for sugars in gene expression, as well as the anoxic response. For example, rates of survival after anoxia increased with exogenous sucrose levels in the media, indicating that sucrose was acting conventionally as a substrate for glycolysis. However, high exogenous sucrose appeared to inhibit *AVP1* expression by an unexplained mechanism. The upregulation in *AVP1* expression in AVP1-1 plants induced by a low-sugar medium was tentatively linked to the observation that the shoots of these seedlings recovered faster after an anoxic treatment.

This study shows that the *AVP1* gene is a major anaerobic stress tolerance gene, either for survival during anoxia or after return to an oxygen-rich atmosphere. Tolerance of *AVP1*- overexpressing plants to anoxia could be predicted based on its role in membrane polarisation; this follows previous claims of a role in

salinity and drought tolerance. The clear phenotypic changes as a result of *AVP1* overexpression were remarkable in that its expression was less than doubled in normoxia. Furthermore, plants were allowed to recover in air in order to assess survival and growth after anoxia; more strongly overexpressed copies of this gene in a more tolerant genetic background might be expected to elicit even greater levels of anoxia tolerance. Finally, the importance of *AVP1* expression in roots appeared to play out most strongly in shoots: in plants where roots are flooded and shoots are oxygenated, different tolerance patterns may be revealed.

REFERENCES

Albrecht G, Mustroph A, Fox TC. **2004**. Sugar and fructan accumulation during metabolic adjustment between respiration and fermentation under low oxygen conditions in wheat roots. *Physiologia Plantarum* **120**: 93–105.

Armstrong W. **1980**. Aeration in Higher Plants. *Advances in Botanical Research* **7**: 225–332.

Asaoka M, Segami S, Maeshima M. **2014**. Identification of the critical residues for the function of vacuolar H+-pyrophosphatase by mutational analysis based on the 3D structure. *Journal of Biochemistry* **156**: 333–344.

Atwell BJ, Greenway H, Colmer TD. **2015**. Efficient use of energy in anoxia-tolerant plants with focus on germinating rice seedlings. *New Phytologist* **206**: 36–56.

Atwell BJ, Waters I, Greenway H. **1982**. The effect of oxygen and turbulence on elongation of coleoptiles of submergence-tolerant and -intolerant rice cultivars. *Journal of Experimental Botany* **33**: 1030–1044.

Bailey-Serres J, Chang R. **2005**. Sensing and signalling in response to oxygen deprivation in plants and other organisms. *Annals of Botany* **96**: 507–518.

Bailey-Serres J, Fukao T, Gibbs DJ, *et al.* 2012. Making sense of low oxygen sensing. *Trends in Plant Science* **17**: 129–138.

Bailey-Serres J, Voesenek LACJ. **2008**. Flooding Stress: Acclimations and Genetic Diversity. *Annual Review of Plant Biology* **59**: 313–339.

Bailey-Serres J, Voesenek LACJ. **2010**. Life in the balance: a signaling network controlling survival of flooding. *Current Opinion in Plant Biology* **13**: 489–494.

Bao AK, Wang SM, Wu GQ, Xi JJ, Zhang JL, Wang CM. **2009**. Overexpression of the Arabidopsis H+-PPase enhanced resistance to salt and drought stress in transgenic alfalfa (Medicago sativa L.). *Plant Science* **176**: 232–240.

Branco-Price C, Kawaguchi R, Ferreira RB, Bailey-Serres J. **2005**. Genome-wide analysis of transcript abundance and translation in arabidopsis seedlings subjected to oxygen deprivation. *Annals of Botany* **96**: 647–660.

Brini F, Hanin M, Lumbreras V, *et al.* 2007. Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in Arabidopsis thaliana. *Plant Cell Reports* **26**: 2017–2026.

Carystinos GD, MacDonald HR, Monroy AF, Dhindsa RS, Poole RJ. **1995**. Vacuolar H(+)-translocating pyrophosphatase is induced by anoxia or chilling in seedlings of rice. *Plant physiology* **108**: 641–9.

Colmer TD, Huang S, Greenway H. **2001**. Evidence for down-regulation of ethanolic fermentation and K+ effluxes in the coleoptile of rice seedlings during prolonged anoxia. *Journal of experimental botany* **52**: 1507–17.

Dennis ES, Dolferus R, Ellis M, *et al.* 2000. Molecular strategies for improving waterlogging tolerance in plants. *Journal of experimental botany* **51**: 89–97.

Dolferus R, Klok EJ, Delessert C, *et al.* 2003. Enhancing the anaerobic response. *Annals of Botany* **91**: 111–117.

Van Dongen JT, Fröhlich A, Ramírez-Aguilar SJ, *et al.* 2009. Transcript and metabolite profiling of the adaptive response to mild decreases in oxygen concentration in the roots of arabidopsis plants. *Annals of Botany* **103**: 269–280.

Drew M. **1997**. Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia.

Annual Review of Plant Physiology and Plant Molecular Biology **48**: 223–250.

Drozdowicz YM, Kissinger JC, Rea PA. **2000**. AVP2, a Sequence-Divergent, K+-Insensitive H+ -Translocating Inorganic Pyrophosphatase from Arabidopsis. **123**: 353–362.

Edwards JM, Roberts TH, Atwell BJ. **2012**. Quantifying ATP turnover in anoxic coleoptiles of rice (Oryza sativa) demonstrates preferential allocation of energy to protein synthesis. *Journal of Experimental Botany* **63**: 4389–4402.

Ellis MH, Dennis ES, Peacock WJ. **1999**. Arabidopsis Roots and Shoots Have Different Mechanisms for Hypoxic Stress Tolerance. *Plant physiology* **119**: 57–64.

Felle HH. **2005**. pH regulation in anoxic plants. *Annals of Botany* **96**: 519–532.

Ferjani A, Segami S, Horiguchi G, Muto Y, Maeshima M, Tsukaya H. **2011**. Keep an Eye on PPi: The Vacuolar-Type H+-Pyrophosphatase Regulates Postgerminative Development in Arabidopsis. *The Plant Cell* **23**: 2895–2908.

Gaxiola RA, Edwards M, Elser JJ. **2011**. A transgenic approach to enhance phosphorus use efficiency in crops as part of a comprehensive strategy for sustainable agriculture. *Chemosphere* **84**: 840–845.

Gaxiola RA, Li J, Undurraga S, *et al.* 2001. Drought- and salt-tolerant plants result from overexpression of the AVP1 H+-pump. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 11444–11449.

Gaxiola RA, Rao R, Sherman A, Grisafi P, Alper SL, Fink GR. **1999**. The Arabidopsis thaliana proton transporters , AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 1480–1485.

Gaxiola RA, Sanchez CA, Paez-Valencia J, Ayre BG, Elser JJ. **2012**. Genetic Manipulation of a "Vacuolar" H+-PPase: From Salt Tolerance to Yield Enhancement under Phosphorus-Deficient Soils. *Plant Physiology* **159**: 3–11.

Gibbs J, Greenway H. **2003**. Review : Mechanisms of anoxia tolerance in plants . I . Growth , survival and anaerobic catabolism. *Functional Plant Biology* **30**: 1–47.

Gibbs DJ, Lee SC, Isa NM, *et al.* 2011. Homeostatic response to hypoxia is regulated by the N-end rule pathway in plants. *Nature* **479**: 415–8.

Gonzali S, Loreti E, Novi G, Poggi A, Alpi A, Perata P. **2005**. The use of microarrays to study the anaerobic response in Arabidopsis. *Annals of Botany* **96**: 661–668.

Greenway H, Gibbs J. **2003**. Mechanisms of anoxia tolerance in plants. II. Energy requirements for maintenance and energy distribution to essential processes. *Functional Plant Biology* **30**: 999–1036.

Greenway H, Waters I, Newsome J. **1992**. Effects of Anoxia on Uptake and Loss of Solutes in Roots of Wheat. *Australian Journal of Plant Physiology* **19**: 233.

Guescini M, Sisti D, Rocchi MBL, Stocchi L, Stocchi V. **2008**. A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. *BMC bioinformatics* **9**: 326.

Gutierrez L, Mauriat M, Stephanie G, *et al.* 2008. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnology Journal* **6**: 609–618.

Hattori Y, Nagai K, Ashikari M. **2011**. Rice growth adapting to deepwater. *Current Opinion in Plant Biology* **14**:

100–105.

Huang S, Greenway H, Colmer TD. **2003**. Anoxia tolerance in rice seedlings: Exogenous glucose improves growth of an anoxia-'intolerant', but not of a "tolerant" genotype. *Journal of Experimental Botany* **54**: 2363–2373.

Huang S, Greenway H, Colmer TD, Millar AH. **2005**. Protein synthesis by rice coleoptiles during prolonged anoxia: Implications for glycolysis, growth and energy utilization. *Annals of Botany* **96**: 703–715.

Hunt PW, Klok EJ, Trevaskis B, *et al.* 2002. Increased level of hemoglobin 1 enhances survival of hypoxic stress and promotes early growth in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 17197–17202.

Igamberdiev AU, Kleczkowski LA. **2011**. Magnesium and cell energetics in plants under anoxia. *The Biochemical journal* **437**: 373–9.

Kim EJ, Zhen RG, Rea PA. **1994**. Heterologous expression of plant vacuolar pyrophosphatase in yeast demonstrates sufficiency of the substrate-binding subunit for proton transport. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 6128–6132.

Klok EJ, Wilson IW, Wilson D, *et al.* 2002. Expression profile analysis of the low-oxygen response in Arabidopsis root cultures. *The Plant cell* **14**: 2481–2494.

Kole C. **2013**. *Genomics and Breeding for Climate- Resilient Crops Vol. 1 Concepts and Strategies*.

Kulichikhin KY, Greenway H, Byrne L, Colmer TD. **2009**. Regulation of intracellular pH during anoxia in rice coleoptiles in acidic and near neutral conditions. *Journal of Experimental Botany* **60**: 2119–2128.

Lasanthi-Kudahettige R, Magneschi L, Loreti E, *et al.* 2007. Transcript profiling of the anoxic rice coleoptile. *Plant Physiol* **1441**: 218–231.

Li J, Yang H, Peer WA, *et al.* 2005. Arabidopsis H+-PPase AVP1 regulates auxin-mediated organ development. *Science* **310**: 121–5.

Licausi F, Kosmacz M, Weits DA, *et al.* 2011. Oxygen sensing in plants is mediated by an N-end rule pathway for protein destabilization. *Nature* **479**: 419–422.

Lin YC, Benner C, Mansson R, *et al.* 2012. Global changes in the nuclear positioning of genes and intra- and interdomain genomic interactions that orchestrate B cell fate. *Nature Immunology* **13**: 1196–1204.

Liu W, Saint D. **2002**. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. *Anal Biochem* **302**: 52–59.

Liu F, Vantoai T, Moy LP, Bock G, Linford LD, Quackenbush J. **2005**. Global transcription profiling reveals comprehensive insights into hypoxic response in Arabidopsis. *Plant physiology* **137**: 1115–1129.

Liu Q, Zhang Q, Burton RA, Shirley NJ, Atwell BJ. **2009**. Vacuolar pyrophosphatases confer anoxia tolerance in rice by pumping protons across the tonoplast. *Thesis. Department of Biological Science, Macquarie University.*: 1–14.

Liu Q, Zhang Q, Burton RA, Shirley NJ, Atwell BJ. **2010**. Expression of vacuolar H+-pyrophosphatase (OVP3) is under control of an anoxia-inducible promoter in rice. *Plant Molecular Biology* **72**: 47–60.

Loreti E, Yamaguchi J, Alpi A, Perata P. **2003**. Sugar modulation of α-amylase genes under anoxia. *Annals of Botany* **91**: 143–148.

Maeshima M. **2000**. Vacuolar H+ -pyrophosphatase. *Biochemica et Biophysica Acta* **1465**: 37–51.

Maeshima M. **2001**. Tonoplast Transporters : Organization and Function. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**: 469–497.

Matsumura H, Takano T, Takeda G, Uchimiya H. **1998**. Adh1 is transcriptionally active but its translational product is reduced in a rad mutant of rice (Oryza sativa L.), which is vulnerable to submergence stress. *Theoretical and Applied Genetics* **97**: 1197–1203.

Mitsuda N, Enami K, Nakata M, Takeyasu K, Sato MH. **2001**. Novel type Arabidopsis thaliana H + -PPase is localized to the Golgi apparatus. *FEBS Letters* **488**: 29–33.

Mohanty S. **2013**. IRRI - Trends in global rice consumption. *Rice Today*: 44–45.

Muto Y, Segami S, Hayashi H, *et al.* 2011. Vacuolar Proton Pumps and Aquaporins Involved in Rapid Internode Elongation of Deepwater Rice. *Bioscience, Biotechnology, and Biochemistry* **75**: 114–122.

Narsai R, Edwards JM, Roberts TH, Whelan J, Joss GH, Atwell BJ. **2015**. Mechanisms of growth and patterns of gene expression in oxygen-deprived rice coleoptiles. *The Plant Journal* **82**: 25–40.

Narsai R, Howell KA, Carroll A, Ivanova A, Millar AH, Whelan J. **2009**. Defining Core Metabolic and Transcriptomic Responses to Oxygen Availability in Rice Embryos and Young Seedlings. *Plant Physiology* **151**: 306–322.

Narsai R, Rocha M, Geigenberger P, Whelan J, Van Dongen JT. **2011**. Comparative analysis between plant species of transcriptional and metabolic responses to hypoxia. *New Phytologist* **190**: 472–487.

Normile D. **2008**. Agricultural research. Reinventing rice to feed the world. *Science* **321**: 330–333.

Peirson SN, Butler JN, Foster RG. **2003**. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res* **31**: e73.

Pfaffl MW. **2001**. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research* **29**: 42–45.

Ramakers C, Ruijter JM, Lekanne Deprez RH, Moorman AFM. **2003**. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters* **339**: 62–66.

Rea P a, Poole RJ. **1993**. Vacuolar H+ -Translocating Pyrophosphatase. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**: 157–180.

Ruijter JM, Ramakers C, Hoogaars WMH, *et al.* 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research* **37**.

Rutledge RG. **2004**. Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications. *Nucleic acids research* **32**: e178.

Sachs MM, Freeling M, Okimoto R. **1980**. The anaerobic proteins of maize. *Cell* **20**: 761–767.

Sanchez-Serrano JJ, Salinas J. **2014**. Arabidopsis Protocols. *Methods in Molecular Biology* **1062**: 225–239.

Sarafian V, Kim Y, Poole RJ, Rea PA. **1992**. Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 1775–9.

Setter TL, Waters I. **2003**. Review of prospects for germplasm improvement for waterlogging tolerance in wheat, barley and oats. *Plant and Soil* **253**: 1–34.

Tognetti JA, Horacio P, Martinez-Noel G. **2013**. Sucrose signaling in plants: A world yet to be explored. *Plant*

Signaling & Behavior **8**: e233161-e233161-10.

Undurraga SF, Santos MP, Paez-Valencia J, *et al.* 2012. Arabidopsis sodium dependent and independent phenotypes triggered by H+-PPase up-regulation are SOS1 dependent. *Plant Science* **183**: 96–105.

Vashisht D, Hesselink A, Pierik R, *et al.* 2011. Natural variation of submergence tolerance among Arabidopsis thaliana accessions. *New Phytologist* **190**: 299–310.

Wang X, Lee RS, Alver BH, *et al.* 2016. SMARCB1-mediated SWI/SNF complex function is essential for enhancer regulation. *Nature Genetics* **49**: 289–295.

Webb T, Armstrong W. **1983**. The effects of anoxia and carbohydrates on the growth and viability of rice, pea and pumpkin roots. *Journal of Experimental Botany* **34**: 579–603.

Wolf C, Scherzinger M, Wurz A, Pauli U, Hubner P, Luthy J. **2000**. Detection of cauliflower mosaic virus by the polymerase chain reaction: testing of food components for false-positive 35S-promoter screening results. *European Food Research and Technology* **210**: 367–372.

Xia JH, Roberts JKM. **1996**. Regulation of H+ Extrusion and Cytoplasmic pH in Maize Root Tips Acclimated to a Low-Oxygen Environment. *Plant physiology* **111**: 227–233.

Yang H, Knapp J, Koirala P, *et al.* 2007. Enhanced phosphorus nutrition in monocots and dicots over-expressing a phosphorus-responsive type I H+-pyrophosphatase. *Plant Biotechnology Journal* **5**: 735–745.

Yeo AR, Flowers TJ. **1986**. Salinity resistance in rice (*Oryza sativa* L.) and a pyramiding approach to breeding varieties for saline soils. *Functional Plant Biology* **13**: 161–173.

Zhao S, Fernald RD. **2005**. Comprehensive Algorithm for Quantitative Real-Time Polymerase Chain Reaction. *J Comput Biol* **12**: 1047–1064.

SUPPLEMENTARY MATERIAL

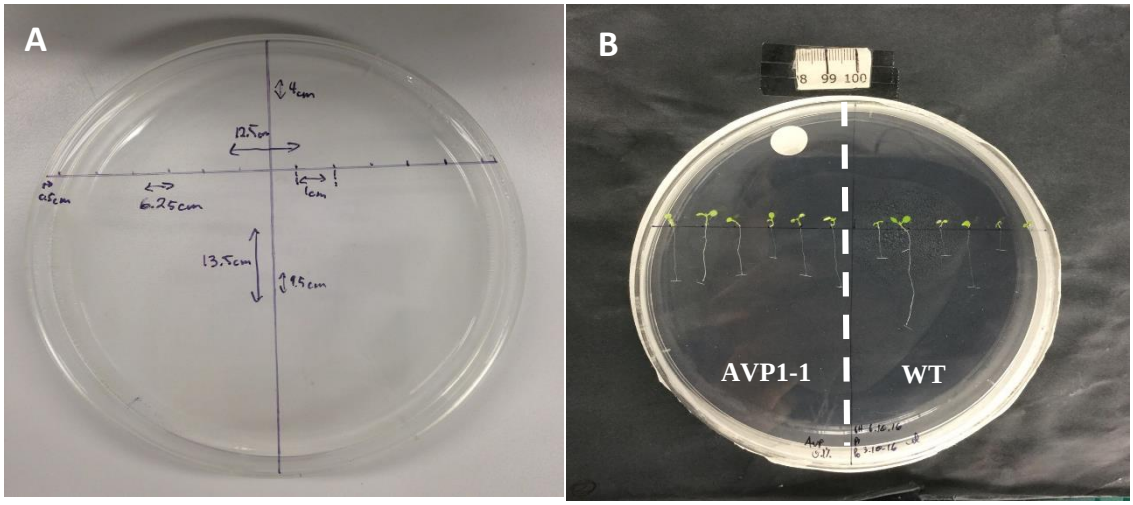

FIG. S-1. Examples demonstrating how plants were grown on plates; (A) a schematic diagram demonstrating how plants were arranged on petri plates; (B) an example of plants plated using this method. A horizontal line was drawn across the plate at a width of 12.5cm, and a vertical line was drawn down the middle of the plate separating both sides. Seeds were spaced 1cm apart, beginning 0.5cm from the edge of the plate, with six seeds per side. Both genotypes were grown on a single plate, each on separate sides of the dividing line.

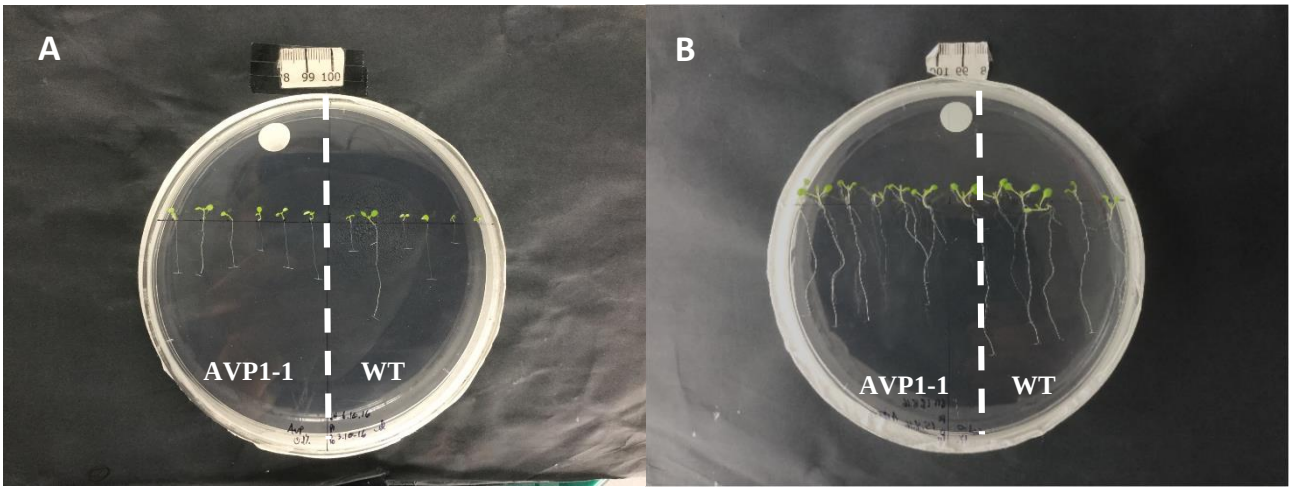

FIG. S-2. Comparison of sizes of AVP1-1 transgenic and WT *Arabidopsis* grown upright on plates across both media sucrose levels immediately prior to treatments; (A) seven-day-old plants grown on 0.1% sucrose media; (B) six-day-old Arabidopsis grown on 1% sucrose media.

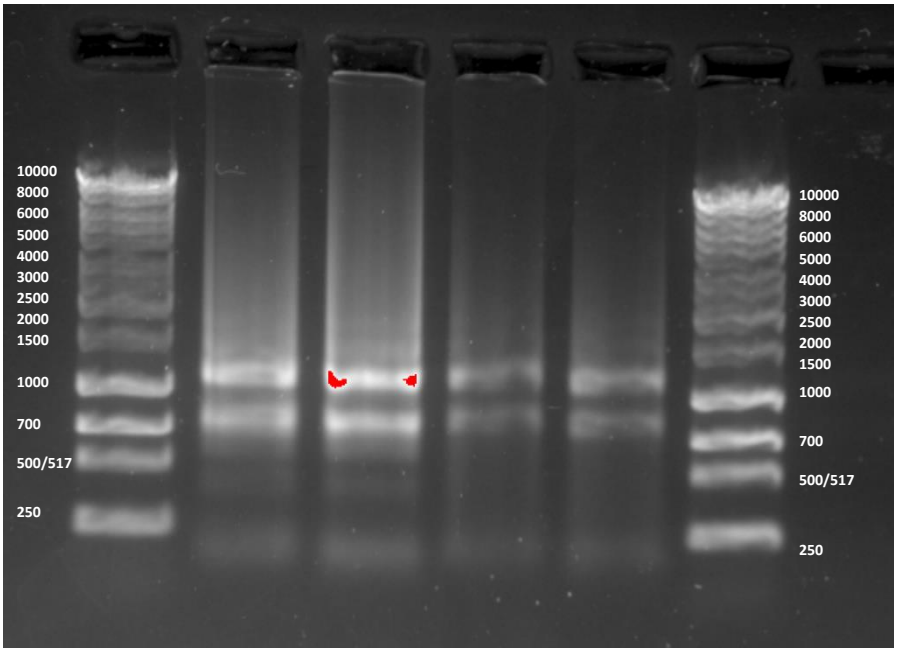

FIG. S-3. Aliquots of freshly-extracted total RNA on a 1% (w/v) agarose gel with a 1kb plus hyperladder (Bioline) stained with ethidium bromide. Two bright bands containing 18S and 28S rRNA are clearly visible, indicating RNA integrity remains intact.

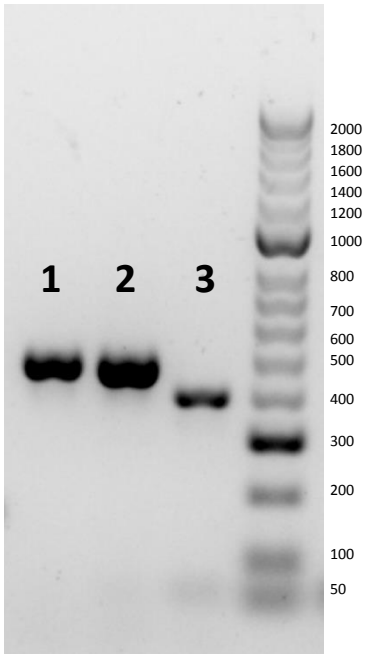

FIG. S-4**.** Gel electrophoresis of PCR products from (1) AVP1-1 DNA; (2) WT DNA and (3) AVP1-1 cDNA using a 50bp Hyperladder (Bioline). A PCR reaction amplifying *Actin* was run using a primer set (denoted Act) which discriminates between genomic and cDNA. The expected genomic DNA amplicon size is 490 bp, and the expected cDNA size is 390 bp.

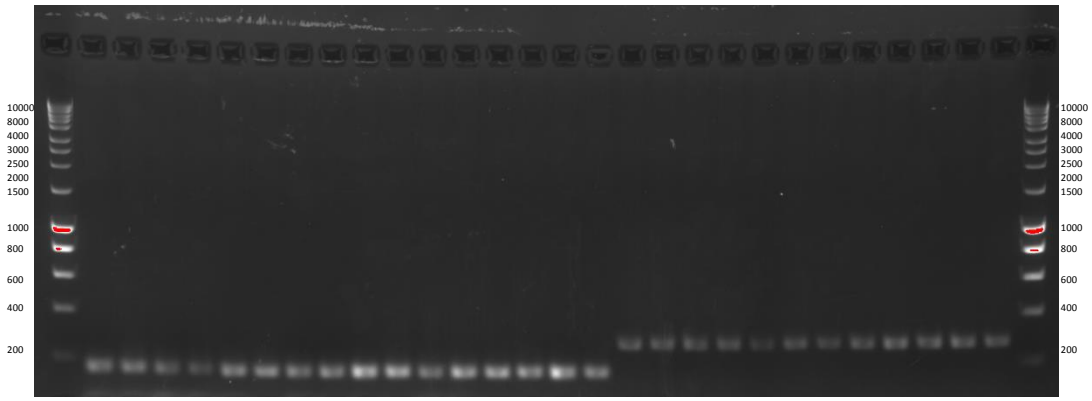

FIG. S-5**.** Gel electrophoresis of qPCR products from 36 h of anoxia using a 1kb Hyperladder (Bioline), ensuring that a single band of the expected size was present. The left side is displaying 141 bp bands from *AVP1*-specific primers as the target (denoted AVP1b) and *EF1α*-specific primers (denoted EF1α) as a reference gene.

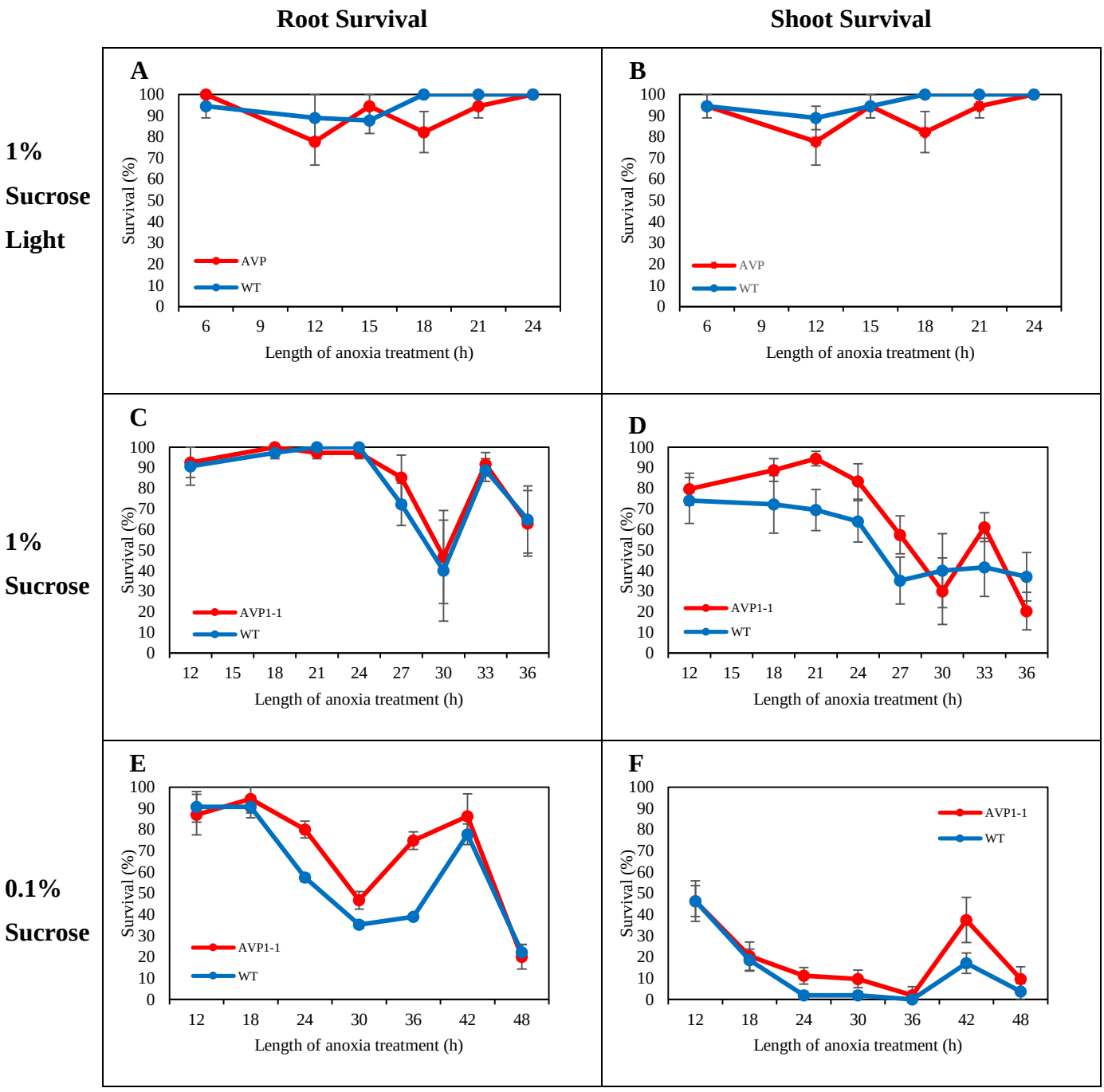

FIG. S-6. Survival percentages over various periods of time in anoxia for roots and shoots time of anoxically-treated roots and shoots of AVP1-1 and WT. Figures show: 1% sucrose in light for (A) roots and (B) shoots; 1% sucrose in darkness for (C) roots and (D) shoots; and 0.1% sucrose in darkness for (E) roots and (F) shoots. Survival was scored as the ability to initiate a new leaf or elongate roots past their initial length 2 weeks after the anoxic treatment. Note differences between x-axes as treatment lengths vary. Error bars represent SE averaged between both genotypes. Significant genotype differences were observed ($p < 0.05$) for graphs D, E and F.

TABLE S-1. *Summary of anaerobic treatment lengths, use of light or darkness, the percentage of media sucrose, the age of plants when undergoing treatments and the number of control and treatment plates replicated for each given set of conditions.*

Media Sucrose (%)	Treatment Length (h)	Light/ Dark	No. Replicates (treatment)	No. Replicates (Control)	Plant age at treatment (d)
1	6	light	3	0	7
1	9	light	3	0	7
1	12	light	3	0	7
1	15	light	3	0	7
1	18	light	3	0	7
1	21	light	3	0	7
1	24	light	3	0	7
1	12	dark	9	6	6
1	18	dark	6	6	6
1	21	dark	6	6	6
1	24	dark	6	6	6
1	27	dark	9	9	6
1	30	dark	5	5	6
1	33	dark	6	6	6
1	36	dark	9	9	6
0.1	12	dark	9	9	7
0.1	18	dark	9	9	7
0.1	24	dark	9	9	7
0.1	30	dark	9	9	7
0.1	36	dark	9	9	7
0.1	42	dark	9	9	7
0.1	48	dark	9	9	7

TABLE S-2. *Mean dry weights of roots and shoots for normoxic controls grown on 1% and 0.1% sucrose medium and treated for varying periods in darkness. Plants were given a 2-week recovery period once treatments were complete, after which they were harvested and dried. Shoots were separated from roots, and the dry weight was averaged across plants from a single genotype on a single plate. Note that treatment times of 21, 27 and 33 h on 1% sucrose media were excluded in order to produce equivalent time points between both media sucrose levels. The percentage change was calculated as |x| = [(0.1% sucrose weight – 1% sucrose weight)/1% sucrose weight]*100. Significance was determined using a two-sample t-test; all differences between means of 1% sucrose and 0.1% sucrose tissues were significant (p <0.001).*

Time in darkness (h)	Mean dry weight (mg) 1% Sucrose Medium	Mean dry weight (mg) 0.1% Sucrose Medium	Percent difference (%)
AVP1-1 Roots			
12	2.65	0.65	75
18	2.23	0.57	75
24	2.14	0.50	76
30	2.92	0.60	80
36	1.84	0.39	79
AVP1-1 Shoots			
12	7.14	3.20	55
18	6.74	2.83	58
24	7.10	2.39	66
30	5.72	2.47	57
36	6.53	2.08	68
WT Roots			
12	1.58	0.18	89
18	1.86	0.26	86
24	1.51	0.24	84
30	1.88	0.36	81
36	1.69	0.17	90
WT Shoots			
12	5.58	1.24	78
18	5.53	1.34	76
24	5.71	1.19	79
30	5.60	1.53	73
36	5.60	0.95	83

TABLE S-3. *Summary of survival and recovery rates (where applicable) for Arabidopsis AVP1-1 transgenics and WT (Col-0) treated in either anoxic treatments or normoxic controls in light or darkness in two different sucrose percentages for a duration of 6 – 48 h. Survival percentages and recovery rates are averages from 6 – 9 plates for each set of treatment conditions consisting of 6 plants of each genotype. Percentages were rounded to the nearest whole number.*

				Anoxia Treatments				Normoxic Controls			
Duration of treatment (h)	Genotype	Light/ dark	Media Sucrose (%)	Shoot Survival (%)	Root Survival (%)	Shoot Recovery (day-1)	Root Recovery (day-1)	Shoot Survival (%)	Root Survival (%)	Shoot Recovery (day-1)	Root Recovery (day-1)
12	avp1-1	dark	0.1	46	87	0.22	0.35	100	100	0.50	0.50
18	avp1-1	dark	0.1	20	94	0.17	0.45	100	100	0.50	0.50
24	avp1-1	dark	0.1	11	80	0.13	0.35	100	100	0.49	0.50
30	avp1-1	dark	0.1	10	47	0.11	0.34	100	100	0.48	0.50
36	avp1-1	dark	0.1	2	75	0.01	0.32	100	100	0.49	0.50
42	avp1-1	dark	0.1	37	86	0.17	0.39	98	100	0.50	0.49
48	avp1-1	dark	0.1	10	20	#N/A	0.17	100	100	0.46	0.50
12	col-0	dark	0.1	46	91	0.19	0.36	100	100	0.50	0.50
18	col-0	dark	0.1	19	91	0.23	0.43	100	100	0.49	0.50
24	col-0	dark	0.1	2	57	0.06	0.37	100	100	0.48	0.50
30	col-0	dark	0.1	2	35	0.01	0.25	100	100	0.45	0.50
36	col-0	dark	0.1	0	39	0.00	0.34	100	100	0.47	0.47
42	col-0	dark	0.1	17	78	0.19	0.39	98	100	0.48	0.47
48	col-0	dark	0.1	4	22	0.01	0.16	100	100	0.44	0.44
12	avp1-1	dark	1	80	93	0.34	0.32	100	100	0.38	0.50
18	avp1-1	dark	1	89	100	0.40	0.25	100	97	0.49	0.57
21	avp1-1	dark	1	94	97	0.33	0.23	100	100	0.49	0.49
24	avp1-1	dark	1	83	97	0.29	0.23	97	97	0.48	0.50
27	avp1-1	dark	1	57	85	0.19	0.16	89	85	0.31	0.43
30	avp1-1	dark	1	30	47	0.11	0.08	100	100	0.28	0.50
33	avp1-1	dark	1	61	92	0.30	0.20	100	100	0.43	0.48
36	avp1-1	dark	1	20	63	0.12	0.15	100	100	0.41	0.48
12	col-0	dark	1	74	91	0.27	0.35	100	100	0.42	0.49
18	col-0	dark	1	72	97	0.26	0.32	100	100	0.48	0.40
21	col-0	dark	1	69	100	0.26	0.26	100	100	0.47	0.50
24	col-0	dark	1	64	100	0.22	0.24	100	100	0.47	0.48
27	col-0	dark	1	35	72	0.13	0.18	89	89	0.27	0.44
30	col-0	dark	1	40	40	0.15	0.09	100	100	0.28	0.50
33	col-0	dark	1	42	89	0.19	0.20	100	100	0.33	0.50
36	col-0	dark	1	37	65	0.12	0.15	100	98	0.40	0.48
6	avp1-1	light	1	94	100	-	-	-	-	-	-
12	avp1-1	light	1	78	78	-	-	-	-	-	-
15	avp1-1	light	1	94	94	-	-	-	-	-	-
16	avp1-1	light	1	82	82	-	-	-	-	-	-
21	avp1-1	light	1	94	94	-	-	-	-	-	-
24	avp1-1	light	1	100	100	-	-	-	-	-	-
								-	-	-	-
6	col-0	light	1	94	94	-	-	-	-	-	-
12	col-0	light	1	89	89	-	-	-	-	-	-
15	col-0	light	1	94	88	-	-	-	-	-	-
18	col-0	light	1	100	100	-	-	-	-	-	-
21	col-0	light	1	100	100	-	-	-	-	-	-
24	col-0	light	1	94	100	-	-	-	-	-	-

TABLE S-4. *Log 2 of* AVP1 expression *in normoxia, expressed as a fold-change relative to the reference gene* EF1α.

Media Sucrose	AVP1-1 Roots	WT Roots	AVP1-1 Shoots	WT Shoots
0.1%	-1.11	-1.40	-1.37	-2.73
1%	0.09	-0.52	-1.18	-1.40
Difference	-1.19	-0.88	-0.19	-1.34

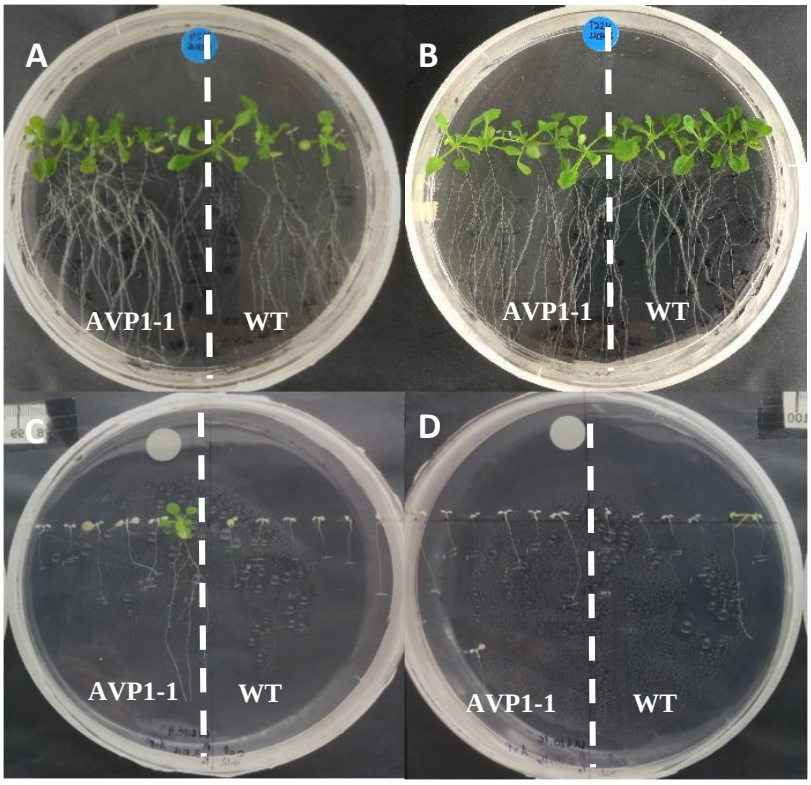

FIG. S-7. Comparison of *Arabidopsis* grown on different media sucrose levels anoxically-treated in the dark for 24 h after a 10 d recovery period; (A, B) plants grown on 1% sucrose media; (C, D) plants grown on 0.1% sucrose medium.

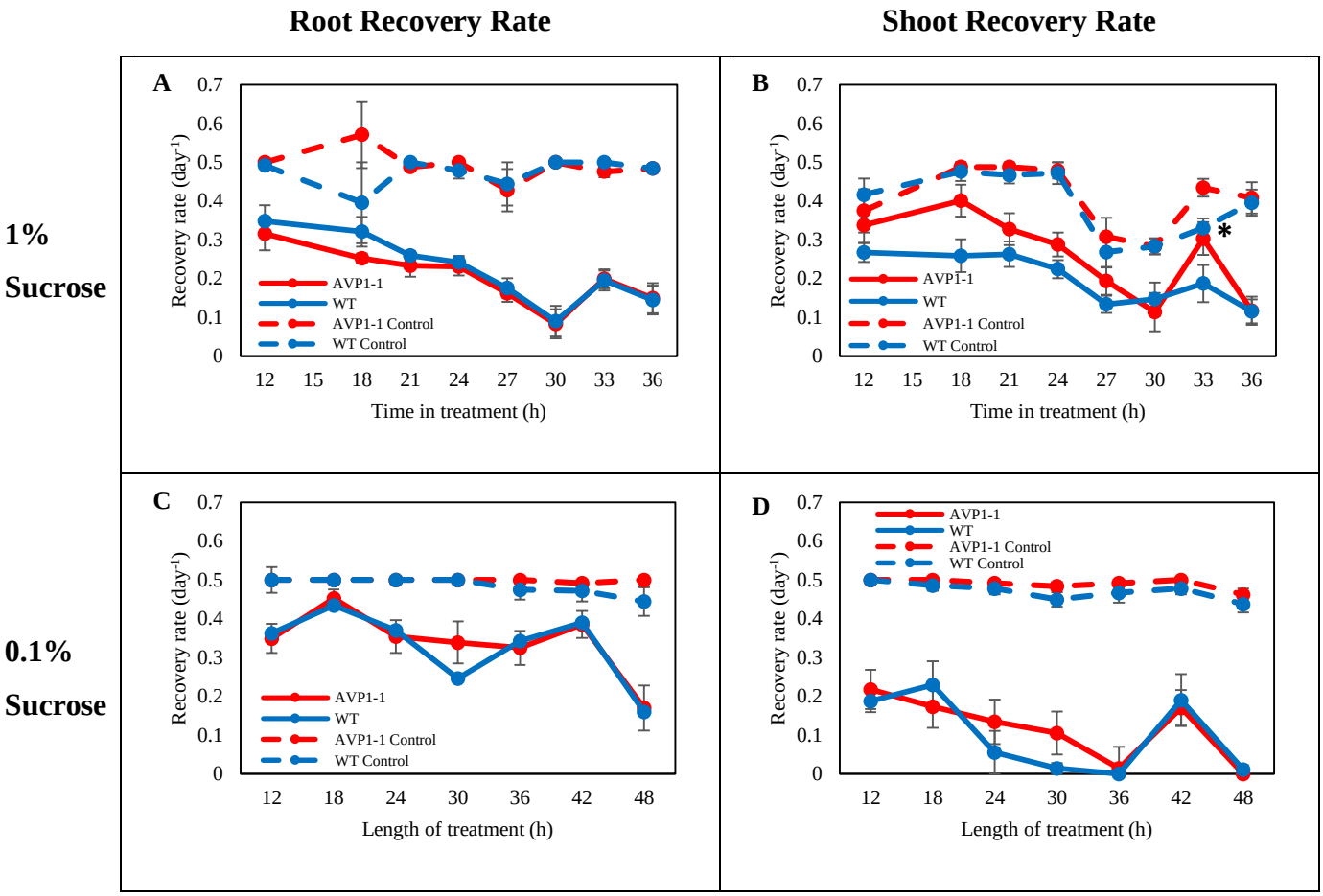

FIG. S-8. The average rate of recovery plotted over time for both AVP1-1 and WT. Figures show: 1% sucrose in light for (A) roots and (B) shoots; 1% sucrose in darkness for (C) roots and (D) shoots; and 0.1% sucrose in darkness for (E) roots and (F) shoots. Recovery rate was measured as the inverse of the first day that plants produced a new leaf or roots elongated past their initial length after anoxia. Note differences in x-axes as treatment lengths vary. Using a two-way ANOVA, significant genotypic effects occurred between anoxic treatments for B and D, and between normoxic controls for D.