

ORIGINAL ARTICLE

Extreme drought can deactivate ABA biosynthesis in embolism-resistant species

Joel A. Mercado-Reyes | Talitha Soares Pereira | Anju Manandhar  |
Ian M. Rimer | Scott A. M. McAdam 

Purdue Center for Plant Biology, Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana, USA

Correspondence

Scott A. M. McAdam, Purdue Center for Plant Biology, Department of Botany and Plant Pathology, Purdue University, 915 W. State St, West Lafayette, IN 47907, USA.
Email: smcadam@purdue.edu

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Abstract

The phytohormone abscisic acid (ABA) is synthesised by plants during drought to close stomata and regulate desiccation tolerance pathways. Conifers and some angiosperms with embolism-resistant xylem show a peaking-type (p-type) response in ABA levels, in which ABA levels increase early in drought then decrease as drought progresses, declining to pre-stressed levels. The mechanism behind this dynamic remains unknown. Here, we sought to characterise the mechanism driving p-type ABA dynamics in the conifer *Callitris rhomboidea* and the highly drought-resistant angiosperm *Umbellularia californica*. We measured leaf water potentials (Ψ_l), stomatal conductance, ABA, conjugates and phaseic acid (PA) levels in potted plants during a prolonged but non-fatal drought. Both species displayed a p-type ABA dynamic during prolonged drought. In branches collected before and after the peak in endogenous ABA levels in planta, that were rehydrated overnight and then bench dried, ABA biosynthesis was deactivated beyond leaf turgor loss point. Considerable conversion of ABA to conjugates was found to occur during drought, but not catabolism to PA. The mechanism driving the decline in ABA levels in p-type species may be conserved across embolism-resistant seed plants and is mediated by sustained conjugation of ABA and the deactivation of ABA accumulation as Ψ_l becomes more negative than turgor loss.

KEYWORDS

abscisic acid, abscisic glucose ester, catabolism, water deficit

1 | INTRODUCTION

Drought is a leading cause of plant mortality (Brodribb et al., 2020) with the severity and frequency of droughts driving plant evolution and species distributions (Bowles et al., 2021; Engelbrecht et al., 2007). Death caused by drought is largely due to the formation of embolism in the xylem which blocks water transport leading to

hydraulic failure and tissue desiccation (Brodribb et al., 2021; Brodribb & Cochard, 2009; Cardoso et al., 2020; Uri et al., 2013). A key adaptation in vascular plants that prevents declines in water potential (Ψ_l) and thus embolism formation during drought is the closure of stomata (Brodribb et al., 2021; Martin-StPaul et al., 2017). Stomata are dynamic valves on the surface of leaves that open and close in response to environmental and endogenous signals (Raschke,

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1975). During drought stomata close to prevent excessive evaporation. The mechanisms that drive stomatal closure during drought have long been debated (McAdam & Brodribb, 2014; Tardieu & Davies, 1993). In seed plants the phytohormone abscisic acid (ABA) plays a critical role in closing stomata during drought stress (Jones & Mansfield, 1970; McAdam & Brodribb, 2012; Mittelheuser & Van Steveninck, 1969). ABA biosynthesis during drought is believed to be triggered by a loss of cell turgor as leaves dehydrate, with peak ABA biosynthesis occurring at a Ψ_l that is close to turgor loss point (Ψ_{tlp}) in herbaceous species (Creelman & Mullet, 1991; Davies et al., 1981; McAdam & Brodribb, 2016; Pierce & Raschke, 1980).

In most herbaceous species, as well as tree species with relatively vulnerable xylem, as drought progresses ABA levels increase in a near-linear function relative to declining Ψ_l or rise to a plateau and remain high, termed a “rising-type” (r-type) ABA dynamic (Brodribb et al., 2014; Zeevaart, 1980). In these species once embolism forms ABA levels increase by an order of magnitude at the onset of leaf death (McAdam et al., 2022). Brodribb and McAdam (2013), investigating ABA dynamics under long-term drought in two conifer species, discovered a divergent ABA dynamic. In the highly drought tolerant, anisohydric, Cupressaceae species *Callitris rhomboidea* R. Br. ex Rich. & A. Rich. (Cupressaceae) native to arid regions of southeastern Australia (Crisp et al., 2019), stomata closed at the onset of drought stress driven by an increase in ABA levels (Brodribb & McAdam, 2013). However, once plants were dehydrated to -4 MPa, ABA levels stopped increasing, and over a subsequent 10 days of soil drought declined to prestress levels (Brodribb & McAdam, 2013). The reduction in ABA levels under long-term drought in *C. rhomboidea* meant that stomata transitioned from closure being driven by ABA to closure being the result of a passive reduction in guard cell turgor, similar to the mechanism of stomatal closure under drought in ferns and lycophytes which have stomata that are insensitive to endogenous ABA (McAdam & Brodribb, 2012). This dynamic of ABA levels during drought was termed a “peaking-type” (p-type) ABA dynamic and has subsequently been discovered in a diversity of conifer species, being associated with the evolution of highly resistant xylem, defined as xylem requiring at least -4 MPa of tension to induce embolism in at least 50% of the xylem (Brodribb et al., 2014). Conifer species from both the Southern Hemisphere callitroid and sister Northern Hemisphere cupressoid clades as well as Taxaceae have evolved embolism-resistant xylem and a p-type ABA dynamic under long-term drought (Brodribb et al., 2014). P-type ABA dynamics have been observed in the field in *C. columellaris* F. Muell in which after 6 months of no rainfall each year in the dry season in Northern Australia stomata are closed yet ABA levels are as low as levels measured in plants in the middle of the wet season when Ψ_l and stomatal aperture are highest (McAdam & Brodribb, 2015). Three studies so far have documented a p-type ABA dynamic in angiosperm species (as recently reviewed by Hasan et al., 2021). One study documented this response in the considerably drought tolerant Central Australian native tree *Acacia aptaneura* Maslin and J.E. Reid (Fabaceae) (Nolan et al., 2017) while two studies have documented the response across six species of arid adapted *Caragana* (Fabaceae) native to Inner Mongolia, China (Yao, Li, et al., 2021;

Yao, Nie, et al., 2021). All of the angiosperm species in which a p-type ABA dynamic during drought has been observed have highly resistant xylem to embolism formation with the Ψ_l of peak ABA occurring between -3.5 and -4 MPa (Nolan et al., 2017; Yao, Li, et al., 2021; Yao, Nie, et al., 2021). From these observations we would hypothesise that highly resistant xylem is required for the evolution of a p-type ABA dynamic across seed plants, and not just in gymnosperms (Brodribb et al., 2014). Resolving the mechanistic unknown driving the p-type ABA dynamic during drought remains challenging because, while highly resistant xylem has evolved independently in at least 130 species from 62 genera and 20 orders of seed plants (McAdam & Cardoso, 2018), there remain very few species with resistant xylem that have a sequenced genome. This lack of genetic information, and recent molecular work in conifers which has not been able to definitively elucidate the driver of p-type ABA dynamics from gene expression profiles (Rizzuto et al., 2023), means that resolving the mechanism driving ABA level decline under long-term drought requires additional, classical physiological and biochemical approaches (Hasan et al., 2021).

There are a number of possible drivers for the p-type dynamic in ABA levels during long-term drought. Given that more than 90% of accumulated ABA synthesised under drought is catabolized into the primary catabolite phaseic acid (PA) when plants are rewatered (Milborrow, 1974), one explanation for the decline in ABA levels during long-term drought in p-type species could be activated ABA catabolism. ABA is catabolized into PA by two biochemical steps encoded by cytochrome P450 CYP707A genes, the expression of these genes is upregulated when plants are rewatered during drought stress, and when plants are exposed to high humidity (Kushiro et al., 2004; Okamoto et al., 2009). ABA can also be reversibly inactivated by conjugation with UPD-glucose to abscisic acid-glucose ester (ABA-GE) (Lee et al., 2006; Milborrow, 1970). ABA-GE can be stored in the vacuole (Burla et al., 2013), or is primarily exported from the leaf in the phloem (Zeevaart & Boyer, 1984). Conjugation occurs by a single biochemical step, catalysed by two isoforms of β -glucosidase (Lee et al., 2006; Xu et al., 2012). There could be an enhanced rate of conjugation of ABA to inactive forms under long-term drought and export of these conjugates in the phloem that could account for the decline in ABA levels. An additional explanation for the decline in ABA levels under long-term drought could be the cessation of de novo biosynthesis of ABA. The loss of cell turgor is a well-described trigger for increasing the expression the gene encoding the rate limiting step in ABA biosynthesis in angiosperms, 9-cis-epoxycarotenoid deoxygenase (NCED3 in *Arabidopsis*) (Bacete et al., 2022; Qin & Zeevaart, 1999; Sussmilch et al., 2017), only a relief of low cell turgor, via rehydration, is known to decrease the expression of this gene (Qin & Zeevaart, 1999). While never before described the cessation of de novo ABA biosynthesis at a threshold Ψ_l under drought would lead to a decrease in ABA levels that is independent of changes in the rate of ABA catabolism or conjugation. A final explanation, is an increase in the rate of ABA export from the leaf via the phloem; phloem flux from the leaf is a major sink for foliage-derived ABA and conjugates (Castro et al., 2019; Jeschke et al.,

1997). While it is often assumed that the rate of phloem flux is low or non-existent when assimilation has ceased during drought (Sevanto, 2014), a common occurrence when stomata are closed, there is a recent report in *Ricinus* that indicates phloem loading occurs even after turgor loss (Gersony & Holbrook, 2022), consequently this hypothesis requires testing.

Here, we sought to characterise the mechanism driving declines in ABA levels under long-term drought in p-type seed plant species. We conducted experiments on two species, the model system for characterising p-type ABA dynamics, the gymnosperm species *C. rhomboidea* (Cupressaceae) native to southeastern Australia and the highly drought-resistant evergreen angiosperm species *Umbellularia californica* (Hook. & Arn.) Nutt. (Lauraceae) native to coastal forests and the foothills of the Sierra Nevada in Western North America (DiLeo et al., 2014). Ψ_l , canopy conductance (g_c), foliage ABA, PA, and conjugate levels were measured in potted plants of each species through a prolonged drought treatment until a non-fatal Ψ_l was reached. We tested four mechanistic hypotheses for the p-type ABA dynamic: (1) increased catabolism of ABA into either PA or (2) conjugation, occurs at a threshold Ψ_l , (3) a cessation of ABA biosynthesis, driven by turgor loss and/or (4) the phloem export of ABA from leaves. The role of export of ABA and conjugates was assessed by girdling branches in drought stressed plants. We developed a technique based on bench dehydration to assess the ability of shoots to rapidly synthesise ABA to assess whether ABA biosynthesis was deactivated under long-term drought in both species as well as the r-type Lauraceae species *Persea americana* Mill. as a control.

2 | MATERIALS AND METHODS

2.1 | Plant material

Individuals of all species were grown from seed in 5 L pots in a mix of Indiana Miami topsoil, ground pine bark and sand at a 0.5:1:0.5 ratio. Plants were between 3 and 5 years of age at the time of experiment. The plants were grown at Purdue University under controlled glasshouse conditions with approximately 12 h natural light supplemented with LEDs (16 h day, 8 h night; Illumitex Power Harvest I4) providing a minimum photon flux at pot height of $150 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Under well-watered conditions, plants received daily irrigation and complete liquid nutrients (Miracle-Gro® Water-Soluble All Purpose Plant Food, The Scotts Company LLC) once every month. Glasshouse temperatures were set at 28°C during the day and 22°C during the night. Air circulation fans in the glasshouse ensured continual air circulation, reducing boundary layer conductance.

2.2 | Canopy conductance and hormone levels

To measure midday whole plant transpiration pots were enclosed in a black plastic bag and covered in aluminium foil secured around the

stem with a releasable cable tie to eliminate evaporation from the soil medium. During drought, pots were weighed (Mettler Toledo) 30 min before and after solar midday. Drought was initiated by withholding water. Samples for Ψ_l determination and foliage hormone analysis were collected 30 min after solar midday after final masses were taken. Samples were wrapped in damp paper towel, then aluminium foil, placed inside an individual ziplock bag and then an insulated box, for transportation to the lab. Ψ_l was measured using a Scholander pressure chamber (PMS Instrument Company) and microscope for accurate determination of balance pressure, by slowly pressurising and depressurising the chamber. After measuring Ψ_l , a subsample of tissue was then taken for hormone analysis (see below). Water was withheld from plants until they reached -6 MPa (at this Ψ_l there were no signs of leaf death or damage). Once this threshold Ψ_l was reached (at least 10 days after a peak in ABA levels was measured), plants were rewatered to soil pot capacity. Measurements were made on rehydrated plants until whole plant transpiration approached the levels measured before drought (4 days). At the end of the experiment total plant foliage area was determined. In *U. californica* and *P. americana* leaf area was calculated by scanning leaves (Epson Perfection V39 Scanner; Epson America, Inc.) and quantifying leaf area using ImageJ (NIH). A mean leaf area for an individual leaf was determined from these images (12.68 cm^2) which was used to adjust total plant leaf area during the experiment to account for leaves periodically harvested for Ψ_l and foliage hormone analysis. In *C. rhomboidea* leaf area was calculated from the ratio of leaf dry weight to leaf area. Whole plant leafy branch area was harvested and dried to completeness at 70°C for 48 h, after which dry mass was taken. Total leaf area was calculated from the ratio of dry mass to leaf area ($70.80 \text{ cm}^2/\text{g}$) determined from sub-samples prepared in the same way. In *C. rhomboidea* a mean area of sample collected for Ψ_l and hormone analysis was determined from 10 random samples (2.86 cm^2). This mean sample area was used to correct whole plant leaf area for declines caused by sampling. Temperature and relative humidity measurements were recorded every 10 min using a HOBO MX2301A Data Logger (Onset Computer Corporation), suspended at plant height in the glasshouse. The gravimetric determination of whole plant water loss was then used to calculate canopy conductance (g_c) by calculating whole plant transpiration (E , $\text{mol m}^{-2} \text{s}^{-1}$) using Equation (1) and the leaf area determined above.

$$E = \frac{\text{moles of water lost}}{\text{area} \times s} \quad (1)$$

Mean vapour pressure deficit (VPD) of the atmosphere for the hour during which E was measured (Sadler & Evans, 1989) and g_c was then calculated from E and VPD using Equation (2):

$$g_c = \frac{E}{\text{VPD} \times P_{\text{atm}}}, \quad (2)$$

where P_{atm} is atmospheric pressure. We assumed negligible boundary layer conductance due to the constant air circulation in the glasshouse, and that leaf temperature approximated air temperature.

2.3 | Girdling experiments

To test for an effect of reduced phloem transport during drought on foliage hormone levels, before drought a single, large branch on three individuals was girdled. Branches were girdled by carefully removing 2–3 cm of periderm just above the intersection of a side branch and the main, leading stem. Once periderm was removed a hydrogel was applied to the xylem (Tensive; Parker Labs). Samples from girdled and non-girdled branches were harvested concurrently to determine Ψ_l using a Scholander pressure chamber and to collect a subsample of tissue for hormone quantification.

2.4 | Rapid bench dehydration assay to assess ABA biosynthetic capacity

A method was developed to test for the capacity of leaves to rapidly synthesise ABA, relying on the well described effect of bench dehydration of excised branches inducing ABA biosynthesis in leaves (Wright & Hiron, 1969). Branches were excised from individuals of each species at cardinal timepoints before water stress imposition and 10 days after peak ABA levels, or in the r-type species *P. americana* 25 days following stomatal closure. To test whether tissues were capable of ABA biosynthesis branches were excised under water and rehydrated overnight, the following morning branches were dehydrated on a bench under low light levels ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 12 h in *C. rhomboidea* and 7 h in *U. californica* and *P. americana*. Samples from the drying branches were collected periodically in *U. californica*, every 3 h in *C. rhomboidea*, or 2 h in *P. americana*, to determine Ψ_l and foliage ABA and ABA-GE levels. Laboratory environmental data was logged to ensure a constant VPD (2.1 kPa) was maintained during the experiment.

2.5 | Quantification of ABA, PA and conjugates

Samples for hormone analysis were processed according to McAdam (2015). After Ψ_l was measured and the pressure chamber was slowly depressurised, approximately 0.15 g of tissue was taken from the leaf, avoiding the midrib, fresh weight was immediately measured (± 0.0001 g) and covered in approximately 8 mL of cold (-20°C) 80% methanol in water (vol/vol) with 250 g/L added butylated hydroxytoluene as an antioxidant and stored overnight at -20°C . Leaf tissue was homogenised and hormones extracted at 4°C overnight. Fifteen nanograms of $[^2\text{H}_6]\text{ABA}$ and $[^2\text{H}_3]\text{PA}$ (OChemim Ltd.) were added to each sample as an internal standard. To measure ABA-GE levels, an additional aliquot was taken and an alkaline hydrolysis method was used (Hansen & Dörffling, 1999). An aliquot of extract was dried to completeness under vacuum and hormones were resuspended in 200 μL of 2% acetic acid and 10% acetonitrile in H_2O (vol/vol). Each sample was then centrifuged at 14 800 RPM for 3 min and a 100 μL aliquot was taken for analysis. Hormone levels were quantified using an

Agilent 6460 series triple quadrupole LC/MS (Agilent) fitted with an xBridge HPLC column (C18, 2.1×100 mm, $3.5 \mu\text{m}$; Waters Corporation). Solvents used were 2% acetic acid in H_2O (vol/vol, Solvent A) and acetonitrile (Solvent B) at a flow of 0.3 mL/min. The running gradient went from 90% Solvent A and 10% Solvent B to 5% Solvent A and 95% Solvent B at 5 min and then back to initial values. An aliquot of 10 μL of sample was injected. The LC/MS was operated in negative ion electrospray mode with the needle running at 3.5 kV. To detect each metabolite and respective internal standard we used selected reaction monitoring. We used an ion source temperature of 325°C and nitrogen as the desolvation gas flowing at 8 L/min. The tandem transitions were m/z 263.1 to 153, 204 and 219, for ABA; for $[^2\text{H}_6]\text{ABA}$ the transitions monitored were m/z 269.1–159, 207 and 225. For PA, the tandem transitions were m/z 279.3–139.1 and 205; and m/z 282.3–142.1 and 208, for $[^2\text{H}_3]\text{PA}$. The cone voltage was 100 V. For all transitions, the collision energy was 5 V. Dwell time was set to 50 ms for each channel. Hormone levels were analysed in the Agilent Quantitative Analysis software. Quantification was done using the m/z 263.1–153 and corresponding labelled channel for ABA, and m/z 279.3–139.1 and corresponding labelled channel for PA. Hormone levels in terms were calculated as the ratio of endogenous to labelled hormone peak areas, multiplied by the amount labelled ABA added to the sample (in all cases 15 ng), divided by the fresh weight of the sample collected. ABA-GE levels were determined as the difference between ABA levels in a quantified unhydrolyzed sample and the hydrolysed sample (Hansen & Dörffling, 1999).

2.6 | Pressure–volume curves

Pressure–volume curves were undertaken to determine Ψ_{tlp} and the degree of osmotic adjustment in response to long-term drought both before drought and in leaves collected from the same plants that had experienced long-term drought sufficient to reduce ABA levels to initial levels measured before the drought (Tyree & Hammel, 1972). Five individual leaves or leafy shoots from the same individual were collected and rehydrated overnight to a hydrated state (full hydration was considered when initial Ψ_l was greater than -0.05 MPa). Tissue was scanned to obtain hydrated area then leaves or shoots were dehydrated on the bench, and measurements of weight and water potential were periodically recorded as water potential progressively declined (Tyree & Hammel, 1972). Ψ_{tlp} was calculated as the Ψ_l at which the relationship between the inverse of relative water content and Ψ_l deviates from a linear regression.

2.7 | Leaf vulnerability curves

Leaf vulnerability curves in *U. californica* were performed using the optical method (Brodribb et al., 2016) in leaves of an intact individual that had soil washed clear of the roots and slowly dehydrated on the

bench. Ψ_l was measured periodically during the dehydration in excised leaves using a pressure chamber.

2.8 | Data analysis

Generalised additive models (GAM) and standard errors were fitted for ABA level, PA level, and g_c using the `gam()` function in the MGCV package (Wood, 2011) of R software (v.4.0.5; R Core Team, 2018). Analysis of variance was performed for all comparisons using the `aov` function, and significant interactions were determined using the TukeysHSD function of the multcomp package (Hothorn et al., 2008) in R software. Correlation analysis for ABA and PA levels was performed using the `cor.test` function in R software. Graphs were generated using the Sigmaplot software (v.10; Systat Software). Leaf vulnerability curves were plotted as mean Ψ_l at 5% increments of total embolized xylem area as recommended by Cardoso et al. (2022).

3 | RESULTS

3.1 | Peaking-type ABA dynamics in *Umbellularia* and *Callitris*

During a soil drought, the evergreen angiosperm species *U. californica*, like the conifer species *C. rhomboidea*, displayed a peaking-type dynamic in foliage ABA levels (Figure 1). Before drought at a water potential of -0.7 MPa, mean foliage ABA levels in *U. californica* were $0.714 \pm 0.61 \mu\text{g/g FW}$ ($\pm\text{SE}$; Figure 1a). As leaf water potential (Ψ_l) declines, ABA levels in *U. californica* rose to a mean peak of $4.924 \pm 0.4 \mu\text{g/g FW}$ ($\pm\text{SE}$) once Ψ_l had reached to -3.1 MPa (Figure 1a), at a rate of $2.3 \mu\text{g g}^{-1} \text{FW MPa}^{-1}$. As *U. californica* experienced more negative Ψ_l ABA levels gradually declined, decreasing back to a mean of $1.09 \pm 0.6 \mu\text{g/g}$ at a Ψ_l of -6.02 MPa (Figure 1a). ABA levels in *U. californica* decreased at an average rate of $0.15 \mu\text{g g}^{-1} \text{FW MPa}^{-1}$ during this time. In *C. rhomboidea* mean initial ABA level before drought was $0.236 \pm 0.11 \mu\text{g/g FW}$ ($\pm\text{SE}$), this increased to a

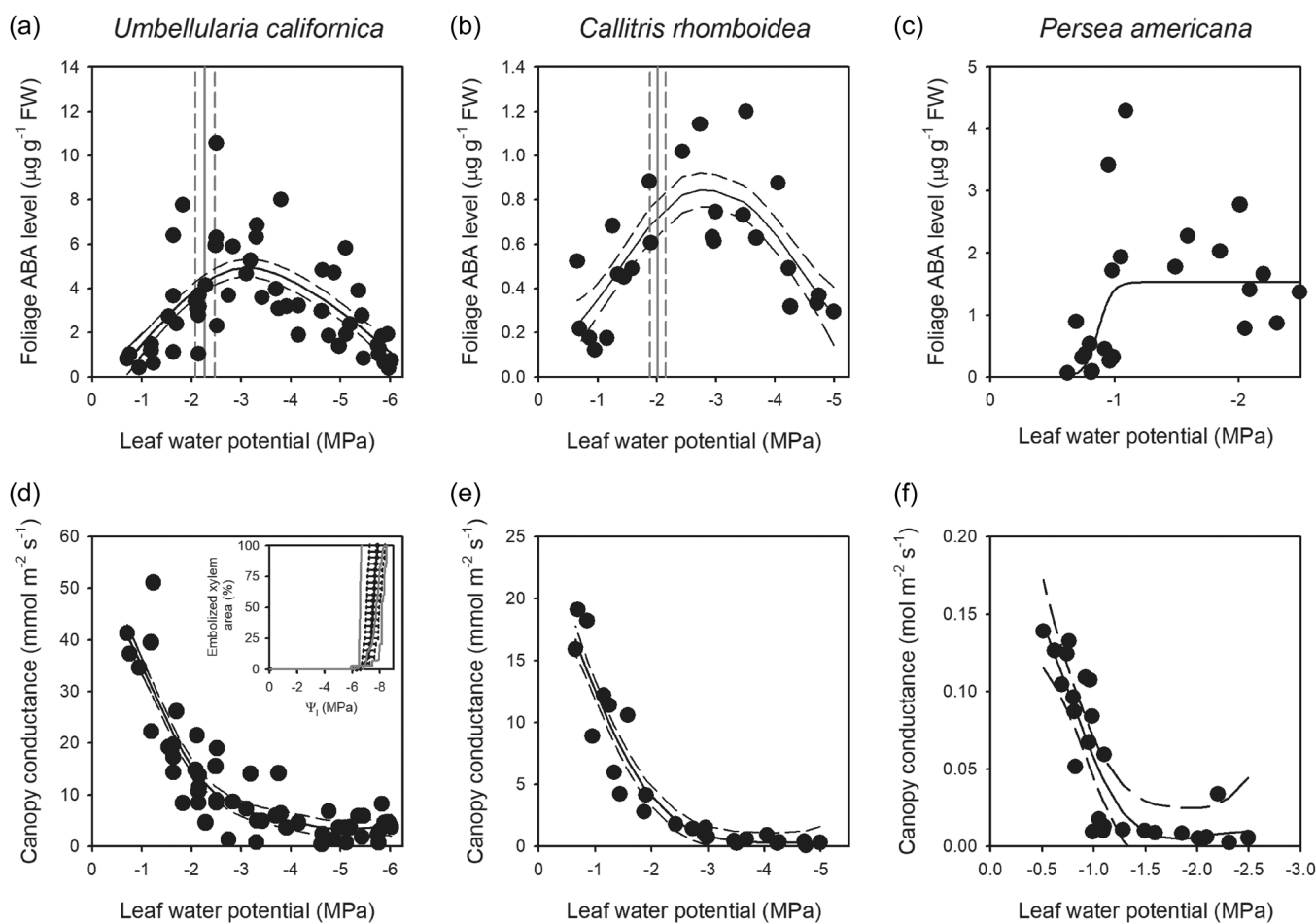


FIGURE 1 Midday foliage abscisic acid (ABA) level as leaf water potential (Ψ_l) declines during soil drought in the angiosperm species *Umbellularia californica* (a), conifer species *Callitris rhomboidea* (b) and angiosperm species *Persea americana* (c). Mean Ψ_l at turgor loss point and standard errors are shown as grey solid and dashed lines vertical dashed grey lines in a and b. Midday canopy conductance during soil drought in *U. californica* (d), *C. rhomboidea* (e) and *P. americana* (f). Generalised additive model (GAM) curves and standard errors are represented in all relationships by solid and dashed black lines, respectively, a sigmoidal regression between ABA levels and Ψ_l is shown for *P. americana*. The insert in d depicts the leaf xylem vulnerability to embolism in *U. californica*, black symbols represent means ($\pm\text{SE}$) for three replicate leaves (grey lines).

mean peak ABA level of $0.845 \pm 0.8 \mu\text{g/g FW}$ ($\pm\text{SE}$) at -2.7 MPa (Figure 1b) at a rate of $0.35 \mu\text{g g}^{-1} \text{ FW MPa}^{-1}$. After ABA levels peaked, they declined by an average of $0.26 \mu\text{g g}^{-1} \text{ FW MPa}^{-1}$ to $0.275 \pm 0.13 \mu\text{g/g FW}$ ($\pm\text{SE}$) at -5 MPa (Figure 1b). In both species, canopy conductance (g_c) declined exponentially as ψ_l declined, with an 83%–99% reduction in g_c once ψ_l had declined to the ψ_l at which peak levels of foliage ABA occurred in *U. californica* and *C. rhomboidea*, respectively (Figure 1c,d). In *U. californica* mean ($\pm\text{SE}$) maximum g_c before drought was $40.9 \pm 0.2 \text{ mmol m}^{-2} \text{ s}^{-1}$, this exponentially declined to a minimum of $0.42 \pm 0.2 \text{ mmol m}^{-2} \text{ s}^{-1}$ by -6.02 MPa (Figure 1d). In *C. rhomboidea*, mean ($\pm\text{SE}$) maximum g_c before drought was $17 \pm 0.1 \text{ mmol m}^{-2} \text{ s}^{-1}$, this declined to a minimum of $0.026 \pm 0.13 \text{ mmol m}^{-2} \text{ s}^{-1}$ by -6.02 MPa (Figure 1e). Canopy conductance remained low for the duration of the drought in both species. *U. californica* was found to have highly embolism-resistant xylem in which the mean ψ_l at which 50% of the leaf xylem was embolized (P_{50}) was $-7.5 \pm 0.47 \text{ MPa}$ (insert in Figure 1d). In *P. americana* foliage ABA levels increased from $0.41 \pm 0.17 \mu\text{g/g FW}$ ($\pm\text{SE}$) at a ψ_l of -0.7 MPa before drought to mean maximum of $2.8 \pm 0.6 \mu\text{g/g FW}$ ($\pm\text{SE}$) at -1.1 MPa , these high levels of ABA in the leaves were maintained above $0.8 \mu\text{g/g FW}$ for the duration of a long-term drought (Figure 1c). In *P. americana* mean ($\pm\text{SE}$) maximum g_c before drought was $0.123 \pm 0.007 \text{ mol m}^{-2} \text{ s}^{-1}$ which declined exponentially to a minimum of $6 \pm 0.001 \text{ mmol m}^{-2} \text{ s}^{-1}$ at -1.52 MPa (Figure 1e).

3.2 | Catabolism of ABA to PA cannot explain declining ABA levels during drought

Foliage PA levels displayed a p-type dynamic as ψ_l declined during drought in the angiosperm species *U. californica* (Figure 2). PA levels were significantly correlated with ABA levels in *U. californica* (Pearson's $r_{57} = 0.74$, $p < 0.0001$) while no significant correlation between PA and ABA levels was observed in the conifer species *C. rhomboidea* (Pearson's $r_{23} = 34$, $p = 0.09234$). In *U. californica* mean foliage PA levels were $0.292 \pm 0.095 \mu\text{g/g FW}$ ($\pm\text{SE}$) at -0.7 MPa before the drought, then as ψ_l decreased to -3.3 MPa foliage PA levels increased reaching a maximum of $0.969 \pm 0.063 \mu\text{g/g FW}$ ($\pm\text{SE}$) (Figure 2). In *U. californica* as drought progressed foliage PA levels declined to a minimum of 0.545 ng/g FW at -6.02 MPa , at a rate of $0.167 \mu\text{g g}^{-1} \text{ FW MPa}^{-1}$ (Figure 2). In the conifer species *C. rhomboidea*, there was very little change in PA levels as drought progressed (Figure 2). PA levels before drought were low at a mean of $0.107 \pm 0.1 \mu\text{g/g FW}$ at -0.65 MPa , there was one period during drought where there was a wide variation in PA levels to a maximum of $0.504 \pm 0.08 \mu\text{g/g FW}$ around -3.51 MPa (Figure 2). However, PA levels remained at $0.15 \pm 0.013 \mu\text{g/g FW}$ ($\pm\text{SE}$) once ψ_l had declined to -5 MPa .

3.3 | Rehydration activates ABA catabolism to PA

The rewatering of drought stressed *U. californica* and *C. rhomboidea* plants activated the catabolism of ABA to PA (Figure 3). In the

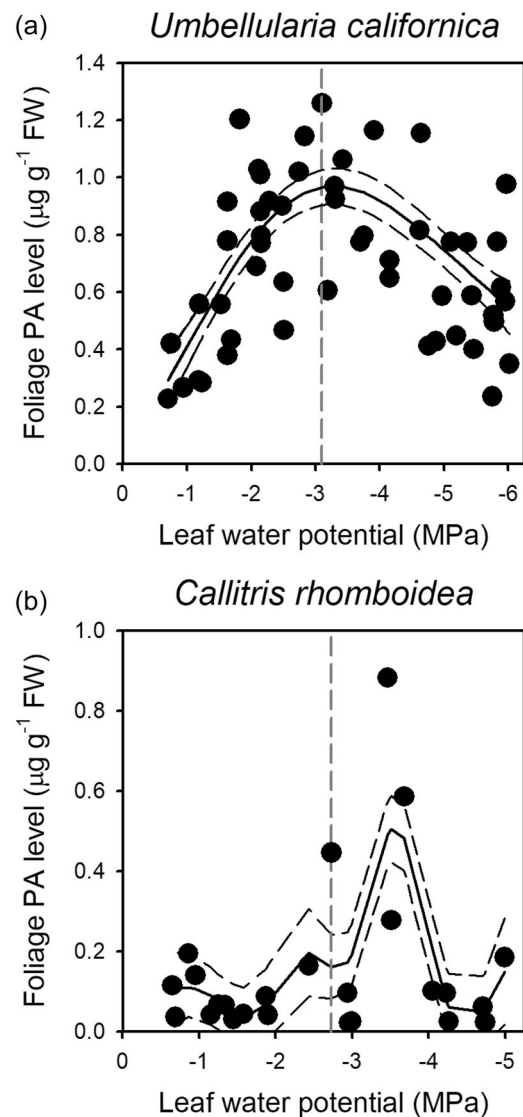


FIGURE 2 Foliage phaseic acid (PA) levels as leaf water potential (ψ_l) declines during soil drought in the angiosperm species *Umbellularia californica* (a) and conifer species *Callitris rhomboidea* (b). Generalised additive model (GAM) curves and standard errors are represented by solid and dashed lines, respectively. The grey vertical line depicts the ψ_l at peak foliage ABA level.

angiosperm species *U. californica* rewatering whole plants from a ψ_l of -6 MPa , when foliage ABA levels were the lowest of the entire drought period, resulted in a further reduction in foliage ABA levels over 4 days from 1.62 ± 0.35 to $0.297 \pm 0.025 \mu\text{g/g FW}$ (Figure 3b). This reduction in foliage ABA levels in *U. californica* corresponded to an increase in foliage PA levels from 0.42 ± 0.08 to $1.08 \pm 0.076 \mu\text{g/g FW}$ in the 24 h after rewatering (Figure 3a). In the conifer species *C. rhomboidea* rewatering whole plants from a similar initial ψ_l at the angiosperm *U. californica* restored water status and also drove a decline in foliage ABA levels from 0.54 ± 0.15 to $0.16 \pm 0.03 \mu\text{g/g FW}$ in 48 h (Figure 3e). Like in the angiosperm species, PA levels in *C. rhomboidea* increased from 0.057 ± 0.03 to $0.4 \pm 0.13 \mu\text{g/g FW}$ (Figure 3d).

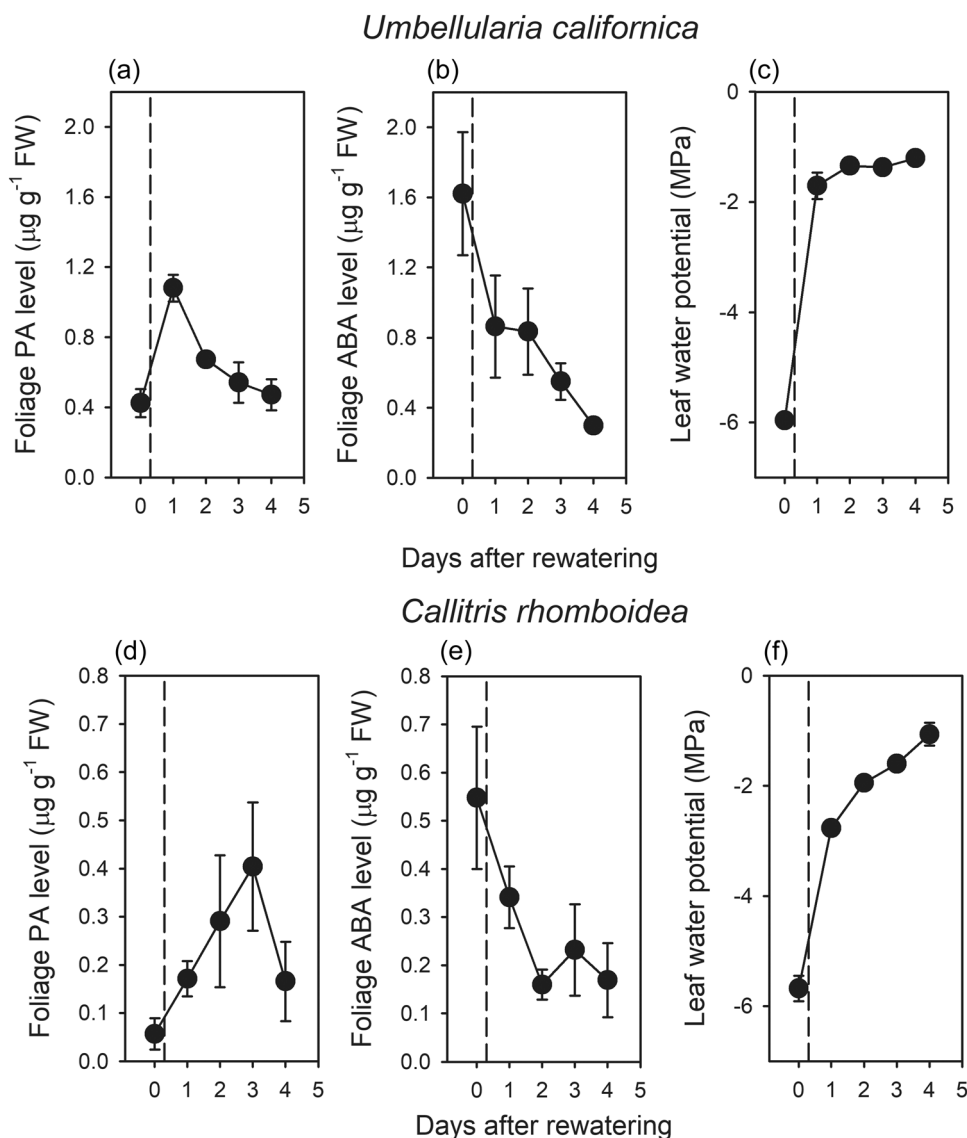


FIGURE 3 Mean midday foliage phaseic acid (PA) levels (a, d), foliage abscisic acid (ABA) levels (b, e) and leaf water potential (c, f) in plants of the angiosperm species *Umbellularia californica* (a–c) and conifer species *Callitris rhomboidea* (d–f) that were rewatered from a drought-stressed state (denoted by dashed vertical lines) ($n = 5$, $\pm$ SE).

3.4 | Accumulation of conjugated forms of ABA occurred during drought

In both species foliage ABA conjugate levels increased as drought progressed to a maximum 10 days after peak ABA levels were measured in leaves and when foliage ABA levels were a minimum (Figure 4). In the angiosperm species *U. californica* mean foliage ABA conjugate levels increased from 0.2 to 2.63 $\mu\text{g/g}$ FW before drought to when ABA levels were the lowest at the end of the drought period (Figures 1 and 4). Similar patterns in foliage ABA conjugate level during drought were observed in the conifer species *C. rhomboidea*, increasing to a much higher level than *U. californica*, from 1.5 to 23 $\mu\text{g/g}$ FW (Figure 4).

3.5 | Phloem transport did not play a role in ABA dynamics during drought

Girdling branches and ceasing phloem transport had no effect on the average foliage ABA level dynamics during drought (Figure 5), or on foliage ABA conjugate levels (Figure 4), in either species. In the angiosperm species *U. californica* the mean GAM fitted through foliage ABA data as Ψ_l declined was slightly higher over the Ψ_l period at which there was a peak in foliage ABA levels, however foliage ABA levels did decline to levels measured in unstressed leaves once plants had reached a Ψ_l of -6 MPa (Figure 5a). Mean ABA levels in girdled branches of *C. rhomboidea* increased from 0.2 ± 0.1 $\mu\text{g/g}$ FW at -0.59 MPa to 0.775 ± 0.084 $\mu\text{g/g}$ FW at

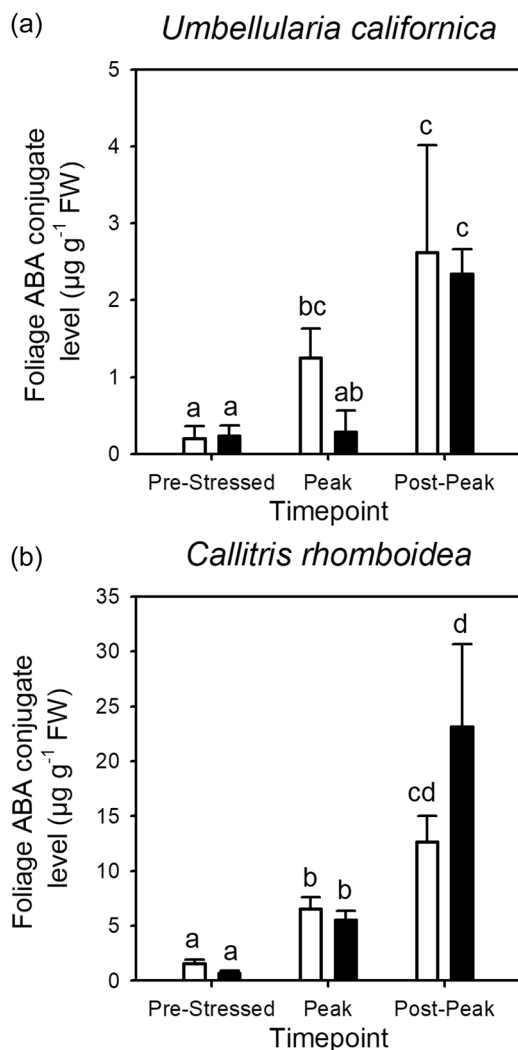


FIGURE 4 Mean foliage ABA conjugate levels ($n = 3$, $\pm$ SE) in leaves taken from branches that were intact (white) or girdled (black) from plants of the angiosperm species *Umbellularia californica* (a) and conifer species *Callitris rhomboidea* (b) before drought stress (pre-stressed), at peak ABA levels (peak) and 10 days after peak ABA levels (post-peak). Different letters denote significant differences ($p < 0.05$) in mean ABA-GE levels.

-3.16 MPa (Figure 5), this was similar to levels of ABA in ungirdled branches (t -test, $p = 0.3937$).

3.6 | Dehydration triggered ABA accumulation is temporarily deactivated in leaves that have experienced low Ψ_l

In the p-type angiosperm species *U. californica*, we found that rapid bench dehydration of never-before stressed branches triggered a fast and considerable accumulation of foliage ABA levels (Figure 6b). In *U. californica*, foliage ABA levels increased by $0.345 \mu\text{g g}^{-1} \text{FW h}^{-1}$ as branches were dehydrated on the bench (Figure 6b). This was very similar to the increase in ABA levels observed in branches of the r-type

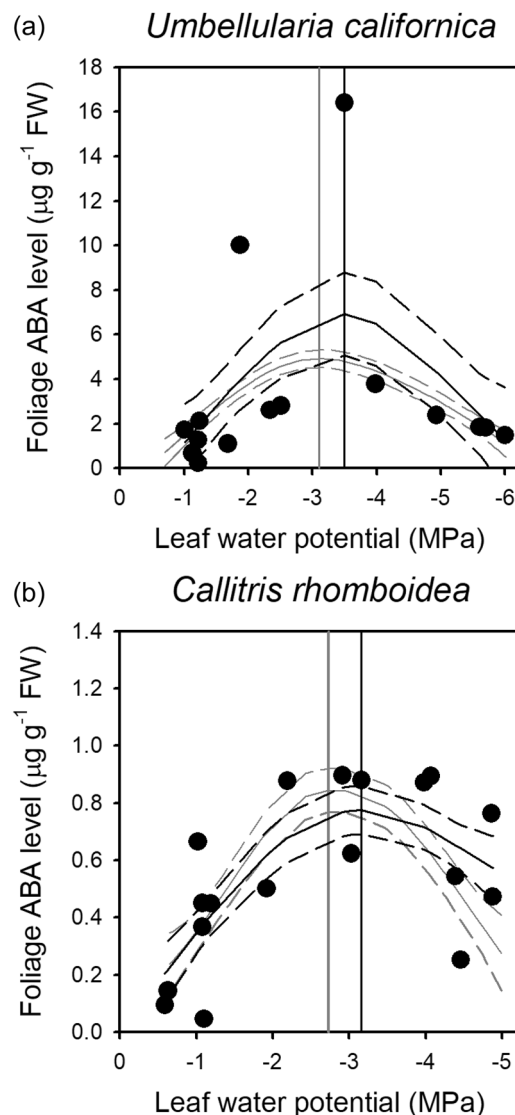


FIGURE 5 Foliage abscisic acid (ABA) levels as leaf water potential (Ψ_l) declines during soil drought in leaves taken from girdled branches in the angiosperm species *Umbellularia californica* (a) and conifer species *Callitris rhomboidea* (b). Generalised additive model (GAM) curves and standard errors are represented by solid and dashed black lines, respectively, for the ABA data from girdled branches, and in grey from ABA data taken from intact branches. The black vertical line depicts the Ψ_l at peak foliage ABA level in girdled branches, and the grey vertical line the Ψ_l at peak foliage ABA level in intact branches.

angiosperm species *P. americana* which increased by $0.45 \mu\text{g g}^{-1} \text{FW h}^{-1}$ (Figure 6c). There was no difference in the accumulation rate of ABA in dehydrated branches of *P. americana* regardless of whether they were collected from never-before stressed branches or from plants that had sustained long-term drought for 25 days (Figure 6c). In contrast, in *U. californica* in branches collected from plants under long-term drought at least 10 d after peak foliage ABA levels and rehydrated overnight, Ψ_l decreased rapidly when bench dried while ABA levels remained low and unchanged (less than $0.767 \pm 0.092 \mu\text{g/g FW}$, mean $\pm$ SE; Figure 6b). Ψ_l in never-before stressed branches of *U.*

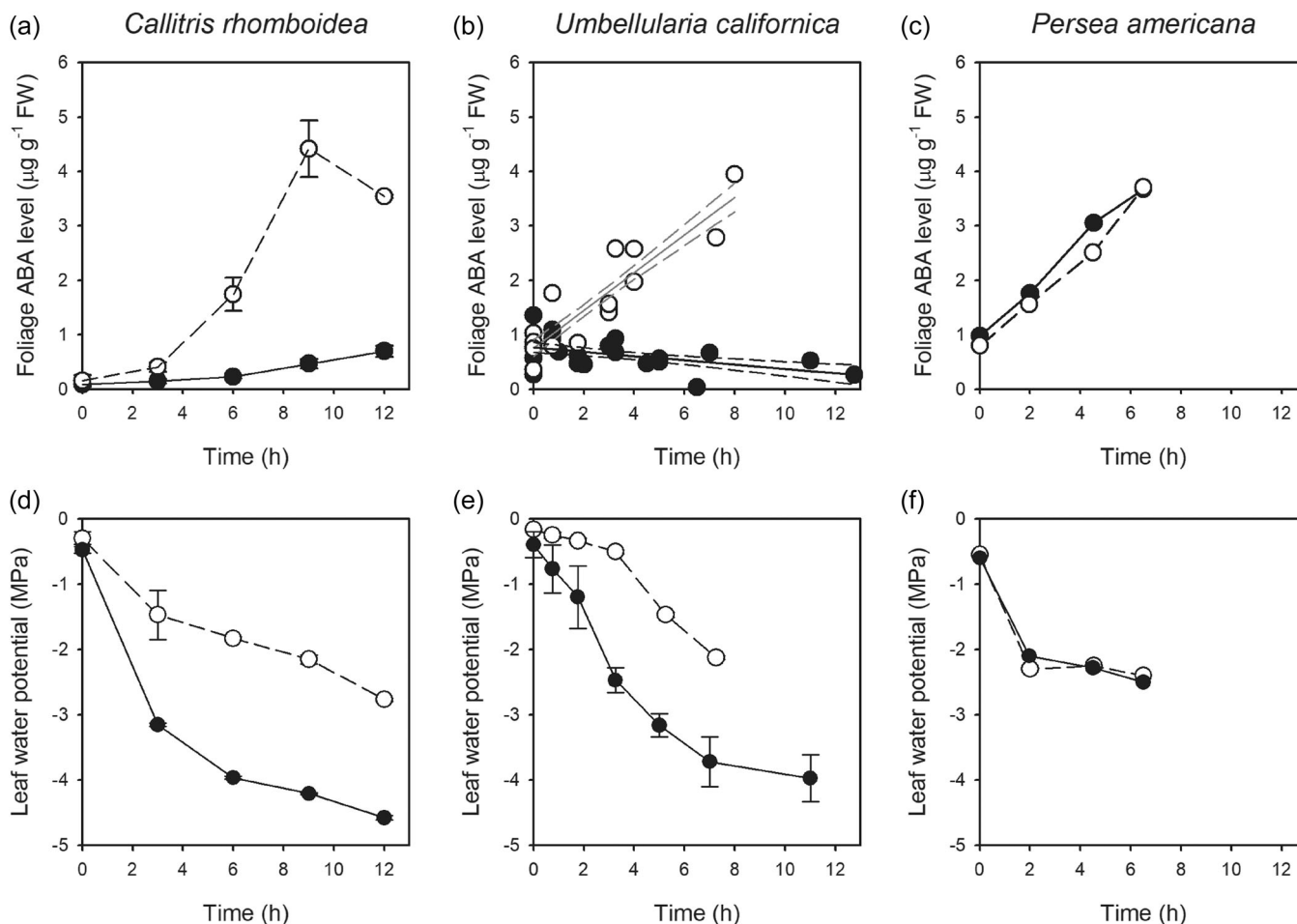


FIGURE 6 Foliage abscisic acid (ABA) levels (a–c) and leaf water potentials (d, e) as excised branches of the p-type conifer species *Callitris rhomboidea* (a, d) and angiosperm species *Umbellularia californica* (b, e) and r-type angiosperm species *Persea americana* (c), that were rehydrated overnight, are subsequently dehydrated on the laboratory bench. Bench dehydration started at time = 0. Branches were taken from either unstressed plants (white) or plants that were exposed to a soil drought for 10 days after peak ABA levels were measured (black) or in *P. americana* 25 days after the onset of drought when ABA levels were still high. Data represent means and standard errors ($n = 5$) in *C. rhomboidea*, pooled data for five branches in *U. californica* and single leaves in *P. americana*. Generalised additive model (GAM) curves and standard errors are represented by black solid and dashed lines for ABA levels in branches harvested from *U. californica* plants exposed to drought for 10 days after peak ABA and grey lines for ABA levels in branches harvested from unstressed *U. californica* plants.

californica which accumulated considerable levels of ABA on rapid dehydration decreased at a slower rate than for branches that were collected from plants that had experienced long-term drought, with WP at 3.25 h after the initiation of dehydration being -0.50 MPa in never-before stressed branches and -2.47 MPa in branches collected from plants experiencing long-term drought that were not accumulating foliage ABA levels (Figure 6e). Similarly, in the conifer species *C. rhomboidea* foliage ABA levels increased in unstressed branches from 0.153 ± 0.1 to $1.743 \pm 0.3 \mu\text{g g}^{-1}$ FW at 6 h while in branches collected from plants under long-term drought at least 10 days after peak foliage ABA levels were measured displayed relative low levels of foliage ABA increasing from $0.090 \pm 0.02 \mu\text{g/g}$ FW to just $0.225 \pm 0.024 \mu\text{g/g}$ FW at 6 h (Figure 6a). Ψ_l in never-before stressed branches of the conifer *C. rhomboidea*, like those of *U. californica*, decreased at a slower rate than that of branches in which ABA levels did not accumulate on rapid bench dehydration. Ψ_l decreased to

-1.83 MPa in never-before stressed branches and -3.96 MPa in branches from plants under long-term drought at 6 h (Figure 6d). The ability to synthesise ABA on rapid bench dehydration recovers in *U. californica* plants after rewatering (Figure 7). In plants that were drought stressed for 10 days after peak ABA levels were measured then rewatered to saturating soil water capacity, branches harvested 3 days after rewatering and then rapidly dehydrated on the bench were able to synthesise ABA over 10 h (Figure 7). Mean foliage ABA levels in these branches increased from 0.26 to $1.97 \mu\text{g/g}$ FW in 10 h (Figure 7).

3.7 | Ψ_l at peak foliage ABA level corresponds to Ψ_{tlp}

In the angiosperm species *U. californica* mean turgor loss point (Ψ_{tlp}) in never-before stressed plants was -2.46 ± 0.2 MPa (Figure 8). In

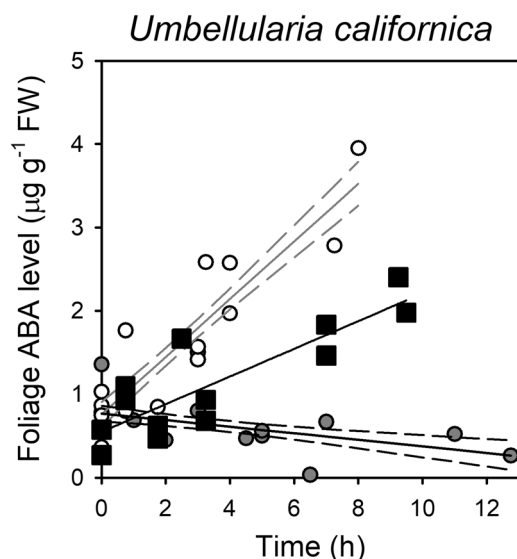


FIGURE 7 Foliage abscisic acid (ABA) levels as branches of the angiosperm species *Umbellularia californica* that were rehydrated overnight were subsequently dehydrated on the laboratory bench for 12 h. Bench dehydration started at time = 0. Branches were taken from either unstressed plants (white), plants that were exposed to a soil drought for 10 days after peak ABA levels were measured (grey) (data from Figure 4), or plants exposed to soil drought for 10 days after peak ABA levels were measured then rewatered and maintained in saturated soil for 3 days (black squares). Data are taken from leaves collected from five branches. Generalised additive model (GAM) curves and standard errors are represented by black solid and dashed lines for ABA levels in branches harvested from *U. californica* plants exposed to drought for 10 days after peak ABA and grey lines for ABA levels in branches harvested from unstressed *U. californica* plants, a significant linear regression is shown for the data collected from a rehydrated plant.

leaves collected from plants approximately 10 days after peak foliage ABA level, when Ψ_l had declined to -6 MPa, Ψ_{tlp} was not significantly lower than never-before stressed plants ($t(6) = 1.896$, $p = 0.1067$), with a mean Ψ_{tlp} of -2.06 ± 0.15 MPa (Figure 8). Similarly, in the conifer species *C. rhomboidea*, mean Ψ_{tlp} in never-before stressed plants was -1.922 ± 0.13 MPa (Figure 8), which was not significantly different than mean Ψ_{tlp} in branches of plants that had experienced drought for at least 10 days after peak foliage ABA levels (-2.016 ± 0.14 MPa, $t(6) = 0.494$, $p = 0.6387$).

4 | DISCUSSION

Here, we document the occurrence of p-type ABA dynamics in an angiosperm species outside of Fabaceae, the anisohydric, highly embolism-resistant, evergreen Lauraceae species *U. californica*. This result, coupled with previous reports of p-type ABA dynamics in species from two genera in Fabaceae (Nolan et al., 2017; Yao, Li, et al., 2021; Yao, Nie, et al., 2021), all adapted to seasonally dry or arid environments and with highly embolism-resistant xylem, suggests that the evolution of the p-type ABA response to long-

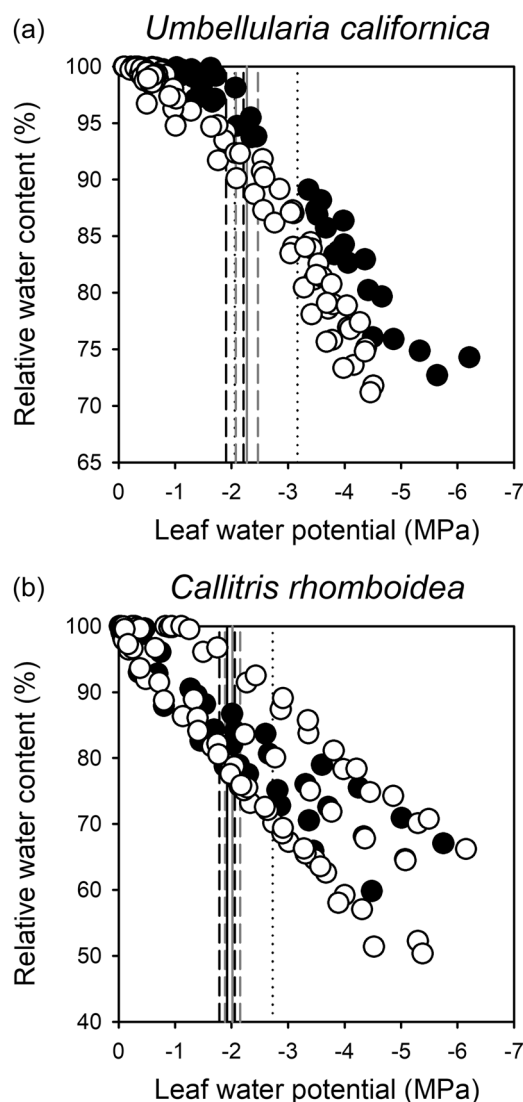


FIGURE 8 Pressure volume curves for leaves of the angiosperm *Umbellularia californica* (a) and small branches of the conifer *Callitris rhomboidea* (b) ($n = 5$) in never-before stressed plants (black) and plants that had experienced long-term drought and at least 10 days at a leaf water potential (Ψ_l) more negative than when peak foliage abscisic acid (ABA) level occurred (open circles). Dotted vertical lines represent the Ψ_l at peak foliage ABA level. Black vertical and dashed lines represent mean Ψ_l at turgor loss point (Ψ_{tlp}) and standard error for tissue collected from plants under long-term drought. Grey solid and dashed vertical lines represent mean Ψ_{tlp} and standard error for leaves of never-before stressed plants.

term drought is linked to the evolution of highly resistant xylem and anisohydric stomatal regulation during drought (Brodribb & McAdam, 2013). Highly resistant xylem has evolved frequently across angiosperm species (McAdam & Cardoso, 2018) suggesting that this ABA response to drought may be commonly observed across angiosperms. Our results demonstrate the occurrence of a p-type ABA response now in two highly divergent angiosperm families, the Magnoliid lineage Lauraceae and the Fabaceae (Nolan et al., 2017; Yao, Li, et al., 2021; Yao, Nie, et al., 2021). The absence of high levels of ABA under

long-term drought in some angiosperm species implies that, like in conifers, the stomata of p-type species may be closed passively by low Ψ_l under long-term drought (Brodrribb & McAdam, 2013; McAdam & Brodrribb, 2015). This is a controversial hypothesis for angiosperm stomatal biology (Franks, 2013; Merilo et al., 2017), especially given that angiosperm ABA biosynthetic and signalling mutants have stomata that are insensitive to changes in leaf water status (Brodrribb et al., 2021; Cernusak et al., 2019; McAdam et al., 2016). It has been suggested that passive regulation of stomatal aperture in response to changes in leaf water status is absent from this group of land plants (McAdam & Sussmilch, 2020). Recently in characterising p-type ABA dynamics in *Caragana*, Yao, Li, et al. (2021) suggested that ethylene might be closing stomata during drought and on recovery from drought when ABA levels are low but stomata are not yet open to maximum apertures. Further work is required to address whether the stomata of p-type angiosperms are closed at low Ψ_l passively via low cell turgor pressure, or via an alternative metabolic signal such as ethylene (Hasan et al., 2021).

Similarities in the dynamics of ABA, catabolite and conjugate levels, as well as the inhibition of dehydration-induced ABA biosynthesis during drought between the p-type angiosperm and conifer species in this study suggests that there is a shared mechanism driving the decline in ABA levels under long-term drought stress in seed plants. Of the four mechanistic hypotheses for the p-type ABA dynamic that we tested we found that a cessation of ABA biosynthesis, driven by turgor loss, is the most likely explanation for a p-type ABA dynamic. By rapidly dehydrating branches on the bench we could assess the ability of leaf tissue to rapidly synthesise ABA in response to dehydration (Wright & Hiron, 1969). This technique allowed us to study ABA accumulation or biosynthetic capacity without the need to quantify the expression of key ABA biosynthetic genes, which can be costly and time consuming, requiring a detailed understanding of the homologues of key genes identified in model angiosperms (Sussmilch et al., 2019). We find that ABA accumulation is highly active and rapid at all times in r-type species like *P. americana* and in unstressed branches of p-type species, like numerous early studies into ABA biosynthesis in herbaceous plants (Davies et al., 1981; Pierce & Raschke, 1980; Wright & Hiron, 1969). In p-type species this ability is eliminated in branches that are taken from plants when ABA levels are low under long-term drought and rehydrated overnight on the bench before dehydration (Figure 6). Coincidentally, we found that the Ψ_l at which peak ABA levels occurred after Ψ_{tip} in the p-type species (Figure 1). Work is required to confirm if the expression of key ABA biosynthetic genes such as 9-*cis*-epoxycarotenoid deoxygenase (*NCED*) genes are no longer upregulated on rapid dehydration in branches from p-type species that do not synthesise ABA when dehydrated on the bench (Hasan et al., 2021). ABA biosynthesis is triggered as cells lose turgor or volume (Creelman & Mullet, 1991; Davies et al., 1981; McAdam & Brodrribb, 2016; Pierce & Raschke, 1980; Sack et al. 2018), yet there has been very little work conducted on plant tissue that has been dehydrated to a Ψ_l more negative than Ψ_{tip} . The potential causes of ABA biosynthesis cessation at a Ψ_l more negative than Ψ_{tip} are highly speculative. Explanations range from an absent trigger for *NCED*

expression once membrane pressure on the cell wall ceases (Bacete et al., 2022); cellular processes such as transcription and translation of RNA ceasing at a Ψ_l more negative than Ψ_{tip} (Dhindsa & Cleland, 1975); or carotenoid precursors for ABA biosynthesis, often stored in chloroplasts, may be depleted because of increases in the de-epoxidation state of the xanthophyll cycle (Munné-Bosch & Alegre, 2000), reducing availability for conversion to ABA. Munné-Bosch and Alegre (2000) found in the extremely drought-resistant *Rosmarinus officinalis*, in which 50% of the xylem experiences embolism at a Ψ_l at -8 MPa (Brodrribb et al., 2017) during a severe summer drought the levels of ABA carotenoid precursors violaxanthin and neoxanthin declined by more than 85% maximum levels. We show that the ability to recover ABA biosynthesis can occur in p-type species, with ABA levels accumulating, but not to levels in never-before stressed branches 4 days after rewatering. This recovery might reflect the rapid recovery of carotenoid levels upon rehydration (Munné-Bosch & Alegre, 2000).

Once ABA biosynthesis ceases at a Ψ_l more negative than Ψ_{tip} in p-type species our results suggest that continual conjugation of ABA, presumably into ABA-GE, which is the primary, if not only, conjugate for ABA (Milborrow, 1970), and not catabolism of the remaining ABA is the main driver for a decline in foliage ABA levels once ABA biosynthesis ceases. This result rules out our first hypothesis that increased catabolism of ABA into either PA or conjugation, occurs at a threshold Ψ_l to drive the p-type response. It is believed that phloem flux is greatly reduced during drought in most species (Hartmann et al., 2013; Sevanto, 2014; although cf. Gersony & Holbrook, 2022), and our results demonstrate that girdling the phloem does not change the p-type ABA dynamic during drought or the accumulation of ABA conjugates, which rules out our final hypothesis that phloem export of ABA during drought was reducing ABA levels. By quantifying the levels of PA during long-term drought, we are able to rule out catabolism was the primary driver of a decrease in ABA levels in these species. The increase in catabolism of low levels ABA after rewatering suggests that catabolism of ABA was downregulated under long-term drought (Figure 3). Our results demonstrate that catabolism of ABA into PA did not significantly increase after peak ABA levels.

4.1 | Conclusion

Here we attempt to characterise the mechanism driving a p-type response of ABA dynamics exhibited by highly drought tolerant angiosperm and gymnosperm species under long-term drought. The use of rapid bench dehydration to assess ABA biosynthesis revealed that ABA biosynthesis on dehydration is inhibited under long-term drought, a phenomenon unique to p-type species. Continual conjugation of ABA appears to drive a decline in ABA levels once biosynthesis is inactivated. Future work is needed to assess the occurrence of the p-type behaviour in a wider range of species of seed plants, including in herbaceous species which usually die after a slight decrease in Ψ_l below Ψ_{tip} (Skelton et al. 2017).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Anju Manandhar  <https://orcid.org/0000-0001-5687-2957>

Scott A. M. McAdam  <https://orcid.org/0000-0002-9625-6750>

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