Investigating the role of phytohormones during desiccation in two evolutionarily distinct resurrection plants

By

Sabine Maye Kirchner

KRCSAB002

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In fulfilment of the requirements for the degree

MSc Molecular and Cell Biology

Faculty of Science

UNIVERSITY OF CAPE TOWN

University of Cape Town

Date of submission: 04/02/2025

Supervisors: Prof. Jill Farrant[1], Prof. Dr. Ute Vothknecht[2] and Dr. Llewelyn van der Pas[1]

[1] Faculty of Science, Department of Molecular and Cell Biology

[2] Institute for Cellular and Molecular Botany (IZMB), Bonn University

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

PLAGIARISM DECLARATION

I, **Sabine Kirchner**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce, for the purpose of research, either the whole or any portion of the contents in any manner whatsoever.

Signature: …. Signed by candidate ……

Date: 11/01/2025

ABSTRACT

Most plants encounter challenges brought on by various abiotic stressors which subsequently prompt adaptations to mitigate these challenges. Water deficit brought about by drought conditions is a significant abiotic stressor that impacts crop productivity, ultimately threatening global food security. Significant effort has thus gone into researching the unique adaptations that have allowed a remarkable group of angiosperms termed *resurrection plants*, to survive extreme water loss with minimal damage. This thesis focused on profiling phytohormone abundances in two evolutionarily distinct resurrection plant species; *Craterostigma pumilum* and *Xerophyta schlechteri*, during a dehydration time course. The objective was to elucidate some of the molecular processes that occur in the context of differing desiccation tolerance strategies, namely homoiochlorophylly and poikilochlorophylly. Hormone profiles were then followed up with gene expression analysis of desiccation-responsive genes *RD29B* and *GASA3* in *C. pumilum* to further explore hormone signalling associated with jasmonates. In order to do this, and because the major findings on the roles of these genes in relation to jasmonate signalling emanate from work on the model plant *Arabidopsis thaliana*, protocols designed for their analyses in that species were tested against *C. pumilum* to investigate their effectiveness when implemented in a non-model species. Species-specific hormone profiles were identified not only between the two resurrection plants, but also within their leaf and root tissues. Abscisic acid (ABA) emerged as a central regulator of stress responses, while jasmonic acid (JA) appeared to play more of a supporting role, and the dynamics of 12-oxo-phytodienoic acid (OPDA), a precursor of JA, suggested a potential alternative signalling pathway that may occur in resurrection plants during desiccation. Overall, the findings point to species-specific hormone profiles that may be unique to resurrection plants and underscore the complexity of hormonal interactions in plant responses to water deficit stress. Additionally, results highlight the need for further optimization of laboratory protocols designed with specific species in mind and warn against a potential over-reliance on protocols designed for model species.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Prof. Jill Farrant, Prof. Ute Vothknecht and Dr. Llewelyn van der Pas for their passion and commitment towards this project and for their guidance and limitless support throughout this process. In addition, I would like to thank Prof. Jill Farrant particularly for her academic support as well as significant funding contribution from her Georg Forster Research Award granted by the Alexander von Humboldt Foundation. Also, thank you to the UCT Postgraduate Funding Office for helping to fund this project through the Masters Research Scholarship. I would also like to thank Dr. Katharina Gutbrod from the AG Dörmann for her expertise in the handling of my HPLC-ESI-MS/MS analysis during phytohormone profiling and Eugene Kabwe Ntuntu Kabamba for his technical skill and assistance in isolating homologous regions of candidate genes in *A. thaliana*. Additionally, I need to thank Lu and fellow MSc student Liam Lumley in particular, for acting as fantastic soundboards and for lifting my spirits during rough patches. Thank you to all members of the Plant Stress Laboratory for bringing me into this family and for igniting my passion for this subject. Lastly, I would like to thank my family, specifically my parents and loving partner, for their words of encouragement as well as for their emotional support, for their patience, tolerance, and for always being in my corner.

TABLE OF CONTENTS

PLAGIARISM DECLARATION i
ABSTRACT ii
ACKNOWLEDGEMENTS iii
LIST OF TABLES vi
LIST OF FIGURES vi
LIST OF ABBREVIATIONS vii
Chapter 1 : Introduction 1

1.1. Overview 1
1.2. Desiccation and resurrection 1

1.2.1. The nature of resurrection plants 3
1.2.2. Desiccation tolerance and phytohormone signalling of resurrection plants 4
1.2.3. Model resurrection plants for research on desiccation tolerance 5
1.2.4. Understanding phytohormone interactions utilizing the model plant *Arabidopsis thaliana* 7

1.3. Research aims and objectives 8

Chapter 2 : Phytohormonal control mechanisms of desiccation responses in plants 9

2.1. Introduction 9

2.1.1. Phytohormones as master signalling molecules during a desiccation response 10
2.1.2. The use of phytohormones for the enhancement of crops through biotechnology 14

2.2. Materials and methods for the analysis of phytohormone profiles in the leaves and roots of two resurrection plant species 16

2.2.1. Hormone profiling in the vegetative tissue of *X. schlechteri* and *C. pumilum* 16

2.3. Results and Discussion 21

2.3.1. *Xerophyta schlechteri* and *Craterostigma pumilum* adopt morphological strategies to circumvent drought stress 21
2.3.2. Assessment of dehydration and relative water content (RWC) 22
2.3.3. Hormone trends 25
2.3.4. Conclusions 37

Chapter 3 : The control of gene expression during desiccation response in plants 39

3.1. Introduction 39
3.1.1. Transcriptomic analyses in the context of desiccation tolerant species 39
3.1.2. Control of the jasmonate pathway during desiccation 40
3.1.3. RT-qPCR in the context of plant research 46
3.2. Materials and methods for gene expression analysis of *MYC2*, *RD29B* and *GASA3* during the desiccation response of *C. pumilum* 47
3.2.1. Overcoming polyploidy 47
3.2.2. Sample preparation, RNA Extraction and cDNA synthesis 49
3.2.3. RT-qPCR setup using the comparative CT Method (ΔΔ CT Method) 50
3.3. Results & Discussion 52
3.3.1. Evaluation of RNA extraction protocols across species 52
3.3.2. Transcript variation in octoploid species 53
3.3.3. Evaluation of primers and reference genes 57
3.3.4. $\Delta\Delta C_T$ validation 58
3.3.5. Gene expression of GASA3 and RD29B in the leaves and roots of *C. pumilum* ... 59
3.3.6. Evaluation of qPCR techniques in the model plant Arabidopsis thaliana 63
3.3.7. Evaluation of MYC2 under drought stress in *A. thaliana* 63
3.3.8. Conclusions 67
Chapter 4 : Overall Conclusions 68
Appendix A 89
Appendix B 90
Appendix C 91
Appendix D 93
Appendix E 94

LIST OF TABLES

Table 2-1: Seed collection data.. 16
Table 3-1: Final primer pair details.. 48
Table 3-2: Significantly differentially expressed genes (SDEGs) during dehydration in *C. pumilum*. 53

LIST OF FIGURES

Figure 2-1: Simplified overview of typical phytohormone synthesis........ 13
Figure 2-2: Schematic representation of phytohormone crosstalk during a dehydration response........ 14
Figure 2-3: Plant tissue sampling timeline........ 17
Figure 2-4: Stages of desiccation in *C. pumilum* (top) and *X. schlechteri* (bottom).. 21
Figure 2-5: Dehydration curves of *C. pumilum* and *X. schlechteri*. 24
Figure 2-6: Hormone abundance trends during desiccation in the leaves of *C. pumilum* and *X. schlechteri*........ 27
Figure 2-7: Log2FC of hormone abundances in the leaves and roots of *C. pumilum* (a) and *X. schlechteri* (b) during drying. 43
Figure 3-2: ABA-independent and ABA-dependent abiotic stress response of RD29A and RD29B. 44
Figure 3-3: Percentage identity matrix presenting trends in sequence similarity of transcript variants of MYC2, RD29B, UBQ10 and GAPDH to determine potential biological significance. 54
Figure 3-4: A graphical representation of the respective lengths of each transcript variant associated with the genes under investigation. 56
Figure 3-5: Linear regression models of RT-qPCR standard curves to ensure equal amplification efficiency for each gene of interest and their respective reference genes.. 58
Figure 3-6: Relative gene expression of GASA3 and RD29B in the leaves and roots of *C. pumilum* during a dehydration response.. 61
Figure 3-7: Linear regression models of RT-qPCR standard curves to ensure equal amplification efficiency for MYC2 and SAND reference gene in *A. thaliana*........ 65
Figure 3-8: Relative gene expression of MYC2 in the leaves of *A. thaliana* during full turgor and dehydrated conditions with samples gathered in the morning and afternoon. 66
Figure 4-1: Tentative representation of potential signalling pathways during the dehydration and desiccation responses of *C. pumilum*.. 70

LIST OF ABBREVIATIONS

13-LOX – 13-lipoxygenase

ABA – Abscisic acid

ABI3 – Abscisic acid-insensitive 3

ABRE – ABA-responsive element

AD – Airdry

AOC – Allene oxide cyclase

AOS – Allene oxide synthase

AREB – ABA-responsive element-binding protein

bHLH – basic helix loop helix

CBF – C-repeat binding factor

COI1 – Coronatine-insensitive 1

CTAB - Cetyl trimethylammonium bromide

DREB – Dehydration-responsive element-binding protein

DT – Desiccation tolerance

EGL1 – Enhancer of GLABRA3 1

EtBr – Ethidium bromide

ETH – Ethylene

FT – Full turgor

GA – Gibberellic acid

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GASA3 – GA-stimulated in *Arabidopsis*

GAST1 – GA-stimulated transcript 1

HPLC-ESI-MS/MS – High-performance liquid chromatography electrospray ionization tandem mass spectrometry

HSPs – Heat shock proteins

IAA – Indole-3-acetic acid

JA – Jasmonic acid

JA-Ile – Jasmonyl-isoleucine

JAR1 – Jasmonate-resistant 1

JAZ – Jasmonate-ZIM-domain

LEAs – Late-embryogenesis abundant proteins

Me-JA – Methyl jasmonate

MRM – Multiple reaction monitoring

MYC2 - Myelocytomatosis-related 2

OPDA – 12-oxo-phytodienoic acid

OPR3 – OPDA reductase 3

PCR – Polymerase chain reaction

PLA1 – Phospholipase 1

RD29 – Responsive to desiccation

ROS – Reactive oxygen species

RT-qPCR – Real-time quantitative PCR

RWC – Relative water content

SA – Salicylic acid

SDEG – Significantly differentially expressed

SOD – Superoxide dismutase

UBQ10 – Polyubiquitin 10

α-DOX – α-dioxygenase

α-LeA – α-lenolenic acid

Chapter 1 : Introduction

1.1. <u>Overview</u>

Plants are constantly exposed to harsh or unfavourable abiotic and biotic conditions, which has led to numerous biological adaptations to reduce the negative effects associated with different stressors. Among the different abiotic stressors, water deficit has been one of the most widely studied as it presents some of the greatest risks towards crop productivity. Researchers have thus turned their attention to drought tolerant plants in an effort to understand the morphological, physiological and biochemical adaptations that lend them the ability to survive extreme conditions of water deficit in order to potentially confer these to drought sensitive crops.

Agriculture is largely dependent on angiosperms and although the ability to survive severe water scarcity is present in several plant families, full vegetative desiccation tolerance is rare in angiosperms (Farrant, *et al*., 2020). However, in the past few decades a specialized group of angiosperms that display an astonishing resistance to drought, have been discovered. These plants are termed 'resurrection plants' as they possess the ability to survive nearly complete water loss and can resume full metabolic activity following rehydration (Gaff, 1977). Due to their unique nature, these plants have become models for assessing the underlying biological mechanisms used by vascular flowering plants to overcome extreme drought conditions (Oliver, *et al.,* 2000; Hilhorst & Farrant, 2018).

The investigation of vegetative desiccation tolerant plants has uncovered several molecular mechanisms that higher order vascular plants are able to adopt to mitigate the harsh effects of drought stress. Among these, phytohormone interactions are especially compelling due to their involvement in signalling pathways, their complex crosstalk networks, and their effects on the expression patterns of drought-responsive genes (reviewed in Tiwari, *et al*., 2017).

1.2. <u>Desiccation and resurrection</u>

It is well known that water is a necessary component for the development and survival of all organisms. It is the most abundant molecule in most organisms and acts as the driving force for many crucially important biological process such as photosynthesis, cellular respiration,

temperature regulation, biosynthesis of important chemical structures, for example phospholipids, maintaining cell structure, and many more. Thus, it stands to reason that the ability to mitigate water loss in any organism posits major advantages towards the survival of that organism.

Desiccation tolerance (DT) is a term which describes the unique ability of an organism to survive extreme levels of subcellular water loss (Bewley, 1979). In contrast to drought tolerance, desiccation tolerance is defined as the ability of vegetative tissues to survive dehydration to the air-dry state (below the absolute water content of 0.1 g H_2O. g^{-1} dry mass) in an environment at 50% relative humidity and 20°C (Vertucci & Farrant, 1995; Oliver, *et al.,* 2020).

Many plants have developed adaptations which allow them to survive in environments where resources such as water, are scarce. For example, plants classified as *xerophytes* can be found in arid environments and present a greater number of adaptive mechanisms to drought tolerance than *mesophytes* and *hydrophytes,* which are commonly found in semi-arid and humid environments respectively (Warming, *et al*., 1909). Mechanisms such as those adopted by xerophytes are particularly relevant today following the increased prevalence of drought around the world. The effects of drought can severely impact the growth and yield stability of crops in the field due to significant changes in physiological and molecular processes (Anjum, *et al.*, 2011). Under drought conditions, negative soil water potential in relation to the plant and open stomata result in water loss through the roots and leaves (Anjum, *et al*., 2011). Among other negative effects, this leads to low turgidity, dehydration of the protoplast and subsequent detachment of the plasma membrane from the cell wall, as well as an increased concentration of dissolved molecules (Martínez-Vilalta & Garcia-Forner, 2016). The majority of vascular plant species possess the ability to survive desiccation and mitigate drought stress at some point during their lifecycle. However, this ability is typically confined to tissues such as seeds, spores and pollen (Scott, 2000). Full vegetative desiccation tolerance is far less common, with only 600 land species (including bryophytes, ferns and angiosperms) having been identified to possess this trait (Marks, *et al.*, 2021). It is widely believed that the ability of vascular plants to endure desiccation evolved from mechanisms used by reproductive structures such as seeds and pollen to survive long periods of water scarcity, as desiccation tolerance is one of their most fundamental properties (Illing, *et al.,* 2005; Costa, *et al*., 2017; Van Buren, *et al*., 2018). A distinction should be made here between two types of seeds that differ in their physiological

behaviour; orthodox and recalcitrant seeds, which were classified by Roberts (1973). Recalcitrant seeds usually exist in areas where a high moisture content is maintained and do not necessarily rely on desiccation-tolerance mechanisms, whereas orthodox seeds are able to withstand not only water loss down to the air-dry state, but freezing temperatures as well. It is thus believed that many annual species inherited some degree of desiccation tolerance from orthodox seeds. The biological mechanisms of desiccation tolerance in these seeds is believed to have developed during the final maturation stage of seed development and subsequent dormancy, where almost all water is removed, leading to a state of quiescence, which describes the state during which extremely low levels of metabolic activity occurs (Lynch & Clegg, 1986). When water becomes available in the environment again, the seed is then able to germinate. Because seed desiccation is considered necessary for the completion of the orthodox seed life cycle, it stands to reason that this is an adaptive strategy that came about to allow for the survival and propagation of the plant species in inhospitable conditions. Ultimately, understanding how these processes might occur in vascular tissues is vital for addressing challenges posed by climate change and ensuring the sustainability of plant species in the face of increasing water scarcity.

1.2.1. The nature of resurrection plants

Adaptation to drought stress can be traced back to early terrestrial plant species that evolved from aquatic ancestors that overcame numerous abiotic stresses, including those associated with desiccation, in areas where water was scarce (Niklas, 1999). Through synthetic phylogenetic analyses, Oliver *et al*. (2000) determined that the acquisition of vegetative desiccation tolerance was an important step for the colonization of land by aquatic species. The authors also postulate that algae and primitive bryophytes were among the first to obtain this ability, followed by mosses and ferns. Most stress response mechanisms in angiosperms have come about as a result of evolutionary adaptation and molecular acclimation, where adaptation refers to specialized features that have developed through evolution, such as changes in genotypes and ecophysiologies, and acclimation involves the modification of molecular systems such as gene expression and hormone signalling. The current theory for the development of DT in angiosperms posits that DT is a result of the rewiring of regulatory networks in seeds and vegetative tissues that allows them to inhabit areas with seasonal rainfall (Farrant & Moore, 2011).

Before the discovery of resurrection plants, strategies for desiccation tolerance were already being studied in lower order plant families such as bryophytes, which have come to be known as 'drought evaders'. Bryophytes can be characterized as *ectohydric*, meaning that their desiccation strategy involves, among other variables, large relative water quantities situated in external capillaries, as opposed to water quantity in the symplast (Buch, 1947). The benefits of a strategy where most water conduction occurs externally via detailed capillary architecture, involve cellular spaces continuously receiving water through diffusion when needed as a result of differences in water potential in regions where cellular water is low (Birse, 1957; Richards, 1996). Waxy cuticles surrounding all leaf surfaces in bryophytes further enhance this strategy as water is not easily lost through transpiration. Furthermore, the passiveness of this strategy likely contributes to metabolic energy being focused towards full turgor cellular metabolism rather than water conductance or nutrient storage. This is in contrast to *endohydric* thalloid liverworts and vascular plants, like resurrection plants, that adopt internal water conduction through xylem and phloem. Although a seemingly small anatomical adjustment, this difference in physiological adaptation between bryophytes and vascular plants highlights the wide scope of water deficit strategies abundant in plants. Collectively, understanding these differences as they relate to resurrection plants may contribute to a deeper understanding of plant resilience and adaptability in the face of abiotic stressors and provide valuable insights for future research and biotechnological efforts.

1.2.2. Desiccation tolerance and phytohormone signalling of resurrection plants

Under conditions involving severe dehydration during vegetative tissue desiccation, several stresses arise that require ordered responses to mitigate their effects and ensure the continued survival of the plant. These responses typically aim to reduce damage to plant tissues and to rapidly re-establish metabolic activity via photosynthesis, following rehydration (Oliver & Bewley, 1997; Oliver, *et al*., 1998; Alpert & Oliver, 2002). The water content within a vascular plant is typically regulated through the opening and closing of stomata. Under drought stress, stomatal closure acts as a mechanism to reduce water loss through transpiration. The subsequent effect includes the inhibition of CO_2 diffusion to the chloroplasts and mesophyll cells, resulting in limited CO_2 availability for use in the Calvin cycle and the accumulation of reactive oxygen species (ROS) (Chaves, *et al.,* 2009; Ashraf & Harris, 2013). Unregulated build-up of ROS can lead to uncontrolled, irreparable damage to cellular components,

eventually resulting in the death of the cell (Fu & Huang, 2001; Roach & Krieger-Liszkay, 2014). Two strategies are proposed to have been adopted by resurrection plant species as a means to mitigate these negative effects; poikilochlorophylly and homoiochlorophylly (discussed below).

Vegetative desiccation tolerance can be thought of as existing in two general forms; constitutive or inducible. Constitutive vegetative DT (as seen in non-vascular organisms such as algae and bryophytes) utilises post-desiccation repair mechanisms during rehydration, whereas inducible vegetative DT (as seen in vascular plants) requires modification of biochemical and physiological mechanisms during desiccation (Oliver, *et al*., 2000). Among these mechanisms, phytohormone signalling stands out as one of the most involved, but simultaneously understudied aspects of desiccation tolerance in resurrection plants, as evidenced by the numerous studies that have been conducted to understand phytohormone interactions in the model species *A. thaliana (*reviewed in Waadt, *et al*. (2022)). Research focusing on endogenous phytohormone signalling in resurrection plants is scarce and most research has focused on abscisic acid (ABA), which has been put forward as being one of the key regulator hormones in dehydration responses in resurrection plants (Bartels & Hussain, 2011). This identified research gap, along with a comprehensive exploration into phytohormone signalling in resurrection plants is discussed at length in the next chapter.

1.2.3. Model resurrection plants for research on desiccation tolerance

The poikilochlorophyllous species *Xerophyta schlechteri* and homoiochlorophyllous species *Craterostigma pumilum* have both been classified as resurrection plants. Due to their evolutionarily distinct strategies to survive nearly total desiccation, these two species present the unique opportunity to understand the many strategic molecular mechanisms, such as changes in phytohormone abundances, that resurrection plants can call upon to survive desiccation.

i. The poikilochlorophyllous plant Xerophyta schlechteri

The monocot resurrection species *Xerophyta schlechteri* (Baker) N.L. Menezes, previously erroneously called *Xerophyta viscosa* in some instances, has become one of the most well-studied poikilochlorophyllous species to date, with its full genome having been made available

in the past few years. *X. schlechteri* is a perennial herb which forms a part of the Velloziaceae family and is native to Zimbabwe, Botswana and the upper region of South Africa (Herman & Retief, 1997). This resurrection plant tends to occupy mostly grassland and rocky crevices where water is typically scarce (Herman & Retief, 1997).

Similarly to other poikilochlorophyllous resurrection species, *X. schlechteri* mitigates extensive water deficit stress by reducing photooxidative stress through breaking down its photosynthetic apparatus (chlorophyll and thylakoid membranes), which is then reconstituted upon rehydration (Gaff, 1977; Sherwin & Farrant, 1998; Farrant, 2000; Farrant, *et al*., 2003). In this way, photosynthesis is inhibited in the early stages of drying (to ~55% RWC), with an increase in antioxidants and metabolic redirection towards the production of sugars such as sucrose and raffinose (Farrant, *et al*., 2015). During late stage desiccation (to ~10% RWC), the leaves of *X. schlechteri* typically show an increase in proteins involved in signal transduction as well as those that have become known as desiccation or stress-associated proteins such as heat shock proteins (HSPs) and late embryogenesis abundant proteins (LEAs). The disadvantages of this strategy relate to the costly need for reassembly of the photosynthetic apparatus upon rehydration via *de novo* translation, resulting in delayed recovery following a desiccation response (Dace, *et al*., 1998; Ingle, *et al*., 2008).

ii. The homoiochlorophyllous plant Craterostigma pumilum

Craterostigma pumilum Hochst is a perennial dicot resurrection plant originating from the family Linderniaceae. A systematic review of well-studied resurrection plants by Tebele *et al*. (2021) explains that *C. pumilum* remains one of the more understudied dicotylous resurrection plants. This is potentially a result of researchers' attention towards the related species *C. plantagineum,* which was shown to be the most well-studied resurrection in the last few years. *C. plantagineum* was among the earliest resurrection plants to be discovered and was first analysed by Bartels *et al*. (1990). To date, although little research has been done on understanding the molecular mechanisms involved in the desiccation response of *C. pumilum*, there is growing interest in this topic, with Christ *et al*. (2014) and Zia *et al*. (2016) having investigated protection of the photosynthetic apparatus, Oung, *et al*. (2022) having explored ontogenetic effects at the thylakoid membrane level and du Toit *et al*. (2021) having examined physiological desiccation-associated leaf tissue differentiation.

In contrast to poikilochlorophylly, homoiochlorophyllous resurrection species employ a physiological strategy whereby they retain most of their chlorophyll and thylakoid membranes, and instead undergo leaf folding while accumulating anthocyanins, xanthophylls and polyphenols in an effort to reduce chlorophyll exposure to light (Smirnoff, 1993; Sherwin & Farrant, 1998; Farrant, 2000; Farrant, *et al.,* 2003; Georgieva, *et al*., 2009).

1.2.4. Understanding phytohormone interactions utilizing the model plant *Arabidopsis thaliana*

Due to research on phytohormones in resurrection plants being so limited, utilizing extensive data collected on phytohormone signalling in the model plant *A. thaliana* may provide a potential starting avenue for understanding what may be occurring in more complex non-model organisms such as *X. schlechteri* and *C. pumilum*. It has been shown that several hormones are involved in key regulatory stages during dehydration in *A. thaliana*. For example, phytohormones such as ABA (Yang, *et al*., 2014), have been documented to be involved in chlorophyll degradation in *A. thaliana,* which could serve as a foundation on which to understand phytohormone abundances in a pokilochlorophyllous strategy. The mechanisms by which this regulation occurs could be used as a blueprint to understand potentially more complex interactions in resurrection plants. At the time of writing, there are currently no publications exploring how using protocols intended for the analysis of the molecular components of *A. thaliana*, can or should be utilized to explore resurrection plants. Thus, in addition to exploring phytohormone signalling during dehydration and desiccation in resurrection plants, this thesis will also briefly explore the utilization of protocols developed for analysing *A. thaliana* and how effective they may be for further researching resurrection plants.

1.3. Research aims and objectives

Given that much of the groundwork laid in elucidating the specialized mechanisms adopted by plants to mitigate drought stress (including signalling via phytohormones), has focused on studying model organisms such as *A. thaliana*, this research aims to expand on this by evaluating the phytohormone profiles of two evolutionarily distinct resurrection plants as they are subjected to extreme drying. Potential insights into the mechanisms by which homoiochlorophyllous resurrection plants in particular integrate stress signalling will also be expanded upon by assessing expression of particular mediator genes involved in jasmonate stress signalling and how they may be interlinked with other hormone signalling pathways. This project thus has four main aims:

1) Establishing dry-down curves and accompanying morphological characteristics during the early dehydration and more severe desiccation of the homoiochlorophyllous plant *Craterostigma pumilum* and the poikilochlorophyllous plant *Xerophyta schlechteri*.

2) Producing phytohormone abundance profiles during the phases of dehydration and desiccation within these two species by utilizing High Performance Liquid Chromatography Electron Spray Ionization Mass Spectrometry (HPLC-ESI-MS/MS), in order to elucidate potential differences in stress-signalling strategies adopted by homoiochlorophyllous and poikilochlorophyllous resurrection plants.

3) Measuring the expression of genes that have been hypothesized to participate in jasmonate stress signalling and crosstalk with other signalling pathways; myelocytomatosis-2 (*MYC2*), responsive-to-desiccation-29-B (*RD29B*), and gibberellic-acid-stimulated-arabidopsis-3 (*GASA3*).

4) Assessing the effectiveness of relying on data from model species such as *A. thaliana* to predict dehydration response trends in understudied angiosperm species through comparing results of this thesis to those of well-established theories about water deficit responses expanded from research on *A. thaliana*.

Chapter 2 : Phytohormonal control mechanisms of desiccation responses in plants

2.1. Introduction

As mentioned in the introductory chapter, the evolution of plants has led to specialised adaptations whereby they are able to mitigate a variety of stresses brought about by unfavourable abiotic conditions. The ability to do so involves several key steps ranging from the perception of stress by the plant to regulating its effects, typically via signal transduction. There are many strategies plants can adopt to successfully survive severely unfavourable conditions. On a molecular level, they are able to manipulate their gene expression, process transcripts and control protein translation and modification, which in turn affects signalling pathways and the production of specific metabolites (Dong, *et al.*, 2022). One of the most important, yet simultaneously under-researched primary elements of these cascades includes the large variety of phytohormones often associated with abiotic stress responses.

The term phytohormones describes a set of small organic compounds that encompass a number of functions including, *inter alia*, the regulation of plant growth, germination, cell division and stress resistance, with most processes requiring the cooperation of numerous phytohormones at once (summarised in Miransari (2011)). The most well-documented classes of stress-responsive phytohormones include ABA, auxins, cytokinins, gibberellins (GAs), jasmonic acid (JA), salicylic acid (SA) and ethylene (ETH). However, due to limitations regarding acquiring standards for the assessment of each of these via HPLC-ESI-MS/MS, this thesis will only focus on GA, ABA, JA and SA along with some of their derivatives, i.e. GA3 and GA4, jasmonyl-isoleucine (JA-Ile), and 12-oxo-phytodienoic acid (OPDA). Although there are large gaps in our understanding regarding the mechanistic basis of abiotic stress, it is generally accepted that fluctuations of stress-responsive phytohormones, along with their biosynthetic pathways (Figure 2-1), play a central role in the coordination of stress response via signal transduction and crosstalk, resulting in the activation of a common second messenger or a phosphorylation cascade (Khan, *et al.*, 2012).

2.1.1. Phytohormones as master signalling molecules during a desiccation response

i. Gibberellic acid

The gibberellic acids describe a large group of tetracyclic diterpenoid acids that remain functional throughout the lifecycle of a plant. Their main functions include stimulating plant growth and development through cell division and elongation, seed germination and reproductive development (Colebrook, *et al*., 2014). Although these functions are not inherently related to drought tolerance, there has been evidence to suggest a role for GAs during drought response. For example, inhibition of endogenous GA biosynthesis has been reported to result in reduced growth and conferred drought tolerance in beans (Halevy & Kessler, 1963) and *A. thaliana* (Magome, *et al*., 2004).

The predominant bioactive forms of GA include GA1, GA3, GA4 and GA7, all of which are synthesised, beginning in the plastid, from trans-geranylgeranyl diphosphate (Figure 2-1). To date, most evidence seems to suggest that a set of genes termed GA2ox genes are mostly responsible for the response of GA biosynthesis to abiotic stress (Kasahara, *et al*., 2002; Binenbaum, *et al*., 2018). Regulation of GA-mediated gene expression is typically controlled via DELLA proteins (Figure 2-2), which are responsible for the repression of plant growth and development, thus conversely, upon dehydration stress, the abundance of GAs tends to decrease while DELLA proteins accumulate (Achard, *et al*., 2006). Similarly, upstream negative regulation of GA occurs via a protein called SPINDLY (Qin, *et al*., 2011). Through these regulatory pathways, the means by which GAs may be utilised during a dehydration response in many plants seems to be via restriction of growth and subsequent conservation of energy.

ii. Abscisic acid

Abscisic acid is a phytohormone responsible for the regulation of many important processes involved in biotic and abiotic stress responses (Adie, *et al*., 2007; Hirayama & Shinozaki, 2007). Two discoveries by Wright & Hiron (1969), and Mittelheuser & van Steveninck (1969), highlighted the potentially major role of ABA in the plant stress response through stomatal control, and this work was later supported by Artsaenko *et al*. (1995), who found that ABA has an effect on the endoplasmic reticulum and subsequent stomatal closure.

The angiosperm *A. thaliana* has served as the model organism under study for the fundamental understanding of ABA signal transduction with three major core components having been identified. Briefly, in the basic model of ABA-signalling during abiotic stress response, when endogenous ABA is present, a family of START proteins, (1) PYR/PYLs, inhibits phosphatase activity through interacting with clade A PP2Cs (Ma, *et al.,* 2009; Park, *et al.*, 2009). This allows for the activation of a group of (2) kinases (SnRK2s) which then phosphorylate (3) ABF/AREB transcription factors (Mustilli, *et al.*, 2002; Yoshida, *et al.*, 2002; Yoshida, *et al.*, 2006). Active ABFs are then able to bind to ABA-responsive promotor elements (ABREs) to induce expression of ABA-responsive genes.

The functions of ABA during stress response are known to be extremely broad and interconnected with many other pathways, making deciphering their exact mode of action a difficult task and there is extremely limited data available on the effect of ABA (and many other phytohormones) in resurrection plants. However, extensive early research led to the hypothesis that the *Arabidopsis* abscisic acid-insensitive (*ABI3*) gene is responsible for the activation of downstream ABA-inducible genes during dehydration in *A. thaliana* (Ooms, *et al*., 1993; Parcy, *et al*., 1994; Furini, *et al*., 1996). This theory was tested by Chandler & Bartels (1997) who identified that the product of an *ABI3* homolog in the homoiochlorophyllous resurrection plant *C. plantagineum* was indeed able to activate LEA-type genes in transient expression assays. Expression of this gene was not, however, detected in fully developed leaves, leading to the conclusion that additional transcription factors are likely involved in LEA-gene activation. Other very recent publications showcased similar results indicating that tight regulation of ABA occurs during water deficit in the resurrection plant *Selaginella pulvinate* (Yu, *et al*., 2024) and *Barbacenia graminifolia* (Vieira, *et al*., 2024). This research illustrates the complexities associated with polyploid species as well as the intricacies of plant regulatory networks. It also provides some evidence to suggest that *A. thaliana* could be used as an effective model for identifying and isolating genes that may be present in the desiccation response of resurrection plants.

iii. Jasmonic acid

The biosynthesis and relationship of JA with drought response is discussed at length in Chapter 3.

iv. Salicylic acid

Salicylic acid (SA) is a phenolic compound comprising an aromatic ring, carboxyl group and hydroxyl group. The involvement of endogenous SA during water deficit response has been demonstrated in several studies (Munné-Bosch & Peñuelas, 2003; Bandurska & Stroi ski, 2005; Miura, *et al*., 2013). In addition, exogenous application of SA has also been shown to be effective in improving drought resistance in wheat, but only when applied at low concentrations (Al-Hakimi & Hamada, 2001). It is proposed that high levels of SA induce ROS production in photosynthetic tissues, resulting in increased oxidative stress in place of improved drought resistance (Borsani, *et al*., 2001). A recent review by Song *et al*. (2023) summarised several publications having explored the role of SA in abiotic stress responses, many of which conclude that the overall roles of SA as it relates to stress include (1) activating plant defence mechanisms through the production of secondary metabolites (Chávez-Arias, *et al*., 2022) and upregulating antioxidant activity (Alam, *et al*., 2022), (2) improving photosynthetic efficiency (Nazar, *et al*., 2015; Khalvandi, *et al*., 2021), and (3) promoting drought-induced leaf senescence (Abreu & Munné-Bosch, 2008).

The reported effects of SA thus remain somewhat broad and unspecific, with virtually no insights into these mechanisms in resurrection plants. Publications that do comment on the putative roles of salicylic acid in resurrection plants also make mention of the broad effects as listed above, but most attention is given to the mechanisms of ABA and JA (Bartels & Hussain, 2011; Djilianov, *et al*., 2013; Yu, *et al*., 2024; Vieira, *et al*., 2024).

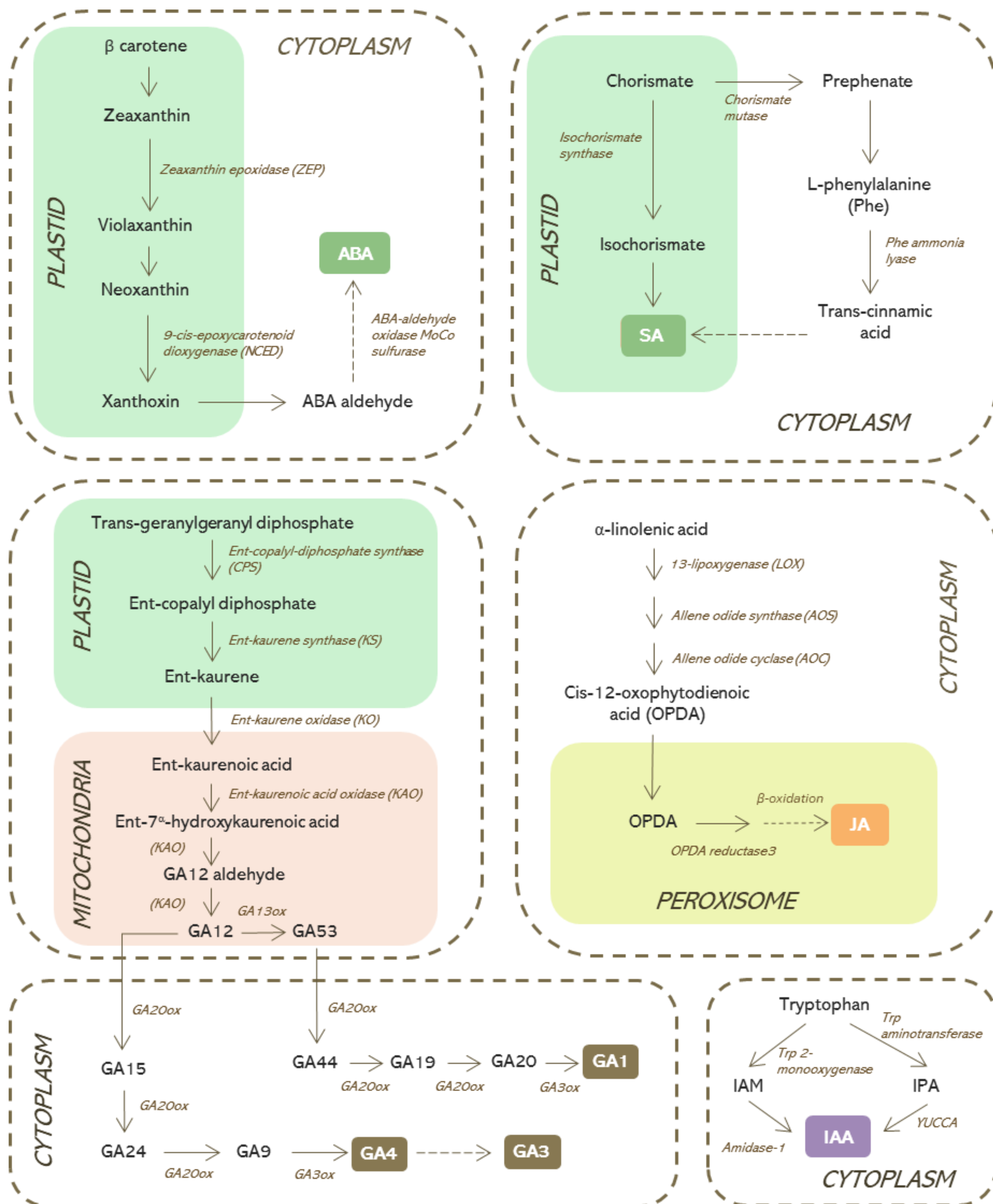


Figure 2-1: Simplified overview of typical phytohormone synthesis. *The above figure depicts the biosynthesis pathways of abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), the gibberellins GA3, GA4 and GA1, and IAA. This figure shows the compartmentalisation of these pathways within subcellular organelles and was adapted from Salvi et al. (2021).*

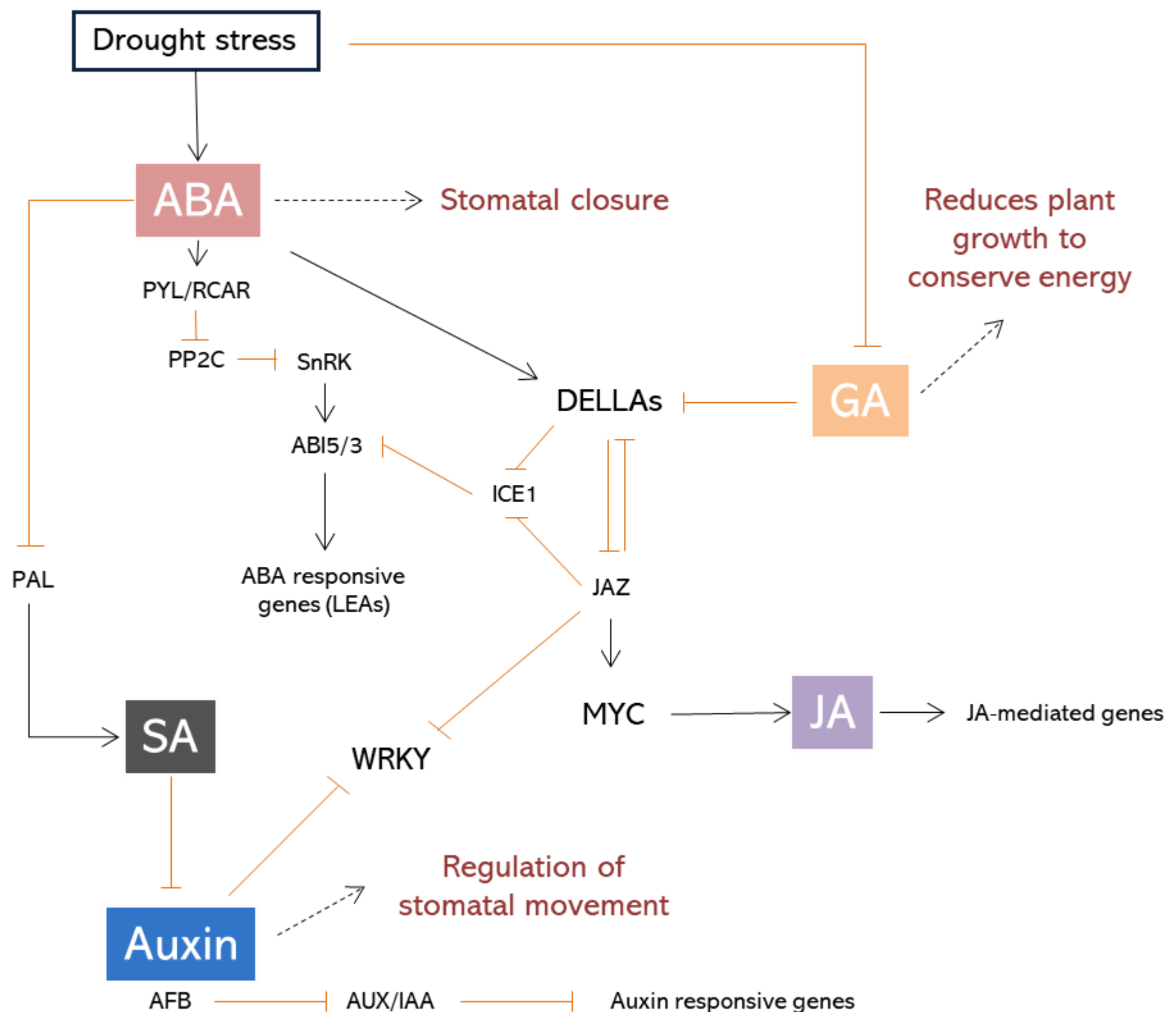


Figure 2-2: Schematic representation of phytohormone crosstalk during a dehydration response. *This figure very briefly illustrates the most commonly referenced pathways for phytohormone crosstalk between abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), the gibberellins (GA) and auxin, that is available in the literature and was adapted from Verma, et al. (2016), Liu & Timko (2021), and Salvi, et al. (2021).*

2.1.2. The use of phytohormones for the enhancement of crops through biotechnology

As the human population continues to grow, so too does the demand for identification of new potential targets for biotechnological crop enhancement that will lead to increased crop survival, nutrition and yield. This need emphasises the demand for innovative biotechnological engineering able to mitigate the effects of biotic and abiotic stresses. Successful utilisation of engineered phytohormones in a number of studies has shown promise.

The overproduction of a GA catabolic enzyme, GA2-oxidase, was used to produce semi-dwarf rice and wheat which allowed for higher-yielding cultivars (Silverstone & Sun, 2000). ABA stress-responsive genes such as C-repeat binding factor (CBF) and dehydration-responsive element-binding (DREB) genes have been modulated to confer enhanced drought tolerance in *A. thaliana* (Park, *et al*., 2008) and tomato (Lee, *et al*., 2003). Overexpression of the YUCCA6 gene, which is involved in tryptophan-dependent IAA (auxin) biosynthesis was associated with rapid shoot growth, reduced ROS and increased drought tolerance in transgenic poplars (Ke, *et al*., 2015). The examples above indicate that most exploration in dehydration tolerance focuses on increasing the yield of cultivars as opposed to improving their survivability during harsh conditions. Furthermore, despite their established association with drought tolerance there are very limited studies focused on implementing GA, JA or SA in biotechnology for the conference of drought tolerance to crop plants. These factors highlight the need for further investigation into these avenues.

2.2. Materials and methods for the analysis of phytohormone profiles in the leaves and roots of two resurrection plant species

2.2.1. Hormone profiling in the vegetative tissue of *X. schlechteri* and *C. pumilum*

i. Plant material, growth conditions and seed germination

Seeds of *X. schlechteri* and *C. pumilum* were germinated on Jiffy® peat pellets within 12-pot propagators with transparent closed lids to maintain humidity. *X. schlechteri* seeds were collected by the Plant Stress Laboratory at University of Cape Town and *C. pumilum* seeds were collected by the Department of Biomolecular Science at the Weizmann Institute of Science in Israel and supplied by a former PSL student. After five months, 15 plants of each species were re-potted in 500 mL plant pots using a blended compost and bark potting soil (Reliance, RSA). Germination and subsequent plant growth occurred under controlled conditions at 24°C, 55% humidity, ~115 PAR ($mol.m^{-2}.s^{-1}$) light intensity, and a 16/8hour light/dark cycle. Plants were allowed to grow under well-watered conditions for a further four months before the commencement of sampling.

Table 2-1: Seed collection data. *X. schlechteri and C. pumilum seed identification numbers and date of collection.*

X. schlechteri		
Seed ID	**Collection Date**	**Collection location**
XV4-LF99	02/2023	South Africa
XV8-LF99	02/2023	South Africa
XV12-LF99	11/2022	South Africa
XS8-LF02	11/2022	South Africa
XS17-LF22	02/2023	South Africa
C. pumilum		
Seed ID	**Collection Date**	**Collection location**
N/A	2020	Israel

ii. Dehydration treatment and sampling

All samples were collected while plants were in their vegetative states at the same time each collection day. At the beginning of the dehydration treatment, plants were watered and root and leaf tissue collected the following day to represent the full turgor (FT) condition. Plants were then subjected to dehydration by withholding water. A Procheck soil probe (Decagon Devices, USA) was used to determine the time point at which the volumetric water content in the soil dropped below 0%. Leaf and root tissue were collected at regular intervals throughout the dehydration and desiccation time course, using morphological cues to represent stages of approximately 80%, 60%, 20% and air-dry (AD) relative water content (RWC). Because the collection of root tissue resulted in the ultimate death of each plant, tissues were only sampled once per plant.

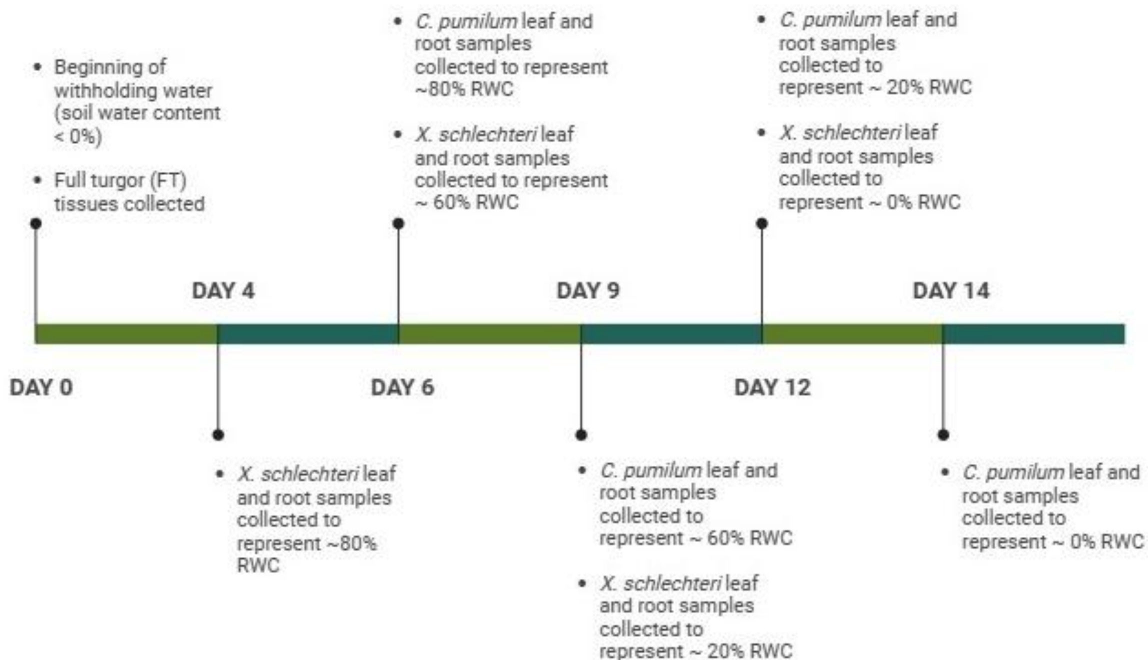


Figure 2-3: Plant tissue sampling timeline. *Visual representation of leaf and root tissue sampling of X. schlecteri and C. pumilum during dehydration treatment from Day 0 to Day 14.*

Tissue was immediately flash frozen in liquid nitrogen and stored at -80°C. Three leaf replicates were also collected at each time point to calculate the relative water content (RWC) using the following equations:

$$Relative\ Water\ Content\ (RWC) = \frac{Actual\ Weight_D\ (AW_D)}{Actual\ Weight_{FT}\ (AW_{FT})} \quad 1$$

$$Actual\ Weight\ (AW) = \frac{Fresh\ Weight\ (FW) - Dry\ Weight\ (DW)}{Dry\ Weight\ (DW)} \quad 2$$

Because leaf tissue of *X. schlechteri* does not readily absorb water during typical incubation in water in darkness, the turgid weight cannot be accurately calculated (Radermacher, *et al*., 2019). Thus, the AWC of fully hydrated leaf and root tissue from both *X. schlechteri* and *C. pumilum* sampled at artificial dawn, was calculated (AWC_{FT}) and used to reflect the full turgor condition. At each sampling point, leaf and root tissue were weighed to determine their fresh weight (FW) and then again following drying at 70°C for 48 hours to obtain the dry weight (DW). These results were then used to determine their actual weight (AW_D) and subsequent RWC.

iii. Phytohormone extraction and preparation

Hormones were extracted from both leaf and root tissues of *C. pumilum* and *X. schlechteri* using the below protocol as outlined by Pan, *et al.* (2010).

Working solutions for all internal standards (Appendix A) were diluted to 1 µg mL^{-1} in methanol and combined to form a single solution containing all hormone standards. A 2:1:0.002 (*v/v/v*) extraction solvent was also made by adding 100 µL of concentrated hydrochloric acid to 100 mL of 2-propanol and 50 mL of distilled water.

Approximately 50 mg of plant tissue was ground to a fine powder in liquid nitrogen using a pestle and mortar and transferred to 2 mL screw-cap vials. To this, 50 µL of the abovementioned working solution of internal standards (Appendix A) was added, followed by 500 µL of the extraction solvent. The samples were then incubated on a shaker at 4°C at 100 RPM for 30 minutes. After incubation, 1 mL of dichloromethane was added to each sample and the samples were left to incubate at the same speed at 4°C for a further 30 minutes. Samples were then placed in a microcentrifuge and spun at 4°C at 13 000 x *g* for 7 minutes. After the formation of distinct phase separation, approximately 900 µL of the lower phase solvent in

each sample was transferred to a new 1.5 mL tube and the subsequent samples were concentrated using a nitrogen evaporator and stored at -20°C for later analysis.

iv. Quantification of phytohormones via High Performance Liquid Chromatography Electrospray Ionisation Tandem Mass Spectrometry (HPLC-ESI-MS/MS)

Hormone analysis on extracted samples was performed in the Quantitative Lipidomics Facility of the University of Bonn. Dried samples were resuspended in 100 µL methanol:H_2O (1:1, *v/v*, with 0.1% formic acid) and then subjected to separation on a reverse-phase C_{18} Gemini HPLC Column (5 µm particle size, 150 x 2.00 mm, Phenomenex) for analysis using HPLC-ESI-MS/MS.

The quantification of phytohormones through mass spectrometry utilized a QTRAP 6500+ LC-MS/MS system (Sciex, Germany) equipped with a Tubo V ion source and an Agilent 1260 Infinity quaternary pump.

v. Equipment setup for High Performance Liquid Chromatography (HPLC)

The HPLC column was set to a temperature of 40°C and the solvent flow rate to 0.3 $mL.min^{-1}$. The mass spectrometry conditions were set as follows: source was set to turbo spray; ion polarity set to negative; needle voltage set at − 4,500 V; ion source temperature set at 150°C; the gas used was nitrogen; the collision gas was set to medium; the curtain gas was set to 25 psi; and the nebulizing gas and heater gas were set at 25 psi.

The parameters for multiple reaction monitoring (MRM) were set as follows: the declustering potential was set at -80 V; the entrance potential was set at -10 V; the cell exit potential was set at -8 V; and collision energies were optimized for each phytohormone (Appendix A).

vi. Data processing and statistics

R version 4.4.1 (R Core Team, 2020) was used in conjunction with the "ggplot2" package (Wickham, 2016) to plot the RWC of leaf and root tissues against the respective day of sampling for each condition for both *X. schlechteri* and *C. pumilum* to generate a visual dehydration curve. The figure was altered using the smoothing function "loess" and the selected theme was "minimal". Individual replicates were plotted along with sample standard deviation to represent variation among the samples.

Phytohormone concentration was captured by MultiQuant 3.0.2 software (Sciex, Germany) under different RWC conditions and this data was transferred to Microsoft® Excel® and finally imported to R using the “read.table” function. Using R version 4.4.1. (R Core Team, 2020), the individual datasets for hormone abundance in each species were merged and annotated with dataset identifiers using the “mutate” function from the “tidyverse” package (Wickham, *et al.*, 2023).

Summary statistics including the mean concentration and standard deviation were computed for each hormone class across the different dehydration treatments. The “ggplot2” package (Wickham, 2016) was used for plotting.

Normality of the concentration of each phytohormone was assessed using the Shapiro-Wilk test. It was concluded that all individual hormone data sets showed non-normal distribution and thus Kruskal-Wallis tests were performed to compare phytohormone abundance across the different RWC conditions, followed by the post-hoc Dunn test.

2.3. Results and Discussion

2.3.1. *Xerophyta schlechteri* and *Craterostigma pumilum* adopt morphological strategies to circumvent drought stress

To determine the differences in morphological phenotype between *X. schlechteri* and *C. pumilum*, photographs were taken during a dehydration time course (Figure 2-4). It should be noted that this chapter refers to both dehydration and desiccation stages, where early dehydration refers to a RWC around 80%, mid dehydration refers to around 60%, late stage dehydration referring to below 20% and desiccation describing the air-dry stage. At full turgor, the whorled leaves of *C. pumilum* appeared broadly ovate, with a narrow base resembling the petiole. The leaf cuticle also presented as slightly waxy and individual leaves contained trichomes near the leaf tips.

Figure 2-4: Stages of desiccation in C. pumilum (top) and X. schlechteri (bottom). *Photographs were taken at a side-angle immediately following the plants' removal from the soil at (a) full turgor (≥ 80% RWC), (b) ~80% RWC, (c) ~60% RWC, (d) ~20% RWC and (e) air-dry state (≤ 20% RWC).*

Following cessation of watering, desiccation occurred more slowly in *C. pumilum* than *X. schlechteri,* with *C. pumilum* only reaching the airdry state two days after *X. schlechteri.* These trends are similar to those reported by Farrant, *et al*. (2015), Radermacher, *et al* (2019), Gabier, *et al*. (2021), and du Toit, *et al*. (2021). Similarly, *X. schlechteri* began to show initial signs of dehydration stress at ~80% RWC, two days before *C. pumilum*, in the form of yellowing at the tips of the leaves. During this phase, both plants showed slight discolouration in their leaves

and roots. The roots started to become more rigid and held less soil when extracted from their pots.

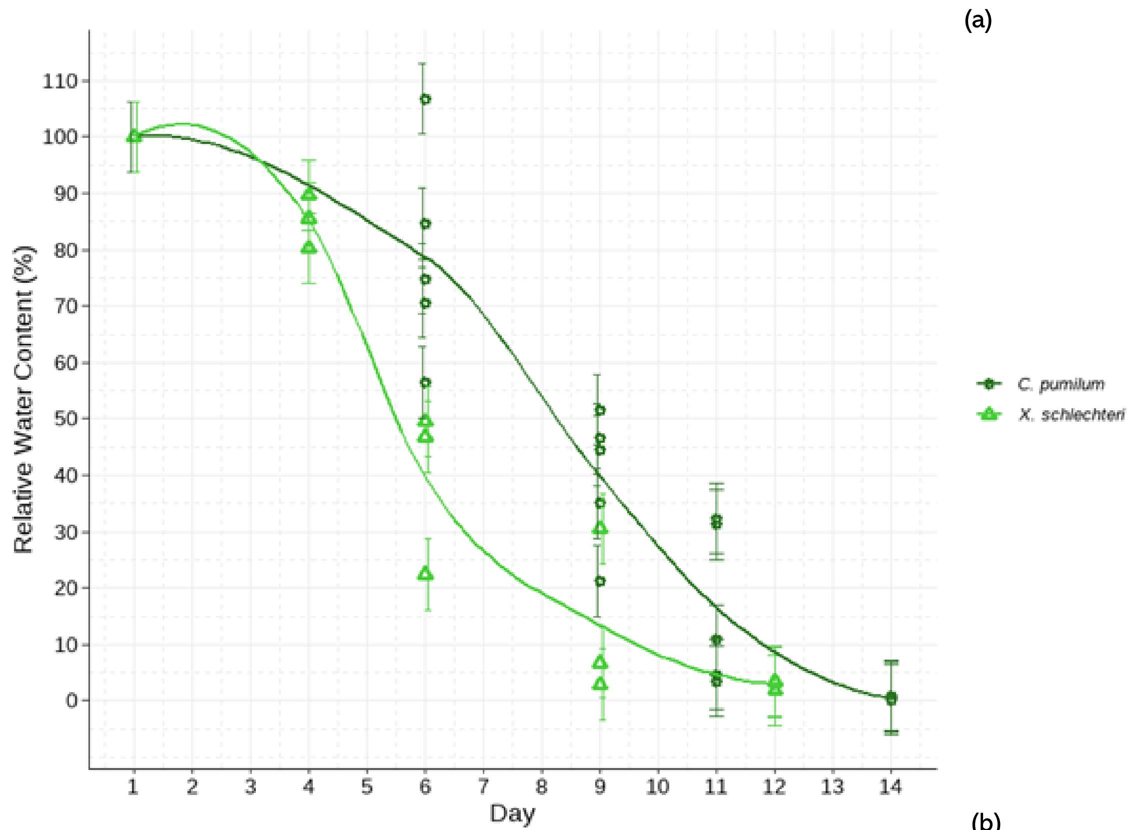
During the stage of mild stress (~60% RWC), the leaves of *C. pumilum* showed a more substantial loss of pigmentation accompanied by a clear loss of turgidity as well as leaf shrinkage while folding into themselves. A characteristic trait of anthocyanin accumulation was evident in *C. pumilum* at ~20% RWC. In addition, the leaves of *C. pumilum* adopted an elastic type of morphology where they became 'rubbery' and malleable, making them more difficult to rip or tear. At this point, *X. schlechteri* also began to show more apparent characteristics of desiccation stress in the form of leaves beginning to appear senescent and anthocyanin accumulation along the longitudinal dimension of the leaves, accompanied by folding along the midrib, exposing only the abaxial surface. During the committed desiccation response (≤ 20% RWC), the roots of both species, especially *C. pumilum*, became brittle and fragile with far fewer complex networks (Figure 2-4d). The leaves of desiccated *C. pumilum* plants showed significant shrinkage, hardening and anthocyanin accumulation in relation to their full turgor counterparts, and the mostly senesced leaves of *X. schlechteri* became pale and showed further signs of anthocyanin accumulation.

2.3.2. Assessment of dehydration and relative water content (RWC)

The leaves of *C. pumilum* displayed a slightly more gradual rate of water loss when compared to the leaves of *X. schlechteri* (Figure 2-5a). This trend is also reflected in Figure 2-4 where the leaves and roots of *C. pumilum* showed markedly fewer signs of dehydration stress around ~60% RWC, i.e. less visible anthocyanin accumulation, less discolouration, and a denser root network.

It is not typical in desiccation tolerance research to assess the relative water content in the roots of plants; however, in the interest of thoroughness and to potentially better explain gene expression or hormone changes present in the roots in downstream experiments, a similar dehydration curve (Figure 2-5b) was produced for the roots in both plants. The rate of water loss is greater in the roots of both species relative to the leaves. The relative pattern of water loss is also similar between the leaves and roots of *X. schlechteri*, meaning that the rate of water loss is comparable at the relevant time points e.g. there is a similarly steep drop in RWC from

~80% to ~60% in a short space of time in both tissues. Conversely, the pattern of water loss in the roots of *C. pumilum* did not follow the same shape as the leaves, with a notably rapid loss of water directly following cessation of watering (Figure 2-5b).



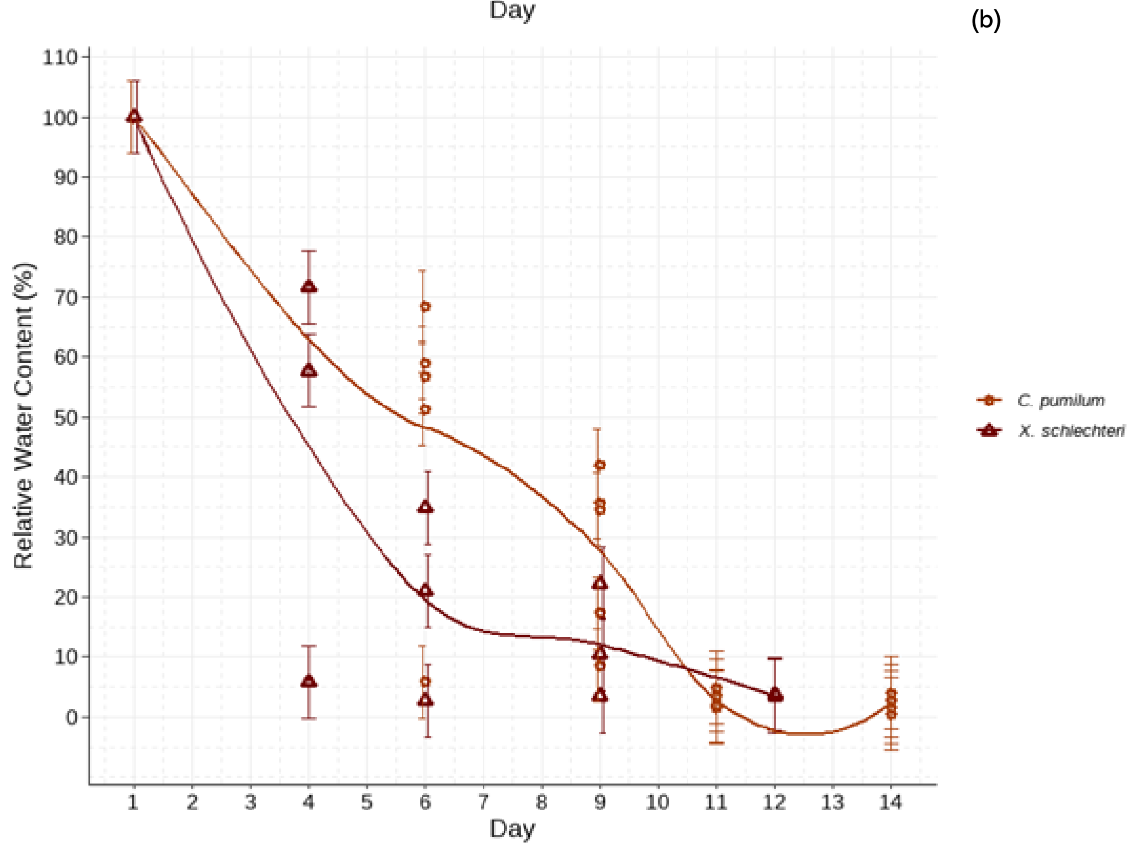


Figure 2-5: Dehydration curves of C. pumilum and X. schlechteri. *Shown above are the representative stages of desiccation of the (a) leaves and (b) roots of C. pumilum and X. schlechteri as evidenced by their relative water content (RWC) in relation to the number of days following*

cessation of watering. C. pumilum n = 4, X. schlechteri n = 3. Individual replicates are plotted with standard deviation.

2.3.3. Hormone trends

In order to investigate the hormonal profiles that emerge during different stages of desiccation in the homoiochlorophyllous plant *C. pumilum* and the poikilochlorophyllous plant *X. schlechteri,* phytohormones were isolated and analysed via HPLC-ESI-MS/MS at various time points. The multitude of hormones under study (ABA, GA3, GA4, JA, JA-Ile, OPDA and SA), showed differences in abundance and expression trends during desiccation in both *C. pumilum* and *X. schlechteri*. Additionally, it was found that there were large variations in the abundance of these hormones in relation to one another specifically, as evidenced by the notably different axes scales seen in Figure 2-6. In addition to the statistically non-normal distribution of hormone data mentioned in Section 2.2., these results indicate that the hormone abundance tests show high sensitivity, picking up high degrees of variation between samples. Future tests should aim to mitigate this by including additional replicates for non-model species.

i. The gibberellins

Two gibberellins (GA3 and GA4) were assessed here to elucidate their potential functions as water deficit stress signalling molecules as they relate to differing plant stress response strategies.

The abundance of GA3 in the leaf tissue of *C. pumilum* exhibits a pronounced increase during the initial stages of dehydration, followed by a decline as the plant enters the mid dehydration phase below 60% RWC (Figure 2-6a & Figure 2-7a). In contrast, GA4 shows a gradual reduction corresponding with a decrease in water content of the leaves. These individual trends imply that GA3 and GA4 may engage in distinct signalling pathways and modulate separate mechanisms associated with desiccation tolerance. Lee & Kende (2001) have demonstrated that GA3 influences the plasticity of cell walls in rice by promoting the expression of expansin genes, which govern the long-term extension and relaxation of cell wall structures. Expansin proteins have also been mentioned by Moore *et al*. (2013) and Moore *et al*. (2024), who suggest a subtle remodelling approach that is adopted by *C. wilmsii* and *C. plantagineum*, wherein expansin proteins along with xyloglucan transhydrolases are utilized to plasticize cell walls during desiccation-induced cell wall folding. In light of this, it could be argued that gibberellin plays a role in priming cell walls in response to stress induced by water loss through the

promotion of expansins. In addition, several studies have suggested that the most bioactive form, GA3, is involved in enhancing antioxidant enzymes, increasing the content of proteins, glucosinolate, and unsaturated fatty acids such as linolenic acid, which is the precursor of JA, another important phytohormone.

Both gibberellins expressed similar patterns of abundance in the root tissue, with both showing significantly reduced levels during both early and late desiccation stages (Figure 2-6b & Figure 2-7a). This suggests that GA3 and GA4 may interact with the same or similar pathways during a desiccation response in the roots of *C. pumilum* and perform similar functions. By reducing growth in the roots during water deficit stress, the plant is able to put more energy towards mechanisms that reduce the effects of the stress; for example, through promoting the removal of ROS. In addition, many phytohormones tend to be transported from the roots to the leaves via the xylem during a stress response (Stoll, *et al*., 2000) and so it is again likely that the expansion of the root system is not a priority to mitigate drought stress in this species. However, this decrease in gibberellin does coincide with findings from Norwood *et al*. (2003), who posit that poikilohydric plants such as *C. plantagineum,* a close relative of *C. pumilum,* experience damage to secondary and tertiary roots during desiccation, but are able to maintain their primary roots which then go on to produce an entirely new root network upon rehydration.

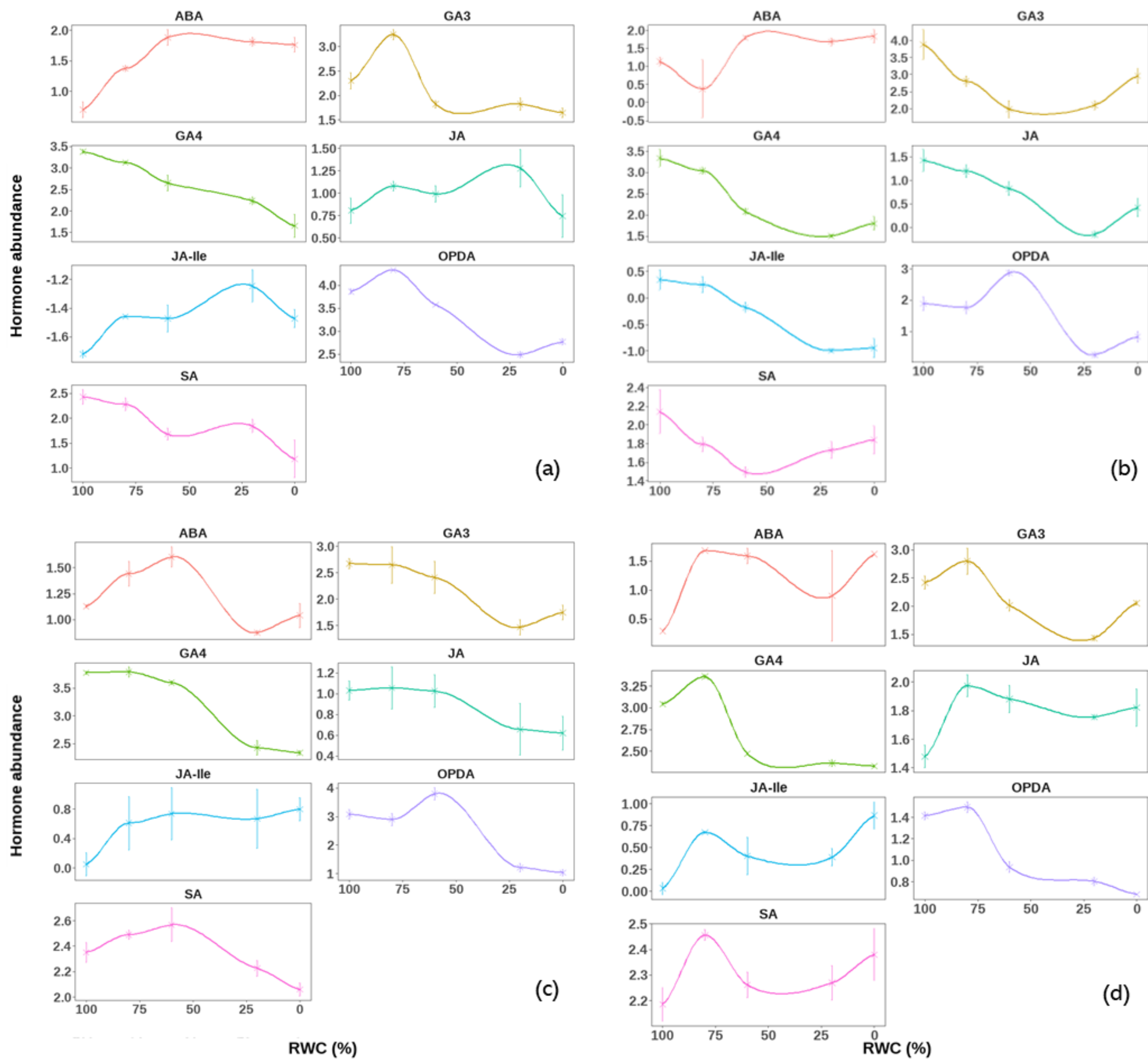


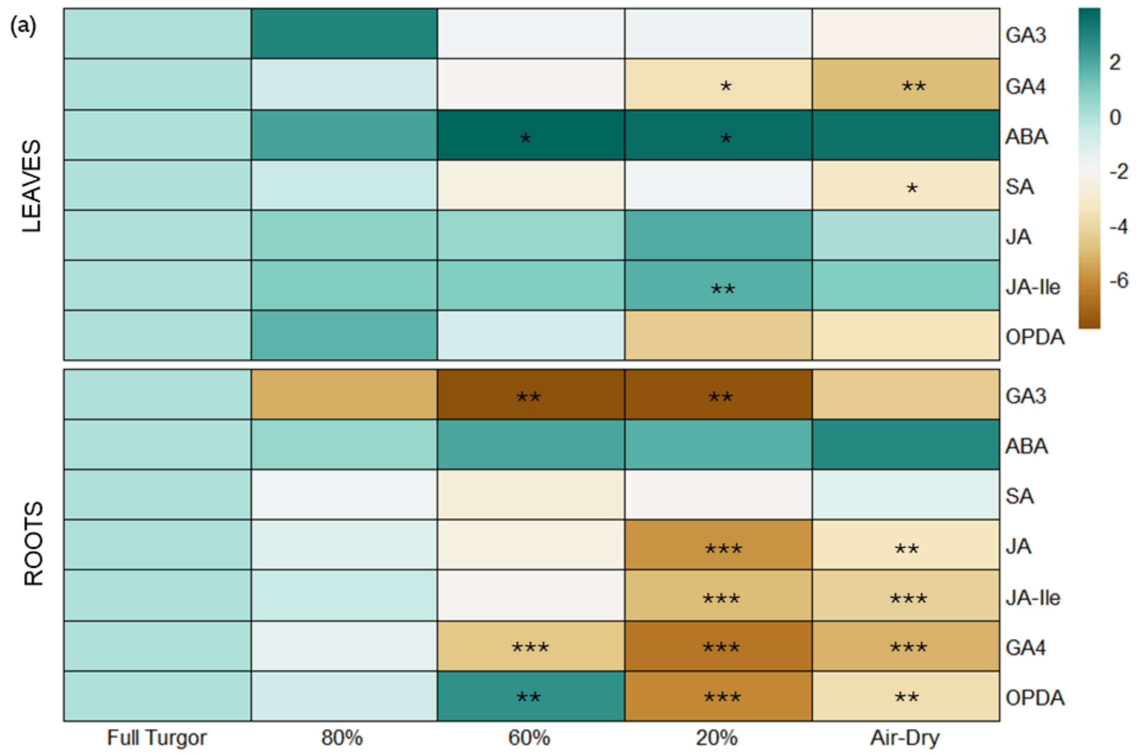
Figure 2-6: Hormone abundance trends during desiccation in the leaves of C. pumilum and X. schlechteri. *The trendlines represent log-transformed abundances (ng g FW^{-1}) of the seven relevant groups of hormones for this study in the (a) leaves and (b) roots of C. pumilum, and the (c) leaves and (d) roots of X. schlechteri, during a dehydration period encompassing various stages: full turgor (100%), 80%, 60%, 20% RWC, and the desiccated (air-dry) state (0%).*

The concentration of gibberellins in the leaves of *X. schlechteri* exhibits a progressive decline (Figure 2-6c & Figure 2-7b). However, unlike the findings observed in *C. pumilum*, this reduction occurs only under conditions of more prominent dehydration, at approximately 60% RWC. This observation implies that the cessation of growth in favour of alleviating drought-induced stress only becomes significant for *X. schlechteri* during instances of more severe stress, in contrast to the behaviour exhibited by *C. pumilum*. This phenomenon could be attributed to the fact that *X. schlechteri* may not be as inclined towards initiating a stress response, as poikilochlorophyllous plants dismantle their photosynthetic structures and are required to reconstruct them upon rehydration (Gaff, 1977; Sherwin & Farrant, 1998; Farrant, 2000; Farrant, *et al.*, 2003).

Within the roots of *X. schlechteri*, both GA3 and GA4 increased during early dehydration at approximately 80% RWC, followed by a sharp decrease during mid dehydration down to the air-dry state (Figure 2-6d & Figure 2-7b). Similarly to *C. pumilum*, the trends between the two gibberellins are much the same, suggesting that they function similarly during a desiccation response in the roots. The increase of these gibberellins during drying may occur as a result of the "drought rhizogenesis" hypothesis, which posits that plants, specifically Brassicaceae, develop short tuberized hairless roots that can withstand long periods of drought and then develop a new functional root system upon rehydration (Vartanian, *et al*., 1994). This idea has also been researched in seedlings of *X. schlechteri* (Lyall, *et al*., 2014). Similarly to *C. plantagineum* as discussed above, the primary root of *X. schlechteri* seedlings also appear to survive desiccation and prompt the development of secondary roots upon rehydration. Thus, the accumulation of gibberellins during early drying in the roots of *X. schlechteri* may occur as a result of priming the primary roots in preparation for desiccation, later being able to easily prompt the development of new secondary roots when water becomes available once again.

ii. Abscisic acid

ABA was assessed in this study as it is largely considered one of the most important factors in a dehydration response and may influence the strategies of homoiochlorophyllous and poikilochlorophyllous species. It has also been linked to dependent and independent interactions with the JA signalling pathway in particular and its assessment could shed light on the various mechanisms adopted by this pathway during a stress response in different species.



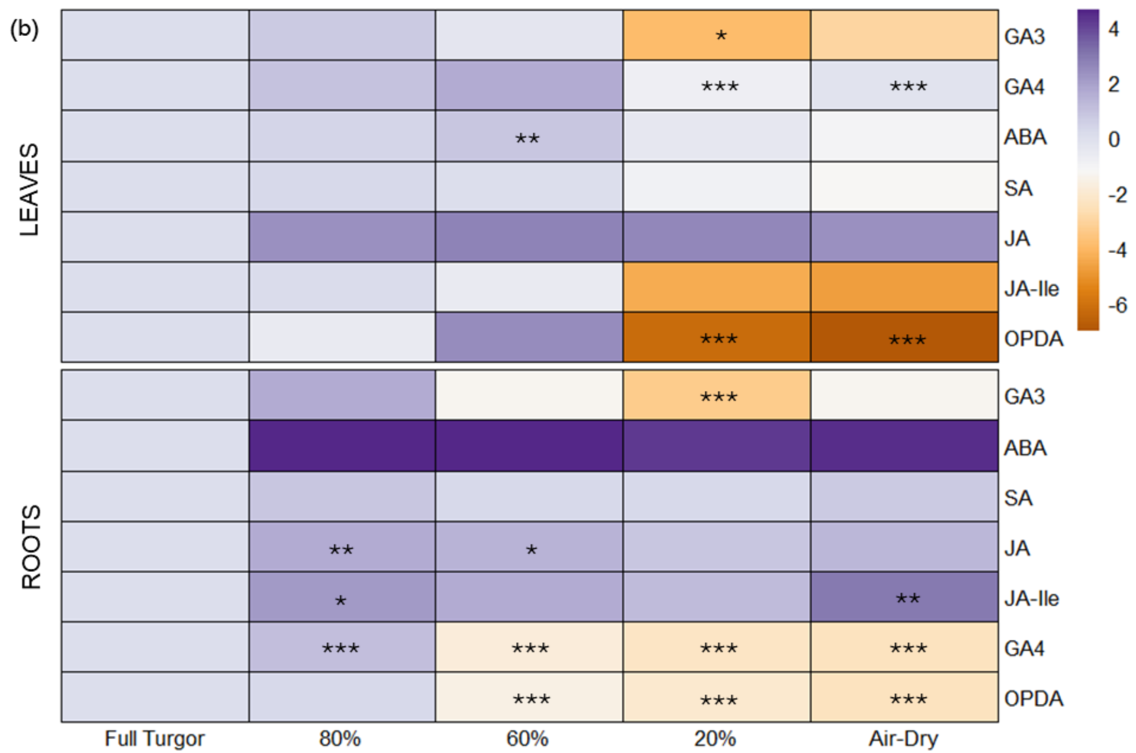


Figure 2-7: Log2FC of hormone abundances in the leaves and roots of C. pumilum (a) and X. schlechteri (b) during drying. *The above heat map shows the log2 fold change of phytohormone*

*abundances in relation to samples at the full turgor state. Changes that were found to be statistically significant are denoted as <0.001 = “***”, <0.01=”**”, <0.05=”*”.*

The abundance of ABA in leaves of *C. pumilum* showed a marked increase during mid-stage dehydration at around 60% RWC and levels remained high all the way down to the air-dry state, showing a similar trend to another homoiochlorophyllous plant *Selaginella pulvinate* (Yu, *et al*., 2024). Increased abundance of ABA is associated with inducing leaf senescence brought about by dehydration stress and typically also results in the breakdown of chlorophyll in non-resurrection plants. In desiccation-tolerant poikilochlorophyllous monocots, the correlation between dehydration-induced chlorophyll degradation and leaf senescence is believed to be uncoupled, as discussed by Tuba (2008). This means that the processes of age-dependent leaf senescence and chlorophyll degradation are distinct from those used by poikilochlorophyllous plants during a desiccation response. Conversely, in ABA-dependent homiochlorophyllous resurrection plants, exemplified by *C. plantagineum* (Petersen, *et al*., 2012), it seems that a mechanism exists that mitigates both ABA-mediated chlorophyll degradation and senescence during the dehydration process. Thus, it is likely that, similar to its sister species *C. plantagineum*, *C. pumilum* may show increased levels of ABA during drying, but not for the purpose of breaking down chlorophyll. Giarola, *et al*. (2020) discovered a novel germin-like protein (CpGLP1) that was shown to accumulate in response to dehydration as well as ABA treatment in *C. plantagineum*, suggesting its involvement in desiccation responses. The protein was shown to exhibit superoxide dismutase (SOD) activity, which is essential in managing ROS accumulation and interaction with pectins, which form a major component of plant cell walls. These findings suggest that CpGLP, induced by ABA, may facilitate cell wall plasticity which inherently may support cell-longevity in homoiochlorophyllous species that do not dismantle their photosynthetic apparatus during desiccation. These findings also assist in contrasting the strategies adopted by poikilochlorophyllous vs. homoiochlorophyllous plants, as the accumulation of ABA may result in desiccation-induced leaf senescence and break down of chlorophyll in the former, or the facilitation of cell wall plasticity in the latter.

The abundance of ABA shows a gradual increase from mid-stage dehydration onwards with its peak appearing at the airdry stage in the roots of *C. pumilum*. This result is similar to those found by Djilinov *et al*. (2013) who noted a peak in ABA at the air-dry stage as well. Although very little data on the management of root architecture has been established in resurrection plants during a desiccation response, it has been shown that ABA works in conjunction with auxin and reduces primary root growth (Zhang, *et al*., 2010), but enhances lateral root growth

(Lee, *et al*., 2012) in *A. thaliana.* Research into *C. plantagineum* has shown that its root system capable of surviving desiccation, however the roots senesce shortly after rehydration. The plant is then able to regrow an entirely new root system around 2 weeks following rehydration (Norwood, *et al*., 2003). Thus, gradual increasing of root ABA during desiccation of *C. pumilum* may be indicative of an attempt to act as a stasis regulator which inhibits root growth during drying in favour of potentially allowing for the reestablishment of a new root system when water becomes available again. However, to fully test this theory, additional research incorporating a rehydration time curve would be necessary in future research.

The abundance of ABA within the leaves of *X. schlechteri* shows faster accumulation during early dehydration than in the leaves of *C. pumilum*, followed by a sharp decline after mid-stage dehydration, maintaining low levels during late-stage dehydration at roughly 20% RWC and the air-dry state. This trend is reflective of a previous study that was conducted on *X. schlechteri* (Gabier, *et al*., 2021), and another conducted on the resurrection fern *Pleopeltis polypodioides (*John*, et al.*, 2023), wherein the authors found that endogenous levels of ABA increased during the initial stages of dehydration, but then decreased significantly to roughly one-fourth of that in hydrated fronds and increase again upon rehydration. The authors also discovered that stomatal closure, a characteristic mechanism of ABA-induction, did in fact operate independently of ABA and was rather influenced by environmental factors such as blue light and calcium ions. Their findings highlighted the fact that ABA does not always influence expected dehydration responses such as stomatal closure and non-model organisms show adaptations for dealing with water loss. Mercado-Reyes, *et al*. (2023) also found this pattern of 'p-type' ABA levels during dehydration in conifers and the embolism-resistant angiosperm *Umbellularia californica*, and linked the phenomenon to conversion of ABA to conjugates during late dehydration brought about by turgor loss. With this in mind, it is possible that *X. schlechteri* adopts similar mechanisms in which accumulation of ABA is more prominent in the very early stages of dehydration. It has already been proposed that *X. schlechteri* adopts separate early- and late-stage dehydration responses wherein early-stage dehydration involves the shutting down of photosynthesis and an increase in antioxidants in preparation for a committed desiccation response (reviewed in Farrant, *et al*. (2007)). Building on work by Kariola *et al*. (2006) and Aalto *et al*. (2012), it has been proposed that the protein ERD15 in particular provides an ABA regulated threshold which determines when *X. schlechteri* will commit to a large scale response to desiccation from early stage to late stage.

Similarly to expression levels in the leaf tissue, the abundance of ABA increased significantly during early-stage dehydration in the roots of *X. schlechteri*, but rather than decreasing during the desiccation stage as was the case in the leaves, ABA levels remained higher than the initial full turgor state all the way from early dehydration down to the airdry state. This suggests that ABA may play a larger role in controlling root architecture in *X. schlechteri* than *C. pumilum*, whose root ABA content only increased gradually during desiccation to the airdry state.

In summary, these findings underscore the critical role of ABA in mediating the dynamic responses of homoiochlorophyllous and poikilochlorophyllous plants. The results illustrate that the effects of ABA appear to be more dominant during mid- to late-stage dehydration and desiccation in *C. pumilum*, suggesting its involvement in cell wall plasticity and root architecture adjustments. Conversely, *X. schlechteri* exhibits a distinct pattern of ABA accumulation, with elevated levels during early dehydration stages that drop in the leaf tissue, but persist through desiccation in the root tissue, highlighting the characteristic 'commitment' of *X. schlechteri* to a desiccation response that involves dismantling the photosynthetic apparatus past a certain water deficit threshold.

iii. The jasmonates

JA and one of its derivatives, JA-Ile were assessed in this paper, along with one of its precursors, OPDA to understand how these elements might work in conjunction to coordinate a desiccation response in *C. pumilum* and *X. schlechteri*. The expression of relevant genes associated with JA signalling is also explored in later Chapters to further elucidate the intricacies of this system.

The abundance of JA seemed to peak at roughly 20% RWC in the leaves of *C. pumilum*, but it decreased again sharply at the air-dry state, indicating a potential final response to attempt to mitigate stress during desiccation through JA-signalling before the air-dry state. A similar trend was also observed regarding the JA derivative JA-Ile, suggesting that the two compounds are indeed linked and work in unison during a desiccation response. Studies by Djilianov, *et al*. (2013) and Yu, *et al*. (2024) showed somewhat contrasting results in the homoiochlorophyllous resurrection plants *Haberlea rhodopensis* and *Selaginella pulvinata* respectively. The authors suggest that JA may act as an early signal that eventually helps to induce a desiccation response and showed that the highest recorded levels of JA ocurred during early- to mid-stage dehydration at roughly 60% RWC. This could indicate that *C. pumilum* may adopt a slightly

different strategy in terms of signalling a stress response, potentially through the utilization of a different phytohormone such as SA or perhaps a derivative of JA such as its precursor OPDA.

As was the case within the leaf tissue, the results of JA and JA-Ile accumulation in the roots of *H. rhodopensis* (Djilanov *et al*., 2013) departed from those seen in this thesis. The authors revealed a dynamic increase which occurred again around 60% RWC, with JA-Ile increasing steadily to the air-dry state. However, a significant difference was observed between the FT and both 20% RWC and air-dry states in the roots of *C. pumilum*, meaning that an opposing trend was observed where JA and JA-Ile show a steady decrease during dehydration. It should be noted that high variability among these samples may indicate less accurate findings. High variability may have come about as a result of the generally low concentration of JA and JA-Ile observed when compared to all other hormones assessed. Tentatively, it does appear that neither JA nor its derivative play a pivotal role in a drought response in the roots of *C. pumilum*.

Despite a large degree of variability, a slight increase of JA or JA-Ile occurred in the leaves of *X. schlechteri* during early dehydration, indicating a potential early response which persists through to the desiccated state. Poikilochlorophyllous plants such as *X. schlechteri* demonstrate the ability to 'degreen' or break down their chlorophyll in response to dehydration and there is evidence to suggest that JA and/or its derivatives play a role in this function. For example, a study by Zhu *et al.* (2015) revealed a mechanism whereby MYC proteins mediate jasmonic acid-induced chlorophyll degradation by activating major chlorophyll catabolic genes in the model plants *A. thaliana* and *Nicotiana benthamiana*. The authors expressed that this mechanism was induced by a different JA derivative; methyl-jasmonate (MeJA). So, although the results from the current study suggest that JA might play a very miniscule role in a desiccation response, it would be valuable for future studies to assess the content of MeJA and other potential derivatives that may come into play during desiccation in place of JA.

A high degree of variability was once again observed in JA and JA-Ile results in the roots of *X. schlechteri*. However, the concentration of JA and JA-Ile increased initially at roughly 80% RWC and then again at the airdry state. Tentatively, the results may suggest that the JA signalling pathway plays a role in a dehydration response during the early stage dehydration and desiccated state in the roots of *X. schlechteri.*

The investigation into the concentration dynamics of JA and its derivatives during desiccation reveals distinct patterns that suggest potential coordination of JA signalling in response to

drought stress. Notably, the findings indicate that while JA and JA-Ile concentrations are relatively low, their expression peaks at critical moisture levels, particularly in the leaves of *C. pumilum*. However, the high variability observed in JA and JA-Ile concentrations, especially in the roots, underscores the need for further refinement of extraction protocols for non-model species to improve accuracy in quantification. As there are no dedicated protocols for the extraction of hormones from non-model species such as *C. pumilum* and *X. schlechteri*, the protocol used for this analysis was adapted from Pan, *et al.* (2008), who used *A. thaliana* as their model when developing this protocol. The results obtained through this assessment emphasise the necessity for the refinement of protocols applicable to non-model organisms and/or for an expanded diversity of species to ensure accurate quantification, thereby also illuminating one of the complexities inherent in the examination of novel species such as *C. pumilum*. Future research should also consider the role of other JA derivatives such as MeJA to fully understand the mechanisms of JA-signalling in resurrection plants and other non-model organisms.

iv. 12-oxo-phytodienoic acid (OPDA)

OPDA serves as a key intermediate in the biosynthesis of JA and its active form, JA-Ile, (Wasternack & Hause, 2013). While JA-Ile is recognized by the COI1 receptor to activate downstream signalling, OPDA can also induce gene expression independently of this pathway (Wasternack & Hause, 2016).

In contrast with JA and JA-Ile abundance, OPDA levels showed a marked increase during the early stages of dehydration in the leaves of *C. pumilum*, followed by a significant decrease during the later stages. Typically, the JA-signalling pathway involves the perception of JA-Ile by the COI1 receptor, which promotes the degradation of JAZ proteins that act as repressors of JA-responsive genes (Thines, *et al*., 2007). In contrast, relatively new evidence suggests that that OPDA and its conjugate OPDA-Ile can induce gene expression through a COI1-independent mechanism (Wasternack & Hause, 2016), and so it could be argued that *C. pumilum* adopts this strategy in place of typical JA-signalling to invoke a desiccation response utilising similar genes. The genes targeted by OPDA-Ile are presumed to be similar to those induced by JA-signalling, but the exact function of these genes is not known at the time of writing.

There appears to be another peak of OPDA accumulation during mid dehydration at around 60% RWC in the roots of *C. pumilum*, which continues to suggest that OPDA signalling is more prominent in this species than is JA signalling, though this signalling seems to occur at later stages of drying in the roots than in the leaves.

A similar trend can be found in the leaves of *X. schlechteri*, where OPDA abundance increases around mid-stage dehydration (60% RWC) and decreases again during later stages. As is also the case in *C. pumilum*, it can be observed that OPDA seems to show much greater fluctuations than JA or JA-Ile in the leaves of *X. schlechteri* and likely also depends more on an OPDA-mediated response during dehydration.

OPDA also showed a peak during early dehydration in the roots of X. *schlechteri*, indicating that a response occurs earlier in the roots than in the leaves of this species, which is contrasted in *C. pumilum*. The role of OPDA as it relates to dehydration responses in the roots has not been fully elucidated, however a study by Schulze, *et al*. (2019) demonstrated the ability of OPDA to undergo long-distance translocation from the shoots to the roots of *A. thaliana* via the phloem. Here OPDA participates in a signalling network towards enhancing gene expression of stress-responsive genes and consequently inhibiting root growth, allowing the plant to allocate resources towards defence rather than growth.

The findings presented indicate that OPDA plays a crucial role in the desiccation response of *C. pumilum* and *X. schlechteri*, serving as a key signalling molecule that likely operates independently of the traditional jasmonic acid signalling pathway. The marked increase in OPDA levels during early dehydration stages suggests a unique regulatory mechanism that may prioritize OPDA-mediated responses over JA signalling in these species. This is particularly evident in the differential OPDA accumulation in the roots and leaves. In addition, the potential for OPDA to induce gene expression through a COI1-independent pathway raises important questions about the distinct roles of OPDA and its conjugates such as OPDA-Ile, in regulating stress-responsive gene expression. Future research should aim to elucidate the precise functions of OPDA-targeted genes and the broader implications of OPDA signalling during abiotic stress.

v. Salicylic acid

The abundance of SA showed a gradual decrease from the full turgor state down to the air-dry state in the leaves of *C. pumilum*, contrasting findings from Djilinov *et al*. (2013), who found that SA increased gradually towards the air-dry state but showed no significant fluctuations

during dehydration, reaching only three times the abundance under control conditions at its peak. The authors remark on the pattern resembling that of compounds such as phenols which are involved in oxidative stress tolerance in *H. rhodopensis* (Djilianov, et al., 2011), indicating SA's role as an antioxidant during a desiccation response. Thus, the seemingly opposite trend observed in C. pumilum may suggest an alternative role for SA during desiccation or may indicate that its involvement in desiccation responses is not uniform across species. Typically, homoiochlorophyllous plants do not undergo significant leaf senescence during drying, relying more heavily on morphological changes and anthocyanin accumulation to protect their photosynthetic apparatus against photooxidative stress (Farrant, 2000). Thus, the depletion of SA during the drying process is also necessarily not out of place here and it highlights the involvement of the morphological strategy adopted by *C. pumilum*. Additionally, SA's potential role in a drought or desiccation response in *C. pumilum* should also not be entirely discredited as it has been suggested that high levels of SA can induce photooxidative damage and may adversely affect photosynthetic tissue *(*Borsani*, et al.*, 2001), further bolstering its potential role in dehydration responses at regulated levels. Another study by Al-Hakimi & Hamada (2001) also showed that exogenous SA applied to wheat resulted in effective drought resistance, but again only at low levels. So, it is possible that low levels of SA in *C. pumilum* may yet contribute some form of stress tolerance by a distinct mechanism that is currently not fully explored.

A similar trend can be found in the roots of *C. pumilum*, where levels of SA are slowly decreased as drying occurs, further suggesting a lack of involvement of SA in the desiccation response of *C. pumilum* as it pertains to stress signalling, though it is again possible that SA may indeed play a role in mitigating stress via mechanisms induced at low levels.

Conversely, the abundance of SA shows a gradual increase from early to mid-dehydration, followed by a gradual decline during the desiccation stage in the leaves of *X. schlechteri*. The accumulation of SA during an early dehydration response may be indicative of pre-emptive leaf senescence, which was also noted morphologically in Figure 2-4 as the plant attempts to prepare for a more extreme commitment to a desiccation response, a characteristic trait of poikilochlorophyllous plants (Farrant, 2000). In addition, Rai, *et al*. (1986) originally discovered that the application of SA reversed stomatal closure induced by ABA and this was later expanded upon by Waseem, *et al* (2006) and Saruhan, *et al*. (2011) who revealed that a

diminished decrease in stomatal conductance in SA-treated plants was present under minor drought conditions.

Accumulation of SA was noted during early dehydration in the roots of *X. schlechteri*, followed by a sharp decline thereafter during late dehydration, with another small increase during the air-dry condition. The results thus indicate that SA accumulates more readily in the roots under less severe water deficit, with leaf SA playing a role during more severe water stress at the early- or mid-stage of dehydration. Similar results pertaining to early accumulation of SA in the roots in comparison to leaves were also found by Bandurska & Stroiski (2005) in barley. Two enzymes involved in the synthesis of SA from phenylalanine (PAL and BA2H) have also been shown to increase in activity under moderate water stress as opposed to severe water stress, which in turn triggered the accumulation of SA in the roots of barley seedlings, indicating its role in a dehydration response during mild stress.

In summary, the evidence presented in this study suggests that while SA levels decrease in *C. pumilum* during dehydration, indicating a limited role in stress signalling, there remains the possibility of its involvement in stress tolerance at low concentrations. Conversely, in *X. schlechteri*, this study bolsters the notion that SA accumulation during early dehydration appears to facilitate pre-emptive dehydration-induced leaf senescence, demonstrating a more pronounced role in managing water stress via this strategy.

2.3.4. Conclusions

This chapter aimed to produce a dehydration/desiccation profile for several relevant phytohormones in the evolutionarily distinct resurrection species *C. pumilum* and *X. schlechteri* in an attempt to understand their involvement during stress response in these desiccation tolerant plants. Results showed that different phytohormone profiles exist during desiccation in *C. pumilum* and *X. schlechteri*, emphasising their distinctive strategies for mitigating water deficit stress. The differential accumulation patterns of gibberellins GA3 and GA4, indicate their involvement in specific signalling pathways associated with desiccation tolerance, with GA3 exhibiting a pronounced increase during the initial dehydration stages in *C. pumilum*, while GA4 displays a more gradual decline, highlighting a potential mechanism during which gibberellins prime cell walls in response to stress to reduce cell turgidity and the consequent collapse of the cell wall during drying in homoiochlorophyllous species. ABA emerged as a central regulator of stress responses, with its levels significantly increasing during

mid-stage dehydration in *C. pumilum*, highlighting its role in enhancing cell wall plasticity and potentially lateral root growth. The jasmonates, although present in lower concentrations, appear to play more of a supporting role in drought stress tolerance, particularly in *X. schlechteri*, while OPDA's dynamics suggest a potential alternative signalling pathway during desiccation. Lastly, the observed trends in SA levels indicated limited involvement in stress signalling in *C. pumilum*, while *X. schlechteri* exhibited more pronounced accumulation during early dehydration. Analysing these results should also take into account the plants' monocot and dicot nature. For example, GA has been found to accumulate primarily in active proliferation sites of monocot leaves during development and it decreases gradually according to distance from the leaf origin, whereas the distribution of dicot hormones may be more similar across the entire length of the leaf as the cells of dicot leaves are more similar in age (Gonzalez, *et al*., 2012). Future research into potential hormone gradients across leaves in monocot resurrection plants would be particularly useful in ascertaining more specific roles of certain hormones. Overall, the findings in this chapter underscore the complexity of hormonal interactions in plant responses to water deficit stress and highlight the need for further investigation into the specific mechanisms employed by resurrection plants to overcome these stresses.

Chapter 3 : The control of gene expression during desiccation response in plants

3.1. Introduction

3.1.1. Transcriptomic analyses in the context of desiccation tolerant species

Stress responses by plants are complex, multifaceted and interconnected, meaning that the evaluation of singular systems involved in their stimulation and control is insufficient to fully grasp the many mechanisms that work in unison to confer desiccation tolerance. Thus, in the past few decades, many "desiccomics" studies have been carried out across a wide range of taxonomically diverse resurrection plants in an effort to understand the modulation of physiological, anatomical, genomic, proteomic, metabolomic and transcriptomic processes responsible for defence against desiccation-induced damage (Mitra, *et al*., 2013; Zhang & Bartels, 2018; Pardo, *et al.*, 2020; Farrant, *et al*., 2020; Xu, *et al*., 2021).

There are three main categories of "omics" data; proteomics, metabolomics and transcriptomics. The results presented in this chapter use the principles transcriptomics, in a small-scale analysis of gene expression of three key genes associated with the jasmonic acid pathway, supplementing the metabolomics-based approach to understand hormone interactions in the previous chapter.

Due to advances in the accessibility of next-generation RNA sequencing, numerous studies have been released in recent years that aim to understand the effects of the transcriptomes of various resurrection plants (Lyall & Gechev, 2020). Currently, the majority of transcriptomics publications within the realm of resurrection plants are focused around desiccation and subsequent rehydration experiments involving leaf tissue, with little having been done on root tissue (Tabele *et al*., 2021). It should also be noted that transcriptomic analysis has been reported for all major taxonomic groups displaying vegetative desiccation tolerance, including dicotyledons, monocotyledons, ferns, lycophytes, algae and bryophytes (Gechev, *et al.*, 2021).

To date, most publications involving transcriptomic analysis of resurrection plants are targeted towards angiosperm species as they are most closely related to economically important crops such as wheat and maize (Gechev, *et al*., 2021). The first resurrection plant to have its transcriptome analyzed through next-generation sequencing was *Craterostigma plantagineum,* a homoiochlorophyllous resurrection plant (Rodriguez, *et al*., 2010). The analysis concluded that there are conserved genetic pathways within other DT species that are shared by *C. plantagineum*, however, resurrection plants possess genetic pathways that are highly up- or downregulated during a desiccation response (Gechev, *et al.*, 2021; Kwon, *et al*., 2021), making their transcriptomic analysis all the more important.

Transcriptional modification can be found in a large number of adaptation responses and is involved in numerous molecular processes. One of the most well-known regulators of transcription involves the utilization of transcription factors, which can be influenced by phytohormones. One example of the use of transcription factors can be seen in the regulation of stomatal closure, which is mainly governed by abscisic acid (ABA), protein kinases, reactive oxygen species, and lipid messengers (Peters, *et al*., 2010). In dicotylous resurrection plants such as *C. plantagineum*, some of the most abundant transcripts are involved in maintaining photosynthesis and cell wall integrity during no-stress conditions, whereas the reverse is true during dehydration stress, instead favouring upregulation of genes associated with ROS-scavenger proteins and other proteins such as LEAs that are typically involved in stress responses (Dinakar & Bartels, 2013).

3.1.2. Control of the jasmonate pathway during desiccation

Extensive research into the mechanisms of jasmonic acid (JA) stress signalling has been conducted in the model plant *A. thaliana*. The term 'jasmonates' describes a group of naturally occurring compounds with various functions and derivatives that are produced as a result of ß-oxidation of polyunsaturated fatty acids, such as free jasmonic acid (JA), an amino acid derivative called jasmonyl isoleucine (JA-Ile), jasmonyl ACC (JA-ACC), and a methyl ester derivative (MeJA) (Creelman & Mullet, 1995). Jasmonates are known to play a role in various regulatory pathways for the up- and downregulation of gene expression. The oxylipin, jasmonic acid, acts as a regulator for many aspects of plant physiology, including reproductive development, as well as defence against pathogens and abiotic stressors such as UV radiation and drought (Conconi, *et al*., 1996; Browse, 2005; Glazebrook, 2005). As is the case with

several other abiotic stresses, stress induced by water deficit impacts multiple physiological processes and defence signalling pathways (Kuromori, *et al*., 2018), and although the exact role of JA during drought stress remains unclear, it is possible that it is implicated in signalling cell death (Kohli, *et al*., 2013).

i. JA biosynthesis

In order to understand the role of jasmonic acid in the desiccation response of any plant, it is necessary to understand its origin and biosynthesis. The jasmonate biosynthesis pathway was first outlined by Vick and Zimmerman (1983), and typically involves the latter of the two main chemical pathways that occur during oxidation of polyunsaturated fatty acids; (1) the α-Dioxygenase (α-DOXs) pathway and (2) the Lipoxygenase (LOX) pathway. During the LOX pathway, jasmonates are derived from α-linolenic acid (α-LeA), a compound released from galactolipids of chloroplast membranes via phospholipase 1 (PLA1) (Scherer, *et al*., 2010) (Wasternack & Hause, 2013). During this reaction cascade, α-LeA is converted to cts-(+)-OPDA in the plastid and then transported to the peroxisome where it undergoes further multienzymatically-catalyzed ß-oxidation to become jasmonic acid. From here, JA can also be converted to several of its derivatives such as jasmonyl-L-isoleucine (JA-Ile) and methyl jasmonate (MeJA) (Browse, 2009; Wasternack & Hause, 2013). The notable enzymes involved in LOX pathway synthesis include (1) 13-lipoxygenase (13-LOX), which is responsible for the oxygenation of α-LeA, (2) 13-allene oxide synthase (AOS), which converts this oxygenated JA substrate to an epoxide, (3) allene oxide cyclase (AOC), which cyclises the epoxide to OPDA inside the plastid, and (4) OPDA reductase 3 (OPR3), which reduces OPDA in preparation for ß-oxidation to produce (+)-7-iso-JA (jasmonic acid) (Wasternack & Hause, 2013). Regulation of JA biosynthesis typically occurs via a positive feedback loop and tends to be tissue specific (Wasternack, 2007), and has also been shown to occur in localized regions following stresses such as wounding by pathogens (Koo, *et al*., 2009).

ii. The role of JA in a water deficit response in Arabidopsis thaliana

The functions of jasmonates, like most other phytohormones, are broad, meaning that their role in desiccation responses of both desiccation tolerant and desiccation sensitive plants is difficult to pinpoint. Adding to this is the wide variety of plant tissue within which JA is typically found; the highest concentrations of JA are usually found in meristematic tissue, reproductive tissue

and flowers, with the lowest concentrations occuring in mature leaves (Pandey, 2017), despite the latter being the main site of JA biosynthesis (Mueller, 1997).

It has been suggested that jasmonates could mediate stress responses to several abiotic stresses in *A. thaliana,* which has largely been considered a model plant for the investigation of dehydration tolerance and most other stressors (Wang, *et al*., 2001). Shinozaki and Yamaguchi-Shinozaki (2007) found that two main pathways contribute to plant defence against dehydration; one being ABA-dependent and the other ABA-independent. Within the latter, JA acts as an endogenous phytohormone signal which induces a defensive water deficit response through the expression of JA-mediated genes. Although the JA signalling cascade is effective in protecting the plant from external stressors, Zander *et al*. (2020) posits that the over-defensive nature of this pathway may impede the growth and development of the plant. Interestingly, previous studies have also found that exogenous application of JA improves the antioxidant ability of soybean (Anjum, *et al*., 2011).

iii. MYC2 (Myelocytomatosis-related 2)

Signal transduction of JA is outlined in Figure 3-1. Under conditions where no significant stressors are present, the jasmonate response is repressed by a group of proteins known as Jasmonate-Zim-Domain (JAZ) repressors, which interact with a number of JA-responsive transcription factors including MYC2 (Chini, *et al*., 2007; Thines, *et al*., 2007). The MYC family forms part of the basic helix-loop-helix (bHLH) superfamily of transcription factors, whose members are largely involved in plant stress response and secondary metabolism, and can be found in many angiosperms (Peñuelas, *et al*., 2019). These proteins play a central role as transcription factors in the JA signalling pathway. MYC2, MYC3, MYC4 and MYC5, belong to the IIIe bHLH clade in *A. thaliana*. In contrast to the other members, MYC2 has a stronger activation capacity as a result of its ability to form DNA looping structures, lending it an increasing affinity for DNA and subsequent gene expression (Kazan & Manners, 2013). When no stressors are acting on the plant, the JA signalling pathway is suppressed via the suppression of MYC2 by JAZ proteins (Pauwels & Goossens, 2011; Kazan & Manners, 2012; Wager & Browse, 2012). Conversely, when abiotic stressors such as drought are detected, coronatine-insensitive 1 (COI1) regulates ubiquitin-dependent JAZ degradation, thereby

releasing MYC2 from repression, resulting in the expression of JA-mediated genes (Thines, *et al*., 2007).

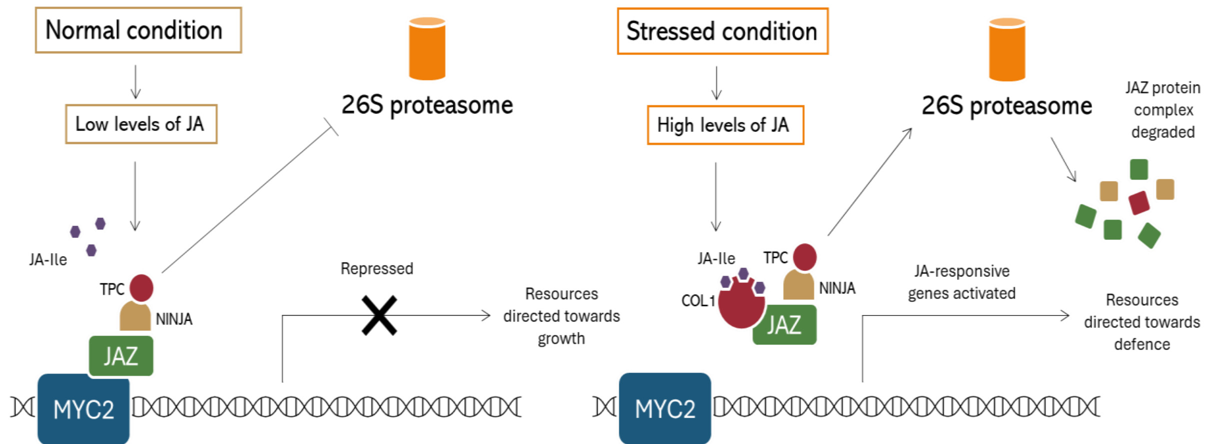


Figure 3-1: Model for jasmonic acid (JA) signal transduction during an abiotic stress response. *Under normal conditions, a group of repressor proteins (JAZ) bind to the transcription factor MYC2 and recruit corepressors such as NINJA and TPC to suppress gene transcription when the active form of JA (JA-Ile) is present at low concentrations. In response to stressed conditions, JA-Ile binds to the F-box protein COL1 for ubiquitination of JAZ, releasing its repression of MYC2 and facilitating its degradation via the 26S proteasome. The removal of the JAZ complex from MYC2 results in JA-responsive genes being activated for downstream defence against abiotic stress. This figure was adapted from (Geetika, et al., 2017) and (Raza, et al., 2021).*

iv. RD29A and RD29B

Yamaguchi-Shinozaki, *et al*. (1992) isolated nine *RD* genes from *A. thaliana* under dehydration stress utilizing differential hybridization, two of which include *RD29A* and *RD29B*. They were originally thought to occur on the same locus, but extensive restriction analysis revealed that they are tandem gene duplications and encode very similar proteins (Yamaguchi-Shinozaki & Shinozaki, 1993). The respective proteins encoded by *RD29A* and *RD29B* each contain one cysteine residue which suggests structural similarity to late embryogenesis abundant (LEA) proteins, which are known to be involved in desiccation tolerance (Jia, *et al*., 2012). However, reverse genetic analyses have revealed that *RD29A* and *RD29B* are unlikely to act directly as protective molecules, but rather as warning signals of abiotic stress (Msanne, *et al*., 2011).

ABA-responsive element-binding protein (AREB) and dehydration-responsive element-binding protein (DREB) are two transcription factors that play an important role in signal transduction during abiotic stress responses. During a stress response, AREB is responsible for binding to the ABA-responsive element (ABRE), a *cis*-acting element in the promotor region of ABA-dependent genes (Fujita, *et al*., 2005), whereas DREB is responsible for binding to the dehydration-responsive element (DRE) in the promotor of ABA-dependent/independent genes

(Baker, *et al*., 1994). Both these elements are involved in downstream expression of *RD29A* and *RD29B*, as outlined in Figure 3-2.

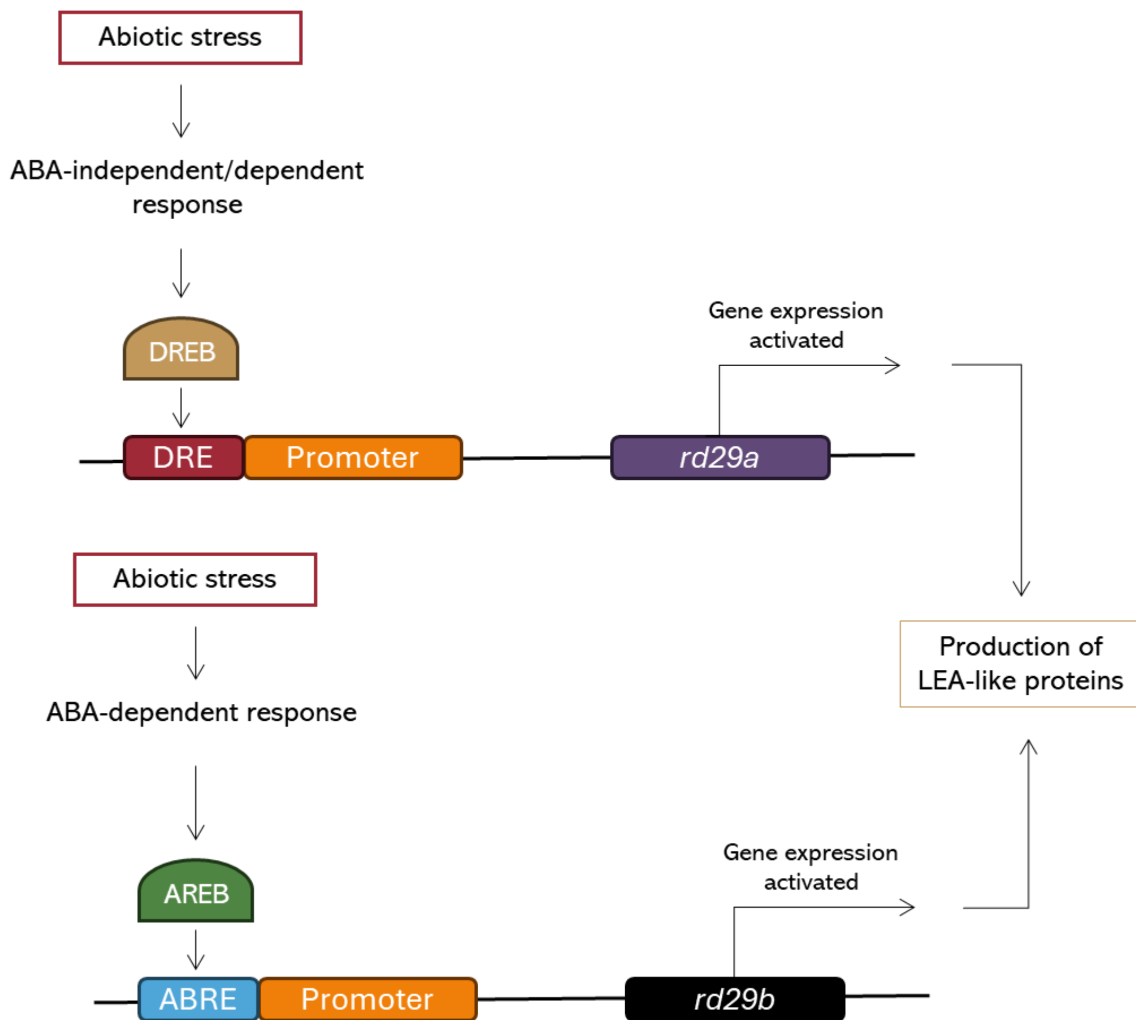


Figure 3-2: ABA-independent and ABA-dependent abiotic stress response of RD29A and RD29B. *Schematic representation of a typical response of RD29A and RD29B to abiotic stress where the expression of RD29A is not necessarily reliant on the abundance of ABA, whereas the expression of RD29B is entirely dependent on high levels of ABA. During a stress response, dehydration-responsive element-binding protein (DREB) binds to the dehydration-responsive element (DRE) motif in the promoter region of ABA-dependent/independent genes, resulting in the expression of RD29A. Conversely, the transcription factor ABA-responsive element-binding protein (AREB) acts only when ABA is present in high concentrations and binds to the ABA-responsive element (ABRE) in the promoter region of ABA-responsive genes, resulting in the expression of RD29B. Both RD29A and RD29B produce LEA-like proteins, which are known to accumulate during a desiccation response.*

v. GASA3 (GA-stimulated in Arabidopsis 3)

The *GASA* gene family plays an important role in plant growth and development, particularly regarding cell differentiation, seed germination, stem elongation and flowering (Fleet & Sun, 2005). Shi *et al*. (1992) first reported the *GAST1* (GA-stimulated transcript 1) gene from a GA-deficient tomato plant and homologs of this gene were later uncovered in various other plants,

including the *GASA* genes in *A. thaliana* (Herzog, *et al*., 1995). These genes typically encode small polypeptides with conserved GASA domains and 12 conserved cysteine residues. Of the 15 known *GASA* genes in *A. thaliana*, *GASA1* and *GASA4* have been most extensively researched and are dependent on GA-signalling, whereas the lesser-studied *GASA2* and *GASA3* genes are seemingly not affected by the abundance of GA (Herzog, *et al*., 1995). These findings were confirmed more recently by Zhang & Wang (2008) who posit that *GASA* genes in *A. thaliana* may act as integrators of GA and ABA signalling pathways. The *GASA* gene family has been shown to play a role in the crosstalk between GA and JA through their interactions with DELLA proteins, particularly *GASA1*, *GASA4*, *GASA6* and *GASA9* (Zhang & Wang, 2008), however very little research is available regarding the functionality of other *GASA* genes such as *GASA3*.

The relationship between GA and JA addresses the plant quandary between 'growing' and 'defending' in response to abiotic stimuli. Hou *et al*. (2010) described a "relief of repression" model to describe how DELLAs, a prominent group of proteins involved in GA-signalling, affect JA signalling through competitive binding to JAZ proteins. A high abundance of JA-Ile results in the degradation of JAZ proteins and allows for the subsequent activation of MYC2, a transcriptional activator of JA-induced gene expression. Under normal conditions, high levels of GA result in the degradation of DELLAs, however, during a stress response, as the abundance of GA declines, the inhibition of DELLAs is also eliminated, resulting in their activated states competing with MYC2 for binding to JAZ proteins. The liberation of MYC2 then results in the expression of downstream JA-induced genes and the accompanying response to stresses such as dehydration.

Mahmud *et al*. (2022) revealed that *GASA3* is upregulated during drought in *A. thaliana,* which inherently contradicts the expected effects of a gene in this family, indicating that it may play a role in drought stress and warrants further investigation. The results from this study indicated that overexpression of Jasmonate-resistant 1 (*JAR1*) hindered the upregulation *GASA3* during a drought response, which may suggest a link between the two. The exploration of this hypothesis may reveal new insights into transcriptional regulation and crosstalk between GA and JA during a dehydration and further desiccation response.

3.1.3. RT-qPCR in the context of plant research

Polymerase Chain Reaction (PCR) is well known as a ground-breaking technique used for various molecular diagnostics and was developed by Kary B. Mullis in 1985. Then, a few years later, an approach called 'kinetic PCR analysis' was introduced by Higuchi *et al*. (1993). Since then, PCR techniques have become fully quantitative, hence the development of RT-qPCR (real-time quantitative PCR), which has been highly regarded as the gold standard for quantitative analysis of nucleic acids.

However, despite characteristically high levels of sensitivity and selectivity for the evaluation of gene expression using RT-qPCR, the apparent simplicity of the technique remains susceptible to a lack of clarity and reproducibility in literature, with few publications outlining detailed acquisition of results (Die & Román, 2012). In line with this, further concerns over the assessment of RNA quality and data normalization have prompted researchers, particularly in the medical field, to attempt to develop protocols that overcome these issues (Bustin, 2002; Huggett, *et al*., 2005; Fleige & Pfaffl, 2006), with the plant research community not far behind (Gutierrez, *et al*., 2008; Udvardi, *et al*., 2008). This chapter aims to utilise the sensitivity and selectivity of an RT-qPCR assay to assess the relative gene expression of desiccation-responsive genes and their relation to jasmonates, while simultaneously evaluating the effectiveness of the technique in exploring polyploid non-model plants. The genes to be analysed include *MYC2*, *RD29B* and *GASA3*.

3.2. Materials and methods for gene expression analysis of *MYC2*, *RD29B* and *GASA3* during a desiccation response

3.2.1. Overcoming polyploidy

Due to the polyploid nature of both *X. schlechteri* and *C. pumilum*, assessing their gene expression poses various considerations. Both species are considered to be octoploid in nature (Costa, *et al*., 2017; du Toit, *et al*., 2021), meaning that there is potential for the existence of eight gene variants per species. These variants may act similarly or entirely differently to one another under any given condition, thus the need to identify these variants and understand their functions individually and as a collective whole is crucial.

In this study, several gene variants were identified for *C. pumilum* and *X. schlechteri* using existing data. At the time of this experiment, *C. pumilum* data was not available on the NCBI database. Thus, *C. pumilum* variants were identified using annotated transcriptomic data from du Toit *et al*. (2021) (Appendix B).

Following identification of the relevant transcripts, a multiple sequence alignment was conducted using Clustal Omega (Sievers, *et al*., 2011) to analyse conserved regions and sequence similarity. From these alignments, the transcript sequence sharing the most common conserved regions with all others was selected for subsequent RT-qPCR analysis for gene expression and accompanying primer design. In the cases concerning the reference or 'housekeeping' genes: *GAPDH* and *UBQ10*, significantly differentially expressed gene (SDEG) data from du Toit *et al*. (2021) was analysed and transcript variants showing the lowest amount of differential expression between the full turgor and late dehydration stages were selected as the target sequences for downstream experiments and primer design.

i. Primer design

The chosen transcript sequence for each gene was entered into the PrimerQuest™ Tool (Integrated DNA Technologies, 2012), and five potential primer pairs were suggested per gene. A single primer pair was selected based on meeting the following criteria: a T_m between 60°C and 62°C, GC content above 45% for both primers, an amplicon length between 100bp and

150bp, and primer length between 20bp and 24bp. Each primer pair was then assessed using CLC Sequence Viewer v8.0.0 (Qiagen Bioinformatics, 2017) In addition, the chosen primer pairs were compared to all sequenced transcripts available in FASTA format from the transcriptome (du Toit, *et al*., 2021), to check for homologous sequences in order to avoid non-specific PCR products as far as possible. PerPrimer v1.1.21 (Marshall, 2003) was used to evaluate primer-dimer formation. The final primer sequences can be seen in Table 3-1.

Table 3-1: Final primer pair details. *The table below outlines the sequences of each primer pair used for downstream RT-qPCR applications with accompanying details including the melting temperature (T_m), GC content and amplicon length.*

C. pumilum					
Gene	**Direction**	**Sequence (5'→3')**	**T_m (°C)**	**GC (%)**	**Length (bp)**
MYC2	Forward	GATTCTTGCCGAGGATGTATGT	60	45	22
	Reverse	CCTCTGGATACTTGGAGCAAAC	61	50	22
GASA3	Forward	GTAGCAAGGCAAGGAGGAAG	60	55	20
	Reverse	CAGAGGTGTGTGTTTCCGTAG	61	52	21
RD29B	Forward	GCGGATAGGGTTAAGGATGC	60	55	20
	Reverse	CCTCCACCTCAGCCATTATTC	60	52	21
GAPDH	Forward	CTCTTCTCAAGTCGGCAAGTTA	60	45	22
	Reverse	AAGGCTGTAGGGAAAGTTCTTC	60	45	22
UBIQ10	Forward	GAGTTCGGACACCATAGACAAT	60	45	22
	Reverse	CTACCATCCTCCAACTGCTTAC	60	50	22
X. schlechteri					
Gene	**Direction**	**Sequence (5'→3')**	**T_m (°C)**	**GC (%)**	**Length (bp)**
MYC2	Forward	GGAGCTTAACTCCCTCATATCC	60	50	22
	Reverse	CGTCATGGAGACCAAGAAGAA	60	47	21
GASA3	Forward	GAAGGTGCTCTAGAACAAGGAG	60	50	22
	Reverse	AGGGCACATGTTGTGGTTTG	61	50	20
RD29B	Forward	GCAGGCGAAGCTAGGAATATAG	60	50	22
	Reverse	AGAAGGCTAATGAACTGAAGGG	60	45	22
GAPDH	Forward	GGAAGTCAACGGAGACTAAAGG	60	50	22
	Reverse	CTTTCGCTGAGGAAGTTAATGC	60	45	22
UBIQ10	Forward	GAAGGCGAAGATACAGGATAAGG	60	47	23
	Reverse	TCTGCTAGAGTACGTCCATCC	60	52	21

ii. Primer confirmation via end-point PCR

As a preliminary experiment, Polymerase Chain Reaction was used to ensure that the primer pairs bind correctly to their targets. Samples were run at six different annealing temperatures

using undiluted cDNA to determine the most applicable temperature for all amplicons derived from the different primer pairs.

cDNA obtained using RNA extracted from both the roots and leaves of the mature *X. schlechteri* and *C. pumilum* plants, was used as template DNA in the reaction setup for end-point PCR analysis. The PCR assay was conducted using One*Taq* Hot Start DNA Polymerase (M0481) (New England Biosystems).

3.2.2. Sample preparation, RNA Extraction and cDNA synthesis

RNA was extracted from three biological replicates per condition for each tissue type and plant using TRIzol Reagent (Thermofisher Scientific, USA), and the CTAB (cetyl trimethylammonium bromide) method as outlined by Majidi & Bahmani (2017).

For the TRIzol Reagent method: Frozen plant samples were treated with 1 mL of TRIzol reagent, vortexed and left to incubate at room temperature for 5 min. Thereafter 200 µL chloroform was added and samples were vortexed until homogenous, then centrifuged at 12 000 RPM. for 15 min at 4°C using a benchtop centrifuge. The upper aqueous phase was removed to a clean tube to which 500 µL of isopropanol was added and the samples were incubated for 10 min at room temperature and then centrifuged at 12 000 RPM. for 15 min at 4°C. The supernatant was removed and 1 mL 75% ethanol (*v/v*) was added and briefly vortexed. The samples were centrifuged at 6 000 RPM. for 5 min at 4°C. The ethanol wash was repeated. The pellets were left to dry until no liquid remained in the tubes and then resuspended in 30 µL of RNase-free water.

For the CTAB (cetyl trimethylammonium bromide) method: A Lysis buffer (100 nM Tris-HCℓ (pH = 8), 25 mM Na_2ETDA (disodium ethylenediaminetetraacetate) (pH = 8), 3% CTAB *(w/v)*, 2 M NaCℓ, and 3% PVP (polyvinylpyrrolidone) *(w/v)*) was prewarmed for 15 minutes at 65°C in a waterbath and then 1 mL buffer was added to approximately 100 mg of frozen plant material, followed by 50 µL 1 M sodium citrate. Samples were vortexed briefly and then treated with 50 µL ß-mercaptoethanol, vortexed again for 20 seconds and incubated in a waterbath at 65°C for 30 min with intermittent vortexing for 20 seconds every 10 min. Samples were removed from the waterbath and 50 µL helper buffers I (25 mM Tris-HCℓ (pH = 8), 10 mM Na_2EDTA (pH = 8), and 50 mM glucose) and II (60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid, and 28.5 mL 0.1% DEPC-treated water) were added sequentially, followed by 20

seconds of vortexing and incubation for 5 min at room temperature. Thereafter, 600 µL ice-cold 24:1 (*v/v*) chloroform-isoamyl alcohol was added and samples were vortexed once again for 20 seconds and then centrifuged at 12 000 RPM. for 10 min at 4°C. The upper aqueous phase was removed and 1 mL ice-cold chloroform-isoamyl alcohol was added. Samples were mixed by inversion and centrifuged at 12 000 RPM. for 10 min at 4°C. The supernatant was then transferred to clean 1.5 mL tubes and 400 uL ice-cold 10 M LiCl was added. Samples were then inverted several times and stored at 4°C overnight. Samples were centrifuged at 12 000 RPM. for 30 min at 4°C. The supernatant was discarded and 1 mL 75% ethanol was added. Samples were washed by inversion and then centrifuged again at 12 000 RPM. for 10 min at 4°C. The ethanol wash step was repeated. The pellets were dried until no liquid remained in the tubes and suspended in 30 µL RNase-free water and stored at -80°C until further processing.

Following extraction, the quality of the RNA samples was determined using 0.8% agarose gel electrophoresis with ethidium bromide (EtBr) (Appendix C).

RNA was treated with *DNase l* using the Invitrogen *DNase l* Amplification Grade kit (ThermoFisher Scientific, USA), according to the manufacturer's instructions and stored at -80°C until later use in cDNA synthesis.

DNase I treated RNA samples were aliquoted into 200 µL PCR tubes and converted to cDNA using the protocol included with the Invitrogen RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions.

3.2.3. RT-qPCR setup using the comparative CT Method (ΔΔ CT Method)

RNA extracted from leaves and roots under each dehydration condition was pooled in a single 200 µL tube and then diluted by a factor of 100 to be used for the setup of noRT (no reverse transcriptase) controls in downstream RT-qPCR runs. cDNA produced from the RNA was also pooled and diluted in a five-step dilution series using a 10-fold dilution factor in order to produce standard curves for quantitative analysis.

The annealing temperature of all genes of interest was optimized by running standard curves using each primer set at three different annealing temperatures on a RotorGene-Q thermocycler

(Qiagen, Netherlands). The RT-qPCR products were run on a 2% agarose gel treated with EtBr to confirm the integrity and size of each amplicon.

i. Validation experiment

To ensure the validity of results produced using the $\Delta\Delta\ C_T$ Method, the efficiencies of both the target and reference genes should be approximately equal. Thus, standard curves using primer sets from both reference genes and all three target genes were set up through utilizing the pooled cDNA sample via the RotorGene Thermocycler (Qiagen, Netherlands) (Appendix D).

Once it was confirmed that the amplification efficiencies of all primer pairs were recorded as approximately equal and within the range between 95% and 105% using RotorGene-6000 series software (Qiagen, Netherlands), a validation test was conducted utilizing the linear regression function in Microsoft® Excel® to further consolidate valid PCR efficiencies. This was done by calculating the mean and standard deviation of replicate sample ΔC_T values of each target gene against each reference gene using the equation $\Delta C_T = C_{T\,target} - C_{T\,reference}$, and then plotting these values against the log of input RNA. Typically, a slope value less than 0.1 indicates a passing validation experiment.

Following confirmation regarding the appropriate use of *GAPDH* and *UBQ10* as reference genes for effective RT-qPCR analysis, data analysis utilizing the $\Delta\Delta C_T$ method was carried out to determine relative gene expression of *MYC2*, *GASA3* and *RD29B* across the four experimental conditions; (1) ~80% RWC, (2) ~60% RWC, (3) ~20% RWC, and (4) air-dry, by examining the fold change in comparison to full turgor (FT) calibrator samples.

The C_T values of *MYC2*, *GASA3* and *RD29B* expression for both dehydrated samples and full turgor calibrator samples were recorded using RotorGene 6000 series software. Following which, the mean $\Delta\Delta C_T$ values and standard deviation were calculated using the equation $\Delta\Delta C_T = \Delta C_{T\,dehydration\,condition} - \Delta C_{T\,full\,turgor}$, to determine the fold-change in gene expression from full turgor samples to various degrees of desiccation.

3.3. Results & Discussion

3.3.1. Evaluation of RNA extraction protocols across species

Protocols involving RNA extraction, gene expression analyses and functional studies have been standardised in the model plant *A. thaliana*, however the typical issues surrounding the reproducibility of RT-qPCR results are exacerbated when developing protocols for non-model plants, due to a lack of genomic or transcriptomic sequencing data and added complications as a result of their polyploid nature. It is thus imperative that potential confounding factors be considered and evaluated when optimizing RT-qPCR assays for non-model species such as those included in this research. Several protocols addressing non-model species standardization of RT-qPCR were very recently compiled by Pabón-Mora *et al*. (2023). However, there is still a significant lack of method development targeting gene expression analysis in resurrection plants specifically.

In order to understand the most effective method for the extraction of RNA from the leaf and root tissues of *C. pumilum* and *A. thaliana*, RNA extraction was conducted utilizing widely recognized techniques, and samples were assessed for quality and integrity through electrophoresis on 0.8% agarose gels. This evaluation allowed for a detailed comparison of the effectiveness of each method, providing insights into the optimal conditions for RNA extraction tailored to specific species. Unfortunately, *X. schlechteri* samples did not yield sufficient tissue during their growth to allow for the analysis of hormone profiles as well as gene expression analysis, thus gene expression was only conducted using tissue from *C. pumilum* and *A. thaliana*.

A comparative analysis of TRIzol and CTAB methodologies employed in the extraction of RNA from *C. pumilum* demonstrated that RNA obtained through the TRIzol reagent exhibited a significantly reduced level of degradation across both tissue types, with a particularly pronounced effect noted in the root tissues (Appendix C). However, both extraction techniques yielded substantial quantities of RNA as determined by spectrophotometric assessments. Notably, neither extraction technique succeeded in yielding high-quality RNA across all three replicates of samples documented at 80% relative water content (RWC), suggesting that degradation of these particular samples may have occurred prior to RNA extraction.

3.3.2. Transcript variation in octoploid species

The polyploid nature of many plants results in complex interactions between genetic components and consequently the optimization of methods for the analysis of gene expression via methods such as qPCR involves a few additional steps to ensure the development of contextually accurate results. *C. pumilum* is described as an octoploid species, which may raise questions regarding the biological significance of each transcript as not all variations may behave similarly. Recently, du Toit *et al*. (2021) revealed eight significantly differentially expressed (SDE) transcripts for *MYC2*, however only one for *GASA3*, three for *RD29B*, two for reference gene *UBQ10* and six for reference gene *GAPDH* (Appendix B), enhancing the idea that not all versions of a gene may participate in the same metabolic/molecular processes. Despite the fact that not all eight transcript variants associated with each gene exhibited differential expression in du Toit *et al*. (2021), their findings revealed that the transcript variants which did demonstrate differential expression during desiccation exhibited similar expression patterns to one another, implying that each SDE transcript would likely produce similar expression patterns in this experiment. Thus, this chapter only focused on assessing the expression patterns of a single transcript variant per gene of interest.

Table 3-2: Significantly differentially expressed genes (SDEGs) during dehydration in C. pumilum. *du Toit et al. (2021) revealed the number of differentially expressed transcripts of MYC2, GASA3 and RD29B during a desiccation response in C. pumilm. Although this is an octoploid species, not all theoretical transcript variants are available for every gene. The transcripts chosen for reference genes UBQ10 and GAPDH were selected based on the lowest degree of differential expression found in du Toit et al. (2021). This table outlines which transcript variant of each gene in C. pumilum was chosen for downstream experiments.*

GOI	*A. thaliana* tag	No. of SDEGs found in *C. pumilum*	Transcript no. chosen for downstream experiments
MYC2	AT1G32640	8	6
GASA3	AT4G09600	1	1
RD29B	AT5G52300	3	2
UBQ10	AT4G05320	2	1
GAPDH	AT1G16300	6	4

As primers utilized for qPCR analysis are restricted to the design for a singular transcript, subsequent to the identification of SDE transcripts for each gene, the sequences of these transcripts were aligned to facilitate the identification of conserved regions and the construction of a percentage identity matrix (Figure 3-3), thereby enabling an assessment of which transcript exhibits the highest degree of similarity with others, ultimately to evaluate which transcript may hold the greatest biological significance.

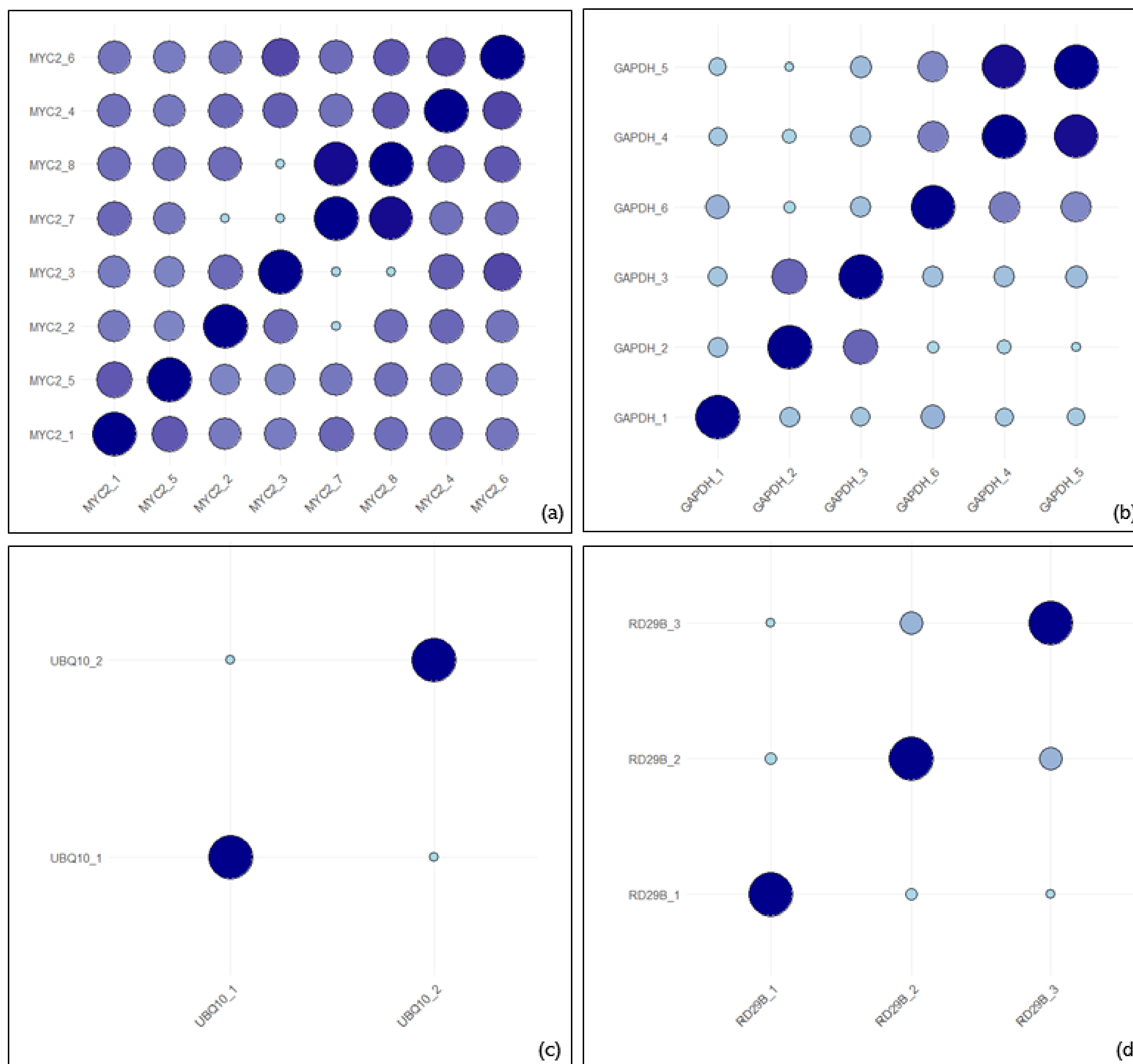


Figure 3-3: Percentage identity matrix presenting trends in sequence similarity of transcript variants of MYC2, RD29B, UBQ10 and GAPDH to determine potential biological significance. *The average percentage identity of each transcript was calculated and used to reveal which transcript showed the highest degree of similarity with all others to ultimately deduce which variant may hold the greatest biological relevance. Data showed that the transcripts with the highest sequence similarities across the four genes were MYC2_6, RD29B_2, UBQ10_1 and GAPDH_4. These variants were then utilized for downstream primer design. Only a single transcript variant of GASA3 was identified by du Toit et al. (2021), thus a percentage identity matrix was not constructed for GASA3.*

Transcripts with the highest degree of sequence similarity for reference genes *UBQ10* and *GAPDH* (Figure 3-3) did not appear to correlate directly with those identified to present with the lowest degree of differential expression in du Toit *et al*. (2021), however, as their purpose for this study was to act primarily as reference genes in RT-qPCR experiments, the selection of each of their respective transcripts for primer design was based solely on which transcript showed the lowest degree of differential expression (UBQ10_1 and GAPDH_4). To further compare transcripts, the length of each transcript of interest was plotted against the others.

Analysis of the lengths of each transcript (Figure 3-4) revealed a large degree of variation, alluding to potentially high degrees of splicing or posttranscriptional modification that likely occurs during expression of these genes. Despite du Toit *et al*. (2021) revealing similar trends of expression for all variants, it is still possible that individual transcripts may possess different functions and although beyond the scope of this thesis, studies involving gene expression analyses of polyploid species should always consider this possibility.

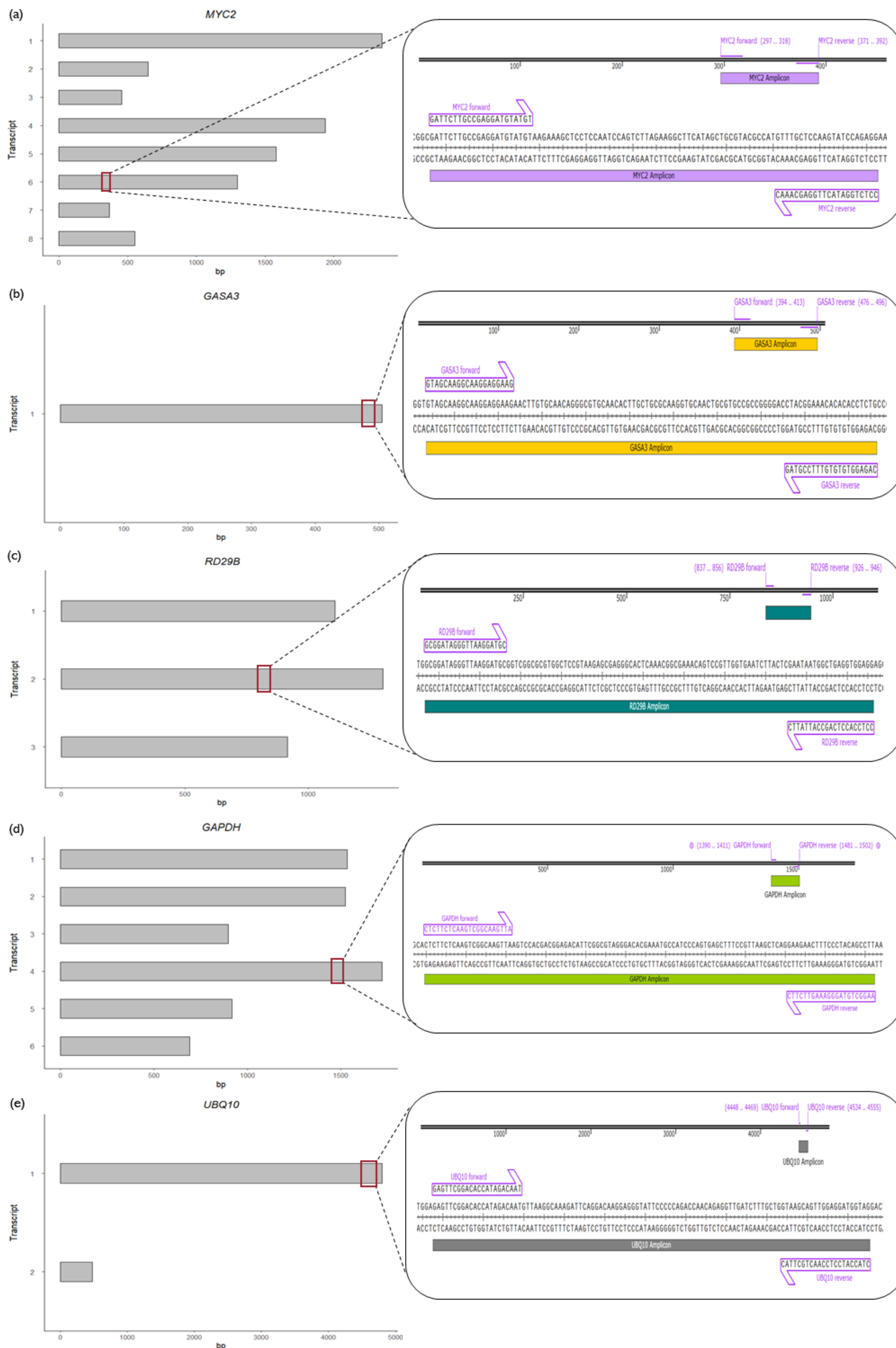


Figure 3-4: A graphical representation of the respective lengths of each transcript variant associated with the genes under investigation. *Subsequent examination of a multiple sequence alignment identified conserved regions within each transcript and these areas were then used towards the design of primers to be used in downstream qPCR gene expression assays. Each red box outlines*

the conserved region of the transcript selected for primer design and closer inspection of each region reveals the genetic location of each primer pair.

3.3.3. Evaluation of primers and reference genes

Primer pairs for each chosen transcript were subjected to temperature testing to ensure optimization (Appendix E). All primer pairs showed the highest efficacy at 61°C. However, reference genes *UBQ10* and *GAPDH, UBQ10* did not show sufficient amplification in the leaf tissue, whereas *GADPH* did not show sufficient amplification in the root tissue. The reason for this remains relatively unclear, but it is reasonable to postulate these transcripts are not necessarily expressed uniformly or indeed at all, across all tissue types. Another possibility is that different transcript variants become active in different tissues, thus transcripts for which primers were not optimised may become active in place of those used in this study. This only further suggests that a gap may exist in our fundamental understanding of gene expression in polyploid organisms and great care should be taken when evaluating results to ensure biological relevancy when dealing with genetically complex organisms.

Despite this, primer pairs targeting *GAPDH* were indeed sufficiently amplified in the leaves and those for *UBQ10* were effectively amplified in the roots, and so *GAPDH* was retained as the reference gene for the experimental analysis of leaf tissue, while *UBQ10* was designated the reference gene for the analysis of root tissue.

In addition, results indicated that *MYC2* did not amplify efficiently (Appendix E), suggesting potential issues with reaction conditions or primer design. However, despite the development of additional sets of primers and subsequent troubleshooting, amplification of *MYC2* still did not occur (data not shown). This persistent difficulty in amplifying in *MYC2* in *C. pumilum* in comparison to *A. thaliana* highlights the potential problems that can arise when adopting protocols designed for model species for the analysis of more complex non-model species. These discrepancies may also indicate a divergence in the jasmonate signalling pathways between *C. pumilum* and *A. thaliana*, indicating potential differences in how these organisms respond to dehydration stress. Moreover, studies have shown that variations in bHLH transcription factors such as *MYC2,* which shares strong homology with *MYC3*, *MYC4* and other TFs such as *EGL1* (ENHANCER OF GLABRA3 1), can dramatically influence plant defence mechanisms (reviewed in Kazan & Manners (2013)), suggesting a high degree of functional diversity within this group of TFs and interference with *MYC2*'s multiple regulatory pathways. Although *MYC2* seemingly governs the expression of a multitude of genes, the

understanding regarding whether *MYC2* interacts directly or indirectly with the promoters of these genes is relatively limited considering its interaction with so many other molecules. Conversely, the assumption that *MYC2* plays a less critical role in *C. pumilum* based on amplification failure may overlook its potential functions that are not yet understood in non-model organisms. The JA signalling pathway is complex even in dicot model species such as *A. thaliana*, and unexpected variations in gene expression in more genetically complex organisms could mean that *MYC2* is involved in different capacities or under specific conditions that were not tested in this thesis.

3.3.4. $\Delta\Delta C_T$ validation

Following the identification of relevant transcripts for each gene and ensuring the efficacy of their respective primers (Appendix B & E), linear regression models were set up using RT-qPCR standard curves for each gene of interest and the reference genes to ensure that their efficiencies were approximately equal for valid use of the $\Delta\Delta C_T$ method of relative quantification (Figure 3-5).

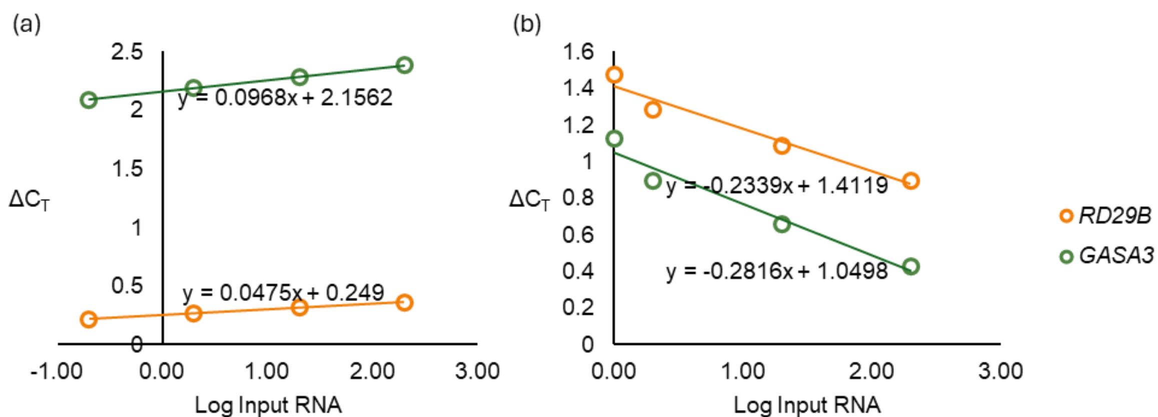


Figure 3-5: Linear regression models of RT-qPCR standard curves to ensure equal amplification efficiency for each gene of interest and their respective reference genes. *The results in panel (a) show those derived from amplification from leaf tissue and (b) from root tissue. Evidently, both genes display approximately equal amplification efficiencies when evaluated against GAPDH in the leaf tissue as noted by both slopes being -0.1 ≤ 0.1, however the opposite is true for results obtained against UBQ10 in the roots as both slopes deviate from the acceptable range.*

Results of this test revealed that when both GOIs were evaluated against *GAPDH* in the leaf tissue (Figure 3-5a), their amplification efficiencies were approximately equal, validating their appropriateness for analysis via the $\Delta\Delta C_T$ method. However, observed discrepancies in amplification efficiency when using *UBQ10* (Figure 3-5b) as a reference in root tissues necessitated a modification in the approach to data analysis. Thus, downstream analysis of root-

derived results was carried out via a less stringent method of relative quantification being the relative standard curve method.

3.3.5. Gene expression of GASA3 and RD29B in the leaves and roots of *C. pumilum*

i. Expression of GASA3 in the leaves

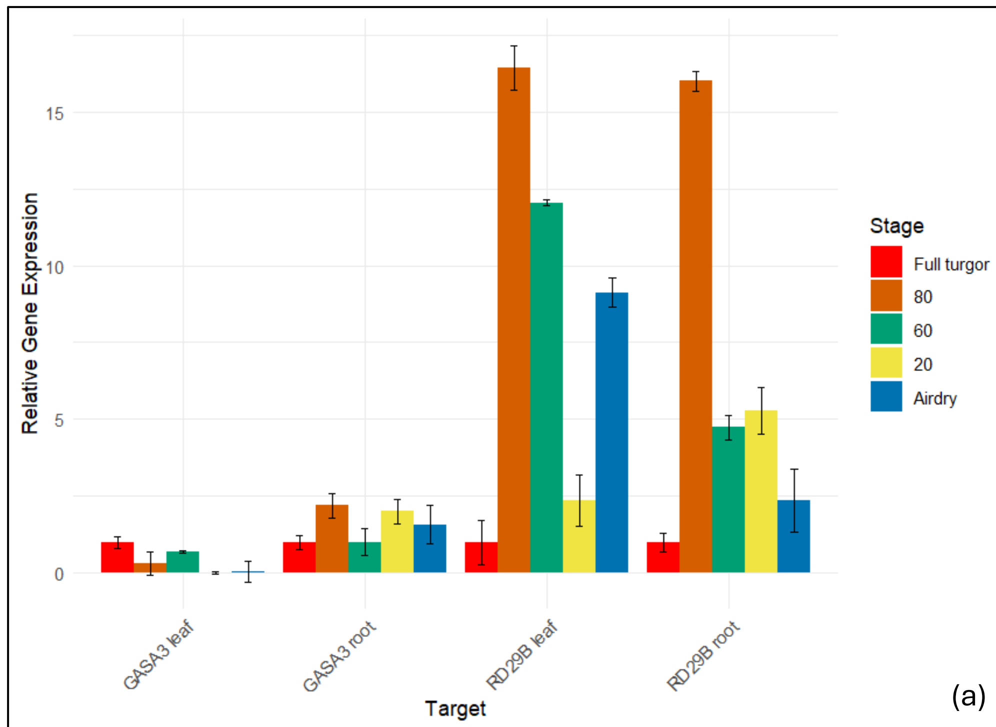
As mentioned in Chapter 2, as GA levels decrease under abiotic stress conditions, the reduced inhibition of DELLAs may enhance stress tolerance by allowing for more robust activation of JA-signalling. To explore this, relative gene expression of *GASA3*, a potential mediator between GA- and JA-signalling was explored during a dehydration response in *C. pumilum*. Relative gene expression of *GASA3* and *RD29B* during desiccation in the leaves and roots of *C. pumilum* are shown in Figure 3-6. The expression of *GASA3* in leaf tissue exhibited downregulation during dehydration until reaching 60% RWC and demonstrated significant downregulation during the period of late dehydration from 20% RWC onward. These findings are in contrast to those published by Mahmud *et al*. (2022), who found that *GASA3* is upregulated in the leaves of *A. thaliana* during drought. However, results from their paper also showed that *GASA3* is supressed during drought in genetic lines that overexpress JAR1, the enzyme that catalyses the conversion of JA to JA-Ile. Thus, when comparing this data to results from Chapter 2, where there is evidently a slight increase in JA-Ile during drought stress in the leaves of *C. pumilum* and a decrease in the roots, it stands to reason that this accumulation of JA-Ile in the leaves may be indicative of upregulation of a JAR1 homolog in *C. pumilum*, which may then also elude to a relationship and potential desiccation-tolerance mechanism between JAR1 and *GASA3* in *C. pumilum*, representative of GA- and JA-mediated crosstalk.

On the other hand, this decrease in *GASA3* in the leaves aligns with the trend exhibited by the phytohormone derivative GA4 as discussed in Chapter 3.3., suggesting a potential correlation between GA4 signalling and *GASA3* expression, particularly since this trend is not observed in the levels of GA3, which is generally considered to be the predominant form of gibberellic acid found in plants such as *A. thaliana*. This close mirroring of *GASA3* activity to a secondary derivative of gibberellic acid suggests that it may not be involved in the main signalling pathway or crosstalk of GA; GA-DELLA-JA communication such as derivatives like *GASA4* or *GASA1*, but may instead simply enhance these processes, participate in similar

communications on a smaller scale or may be involved in entirely different abiotic stress responses such as cell wall strengthening.

ii. Expression of GASA3 in the roots

Conversely, the expression of *GASA3* in the roots was slightly upregulated across all stages of dehydration when compared to the full turgor state. This follows an entirely divergent trend from that of GA3 and GA4 in the roots, indicating a potentially independent role of *GASA3* with respect to gibberellic acid signalling, ie. GA4 and GA3 do not impact the abundance of proteins produced by *GASA3* in the roots. Gibberellic acid plays a pivotal role in modulating root architecture under drought conditions by promoting cell elongation and influencing lateral root development, enhancing the plant's ability to branch out in search of water (Ranjan, *et al*., 2022). However, the downregulated levels of the gibberellins in the roots of *C. pumilum* suggest that it likely does not adopt this strategy. The elevation of *GASA3* levels within the root system appears to be inconsistent with the observed root expansion, as observed through morphological analysis, and both GA3 and GA4 exhibit a decrease in abundance and so it is again plausible to infer that *GASA3* may function independently of gibberellin signalling pathways.



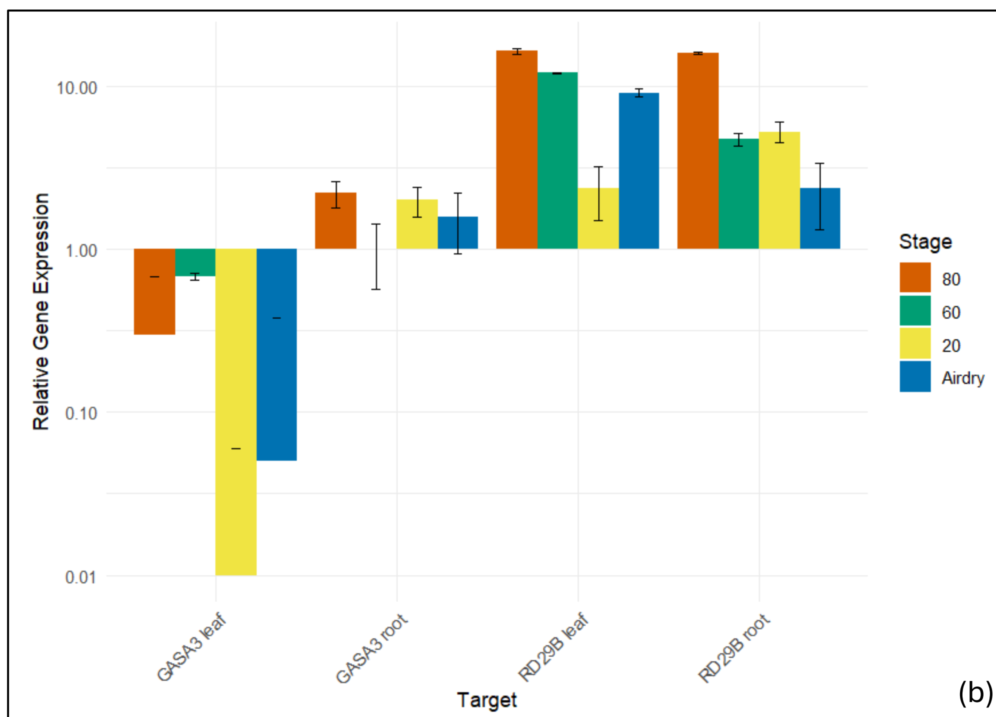


Figure 3-6: Relative gene expression of GASA3 and RD29B in the leaves and roots of C. pumilum during a dehydration response. *The above figures illustrate the expression of GASA3 and RD29B at multiple stages of dehydration (80-, 60-, 20% RWC and at the airdry stage). Panel (a) is plotted on a linear scale, whereas panel (b) is plotted on a logarithmic scale and data analysis of relative gene expression was done using (1) the ΔΔCT method for leaf tissue, using GAPDH as the reference gene and (2) the relative standard curve method for root tissue, using UBQ10 as the reference gene.*

In summary, overall *GASA3* expression during dehydration showed downregulation in leaves and slight upregulation in the roots of *C. pumilum*. This contrasts with findings published in research involving *A. thaliana*, suggesting species-specific drought responses. In addition, the accumulation of JA-Ile in leaves may indicate upregulation of a JAR1 homolog, which links JA and *GASA3* in a dehydration/desiccation-tolerance mechanism. The independent expression of *GASA3* in roots, seemingly unaffected by levels of gibberellic acid, suggests it may play a role in modifying root architecture under drought through distinct mechanisms.

iii. Expression of RD29B in the leaves

RD29B was analysed in this Chapter as a result of its reportedly high dependence on ABA signalling (Uno, *et al*., 2000). *RD29B* exhibited significant upregulation during the early stages of dehydration in the leaves (around 80% to 60% RWC) (Figure 3-6), followed by a relatively sharp decrease at 20% RWC and an additional peak observed during the air-dry stage. This pattern somewhat resembles the fluctuations in ABA levels within the leaves recorded in Chapter 2, complementing findings that *RD29B* is indeed ABA-dependent, at least in the leaves, and likely plays a role in the desiccation response of *C. pumilum* potentially by expressing proteins similar to LEA proteins, which are well-known to participate in a desiccation response in several organisms (Jia, *et al*., 2012).

iv. Expression of RD29B in the roots

RD29B expression in the roots of C. *pumilum* was shown to be upregulated across all stages when compared to samples at full turgor; however, it exhibited a gradual decline as dehydration progressed after the pronounced increase during the early drying stage at 80% RWC. In contrast to the leaves, this observation is noticeably different to the abundance of ABA in the roots, which undermines the assumption that *RD29B* is entirely dependent on ABA signalling. The gradual increase of ABA may suggest that a desiccation response may still be occurring in the roots, but it may be prioritizing other signalling cascades and is not as pronounced as within the leaves.

To summarise, the observed upregulation of *RD29B* during early dehydration stages, alongside its correlation with levels of abscisic acid, underscores its potential role in the desiccation response in *C. pumilum*, particularly in leaf tissues. However, the contrasting expression dynamics in the roots suggest a more complex array of molecular mechanisms that may involve factors beyond ABA, highlighting its multifaceted adaptive strategy to desiccation. This study

reinforces the significance of *RD29B* in the context of drought tolerance, but further research is needed into the role of other genes, for example *RD29A*, that are related to these cascades and can help to paint a more holistic picture of the intricacies of gene regulation during abiotic stress responses in not only non-model organisms, but different tissues as well.

3.3.6. Evaluation of qPCR techniques in the model plant *Arabidopsis thaliana*

As a result of consistent issues during RT-qPCR optimization to assess gene expression of the chosen *MYC2* transcript in *C. pumilum*, evaluation of species-specific trends and/or lack thereof was required. Since the reference gene *GAPDH*, and the two remaining genes of interest, *GASA3* and *RD29B* showed little to no difficulty in producing utilizable qPCR data, it is not unreasonable to postulate that more detailed species-specific molecular interaction involving *MYC2* may have been occurring in *C. pumilum*. Conversely, optimization conditions may not have been set up appropriately to accommodate for *MYC2* expression analysis in *C. pumilum*, constituting a methodological error.

To test this, a shortened RT-qPCR protocol equivalent to that done for the analysis of *C. pumilum* was set up to assess the relative gene expression of *MYC2* (AT1G32640) in the model organism *A. thaliana*, thereby confirming whether inconsistencies arising from the evaluation of *MYC2* in *C. pumilum* came about as a result of methodological error or a potential mechanism adopted by *C. pumilum* as a result of its polyploid nature.

3.3.7. Evaluation of MYC2 under drought stress in *A. thaliana*

A. thaliana, ecotype Col-0 seeds were germinated and allowed to reach maturity over four weeks under well-watered conditions at 24°C, 55% humidity, ≈115 PAR (mol.m-2.s-1) light intensity, and a 16/8-hour light/dark cycle. The plants were thoroughly watered for a final time and the first leaf tissue samples were collected the next day at the same time to represent the full turgor (100% RWC) condition. The plants were then subjected to dehydration by withholding water for the rest of the experiment. Leaf tissue was collected and immediately frozen in liquid nitrogen for storage at -80°C until needed for downstream processing.

Given the uncertainty around whether *MYC2* amplification errors in *C. pumilum* were methodological or biological, RNA was extracted from three *A. thaliana* leaf replicates per

condition (morning vs. afternoon, full turgor vs. dehydrated), via three RNA-extraction methods; (1) extraction using TRIzol Reagent (Thermofisher Scientific, USA), (2) CTAB (cetyl trimethylammonium bromide) method as outlined by Majidi & Bahmani (2017); and (3) via the RNeasy Mini Kit (Qiagen, Netherlands) as per the manufacturer's recommendations. Extracted RNA from all three methods was suspended in RNase-free water and stored at -80°C.

The evaluation of the integrity of RNA indicated that, even when compared with RNA extracted via the Qiagen RNeasy kit, the TRIzol method resulted in the highest level of integrity (Appendix B). When compared to the findings that the CTAB method showed better results within the tissues of *C. pumilum*, these results highlight that because plants, particularly angiosperms, are complex and highly diverse, protocols for the extraction of genetic material from different species necessitate rigorous evaluation and validation to ensure their efficacy across different species.

Additional steps for primer confirmation in *A. thaliana* are not typically as rigorous as those used to isolate transcripts and design primers appropriately in non-model organisms such as *C. pumilum*, as it is a diploid species with readily available primers. Consequently, this raises concerns over using genes identified in *A. thaliana* and attempting to identify and isolate homologs in understudied species as there are many additional steps required that make the analysis prone to error and do not necessarily provide biologically accurate data as it is inherently difficult to compare diploid and polyploid species using the same lens.

Evaluation of *MYC2* and *SAND* primers for the assessment of gene expression in full turgor and dehydrated leaf tissue of *A. thaliana* yielded positive amplification and *SAND* was shown to be constantly expressed under all conditions, confirming its appropriate selection as a reference gene (Appendix D).

A regression test was also done to confirm approximately equal amplification efficiencies between *MYC2* and *SAND* in *A. thaliana* (Figure 3-7). The results showed that their efficiencies were indeed approximately equal and the data was appropriate to assess via the $\Delta\Delta C_T$ method.

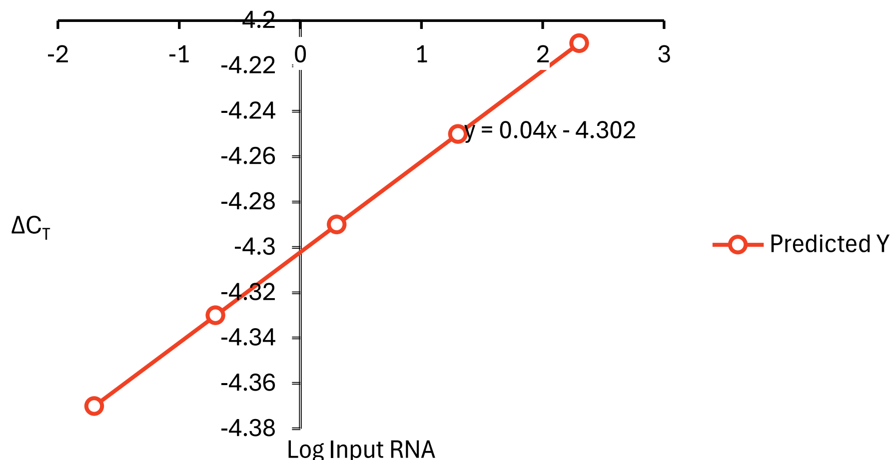


Figure 3-7: Linear regression models of RT-qPCR standard curves to ensure equal amplification efficiency for MYC2 and SAND reference gene in A. thaliana. *The results show the linear regression incorporating C_T values to determine amplification efficiency. The slope is -0.1 ≤ 0.1 and so it can be concluded that the amplification efficiencies of MYC2 and SAND are approximately equal and the data is appropriate to use for gene expression analysis utilizing the $\Delta\Delta C_T$ method of relative quantification.*

To test for the expression of *MYC2* under dehydration stress conditions in *A. thaliana*, plants were subjected to dehydration and sampled at morning and afternoon time points to account for circadian differences in expression. This experiment was set up as a derivative of the methods explained in the previous sections when assessing gene expression in *C. pumilum* in order to understand whether the inability to amplify *MYC2* in that experiment came about due to methodological error or a potential mechanism adopted by *C. pumilum*.

Results from this analysis revealed that *MYC2* was amplified easily in *A. thaliana* under similar conditions and showed expected expression patterns, where there was high accumulation of *MYC2* in dehydrated tissue in the morning samples, but lessened accumulation in the afternoon samples in comparison to full turgor samples (Figure 3-8). In conjunction with the role of *MYC2* in jasmonate signal transduction during a drought response in *A. thaliana*, research has also shown that *MYC2*, along with *MYC3* and *MYC4*, is crucial in regulating time-of-day variations in susceptibility to pathogens (Joseph, *et al*., 2021). The authors demonstrated that accumulation of *MYC*s at subjective dawn enhances *A. thaliana*'s defence against *Botrytis cinerea*. With this in mind, the results are not unexpected and underscore this phenomenon. It can be concluded that *MYC2* expression in *A. thaliana* followed expected trends and the inconsistencies brought about by the lack of *MYC2* amplification in *C. pumilum* is likely not as a result of poor methodological design but may point to more intricate regulation of specific genes.

Because *C. pumilum* is an octoploid species and may display eight transcript variations of the same gene, it is possible that some of these variations are non-functional or perhaps they work together in unexplored ways to facilitate a drought response or an entirely separate function.

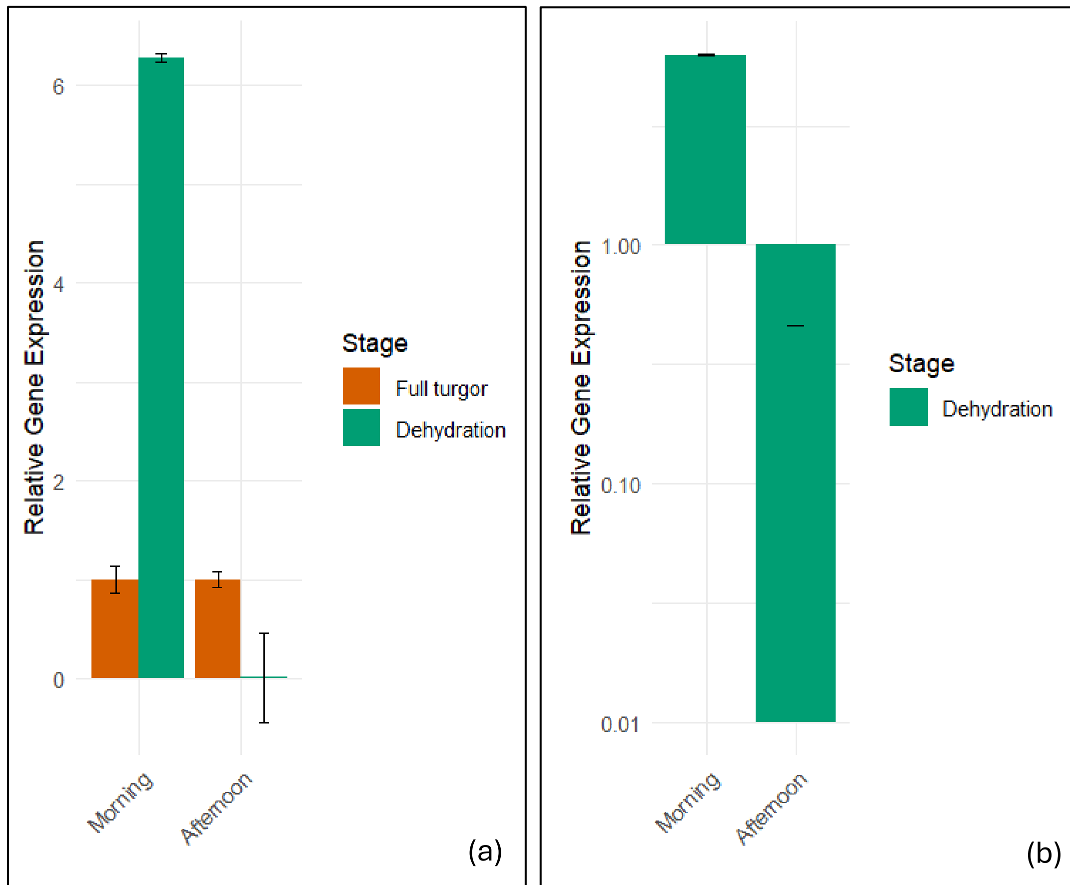


Figure 3-8: Relative gene expression of MYC2 in the leaves of A. thaliana during full turgor and dehydrated conditions with samples gathered in the morning and afternoon. *Panel (a) is plotted on a linear scale, while panel (b) is plotted on a logarithmic scale, thereby showing the fold change in gene expression during dehydration in comparison to full turgor for both morning and afternoon samples.*

3.3.8. Conclusions

This chapter presents a more nuanced examination of the gene expression patterns of *GASA3* and *RD29B* in both the leaves and roots of *C. pumilum* subjected to dehydration stress and underscores the complex roles of signalling molecules in hormonal networks that play a pivotal role in plant responses to abiotic stressors. The observed downregulation of *GASA3* in leaf tissues during desiccation stands in contrast to findings reported in *A. thaliana*, indeed suggesting species-specific mechanisms adopted by *C. pumilum* and potentially other resurrection plants may involve intricate interactions between gibberellin and jasmonic acid signaling pathways. This divergence does infer the possibility that different plant species have evolved unique regulatory responses to cope with drought stress, reflecting their adaptive strategies in varying ecological niches. Additionally, significant upregulation of *GASA3* in the root tissue points to another potentially distinct role in the modulation of root architecture under drought conditions. This finding adds a layer of complexity to our understanding of *GASA3*'s function, particularly in relation to gibberellin signalling, and highlights that its role is not uniform across different plant tissues. Furthermore, the expression patterns of *RD29B* reveal a particularly intriguing aspect of plant stress responses with regard to phytohormone signalling; the ABA-dependence of *RD29B* expression in leaves, contrasted with its varying expression patterns in roots, underscores the multifaceted nature of molecular responses to abiotic stress and suggests that *RD29B* and subsequent downstream effects are not solely dependent on ABA in different tissues. In addition, the challenges faced in amplifying *MYC2* transcripts in *C. pumilum* raise critical concerns regarding the methodological approaches currently utilized for studying gene expression in polyploid species. This issue points to the necessity for more targeted techniques to investigate the mechanisms that govern gene expression in polyploid organisms, as they may differ significantly from those observed in diploid models.

Chapter 4 : Overall Conclusions

During periods of drought, crops lose water through transpiration, leading to stunted growth, low yields, or death, which threatens global food security. Plants face various abiotic and biotic stressors, which has prompted adaptations to mitigate these effects. Water deficit in particular is a significant abiotic stressor effecting crop productivity and has led to extensive efforts to understand how its harsh effects impact plants and how plants have evolved to mitigate some of these negative effects. *Arabidopsis thaliana* has long been considered the model plant species for the assessment of a huge variety of factors in plants, including drought tolerance research. This is due to its unique characteristics that facilitate extensive research in plant biology. It has a relatively small genome, rapid life cycle and a diverse availability of ecotypes, making it an ideal candidate for studying the fundamental processes of plants. However, it is possible that its simplicity can lead to oversimplified conclusions that may not reflect true for more complex plant species in a biological or ecological context. To date, there is very little research that has been done to study hormonal changes in resurrection plants specifically, and how they relate to understanding desiccation tolerance. Thus, the primary objective of this thesis was to uncover the phytohormone profiles associated with two evolutionarily distinct resurrection plant species, *Craterostigma pumilum* and *Xerophyta schlechteri*, in order to gain a more comprehensive understanding of the general involvement of phytohormones in each of the two main desiccation tolerance strategies typically adopted by resurrection plants; homoiochlorophylly and poikilochlorophylly.

Evidently, distinct hormone profiles exist not only between the species, but within the leaf and root tissues of each as well (Chapter 2). Some tentative conclusions could be drawn here, but because the exact relevance of these hormonal fluctuations could not be ascertained, further analysis was done wherein homologous versions of candidate genes isolated in *A. thaliana,* believed to participate in hormone signalling, were evaluated in *C. pumilum*, this being a dicot and more similar to *A. thaliana*. Results incorporating these findings are discussed in Chapter 3 and summarized below in Figure 4-1. In addition, several comparisons can also be drawn between these findings and similar data collected from *A. thaliana* and other (summarized in Figure 2-2). Results from Chigri & Bhattacharyya (unpublished) suggest that ABA is a regulator of *GASA3* and its subsequent dehydration response in *A. thaliana*, however results from this thesis suggest that ABA does not have a direct effect on the expression of *GASA3*

during a desiccation response within the leaves of *C. pumilum*. Despite this, the possibility remains that an ABA-dependent signalling pathway involving *GASA3* may indeed play a role in desiccated roots in *C. pumilum* (Figure 4-1). Typically, the abundance of GA decreases during dehydration, while JA increases in *A. thaliana* (Figure 2-2), resulting in the inhibition of DELLA proteins, release of the inhibitory effects of *MYC2* and subsequent expression of JA-mediated genes. However the potential effects of GA and JA abundances on *GASA3* have not yet been fully elucidated. Results from *C. pumilum* suggest that GA may loosely play a role in the expression of *GASA3*, but the effects would likely vary by tissue type (Figure 4-1). Similarly, the overall low abundance of JA and JA-Ile during desiccation compounded with significantly higher levels of OPDA may be indicative of a unique signalling cascade that may involve mirroring of typical jasmonate signalling through mediators such as JAR1 (Figure 4-1), an inhibitor of *GASA3*. Lastly, similarly to ABA signalling in *A. thaliana* (Figure 2-2), *RD29B* may be involved as a messenger invoked by ABA to signal the accumulation of LEA proteins in *C. pumilum*, which are known to be involved during desiccation in many plants.

Finally, data incorporating the protocols used in this thesis also underscore potential pitfalls of relying on model species such as *A. thaliana* to draw conclusions about more complex non-model plants such as resurrection plants. Future development of species-specific protocols, particularly relating to highly rigid techniques such as RT-qPCR would be greatly beneficial to the analysis of resurrection plants. Furthermore, due to the highly complex nature of hormone signalling as well as the paucity of research into phytohormone interactions in resurrection plants, additional future research should focus on generating more hormone profiles incorporating additional resurrection species to flesh out a more comprehensive understanding of the different DT strategies adopted by resurrection plants.

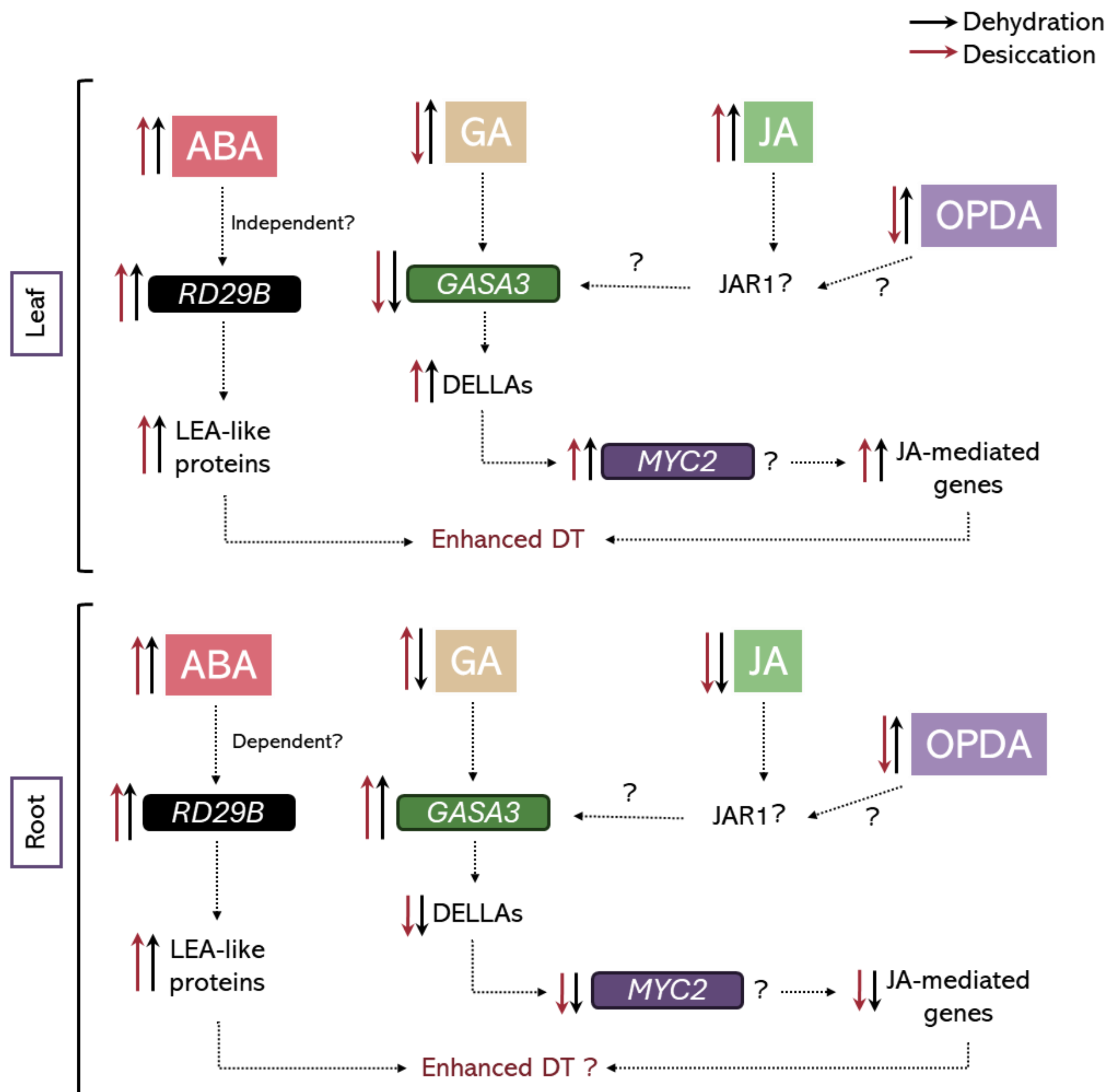


Figure 4-1: Tentative representation of potential signalling pathways during the dehydration and desiccation responses of C. pumilum. *The abundance of phytohormones and some of their potentially associated genes show some differences between the leaf and root tissues. The findings of this thesis highlights the complexities of understanding phytohormone crosstalk and the extent of their involvement with genes potentially involved in DT responses. There are many unknowns and significant future research is needed to compile complete phytohormone profiles of resurrection plants to comprehensively understand their roles in various DT strategies.*

References

1. Aalto, M. K. et al., 2012. ERD15—An attenuator of plant ABA responses and stomatal aperture. *Plant Science,* Volume 182, pp. 19-28. doi: 10.1016/j.plantsci.2011.08.009.
2. Abreu, M. E. & Munné-Bosch, S., 2008. Salicylic acid may be involved in the regulation of drought-induced leaf senescence in perennials: A case study in field-grown Salvia officinalis L. plants. *Environmental and Experimental Botany,* Volume 64, pp. 105-112. doi: 10.1016/J.ENVEXPBOT.2007.12.016.
3. Achard, P. et al., 2006. Integration of Plant Responses to Environmentally Activated Phytohormonal Signals. *Science,* 311(5757), pp. 91-94. doi: 10.1126/science.1118642.
4. Adie, B. A. T. et al., 2007. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. *Plant Cell,* 19(5), pp. 1665-1681. doi: 10.1105/tpc.106.048041.
5. Alam, M. et al., 2022. Seed priming upregulates antioxidant defense and glyoxalase systems to conferring simulated drought tolerance in wheat seedlings. *Plant Stress,* Volume 6, p. doi: 10.1016/j.stress.2022.100120.
6. Al-Hakimi, A. & Hamada, A., 2001. Counteraction of Salinity Stress on Wheat Plants by Grain Soaking in Ascorbic Acid, Thiamin or Sodium Salicylate. *Biologia Plantarum ,* Volume 44, p. 253–261. doi: 10.1023/A:1010255526903.
7. Alpert, P. & Oliver, M. J., 2002. Drying without dying. In: *Desiccation and Survival in Plants .* Wallingford: CABI Publishing, pp. 3-43.
8. Anjum, S. A. et al., 2011. Methyl Jasmonate-Induced Alteration in Lipid Peroxidation, Antioxidative Defence System and Yield in Soybean Under Drought. *Journal of Agronomy and Crop Science,* 197(4), pp. 296-301. doi: 10.1111/j.1439-037X.2011.00468.x.
9. Anjum, S. A. et al., 2011. Morphological, physiological and biochemical responses of plants to drought stress. *African Journal of Agricultural Research,* 6(9), pp. 2026-2032. doi: 10.5897/AJAR10.027.
10. Artsaenko, O. et al., 1995. Expression of a single-chain Fv antibody against abscisic acid creates a wilty phenotype in transgenic tobacco. *The Plant Journal,* 8(5), pp. 745-750. doi: 10.1046/j.1365-313X.1995.08050745.x.
11. Ashraf, M. & Harris, P. J. C., 2013. Photosynthesis under stressful environments: An overview. *Photosynthetica,* 51(2), pp. 163-190. doi: 10.1007/s11099-013-0021-6.

12. Baker, S., Wilhelm, K. & Thomashow, M., 1994. The 5′-region of Arabidopsis thaliana cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Molecular Biology,* Volume 24, p. 701–713. doi: 10.1007/BF00029852.
13. Bandurska, H. & Stroi ski, A., 2005. The effect of salicylic acid on barley response to water deficit. *Acta Physiologiae Plantarum,* Volume 27, pp. 379–386. doi: 10.1007/s11738-005-0015-5.
14. Bartels, D. & Hussain, S., 2011. Resurrection Plants: Physiology and Molecular Biology. In: *Plant Desiccation Tolerance. Ecological Studies.* Berlin: Springer, pp. doi: doi.org/10.1007/978-3-642-19106-0_16.
15. Bartels, D. et al., 1990. Molecular cloninig of absisic acid-modulated genes which are induced during desiccation of the resurrection plant Craterostigma plantagineum. *Planta,* 181(1), pp. 27-34. doi: 10.1007/BF00202321.
16. Bewley, J. D., 1979. Physiological aspects of desiccation tolerance. *Annual Review of Plant Physiology,* pp. 195-238.
17. Binenbaum, J., Weinstain, R. & Shani, E., 2018. Gibberellin localization and transport in plants. *Trends in Plant Science,* Volume 23, p. 410–421. doi: 10.1016/j.tplants.2018.02.005.
18. Birse, E. M., 1957. Ecological Studies on Growth-Form in Bryophytes: II. Experimental Studies on Growth-Form in Mosses. *Journal of Ecology,* 45(3), p. 721–733. doi: doi.org/10.2307/2256954.
19. Borsani, O., Valpuesta, V. & Botella, M. A., 2001. Evidence for a Role of Salicylic Acid in the Oxidative Damage Generated by NaCl and Osmotic Stress in Arabidopsis Seedlings. *Plant Physiology,* 126(3), p. 1024–1030. doi: 10.1104/pp.126.3.1024.
20. Browse, J., 2005. Jasmonate: an oxylipin signal with many roles in plants. *Vitamins and Hormones,* Volume 72, pp. 431-456. doi: 10.1016/S0083-6729(05)72012-4.
21. Browse, J., 2009. Jasmonate passes muster: a receptor and targets for the defense hormone. *Annual Review of Plant Biology,* Volume 60, pp. 183-205. doi: 10.1146/annurev.arplant.043008.092007.
22. Buch, H., 1947. Über die wasser-und mineralstoffversorgung der moose. *Commentationes Biologici Societas Scientiarum,* 9(20), pp. 1-61.
23. Bustin, S. A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology,* Volume 29, pp. 23-39.

24. Bustin, S. A. & Nolan, T., 2004. Pitfalls of Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction. *Journal of Biomolecular Technology,* 15(3), p. 155–166..

25. Chandler, J. W. & Bartels, D., 1997. Structure and function of the vp1 gene homologue from the resurrection plant Craterostigma plantagineum Hochst.. *Molecular and General Genetics ,* Volume 256, p. 539–546. doi: 10.1007/s004380050599.

26. Chaves, M., Flexas, J. & Pinheiro, C., 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany,* 103(4), pp. 551-560. doi: 10.1093/aob/mcn125.

27. Chávez-Arias, C., Ramírez-Godoy, A. & Restrepo-Díaz, H., 2022. Influence of drought, high temperatures, and/or defense against arthropod herbivory on the production of secondary metabolites in maize plants. A review. *Current Plant Biology,* Volume 32, p. doi: 10.1016/j.cpb.2022.100268.

28. Chini, A. et al., 2007. The JAZ family of repressors is the missing link in jasmonate signalling. *Nature,* Volume 448, pp. 666-671. doi: 10.1038/nature06006.

29. Christ, B. et al., 2014. Water deficit induces chlorophyll degradation via the 'PAO/phyllobilin' pathway in leaves of homoio- (Craterostigma pumilum) and poikilochlorophyllous (Xerophyta viscosa) resurrection plants. *Plant, Cell & Environment,* 37(11), pp. 2521-2531. doi: 10.1111/pce.12308.

30. Colebrook, E., Thomas, S., Phillips, A. & Hedden, P., 2014. The role of gibberellin signaling in plant responses to abiotic stress. *Journal of Experimental Biology,* Volume 217, p. 67–75. doi: 10.1242/jeb.089938.

31. Conconi, A., Smerdon, M. J., Howe, G. A. & Ryan, C. A., 1996. The octadecanoid signalling pathway in plants mediates a response to ultraviolet radiation. *Nature,* 383(6603), pp. 826-829. doi: 10.1038/383826a0.

32. Costa, M. et al., 2017. A footprint of desiccation tolerance in the genome of Xerophyta viscosa. *Nature Plants,* Volume 3, p. 17038. doi: 10.1038/nplants.2017.38.

33. Creelman, R. A. & Mullet, J. E., 1995. Jasmonic acid distribution and action in plants :Regulation during development and response to biotic and abiotic stress. *Proceedings of the National Academy of Sciences,* 92(10), pp. 4114-4119. doi: 10.1073/pnas.92.10.411.

34. Dace, H., Sherwin, H. W., Illing, N. & Farrant, J. M., 1998. Use of metabolic inhibitors to elucidate mechanisms of recovery from desiccation stress in the

resurrection plant Xerophyta humilis. *Plant Growth Regulation,* Volume 24, p. 171–177. doi: 10.1023/A:1005883907800.

35. Die, J. V. & Román, B., 2012. RNA quality assessment: a view from plant qPCR studies. *Journal of Experimental Botany,* 63(17), p. 6069–6077. doi: 10.1093/jxb/ers276.

36. Dinakar, C. & Bartels, D., 2013. Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome and metabolome analysis. *Frontiers in Plant Science,* Volume 4, p. doi: 10.3389/fpls.2013.00482.

37. Djilianov, D. L. et al., 2013. Dynamics of Endogenous Phytohormones during Desiccation and Recovery of the Resurrection Plant Species Haberlea rhodopensis. *Journal of Plant Growth Regulation,* Volume 32, pp. 564–574. doi: 10.1007/s00344-013-9323-y.

38. Dong, Q., Wallrad, L., Almutairi, B. O. & Kudla, J., 2022. Ca2+ signaling in plant responses to abiotic stresses. *Journal of Integrative Plant Biology,* 64(2), pp. 287-300. doi: 10.1111/jipb.13228.

39. du Toit, S. F. et al., 2021. Physiological characterisation of tissue differentiation in response to desiccation in the homoiochlorophyllous dicot resurrection plant Craterostigma pumilum Hochst. *Environmental and Experimental Botany,* Volume 192, p. doi: 10.1016/j.envexpbot.2021.104650.

40. Farrant, J., Brandt, W. & Lindsey, G., 2007. An overview of mechanisms of desiccation tolerance in selected angiosperm resurrection plants. *Plant Stress,* Volume 1, pp. 72-84.

41. Farrant, J. M., 2000. A comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plant species. *Plant Ecology,* Volume 151, pp. 29-39. doi: 10.1023/A:1026534305831.

42. Farrant, J. M. et al., 2015. A molecular physiological review of vegetative desiccation tolerance in the resurrection plant Xerophyta viscosa (Baker). *Planta,* Volume 242, pp. 407-426. doi: 10.1007/s00425-015-2320-6.

43. Farrant, J. M. & Moore, J. P., 2011. Programming desiccation-tolerance: from plants to seeds to resurrection plants. *Current Opinion in Plant Biology,* 14(3), pp. 340-345. doi: 10.1016/j.pbi.2011.03.018.

44. Farrant, J. M., Moore, J. P. & Hilhorst, H. W. M., 2020. Editorial: Unifying Insights into the Desiccation Tolerance Mechanisms of Resurrection Plants and Seeds. *Frontiers Plant Science,* Volume 11, p. 1089. doi: 10.3389/fpls.2020.01089.

45. Farrant, J. M. et al., 2003. An investigation into the role of light during desiccation of three angiosperm resurrection plants. *Plant, Cell & Environment,* 26(8), pp. 1275-1286. doi: 10.1046/j.0016-8025.2003.01052.x.
46. Fleet, C. & Sun, T., 2005. A DELLAcate balance: the role of gibberellin in plant morphogenesis. *Current Opinion in Plant Biology,* 8(1), pp. 77-85. doi: 10.1016/j.pbi.2004.11.015.
47. Fleige, S. & Pfaffl, M. W., 2006. RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine,* 24(2-3), pp. 126-139. doi: 10.1016/j.mam.2005.12.003.
48. Fu, J. & Huang, B., 2001. Involvement o fantioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. *Environmental and Experimental Botany,* 45(2), pp. 105-114. doi: 10.1016/S0098-8472(00)00084-8.
49. Fujita, Y. et al., 2005. AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. *The Plant Cell,* 17(12), pp. 3470-8348. doi: 10.1105/tpc.105.035659.
50. Furini, A., Parcy, F., Salamini, F. & Bartels, D., 1996. Differential regulation of two ABA-inducible genes from Craterostigma plantagineum in transgenic Arabidopsis plants. *Plant Molecular Biology,* Volume 30, p. 343–349. doi: 10.1007/BF00020120.
51. Gabier, H., Tabb, D. L., Farrant, J. M. & Rafudeen, M. S., 2021. A Label-Free Proteomic and Complementary Metabolomic Analysis of Leaves of the Resurrection Plant Xerophyta schlechteri during Dehydration. *Life,* 11(11), p. 1242. doi: 10.3390/life11111242.
52. Gaff, D. F., 1977. Desiccation tolerant vascular plants of southern Africa. *Oecologia,* Volume 31, pp. 95-109. doi: 10.1007/BF00348713.
53. Gechev, T., Lyall, R., Petrov, V. & Bartels, D., 2021. Systems biology of resurrection plants. *Cellular and Molecular Life Sciences,* Volume 78, pp. 6365–6394. doi: 10.1007/s00018-021-03913-8.
54. Geetika, S. et al., 2017. Jasmonate Signaling and Stress Management in Plants. In: G. Pandey, ed. *Mechanism of Plant Hormone Signaling under Stress.* New Jersey: JohnWiley & Sons, Inc..
55. Georgieva, K., Röding, A. & Büchel, C., 2009. Changes in some thylakoid membrane proteins and pigments upon desiccation of the resurrection plant Haberlea rhodopensis. *Journal of Plant Physiology,* 166(14), pp. 1520-1528. doi: 10.1016/j.jplph.2009.03.010.

56. Giarola, V. et al., 2020. The dehydration- and ABA-inducible germin-like protein CpGLP1 from Craterostigma plantagineum has SOD activity and may contribute to cell wall integrity during desiccation. *Planta,* 252(5), pp. 1-13. doi: 10.1007/S00425-020-03485-0.
57. Glazebrook, J., 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology,* Volume 43, pp. 205-227. doi: 10.1146/annurev.phyto.43.040204.135923.
58. Gutierrez, L. 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(6), pp. 609-618. doi: 10.1111/j.1467-7652.2008.00346.x.
59. Halevy, A. & Kessler, B., 1963. Increased tolerance of bean plants to soil drought by means of growth-retarding substances. *Nature,* Volume 197, p. 310–311. doi: 10.1038/197310a0.
60. Herman, P. & Retief, E., 1997. *Plants of the northern provinces of South Africa: Keys and diagnostic characters.* 6 ed. Pretoria: National Botanical Institute.
61. Herzog, M., Dorne, A. & Grellet, F., 1995. GASA, a gibberellin-regulated gene family from Arabidopsis thaliana related to the tomato GAST1 gene. *Plant Molecular Biology,* 27(4), pp. 743-752. doi: 10.1007/BF00020227.
62. Higuchi, R., Fockler, C., Dollinger, G. & Watson, R., 1993. Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Reactions. *Nature Biotechnology,* Volume 11, pp. 1026–1030. doi: 10.1038/nbt0993-1026.
63. Hilhorst, H. W. & Farrant, J. M., 2018. Plant Desiccation Tolerance: A Survival Strategy with Exceptional Prospects for Climate-Smart Agriculture. *Annual Plant Reviews,* 1(2), p. doi: 10.1002/9781119312994.apr0637.
64. Hirayama, T. & Shinozaki, K., 2007. Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. *Trends in Plant Science,* 12(8), pp. 343-351. doi: 10.1016/j.tplants.2007.06.013.
65. Hou, X. et al., 2010. DELLAs Modulate Jasmonate Signaling via Competitive Binding to JAZs. *Developmental Cell,* 19(6), pp. 884-894. doi: 10.1016/j.devcel.2010.10.024.
66. Huggett, J., Dheda, K., Bustin, S. & Zumla, A., 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes & Immunity,* Volume 6, p. 279–284.

67. Illing, N. et al., 2005. The Signature of Seeds in Resurrection Plants: A Molecular and Physiological Comparison of Desiccation Tolerance in Seeds and Vegetative Tissues. *Integrative and Comparative Biology,* 45(5), p. 771–787. doi: 10.1093/icb/45.5.771.
68. Ingle, R. A. et al., 2008. Chloroplast biogenesis during rehydration of the resurrection plant Xerophyta humilis: parallels to the etioplast–chloroplast transition. *Plant, Cell & Environment,* 31(12), pp. 1813-1824. doi: 10.1111/j.1365-3040.2008.01887.x.
69. Integrated DNA Technologies, 2012. *PrimerQuest™ Tool.* [Online] Available at: https://eu.idtdna.com/PrimerQuest/Home/Index
70. Jia, H. et al., 2012. Analysis and application of RD29 genes in abiotic stress response. *Acta Physiologiae Plantarum,* Volume 34, pp. 1239–1250. doi: 10.1007/s11738-012-0969-z.
71. John, S., Svihla, Z. T. & Hasenstein, K. H., 2023. Response of endogenous abscisic acid and stomata of the resurrection fern, Pleopeltis polypodioides to de- and re-hydration. *American Journal of Botany,* 110(4), p. doi: 10.1002/ajb2.16152.
72. Joseph, R., Ingle, R. A. & Roden, L. C., 2021. *The MYC transcription factors are involved in regulating the time-of-day variations in susceptibility to Botrytis cinerea in Arabidopsis thaliana.* s.l.:PhD thesis. University of Cape Town.
73. Kariola, T. et al., 2006. EARLY RESPONSIVE TO DEHYDRATION 15, a Negative Regulator of Abscisic Acid Responses in Arabidopsis. *Plant Physiology,* 142(4), p. 1559–1573. doi: 10.1104/pp.106.086223.
74. Kasahara, H. et al., 2002. Contribution of the Mevalonate and Methylerythritol Phosphate Pathways to the Biosynthesis of Gibberellins inArabidopsis. *Metabolism and Bioenergetics,* 277(47), pp. 45188-45194. doi: 10.1074/jbc.M208659200.
75. Kazan, K. & Manners, J. M., 2012. JAZ repressors and the orchestration of phytohormone crosstalk. *Trends in Plant Science,* 17(1), pp. 22-31. doi: 10.1016/j.tplants.2011.10.006.
76. Kazan, K. & Manners, J. M., 2013. MYC2: The Master in Action. *Molecular Plant,* 6(3), pp. 686-703. doi: 10.1093/mp/sss128.
77. Ke, Q. et al., 2015. Transgenic poplar expressing Arabidopsis YUCCA6 exhibits auxin-overproduction phenotypes and increased tolerance to abiotic stress. *Plant Physiology and Biochemistry,* Volume 94, pp. 19-27. doi: 10.1016/j.plaphy.2015.05.003.

78. Khalvandi, M., Siosemardeh, A., Roohi, E. & Keramati, S., 2021. Salicylic acid alleviated the effect of drought stress on photosynthetic characteristics and leaf protein pattern in winter wheat. *Heliyon,* 7(1), p. doi: 10.1016/j.heliyon.2021.e05908.

79. Khan, N., Nazar, R., Iqbal, N. & Anjum, N., 2012. Cross-talk between phytohormone signaling pathways under both optimal and stressful environmental conditions. In: M. A. Harrison, ed. *Phytohormones and Abiotic Stress Tolerance in Plants.* Berlin Heidelberg: Springer-Verlag, p. 49–76.

80. Kohli, A., Sreenivasulu, N., Lakshmanan, P. & Kumar, P. P., 2013. The phytohormone crosstalk paradigm takes center stage in understanding how plants respond to abiotic stresses. *Plant Cell Reports,* Volume 32, pp. 945–957. doi: 10.1007/s00299-013-1461-y.

81. Koo, A. J. K., Gao, X., Jones, A. D. & Howe, G. A., 2009. A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. *The Plant Journal,* 59(6), pp. 974-986. doi: 10.1111/j.1365-313X.2009.03924.x..

82. Kuromori, T., Seo, M. & Shinozaki, K., 2018. ABA Transport and Plant Water Stress Responses. *Trends in Plant Science,* 23(6), pp. 513-522. doi: 10.1016/j.tplants.2018.04.001.

83. Kwon, E. et al., 2021. Identification of resurrection genes from the transcriptome of dehydrated and rehydrated Selaginella tamariscina. *Plant Signaling & Behavior,* 16(12), p. doi: 10.1080/15592324.2021.1973703.

84. Lee, J.-T.et al., 2003. Expression of Arabidopsis CBF1 regulated by an ABA/stress inducible promoter in transgenic tomato confers stress tolerance without affecting yield. *Plant, Cell & Environment,* 26(7), pp. 1181-1190. doi: 10.1046/j.1365-3040.2003.01048.x.

85. Lee, M. et al., 2012. Activation of a flavin monooxygenase gene YUCCA7 enhances drought resistance in Arabidopsis. *Planta,* Volume 235, pp. 923–938. doi: 10.1007/s00425-011-1552-3.

86. Lee, Y. & Kende, H., 2001. Expression of β-Expansins Is Correlated with Internodal Elongation in Deepwater Rice. *Plant Physiology,* 127(2), p. 645–654. doi: 10.1104/pp.010345.

87. Liu, H. & Timko, M. P., 2021. Jasmonic Acid Signaling and Molecular Crosstalk with Other Phytohormones. *International Journal of Molecular Science,* 22(6), p. 2914. doi: doi.org/10.3390/ijms22062914.

88. Lyall, R. & Gechev, T., 2020. Multi-omics insights into the evolution of angiosperm resurrection plants. *Annual Plant Reviews,* 3(1), p. doi: 10.1002/9781119312994.apr0730.
89. Lyall, R., Ingle, R. A. & Illing, N., 2014. The Window of Desiccation Tolerance Shown by Early-Stage Germinating Seedlings Remains Open in the Resurrection Plant, Xerophyta viscosa. *PLoS ONE,* 9(3), p. doi: 10.1371/journal.pone.0093093.
90. Lynch, R. & Clegg, J., 1986. Study of metabolism in dry seeds of Avena fatua L. evaluated by incubation with ethanol-1-14C. In: *Membranes, metabolism, and dry organisms.* New York: Cornell University Press, pp. 50-58.
91. Magome, H. et al., 2004. dwarf and delayed-flowering 1, a novel Arabidopsis mutant deficient in gibberellin biosynthesis because of overexpression of a putative AP2 transcription factor. *The Plant Journal,* 37(5), pp. 720-729. doi: 10.1111/j.1365-313X.2003.01998.x.
92. Mahmud, S. et al., 2022. Constitutive expression of JASMONATE RESISTANT 1 induces molecular changes that prime the plants to better withstand drought. *Plant, Cell & Environment,* 45(10), pp. 2906-2922. doi: 10.1111/pce.14402.
93. Majidi, M. & Bahmani, Y., 2017. Isolation of high-quality RNA from a wide range of woody plants. *Journal of Plant Molecular Breeding,* 5(2), pp. 50-59. doi: 10.22058/JPMB.2018.82001.1157.
94. Marks, R. A., Farrant, J. M., McLetchie, D. N. & VanBuren, R., 2021. Unexplored dimensions of variability in vegetative desiccation tolerance. *American Journal of Botany,* 108(2), pp. 346-358. doi: 10.1002/ajb2.1588.
95. Marshall, O., 2003. *PerlPrimer v1.1.21.* [Online]
Available at: http://perlprimer.sourceforge.net
96. Martínez-Vilalta, J. & Garcia-Forner, N., 2016. Water potential regulation, stomatal behaviour and hydraulic transport under drought: deconstructing the iso/anisohydric concept. *Plant, Cell & Environment,* 40(6), pp. 962-976. doi: 10.1111/pce.12846.
97. Ma, Y. et al., 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science,* 324(5930), pp. 1064-1068. doi: 10.1126/science.1172408.
98. Mercado-Reyes, J. A. et al., 2023. Extreme drought can deactivate ABA biosynthesis in embolism-resistant species. *Plant, Cell & Environment,* 47(2), pp. 497-510. doi: 10.1111/pce.14754.

99. Miransari, M., 2011. Role of Phytohormone Signaling During Stress. In: P. Ahmad & M. Prasad, eds. *Environmental Adaptations and Stress Tolerance of Plants in the Era of Climate Change.* s.l.:Springer Science & Business Media, pp. 381-393.
100. Mitra, J. et al., 2013. Understanding desiccation tolerance using the resurrection plant Boea hygrometrica as a model system. *Frontiers in Plant Science,* Volume 4, p. 446. doi: 10.3389/fpls.2013.00446.
101. Mittelheuser, C. J. & van Steveninck, R. F. M., 1969. Stomatal Closure and Inhibition of Transpiration induced by (RS)-Abscisic Acid. *Nature,* 221(5177), pp. 281-282. doi: 10.1038/221281a0.
102. Miura, K. et al., 2013. SIZ1 deficiency causes reduced stomatal aperture and enhanced drought tolerance via controlling salicylic acid-induced accumulation of reactive oxygen species in Arabidopsis. *The Plant Journal,* 73(1), pp. 91-104. doi: 10.1111/tpj.12014.
103. Moore, J. P. et al., 2024. Cell Wall Profiling of the Resurrection Plants Craterostigma plantagineum and Lindernia brevidens and Their Desiccation-Sensitive Relative, Lindernia subracemosa. *Plants,* 13(16), p. 2235. doi: 10.3390/plants13162235.
104. Moore, J. P. et al., 2013. Arabinose-rich polymers as an evolutionary strategy to plasticize resurrection plant cell walls against desiccation. *Planta,* Volume 237, pp. 739–754. doi: 10.1007/s00425-012-1785-9.
105. Msanne, J., Lin, J., Stone, J. M. & Awada, T., 2011. Characterization of abiotic stress-responsive Arabidopsis thaliana RD29A and RD29B genes and evaluation of transgenes. *Planta,* Volume 234, pp. 97–107. doi: 10.1007/s00425-011-1387-y.
106. Mueller, M. J., 1997. Enzymes involved in jasmonic acid biosynthesis. *Physiologia Plantarum,* 100(3), pp. 653-663. doi: 10.1111/j.1399-3054.1997.tb03072.x.
107. Munné-Bosch, S. & Peñuelas, J., 2003. Photo- and antioxidative protection, and a role for salicylic acid during drought and recovery in field-grown Phillyrea angustifolia plants. *Planta,* Volume 217, pp. 758–766. doi: 10.1007/s00425-003-1037-0.
108. Mustilli, A.-C.et al., 2002. rabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell,* 14(12), pp. 3089-3099. doi: 10.1105/tpc.007906.
109. Nazar, R., Umar, S., Khan, N. & Sareer, O., 2015. Salicylic acid supplementation improves photosynthesis and growth in mustard through changes in proline accumulation and ethylene formation under drought stress. *South African Journal of Botany,* Volume 98, pp. 84-94. doi: 10.1016/j.sajb.2015.02.005.

110. Niklas, K. j., 1999. *The Evolutionary Biology of Plants.* 1 ed. Chicago: University of Chicago Press.

111. Norwood, M., Toldi, O., Richter, A. & Scott, P., 2003. Investigation into the ability of roots of the poikilohydric plant Craterostigma plantagineum to survive dehydration stress. *Journal of Experimental Botany,* 54(391), p. 2313–2321. doi: 10.1093/jxb/erg255.

112. Oliver, M. J. & Bewley, J. D., 1997. Desiccation-Tolerance of Plant Tissues: A Mechanistic Overview. *Horticultural Reviews,* Volume 18., pp. 171-214. doi: 10.1002/9780470650608.ch3.

113. Oliver, M. J. et al., 2020. Desiccation Tolerance: Avoiding Cellular Damage During Drying and Rehydration. *Annual Review of Plant Biology ,* Volume 71, pp. 435-460. doi: 10.1146/annurev-arplant-071219-105542.

114. Oliver, M. J., Tuba, Z. & Mishler, B. D., 2000. The evolution of vegetative desiccation tolerance in land plants. *Plant Ecology,* Volume 151, pp. 85-100. doi: 10.1023/A:1026550808557.

115. Oliver, M., Velten, J. & Mishler, B., 2005. Desiccation tolerance in bryophytes: a reflection of the primitive strategy for plant survival in dehydrating habitats?. *Integrative and Comparative Biology (ICB),* 45(5), p. 788–799. doi: 10.1093/icb/45.5.788.

116. Oliver, M., Wood, A. & O'Mahony, P., 1998. To dryness and beyond preparation for the dried state and rehydration in vegetative desiccation-tolerant plants. *Plant Growth Regulation ,* Volume 24, pp. 193-201. doi: 10.1023/A:1005863015130.

117. Ooms, J. et al., 1993. Acquisition of Desiccation Tolerance and Longevity in Seeds of Arabidopsis thaliana (A Comparative Study Using Abscisic Acid-Insensitive abi3 Mutants). *Plant Physiology,* 102(4), p. 1185–1191. doi: 10.1104/pp.102.4.1185.

118. Oung, H. M. O. et al., 2022. Differential response of the photosynthetic machinery to dehydration in older and younger resurrection plants. *Journal of Experimental Botany,* 73(5), p. 1566–1580. doi: 10.1093/jxb/erab485.

119. Pabón-Mora, N. et al., 2023. Expression and Functional Studies of Leaf, Floral, and Fruit Developmental Genes in Non-model Species. *Flower Development,* Volume 2686, pp. 365–401. doi: 10.1007/978-1-0716-3299-4_19.

120. Pandey, G. K., 2017. *Mechanism of plant hormone signaling under stress.* New Jersey: John Wiley & Sons Inc..

121. Pan, X., Welti, R. & Wang, X., 2008. Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography–electrospray tandem mass spectrometry. *Phytochemistry,* 69(8), pp. 1773-1781. doi: 10.1016/j.phytochem.2008.02.008.

122. Pan, X., Welti, R. & Wang, X., 2010. Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. *Nature Protocols,* 5(6), pp. 986-992. doi: 10.1038/nprot.2010.37.

123. Parcy, F. et al., 1994. Regulation of gene expression programs during Arabidopsis seed development: roles of the ABI3 locus and of endogenous abscisic acid. *The Plant Cell,* 6(11), p. 1567–1582. doi: 10.1105/tpc.6.11.1567.

124. Pardo, J. et al., 2020. Intertwined signatures of desiccation and drought tolerance in grasses. *Proceedings of the National Academy of Sciences of the United States of America,* 117(18), pp. 10079-10088. doi: 10.1073/pnas.2001928117 .

125. Park, H. et al., 2008. Overexpression of Arabidopsis ZEP enhances tolerance to osmotic stress. *Biochemical and Biophysical Research Communications,* 375(10), pp. 80-85. doi: 10.1016/j.bbrc.2008.07.128.

126. Park, S. et al., 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science,* 324(5930), pp. 1068-1071. doi: 10.1126/science.1173041.

127. Pauwels, L. & Goossens, A., 2011. The JAZ Proteins: A Crucial Interface in the Jasmonate Signaling Cascade. *The Plant Cell,* 23(9), p. 3089–3100. doi: 10.1105/tpc.111.089300.

128. Peñuelas, M. et al., 2019. Jasmonate-Related MYC Transcription Factors Are Functionally Conserved in Marchantia polymorpha. *The Plant Cell,* 31(10), p. 2491–2509. doi: 10.1105/tpc.18.00974.

129. Peters, C. et al., 2010. Nonspecific phospholipase C NPC4 promotes responses to abscisic acid and tolerance to hyperosmotic stress in Arabidopsis. *Plant Cell,* 22(8), p. 2642–2659. doi: 10.1105/tpc.109.071720.

130. Petersen, J. et al., 2012. The lysine-rich motif of intrinsically disordered stress protein CDeT11-24 from Craterostigma plantagineum is responsible for phosphatidic acid binding and protection of enzymes from damaging effects caused by desiccation. *Journal of Experimental Botany,* 63(13), p. 4919–4929. doi: 10.1093/jxb/ers173.

131. Qiagen Bioinformatics, 2017. *CLC Sequence Viewer:*. [Online] Available at: https://digitalinsights.qiagen.com/products/clc-sequence-viewer/latest-improvements/

132. Qin, F. et al., 2011. SPINDLY, a Negative Regulator of Gibberellic Acid Signaling, Is Involved in the Plant Abiotic Stress Response. *Plant Physiology,* 157(4), p. 1900–1913. doi: 10.1104/pp.111.187302.

133. R Core Team, 2020. R: A language and environment for statistical computing.. *R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.*

134. Radermacher, A. L., Toit, S. F. d. & Farrant, J. M., 2019. Desiccation-Driven Senescence in the Resurrection Plant Xerophyta schlechteri (Baker) N.L. Menezes: Comparison of Anatomical, Ultrastructural, and Metabolic Responses Between Senescent and Non-Senescent Tissues. *Frontiers in Plant Science,* p. doi: 10.3389/fpls.2019.01396.

135. Rai, V. K., Sharma, S. S. & Sharma, S., 1986. Reversal of ABA-Induced Stomatal Closure by Phenolic Compounds. *Journal of Experimental Botany,* Volume 37, pp. 129-134.

136. Ranjan, A. et al., 2022. Shaping the root system architecture in plants for adaptation to drought stress. *Physiologia Plantarum,* 174(2), p. 13651. doi: 10.1111/ppl.13651.

137. Raza, A. et al., 2021. Jasmonic acid: a key frontier in conferring abiotic stress tolerance in plants. *Plant Cell Reports,* Volume 40, pp. 1513–1541. doi: 10.1007/s00299-020-02614-z.

138. Richards, P. W., 1996. *The tropical rain forest : an ecological study.* 2nd ed. New York: Cambridge University Press.

139. Roach, T. & Krieger-Liszkay, A., 2014. Regulation of photosynthetic electron transport and photoinhibition. *Current Protein & Peptide Science,* 15(4), pp. 351-362. doi: 10.2174/1389203715666140327105143.

140. Roberts, E., 1973. Predicting the storage life of seeds. *Seed Science and Technology,* 1(3), pp. 499-514.

141. Rodriguez, M. C. S. et al., 2010. Transcriptomes of the desiccation-tolerant resurrection plant Craterostigma plantagineum. *The Plant Journal,* 63(2), pp. 121-228. doi: 10.1111/j.1365-313X.2010.04243.x.

142. Salvi, P. et al., 2021. Phytohormone signaling and crosstalk in regulating drought stress response in plants. *Plant Cell Reports,* Volume 40, pp. 1305–1329. doi: 10.1007/s00299-021-02683-8.

143. Saruhan, N., Saglam, A. & Kadioglu, A., 2011. Salicylic acid pretreatment induces drought tolerance and delays leaf rolling by inducing antioxidant systems in maize genotypes. *Acta Physiologiae Plantarum,* Volume 34, pp. 97–106. doi: 10.1007/s11738-011-0808-7.

144. Scherer, G. et al., 2010. Patatin-related phospholipase A: nomenclature, subfamilies and functions in plants. *Trends in Plant Science,* 15(12), p. 693–700. doi: 10.1016/j.tplants.2010.09.005.

145. Schulze, A. et al., 2019. Wound-Induced Shoot-to-Root Relocation of JA-Ile Precursors Coordinates Arabidopsis Growth. *Molecular Plant,* 12(10), pp. 1383-1394. doi: 10.1101/517193..

146. Sherwin, H. W. & Farrant, J. M., 1998. Protection mechanisms against excess light in the resurrection plants Craterostigma wilmsii and Xerophyta viscosa. *Plant Growth Regulation,* Volume 24, pp. 203-210. doi: 10.1023/A:1005801610891.

147. Shi, L., Gast, R., Gopalraj, M. & Olszewski, N., 1992. Characterization of a shoot-specific, GA3- and ABA-regulated gene from tomato. *Plant Journal,* 2(2), pp. 153-159.

148. Sievers, F. et al., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology,* 7(539), p. doi: 10.1038/msb.2011.75.

149. Silverstone, A. L. & Sun, T.-p., 2000. Gibberellins and the Green Revolution. *Trends in Plant Science,* 5(1), pp. 1-2. doi: 10.1016/S1360-1385(99)01516-2.

150. Smirnoff, N., 1993. The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist,* 125(1), pp. 27-58. doi: 10.1111/j.1469-8137.1993.tb03863.x.

151. Song, W. et al., 2023. Advances in Roles of Salicylic Acid in Plant Tolerance Responses to Biotic and Abiotic Stresses. *Plants,* 12(19), p. 3475. doi: 10.3390/plants12193475.

152. Stoll, M., Loveys, B. & Dry, P., 2000. Hormonal changes induced by partial rootzone drying of irrigated grapevine. *Journal of Experimental Botany,* Volume 51, p. 1627–1634. doi: 10.1093/jexbot/51.350.1627.

153. Tebele, S. M., Marks, R. A. & Farrant, J. M., 2021. Two Decades of Desiccation Biology: A Systematic Review of the Best Studied Angiosperm Resurrection Plants. *Plants,* 10(12), p. 2784. doi: 10.3390/plants10122784.

154. Thines, B. et al., 2007. JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling. *Nature,* Volume 448, pp. 661-665. doi: 10.1038/nature05960.

155. Tiwari, S. et al., 2017. A Functional Genomic Perspective on Drought Signalling and its Crosstalk with Phytohormone-mediated Signalling Pathways in Plants. *Current Genomics,* 18(6), pp. 469-482. doi: 10.2174/1389202918666170605083319.

156. Tuba, Z., 2008. Notes on the poikilochlorophyllous desiccation-tolerant plants. *Acta Biologica Szegediensis,* 52(1), pp. 111-113.

157. Udvardi, M. K., Czechowski, T. & Scheible, W.-R., 2008. Eleven Golden Rules of Quantitative RT-PCR. *The Plant Cell,* 20(7), p. 1736–1737. doi: 10.1105/tpc.108.061143.

158. Uno, Y. et al., 2000. rabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proceedings of the National Academy of Sciences of the United States of America,* 97(21), pp. 11632-11637. doi: 10.1073/pnas.190309197.

159. Van Buren, R. et al., 2018. Desiccation Tolerance Evolved through Gene Duplication and Network Rewiring in Lindernia. *The Plant Cell,* 30(12), p. 2943–2958. doi: 10.1105/tpc.18.00517.

160. Vartanian, N., Marcotte, L. & Giraudat, J., 1994. Drought Rhizogenesis in Arabidopsis thaliana (Differential Responses of Hormonal Mutants). *Plant Physiology,* 104(2), p. 761–767. doi: 10.1104/pp.104.2.761.

161. Verma, V., Ravindran, P. & Kumar, P. P., 2016. Plant hormone-mediated regulation of stress responses. *BMC Plant Biology,* Volume 16, pp. 86. doi: 10.1186/s12870-016-0771-y.

162. Vertucci, C. & Farrant, J., 1995. Acquisition and Loss of Desiccation Tolerence. In: *Seed development and germination.* New York: Marcel Dekker, pp. 237-271.

163. Vick, B. & Zimmerman, D., 1983. The biosynthesis of jasmonic acid: a physiological role for plant lipoxygenase. *Biochemical and Biophysical Research Communications,* 111(2), pp. 470-477. doi: 10.1016/0006-291X(83)90330-3.

164. Vieira, E. A. et al., 2024. Desiccation tolerance in the resurrection plant Barbacenia graminifolia involves changes in redox metabolism and carotenoid oxidation. *Frontiers in Plant Science,* Volume 15, p. doi: 10.3389/fpls.2024.1344820.

165. Waadt, R. et al., 2022. Plant hormone regulation of abiotic stress responses. *Nature Reviews Molecular Cell Biology,* Volume 23, p. 680–694.

166. Wager, A. & Browse, J., 2012. Social network: JAZ protein interactions expand our knowledge of jasmonate signaling. *Frontiers Plant Physiology,* 8(3), p. 41. doi: 10.3389/fpls.2012.00041.

167. Wang, S., Mopper, S. & Hasenstein, K. H., 2001. Effects of salinity on endogenous ABA, IAA, JA, AND SA in Iris hexagona. *Journal of Chemical Ecology,* 27(3), pp. 327-342. doi: 10.1023/a:1005632506230.

168. Warming, E., Vahl, M., Groom, P. & Balfour, I. B., 1909. *Oecology of Plants: An Introduction to the Study of Plant-communities.* 1 ed. London: Clarendon Press.

169. Waseem, M., Athar, H.-u.-R. & Ashraf, M., 2006. Effect of salicylic acid applied through rooting medium on drought tolerance of wheat. *Pakistan Journal of Botany,* 38(4), pp. 1127-1136.

170. Wasternack, C., 2007. Jasmonates: An Update on Biosynthesis, Signal Transduction and Action in Plant Stress Response, Growth and Development. *Annals of Botany,* 100(4), p. 681–697. doi: 10.1093/aob/mcm079.

171. Wasternack, C. & Hause, B., 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. *Annals of Botany,* 111(6), p. 1021–1058. doi: 10.1093/aob/mct067.

172. Wasternack, C. & Hause, B., 2016. OPDA-Ile – a new JA-Ile-independent signal?. *Plant Signaling & Behaviour,* 11(11), p. e1253646. doi: 10.1080/15592324.2016.1253646.

173. Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. *Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.*

174. Wickham, H. et al., 2023. dplyr: A Grammar of Data Manipulation. *R package version 1.1.4, https://github.com/tidyverse/dplyr, https://dplyr.tidyverse.org.*

175. Wright, S. & Hiron, R., 1969. (+)-Abscisic Acid, the Growth Inhibitor induced in Detached Wheat Leaves by a Period of Wilting. *Nature,* Volume 224, pp. 719-720. doi: 10.1038/224719a0.

176. Xu, X. et al., 2021. Molecular insights into plant desiccation tolerance: transcriptomics, proteomics and targeted metabolite profiling in Craterostigma plantagineum. *Plant Journal,* 107(2), pp. 377-398. doi: 10.1111/tpj.15294.

177. Yamaguchi-Shinozaki, K., Koizumi, M., Urao, S. & Shinozaki, K., 1992. Molecular Cloning and Characterization of 9 cDNAs for Genes That Are Responsive to Desiccation in Arabidopsis thaliana: SequenceAnalysis of One cDNA Clone That Encodes a Putative Transmembrane Channel Protein. *Plant and Cell Physiology,* 33(3), pp. 217–224. doi: 10.1093/oxfordjournals.pcp.a078243.

178. Shinozaki, K. & Yamaguchi-Shinozaki. 2007. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review of Plant Biology*, Volume 57, pp. 781-803. doi: 10.1146/annurev.arplant.57.032905.105444

179. Yamaguchi-Shinozaki, K. & Shinozaki, K., 1993. Characterization of the expression of a desiccation-responsive rd29 gene of Arabidopsis thaliana and analysis of its promoter in transgenic plants. *Molecular and General Genetics,* 236(2-3), pp. 331-340. doi: 10.1007/BF00277130.

180. Yang, J., Worley, E. & Udvardi, M., 2014. A NAP-AAO3 regulatory module promotes chlorophyll degradation via ABA biosynthesis in Arabidopsis leaves. *Plant Cell,* 26(12), pp. 4862-4874. doi: 10.1105/tpc.114.133769.

181. Yoshida, R. et al., 2002. ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis. *Plant Cell Physiology,* 43(12), pp. 1473-1483. doi: 10.1093/pcp/pcf188.

182. Yoshida, R. et al., 2006. The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in Arabidopsis. *Journal of Biological Chemistry,* 24(281), pp. 5310-5318. doi: 10.1074/jbc.M509820200.

183. Yu, R. et al., 2024. Desiccation tolerance mechanisms of resurrection plant Selaginella pulvinata. *Environmental and Experimental Botany,* Volume 218, p. doi: 10.1016/j.envexpbot.2023.105602.

184. Zander, M. et al., 2020. Integrated multi-omics framework of the plant response to jasmonic acid. *Nature Plants,* Volume 6, pp. 290–302. doi: 10.1038/s41477-020-0605-7.

185. Zhang, H. et al., 2010. ABA promotes quiescence of the quiescent centre and suppresses stem cell differentiation in the Arabidopsis primary root meristem. *Plant Journal,* Volume 64, pp. 764–774. doi: 10.1111/j.1365-313X.2010.04367.x.

186. Zhang, Q. & Bartels, D., 2018. Molecular responses to dehydration and desiccation in desiccation-tolerant angiosperm plants. *Journal of Experimental Botany,* 69(13), p. 3211–3222. doi: 10.1093/jxb/erx489.

187. Zhang, S. C. & Wang, X., 2008. Expression pattern of GASA , downstream genes of DELLA, in Arabidopsis. *Chinese Science Bulletin,* 53(24), pp. 3839-3846. doi: 10.1007/s11434-008-0525-9.

188. Zhu, X. et al., 2015. Jasmonic acid promotes degreening via MYC2/3/4- and ANAC019/055/072-mediated regulation of major chlorophyll catabolic genes. *The Plant Journal,* 84(3), pp. 597-610. doi: 10.1111/tpj.13030.

189. Zia, A. et al., 2016. Protection of the photosynthetic apparatus against dehydration stress in the resurrection plant Craterostigma pumilum. *The Plant Journal,* 87(6), pp. 664-680. doi: 10.1111/tpj.13227.

Appendix A

Phytohormone signalling compounds. *Find below the standards/signalling compounds used to represent each phytohormone class for accurate quantification during HPLC-MS.*

Hormone	Standard
Jasmonic acid (JA)	H2-JA
Abscisic acid (ABA)	d6-ABA*
Auxin	d5-IAA*
Salicylic acid (SA)	d6-SA*
Gibberelin (GA_4)	d2-GA4*
Jasmoic acid isoleucine (Ja-Ile)	Ja-Ile
13-epi-12-oxo-phytodienoic acid (OPDA)	OPDA
*** Deuterated**	

Multiple reaction monitoring (MRM) parameters for phytohormone analysis. *This table lists the following MRM parameters that were used for the identification and quantification of each phytohormone class: retention times (RT), mass-to-charge ratios at each mass filter (Q1 and Q3), and the collision energy at the collision cell (Q2).*

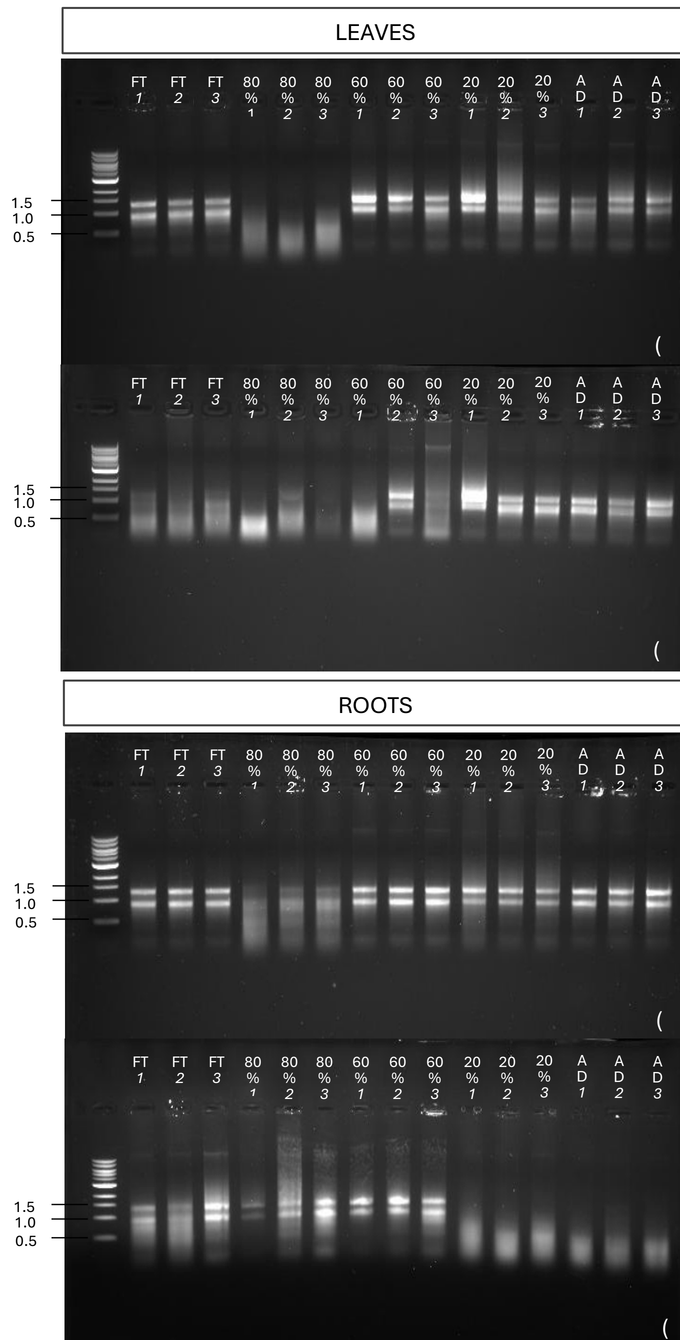
Analyte	Retention Time (min)	Quadrupole 1 (m/z)	Quadrupole 3 (m/z)	Collision energy (V)
GA3	9	345.1	142.7	-32
ABA	13.1	262.8	152.6	-14
SA	12.9	136.6	92.8	-22
JA	14.9	209	59	-24
JA-Ile	18	321.9	129.8	-26
GA4	17.5	331.1	212.7	-24
OPDA	20.6	291.1	164.6	-30
d6-ABA	13	269.1	158.8	-14
d6-SA	12.7	141.9	97.8	-22
H2JA	16.4	211	58.8	-24
d2-GA4	17.5	333.1	214.7	-24

Appendix B

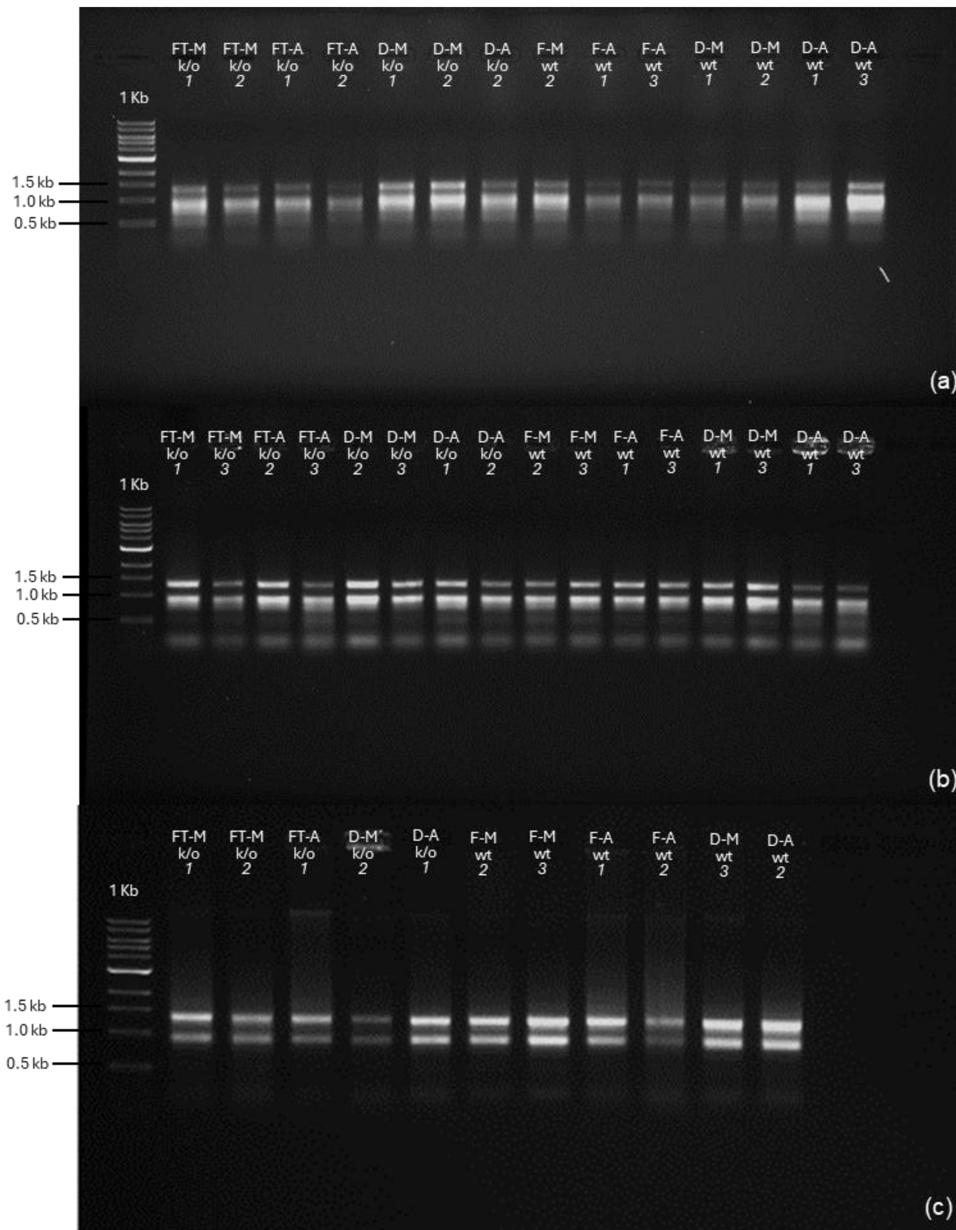
Chosen transcripts to be used in downstream primer design and further RT-qPCR analysis*. The table below highlights the transcript variants identified to be homologs of MYC2, GASA3, RD29B, UBQ10 and GAPDH in A. thaliana for C. pumilum and X. schlechteri. The homologs identified were found using transcriptomic data from (Francois), Orthofinder and NCBI tblastn. Clustal Omega and CLC Sequence Viewer were used to align each group of transcripts to identify those with the most conserved regions to then be used for downstream primer design and RT-qPCR analysis.*

GOI	*A. thaliana* tag	*C. pumilum* transcript tags found	*X.schlechteri* transcript tags found
MYC2	AT1G32640	1) evgpacbUnnamedSample_HQ_transcript/4084 2) evgtrinLocDN49871c0g3t1 3) rnaspadesvelvLoc173800ct1 4) evgtrinLocDN43779c0g1t3 5) evgpacbUnnamedSample_HQ_transcript/10431 6) evgtrinLocDN43779c0g4t1 7) evgtrinLocDN51935c1g3t1 8) evgtrinLocDN51935c1g1t2	1) Xvis03_218694-PA 2) Xvis03_218787-PA 3) Xvis03_216855-PA 4) Xvis03_201608-PA 5) Xvis03_216854-PA
GASA3	AT4G09600	1) rnaspadesvelvLoc166056ct1	1) Xvis03_217671-PA 2) Xvis03_217671-PA 3) Xvis03_217671-PA 4) Xvis03_202226-PA 5) Xvis03_219123-PA 6) Xvis03_213980-PA 7) Xvis03_209624-PA
RD29B	AT5G52300	1) evgpacbUnnamedSample_HQ_transcript/17283 2) rnaspadesvelvLoc87433ct1 3) evgtrinLocDN43048c1g1t4	1) Xvis03_210802-PA 2) Xvis03_214128-PA 3) Xvis03_216302-PA 4) Xvis03_218117-PA
UBQ10	AT4G05320	1) evgpacbUnnamedSample_HQ_transcript/520 2) rnaspadesvelvLoc169748ct1	1) Xvis03_201354-PA 2) Xvis03_205494-PA 3) Xvis03_224577-PA 4) Xvis03_212281-PA 5) Xvis03_216222-PA
GAPDH	AT1G16300	1) evgpacbUnnamedSample_HQ_transcript/11167 2) evgpacbUnnamedSample_HQ_transcript/11535 3) evgtrinLocDN53141c1g1t1 4) evgtrinLocDN53141c2g1t4 5) evgtrinLocDN53141c2g1t10 6) evgtrinLocDN52923c0g1t7	1) Xvis03_219850-PA

Appendix C



Integrity of RNA samples extracted from C. pumilum. *Evaluation of RNA integrity using the most viable leaf and root samples (according to data obtained from Nanodrop spectrophotometry) from extraction via (a) & (c) CTAB extraction and (b) & (d) TRIzol reagent, at each level of dehydration from full turgor (FT) to the air-dry (AD) state in C. pumilum.*

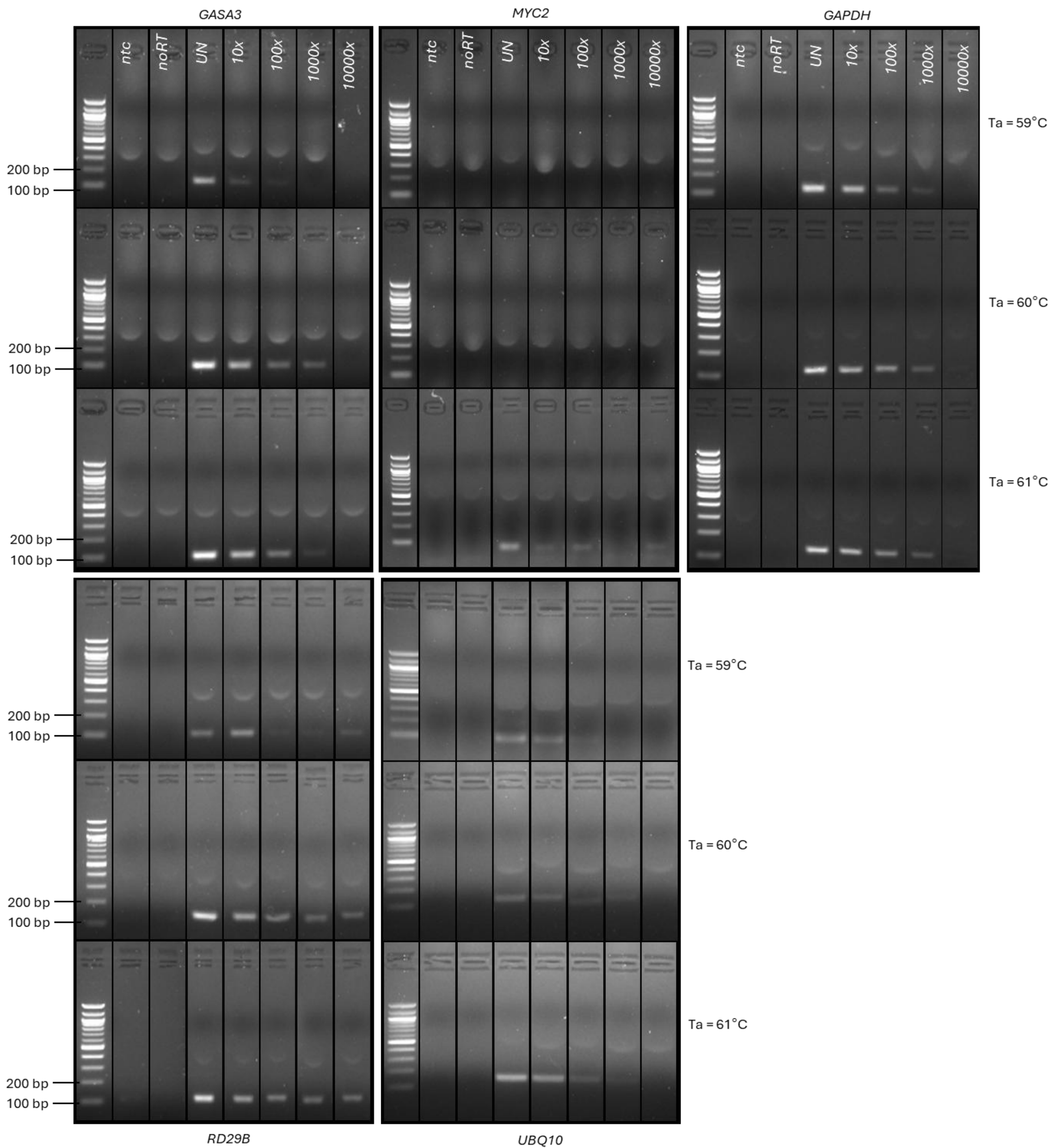


Integrity of RNA samples extracted from A. thaliana. *Panel (a) shows samples extracted using the CTAB method, while panels (b) and (c) show samples extracted using TRIzol reagent and Zymo purification kit respectively.*

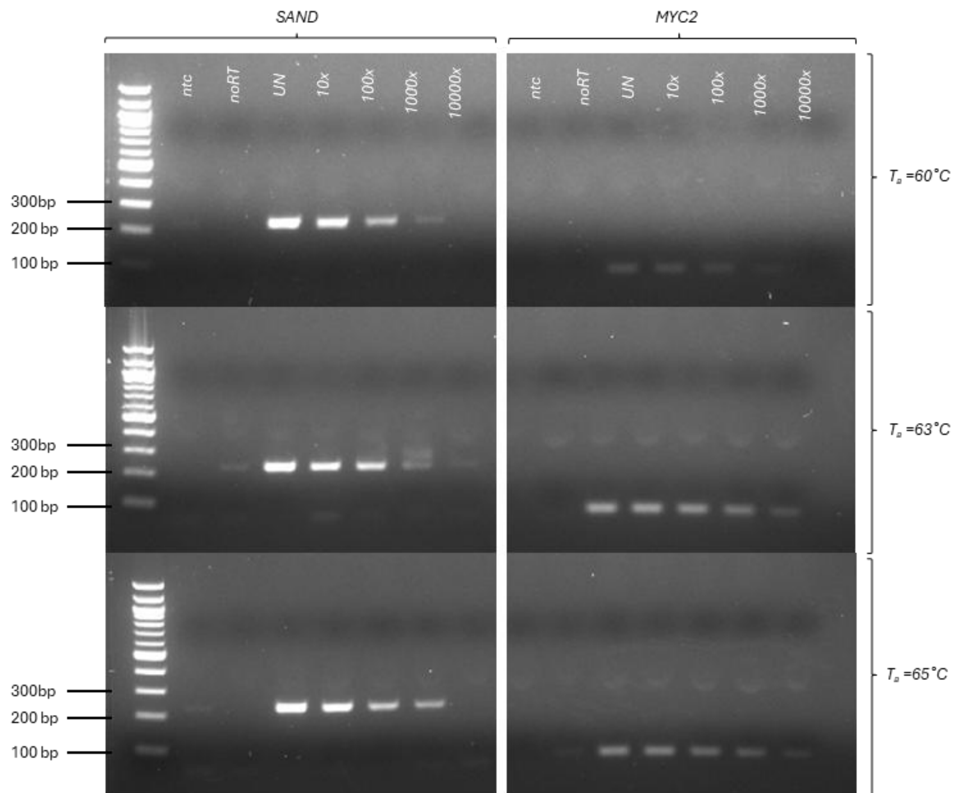
Appendix D

RT-qPCR cycling conditions for C. pumilum and X. schlechteri samples.		
Step	**Temperature (°C)**	**Time**
Initial denaturation	95	15 minutes
Denaturation	95	30 seconds
Annealing	61	30 seconds
Extension	72	30 seconds
Final Extension	72	5 minutes
Cooling	4	5 minutes

Appendix E



qPCR products of temperature tests for reference genes and genes of interest in C. pumilum. *qPCR products of a 10X dilution series were run on 2% agarose gel to assess their integrity. Evidently, MYC2 showed no amplification and was not able to be assessed further.*



qPCR products of temperature tests for reference genes and genes of interest in A. thaliana. *qPCR products of a 10X dilution series were run on 2% agarose gel to assess their integrity. MYC2 showed improved amplification at higher annealing temperatures.*