

DRY AND BACK AGAIN:
Characterization of Desiccation-Associated
Differentiation of Leaf Tissues in *Craterostigma*
***pumilum* Hochst**

By

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Declaration

I, **Stephanus Francois du Toit**, 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.

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Abstract

Resurrection plants are a polyphyletic group of angiosperms which display true desiccation tolerance - the ability to survive near complete loss of cellular water for extended periods, while recovering metabolic competence upon watering. This is achieved by employing tailored protection behaviours depending on the relative state of (de)hydration. Recent work has raised interest in desiccation-associated changes related to tissue destiny in desiccation tolerant vegetative tissues. In this thesis, physiological and transcriptomic techniques were used to characterize such a phenomenon in the homoiochlorophyllous dicot resurrection plant *Craterostigma pumilum*. Detailed phenotypic observation and pulse-amplitude-modulation fluorometry were used to identify the critical water contents at which key physiological changes occur in leaves of *C. pumilum* and how this relates to desiccation-associated differentiation between leaf Tip and Base tissues. This was followed by transcriptomic analyses and comparison between these two tissues, to identify potentially key processes involved in desiccation associated tissue differentiation. All findings were then synthesised with existing information reported on for other resurrection plant species to create a theoretical model of desiccation-associated tissue differentiation. This differentiation phenomenon is shown to be transcriptionally initiated during the desiccation commitment stage of the *C. pumilum* dehydration cycle, but is only realised phenotypically during early rehydration and after initial water movement through the leaf tissues. This work provides strong evidence for the existence of desiccation-associated tissue differentiation in *C. pumilum* and highlights the potential involvement of the phytohormone auxin in the determination of leaf tissue responses to progressive dehydration and anhydrobiosis in resurrection plants.

CHAPTER 1:
Introduction & Background

What is a resurrection plant?

Resurrection plants are a small polyphyletic group of land plant species, capable of desiccation tolerance (DT) in their vegetative tissues. DT is the ability of an organism to survive loss of as much as 95% of its cellular water, for prolonged periods, while being able to recover full metabolic competence within hours to days following rehydration (Gaff, 1977). This is a rare ability in land plants, being recorded in approximately 600 species to date, only ≈ 245 of which being angiosperm species (Gaff & Oliver, 2013; Marks *et al.*, 2021). Many of these species occupy xeric habitats, often with frequent dry seasons and/or very rocky soils, outcrops or inselbergs – thus habitats with high hydraulic conductivities and/or high evaporative indices (Brakensiek *et al.*, 1986; Alpert, 2000; Porembski & Barthlott, 2000, Farrant *et al.*, 2012). The evolution of vegetative desiccation tolerance has thus likely been selected for as a strategy to survive periodically water deficient environments, either in extant species or in ancestral ones (Farrant *et al.*, 2012). The current thesis focusses on one such an angiosperm species, *Craterostigma pumilum* Hochst, so for the sake of simplicity from here onward ‘resurrection plants’ will be used to refer to the desiccation tolerant angiosperm species.

The means of DT implemented by resurrection plants has canonically been proposed to be derived from the same, or similar, processes which allow DT in orthodox seeds (Oliver *et al.*, 2000, 2020; Costa *et al.*, 2017; VanBuren *et al.*, 2017; Farrant & Hilhorst, 2022). Most works agree that this likely occurred through co-option of the processes in question, with recent work by Lyall *et al.* (2020) suggesting that novel transcription factor binding sites may have been introduced by some species, to differentially regulate these processes during extensive drying in vegetative tissues.

Briefly, in orthodox seeds during the maturation of the embryo, various storage compounds and osmoprotectants accumulate, which serve to both protect the embryo during desiccation and provide source nutrients during germination (Vertucci & Farrant, 1995; Angelovici *et al.*, 2010; Bewley *et al.*, 2013; Leprince *et al.*, 2017). Accumulation of storage compounds such as sugars, amino acids and organic acids aid in maintaining the structural integrity of the cells as mechanical forces associated with progressive water loss set in (Iljin, 1957; Levitt, 1980; Bewley *et al.*, 2013; Ballesteros & Walters, 2019). This is achieved through the emergent property of these metabolites to form extremely viscous, glass-like mixtures, as the progressive loss of water allows novel hydrogen bonding to form – vitrification (Vanhel & Blond, 1999; Vertucci, 1990; Sakurai *et al.*, 2008; Choi *et al.*, 2011; Dai *et al.*, 2013). Accumulation and production of intrinsically disordered proteins, such as *inter alia* late embryogenesis abundant (LEA) proteins and heat shock proteins (HSP), serve as additional osmoprotectants (Close, 1997; Farrant *et al.*, 2015; Artur *et al.*, 2019; Oliver *et al.*, 2020). These proteins are proposed to alter in conformation as the cellular environment becomes more anhydrous, adopting *de novo* functionality, such as stabilization of membranes, proteins and RNAs (Tunnacliffe & Wise, 2007). These accumulation processes are prevalent in resurrection plants and largely considered as fundamental to the acquisition of DT (Farrant & Hilhorst, 2022).

Unlike orthodox seeds however, vegetative tissues face the additional danger of aberrant photosynthesis, an inherently water dependent process, with progressive water loss. Ongoing exposure to photosynthetically active radiation results in the continued excitation of photochemicals, like chlorophylls. Under ample hydration, these would be quenched via the photosystems and converted into energy rich molecules, or converted into lower energy radiation and heat, via non-photochemical quenching mechanisms. In the absence of ample water, photosystem II is unable to return to the ground state and continually quench photochemicals. Non-photochemical quenching mechanisms, such as the xanthophyll cycle (reviewed in Demmig-Adams & Adams, 1996), will also eventually become saturated as their enzymatic cycling slows due to continued water loss. Ultimately resulting in accumulation of excess excitation energy, most commonly in the form of excited singlet chlorophylls, and the consequential formation of reactive species of oxygen (ROS) and nitrogen (RNS) (Demmig-Adams & Adams, 1996; Reviewed in Oliver *et al.*, 2020). If not managed, these reactive species will interact with membranes, proteins and DNA indiscriminately, resulting in their damage (Rout & Shaw, 2001; Oliver *et al.*, 2020). Accumulated damage to important cellular components will eventually result in the cessation of biological process, the death of cells and ultimately the organism.

Successful DT strategies thus need to address every step of this process to ensure survival in the anhydrobiotic state. Poikilochlorophyllous species address excess excitation energy by actively breaking down chlorophylls and dismantling their chloroplasts, after a moderate amount of water loss, thus ceasing photosynthetic activity (Tuba *et al.*, 1998). Homoiochlorophyllous species maintain the majority of their chlorophyll and photosynthetic machinery and instead mitigate the excitation energy by regulated decoupling of their light harvesting complexes (Zia *et al.*, 2016). Both groups attempt to avoid the excess ROS generation through production of antioxidant molecules, such as carotenoids, both constitutively and upon water stress (Oliver *et al.*, 2020). Both additionally produce a variety of ROS scavenging enzymes, such as super oxide dismutase and glutathione reductase, to decrease any ROS that were produced (Farrant *et al.*, 2007; Challabathula *et al.*, 2016; Zhang & Bartels, 2018). The production of early light inducible proteins (ELIPS), typically light stress associated chlorophyll-binding proteins, have also been highly associated with DT in most resurrection plants (Bartels *et al.*, 1992; Alamillo & Bartels, 2001; Zeng *et al.*, 2002; Hutin *et al.*, 2003; Gechev *et al.*, 2013; Yobi *et al.*, 2017; Challabathula *et al.*, 2018; VanBuren *et al.*, 2019). Their accumulation has been proposed to play a role in photooxidative damage reduction during prolonged anhydrobiosis, although to date the understanding of the function of ELIPS in resurrection plants is limited (Bartels *et al.*, 1992). A further commonality is the accumulation of anthocyanins, typically in photosynthetic tissues with the most exposure to light in the anhydrobiotic state (Farrant, 2000; Vander Willigen *et al.*, 2001; Farrant *et al.*, 2007; Charuvi *et al.*, 2019; Radermacher *et al.*, 2019). These prevent excitation energy and ultraviolet radiation, to a degree, from reaching available photosynthetic

molecules while the organism is in the anhydrous state, and thus unable to respond actively (Hopkins, 1992; Sherwin & Farrant, 1998).

Water plays a structural role in plants, providing turgidity to cells through positive pressure from a central water containing vacuole. Major physical strains on the cell walls and plasmalemma are thus associated with extreme water loss, resulting in plasmolysis, and ultimately cytorrhesis and cell death (Iljin, 1957; Levitt, 1980). At the organ and organism scale, this may additionally result in loss of structural integrity, and thus crumpling and wilting, as the structural support of water is lost. Particularly in tissues which do not inherently serve a mechanical support function i.e., collenchyma and sclerenchyma (Niklas, 1992; Leroux, 2012). Resurrection plants employ two main strategies to prevent such mechanical stresses, the degree of which being species specific and often in inverse proportions (Farrant *et al.*, 2007). They may increase the plasticity of their cell walls during dehydration, enabling folding to minimize sheering of the plasmalemma and/or increase their degree of vacuolation in order to supplement the loss of internal positive pressure (Oliver *et al.*, 2020; Farrant & Hilhorst, 2022).

The ability to rehydrate from the anhydrous state, and once again achieve metabolic competence is a necessity of a successful DT strategy. While potential for structural damage to tissues, through sudden inter cellular pressure changes, seem to be an inevitable challenge associated with a rapid influx of water in dry tissues, to our knowledge no work has been produced investigating this in resurrection plants. However, much is known about the molecular investments made by resurrection plants species to recover on a cellular level. Resurrection plants create and store many transcripts and enzymes during the late stages of dehydration so these may be available once water becomes available again (Reviewed in Farrant and Hilhorst 2022; Oliver *et al.*, 2020). These are vital in the early stages of rehydration, where the organism must assemble its chloroplasts (poikilochlorophyllous species) or repair any damaged chloroplasts (homiochlorophyllous species), before it is able to photosynthesize (Ingle *et al.*, 2008). Recent speculative work by du Toit *et al.* (2021) proposed that these early rehydration processes may further be regulated by the degree of cellular hydration. They hypothesized that changes in the chemophysical properties of the previously anhydrous cellular matrix, such as viscosity and hydrogen bonding potential, may affect conformation and thus activation timing of various enzymes required for recovery and re-initiation of metabolism. Such chemophysical effects have been demonstrated *in vitro* by Choi *et al.* (2011) and Huang *et al.* (2014).

What is desiccation associated tissue differentiation?

Like any other plant, resurrection plants must engage in some form of senescence processes during their life history, to *inter alia* cycle nutrients from damaged tissues or tissues of substantial age. However, where desiccation sensitive species often undergo senescence during progressive water loss (reviewed in Munné-Bosch & Algre, 2004), resurrection plants have been shown to suppress this form of senescence in the bulk of their tissues (Christ *et al.*, 2014;

Griffiths *et al.*, 2014; Williams *et al.*, 2015; Zhang & Bartels, 2016; Radermacher *et al.*, 2019; 2021; Okemo *et al.*, 2021).

Radermacher *et al.* (2019; 2021) demonstrated that in the poikilochlorophyllous monocot *Xerophyta schlechteri*, desiccation driven senescence of only the oldest (leaf apex) tissues occurs. This process was observed to be transcriptionally and metabolically induced at relative water contents below 35%, but only being phenotypically actualized during early rehydration. Thus, fundamentally associated with the DT process of this species. Radermacher *et al.* (2019, 2021) further proposed that their defined “senescent tissues” undergo a delayed form of senescence in response to progressive dehydration and additional differential oxidative stress between the two tissues.

If this phenomenon presents itself in other, unrelated resurrection plants it would raise the question as to whether this reflects an additional form of convergent evolution among the resurrection plants, or an unconsidered cost-benefit interaction associated with evolving DT in angiosperms. Given senescence and related processes in angiosperms can vary greatly in their presentation, both at the molecular and phenotypic scales (Kabbage *et al.*, 2017), the phenomenon of desiccation driven senescence will be referred to as ‘desiccation associated tissue differentiation’ in this thesis.

Why *Craterostigma pumilum*?

Craterostigma pumilum is a small, herbaceous, evergreen dicot plant from central Africa (Ward & Dale, 1899). It produces many seeds, can be grown in hothouse conditions, has a clear dehydration phenotype, under experimental conditions, and is established as a homoiochlorophyllous species (Christ *et al.*, 2014; Charuvi *et al.*, 2015, 2019; Zia *et al.*, 2016).

It is an appealing model to investigate because it is one of the most sparsely studied species to date, while still sharing a close phylogenetic relation to one of the most studied resurrection plant species *C. plantagineum* (Fischer *et al.*, 2013; Tebele *et al.*, 2021). This allows for ample room for exploration and discovery, while still having enough source material from a sister species to relate findings too.

Finally, successfully demonstrating the presence of desiccation associated tissue differentiation in a species with a great evolutionary distance from *X.schlechteri*, not only phylogenetically but also in its DT strategy and life history (Sherwin & Farrant, 1998; Farrant *et al.*, 1999, 2003, 2007, 2012; Farrant, 2000; Deng *et al.*, 2003; Christ *et al.*, 2014), would provide very stable grounds to motivate the investigation of this phenomenon as an additional trait which links the angiosperm resurrection plants.

Why care?

In recent years, the importance of resurrection plants as valuable models for understanding mechanisms associated with the mitigation of severe water deficit, and associated stresses, has been brought to light (Farrant *et al.*, 2015; Hilhorst & Farrant, 2018; Farrant & Hilhorst, 2022).

Such knowledge may provide insight to the potential improvement of drought tolerance in crop plants; a facet with increasing importance as global temperatures increase and food security declines.

In desiccation-sensitive plants, such as cereal crops, drought induces several stress responses including leaf senescence. This abiotic stress-induced leaf senescence, and ultimate leaf death and abscission, promotes the survival of these plants by allowing for the remobilization of nutrients and maintenance of a favorable water balance through the reduction in transpiration area (reviewed in Munné-Bosch & Algre, 2004; Woo *et al.*, 2019). While these processes may aid in the survival of the organism under stress conditions, this form of accelerated senescence has been shown to negatively impact crop yields (Gregersen *et al.*, 2013; Hafsi *et al.*, 2013).

Understanding an arguably novel, senescence-like process in the resurrection plants has the potential to both: highlight new ways to mitigate abiotic stress-induced senescence; and highlight an unforeseen consequence of the DT strategy. Both aspects having relevance in the context of using knowledge from resurrection plants in the development of more drought resistant crops. Furthermore, demonstrating additional convergent evolutionary traits in angiosperm species, which already display the very rare DT trait, may garner interest in these species. Potentially promoting research and conservation efforts. Finally, successfully demonstrating the presence of such a phenomenon suggests its oversight in many preceding works in the field and creates a new context to view these results in.

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CHAPTER 2:

Physiological characterisation of tissue differentiation in response to desiccation in the homoiochlorophyllous dicot resurrection plant *Craterostigma pumilum* Hochst

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Introduction

Resurrection plants are a small polyphyletic group of plant species capable of desiccation tolerance (DT) in their vegetative tissues. DT is the ability of an organism to survive loss of as much as 95 % of its cellular water for a prolonged period, while being able to recover full metabolic competence within hours to days following rehydration (Gaff, 1977). In recent years, the importance of resurrection plants as valuable models for understanding mechanisms associated with the mitigation of severe water deficit and associated stresses has been brought to light (Farrant *et al.*, 2015; Hilhorst & Farrant, 2018). Such knowledge may provide insight to the potential improvement of drought tolerance in crop plants; a facet with increasing importance as global temperatures increase and food security declines.

In desiccation-sensitive plants, such as cereal crops, drought induces several stress responses including leaf senescence. This abiotic stress-induced leaf senescence, and ultimate leaf death and abscission, promotes the survival of these plants by allowing for the remobilization of nutrients and maintenance of a favourable water balance through reduction in transpiration area (reviewed in Munné-Bosch & Alegre, 2004; Woo *et al.*, 2019). While these processes may aid in the survival of the organism under stress conditions, this form of accelerated senescence has been shown to negatively impact crop yields (Gregersen *et al.*, 2013; Hafsi *et al.*, 2013).

It has been proposed that resurrection plants are able to suppress drought-induced senescence in the bulk of their tissues by employing signal blocking mechanisms (Griffiths *et al.*, 2014). However, recent work by Radermacher *et al.* (2019) reported the existence of a desiccation-driven senescence, which occurs in only the oldest tissues of *Xerophyta schlechteri* (Baker) NL Menezes. This work raises the question as to whether similar processes exist in other resurrection plants, reflecting an additional form of convergent evolution in these species.

Here we report on the presence of desiccation-associated differentiation of leaf tissues in *Craterostigma pumilum* Hochst and its' progression during dehydration and rehydration. This homoiochlorophyllous dicot resurrection plant species was selected for its evolutionary distance from the poikilochlorophyllous monocot *X. schlechteri*, not only phylogenetically but also in its DT strategy and life history (Sherwin & Farrant, 1998; Farrant *et al.*, 1999, 2003, 2007, 2012; Farrant, 2000; Deng *et al.*, 2003; Christ *et al.*, 2014). Our data shows similarities in desiccation-associated leaf tissue differentiation between these species, pointing to its potential significance in the DT strategy.

Abbreviations:

PAR, Photosynthetically Active Radiation; *F*, chlorophyll fluorescence; *ETR*, (apparent) electron transport rate; *YII*, effective quantum yield of *PSII*; *YNPQ*, quantum yield of regulated energy dissipation; *YNO*, quantum yield of non-regulated energy dissipation.

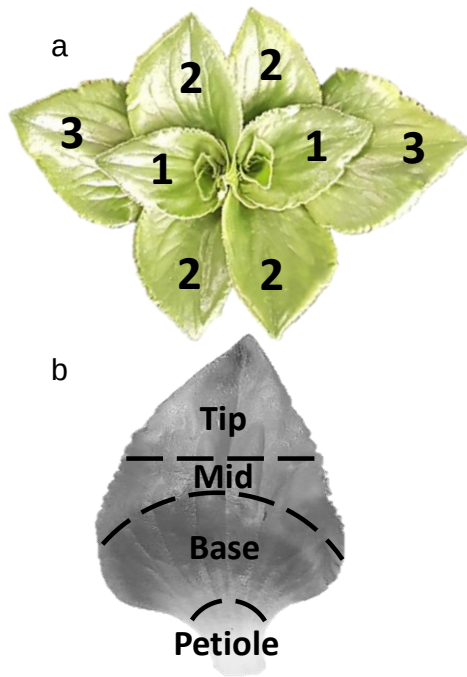


Figure 1: Tissue sampling schematic for *C. pumilum*. Leaf whorl numbering scheme followed when sampling (a), with youngest fully formed leaf pair assigned the lowest value. Leaves which share a whorl, share an assigned number as proxy for similar age. Tissues were sampled from the leaves according to (b), with the ‘Tip’ and ‘Base’ being the furthest and closest $\frac{2}{3}$ ths of the total green area of the leaf, respectively. The ‘Tip’ and ‘Base’ is separated by the ‘Mid’ tissue; the non-green tissue adjacent to the ‘Base’ is the ‘Petiole’.

Materials and Methods

Plant growth and sampling

Seeds of *Craterostigma pumilum* were germinated on peat pellets (Jiffy®), in a closed container with transparent lid to maintain humidity, for two weeks. Thereafter, seedlings were carefully separated, as to not disturb the peat surrounding the roots, and transferred to individual 350ml plant tray cells containing vermiculite-containing potting soil. Plants were germinated and grown at ≈ 115 PAR ($\text{mol.m}^{-2}.\text{s}^{-1}$) at 24°C, with 16h light period. Plants were allowed to grow under well-watered conditions for 6 months prior to the start of the experiment. Plants were dehydrated by withholding water, and rehydration was carried out by soaking pots in water for approximately 30 min, after which excess water was removed. Leaf whorls were assigned age ranks according to Figure 1a and leaf tissues sampled according to Figure 1b. The inclusion of a Mid tissue section in our sampling scheme prevents signal contamination between Tip and Base tissues. The boundary line between Mid and Base tissues is curved to compensate for cells on leaf margins possibly being more similar developmentally to Tip tissue than Base tissue (Reviewed in Xiong & Jiao, 2019). Relative Water Content (RWC) of excised leaf tissues was determined according to Barrs & Weatherly (1962). At the start of the experiment and, again at each sampling point, top-down photographs

were taken of individual plants to track observable phenotypic changes associated with dehydration and rehydration. All sampling took place 2 hours after the start of the light period.

RWC time correction using soil moisture measurements

Prior to experimentation, a small representative population of 20 plants were selected to determine the effect of soil moisture on the onset of dehydration stress in leaf tissues. An EM50 data logger (ICT International), equipped with EC-5 soil volumetric water content (VWC) probes, recorded the VWC of the soil adjacent to the roots every 30min for one dehydration-rehydration cycle. The select population was sampled as stated above for RWC determination and subjected to PAM measurements as stated below. The VWC readings were averaged for all probes and used to determine the best-fit water loss equation against time, using Microsoft® Excel®, for the soil mix and container dimensions used. Photographs were used to determine

the dates when individual plants showed first signs of stress (stress-induced senescence), and to approximate the soil VWC at which these stress signs should begin appearing for the given growth conditions. During subsequent experiments, the VWC of the plants sampled was determined at the time of sampling. The previously determined water loss rate was then used to approximate how many days had elapsed, for each individual plant at the time of sampling, since the respective soil VWC dropped below the predicted stress onset VWC.

Pulse-Amplitude Modulated (PAM) chlorophyll fluorescence measurements

Prior to leaf excision for RWC determination, sampled plants (n=77) were dark adapted for 30min. Whole plants were placed in an Imaging-PAM-fluorometer (Heinz Walz GmbH, Effeltrich, Germany) MAXI-Version. Minimum (F_o) and maximum (F_m) fluorescence were determined, followed by standard excitation (115PAR, 260s, 20s reading interval) and relaxation (0PAR, 180s, 20s reading interval) protocols. After completion, light curves were recorded, with light intensity increasing stepwise from 0PAR to 1250PAR every 20s. Whole plant fluorescence and image data were exported as .pim files using the associated ImagingWin software (v2.47, Heinz Walz GmbH, Effeltrich, Germany) for later analyses.

Water movement tracking

Whorl 2 leaves with intact petioles (Figure 1a) were dissected from air-dry plants. The leaves were positioned upright in 5ml glass vials, such that only the cut site on the petiole was submerged in either 1.6mg/ml 4.4kDa fluorescein isothiocyanate - dextran (FITC-Dextran) (Sigma-Aldrich) solution or double distilled water (DDW). The leaves were allowed to rehydrate for 48h in the dark (to prevent degradation of the fluorophore) and were visualized using a Nikon SMZ25 Stereo Microscope with P2-EFLM Motorized Epi Fluorescence Attachment under both bright-field and 3s GFP-excitation.

Data processing and statistics

The RWC for each tissue of interest (Figure 1b), as well as the whole leaf (sum of parts), was calculated as stated above and recorded using Microsoft® Excel®. Using the ImagingWin software (v2.47, Heinz Walz GmbH, Effeltrich, Germany), areas of interest (AOIs) were selected on two leaves, belonging to whorl 2 (Figure 1). Whorl 2 leaves were selected as, according to our definitions (Figure 1a), leaves of this whorl represent the youngest fully formed leaf pair and thus most likely of all leaf pairs to display DT across all its tissues. The fluorescence data of interest were derived using prescribed internal relations and exported to .csv format.

Using R version 4.0.1 (R Core Team, 2020) with the “ggplot2” (Wickham, 2016) package, the RWC of Tip and Base tissues was plotted against the respective predicted days post onset of the stress, based on soil VWC (described above). This produced a calibrated dehydration curve, which was subjected to a 1st degree LOESS (30% smoothing) regression. The RWC for the rehydration curve was plotted against hours post imbibition. A moving average was applied along with boxplot presentations, to illustrate variation among samples. F_v/F_m was derived for each tissue of interest during the dehydration curve and plotted against RWC of the respective leaves, using ggplot2. Additionally, 2nd degree loess smoothing (30%) curves were applied with 95% confidence intervals using the same package. Core R was used to interpolate LOESS

surfaces (15% smoothing, degree 2) for the variable fluorescence ($F_m' - F_o'$) for the induction curve (excitation & relaxation curves) data vs illumination time and RWC, as well as the apparent electron transport rate (ETR), effective quantum yield of PSII (YII), quantum yield of regulated energy dissipation (YNPQ), and quantum yield of non-regulated energy dissipation (YNO) from the light curve data vs illumination intensity and RWC. The smoothing span of 15% for the surfaces was selected to reflect as close to the 'true' pattern without overfitting. LOESS surfaces were visualized using the "plot3Drgl" (Soetaert, 2016) and "rgl" (Adler *et al.*, 2020) packages with the mean error, σ , given by:

$$(1) \quad \sigma = \frac{1}{n} \sqrt{\sum_{i=1}^n (z_i - f(x_i, y_i))^2}$$

where n is the number of data points used for the regression, z_i is the i^{th} data point and $f(x_i, y_i)$ its value, as estimated by a LOESS regression using the measured values of x_i and y_i . The mean error for all LOESS surfaces suggested good fit to the recorded data (Supplementary Figure 2 & 3) at smoothing span of 15%.

Results

The majority of the dehydration-related data presented in this work is given on a continuous scale. For ease of interpretation, the data have been divided into discrete response stages, with RWC ranges. Assignment of these are supported by changes to physiology, observed here (Figure 2 & 3), and similar stages reported in literature (Navari-Izzo *et al.*, 2000; Farrant *et al.*, 2007, 2015; Georgieva *et al.*, 2008; Charuvi *et al.*, 2015, 2019; Mladenov *et al.*, 2015; Zia *et al.*, 2016; Tan *et al.*, 2017; Oliver *et al.*, 2020).

Anatomical changes in C. pumilum leaves during dehydration

The leaves of *C. pumilum* grow in pairs of approximately equal developmental age. Each new pair grows perpendicular to the preceding pair (Figure 2). Plants utilized in the present study typically had 5 to 6 whorls, with leaves of whorl 7 normally succumbing to age related senescence under well-watered conditions (data not shown). The first visual indicator of water deficit stress in *C. pumilum* came in the form of senescence in leaves of whorl 5 and older, with the leaves becoming progressively yellower as plants continued to dehydrate (Figure 2b). The onset of senescence in these whorls was therefore used as a physiological marker of the early-stage (80% > to 60% RWC) dehydration response. This form of senescence concluded by the end of the mid stages of dehydration (60% > to 30% RWC), with these older leaves having been abscised (Figure 2c). The mid-stage response was also characterized by loss of leaf turgidity and shrinkage of the leaves, while maintaining a green colour (Figure 2c). The late-stage response (30% > to 15% RWC) was characterized by the visible accumulation of anthocyanins in the adaxial surfaces of the leaves (Figure 2d). This typically occurred over approximately two days (Figure 3). The approach to the air-dry stage (15% > to ~8% RWC) involved additional shrinkage

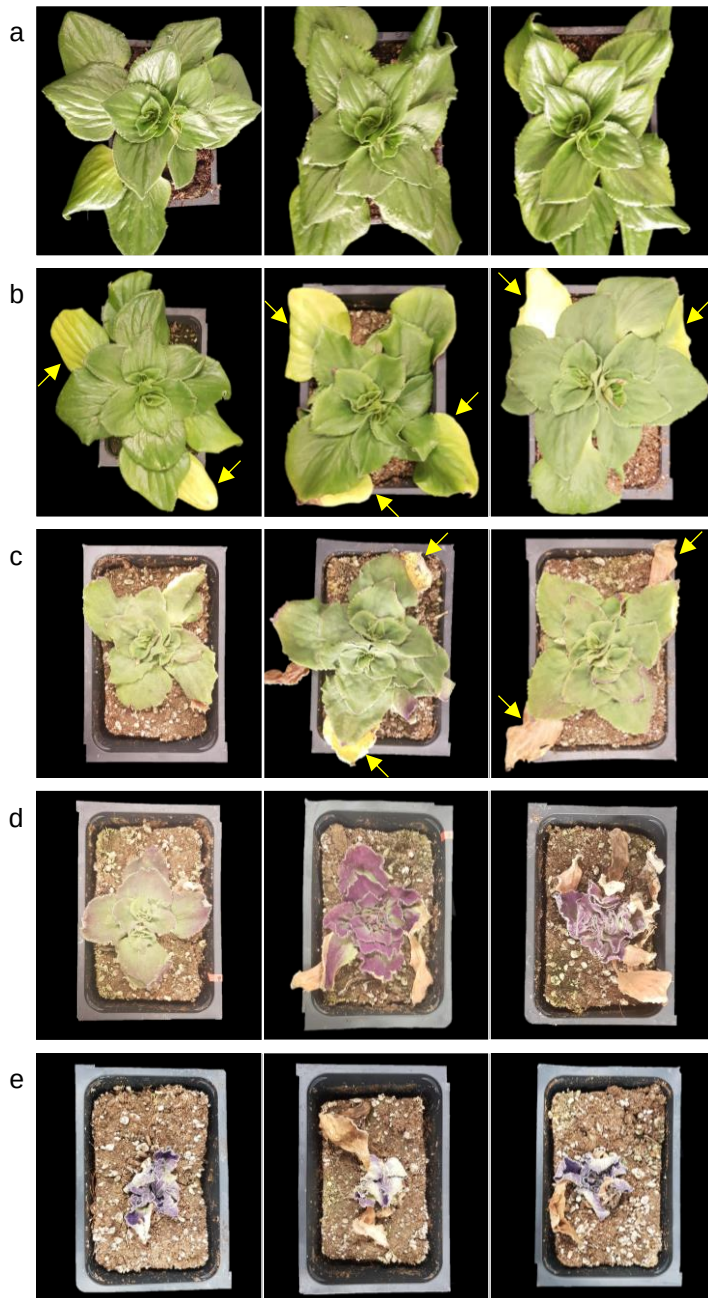


Figure 2: Stages of dehydration in *C. pumilum*. Top-down photographs were taken of potted plants at full turgor (a, $\geq 80\%$ RWC); early-stage dehydration (b, $80\% >$ to 60% RWC); mid-stage dehydration (c, $60\% >$ to 30%); late-stage dehydration (d, $30\% >$ to 15%) and air-dry stage (e, $15\% >$ to $\approx 8\%$). Yellow arrows highlight instances of water stress induced senescence in b & c.

of the leaves, as well as noticeable leaf curling such that the trichome-rich abaxial surfaces were exposed and the majority of the youngest leaves completely masked (Figure 2e). With exception of the presence of senescence, which occurred in early dehydration, the leaves of *C. pumilum* appeared to respond largely uniformly to dehydration with no clear visible differences between Tip and Base tissues (Figure 2).

Subsequently, a total 77 plants of similar age and development were studied during progressive dehydration, using PAM and RWC determination. None of the plants had been exposed to drought stress conditions prior to sampling and each plant was sampled only once. Whorl 2 leaves were subjected to PAM analysis, comprising in total 154 sampling points dispersed along an RWC range of 90 to 8% (Supplementary Figure 1). The inclusion of soil VWC data in the construction of the dehydration curve has allowed for a calibrated view of *C. pumilum* water loss. While measured VWC values greatly depend on texture, composition and volume of the growth soil, the inclusion thereof has allowed for the removal of much of the variation typically seen in dehydration

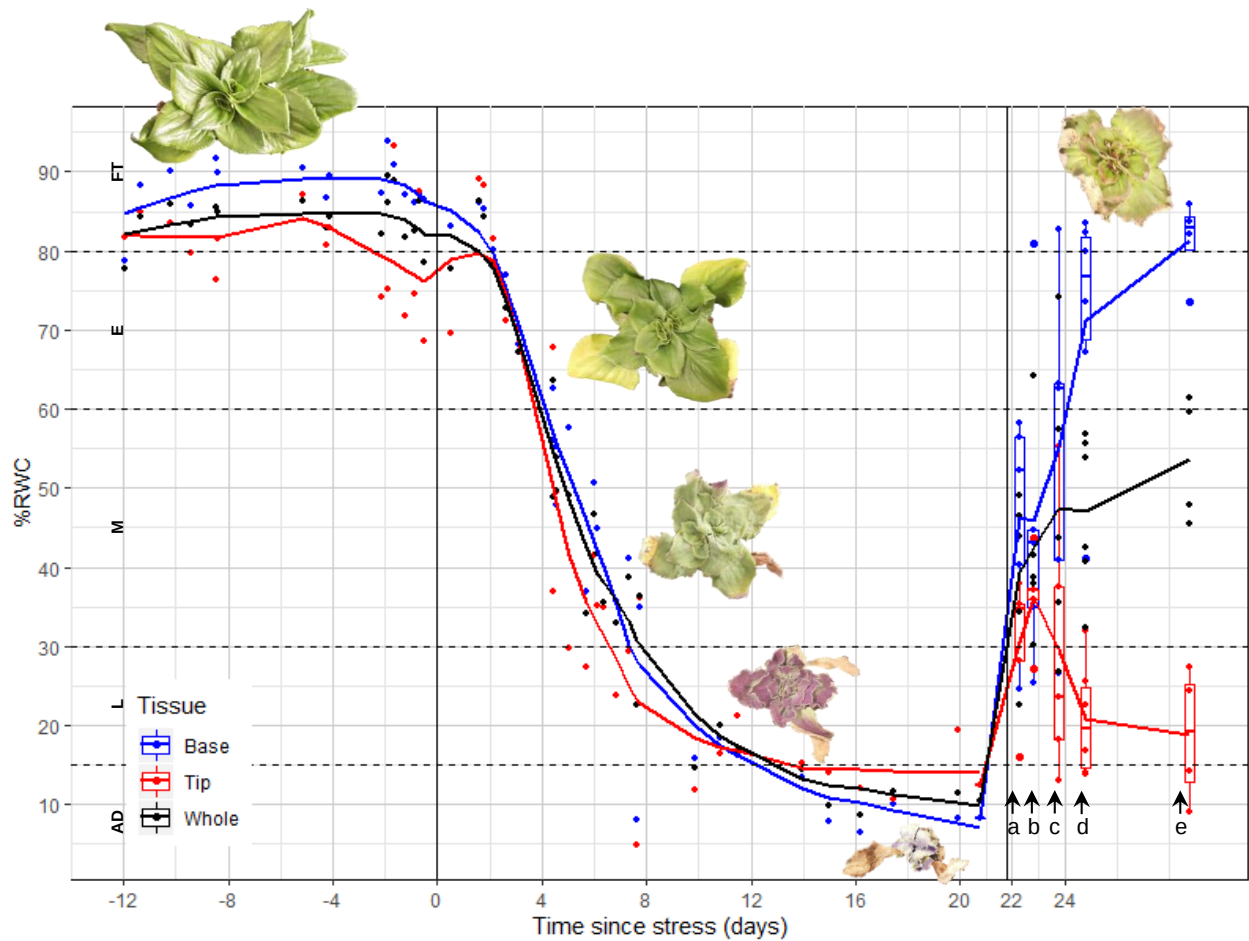


Figure 3: Time-corrected dehydration-rehydration curves of *C. pumilum*. RWC of sample points, as well as respective Base and Tip tissues, are given against corrected days post stress based on soil moisture. A loess curve (30% smoothing, degree 1) is fitted for each type of tissue of the dehydration curve ($n = 44$ plants). Moving average lines are fitted for the rehydration curve ($n = 25$ plants) along with boxplots for each time point. 12 hours rehydration (a); 24 hours rehydration (b); 48 hours rehydration (c); 72 hours rehydration (d); 1 week rehydration (e). Stages of dehydration (see Figure 2) are indicated by horizontal dashed lines and abbreviations “FT” (full turgor), “E” (early-stage), “M” (mid-stage), “L” (late-stage) and “AD” (air-dry stage) respectively. Representative plant images of each stage are provided, as well as for 72 hours post rehydration.

curves of resurrection plants (Farrant *et al.*, 1999; Vander Willigen *et al.*, 2001; Christ *et al.*, 2014; Zia *et al.*, 2016; Radermacher *et al.*, 2019), by controlling for the effect of soil hydration on plant dry-rate. Under the conditions used in this study, *C. pumilum* plants took approximately 16 days from entrance into the early-stage response to reach the airdry stage (minimum RWC stabilization; Figure 3). Loss of water was most rapid and consistent during the early- and mid-stages ($80\% \geq \& \leq 30\%$ RWC) of dehydration and slowed in the late-stage until the air-dry stage was reached. No clear difference in the rate and pattern of drying was observed between the Tip and Base tissues during dehydration, despite the Tip tissues consistently having a marginally lower water content. The average RWC of the whole leaf was therefore used in subsequent data analysis associated with dehydration.

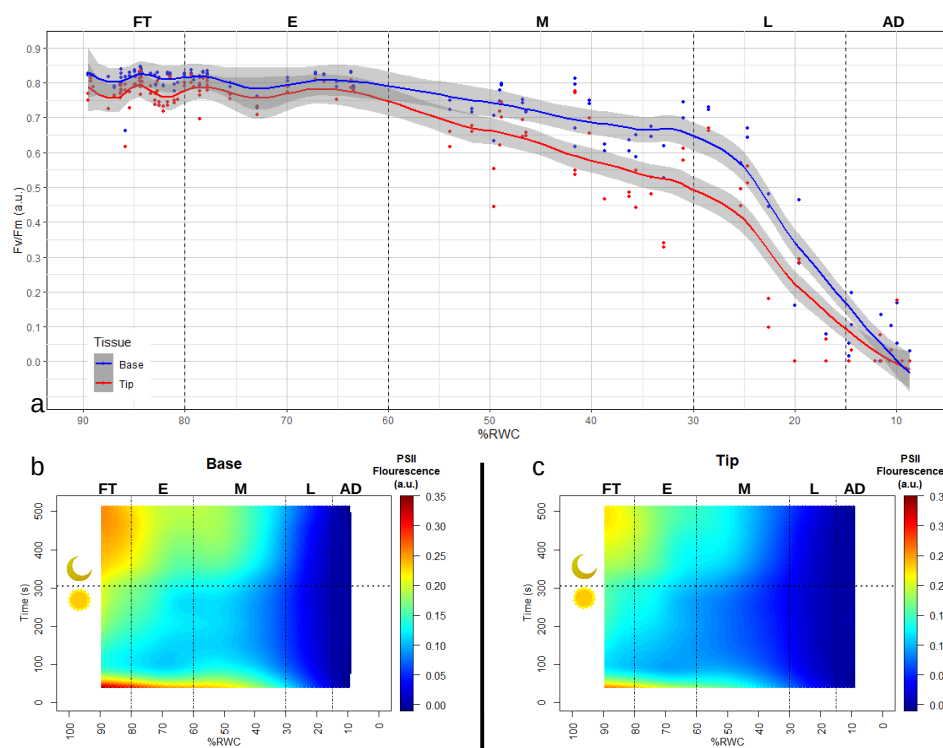


Figure 4: Changes in PSII maximal quantum yield during dehydration. Recordings were taken after 30min dark adaptation, with actinic light equal to growth conditions at 115 PAR (mol.m⁻².s⁻¹). Change in Fv/Fm are shown against RWC (a) for Base and Tip tissues (n = 77 plants). A loess regression is applied for each tissue (30% smoothing, degree 2) to highlight trends. A 95% confidence interval is shown for each regression. Loess surfaces (15% smoothing, degree 2) for the Base and Tip tissues (b and c respectively) are shown. Time 0 to 300s denotes the period where actinic light was on (sun symbol), while the remaining time denotes the recovery period after the light was switched off (crescent moon symbol). Stages of dehydration (see Figure 2) are indicated by vertical dashed lines and abbreviations “FT” (full turgor), “E” (early-stage), “M” (mid-stage), “L” (late-stage) and “AD” (air-dry stage) respectively. The time where actinic light is switched off is indicated by horizontal dashed line. For distribution of residuals and sample points see Supplementary material.

approximately the same RWC.

Fluorescence induction patterns of Base and Tip tissues are shown in Figures 4 b and c, respectively. Both figures depict the induction (sun symbol) and relaxation (crescent moon symbol) phases as a surface, to approximate the corresponding changes that occur during dehydration. As can be seen, PSII fluorescence was at a maximum immediately after the start of induction. Fluorescence then declined rapidly before showing some recovery after 150s. This initial pattern persisted in both tissues until the end of the early-stage response. During the mid-stage, emitted fluorescence remained relatively high, although lower than at higher water contents. Following further decrease, a plateau was reached, this pattern being consistent for

PAM analyses of photosynthesis during dehydration

At full turgor, PSII maximum potential quantum yield, given by Fv/Fm, appeared to have remained consistent at around 0.8 (Figure 4a). During the early-stage response, values recorded for both Tip and Base tissues appeared largely unchanged (Figure 4a). A gradual decline in Fv/Fm was obtained only during the mid-stage of dehydration, followed by a steeper decline during the late-stage (Figure 4a). During these mid to late stages, the two tissues exhibited the greatest deviation in their response to dehydration, as probed by decrease in Fv/Fm, despite both presenting minimal values at

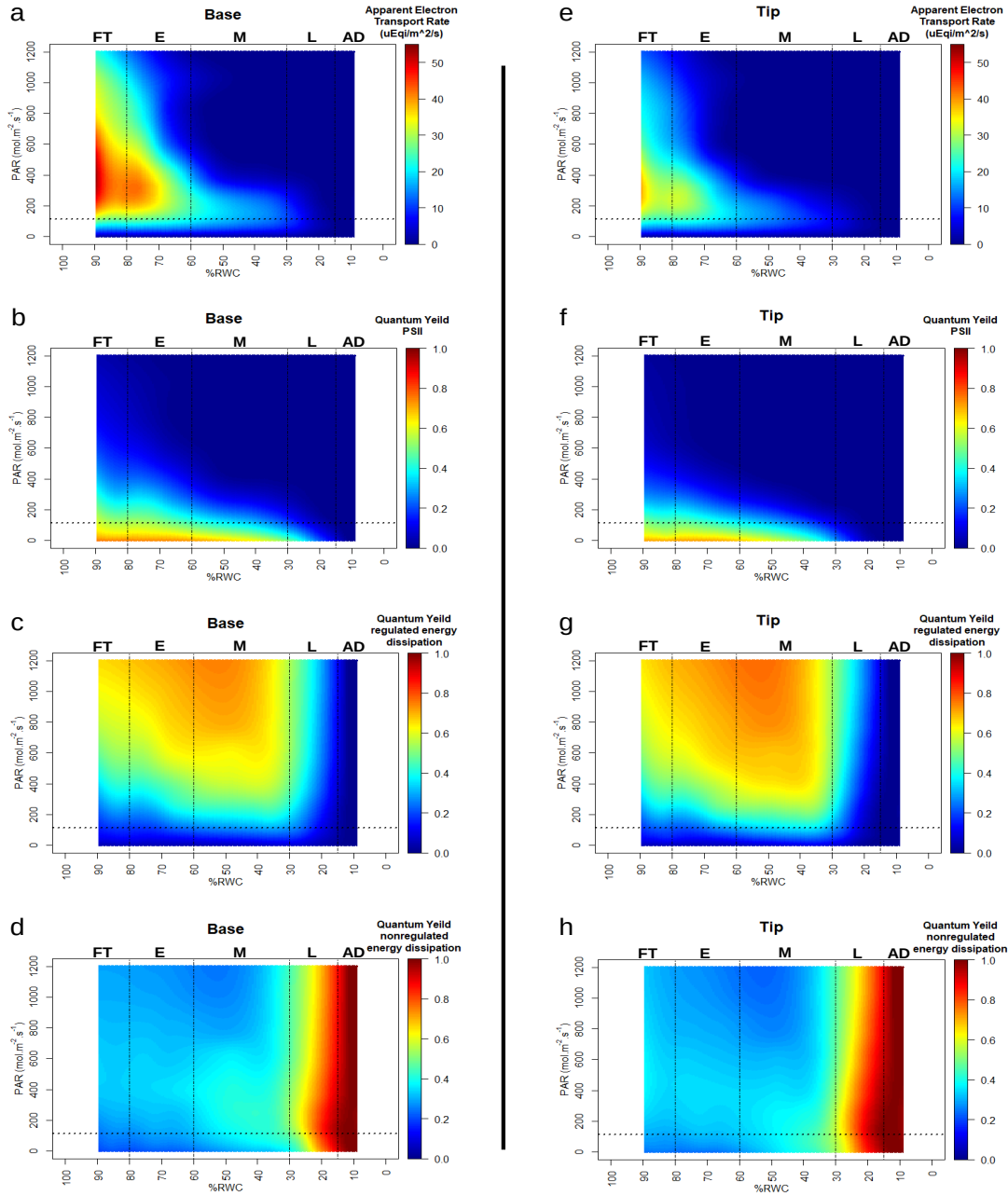


Figure 5: Changes in photosynthetic parameters during dehydration. Light curves were conducted post induction curves (see Figure 4). Loess surfaces for each figure were interpolated independently (15% smoothing, degree 2). For distribution of residuals and sample points see Supplementary material. Stages of dehydration (see Figure 2) are indicated by vertical dashed lines and abbreviations “FT” (full turgor), “E” (early-stage), “M” (mid-stage), “L” (late-stage) and “AD” (air-dry stage) respectively. Growth light, 115 PAR (mol.m⁻².s⁻¹) is indicated by horizontal dashed line.

both tissues. Fluorescence largely ceased during the late-stage. Once the actinic light was turned off, recovery of fluorescence was observed in both tissues. The most rapid one was seen in leaves at full turgor and during early-stages of drying. Thereafter, recovery progressively

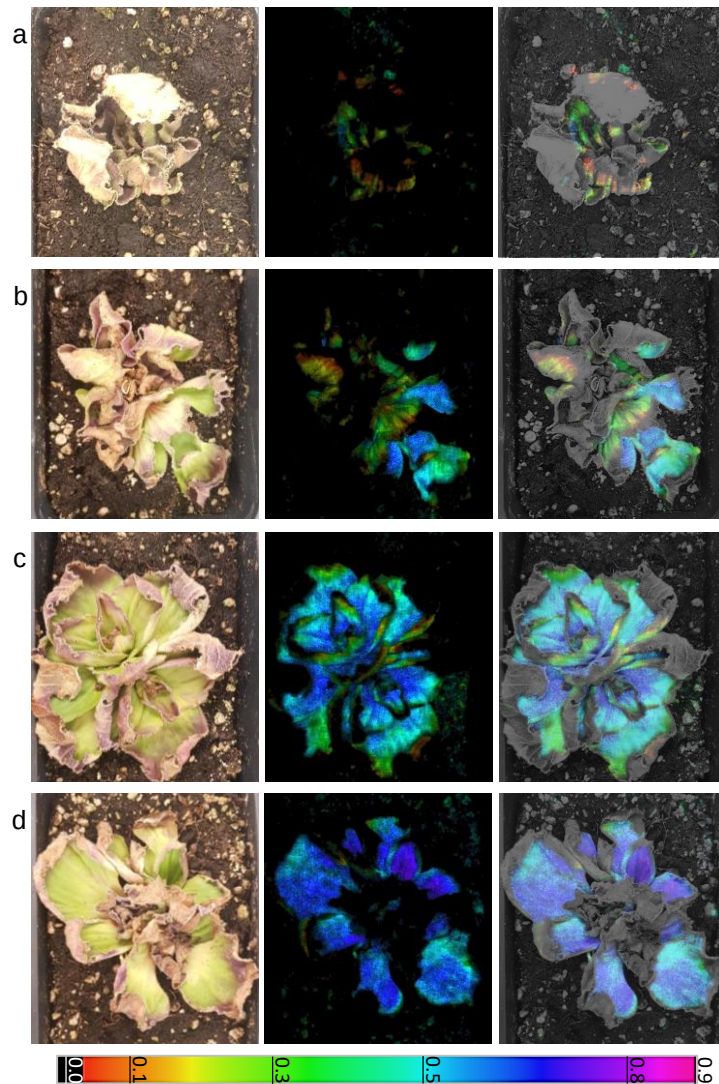


Figure 6: Max quantum yield (F_v/F_m) during rehydration. Recordings and images were taken after 30min dark adaptation, with actinic light equal to growth conditions at 115 PAR ($\text{mol.m}^{-2}.\text{s}^{-1}$). Hours post imbibition, 12 (a), 24 (b), 48 (c) and 72 (d) are shown. Top-down photograph of plants at respective time points are depicted (left) with F_v/F_m reading distribution (center) and an overlay (right). A colour bar is provided with guide readings for ease of understanding.

stage, YII only began declining as of $\leq 70\%$ RWC. YII vanished below 15% and 20% RWC for the Base and Tip tissues, respectively. Responding nearly inversely to YII, the quantum yield of regulated energy dissipation (YNPQ) increased as light intensity increased and RWC decreased (Figure 5c & g). The increase in YNPQ, which accounts for the efficiency of thermal dissipation of absorbed light energy, contrasts the findings reported by Zia *et al.* (2016). YNPQ dropped upon entrance to the late-stage ($\approx 20\%$ RWC), for both tissue types (Figure 5c & g). The expected remaining fraction of absorbed light energy, the quantum yield of non-regulated

declined until the late-stage, where fluorescence ceased altogether. The Tip tissues largely replicated the patterns seen in the Base tissues, albeit at consistently lower fluorescence.

Rapid light curves provide valuable information on the saturation characteristics of photosynthesis, particularly (apparent) electron transport rates (ETR). ETR peaked at light intensities marginally above actinic light at high RWC, in both Tip and Base tissues, and showed a decline as light intensities increased (Figure 5a & e). As RWC decreased, the ETR dropped at lower and lower light intensities, with the steepest decline observed between 80% and 50% RWC. This steep decline is likely due to the progressive breakdown of cytochrome *f* subunit (Charuvi *et al.*, 2015; Zia *et al.*, 2016), with the current study providing a more complete time and RWC window during which this process occurs.

At higher RWC ($>70\%$), the effective quantum yield of PSII (YII) remained high, in both Tip and Base tissues (Figure 5b & f). Despite exhibiting a small dip near the end of the FT

energy dissipation (YNO), reached a maximum during the late-stage (Figure 5d & h). This coincides with the visible accumulation of anthocyanins in adaxial leaf surfaces and represents a previously overlooked aspect of photosynthetic protection in this species (Figure 2d, Zia *et al.*, 2016). In all light curve recordings, the Tip tissue consistently reproduced the same pattern as the Base tissue, albeit with lower values for ETR and YII and higher values for YNPQ (Figure 5 e - h).

Photosynthesis, tissue differentiation and water movement during rehydration

Both tissues increased in RWC for the first 24 hours post imbibition (Figure 3, point a & b). Thereafter the Base tissues continued to increase in RWC until full turgor values were again reached approximately seven days post imbibition (Figure 3, point c to e). Interestingly, the Tip tissue decreased in RWC after 24 hours (Figure 3, point b) and stabilized after 72 hours (Figure 3, point d). The minimum water content recorded for of the Tip tissue, during rehydration, was recorded seven days post imbibition, and was only marginally higher than the minimum RWC recorded for the tissue in the air-dry stage (Figure 3, point e).

Leaf uncurling and expansion became evident around 12 hours post imbibition (Figure 6a). Exposed green tissues, however, still presented very low Fv/Fm values at this point. Uncurling of the leaves continued to progress rapidly until approximately 72 hours post imbibition (Figure 6b-d), thereafter little to no significant changes were evident. Beyond this stage, growth appeared to be focused on younger leaves (data not shown). Despite green tissues being exposed to light from 24 hours after watering onward, Fv/Fm values similar to those recorded at the full turgor (Figure 4a) became apparent in the majority of exposed Base tissues only around 72 hours post imbibition (Figure 6d). Chlorophyll fluorescence was detected in Tip tissues until 48 hours post imbibition, albeit it was noticeably lower than that measured for Base tissues. It became undetectable by 72 hours in Tip tissues, which also appeared differently coloured compared to the Base tissues, resembling tissues in the air-dry stage. This suggests that tissue differentiation progressed soon after imbibition with major changes having occurred by 72 hours post imbibition.

The movement of water through whorl 2 leaves was monitored through the introduction of a water-soluble fluorescent marker. After 48 hours, with only the petioles of leaves being exposed to double-distilled water (DDW), or DDW containing the fluorescent marker (Figure 7), leaves were able to rehydrate such that they resembled those of intact plants at a similar stage of rehydration (Figure 6c). The fluorescent marker was effectively transported throughout the leaf, confirming that water is adequately transported to the Tip tissues during the early stages of rehydration. The leaves exposed to water with and without the marker showed no significant difference during rehydration progression (Figure 7a & d).

Discussion

The phenomenon of desiccation-associated leaf tip differentiation and senescence has long been anecdotally referred to (Beckett *et al.*, 2012; Williams *et al.*, 2015; Artur *et al.*, 2019), but has only recently been demonstrated in resurrection plants (Radermacher *et al.*, 2019). This phenomenon, often written off as incomplete rehydration of leaf tissues (Charuvi *et al.*, 2015; Farrant *et al.*, 2015; Williams *et al.*, 2015; Zia *et al.*, 2016; Zhang & Bartels, 2016), thus requires deeper investigation. The current study is the first to describe this phenomenon in a homoiochlorophyllous dicot resurrection plant.

At full turgor, *C. pumilum* appears largely unremarkable amongst other small herbaceous dicots, with leaf pairs presenting in successive whorls and where leaf shedding occurs above a certain age through age-related senescence (Figure 2a). Upon the onset of dehydration and the accompanying stress, the oldest leaves (typically whorl 5 or 6) undergo senescence (Figure 2b). These leaves ultimately become abscised from the plant during the mid-stage of dehydration (Figure 2c). This abscission likely limits transpiration through canopy reduction, thus slowing down water loss. This appears similar to processes acting in desiccation-sensitive species, and the *C. pumilum* phenotype investigated by Christ *et al.* (2014). The other leaf tissues display changes that are synonymous with most desiccation-tolerant homoiochlorophyllous species: gradual loss of turgor, shrinkage of cells and tissues, and ultimately accumulation of anthocyanins and leaf curling (Figures 2 & 3; and see Bartels *et al.*, 1990; Vicré *et al.*, 2004;

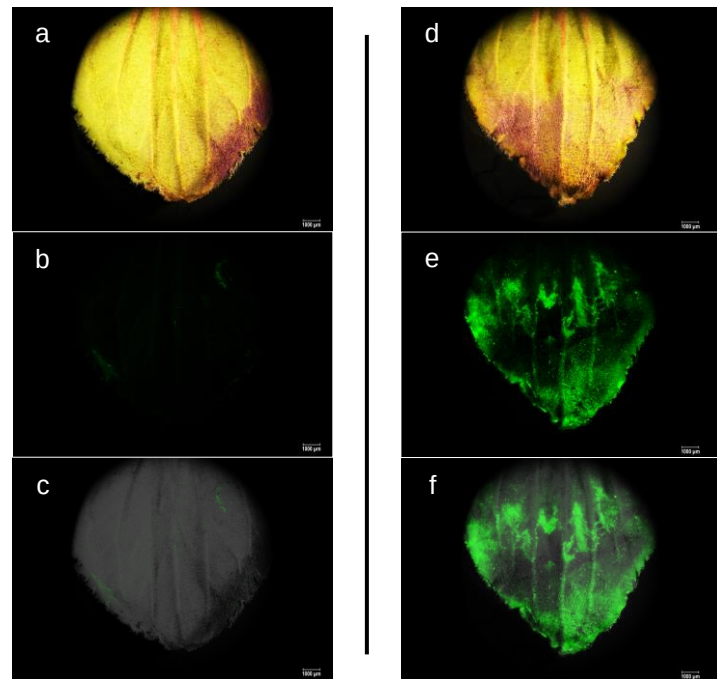


Figure 7: Fluorescent mapping of water distribution in leaves during rehydration. Fluorescent stereo-microscope images of leaves rehydrated in the absence and presence of a water-soluble fluorescent marker. Leaves were dissected from air-dry plants and allowed to rehydrate under controlled conditions for 48 hours. Images (a, b, c) show the abaxial surface of a leaf rehydrated with de-ionized water. Images (d, e, f) show the abaxial surface of a leaf rehydrated with 1.6mg/ml 4.4kDa FITC-Dextran solution. Brightfield (a, d), 3s GFP excitation (b, e) and overlay (c, f) images are shown. Magnification 1x. Scale bars indicated (1000µm). Results were replicated three times.

Farrant *et al.*, 2007; Christ *et al.*, 2014; Charuvi *et al.*, 2015, 2019; Zia *et al.*, 2016; Zhang & Bartels, 2016).

No obvious differences were apparent between the Tip and Base tissues of any of the leaves which successfully achieved the air-dry stage and thus did not succumb to stress-induced senescence during dehydration (Figure 2 & 3). During the rehydration phase, however, a clear difference between the two tissues has emerged (Figure 3 & 6). Where Base tissue accumulates water gradually during rehydration, and ultimately reaches RWC similar to that of full turgor stage tissues, the Tip tissues show an initial accumulation of water, but then lose much of this water and stabilize at a low water content of ~20% RWC (Figure 3). Such a difference in water accumulation by the two tissues was also reported by Radermacher *et al.* (2019) in the leaf tissues of the poikilochlorophyllous monocot, *X. schlechteri*. However, in that species only limited rehydration (to 20% RWC) was observed in leaf tip tissues. Initial influx of water to Tip tissues, followed by sudden loss thereof, may be due to initial passive flow of water followed by induction of water-dependant intra- and extracellular processes, resulting in efflux of water from the tissues. Considering that the Tip tissues showed no recovery following rehydration, even when given substantial time (Figure 3), it is likely that senescence processes follow differentiation; the observed efflux of water may be the first indicator of this. If indeed the case, this would suggest that the transcripts or proteins responsible for differentiation and subsequent senescence-associated processes should have been laid down in the Tip tissues at some point during dehydration.

During the full turgor stage, measured outputs of photosynthetic efficiency, such as F_v/F_m , variable fluorescence and quantum yield of PSII (YII), reflect those expected of healthy plants (Figure 4 & 5). Leaves not undergoing stress induced senescence during the early stage of dehydration had F_v/F_m values similar to those of full turgor plants, with only more in-depth analyses revealing signs that the plants are under stress. Under actinic light illumination, the variable fluorescence from PSII decreased and the leaves exhibited a diminished ability for recovery in the dark (Figure 4b & c). Additionally, the plants showed a decreased ability to tolerate short-term excess excitation energy relative to full turgor plants, exhibiting lower ETR and YII, and higher YNPQ, at progressively lower light intensities (Figure 5). These trends continued as the plants entered the mid-stage of dehydration. Here, the light curves plateaued (Figure 5) and the variable fluorescence and F_v/F_m' declined (Figure 4). This is likely due to ongoing rearrangement of the photosynthetic apparatus and detachment of LHCII from PSII during this stage (Charuvi *et al.*, 2015), with sufficient protection implemented during the higher RWC for the plant to tolerate excess excitation energy and potentially other additional stresses. During the mid-stage, significant deviation between the Tip and Base tissues also became clear as Base tissues exhibited significantly higher F_v/F_m values (Figure 4a). This notwithstanding, F_v/F_m continued decreasing in both tissues at a similar rate, reaching its minimum at the same point during dehydration (Figure 4a). The most noticeable physiological change in the *C. pumilum* dehydration response occurred during the late-stage of dehydration, when all remaining leaf tissues became deep purple as anthocyanins accumulated (Figure 2d), resulting in significant attenuation in the amount of light energy reaching the photosynthetic apparatus.

Water loss slows and ultimately ceases as minimum water contents are reached in the air-dry stage. With this final loss comes final shrinkage of the leaves and change in their orientation, such that the trichome-rich abaxial surfaces of the leaves are presented, and the youngest tissues are masked (Figure 2e & 3; Farrant *et al.*, 2007). The shrinkage of the leaves serves to increase the surface density of trichomes and thus their reflective effect (Ehleringer *et al.*, 1976; Fernández *et al.*, 2014).

The Tip tissues largely replicated the patterns seen in the Base tissues, albeit at consistently lower fluorescence. Despite Tip tissues seemingly under behaving relative to Base tissues in all PAM measurements, patterns of response to dehydration were largely similar between the two tissues. While *C. pumilum* has been suggested to undergo some senescence-like processes during dehydration (Charuvi *et al.*, 2019), it is difficult to select any single measure or difference in tissue reading and attribute them to one tissue experiencing additional stress compared to the other. Both tissues appear to follow the same path of protective photosynthetic responses to reach quiescence, albeit with the Tip tissue showing a somewhat decreased capacity to do so, similar to what was reported in *X.schlechteri* (Radermacher *et al.*, 2019).

Mirroring the RWC data, there is a dichotomy between the two tissues in the distribution of Fv/Fm values across the leaves during rehydration (Figure 6). Base tissues steadily recovered until photosynthetic competence was reached by 72 hours, while the Tip tissue did not (Figure 6). To confirm that the observed tissue differentiation phenomenon is not merely the result of a compromised supply of water to the Tip tissues, water tracking through the use of a water-soluble fluorescent probe (Figure 7) was employed. The results of which indicated that water reaches the extremities of the Tip tissue by at least 48 hours post imbibition and that water supply to all parts of the leaf are not disrupted by potential damage to vasculature during leaf shrinkage and curling.

Taken as a whole, our findings indicate the existence of desiccation-associated tissue differentiation in *C. pumilum*, and that realisation of this phenomenon occurs (or is primarily manifested and completed) only during rehydration. Given the low water contents at which differentiation occurs, it is unlikely that it is resultant of *de novo* transcription and suggests that transcripts laid down during dehydration are translated once cellular hydration becomes sufficiently high (du Toit *et al.*, 2021). The window for the generation of these transcripts or the establishment of differentiation zones are, however, still not compellingly clear, despite the demonstration here of some differences in the particular tissues' response to dehydration. Additionally, as Tip tissues appear to have no obvious obstruction to their water supply, nor severe limitations to their photosynthetic potential, it might suggest that either irreparable extents of subcellular damage occurs in these tissues during desiccation, similar to that observed by Radermacher *et al.* (2019) in *X. schlechteri*, or some other benefit exists for sacrificing leaf tissues during rehydration through a programmed process. Further work is needed in order to identify the processes responsible for this desiccation-associated tissue differentiation and its potential evolutionary role in *C. pumilum* and other homoiochlorophyllous resurrection plants.

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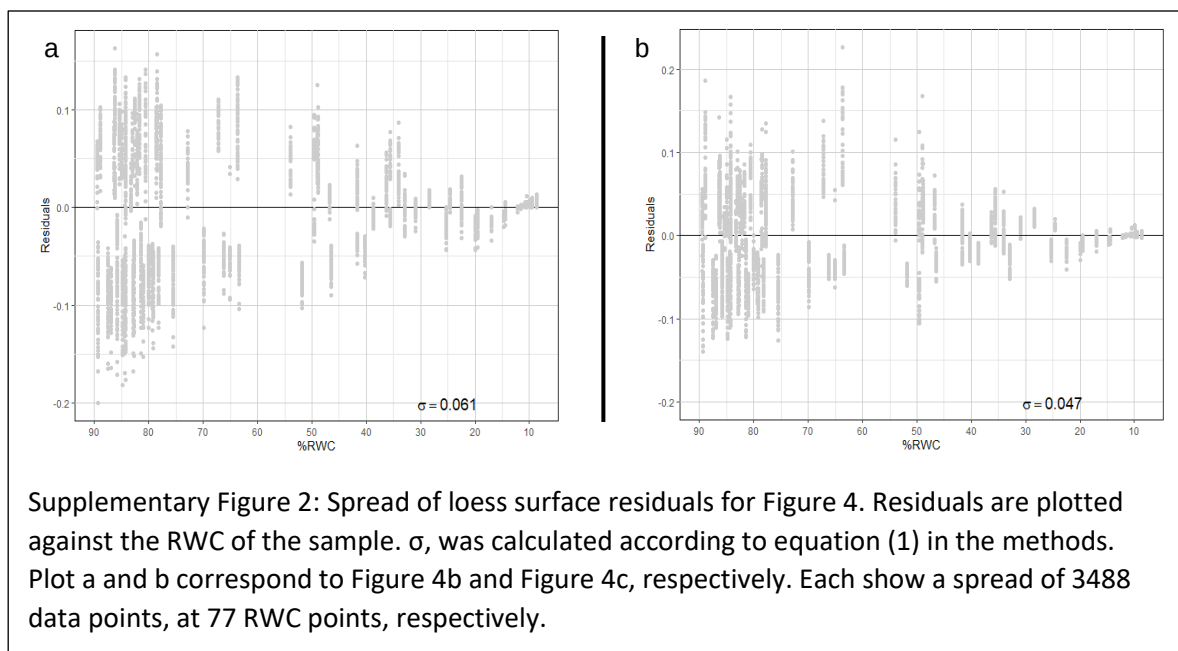
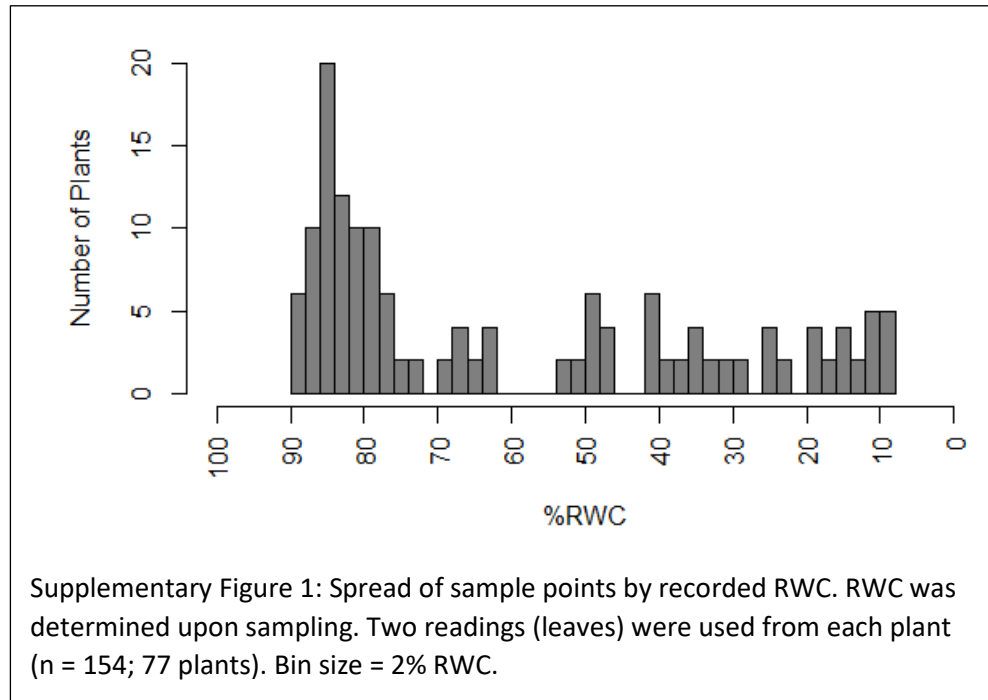
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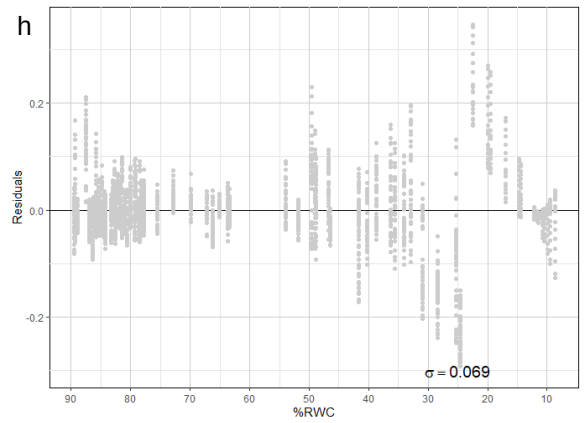
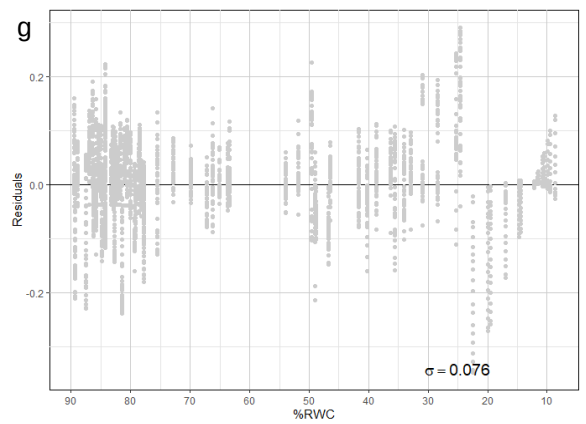
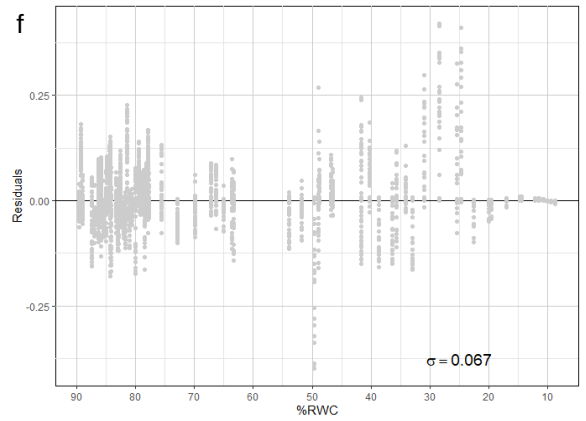
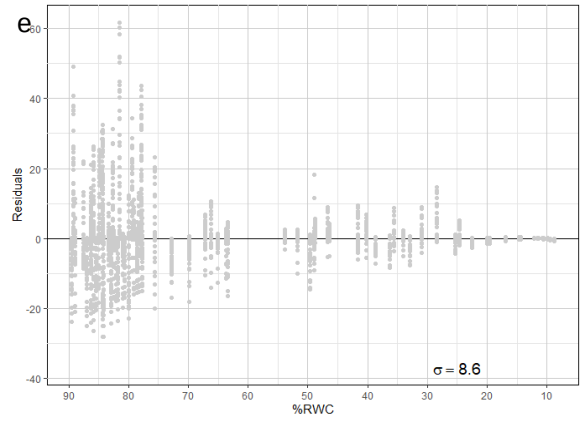
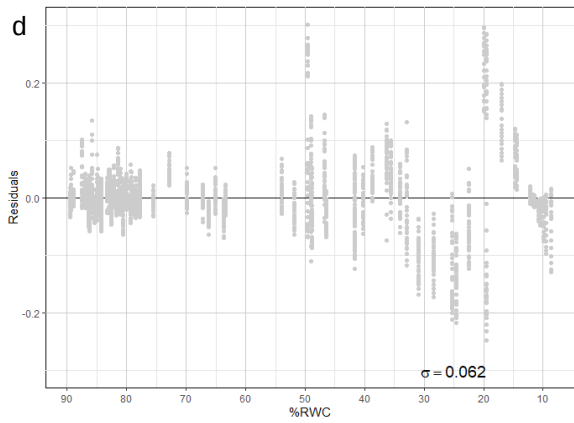
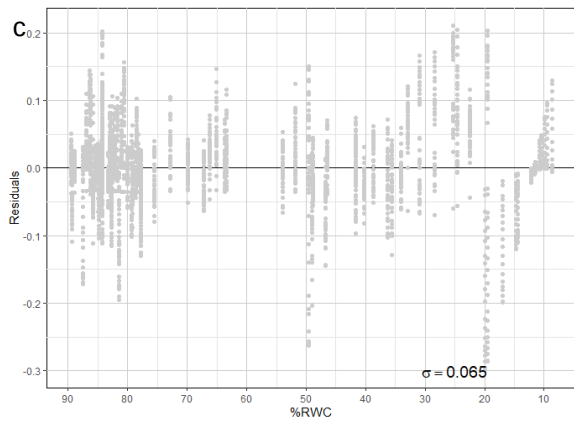
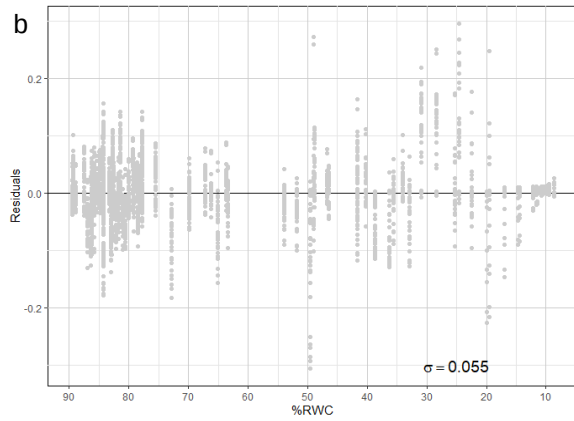
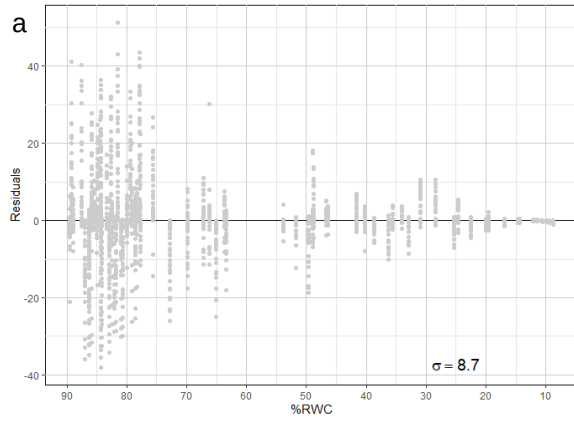
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Supplementary Material





Supplementary Figure 3: Spread of loess surface residuals for Figure 5. Residuals are plotted against the RWC of the sample. σ , was calculated according to equation (1) in the methods. Plot a – h correspond to those in Figure 5. Each show a spread of 3330 data points, at 77 RWC points, respectively.

CHAPTER 3:

Transcriptomic characterisation of tissue differentiation in response to desiccation in the homoiochlorophyllous dicot resurrection plant *Craterostigma pumilum* Hochst

Introduction

The ability of angiosperm resurrection plants to seemingly avoid the damage brought on by extreme water loss in their tissues has highlighted them as species of interest for the development of more drought tolerant crops (Farrant & Hilhorst, 2022). This ability of resurrection plants to lose the bulk of their water for extended periods, while being able to revive with the addition of water, has ample support in literature (reviewed in Tebele *et al.*, 2021). Recent work by Radermacher *et al.* (2019, 2021) has drawn attention to the vegetative tissues of resurrection plants which do seemingly not display this ability of desiccation tolerance – that is to say, the tissues display no or an incomplete rehydration phenotype.

The tissues in question have been suggested to undergo a form of senescence which is tied to the anhydrobiosis process and related to tissue developmental stage (Radermacher *et al.*, 2019). Understanding processes such as this presents an unconventional means of approaching yield loss due to water deficit stress in crop plants (Gregersen *et al.*, 2013; Hafsi *et al.*, 2013).

Here, a transcriptomic approach has been used to further motivate the presence of the desiccation associated tissue differentiation in the homoiochlorophyllous dicot resurrection plant *Craterostigma pumilum* Hochst and qualify it as a senescence-like process (Ward & Dale, 1899; Hoekstra *et al.*, 2001; du Toit *et al.*, 2022 (Chapter 2)).

Materials and Methods

Plant growth and sampling

Plant tissues stored from experiments performed in Chapter 2, were utilized for transcriptomic studies. Briefly, on harvest, tissues were divided into Base and Tip as described (see Chapter 2, Figure 1), flash frozen and stored at -80°C until use. Tissue from all identified stages of dehydration (full turgor, $\geq 80\%$ relative water content (RWC); early-stage, $80\% >$ to 60% RWC; mid stage, $60\% >$ to 30% RWC; late-stage, $30\% >$ to 15% RWC; air-dry stage, $15\% >$ to $\approx 8\%$ RWC; Chapter 2), as well as 12, 24, 48 and 72 hours post rehydration, were processed for sequencing. Leaf RWC was determined in Chapter 2, from respective twin whorl 2 leaves. Three biological replicates were selected per dehydration and rehydration stage and both tissue types originated from the same leaf.

RNA extraction and sequencing

Approximately 100mg of frozen tissue, per sample, was homogenized to a fine powder. Care was taken to limit tissue thawing. RNA was extracted from homogenized tissue with the QIAGEN RNeasy® Plant Mini Kit, using RLT Buffer. RNA integrity and quality was checked via RNA ScreenTape® (Agilent).

The samples were sequenced using the Illumina NovaSeq 6000 at the Mantoux Bioinformatics institute of the Nancy and Stephen Grand Israel National Center for Personalized Medicine (INCPM), Weizmann Institute of Science. Library preparation and sequencing runs were performed using the NovaSeq 6000 S1 Reagent Kit (100 cycles; Illumina), with the addition of Unique Molecular Identifiers (UMIs).

Sequence processing and statistics

Sequence pre-processing and trimming was performed using Trimmomatic (Version 0.39; SLIDINGWINDOW:4:18; LEADING:15; TRAILING:15; MINLEN:50; Bolger *et al.*, 2014) and Prinseq-Lite (Version 0.20.4; trim tail left: 10, trim tail right: 10, custom parameters: “T 80%;A 80%”; Schmieder & Edwards, 2011). Cleaned reads were mapped to a reference transcriptome and expression calculated, with bowtie2 (Langmead & Salzberg, 2012) and RSEM (Li & Dewey, 2011). “Expected” counts were used as raw reads counts. Reference transcriptome provided by Faigon *et al.* (original data, unpublished).

All subsequent analyses and statistics were performed on R (version 4.1.2). Gene and sample outlier detection was performed using the *goodSamplesGenes* function, within the ‘WGCNA’ package (Langfelder & Horvath, 2008, 2012), and the *hclust* function, within core R using gene Euclidean distances, respectively and omitted from further analyses. Gene differential expression was calculated using the ‘DESeq2’ package (Love *et al.*, 2014) between all trait combinations of interest (pair-wise). Any gene which showed significant differential expression (p-value adjusted for multiple testing (padj) <0.05), between any pair of traits were added to a gene candidate list for expression clustering. Raw gene counts were normalised using relativised log transformation (rlog) via the ‘DESeq2’ package.

Transformed counts were used for principal component analyses (PCA), to determine relative sample grouping, using core R functions. For expression clustering, genes from the candidate list and associated read counts were clustered using hierarchical clustering from Euclidean distance. Multiple “cut sites” were selected for the resulting dendrogram, each with differing numbers of resultant clusters. The final number of clusters were selected such that the depicted information was informative without being overly dense or repetitive. Gene Z scores were calculated, divided into the relevant clusters and depicted such that “Base” and “Tip” patterns are separate. Clusters were visualized using the ‘ggplot2’ package (Wickham, 2016).

Gene annotations were provided from Faigon *et al.* (original data, unpublished). Gene Ontology (GO) terms for stress related and hormone response terms were investigated, for pair-wise comparisons of interest, to get an overview of significant transcriptional shifts present. Kyoto Encyclopedia of Genes and Genomes (KEGG) K numbers were used to elucidate metabolic processes and genes of interest attributed to each expression cluster. Metabolism modules were determined using the KEGG online database via the online KEGG Orthology service (<https://www.genome.jp/kegg/ko>). Gene descriptions were extracted for each cluster and the text mined in R, using the ‘tm’ package (Feinerer *et al.*, 2008; Feinerer & Hornik, 2020), to determine unique descriptor frequencies. Gene descriptors were assigned based on alignment to *inter alia* the Uniprot database

Results & Discussion

Suitable RNA for sequencing was extracted from all samples, except from Tip tissues sampled from leaves at air-dry stage and 12 hours rehydration. These tissues consistently showed minute to no RNA yield after extraction and were thus omitted.

The quality of the sequencing run appeared suitable with <5% of reads being dropped during quality trimming. Of the remaining reads, an average overall alignment rate of ≥85% to the reference transcriptome was observed. Sixty-two genes, identified in the reference transcriptome (Faigon, original data, unpublished), were omitted as they had zero representative reads in this study. A total of 81 459 genes were carried forward to statistical analyses. Only one sample was identified as an outlier, based on Euclidean distance from other samples. This was a full turgor Base sample and was removed from further analyses. A PCA with the inclusion of this outlier can be seen in Supplementary Figure 2.

Sample distribution

The PCA plot of normalized counts explained a total of 52.7% of the variation observed with the first two principal components (Figure 1). An additional 7.59% was explained with the addition of the third principal component (Supplementary Figure 1), however this added little in terms of interpretation of the sample spread and grouping. The remaining unexplained variation was a possible result of the high gene number estimated in the reference transcriptome generated by Faigon *et al.* (original data, unpublished), innate biological variation of this non-model species and the high likelihood that *C. pumilum* is polyploid, like its sister species *C. plantagineum* (VanBuren *et al.*, 2022). In addition, samples

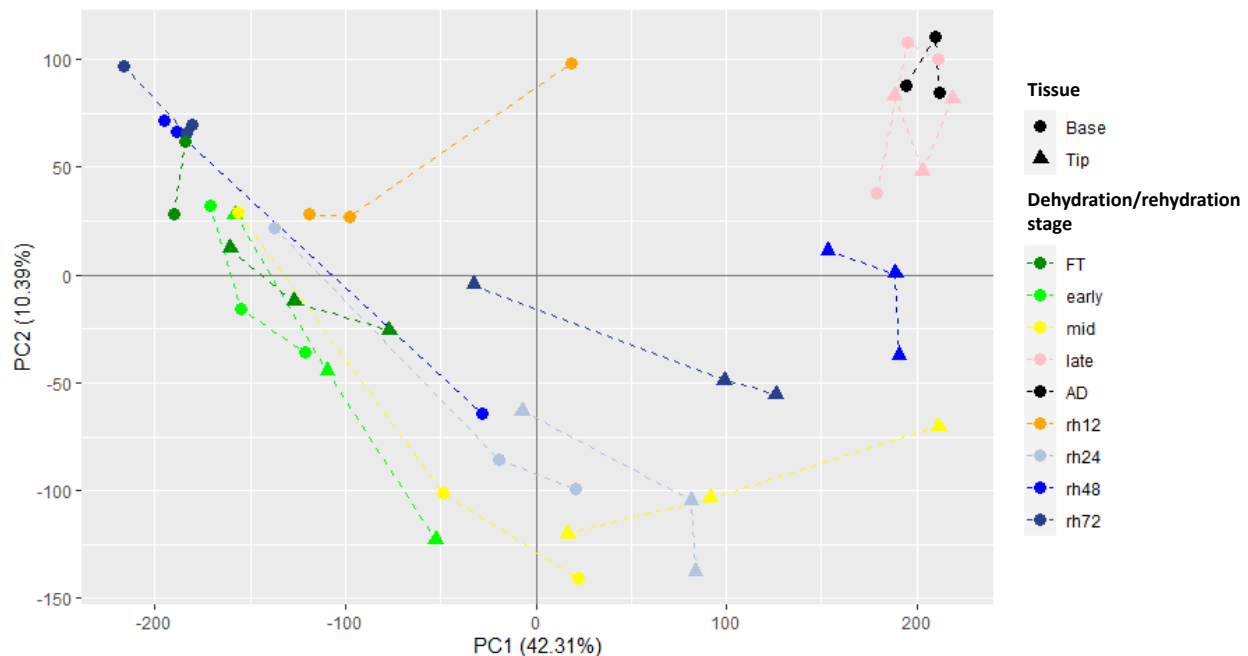


Figure 1: Principle component analysis (PCA) showing inter-sample relations based on normalized (rlog) read counts. “Base” and “Tip” refer to tissue types described in Chapter 2. Abbreviations: “FT”, full turgor; “AD”, air-dry; “rh.”, hours post rehydration.

from dehydration and rehydration stages which have broader defined %RWC ranges, or which are subject to greater variation in individual plant response (ie. rehydration; see Chapter 2 Figure 3), showed greater variation between points in the same dehydration/rehydration stage. While this does not discredit the findings presented here, it instead highlights inherent obstacles in this field of research. Despite this, the PCA revealed valuable trends upon inspection (Figure 1).

Base tissues at full turgor, 48 hours rehydration and 72 hours rehydration grouped together in the far left (negative range) of PC1, while Base tissues at late stage and air-dry stage group with Tip tissues at late stage, in the far right (positive range). This supports the notion that these two groupings represent the two most extreme transcriptional states in the dehydration-rehydration cycle (Farrant & Hilhorst, 2021), namely the hydrated state and the anhydrobiotic state. The dehydration/rehydration stages, which exist between these two groupings, similarly occupy the in-between range of PC1 (Figure 1) and reproduce the pattern anticipated by the dehydration-rehydration cycle. Tip full turgor stage points, however grouped closer to the Base points at early stage dehydration than at full turgor stage and points from 24 hours rehydration, for both tissue types, seem to associate with the respective mid stage points (Figure 1). Interestingly, Tip 48-hours and 72-hours rehydration points grouped independently and separately from points belonging to other groups (Figure 1). This mirrors the %RWC and physiological trends seen in Chapter 2 Figure 3, where the realization of tissue differentiation was

anticipated to occur sometime between 24 hours and 48 hours rehydration, and progress thereafter. No clear pattern was present for the distribution of points along the ranges of PC2 and PC3. Points from full turgor, late, air-dry and Base rehydration stages largely existed in the positive range of PC2 and negative range of PC3 (Figure 1, Supplementary Figure 1). Remaining points largely existed in the opposite ranges as the aforementioned stages, in both PCs respectfully. Only points from the early and mid stages consistently occupied the negative field of both PC2 and PC3 (Figure 1, Supplementary Figure 1).

Overall, the PCA confirmed that sample dehydration/rehydration stage identifiers, as well as tissue identifiers in the case of rehydration stages, show adequate transcriptional differences to motivate their

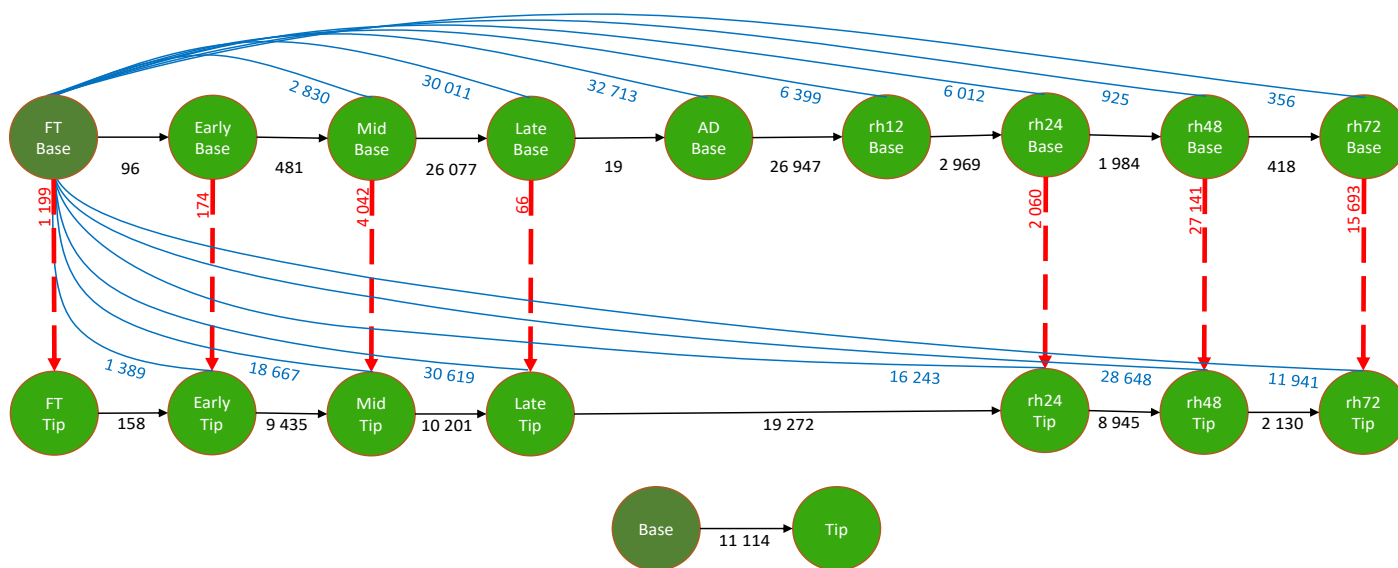


Figure 2: Number of significantly differentially expressed genes between respective RWC classes and tissue type combinations (pair-wise). A total of 58 000 unique genes were differentially expressed among all experiment conditions. Significance: $\text{padj} < 0.05$. “Base” and “Tip” refer to tissue types described in Chapter 2. Abbreviations: “FT”, full turgor; “AD”, air-dry; “rh.”, hours post rehydration.

use as discrete groups, in subsequent analyses. This additionally complements the physiological observations reported in Chapter 2, with regard to classification of these stages.

Differential expression analyses

Dehydration

Of the total genes used for statistical analyses, 58 000 unique genes were determined to be significantly differentially expressed genes ($\text{padj} < 0.05$; SDEGs), between at least one pair of conditions (Figure 2). Of these approximately 62% of genes had GO terms annotated to them (Faigon *et al.*, original data, unpublished). Radermacher *et al.* (2019, 2021) suggested that the desiccation associated differentiation of tissue destiny in *Xerophyta schlechteri* was a specialized form of senescence. As such, all pair wise comparisons were mined for GO terms associated with factors which are suggested to play roles in triggering senescence, such as ‘response to stress’ or ‘response to phytohormones’ (stresses reviewed in Griffiths *et al.*, 2014; phytohormones reviewed in Sarwat *et al.*, 2013) (Table 1 & 2). Since the exact role-players in plant senescence are not entirely understood or shown to vary among plant families (reviewed in Kabbage *et al.*, 2017), terms relating to leaf senescence, autophagy and ubiquitination were also mined. Importantly, the GO Term for ‘Programmed Cell Death’ (GO:0012501) was mined for

but yielded no significant results in the pair-wise comparisons of interest (Figure 2). Gene descriptors for other genes implicated as role-players in senescence (reviewed in Kabbage *et al.*, 2017), such as metacaspases, *VACUOLAR PROCESSING ENZYMES (VPE)*, *SUBTILISIN-LIKE SERINE PROTEASES* (subtilases/saspases), *SUCROSE NONFERMENTING 1-RELATED KINASES (SnRKs)* and *TARGET OF RAPAMYCIN (TOR)* were also mined, however none of these showed notable differences in expression between pair-wise comparisons of interest (Supplementary Data Sheet 1). The lack of SDEGs, with the mentioned annotations, did however not necessarily indicate absence of their associated processes. As such, processes relating to autophagy and senescence were still investigated in subsequent cluster analyses (Figure 3).

In Base tissues, the greatest number of SDEGs occurred between full turgor and the late and air-dry stages respectively (Figure 2). This corroborates the PCA findings that these stages are not only extremes in terms of %RWC but also in terms of transcription patterns. Interestingly, the greatest inter stage transcriptional shifts, in Base tissues, occurs between the mid stage and late stage and the air-dry stage and 12 hours rehydration (Figure 2), respectively. The transition between late and air-dry stages however, has very few SDEGs, suggesting that many of the transcripts necessary for anhydrobiosis were already being expressed prior to the late stage. Of the SDEGs which show an increased abundance, five are associated with membrane and cell wall modification (Supplementary Data Sheet 1). These are the two probable ester hydrolases *MONOGLYCERIDE LIPASE-like (MGLL-like)* and *CARBOXYLESTERASE 15 (CES15)*, the membrane component *AQUAPORIN TIP1-1-like*, cell wall modifying *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 13 (FLA13)* and the pectin hydrolase regulator *POLYGALACTURONASE-1 NON-CATALYTIC SUBUNIT BETA-like (GP1-like; Watson et al., 1994)* (Supplementary Data Sheet 1). Of the two ester hydrolases only *MGLL-like* is confirmed to function on membrane components, but the catalytic activity is shared by both (Aranda *et al.*, 2014) suggesting a potential shared function in membrane modification. Provided these transcripts are translated during this very late stage of dehydration, their increased abundance may suggest that the leaf curling behaviour observed in Chapter 2, requires ongoing modification of the cell wall and membranes. Vitré *et al.* (2004a) showed that desiccating leaf tissues of *C. wilmsii* increased in Ca^{2+} content. They suggested that this is necessary to cross-link cell wall polymers, such as acidic pectins, to modulate the mechanical properties of the cell wall architecture in the dry state (Vitré *et al.*, 2004ab). Modulation of the cell wall properties would aid in mitigating mechanical stresses in the anhydrobiotic state and stresses associated with rehydration, as water enters the cell apoplastically. Alternatively, these transcripts may be translated only upon rehydration and may thus play a role in modifying cell walls as cellular water content and internal pressure increases.

The other smallest inter stage transcriptional shifts occurs between full turgor and the early stage (Figure 2). This suggests little significant transcriptional change was needed for this tissue to transition from the fully hydrated state to one beginning to experience water deficit stress. Notable stress related SDEGs in this transition was a variety of *LATE EMBRYOGENESIS ABUNDANT (LEA)* proteins, *DESICCATION STRESS PROTEIN 22 (DSP22)*, *THIOREDOXIN (TRX)* homologues and *ALDOSE REDUCTASE (ALDR)* homologues (Supplementary Data Sheet 1). An *ALDR* homologue was shown to be expressed in *X. schlechteri* during dehydration and confer osmotic stress tolerance when cloned into *E.coli* (Mundree *et al.*, 2000). *DSP22* was demonstrated to be *EARLY LIGHT INDUCIBLE PROTEIN (ELIP)* like and localized to the chloroplasts of *C. plantagineum* during dehydration (Bartels *et al.*, 1992). Accumulation of *ELIPs* has been proposed to play a role in photooxidative damage reduction during prolonged anhydrobiosis (Bartels *et al.*, 1992). They have also been shown to be significantly proliferated in the genomes of most resurrection plants (VanBuren *et al.*, 2019). *TRX* proteins regulate various redox reactions and play an important role in chloroplasts, regulating photosynthetic reactions to allow for light acclimation (reviewed in Geigenberger *et al.*, 2017). Therefore, during the initial stages of water deficit stress, *C.*

pumilum seemed to prioritise protection of its chloroplasts and regulation of photosynthesis, while accumulating protective proteins like LEAs.

The transcriptional switch from the water deficit stress response to the desiccation response may have occurred between the mid and late stages. Indeed, many of the SDEG terms present between the mid and late stage were annotated to some form of stress response, as well as protein ubiquitination, response to abscisic acid (ABA) and response to auxin (Table 1). Interestingly, more SDEGs annotated with the term 'response to oxidative stress' were present, than SDEGs annotated with other stress associated response terms, such as for water deprivation, salt and heat stress (Table 1). Additionally, far more SDEGs were seen with terms related to the response to auxin than related to the response to ABA. These trends appeared consistent among all pairwise comparisons (Table 1 & 2). Given the plants were subjected to severe water deficit stress, one would expect more differential expression in genes with terms such as 'response to water deprivation' and 'response to salt stress', since soil salts will increase in concentration as solvent water decreases. However, nearly all abiotic stresses have an oxidative stress component, resulting from the over-production and accumulation reactive oxygen species (ROS) (Demidchik, 2015). The many SDEGs with the term 'response to oxidative stress' thus represent an aspect of the overall response to the many stresses associated with desiccation.

ABA is classically classed as a water deficit stress responsive phytohormone in plants (Bray, 1997), while auxin is considered an essential growth hormone, as it plays a vital role in cell wall expansion (reviewed extensively in Majda & Robert, 2018). During the transition from mid to late stage, eleven SDEGs annotated with *ABSCISIC ACID-INSENSITIVE 5-like (ABI5-like)* and isoforms thereof were present (Supplementary Data Sheet 1). *ABI5* is an ABA-dependant bZIP transcription factor which influences expression of stress-response genes, with an increased abundance being associated with elevated ABA content and elevated expression of stress response genes (reviewed in Skubacz *et al.*, 2016). *ABI5*, and the related *ABI3* regulon, has also been implicated as potentially being important to the stress response of resurrection plants, particularly in the *Xerophyta* genus (Costa *et al.*, 2017; Lyall *et al.*, 2020; Oliver *et al.*, 2020, Xu *et al.*, 2021). However, no SDEGs annotated with *ABI3-like* were found in any pairwise comparisons and, while *ABI5-like* transcripts were present throughout the dehydration cycle of *C. pumilum*, all *ABI5-like* SDEGs present in the transition from mid to late stage showed a decreased abundance (Supplementary Data Sheet 1). Additionally, SDEGs annotated with *ABSCISIC ACID 8'-HYDROXYLASE 4-like (CYP707A4-like)*, key enzymes in ABA catabolism (Kushiro *et al.*, 2004), and *SUPPRESSOR OF ABI3-5 (SAU)*, a splicing suppressor of the *ABI3* cryptic intron (Sugliani *et al.*, 2010), showed an increased abundance (Supplementary Data Sheet 1). This suggests that responses to ABA were being suppressed during the Mid to Late stage transition and that ABA contents were being controlled through catabolism. This was further supported by the increased abundance of three *PICKLE (PKL)* annotated SDEGs, a chromatin-remodelling factor which negatively regulates expression of *ABI5* and *ABI3* through histone methylation (Perruc *et al.*, 2007). This finding is in apparent contradiction with work presented in other resurrection plants which increase in their ABA responses with progressive drying (Costa *et al.*, 2017; Gabier *et al.*, 2021; Xu *et al.*, 2021). Notably, recent transcriptomic work in *C. plantagineum* (Xu *et al.*, 2021) did show a significant increase in expression of ABA signalling genes, but only when comparing full turgor to "partial dehydration" (~60% RWC, the upper limit of the defined Mid stage, Chapter 2). These ABA signalling genes showed a significant decrease when they compared "partial dehydration" to samples in the anhydrobiotic state. When we compared full turgor to the mid stage, SDEGs for *ABI5-like* were present and did show an increased abundance (Supplementary Data

Sheet 1). The apparent lack of any SDEGs for *ABI5-like* when an early dehydration stage was included thus suggests that these genes had a slow accumulation during ongoing water deficit stress and changes therefore did not appear significant until their significant decrease between the mid and late stages. This is supported by findings from Vitré et al (2004a) which show that ABA content in *C. wilmsii* increases gradually during early water deficit stress and decreases significantly by $\approx 20\%$ RWC (our defined late stage dehydration).

Table 1: GO terms for intra-tissue pair-wise comparisons of interest. Annotated terms relating to stress and hormone response were selected, as well as terms relating to senescence, from significantly (padj < 0.05) differentially expressed genes. Total number of significantly differentially expressed genes (SDEGs) belonging to a particular GO term are shown. Values in parentheses indicate SDEGs with increased abundance. Abbreviations: “FT”, full turgor; “E”, early; “M”, mid; “L”, late; “AD”, air-dry; “rh”, hours post rehydration.

Biological Process	FT Base vs E Base	E Base vs M Base	M Base vs L Base	L Base vs AD Base	AD Base vs 12H Base	12H Base vs 24H Base	24H Base vs 48H Base	48H Base vs 72H Base	FT Tip vs E Tip	E Tip vs M Tip	M Tip vs L Tip	24H Tip vs 48H Tip	48H Tip vs 72H Tip
Stress related													
Response to oxidative stress (GO:0006979)	-	-	77 (↑41)	-	83 (↑33)	6 (↑5)	6 (↑2)	3 (↑0)	1 (↑0)	38 (↑29)	24 (↑13)	25 (↑11)	4 (↑3)
Response to salt stress (GO:0009651)	-	2 (↑2)	23 (↑8)	-	23 (↑13)	4 (↑4)	1 (↑0)	1 (↑0)	1 (↑0)	8 (↑5)	10 (↑0)	9 (↑3)	2 (↑2)
Response to water deprivation (GO:0009414)	-	1 (↑1)	13 (↑4)	-	16 (↑8)	2 (↑2)	1 (↑0)	-	-	5 (↑1)	6 (↑0)	1 (↑0)	1 (↑1)
Response to wounding (GO:0009611)	5 (↑5)	9 (↑9)	21 (↑7)	-	42 (↑10)	6 (↑5)	2 (↑0)	11 (↑0)	-	21 (↑16)	9 (↑4)	5 (↑3)	1 (↑0)
Response to heat (GO:0009408)	-	-	27 (↑15)	-	33 (↑12)	4 (↑3)	-	-	-	9 (↑7)	12 (↑4)	8 (↑6)	3 (↑1)
Leaf senescence (GO:0010150)	-	-	3 (↑1)	-	4 (↑3)	1 (↑1)	-	-	-	-	1 (↑0)	1 (↑0)	-
Autophagy (GO:0006914)	-	-	19 (↑12)	-	23 (↑4)	3 (↑3)	1 (↑0)	-	-	6 (↑5)	3 (↑2)	6 (↑5)	-
Autophagosome assembly (GO:0000045)	-	-	23 (↑15)	-	20 (↑6)	3 (↑2)	-	-	-	5 (↑4)	10 (↑7)	5 (↑2)	1 (↑1)
Protein ubiquitination (GO:0016567)	1 (↑0)	6 (↑6)	277 (↑158)	-	282 (↑136)	18 (↑9)	16 (↑1)	2 (↑0)	1 (↑0)	129 (↑91)	131 (↑56)	119 (↑61)	17 (↑13)
Ubiquitin-dependent protein catabolic process (GO:0006511)	-	4 (↑4)	134 (↑96)	-	139 (↑32)	11 (↑5)	3 (↑1)	1 (↑0)	-	45 (↑37)	51 (↑24)	35 (↑20)	2 (↑2)

Table 1 continued:

[illegible]

In *Oryza sativa* (rice), ABA was shown to have an inverse relationship to auxin under drought stress, suggesting pathway interactions (Du *et al.*, 2010, 2013; Reviewed in Skubacz *et al.*, 2016). The high number of auxin responsive genes, as well as their expression distribution (Table 1), suggest that this phytohormone plays some role in the water deficit stress response of *C. pumilum*. Work on *C. wilmsii* showed an increase in auxin content during dehydration and, to a lesser extent, during rehydration (Vicré *et al.*, 2004a). Auxin is known to promote cell wall flexibility and extensibility, by acidifying the apoplast, through activation of proton pumps in the plasma membrane (Braybrook & Peaucelle, 2013; Ren & Gray, 2015). This acidification subsequently modifies the cell wall composition through pectin polymerization, hemicellulose xyloglucan depolymerization and activation of cell wall loosening proteins, such as *inter alia* expansins (Nishitani & Masuda, 1981; Cosgrove, 2000). Many resurrection plants exhibit some degree of cell wall folding during drying, typically below 50% RWC, with the most extreme cases being the accordioning of the cell wall in the *Craterostigma* genus (Vicré *et al.*, 1999; Farrant, 2000; Cooper & Farrant 2002; Vicré *et al.*, 2004b; Moore *et al.*, 2013; Jung *et al.*, 2019; Radermacher *et al.*, 2019; Oliver *et al.*, 2020). During the Mid to Late stage transition, auxin responsive SDEGs showed mix of increased and decreased abundances. The SDEGs, which show an increased abundance, included various members of the *DORMANCY-ASSOCIATED GENE/AUXIN-REPRESSED 12.5KDA PROTEIN family (DRM/ARP)*, *AUX/IAA PROTEIN family (AUX/IAA)*, *SMALL AUXIN UP-REGULATED RNA family-like (SAUR-like)* and *AUXIN RESPONSE FACTOR family (ARF)* (Supplementary Data Sheet 1). Notably, increased abundance was also seen for *INDOLE-3-PYRUVATE FLAVIN MONOOXYGENASE YUCCA (YUC)* SDEGs, a class of enzymes which catalyse the tryptophan-dependant biosynthesis of auxin (Zhao *et al.*, 2001; Cheng *et al.*, 2006). Genes of the *ARF* and *IAA* families are auxin dependant transcription factors (Liscum & Reed, 2002). *IAA* transcription regulators interact with *ARF* transcription factors and repress their function, when auxin concentrations are below a particular threshold (Liscum & Reed, 2002). *ARP* genes often contain one or more intrinsically disordered regions (automatic annotation, MobiDB-lite, UniProt) and are induced by auxin (Shi *et al.*, 2013), particularly in the water deficit stress response of embryos and seedlings (Lee *et al.*, 2013; Jing *et al.*, 2017). *ARPs* function in arresting hypocotyl elongation and leaf growth, possibly by inhibiting cell elongation (Lee *et al.*, 2013). Proteins from the *SAUR* family are induced by auxin, albeit not exclusively, and induce plant growth through regulation of cell wall acidification (reviewed in Stortenbeker & Bemer, 2019).

We propose that during progressive dehydration, *C. pumilum* Base tissues respond through gradual accumulation of ABA signalled stress responses. During the transition from mid to late stage, *C. pumilum* suppresses ABA signalled responses, through ABA catabolism and suppression of *ABI5* homologues to promote auxin induced pathways. These pathways are activated by a small group of *ARF* transcription factors, particularly *ARF1*, *14* and *19* homologues (Supplementary Data Sheet 1). *ARF* activity is tightly controlled by *IAA* transcription regulators and is dependent on auxin concentration. Auxin concentration is possibly regulated by *YUC* family enzymes. Without a genome, the exact targets of these *ARF* transcription factors are difficult to determine especially the presence of novel *ARF* targets, which function during desiccation. However, the presence of *SAURs* and *ARPs* strongly suggest a role in cell wall plasticization, while inhibiting auxin induced growth.

Rehydration

The second greatest inter stage transcriptional shift in Base tissues, between the air-dry stage and 12 hours rehydration, had more than 26 000 SDEGs (Figure 2). This was surprisingly large given the low %RWC present in tissues 12 hours post rehydration (average 45% RWC; see Chapter 2 Figure 3). The presence of active transcription machinery therefore seems unlikely unless one assumes a cellular environment, which allows such reactions, despite a low water cellular environment (du Toit *et al.*, 2021). Intriguingly, Cooper & Farrant (2002) showed that *C. wilmsii* plants, rehydrated in inhibitors of

both transcription and translation, were able to revive completely. This suggests that although this transcriptional shift is large, an adequate number of cellular constituents may be present in the anhydrobiotic state to recover from anhydrobiosis. In this transcriptional shift, like in the mid to late stage transition, a large number of SDEGs for auxin and ABA responsive terms were present (Table 1). Interestingly, where in the mid to late stage ABA signalled responses seem suppressed, during the air-dry to 12 hours rehydration transition ABA signalled responses seemed induced. SDEGs for *SAU* and *PKL* show decreased abundances, while *ABI5*-like homologues and various *ABSCISIC ACID RECEPTOR* *PYL* (*PYL*) family transcription factors, particularly *PYL2*, 4 and 8 homologues, showed an increased abundance (Supplementary Data Sheet 1). Homologues of *ABI5* showed additional significant increase in abundance between 12 hours rehydration and 24 hours, however *PYL2* showed a significant decrease in abundance (Supplementary Data Sheet 1). From 24 hours rehydration to 72 hours rehydration no additional ABA responsive genes showed a significant increase in abundance and only one *ABI5* homologue showed a significant decrease in abundance between 48 hours rehydration and 72 hours rehydration (Supplementary Data Sheet 1). This suggests that *C. pumilum* engaged in ABA mediated stress responses during the first 24 hours following rehydration. This may be to induce water conservation mechanisms, such as stomatal closure, or suppress non-essential functions until hydration was again sufficient (Davies, 2012; Skubacz *et al.*, 2016).

Auxin responsive SDEGs which increased in abundance between air-dry and 12 hours rehydration included various members of the *AUXIN BINDING PROTEINS* (*ABP*) family, *SAUR*-like family, *ARF* family and *AUXIN TRANSPORTER*-like proteins (Supplementary Data Sheet 1). This suggests that auxin plays a role during the early rehydration of *C. pumilum*, before hydration levels are such that growth can recommence (Chapter 2). Assuming most of the auxin responsive SDEGs are intended for recovery from anhydrobiosis, auxin may here function in repairing damaged cell walls, through promotion of cell wall constituents containing vesicle trafficking (Blancaflor & Hasenstein, 1995; Takesue & Shibaoka, 1999; Majda & Robert, 2018). Indeed, Cooper & Farrant (2002) showed that when *C. wilmsii* was rehydrated in the presence of translational and transcriptional inhibitors, cell walls failed to ‘unfold’ successfully. This supports the hypothesis that transcription and translation in early rehydration, possibly of auxin responsive genes like the *SAUR* family, is necessary for cell walls to recover their shape.

The comparatively low number of SDEGs between Base tissues at full turgor and at 72 hours rehydration suggest that, at this point during rehydration, this tissue has once again reached a state that resembles the full turgor stage. This agrees with findings in Chapter 2 that this is when full metabolic competence is once again reached in this species and is in line with what has been reported for *C. wilmsii* (Cooper and Farrant, 2002; Farrant 2000). This appears to be in contrast with reportage by Xu *et al.* (2021) in *C. plantagineum*, which suggest that metabolic competence is reached by 24 hours rehydration in this species. However, to our knowledge the aforementioned study did not compare their findings with later stages of rehydration and may therefore have overlooked further recovery in this species.

Inter-tissue comparison

When observed independently of Base tissues, Tip tissues seemingly followed a different transcriptional trend, with more inter-stage transcriptional shifts, albeit at lower comparative magnitudes (Figure 2). These shifts appeared to occur between the early and mid stages, the mid and late stages and 24 hours and 48 hours of rehydration. However, SDEGs associated with ABA and auxin response follow a seemingly identical inter-stage expression pattern to those of base tissue during dehydration (Supplementary Data Sheet 1). The only noticeable exception was that some SDEGs for *CYP707A4* homologues, *SAUR* homologues and some *ARFs* had begun to increase in abundance during the early to mid stage transition. This suggested that Tip and Base tissues followed the same pathways during

dehydration, with regard to ABA and auxin mediated stress response, but Tip tissues initiated these responses earlier in their water deficit stress response.

In Tip tissues no differential expression data was available for the air-dry stage and 12 hours rehydration, due to the lack of adequate quantities of mRNA extracted from these tissues. Given the differential expression seen in Base tissues at the same stages, and the number of SDEGs between Tip tissues at late stage and at 24 hours rehydration, it seemed highly unlikely that no mRNA was present in these tissues. Instead, it may be that a combination of a modified cellular environment and the formation of heat shock granules may have sequestered the available mRNA, such that it was inaccessible to the extraction protocol used (Nover *et al.*, 1983). Indeed, Nover *et al.* (1989), described a potentially related phenomenon where mRNA bound in heat shock granules displayed “sedimentation behaviour” and were inaccessible to molecular markers, unless they were introduced prior to the stress or after recovery. The absence of available transcript information in Tip tissues at these points thus highlights a potential cellular environment difference between Base and Tip tissues during anhydrobiosis that differentially facilitates formation of these granules. Candé *et al.* (2004) suggested that the formation of stress granules in cultured human cells was related with the redox potential of the cellular environment, with a more reduced cellular environment inhibiting formation of these stress granules. This may suggest that the cellular environment in Tip tissues was more oxidative than Base tissues, approaching the anhydrobiotic state. Interestingly, although the Tip and Base tissues show different patterns with regard to expression of SDEGs with the term ‘response to oxidative stress’ during dehydration, only the mid stage showed SDEGs with this term when doing intra-stage comparisons of tissues (Tables 1 & 2). Radermacher *et al.* (2021) suggested that the desiccation driven senescence in *X. schlechteri* might in part be due to differential ROS-related signalling and thus a differential oxidative stress experience. Work by Asami *et al.* (2019) additionally suggested that shoots of *Tripogon loliiformis* experienced greater stress than in its roots. While it is difficult to directly relate the anatomy and life history of the leaf blades of the monocots *X. schlechteri* and *T. loliiformis* to the leaves of our dicot *C. pumilum*, our findings suggest that Tip tissues responded differently, with regard to oxidative stress, relative to Base tissues during the mid stage of dehydration and thus during the preparation for anhydrobiosis (Table 2).

When comparing the pool of Base tissues with the pool of Tip tissues, more than 11 000 SDEGs were present (Figure 2). Interestingly, these included a *PURPLE ACID PHOSPHATASE 22-like (PAP22-like)* and *CELL WALL INVERTASE (CW-INV)* homologues, which increased in abundance in pool Tip tissues (Supplementary Data Sheet 1). Proteins from the *PAP* family catalyse the hydrolysis of phosphate and play an vital role in phosphate recycling during plant phosphate starvation (Tran *et al.*, 2010). *CW-INV* enzymes irreversibly cleave sucrose to yield glucose and fructose and are highly associated with source tissues (reviewed Proels & Hückelhoven, 2014). Therefore, just comparing the pool of Base tissues to the pool of Tip tissues, Tip tissues appear to be source tissues, recycling phosphate containing molecules and sucrose for transport to a sink tissue. Indeed, pool Base tissues showed an increased abundance of sugar transporter SDEGs, such as *inter alia* *BIDIRECTIONAL SUGAR TRANSPORTER SWEET12-like*, when compared to pool Tip tissues (Supplementary Data Sheet 1). Suggesting Base tissues may act, at least in part, as a sink tissue.

Intra-stage tissue comparisons revealed that the highest number of SDEGs occurred during rehydration stages and at the mid stage (Figure 2). During the mid stage, Tip tissues showed a 30-fold increased abundance of the *WD40 REPEAT-CONTAINING PROTEIN HOS15 (HOS15)* SDEG, relative to Base tissues. *HOS15* is a chromatin remodelling deacetylase, that has been shown to negatively regulate stress responses, particularly cold stress and ABA signalled stress responses (Zhu *et al.*, 2008; Ali & Yun, 2020). Upon further investigation, *HOS15* appeared to significantly increase in abundance during the transition

from early to mid stage in Tip tissues (Supplementary Data Sheet 1). This finding highlights *HOS15* as a potential regulator of the desiccation-associated tissue differentiation phenomenon.

Table 2: GO terms for intra-stage pair-wise comparisons of interest. Annotated terms relating to stress and hormone response were selected, as well as terms relating to senescence, from significantly (padj <0.05) differentially expressed genes. Total number of significantly differentially expressed genes (SDEGs) belonging a particular GO term are shown. Values in parentheses indicate SDEGs with increased abundance. Abbreviations: “FT”, full turgor; “E”, early; “M”, mid; “L”, late; “AD”, air-dry; “rh”, hours post rehydration.

<u>Biological Process</u>	Base vs Tip	FT Base vs FT Tip	E Base vs E Tip	M Base vs M Tip	L Base vs L Tip	24H Base vs 24H Tip	48H Base vs 48H Tip	72H Base vs 72H Tip
<u>Stress related</u>								
Response to oxidative stress (GO:0006979)	44 (↑24)	7 (↑6)	-	28 (↑18)	-	5 (↑0)	74 (↑39)	88 (↑35)
Response to salt stress (GO:0009651)	7 (↑7)	2 (↑2)	-	7 (↑5)	-	1 (↑1)	24 (↑12)	21 (↑15)
Response to water deprivation (GO:0009414)	4 (↑0)	-	-	3 (↑0)	-	1 (↑0)	10 (↑2)	8 (↑4)
Response to wounding (GO:0009611)	13 (↑12)	13 (↑11)	2 (↑1)	8 (↑4)	-	2 (↑2)	16 (↑13)	34 (↑34)
Response to heat (GO:0009408)	10 (↑9)	1 (↑1)	-	4 (↑3)	-	-	28 (↑16)	20 (↑14)
Leaf senescence (GO:0010150)	-	-	-	-	-	-	4 (↑1)	3 (↑2)
Autophagy (GO:0006914)	4 (↑3)	-	-	3 (↑3)	-	1 (↑0)	20 (↑10)	11 (↑9)
Autophagosome assembly (GO:0000045)	3 (↑0)	-	-	1 (↑1)	-	-	21 (↑15)	7 (↑6)
Protein ubiquitination (GO:0016567)	110 (↑64)	7 (↑6)	2 (↑2)	53 (↑39)	-	18 (↑9)	293 (↑172)	189 (↑125)
Ubiquitin-dependent protein catabolic process (GO:0006511)	39 (↑28)	1 (↑0)	-	8 (↑7)	-	7 (↑5)	122 (↑90)	58 (↑47)
<u>Hormone related</u>								
Response to abscisic acid (GO:0009737)	17 (↑3)	9 (↑3)	4 (↑0)	10 (↑1)	2 (↑0)	3 (↑1)	45 (↑5)	36 (↑14)
Response to ethylene (GO:0009723)	3 (↑1)	-	-	2 (↑0)	-	1 (↑0)	13 (↑1)	6 (↑0)
Response to auxin (GO:0009733)	109 (↑5)	7 (↑2)	-	24 (↑8)	1 (↑0)	28 (↑1)	123 (↑14)	75 (↑9)
Response to gibberellin (GO:0009739)	2 (↑0)	1 (↑0)	-	-	-	1 (↑0)	4 (↑1)	1 (↑0)

The intra-stage comparisons of the rehydration points showed many SDEGs with terms associated with stress response (particularly to oxidative stress), autophagy, autophagosome assembly, protein ubiquitination and response to the phytohormones ABA and auxin (Table 2). At 24 hours rehydration, Tip tissues showed a decreased abundance of almost all auxin and ABA responsive SDEGs present, when compared to Base tissues at the same stage (Supplementary Data Sheet 1). Only *CYP707A4* homologues and an *AUXIN RESPONSIVE GH3 family (GH3)* gene showed increased abundances. *GH3* family enzymes have been shown to have a wide variety of functions, but generally are involved in hormone homeostasis by catalysing the conjugation of the phytohormones auxin, jasmonic acid (JA) and salicylic acid (SA) to amino acids (Staswick *et al.*, 2002, 2004, 2005). Conjugating phytohormones like auxin to amino acids decreases the free auxin pool and prevents interaction with auxin specific transcription factors (Tam *et al.*, 2000; Staswick *et al.*, 2002). SDEGs for *PAP22*, *17* and a *CHLOROPHYLLASE* gene additionally showed an increased abundance in Tip tissues relative to Base tissues at this stage (Supplementary Data Sheet 1). Intra-stage comparisons of 48 hours and 72 hours showed that during these stages Tip tissues had a consistently high (30-fold) accumulation of *HOS15* SDEGs, further supporting this gene as a potential regulator of the desiccation-associated tissue differentiation phenomenon. Additionally, both rehydration stages showed accumulation of SDEGs for *GH3* family, *CYP707A4*, *SAUR* family and various *ELIP-like* genes, including *DSP22* (Supplementary Data Sheet 1). The 48 hours rehydration comparison included additional accumulation of SDEGs for *CW-INV*, while 72 hours included accumulation of SDEGs for *FLAVANONE 3-HYDROXYLASE (F3H)* (Supplementary Data Sheet 1). *F3H* catalyses the biosynthesis of anthocyanin intermediates (Britsch & Grisebach, 1986). The accumulation of *F3H*, *ELIP-like* genes and genes from the *SAUR* family, during rehydration, suggests that Tip tissues maintain plasticized cell walls, likely to prevent mechanical damage as Base tissues rehydrate and expand, and prevent unwanted ROS production by protecting their photosystems with anthocyanins and *ELIP-like* proteins. Notably, mobilization of sugar monomers by Tip tissues, and an increased import of sugars by Base tissues would have altered the relative hygroscopicity of the tissues and thus their water relations. This may suggest that the efflux of water demonstrated in Tip tissues in Chapter 2, is due to change in the tissues' relative sugar status. However, metabolomics will be needed to confirm this.

These findings suggest that Tip tissues engage in more autophagic and catabolic processes during rehydration and possibly experience more oxidative stress (Table 1 & 2), relative to Base tissues. These process and other hormone signalled processes may be regulated through ABA catabolism, the conjugation of *inter alia* auxins to amino acids and epigenetic silencing of stress response genes by *HOS15*. This is largely in agreement with the findings from Radermacher *et al.* (2019, 2021) and suggests that although the *modus operandi* may vary between these two species, the result is largely similar – Tip tissues undergo nutrient mobilization, and therefore a senescence-like process during rehydration, while Base tissue recover metabolic competence and potentially engage in a source-sink interaction with Tip tissues.

Expression clustering

A hierarchical clustering dendrogram 'cut-off' close to the root was selected for expression cluster representation. This was to allow better discussion of the broader trends in the data and thus better comparison between tissues of interest. Six expression clusters were generated with no unique gene identifier occupying more than one cluster. Gene z-scores were calculated using all SDEGs (Figure 2). Zero thus represents the baseline expression and all y-axis ticks are in standard deviations of this mean expression (Figure 3). Clusters for the Tip tissue contained no information for the air-dry stage and 12 hours rehydration. These points were thus omitted instead of interpolated, as much expression fluctuation was seen in the related Base clusters.

Despite no unique gene identifier occupying more than one cluster there was a high number of duplicate annotations in the expression clusters (Table 3 & 4), this along with the high predicted gene number further supported polyploidy in this species. Interestingly, VanBuren *et al.* (2022) reported more than 220 000 high confidence genes in their final annotation of the *C. plantagineum* genome. They determined that *C. plantagineum* is an octoploid and has 21 713 syntenic gene groups with the closely related diploids *Lindernia brevidens* and *L. subracemosa*. Given that the *C. pumilum* reference transcriptome used here (Faigon *et al.*, original data, unpublished) is likely incomplete, the current gene estimate, of 81 521, suggests that *C. pumilum* has approximately four compliments of the aforementioned syntenic groups, roughly half the gene number of *C. plantagineum* and therefore is likely a tetraploid. This apparent genetic redundancy introduced another obstacle in the interpretation of the various processes at work during dehydration and rehydration, and associated tissue differentiation. Summarizing the potential function of individual expression clusters, based on some key processes, greatly improved their interpretation. Many of the cluster trends observed agree with expression trends suggested in other resurrection plant species (Rodriguez *et al.*, 2010; Farrant *et al.*, 2015; Costa *et al.*, 2017; Gabier *et al.*, 2021; Xu *et al.*, 2021 Radermacher *et al.*, 2021). These trends were however only pronounced in the Base tissues. Base and Tip tissue cluster trends differed in most clusters with the most pronounced being seen in the first five clusters and primarily during rehydration (Figure 3).

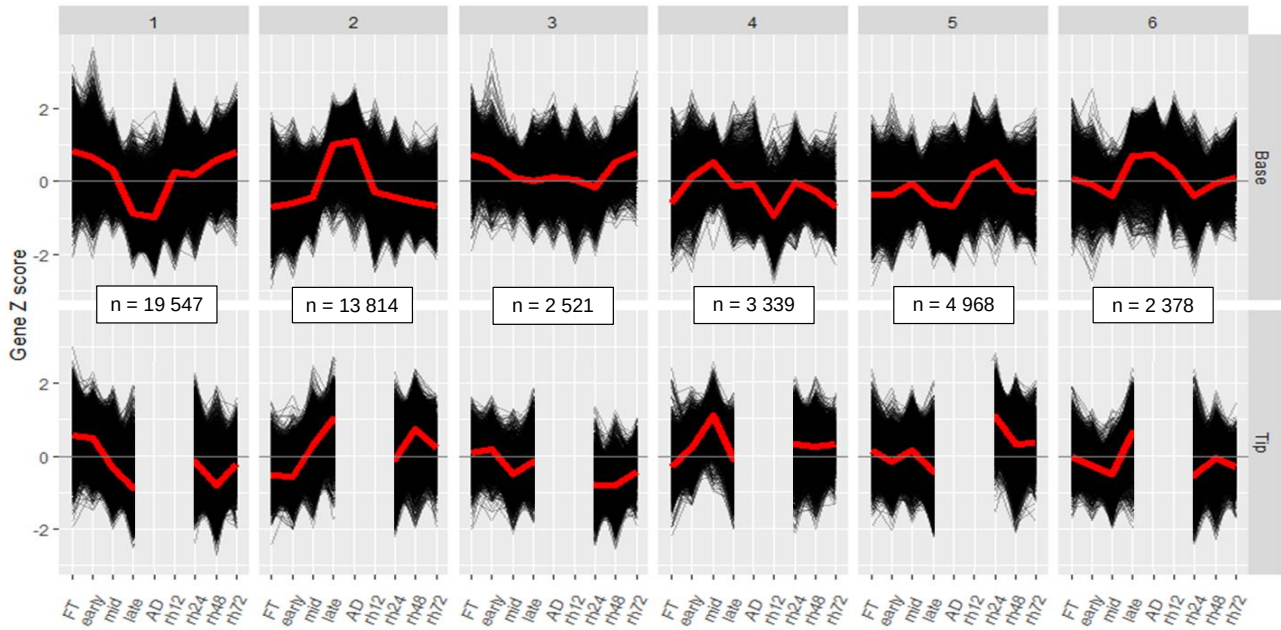


Figure 3: Gene expression clusters. Clusters were identified through hierarchical clustering of expression patterns all unique genes that were differentially expressed among all experimental groups. Genes were selected if they were significantly differentially expressed ($\text{padj} < 0.05$) between at least two experimental groups. Depiction of tissue specific expression patterns was generated after cluster assignment; clusters of the same number thus represent the same group of genes. A total of 58 000 genes were used for cluster determination, of which 46 567 were assigned to one of the depicted clusters. Genes per cluster given by ‘n’. The red line indicates the cluster mean expression. “Base” and “Tip” refer to tissue types described in Chapter 2. Abbreviations: “FT”, full turgor; “AD”, air-dry; “rh.”, hours post rehydration.

Cluster annotations were largely performed using KEGG K numbers, provided by Faigon *et al.* (original data, unpublished). These K numbers were only available for 30.52% of SDEGs, approximately 17 700 genes. Additional cluster annotation was done using gene descriptor frequencies. These were also provided by Faigon *et al.* (original data, unpublished) and were present for 81.43% of SDEGs, approximately 47 230 genes. Complete and near complete metabolic modules were determined from the KEGG database and are presented in Table 3. Additional genes and proteins of interest were retrieved from a combination of the KEGG BRITE database and gene descriptor frequencies and are presented in Table 4. The frequency of gene descriptors for each cluster are presented in Supplementary Data Sheet 2. These lists all contained descriptors for *RETROVIRUS-RELATED POLYMERASE POLYPROTEIN FROM TRANSPOSON TNT 1-94*, *SERINE/THREONINE PROTEIN KINASE*, *PUTATIVE LATE BLIGHT RESISTANCE PROTEIN HOMOLOG R1A-10* and a tag for genes with no descriptors, “---na---”. These were therefore not considered when assigning cluster functions, since they were pervasive (Supplementary Data Sheet 2). Many gene annotations/descriptors were repeated and appeared in more than one cluster. VanBuren *et al.* (2022) similarly saw genes with similar annotations, which have more divergent expression patterns, particularly if multiple homeologs were present. They suggested that the duplicate genes in *C. plantagineum* have partitioned their ancestral function and evolved new roles related to desiccation. The findings reported here are largely in agreement with this conclusion and suggest the same likely happened in *C. pumilum*.

Cluster 1 contained the highest number of complete metabolism modules of all clusters (Table 3). This included biosynthesis modules for various carbohydrates, lipids, amino acids and phytohormones, such as gibberellins and ABA (Table 3). This cluster also includes the complete metabolism modules for the crassulacean acid metabolism (CAM) photosynthesis light and dark reactions as well as the first carbon oxidation reaction of the citric acid cycle (Table 3). This cluster contained the highest number genes with tags for proteins pertaining to enzymes, kinases, peptidases & inhibitors, photosynthesis related, transcription factors, ribosomal and ribosome biogenesis (Table 4). In this regard, the large amount of biosynthesis modules and energy metabolism modules present in Cluster 1 (Figure 3, Table 3), suggested that this cluster functioned in the day-to-day growth and metabolism of the tissues investigated. Interestingly, modules for the initiation of the citric acid cycle were present, but not all genes needed to complete this cycle (Table 3). Instead, all genes for the CAM light and dark reactions were present as well as metabolism modules involving oxaloacetate. This may in part suggest that this species may be a C3-photosynthesis/CAM intermediate species (Winter, 1974), switching between these two modes as needed. However, it is important to note that the KEGG online database only considers the enzymes involved in malate metabolism (eg. *MALATE DEHYDROGENASE*) as being part of the CAM light and dark reactions. Their presence should thus also be considered as evidence of malate metabolism for processes other than photosynthesis. That being said, Xu *et al.* (2021) did suggest based on their transcriptomic study of *C. plantagineum* that that species likely switches from C3 photosynthesis to CAM photosynthesis. They however only reported this during dehydration. It is therefore interesting that the findings presented here show that CAM photosynthesis genes show an increased abundance during full turgor (Figure 3, Table 3). While this may suggest that *C. pumilum* employs CAM photosynthesis even in the hydrated state, based on findings in *C. plantagineum* (Xu *et al.*, 2021), it is more likely that the presence of CAM light and dark modules point to alternative functions of malate in plant homeostasis, such as regulation of stomata (reviewed in Finkemeier & Sweetlove, 2009; and Fernie & Martinoia, 2009).

Table 3. Notable Metabolism modules with matching cluster annotations. Modules were identified from the KEGG data base (see Module#). Key: “**” indicates complete modules represented within a cluster; “*” indicates near complete modules, with one missing gene.

Module#	Description	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
<u>Carbohydrate metabolism</u>							
<u>Central carbohydrate metabolism</u>							
M00001	Glycolysis (Embden-Meyerhof pathway), glucose => pyruvate	**	-	-	*	-	-
M00003	Gluconeogenesis, oxaloacetate => fructose-6P	**	-	-	-	-	-
M00307	Pyruvate oxidation, pyruvate => acetyl-CoA	**	**	-	-	-	-
M00010	Citrate cycle, first carbon oxidation, oxaloacetate => 2-oxoglutarate	**	**	*	**	*	-
<u>Other carbohydrate metabolism</u>							
M00854	Glycogen biosynthesis, glucose-1P => glycogen/starch	**	*	-	*	-	**
M00549	Nucleotide sugar biosynthesis, glucose => UDP-glucose	**	*	-	*	-	-
M00892	UDP-N-acetyl-D-glucosamine biosynthesis, glucose => UDP-GlcNAc	**	-	-	-	-	-
M00132	Inositol phosphate metabolism, Ins(1,3,4)P3 => phytate	-	**	-	-	-	-
<u>Energy metabolism</u>							
<u>Carbon fixation</u>							
M00165	Reductive pentose phosphate cycle (Calvin cycle)	**	-	-	-	-	-
M00168	CAM (Crassulacean acid metabolism), dark	**	*	-	**	*	*
M00169	CAM (Crassulacean acid metabolism), light	**	**	*	*	-	-
<u>Nitrogen metabolism</u>							
M00531	Assimilatory nitrate reduction, nitrate => ammonia	-	**	-	-	**	-
<u>Lipid metabolism</u>							
<u>Fatty acid metabolism</u>							
M00082	Fatty acid biosynthesis, initiation	**	-	-	-	-	-
M00415	Fatty acid elongation in endoplasmic reticulum	**	-	-	-	-	-
M00087	beta-Oxidation	**	**	-	-	-	-
<u>Lipid metabolism</u>							
M00089	Triacylglycerol biosynthesis	*	**	-	**	-	-
M00090	Phosphatidylcholine (PC) biosynthesis, choline => PC	*	*	-	-	-	-
M00092	Phosphatidylethanolamine (PE) biosynthesis, ethanolamine => PE	**	**	-	-	*	-

Table 3 continued :

<u>Amino acid metabolism</u>									
<u>Serine and threonine metabolism</u>									
M00020	Serine biosynthesis, glycerate-3P => serine	*	-	-	-	-	-	-	-
M00018	Threonine biosynthesis, aspartate => homoserine => threonine	*	-	-	-	-	-	-	-
M00555	Betaine biosynthesis, choline => betaine	**	-	-	-	-	-	-	-
<u>Cysteine and methionine metabolism</u>									
M00021	Cysteine biosynthesis, serine => cysteine	**	**	*	-	-	*	*	**
M00368	Ethylene biosynthesis, methionine => ethylene	-	**	-	-	-	-	**	-
<u>Lysine metabolism</u>									
M00527	Lysine biosynthesis, DAP aminotransferase pathway, aspartate => lysine	**	-	-	-	-	-	-	-
<u>Arginine and proline metabolism</u>									
M00844	Arginine biosynthesis, ornithine => arginine	*	-	-	-	-	-	-	-
M00015	Proline biosynthesis, glutamate => proline	*	-	-	-	-	-	-	-
<u>Aromatic amino acid metabolism</u>									
M00910	Phenylalanine biosynthesis, chorismate => arogenate => phenylalanine	**	-	-	-	-	-	**	-
M00040	Tyrosine biosynthesis, chorismate => arogenate => tyrosine	*	-	-	-	-	-	**	-
<u>Other amino acid metabolism</u>									
M00027	GABA (gamma-Aminobutyrate) shunt	*	-	-	-	-	**	-	-
M00118	Glutathione biosynthesis, glutamate => glutathione	**	-	-	-	-	-	*	-
<u>Biosynthesis of terpenoids and polyketides</u>									
<u>Plant terpenoid biosynthesis</u>									
M00097	beta-Carotene biosynthesis, GGAP => beta-carotene	*	-	-	-	-	-	-	-
M00372	Abscicic acid biosynthesis, beta-carotene => abscisic acid	*	-	-	-	-	-	-	-
M00927	Gibberellin A12 biosynthesis, GAPP => GA12	*	-	-	-	-	-	-	-
M00928	Gibberellin A4/A1 biosynthesis, GA12 => GA4 / GA53 => GA1	**	-	-	-	-	-	-	-

Table 4: Notable protein tags in gene clusters. Tags were either assigned from the KEGG BRITE database or via text mining of gene descriptors provided by Faigon *et al.* (original data, unpublished). Numbers indicate the frequency of gene annotations belonging to a group, that was represented in a cluster. Highest frequencies within a group are underlined.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
<u>Metabolism</u>						
Enzymes	<u>787</u>	591	206	279	325	207
Kinases	<u>37</u>	32	12	18	15	12
Phosphatases	23	<u>29</u>	3	8	14	12
Peptidases & inhibitors	<u>51</u>	42	16	16	23	9
Photosynthesis related	<u>38</u>	8	8	-	4	3
<u>Information processing</u>						
Transcription factors	<u>23</u>	16	4	7	8	5
Transcription machinery	26	<u>47</u>	10	8	12	8
mRNA Biogenesis	83	<u>85</u>	28	16	49	32
Spliceosome	38	<u>86</u>	15	8	20	19
Ribosome	<u>113</u>	35	16	7	79	12
Ribosome biogenesis	<u>35</u>	44	9	6	15	19
Translation factors	17	<u>27</u>	5	3	14	7
Membrane trafficking	106	<u>142</u>	29	61	67	30
Ubiquitin system	62	<u>63</u>	20	16	28	19
Proteosome	19	<u>37</u>	3	8	23	5
DNA repair & recombination	53	<u>54</u>	19	13	21	17
<u>Drought stress-related proteins</u>						
Late Embryogenesis Abundant	6	16	1	<u>24</u>	1	-
Dehydrin	1	<u>10</u>	-	5	1	-
Heat Shock	34	<u>39</u>	6	13	8	3
Early Light Inducible	1	<u>26</u>	-	3	-	-

Cluster 2 contained modules relating to carbohydrate, energy and lipid metabolism. Most notably is the presence of the biosynthesis module for phytate and ethylene, as well as the presence of an assimilatory nitrate reduction (Table 3). Cluster 2 contained the highest number of tags for proteins pertaining to phosphatases, transcription machinery, mRNA biogenesis, spliceosome, translation factors, membrane trafficking, ubiquitin system, proteosome and DNA repair & recombination. Notably, this cluster contained a high number of protein tags for *LEA* proteins and the highest number of tags for *DEHYDRIN* family, *HEAT SHOCK*

PROTEIN (HSP) family and *ELIP* family proteins (Table 4). This cluster also contained gene descriptors for an *ELIP*, *LEA* and the *ELIP*-like *DSP22* among highest frequency descriptors (Supplementary Data Sheet 2). This high number of protein tags belonging to known desiccation stress proteins (Farrant *et al.*, 2015; Oliver *et al.*, 2020), along with the pattern of expression (Figure 3), suggests that this cluster functions in the desiccation stress response. Notably, this was the only cluster to contain a descriptor for the *HOS15* gene, further highlighting its involvement in the desiccation stress response of this species (Supplementary Data Sheet 2).

Cluster 3 and onward were more difficult to elucidate the potential function of, based on protein tag frequencies and metabolism modules (Tables 3 & 4). A higher importance was therefore given to the frequency of gene descriptors. Cluster 3 contained no complete metabolism modules (Table 3) and no notable numbers of protein tags (Table 3 & 4), however many of its highest frequency descriptors were for chloroplastic proteins (Supplementary Data Sheet 2). Most notable were the descriptors for

photosystem II repair proteins, thylakoid curvature proteins, chaperone proteins and ribosome binding factors. These suggest that this cluster functioned in chloroplast maintenance and repair, at least in part.

Cluster 4 contained some modules related to photosynthesis, such the CAM light and dark reactions and the first oxidation step of the citric acid cycle, and the GABA shunt (Table 3). Cluster 4 additionally showed a high number of protein tags related to membrane trafficking and showed the highest number of *LEA* protein tags (Table 4). This cluster also contained descriptors related to stress in its highest frequency descriptors (Supplementary Data Sheet 2). This included *LEA* proteins, *ELIP*-like proteins and a *STAY-GREEN* protein. The inclusion of the complete GABA shunt module, which is associated with mitigating ROS stress (reviewed in Ansari *et al.*, 2021), in addition to the high number of protein tags for *LEA* and *ELIP*-like proteins (Thomas & Smart, 1993; Luquez & Guamét, 2002), suggested that this cluster also functioned in the water deficit stress response, similar to Cluster 2. However, the expression pattern seen in this cluster (Figure 3), suggested that this cluster functioned earlier (at a higher %RWC) in the water deficit stress response and gave way for different water deficit stress response around the mid stage of dehydration (Figure 3). This is similar to what was seen in the inter-stage comparisons where stress responses appeared to shift around the mid stage of dehydration. This qualified this as an early water deficit stress response cluster and is possibly reflective of some of the ABA signalled stress responses described during the inter-stage pair-wise comparisons.

The potential function of Cluster 5 was particularly difficult to assign. Cluster 5 was the only cluster, aside from Cluster 2, which contained modules for the biosynthesis of ethylene and assimilatory nitrate reduction (Table 3). While not the highest, Cluster 5 contained a high number of protein tags pertaining to enzymes, peptidases & inhibitors, mRNA biogenesis, ribosomes, ubiquitin system and membrane trafficking (Table 4). In addition, the highest frequency descriptors included descriptors for various ribosomal proteins, ubiquitin ligases and proteinases (Supplementary Data Sheet 2). However, the expression pattern of this cluster suggests that its transcripts largely had a decreased abundance, save for a brief window in Base tissues during the mid stage and during the entire rehydration of Tip tissues. It therefore seemed unlikely that the function of this cluster was mainly translation and protein synthesis based, as one would assume that core cellular processes, like these, would at least have a baseline expression and not be suppressed during most of the dehydration/rehydration cycle (Figure 3). However, work by Martín-Villanueva *et al.* (2021) suggests that a large portion of ubiquitin generation, in eukaryotes, occurs from specific cleavage of a ubiquitin-ribosomal subunit precursor. The presence of ribosomal genes in this cluster may thus be as ubiquitin fodder for the ubiquitin system and, by extension, protein degradation. This is supported by the many SDEGs with GO terms associated with autophagy and ubiquitin-dependent protein catabolic processes seen in the intra-stage pair-wise comparisons between Tip and Base tissues during rehydration stages (Table 2). Notably, this was the only cluster which contained descriptors for *CW-INV* (Supplementary Data Sheet 2). Thus, we suggest that a potential function of this cluster may be in the mass proteolysis of proteins, autophagy and mobilization of *inter alia* amino acids and sugars. This may also be considered the desiccation driven senescence cluster, in the context of Tip tissues (Radermacher *et al.*, 2021), and agrees with findings by Asami *et al.* (2019), in *T. liliiformis*, that autophagy and ubiquitination is upregulated during the mid stage of dehydration.

Cluster 6 contained no notable metabolism modules, besides a starch biosynthesis module (Table 3). This cluster also contained no notably high number of protein tags (Table 4). However, similar to Cluster 3, Cluster 6 had many chloroplast-related descriptors in high frequency, including chaperonins, protein import apparatus and peptide chain release factors (Supplementary Data Sheet 2). Notably, this cluster included the only descriptors for *RUBISCO ACCUMULATION FACTOR 1 (RAF1)* homologues. *RAF1* plays a key role in rubisco biosynthesis and has been proposed to play a role in rubisco stabilization (Feiz *et al.*,

2012). The chloroplast related descriptors present appeared more focussed on the import, movement and modification of proteins in the chloroplast than those seen in Cluster 3 (Supplementary Data Sheet 2). This, coupled with the increased abundance of genes in this cluster during late dehydration (Figure 3), suggested that Cluster 6 had the potential function of chloroplast desiccation stress and subsequent rehydration preparation.

Findings in the context of the dehydration/rehydration cycle

While presented as discrete expression clusters and dehydration stages (Figure 3), the overall pattern of gene expression in *C. pumilum* should be viewed in the context of the dehydration/rehydration cycle as a continuum: beginning at full turgor, sequentially progressing through the various stages of dehydration until the air-dry stage is reached, thereafter progressing through rehydration.

Base

First focussing on the Base tissues, at full turgor the majority of genes with increased abundance are those related to biosynthesis of various metabolites and photosynthesis, as well as chloroplast repair (Table 3 & 4). With the onset of water loss, the plant progresses into the early stage (Chapter 2). This stage appeared largely similar to full turgor with the exception of an increase in transcript abundance of genes in the early water deficit stress cluster (Figure 3, cluster 4). This suggested that the plant had perceived the onset of water deficit stress and had begun transcribing stress associated genes like *inter alia* LEA proteins and *ELIP-like* proteins (Table 4, Supplementary Data Sheet 1; Farrant *et al.*, 2015; Oliver *et al.*, 2020; Xu *et al.*, 2021).

Further water loss transitioned the plants into mid stage, where genes associated with maintaining homeostasis, photosynthesis and chloroplast maintenance began to show a stark decrease in abundance relative to full turgor (Figure 3). Conversely, the early water deficit stress cluster (Cluster 4) was at its highest abundance, while genes in the desiccation stress response cluster (Cluster 2) had also begun increasing in abundance (Figure 3). Until this stage, stress associated genes were likely induced through a gradual increase in ABA content and a subsequent increase of *ABI5* homologues transcription (Supplementary Data Sheet 1). Interestingly, chloroplast focussed expression clusters (Cluster 3 & 6) showed decreased abundances. This collectively suggests that water deficit stress response processes, such as ROS mitigation via the GABA shunt, and protective protein accumulation (LEA, HSP, ELIP), were active and chloroplast function was starting to be suppressed (Ansari *et al.*, 2021). Further loss of water transitioned the plants into the late stage where, interestingly, the early water deficit stress responsive cluster began to show a decrease in abundance, while the desiccation responsive cluster showed a large increase in abundance (Figure 3, Cluster 2 & 4). This translated to an increase in transcripts associated with the production of dehydrins, ELIPs and HSPs. Interestingly, this change in stress response maybe be related to a suppression of ABA related signalling and promotion of a potentially desiccation stress specific auxin response. This response included *inter alia* initiation of cell wall plasticization via *SAUR-like* homologues and activation of tightly controlled *ARF* promoted genes (Table 1, Supplementary Data Sheet 1). The appearance of metabolic modules for the biosynthesis of phytic acid and for nitrate assimilation suggests that, like in seeds nutrients, such as *inter alia* amino acids and phosphates were accumulated during the anhydrobiotic state (Ogawa *et al.*, 1979; reviewed in Sparvoli & Cominelli, 2015). Molecules such as phytic acid additionally act as defence compounds against herbivores (reviewed in Kumar *et al.*, 2021), interactions often overlooked in resurrection plant research, but likely still of importance from the perception of the organism. Despite apparent suppression of chloroplast function in the mid stage, in the late stage, genes associated with chloroplast function increased in abundance (Figure 3, Cluster 6). This suggests that some priming of the chloroplasts for quiescence and

preparation for rehydration likely occurred in this stage (Charuvi *et al.*, 2015). Finally, it was during this stage where a large amount of SDEGs associated with autophagy and protein ubiquitination were expressed, likely as a pro-survival strategy to remove potentially toxic cellular compounds, recycle damaged chloroplasts and thus prevent apoptosis due to toxin and ROS build-up during anhydrobiosis (Williams *et al.*, 2015; Charuvi *et al.*, 2019; Radermacher *et al.*, 2021; Xu *et al.*, 2021). The transcript abundances present at the late stage appeared to continue through to the air-dry stage (Figure 3). This supports the notion the even at very low cellular water contents, the cellular environment is such that some processes can continue, to some extent (Radermacher *et al.*, 2021; du Toit *et al.*, 2021).

Upon rehydration, stress associated transcripts rapidly decreased in abundance, while biosynthesis, photosynthesis, auxin mediated cell wall repair and chloroplast repair genes began to increase in abundance (Tables 1 & 3). Additionally, during the first 12 hours of rehydration ABA related signalling was increased, likely to promote continued mitigation of ROS as hydration increases (Supplementary Data Sheet 1). Also, during the first 24 hours of the rehydration response a decrease in abundance of genes associated with chloroplast protein import, movement and transport was observed, suggesting a possible priority on chloroplast repair versus other functions of the chloroplast (Figure 3). Notably, transcripts from the *ELIP-like* protein contained in Cluster 2 showed a decrease in abundance during rehydration, suggesting a more protective, rather than repair oriented, role for *ELIPS* in *C. pumilum*. Additionally, during this brief rehydration window, an increase of transcript abundance is seen in genes associated with the polyubiquitin system, suggesting rapid protein turn-over (Table 1; Figure 3, Cluster 5). This was likely a very important step in the repair process of this species, removal of proteins damaged during desiccation (reviewed in Del Rio, 2015). From 48 hours post rehydration onwards, very little SDEGs were present, and most expression clusters showed a slow return to expression abundances seen at full turgor (Table 1; Figure 1 & 3). We therefore propose that, while arguably some metabolic competence is reached in this species by 24 hours post rehydration (Chapter 2), up to 72 hours was needed for this tissue to once again be nearly indistinguishable from full turgor tissues and thus be completely recovered.

Tip

Tip tissues responded nearly indistinguishably from Base tissues to early water deficit stress, showing no large transcriptional differences during the full turgor and early stages. Upon entrance to the mid stage a noticeable differential expression of oxidative stress response genes and ubiquitination associated genes become apparent (Table 2; Figure 3). This suggests a different cellular redox potential and general protein ubiquitination status to Base tissues at this stage. During the late stages, however the Tip tissues once again became near indistinguishable from Base tissues transcriptionally. Provided that, as Radermacher *et al.* (2021) suggested in *X. schlechteri*, both Tip and Base tissues transcribed all necessary components to mount an effective DT response, the difference in expression should point to the difference in behaviour following this response, further suggesting that the mid stage of dehydration is where commitment to a desiccation stress response occurred in this species, and the concomitant desiccation associated tissue differentiation was initiated (Chapter 2; Zhang & Bartels, 2018; Radermacher *et al.*, 2019, 2021). Notably, during the mid stage, Tip tissues showed a high accumulation of the chromatin remodelling *HOS15* gene. Translation of *HOS15* would suppress further expression of stress related genes, regulating this would thus allow adequate preparation for anhydrobiosis of this tissue while altering the expression profile of genes for, and following, rehydration. During rehydration, as was suggested in Chapter 2, differentiation of the Tip tissues becomes apparent. During the first 24 hours, little sets Tip and Base tissues apart, however, from 48 hours onwards Tip tissues show large amounts of SDEGs associated with autophagy, nutrient recycling, protein ubiquitination and suppression

of phytohormone responses (Table 2, Figure 3, Supplementary Data Sheet 1). This suggests this tissue engages in senescence like processes and becomes a source tissue following rehydration.

Conclusion

The current study attempted to further motivate the presence of the desiccation associated tissue differentiation in the homoiochlorophyllous dicot resurrection plant *Craterostigma pumilum* Hochst, using a transcriptomic approach.

The data presented here provide good support to the findings presented in Chapter 2. That is: this species follows a multi-stage response to continued water loss in both tissues investigated; the tissues investigated initiated a differential response during the desiccation commitment (mid) stage of dehydration; tissue differentiation is realised approximately 48 hours post rehydration and follows a senescence-like process, involving nutrient mobilization and ubiquitin-dependant catabolic processes. The data presented here further suggest that Tip tissues experience a more oxidative cellular environment during anhydrobiosis, leading to the formation of *inter alia* RNA sequestering heat shock granules, and that the desiccation associated tissue differentiation and subsequent senescence, may be regulated by *HOS15* related chromatin remodelling and negative regulation of stress associated genes. Additionally, these findings suggest that auxin may be an overlooked phytohormone in the desiccation response of *Craterostigma* species, if not resurrection plants in general, and that the duplicate genes in *C. pumilum* may have partitioned their ancestral function and evolved new roles related to desiccation, as was suggested for *C. plantagineum* (VanBuren *et al.*, 2022).

Importantly, this form of desiccation-associated senescence appeared phenotypically different from the stress induced senescence reported in Chapter 2, motivating it as a potentially desiccation-dependant senescence-like process, similar to what is described in *X. schlechteri* (Radermacher *et al.*, 2019, 2021). In fact, considering the vast evolutionary distance between *X. schlechteri* and *C. pumilum*, this phenomenon seems remarkably similar in terms of the realised phenotype and the types and timing of potential processes involved (Radermacher *et al.* 2019, 2021; Chapter 2). The current study thus provides good evidence to suggest that this phenomenon is possibly an additional convergent evolutionary trait shared by angiosperm resurrection plants, although more work, and in a greater variety of species, is necessary to confidently show this.

Finally, the realisation of this phenomenon and the apparent ongoing transcription in Tip tissues, despite a very low %RWC (Chapter 2), provides strong evidence that the cellular environment must still be such that some transcription, translation and enzymatic processes are possible. Supporting claims by du Toit *et al.* (2021) that resurrection plants may allow for ongoing complex cellular processes, in severely limited water conditions, through some acquired property of their metabolite rich cellular matrix.

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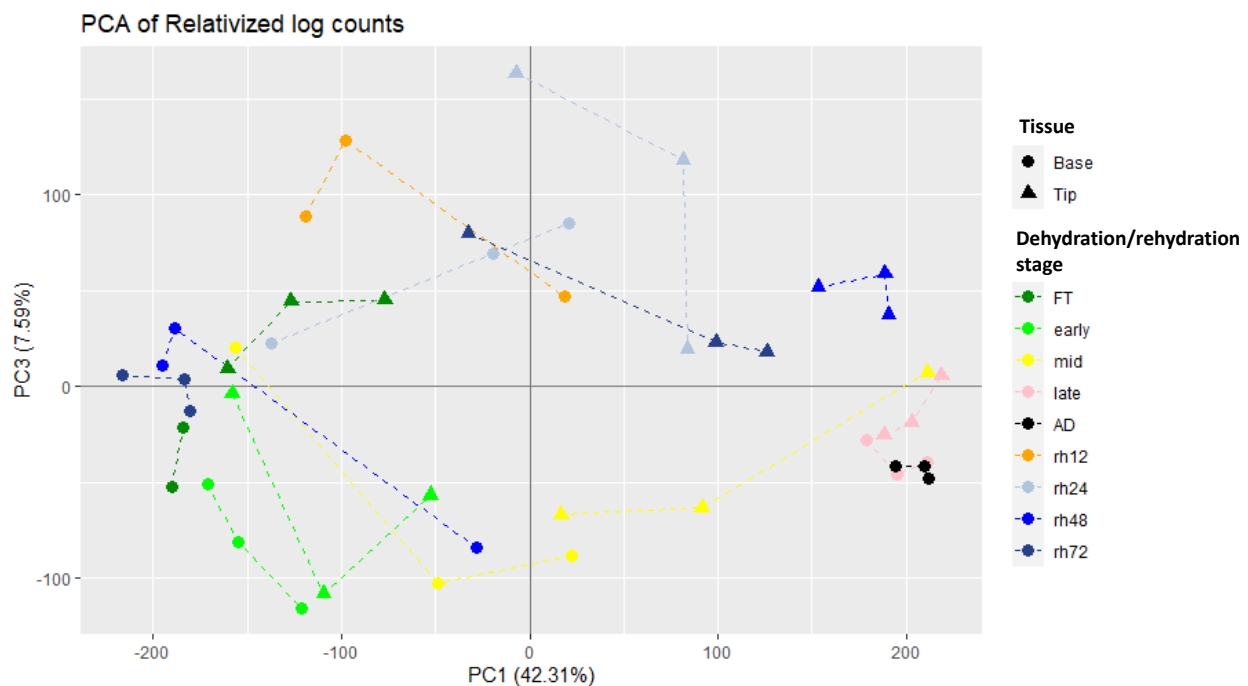
Supplementary Material

Supplementary Data Sheet 1: All pair-wise comparisons depicted in Figure 2. Gene descriptors as well as fold change, significance values and GO annotation are included. Abbreviations: “FT”, full turgor; “E”, early; “M”, mid; “L”, late; “AD”, air-dry; “rh”, hours post rehydration.

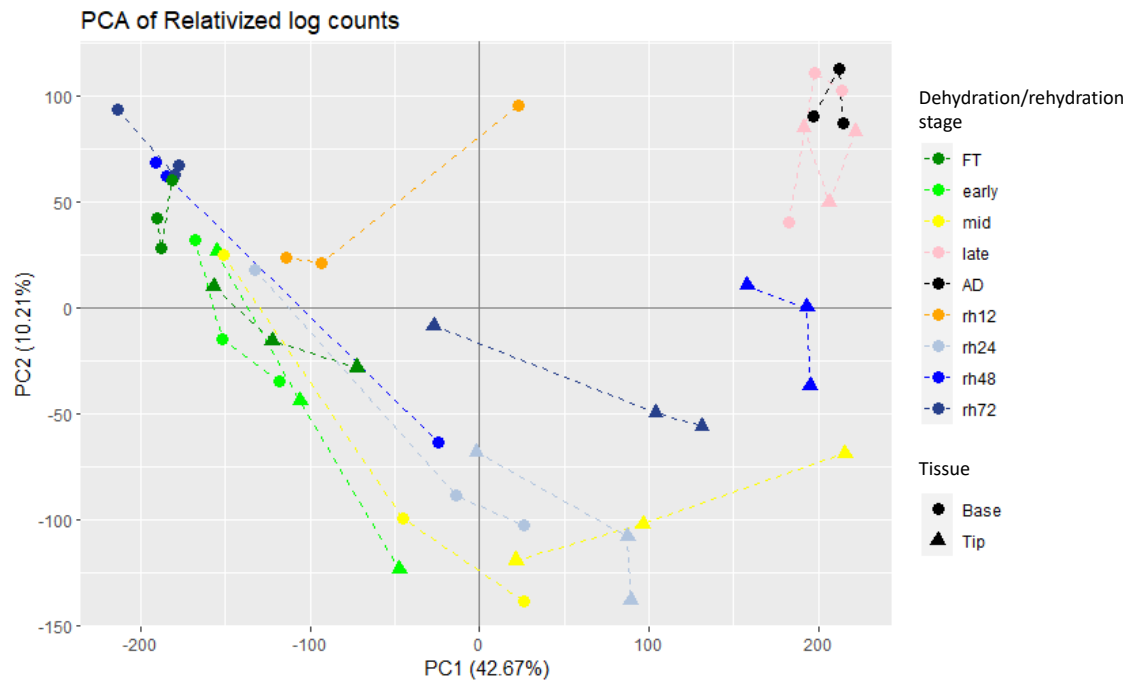
Link: [SDEGs_all_pairwise_comparisons.xlsx](#)

Supplementary Data Sheet 2: Gene descriptors were text mined and the frequency with which genes were annotated with identical descriptors determined by cluster.

Link: [Cluster_Gene_Descriptor_Frequencies.xlsx](#)



Supplementary Figure 1: Principle component analysis (PCA) showing inter-sample relations based on normalized (rlog) read counts. “Base” and “Tip” refer to tissue types described in Chapter 2. Abbreviations: “FT”, full turgor; “AD”, air-dry; “rh.”, hours post rehydration.



Supplementary Figure 2: Principle component analysis (PCA) showing inter-sample relations based on normalized (rlog) read counts. “Base” and “Tip” refer to tissue types described in Chapter 2. Abbreviations: “FT”, full turgor; “AD”, air-dry; “rh.”, hours post rehydration.

CHAPTER 4:
**Model of desiccation associated tissue
differentiation**

Introduction

The purpose of a model is to increase understanding of a complex phenomenon through simplification and approximation, while remaining true to experimental data. Here, we will attempt to condense the body of work on desiccation associated tissue differentiation in *Craterostigma pumilum* leaf tissues, into an explanatory model and hypothesize potential selective pressures which may have benefitted from evolving this sort of phenomenon. Where possible, similarities will be drawn from work by Radermacher *et al.* (2019, 2021) on *Xerophyta schlechteri* to create as unified a model as possible that has potential to be extended to other resurrection plants.

Although the current literature pertaining desiccation associated tissue differentiation in resurrection plants is limited to less than a handful of works (including those presented in this thesis) and spanning only two species with vastly different life histories, there exists a commonality in the tissues investigated (Radermacher *et al.*, 2019, 2021; du Toit *et al.*, 2021 (Chapter 2)). That is: tissues which exist on the same leaf seemingly show differential responses to anhydrobiosis and subsequent rehydration. Most importantly, this differentiation in response between tissues on the same leaf seems to be dependent on the location of tissue relative to the leaf's origin, ie. its connection to the host and thus site of the initial meristem. The closer the tissue is to the origin, the more likely it seems to be to recover upon rehydration. In the case of *X. schlechteri*, a sedge-like monocot, it can be argued that with this distance from the origin comes an increase in age and thus this process may be an age-related phenomenon. However, in *C. pumilum* relative age of tissues on a leaf is not so clear cut and thus begs for a modified explanation.

The unexpectedly high differential expression of auxin-related signalling and response genes, seen in the proposed desiccation-commitment stage (mid stage dehydration; Chapter 3), has led to the hypothesis that this hormone may play a pivotal role in determining the relative fate of *C. pumilum* leaf tissues during desiccation and rehydration. This hypothesis will thus play an integral role in the model to follow.

Background

What shapes a leaf?

In both monocots and eudicots, leaf growth is initiated from the shoot apical meristem (SAM). The emergence of the leaf primordium, its phyllotaxis and its direction of growth is initiated and determined by the formation of discrete auxin maxima (Reinhardt *et al.*, 2000, 2003; Benkova *et al.*, 2003). These auxin maxima are generated by a combination of *de novo* biosynthesis and directional transport of auxin (Benkova *et al.*, 2003, Cheng *et al.*, 2007). Following outgrowth of the leaf primordium, cells in the new leaf undergo multiple rounds of division and differentiate to establish the three main leaf axes i.e., adaxial-abaxial, medio-lateral, and proximal-distal (reviewed in Byrne, 2012; and Xiong & Jiao, 2019). In eudicots, during this phase of rapid cell division and proliferation, the basic shape and size of the leaf is determined, as well as any

complex leaf architecture, such as leaflets (Kaplan & Cooke, 1997; Floyd & Bowman, 2010; Schwartz *et al.*, 2016). The formation of complex architecture is additionally initiated by discrete auxin maxima and zones of auxin response inhibition, which form along the periphery of the leaf primordium (Shwartz *et al.*, 2016).

Following the division phase, leaf cells transition into a phase of expansion and differentiation, and division is limited to meristemoid cells – the cells which will form stomatal guard cells and vasculature (Nadeau & Sack, 2002; Fisher & Turner, 2007; Peterson *et al.*, 2010; Gonzalez *et al.*, 2012). This transition begins at the apex of the leaf and migrates basipetally as a front – the cell cycle arrest front (Muller *et al.*, 2001; Gonzalez *et al.*, 2012; Nelissen *et al.*, 2012, 2016). In eudicot leaves, these fronts progress rapidly, and cell proliferation largely ceases days after the transition front is established at the leaf apex (Andriankaja *et al.*, 2012). In a similar fashion, after a period of expansion, cells sequentially exit the expansion phase in the basipetal direction and enter a state of maturity where no further growth happens (Gonzalez *et al.*, 2012). The coordination of growth processes is thus considered to be temporal and as such, at a given point in time, are linearly organized in the eudicot leaf (Andriankaja *et al.*, 2012, Nelissen *et al.*, 2016).

In monocot leaves, a zone of active proliferation continues to exist at the leaf origin, and is bordered acropetally by a transition front, an expansion zone and mature cells, respectively (Fournier *et al.*, 2005; Nelissen *et al.*, 2012). The position of these fronts is tightly correlated to concentrations of the phytohormones auxin and gibberellin, with auxin being highest at the leaf origin and gibberellin being highest at the transition front to the expansion zone (Nelissen *et al.*, 2012). Both these hormones decrease in concentration gradually with distance from the leaf origin, suggesting spatial and temporal control of leaf development and growth in monocots (Gonzalez *et al.*, 2012; Nelissen *et al.*, 2012, 2016).

While many similarities exist between the early development of eudicot and monocot leaves, distance from the leaf origin is an arguably worse estimate of tissue age in the eudicots. Given the leaves of eudicots develop nearly completely prior to exiting the cell division phase, it can be argued that the cells of the eudicot leaf are all of similar age. What then distinguishes leaf cells at the apex from those closer to the leaf origin is the state of expansion, a process inherently dependent on auxin (Gonzalez *et al.*, 2012; Majda & Robert, 2018).

How do leaves age?

To the best of our knowledge, research interest in leaf age has largely been limited to leaf initiation and eventual senescence – the beginning and end of the eudicot leaf life cycle. Very few studies have investigated the period of maturity which exists between these two extremes, with respect to age or change over time. One of the most complete works on this topic, by Avery (1935), focused on how distribution of auxin in the leaves of two species of *Nicotiana* (eudicots) change with relative distance from the inflorescence, on the stem – a proxy for temporal age.

They produced numerous, well supported, findings and their work was arguably well ahead of its time. They found that, *inter alia*: auxin is present throughout the leaves of *Nicotiana*; the net concentration of auxin, in a leaf, is inversely proportional to the distance of the leaf from the inflorescence, with leaves closer to the inflorescence (younger) having higher concentrations; auxin tends to disappear as leaves mature; a distinct auxin concentration gradient exists within leaves, with concentrations being lowest at the leaf apex and becoming successively higher basipetally; auxin is produced nearly equally throughout the leaf lamina, but is accumulated differentially resulting in the aforementioned gradient; accumulation of auxin and its transport is conducted by leaf vasculature and is polar; and the disappearance of auxin, as leaves mature, begins from the apex and gradually progresses basipetally, as a front (Avery, 1935). The “shape” of the auxin gradient within mature eudicot leaves is thus dependent on the temporal age of the leaves. Presumably the same logic can be applied to monocot leaves, with auxin concentrations in the mature zone progressively decreasing with increasing distance from the leaf origin. To our knowledge this has as yet not been investigated.

With the advent of modern molecular biology techniques, many of the findings of Avery (1935) have received promising support. Notably, work on the *PIN FORMED (PIN)* family of auxin transporters have provided a means by which auxin transport achieves polarity and differentially accumulates in tissues (Friml *et al.*, 2002, 2003; reviewed in Křeček *et al.*, 2009). PIN proteins are membrane bound transporters, with most variants being found in the plasma membrane. They are restricted to specific geometric faces of the cell and therefore allow for directional fluxes of auxin and asymmetric auxin distribution within tissues (Tanaka *et al.*, 2006; Vieten *et al.*, 2007; Petrasek & Friml, 2009). In general, PIN proteins function in the efflux of auxin from cells, however their expression regulation and localization differ among the various groups of these proteins (Křeček *et al.*, 2009). Wiśniewska *et al.* (2006) even demonstrated that manipulation of the coding sequence of PIN proteins was sufficient to change not only their localization in the cell membrane but also concomitant cell auxin polarity. Activity of PIN proteins can additionally be regulated through transcription regulation, degradation, trafficking and transport of the proteins (Tanaka *et al.*, 2006; Vieten *et al.*, 2007; Petrasek & Friml, 2009). Trafficking and recruitment of PIN proteins is related to the phosphorylation status of the protein (Michniewicz *et al.*, 2007). Phosphorylation by the protein kinase, *PINOID* regulates the localization of the protein (Friml *et al.*, 2004) while phosphorylation by the *D6PK* kinase is thought to regulate the activity (Zourelidou *et al.*, 2009). Regulation of PIN proteins thus allows auxin distribution to be altered by various endogenous and exogenous signals, creating the auxin maxima and gradients which underlie most plant developmental processes (Tanaka *et al.*, 2006). Notably, auxin itself has been shown to increase the number and activity of PIN proteins at the plasma membrane (Paciorek *et al.*, 2005). Auxin accumulation itself is thus mediated, in part by, by auxin concentration.

Given that an indicator of maturity in leaf tissues is the cessation of cell expansion, then presumably some feedback mechanism must exist within, or in proximity of, cell walls which signals that a maximum surface area, and thus cell size, has been reached. Without such

feedback the cell wall area to internal cell volume (and by extension pressure) ratio may cease to be favorable for support – plant cells cannot expand indefinitely. Pilling & Höfte (2003) reviewed many potential players in cell wall feedback signalling, however none of these players provide strong evidence for cell wall size feedback. Presumably this form of feedback would need to respond to increasing sheer forces within the cell wall, either from increasing negative pressure from the central vacuole, or positive pressure from other cells in close proximity, and signal for a cessation of expansion.

Provided such a feedback mechanism exists, it would need to regulate auxin response genes downstream, since auxin plays an integral role in promoting cell expansion (Majda & Robert, 2018). Given the findings of Avery (1935), this signal would also need to promote efflux of auxin in the relevant tissues and/or prevent the accumulation of auxin. This may suggest that, since mature areas of leaves have a specific longevity as the mature zone progresses basipetally, zone specific chromatin remodeling occurs within leaf tissues and thus provides a transcriptional tissue identity. To our knowledge, chromatin remodeling as a byproduct of the natural auxin gradients which exists within leaves, and by extension transcriptional tissue identity within zones of maturing leaves, has not yet been investigated. That being said, a good body of work does exist on the differential epigenetic regulation, of *inter alia* auxin biosynthesis and transport genes, within different organs (Lafos *et al.*, 2011; reviewed in Mateo-Bonmati *et al.*, 2019; Casanova-Sáez *et al.*, 2021) and how this may change with change in totipotency and identity (Lafos *et al.*, 2011; He *et al.*, 2012; Chen *et al.*, 2016; Mandel *et al.*, 2022).

Briefly, culturing of differentiated plant tissues (e.g., leaf cuttings), on auxin (or auxin mimicking molecules e.g., 2,4-D) rich media is often sufficient to induce callus formation and subsequent acquisition of totipotency (Duclercq *et al.*, 2011). This is thought to be, in part, as a result of abolishing endogenous auxin gradients and has been shown to result in demethylation of H3K27 (Duclercq *et al.*, 2011; He *et al.*, 2012). Demethylation of H3K27 derepresses auxin response pathways in *Arabidopsis* and enables the leaf-to-callus transition (He *et al.*, 2012; Ikeuchi *et al.*, 2015; Mandel *et al.*, 2022). Inversely, the differentiation of tissues from callus is associated with the acquisition of tissue specific trimethylation of H3K27, which is negatively correlated with tissue specific expression patterns (Lafos *et al.*, 2011; He *et al.*, 2012; Mandel *et al.*, 2022). Mutants deficient in their ability to methylate H3K27 lose the ability to successfully regenerate from callus and thus gain tissue specific transcriptional identity (Mandel *et al.*, 2022). H3K27me3 targets have been demonstrated to be enriched for transcription factors, auxin responsive genes and microRNA genes (Lafos *et al.*, 2011; Mandel *et al.*, 2022). Tissue identity is thus assigned, in part, through H3K27me3 direct suppression of *inter alia* transcription factors and indirect promotion of other transcription factors, through suppression of the microRNA genes which bind then (Lafos *et al.*, 2011). Apart from H3K27me3, other chromatin remodeling mechanisms have also been proposed to play a role in specific tissue development, e.g., H3K4me3 and DNA loops which are derepressed by sufficient auxin concentrations (Ikeuchi *et al.*, 2015; Engelhorn *et al.* 2017; Yamaguchi *et al.*, 2018; reviewed in Mateo-Bonmati *et al.*, 2019; Mandel *et al.*, 2022).

Applying the above knowledge, with temporal age more of the cells within a leaf reach their maximum size and cease expanding. This transition moves as a front, beginning at the apex and gradually progressing basipetally. The initiation of this transition to maturity, is very likely signaled through some marker of maturity, such as cell walls reaching their maximum size. This signal must regulate auxin response genes downstream, to promote efflux of auxin from the affected tissues and prevent further expansion (Avery, 1935). This suggests consistent differential expression of at least auxin transport proteins, like PIN proteins, between leaf cells in the mature zone vs the expansion zone. Mature zones and expansion zones must thus have different transcriptional identities, albeit these differences may be restricted to specific groups of genes. These tissue specific transcriptional identities would additionally need to remain consistent for the duration of a leaf's life cycle. This suggests that the change in transcriptional identity is likely preceded by some sort of chromatin remodeling, such as H3K27me3. This chromatin remodeling would need to be initiated by the initial signal of maturity and may be further reinforced through the accompanied change in internal auxin gradients (Avery, 1935; Mateo-Bonmati *et al.*, 2019).

Model

C. pumilum specific

Applying the knowledge gained through physiological and transcriptomic studies (Chapters 2 & 3), the following model was constructed of the *C. pumilum* leaf life cycle and how it may affect the desiccation associated tissue differentiation phenomenon described.

Leaves of *C. pumilum* grow in pairs of roughly equal age. Pairs grow approximately perpendicularly to preceding pairs (decussate phyllotaxis). As can be seen in Chapter 2, leaf pairs further from the SAM (site of new leaf growth/whorl 1) tend to have successively increased surface area, at full turgor. This provided a good indicator that leaves continue to have active expansion zones as successive leaf pairs are initiated. Given that all plants presented in Chapter 2 were approximately 6 months old, this would suggest that leaves have active expansion zones for at least a number of weeks. It was additionally observed in Chapter 2 that plants rarely had more than six leaf whorls and that leaves of whorl 7 normally succumbed to age related senescence, under well-watered conditions, and whole leaves of whorl 5 and older undergo stress-induced senescence during the early stage of dehydration (80 %> to 60 % RWC).

Following the logic of Avery (1935), the higher the whorl number of a leaf pair, and the further that whorl is from the SAM, the lower its net auxin concentration should be. Additionally, the higher the whorl number of a leaf pair, the higher the proportion of its tissues which should exist in the mature zone and thus no longer be expanding. Leaves of whorl 7, which succumbed to age related senescence under well-watered conditions, would thus have the highest proportion, if not all, of its tissues existing in the mature zone and its age-related senescence

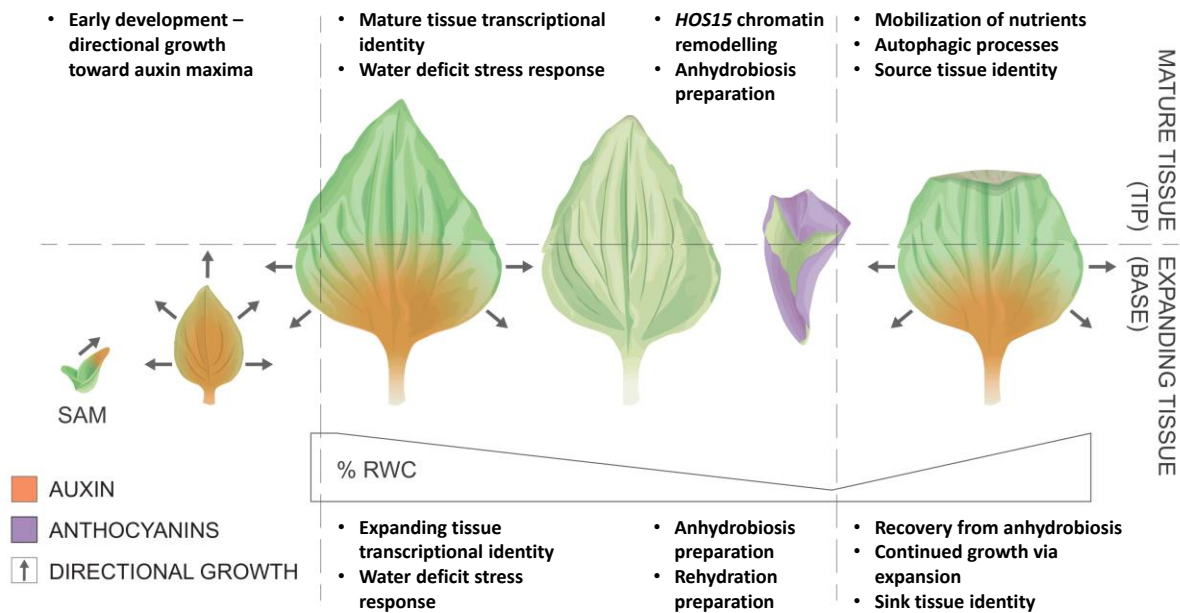


Figure 1: Model of desiccation associated tissue differentiation progression in *C. pumilum* leaves. Abbreviations: SAM, shoot apical meristem; % RWC, relative water content.

induced by a low auxin concentration (Addicott *et al.*, 1955; Kim *et al.*, 2011). Leaves of whorl 5 and older, which succumb to stress-induced senescence, must thus also have the majority of their tissues exist in the mature zone and the inability to activate desiccation tolerance machinery related to a maturity marker. As proposed above, these markers are likely related to chromatin remodelling, either directly or through interactions with auxin related signalling and stress hormones like abscisic acid (ABA) (Skubacz *et al.*, 2016; Chapter 3). Leaves with successively lower whorl number should thus have greater proportions of their leaf tissues existing in the expansion zone and have conforming auxin gradient shapes. That is to say, younger leaves have a greater proportion of their tissues consisting of actively expanding cells and accumulating auxin through polar transport, possibly from maturing acropetal cells of the same leaf.

Entertaining this, the Tip and Base tissues investigated (Chapter 2 & 3) thus represent leaf tissues in the mature and expansion zones, respectively (Figure 1). Tip tissues must therefore have a lower innate concentration of auxin relative to Base tissues at full turgor. These tissues may thus have tissue specific transcriptional patterns, in the absence of stress, and consequently have tissue specific chromatin remodelling (Figure 1). This is supported by the finding in Chapter 3 that, at full turgor, Tip and Base tissues showed significant ($p_{adj} < 0.05$) differential expression of nearly 1200 genes.

With the onset of water deficit stress (early stage dehydration), only 174 genes were significantly differentially expressed between Tip and Base tissues and both tissues had an increased expression of ABA responsive genes (Chapter 3). However, with entrance to the mid

stage of dehydration more than 4000 genes were significantly differentially expressed between Tip and Base tissues and in both tissues repression of ABA responsive genes was present as well as promotion of specific auxin responsive genes (Chapter 3). Putting these findings in context of the model, the genes most affected by the differential chromatin state between the two tissues were likely enriched from auxin response elements. Thus, as ABA related stress responses increased, both tissues were able to respond sufficiently and similarly. That is to say, an increase in ABA responses during the early stage suppresses many auxin responsive pathways (Skubacz *et al.*, 2016) and thus the discrepancy between the Tip and Base tissues is comparatively minute during the early stage of dehydration (Chapter 3). In the transition from early to mid stage, both Tip and Base tissues showed suppression of ABA responses and promotion of auxin responses to *inter alia* promote cell wall plasticization (Chapter 3). Importantly, during this transition Tip tissues showed a significantly high increase in expression of the *HOS15* histone deacetylase, which has been associated with suppression of cold stress and ABA signalled stress responses, via chromatin condensation (Zhu *et al.*, 2008; Ali & Yun, 2020).

Within the scope of the model, Tip specific promotion of *HOS15*, under relatively high water-deficit stress, can come about in a few ways. The chromatin remodelling associated with the transition to cell maturity may directly affect expression of *HOS15*, under water deficit stress, by suppressing expression of a *HOS15* suppressor, thus allowing expression of *HOS15* during transcriptional commitment to anhydrobiosis, in the mid stage. Alternatively, the promotion of the efflux of auxin from Tip tissues, as a result of tissue specific chromatin remodelling, may result in the auxin concentration within Tip tissues to be insufficient to promote the transcription of a *HOS15* suppressor, through IAA transcription regulator interaction. Nevertheless, increase in *HOS15* expression coincided with increased expression of auxin responsive genes and was associated with a mature tissue, strongly suggesting that its expression is associated with the previously discussed leaf auxin gradient (Avery, 1935; Chapter 3).

Increased expression of *HOS15* subsequently deacetylates and suppresses a number of genes associated with the *C. pumilum* dehydration-rehydration cycle, resulting in the desiccation associated tissue differentiation phenotype during rehydration (Figure 1). Given that Tip tissues were suggested to form heat shock granules during anhydrobiosis, likely as a result of a more oxidative cellular environment (Cande *et al.*, 2004; Chapter 3), and that the number of significantly differentially expressed genes between tissues in the late stage is low, we propose that *HOS15* targets do not include genes necessary for successful quiescence. We instead propose that *HOS15* targets include genes necessary for the preparation for rehydration, and subsequent repair and recovery of metabolic competence. Thus, in Tip tissues, where *HOS15* was expressed, these genes were suppressed, and Tip tissues follow a different rehydration path. Similar to what was suggested in *X. schlechteri*, these suppressed genes may include suppressors of senescence and autophagy pathways (Radermacher *et al.*, 2021). This hypothesis is supported by the continued high expression of *HOS15* in Tip tissues during at least

the first 72 hours post rehydration, expression of genes associated with nutrient mobilization in Tip tissues, expression of source tissue associated genes in Tip tissues and failure of Tip tissues to recover photosynthetically (Chapters 2 & 3). Around 48 hours post rehydration the Tip tissues were seemingly established as source tissues (Figure 1), as was evidence by the rapid decline and stabilization of its relative water content (%RWC) – an indicator that the hygroscopicity of the tissue has changed, likely due to movement of sugars (Chapter 3).

Generalization

Assuming the desiccation-associated tissue-differentiation phenomenon can be demonstrated in other homoiochlorophyllous resurrection plants, the general model described above should hold true. Species specific exceptions may however be apparent in the means of senescence and autophagy pathway suppression.

Extending the model to poikilochlorophyllous resurrection plant species is a bit more complex. The model described assumes a means of cells to differentially respond to auxin related stimulus during the commitment to anhydrobiosis stage of their dehydration cycle. A precursor for auxin biosynthesis is the amino acid L-tryptophan, which is produced in chloroplasts via the shikimate pathway (Maeda & Dudareva, 2012; Casanova-Sáez *et al.*, 2021).

Poikilochlorophyllous species dismantle structures in their chloroplasts during their commitment to anhydrobiosis (Sherwin & Farrant, 1996), suggesting that chloroplasts may not have the capacity to produce L-tryptophan for *de novo* auxin biosynthesis. If this is the case, active auxin would need to be converted from inactive storage forms in order for the model to hold (Casanova-Sáez *et al.*, 2021). However, Radermacher *et al.* (2019) showed that in *X. schlechteri* there is an accumulation of L-tryptophan at approximately 35 %RWC in both ST (mature) and NST (expanding) tissues. This provides good evidence that auxin related signalling during the anhydrobiosis commitment stage should be possible in the poikilochlorophyllous resurrection plant *X. schlechteri* and that our proposed model should be applicable.

In applying our generalized model to *X. schlechteri*, the proposed ethylene and WRKY transcription factor controlled signal cascade proposed by Radermacher *et al.*, (2021) can be explained. Ethylene and auxin pathways have synergistic crosstalk during many developmental processes (reviewed in Muday *et al.*, 2012). Elevated concentrations of ethylene have been demonstrated to increase auxin response in roots, affect auxin transport in leaves and roots, and elevated auxin concentrations increase synthesis of ethylene (Addicott *et al.*, 1955; Morgan & Hall, 1962; Lewis *et al.*, 2011; Muday *et al.*, 2012). The findings by Radermacher *et al.* (2019, 2021) may thus represent the species specific means of senescence and autophagy pathway suppression in *X. schlechteri*, through the lens of our model, with differential expression of ethylene signalling and WRKY transcription factors coming about due to tissue specific chromatin remodelling and response to auxin.

Evolutionary relevancies

A fundamental question behind this emerging field is the potential benefit that evolving such a mechanism would provide. Resurrection plants have been proposed to be able to suppress water deficit stress-induced senescence in the bulk of their vegetative tissues by employing signal blocking mechanisms (Griffiths *et al.*, 2014; Williams *et al.*, 2015). Why then alleviate this suppression in mature vegetative tissues and seemingly link this process to anhydrobiosis (Radermacher *et al.*, 2019, 2021; Chapter 2 & 3)?

A minor observation from Chapter 2 was that after 72 hours rehydration, growth in *C. pumilum* appeared focused on young leaves. Given that plants received no direct fertilizer during the rehydration watering, and assuming that there was no change to the soil nutrient status during dehydration, plants would need to obtain the nutrient flush necessary for rapid growth through nutrient mobilization.

The potential benefit of promoting senesce-like processes in some tissues during rehydration may thus be to allow for rapid growth of stalled meristems and leaf primordia. This would provide the evolutionary benefit of quick land or surface colonization following drought and potentially sequestering resources before desiccation sensitive species are able to emerge. Thus, allowing evolution of desiccation tolerance to be competitive following prolonged droughts (Marks *et al.*, 2021).

Indeed, where *C. pumilum* appears to favor rapid vegetative or clonal growth following rehydration, *X. schlechteri*, like its sister species *Xerophyta humilis* (Farrant & Hilhorst, 2022, supplementary video), has been observed to undergo mass flowering following rehydration (personal observation). Mature tissues, having had the most time to accumulate resources, without spending resources on expansion, thus provide a good store of readily available nutrients during rehydration. In addition, in species which favor clonal growth during rehydration, like *C. pumilum*, reduction of canopy size through maintaining mature, senescing tissues at a low %RWC (Chapter 2) allows for greater light availability for new and emerging growth, since these remain in a folded state.

We therefore propose that desiccation associated tissue differentiation may have evolved concurrently with desiccation tolerance in order to promote rapid resources, surface and light sequestering, following prolonged droughts, allowing resurrection plants to compete with desiccation sensitive early land colonizers such as annuals.

Conclusion and Implications

Our proposed model provides a clear and simple mechanism by which a tissue age-dependent response to severe water-deficit stress is possible in resurrection plants. Our model has been generalized, within the scope of current available literature, to allow for future studies on other resurrection plant species. The model has power in that all assumptions made are supported to

a degree by available literature and are clear enough that they can be interrogated in future experiments.

Our model raises a few very important implications for the field of resurrection plant research. That is: besides stress hormones such as ABA, auxin should be considered as vital to the stress response phenotype; careful consideration should be taken of tissue position, both on the organ and plant, when sampling for experiments as this may significantly affect results; aspects of stress response phenotypes may be promoted or suppressed through artificial application of phytohormones like auxin; and that more consideration should be given to tissues which seemingly don't recover during rehydration, as these tissues may still be engaging in complex source-sink relationships at remarkably low water contents.

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Publication List

Radermacher, A.L., **du Toit**, S.F. and Farrant, J.M., 2019. Desiccation-driven senescence in the resurrection plant *Xerophyta schlechteri* (Baker) NL Menezes: Comparison of anatomical, ultrastructural, and metabolic responses between senescent and non-senescent tissues. *Frontiers in plant science*, 10, p.1396.

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