Research Report

Mesophyll Cells Are the Main Site of Abscisic Acid Biosynthesis in Water-Stressed Leaves[1][OPEN]

Scott A.M. McAdam[a,2] and Timothy J. Brodribb[b]

[a]Purdue Center for Plant Biology, Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907

[b]School of Biological Sciences, University of Tasmania, Hobart, Tasmania 7005, Australia

ORCID IDs: 0000-0002-9625-6750 (S.A.M.); 0000-0002-4964-6107 (T.J.B.)

The hormone abscisic acid (ABA) plays a critical role in enhancing plant survival during water deficit. Recent molecular evidence suggests that ABA is synthesized in the phloem companion cells and guard cells. However, the nature of cell turgor and water status in these two cell types cannot easily account for the rapid, water status-triggered ABA biosynthesis observed in leaves. Here, we utilize the unique foliar anatomies of an angiosperm (*Hakea lissosperma*) and four conifer species (*Saxegothaea conspicua*, *Podocarpus latifolius*, *Cephalotaxus harringtonii*, and *Amentotaxus formosana*) in which the mesophyll can be isolated from the vascular tissue to identify the main site of ABA biosynthesis in water-stressed leaves. In all five species tested, considerable ABA biosynthesis occurred in mesophyll tissue that had been separated from vascular tissue. In addition, the removal of the epidermis from the mesophyll in two conifer species had no impact on the observed increase in ABA levels under water deficit. Our results suggest that mesophyll cells are the predominant location of water deficit-triggered ABA biosynthesis in the leaf.

Abscisic acid (ABA) has played a central role in plant responses to osmotic and water stresses since photosynthetic organisms first invaded dry land. The production of ABA is linked to several processes that extend the survival of plants exposed to water stress, stimulating enhanced tolerance to desiccation in bryophyte groups (Takezawa et al., 2011) as well as stomatal closure to delay dehydration in seed plants (Mittelheuser and Van Steveninck, 1969). Despite the remarkable integration of this hormone into plant stress responses, primary questions about the function of ABA remain unanswered. For example, although great progress has been made identifying the various molecular pathways activated by ABA (Geiger et al., 2011; Brandt et al., 2012), the location of ABA biosynthesis remains uncertain.

The earliest work into ABA biosynthesis found that in plants, this compound derives from a carotenoid cleavage reaction in the chloroplasts (Taylor and Smith, 1967; Firn and Friend, 1972; Taylor and Burden, 1972; Loveys, 1977; Lee and Milborrow, 1998). This subcellular localization of the first committed step in the ABA biosynthetic pathway has since been confirmed by molecular and enzymatic studies, which showed that the key enzyme responsible for cleaving the carotenoids 9′-cis-violaxanthin and 9′-cis-neoxanthin to xanthoxin (9′-cis-epoxycarotenoid deoxygenase) is bound to the thylakoid or stroma of the chloroplast (Qin and Zeevaart, 1999; Tan et al., 2001, 2003). While there is no doubt that ABA is a carotenoid derivative and that carotenoid cleavage occurs in the chloroplast, there remains some uncertainty about which tissues are responsible for synthesizing functional levels of ABA.

The earliest studies into ABA biosynthesis found that levels of this hormone increase during water stress, particularly in the mesophyll of the leaf, which contains abundant chloroplasts (Loveys, 1977; Mansfield et al., 1978; Dörffling et al., 1979). ABA biosynthesis may occur outside the leaf (Zhang et al., 1987); ABA can also be transported around the plant via xylem and phloem (Ikegami et al., 2009; McAdam et al., 2016a) and released from chloroplast stores within the leaf (Heilmann et al., 1980; Georgopoulou and Milborrow, 2012). Nevertheless, active foliar ABA biosynthesis predominantly regulates stomatal aperture during water deficit (Holbrook et al., 2002; Sussmilch et al., 2017). A leaf-centric model of ABA synthesis makes sense in terms of providing the rapid response to changes in leaf water status required for efficient stomatal control. In this case, the water status signal triggering ABA biosynthesis in the shoot, related to a loss of cell water content or altered membrane-cell wall interactions (Pierce and Raschke, 1981; McAdam and Brodribb, 2016; Sack et al., 2018), is directly sensitive to changes in both soil water content and evaporation rate (Christmann et al., 2005, 2007).

Recent work in Arabidopsis (*Arabidopsis thaliana*) with a renewed focus on a foliar origin for ABA has been centered on gene promoter, transcript, and protein

[1]This work was supported by the U.S. Department of Agriculture National Institute of Food and Agriculture (Hatch Project 1014908; S.A.M.M.).

[2]Address correspondence to smcadam@purdue.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Scott A.M. McAdam (smcadam@purdue.edu).

S.A.M.M. designed and performed experiments and wrote the article; T.J.B. helped conceive experiments and complemented the writing.

[OPEN]Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.17.01829

localization studies of ABA biosynthetic genes and enzymes, as well as the transgenic overexpression of these genes in specific tissues. Their findings suggest that two leaf tissues are capable of synthesizing ABA, the phloem companion cells and the guard cells (Bauer et al., 2013; Kuromori et al., 2014; Merilo et al., 2018). Through the use of immunofluorescent labels for ABA biosynthetic gene transcripts, as well as in situ hybridization of proteins in leaves dried to an unknown water status, a strong signal was observed in the vascular tissue, indicating ABA biosynthesis in these cells (Koiwai et al., 2004; Endo et al., 2008). This work has been conducted at an increasingly finer scale using promoters of ABA biosynthetic genes driving GFP. The current data from stems, sepals, and petals indicate that phloem companion cells within the vascular tissue are the primary site of ABA biosynthesis in tissues dehydrated to an unknown water status (Kuromori et al., 2014). The concept that guard cells synthesize ABA comes from observations of the expression of ABA biosynthetic genes in guard cells and the apparent restoration of stomata behavior in an ABA biosynthetic mutant with biosynthesis restored in the guard cells under a guard cell-specific promoter (Bauer et al., 2013). Recent work using cell-specific promoters suggests that constitutive expression of an ABA biosynthetic gene in both phloem companion cells and guard cells can restore stomatal responses to vapor pressure deficit (VPD) and that both of these sites are responsible for ABA biosynthesis in the leaf (Merilo et al., 2018). While the promoters used in these studies drive high gene expression in target tissues, it should be noted that none of them has exclusive fidelity to the intended cell types (Truernit and Sauer, 1995; Yang et al., 2008; Oh et al., 2011).

These studies have received much attention; however, they are not supported by the measurement of ABA levels in the hypothesized tissue and do not explain the rapid triggering of ABA biosynthesis by cell turgor or water status in angiosperms (McAdam et al., 2016b). In both of the proposed cell types, cell turgor and water status are autonomously regulated. Phloem turgor is actively regulated by solute loading (Knoblauch et al., 2016); limited evidence and modeling suggest that these tissues only lose turgor in the leaf when xylem water potential declines to a near-lethal level (Sovonick-Dunford et al., 1981; Thompson and Holbrook, 2003; An et al., 2014; Sevanto et al., 2014). Consequently, it is unlikely that a decline in companion cell turgor or water status occurs at high VPD to trigger rapid ABA biosynthesis. Similarly, guard cell turgor is autonomously regulated, changing dramatically in response to a range of environmental signals throughout the day. If cell turgor or water status was the trigger for ABA biosynthesis in guard cells, then this autonomous regulation of cell turgor should result in substantial changes in ABA levels in guard cells over the course of a day, initiating complex positive feedback loops. However, this situation is not observed in stomatal behavior.

In addition to the low likelihood of ABA biosynthesis being triggered by a drop in leaf water status in these tissues, there are more difficult questions that remain unanswered by these two recent models. Companion cells are a unique anatomical feature of angiosperms, being absent from lycophytes and ferns and functionally replaced by Strasburger cells, which have a different ontogeny in conifers (Sauter, 1980; Van Bel, 2003). Therefore, the companion cell model is unable to explain where ABA would be synthesized in species from these lineages. For guard cell autonomous ABA biosynthesis to regulate stomatal responses to water status (altered by either VPD or soil water status), plants would require highly complex hypothetical sensors and signaling pathways to activate ABA biosynthesis. Either, (i) a sensor that can translate humidity in the air and substomatal cavity to a transcriptional regulator of ABA biosynthetic genes, or (ii) a mobile signal from the xylem that rapidly reaches guard cells triggering ABA biosynthesis. Alternatively, guard cell turgor in angiosperm species could drop passively in response to high VPD, triggering localized ABA biosynthesis. However, this model would mean ABA is synthesized in guard cells under all conditions that lower guard cell turgor, like darkness and high CO_2, which seems unlikely. These signaling pathways would have to operate in addition to the water status trigger for ABA biosynthesis that has long been documented in leaves (Pierce and Raschke, 1981). Little evidence exists for these unique, requisite signaling pathways for guard cell autonomous ABA biosynthesis.

An alternative to these recent models is the classical theory that ABA biosynthesis likely occurs outside of the vascular tissue and the guard cells, in the mesophyll (Loveys, 1977; Mansfield et al., 1978; Dörffling et al., 1979). The mesophyll is in close proximity to the guard cells, being a cell type that experiences large changes in turgor or water status as VPD changes (Buckley et al., 2017). Therefore, it is the ideal candidate tissue for translating dynamic changes in water stress into rapid ABA biosynthesis required for angiosperm stomatal responses to VPD (McAdam et al., 2016b). In addition, the abundant chloroplasts in mesophyll cells provide a near limitless source of carotenoid precursors to fuel the continual biosynthesis of ABA, an essential requirement for maintaining stomatal closure during long periods of soil water deficit in many seed plant species (Brodribb et al., 2014; Nolan et al., 2017). To test this theory, we measured ABA levels in simple bench-drying experiments utilizing species with foliar anatomies that allowed us to isolate the mesophyll from the vascular tissue and in some cases the stomata.

RESULTS AND DISCUSSION

The angiosperm species *Hakea lissosperma* (Proteaceae), a native to dry forests in southeastern Australia, has a distinct cylindrical leaf anatomy composed of a central sclerified pith that contains the vascular bundles

that is surrounded by a band of palisade mesophyll interspersed with osteosclerids (Jordan et al., 2013). The manual separation of the palisade mesophyll from the central trace containing the vascular tissue of this species is straightforward. When the isolated palisade mesophyll of *H. lissosperma* was dried on the bench for 30 min, ABA levels significantly increased (Fig. 1). Unlike the chloroplast-rich palisade mesophyll, the central vascular region, which has very few chloroplasts, did not show a significant increase in ABA levels over the same period of bench drying (Fig. 1). Thirty minutes was sufficient time for relative water content in both tissues to decline (Supplemental Table S1). This short period of time was utilized to mimic the time taken for ABA biosynthesis to be significantly up-regulated by an increase in VPD (McAdam and Brodribb, 2015). Despite the recent suggestion that mobile signals from the roots are essential for triggering biosynthesis of ABA in leaves (Takahashi et al., 2018), our data add to a body of work showing that ABA levels in excised leaves can increase over the short period necessary to close stomata in response to an increase in VPD without a signal coming from the roots (Bauerle et al., 2004; McAdam and Brodribb, 2016; Qiu et al., 2017; Sussmilch et al., 2017).

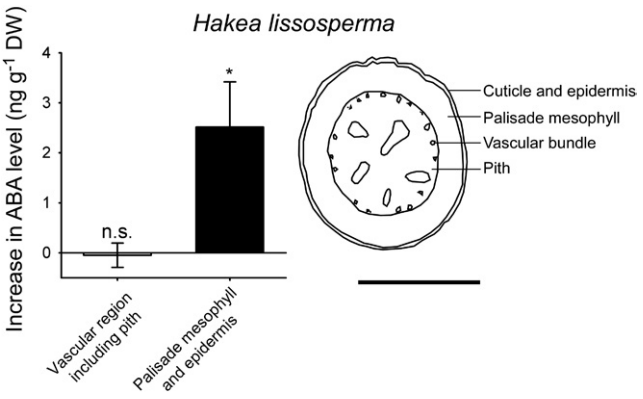

Figure 1. Mean increase in ABA levels in dissected and bench-dried leaf tissue of the angiosperm *H. lissosperma*. Separated tissue included the central vascular region, including vascular bundles and pith (white bar), and the mesophyll with epidermis (black bar). Tissues were dehydrated on the bench for 30 mins (right; $n = 6$ leaves). Error bars indicate 95% confidence interval; asterisk denotes significant changes (n.s., not significant; *, $P < 0.05$; ANOVA with posthoc Tukey's test). A cross-section of the leaf shows the central vascular region and surrounding mesophyll (scale bar = 1 mm).

While there are few angiosperm species with foliar anatomies that allow the easy isolation of the mesophyll from the vascular tissue, conifers provide an excellent experimental system because of the presence of a single vein in the leaf and an abundance of species with comparatively broad leaves (Biffin et al., 2012). Broad-leaved conifers have evolved multiple times, presumably as an adaptation to light-limited, angiosperm-dominated environments (Brodribb and Hill, 1997; Brodribb and Feild, 2008; Biffin et al., 2012). The last common ancestor of gymnosperms and angiosperms was likely the first land plant to have an unambiguous stomatal response to ABA. Extant gymnosperms synthesize this hormone when cells lose turgor (McAdam and Brodribb, 2014), but have been found to only utilize it to close stomata when soil water is limiting and not when VPD increases during the day because of slower rates of ABA biosynthesis compared with angiosperms (Brodribb et al., 2014; McAdam and Brodribb, 2014; Deans et al., 2017). In four diverse conifer species, *Saxegothaea conspicua* (Podocarpaceae), *Podocarpus latifolius* (Podocarpaceae), *Cephalotaxus harringtonii* (Taxaceae), and *Amentotaxus formosana* (Taxaceae), ABA levels in leaf lamina tissue free of xylem or phloem tissue were found to increase significantly after drying on the bench for 2 h (Fig. 2). This length of drying time also resulted in a drop in relative water content in all tissues (Supplemental Table S1). ABA levels also increased in the vascular region of the excised leaves. However, this increase cannot be attributed to synthesis occurring solely in the vascular tissue as the vascular bundle in these excised portions was still surrounded by photosynthetic mesophyll (Fig. 2). There was substantial variation between species in the degree of ABA synthesized in the various tissues, which may be related to the degree of desiccation experienced in the respective tissues. In *C. harringtonii*, more ABA biosynthesis occurred in the section of the leaf containing the vein. However, it should be noted that a very high density of chloroplast-containing mesophyll cells are clustered around the leaf vein of *C. harringtonii*, with the lamina of the leaf containing large intercellular air spaces and comparatively fewer mesophyll cells (Ghimire et al., 2014).

S. conspicua and *A. formosana* were specifically chosen because they have hypostomatic leaves with stomata in distinct abaxial bands on either side of the central vein (Cope, 1998; Mill and Stark Schilling, 2009), which could easily be removed by shallow paradermal section. After removing stomata and excising the vascular region, the remaining tissue was used to test whether mesophyll without stomata synthesizes ABA. We found significant ABA biosynthesis in the mesophyll tissue without stomata-bearing epidermis or veins in both species (Fig. 2). The degree of ABA biosynthesis in astomatal, veinless lamina tissue was similar to veinless leaf lamina that had a stomata-bearing epidermis attached, implying that stomata are not a major source of ABA when leaf water status declines.

Increases in ABA levels have been hypothesized to have a number of origins, including de novo biosynthesis, release from fettered stores in the chloroplasts (Georgopoulou and Milborrow, 2012), and conversion from catabolites (Lee et al., 2006). By extracting ABA in methanol, we can measure total levels of this hormone; however, to test whether increases in ABA levels were attributable to de novo biosynthesis and not catabolite conversion, we concurrently measured the levels of two key catabolites: phaseic acid (PA) and ABA-Glc

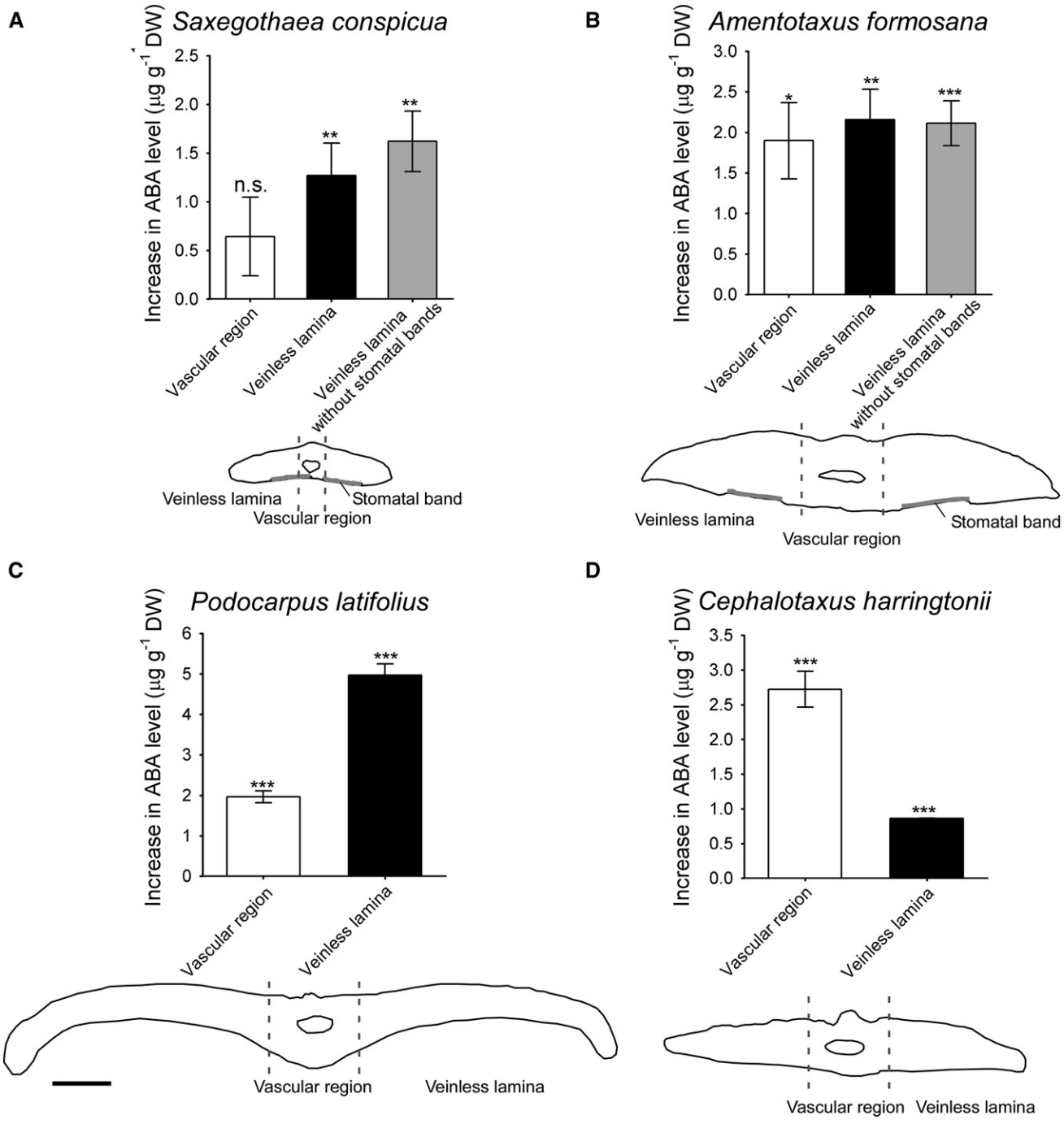

Figure 2. Mean increase in ABA levels in leaf regions following bench drying in four conifer species: A, *S. conspicua*; B, *C. harringtonii*; C, *A. formosana*; and D, *P. latifolius*. Tissue dissected included the region containing the vein (white bars), the remaining veinless lamina (black bars), and in *S. conspicua* and *A. formosana*, the veinless lamina with the stomatal bands excised (gray bars). Tissues were dehydrated on the bench for 120 mins ($n = 6$ leaves). Error bars indicate 95% confidence interval; asterisks denote significant changes (n.s., not significant; **, $P < 0.01$; ***, $P < 0.001$; ANOVA with posthoc Tukey's test). A cross-section of the leaf of each species is shown, including dashed lines depicting the excision zone to separate the respective regions prior to desiccation (scale bar for all sketches = 1 mm).

ester (ABAGE) in *A. formosana*. We found no significant change in ABAGE levels in tissues and a significant reduction in PA levels in only one tissue type. However, this reduction was 10-fold smaller than the increase in ABA levels, likely reflecting a reduction in catabolism rate when ABA is synthesized rather than evidence for catabolite conversion being the source of increased ABA levels (Supplemental Fig. S1).

Through measuring ABA levels in bench-dried leaves and utilizing natural diversity in leaf form, we show that considerable ABA biosynthesis occurs in the mesophyll. In *H. lissosperma*, the only species in which we could completely separate all mesophyll from the vascular tissue, it appeared that no significant ABA biosynthesis occurred in the vascular tissue over 30 mins of dehydration. This result contrasts with those from the labeling of ABA biosynthetic gene promoters, transcripts, and proteins in Arabidopsis leaves and stems bench-dried for 3 to 6 h to an unknown water status, which suggested that significant ABA biosynthesis occurs in the phloem (Endo et al., 2008; Kuromori et al., 2014). There are a number of possible explanations for our contrasting conclusions. No study proposing a phloem or companion cell source of ABA measured levels of ABA in the tissues hypothesized, and all were conducted in leaves either exposed to between 3 and 6 h of bench drying or, in some cases, no water stress at all. It may be that after 6 h of bench drying, a severe loss of turgor in the leaf triggers a loss of turgor in the phloem in Arabidopsis, and subsequently, a transcriptional up-regulation of ABA biosynthetic genes in these cells swamping any signal from mesophyll cells. Regrettably, these molecular-based studies were not conducted over the rapid timeframes (less than 30 mins) known to increase ABA levels in angiosperms, nor have the labeled promoter lines been used to observe biosynthetic location in leaves under natural water stress. The recent observation of a restoration of stomatal phenotype in an ABA biosynthetic mutant background overexpressing ABA biosynthetic genes in either guard cells or phloem companion cells has been cited as strong evidence for guard cell synthesis regulating stomatal behavior. However, the complete restoration of normal foliar ABA levels by these transgenic manipulations (Merilo et al., 2018) given the minor contribution of guard cell ABA levels to the total pool of foliar ABA levels somewhat undermines these conclusions.

In terms of providing a functional translation of leaf water content into ABA levels to regulate stomata, the mesophyll provides an ideal tissue for ABA biosynthesis. The mesophyll cells contain an abundance of chloroplasts and high levels of carotenoid precursors, ensuring that ABA biosynthesis is never substrate limited. Carotenoid substrate limitation severely hampers localized ABA biosynthesis in water-stressed roots, such that biosynthesis is never sufficient to ensure continuous production of ABA in these tissues during water stress (Manzi et al., 2015). Dramatic changes in cell turgor and water status occur in the mesophyll, with these changes in cell water status being most pronounced as the leaf approaches turgor loss (Zhang et al., 2016; Buckley et al., 2017). Modeling suggests that there is a considerable drop in cell water potential and water content across the areole of the leaf during diurnal changes in VPD (Buckley et al., 2017). Moreover, recent high-powered thermal imaging observations of a leaf at high VPD have documented considerable stomatal closure in the center of the areole (Sweet et al., 2017). This patchy pattern may be explained by a large drop in mesophyll water status away from the veins, thereby triggering localized ABA biosynthesis in the center of the areole. Third, the mesophyll cells are in close proximity to the guard cells, meaning that a minimal lag time between ABA biosynthesis and stomatal closure would occur. Finally, an outside guard cell synthesis of ABA would explain stomatal phenotypes in the ABA transporter mutants responsible for the symplastic to apoplastic movement of ABA across the guard cell membrane (Kuromori et al., 2017). In conclusion, our data support early models of ABA biosynthesis occurring in the mesophyll of leaves when water stressed, and that ABA biosynthesis in the companion cells or guard cells is unlikely to contribute to the pool of ABA that regulates stomatal aperture in seed plants.

MATERIALS AND METHODS

Conifers were grown in pots in an open-walled glasshouse with no environmental control, receiving daily watering and weekly applications of liquid nutrients. *Hakea lissosperma* leaves were taken from a mature plant grown on the grounds of the University of Tasmania (Hobart, Tasmania, Australia). All leaf material for experiments was collected before 08:00 in the morning on rainy days to ensure leaves were fully hydrated and initial foliar ABA levels were low.

Excised leaves were dissected on a damp paper towel. For the leaves of conifer species, a razor blade was used to separate the central vein from the veinless lamina on either side. In addition to separating the vascular tissue from the veinless lamina in a subset of *Saxegothaea conspicua* and *Amentotaxus formosana* leaves, shallow paradermal sections were undertaken to remove the stomatal bands on the abaxial surface prior to removing the vein. In *H. lissosperma*, the palisade mesophyll could be readily separated from the central sclerified trace containing the vascular bundles manually. Once dissected and prior to bench drying, isolated tissue was taken for ABA quantification as a prestressed control, and all tissues were weighed to determine turgid weight for the calculation of relative water content after dehydration. Tissue was then dried on the bench for 30 mins in *H. lissosperma* because of the rapid speed of ABA biosynthesis in angiosperms, and for 120 mins in the conifer species due to the reported slower rate of ABA biosynthesis in these species. Once exposed to bench dehydration, tissue was weighed and taken for ABA quantification. ABA in tissue was extracted and purified; for samples from *H. lissosperma*, *Podocarpus latifolius*, *Cephalotaxus harringtonii*, and *S. conspicua*, ABA was quantified by physicochemical methods with an added internal standard using a UPLC-MS/MS according to the method of McAdam (2015). ABA, PA, and ABAGE levels were quantified in samples of *A. formosana* with added deuterated internal standards for each molecule using an Agilent 6400

series triple quadrupole LC/MS. Aliquots of extracts were taken for hormone analysis. Extracts were dried, and dry weight of the sample was determined. Relative water content was calculated as the water content in bench-dried samples as a percentage of the water in a fully turgid sample.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Mean increase in PA levels and ABAGE levels in leaf regions following bench drying in *A. formosana*.

Supplemental Table S1. Mean relative water content in the leaf regions examined after dehydration on the bench for 30 min in the angiosperm *H. lissosperma* and 120 min in four conifer species.

ACKNOWLEDGMENTS

We thank David Nichols for running ABA samples in Tasmania, Amber Jannasch for assistance developing the methods for quantifying hormones and catabolites in Indiana, and John Ross for his freely given, transpacific advice. We acknowledge the use of the facilities of the Bindley Bioscience Center, a core facility of the National Institutes of Health-funded Indiana Clinical and Translational Sciences Institute.

Received January 2, 2018; accepted April 25, 2018; published May 7, 2018.

LITERATURE CITED

An F, Cahill D, Rookes J, Lin W, Kong L (2014) Real-time measurement of phloem turgor pressure in *Hevea brasiliensis* with a modified cell pressure probe. Bot Stud (Taipei, Taiwan) **55**: 19

Bauer H, Ache P, Lautner S, Fromm J, Hartung W, Al-Rasheid KA, Sonnewald S, Sonnewald U, Kneitz S, Lachmann N, (2013) The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. Curr Biol **23**: 53–57

Bauerle WL, Whitlow TH, Setter TL, Vermeylen FM (2004) Abscisic acid synthesis in *Acer rubrum* L. leaves—a vapor-pressure-deficit-mediated response. J Am Soc Hortic Sci **129**: 182–187

Biffin E, Brodribb TJ, Hill RS, Thomas P, Lowe AJ (2012) Leaf evolution in Southern Hemisphere conifers tracks the angiosperm ecological radiation. Proc Biol Sci **279**: 341–348

Brandt B, Brodsky DE, Xue S, Negi J, Iba K, Kangasjärvi J, Ghassemian M, Stephan AB, Hu H, Schroeder JI (2012) Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. Proc Natl Acad Sci USA **109**: 10593–10598

Brodribb TJ, Feild TS (2008) Evolutionary significance of a flat-leaved *Pinus* in Vietnamese rainforest. New Phytol **178**: 201–209

Brodribb T, Hill RS (1997) Light response characteristics of a morphologically diverse group of southern hemisphere conifers as measured by chlorophyll fluorescence. Oecologia **110**: 10–17

Brodribb TJ, McAdam SAM, Jordan GJ, Martins SCV (2014) Conifer species adapt to low-rainfall climates by following one of two divergent pathways. Proc Natl Acad Sci USA **111**: 14489–14493

Buckley TN, John GP, Scoffoni C, Sack L (2017) The sites of evaporation within leaves. Plant Physiol **173**: 1763–1782

Christmann A, Hoffmann T, Teplova I, Grill E, Müller A (2005) Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed Arabidopsis. Plant Physiol **137**: 209–219

Christmann A, Weiler EW, Steudle E, Grill E (2007) A hydraulic signal in root-to-shoot signalling of water shortage. Plant J **52**: 167–174

Cope EA (1998) Taxaceae: the genera and cultivated species. Bot Rev **64**: 291–322

Deans RM, Brodribb TJ, McAdam SAM (2017) An integrated hydraulic-hormonal model of conifer stomata predicts water stress dynamics. Plant Physiol **174**: 478–486

Dörffling K, Tietz D, Streich J, Ludewig M (1979) Studies on the role of abscisic acid in stomatal movements. *In* **F Skoog**, ed, Plant Growth Substances. Springer, Berlin, pp 274–285

Endo A, Sawada Y, Takahashi H, Okamoto M, Ikegami K, Koiwai H, Seo M, Toyomasu T, Mitsuhashi W, Shinozaki K, (2008) Drought induction of Arabidopsis 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. Plant Physiol **147**: 1984–1993

Firn RD, Friend J (1972) Enzymatic production of the plant growth inhibitor, xanthoxin. Planta **103**: 263–266

Geiger D, Maierhofer T, Al-Rasheid KA, Scherzer S, Mumm P, Liese A, Ache P, Wellmann C, Marten I, Grill E, (2011) Stomatal closure by fast abscisic acid signaling is mediated by the guard cell anion channel SLAH3 and the receptor RCAR1. Sci Signal **4**: ra32

Georgopoulou Z, Milborrow BV (2012) Initiation of the synthesis of 'stress' ABA by (+)-[2H6]ABA infiltrated into leaves of *Commelina communis*. Physiol Plant **146**: 149–159

Ghimire B, Lee C, Heo K (2014) Leaf anatomy and its implications for phylogenetic relationships in Taxaceae *s. l.* J Plant Res **127**: 373–388

Heilmann B, Hartung W, Gimmler H (1980) The distribution of abscisic acid between chloroplasts and cytoplasm of leaf cells and the permeability of the chloroplast envelope for abscisic acid. Z Pflanzenphysiol **97**: 67–78

Holbrook NM, Shashidhar VR, James RA, Munns R (2002) Stomatal control in tomato with ABA-deficient roots: response of grafted plants to soil drying. J Exp Bot **53**: 1503–1514

Ikegami K, Okamoto M, Seo M, Koshiba T (2009) Activation of abscisic acid biosynthesis in the leaves of *Arabidopsis thaliana* in response to water deficit. J Plant Res **122**: 235–243

Jordan GJ, Brodribb TJ, Blackman CJ, Weston PH (2013) Climate drives vein anatomy in Proteaceae. Am J Bot **100**: 1483–1493

Knoblauch M, Knoblauch J, Mullendore DL, Savage JA, Babst BA, Beecher SD, Dodgen AC, Jensen KH, Holbrook NM (2016) Testing the Münch hypothesis of long distance phloem transport in plants. eLife **5**: e15341

Koiwai H, Nakaminami K, Seo M, Mitsuhashi W, Toyomasu T, Koshiba T (2004) Tissue-specific localization of an abscisic acid biosynthetic enzyme, AAO3, in Arabidopsis. Plant Physiol **134**: 1697–1707

Kuromori T, Sugimoto E, Shinozaki K (2014) Intertissue signal transfer of abscisic acid from vascular cells to guard cells. Plant Physiol **164**: 1587–1592

Kuromori T, Sugimoto E, Ohiraki H, Yamaguchi-Shinozaki K, Shinozaki K (2017) Functional relationship of AtABCG21 and AtABCG22 in stomatal regulation. Sci Rep **7**: 12501

Lee H-S, Milborrow BV (1998) Endogenous biosynthetic precursors of (+)-abscisic acid. IV. Biosynthesis of ABA from [$2H_n$] carotenoids by a cell-free system from avocado. Funct Plant Biol **24**: 715–726

Lee KH, Piao HL, Kim H-Y, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee I-J, Hwang I (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. Cell **126**: 1109–1120

Loveys BR (1977) The intracellular location of abscisic acid in stressed and non-stressed leaf tissue. Physiol Plant **40**: 6–10

Mansfield TA, Wellburn AR, Moreira TJS (1978) The role of abscisic acid and farnesol in the alleviation of water stress. Philos Trans R Soc Lond B Biol Sci **284**: 471–482

Manzi M, Lado J, Rodrigo MJ, Zacarías L, Arbona V, Gómez-Cadenas A (2015) Root ABA accumulation in long-term water-stressed plants is sustained by hormone transport from aerial organs. Plant Cell Physiol **56**: 2457–2466

McAdam SAM (2015) Physicochemical quantification of abscisic acid levels in plant tissues with an added internal standard by ultra-performance liquid chromatography. Bio Protoc **5**: e1599

McAdam SAM, Brodribb TJ (2014) Separating active and passive influences on stomatal control of transpiration. Plant Physiol **164**: 1578–1586

McAdam SAM, Brodribb TJ (2015) The evolution of mechanisms driving the stomatal response to vapor pressure deficit. Plant Physiol **167**: 833–843

McAdam SAM, Brodribb TJ (2016) Linking turgor with ABA biosynthesis: implications for stomatal responses to vapor pressure deficit across land plants. Plant Physiol **171**: 2008–2016

McAdam SAM, Brodribb TJ, Ross JJ (2016a) Shoot-derived abscisic acid promotes root growth. Plant Cell Environ **39**: 652–659

McAdam SAM, Sussmilch FC, Brodribb TJ (2016b) Stomatal responses to vapour pressure deficit are regulated by high speed gene expression in angiosperms. Plant Cell Environ **39**: 485–491

Merilo E, Yarmolinsky D, Jalakas P, Parik H, Tulva I, Rasulov B, Kilk K, Kollist H (2018) Stomatal VPD response: there is more to the story than ABA. Plant Physiol **176**: 851–864

Mill RR, Stark Schilling DM (2009) Cuticle micromorphology of *Saxegothaea* (Podocarpaceae). Bot J Linn Soc **159**: 58–67

Mittelheuser CJ, Van Steveninck RFM (1969) Stomatal closure and inhibition of transpiration induced by (RS)-abscisic acid. Nature **221**: 281–282

Nolan RH, Tarin T, Santini NS, McAdam SAM, Ruman R, Eamus D (2017) Differences in osmotic adjustment, foliar abscisic acid dynamics, and stomatal regulation between an isohydric and anisohydric woody angiosperm during drought. Plant Cell Environ **40**: 3122–3134

Oh JE, Kwon Y, Kim JH, Noh H, Hong S-W, Lee H (2011) A dual role for MYB60 in stomatal regulation and root growth of *Arabidopsis thaliana* under drought stress. Plant Mol Biol **77**: 91–103

Pierce M, Raschke K (1981) Synthesis and metabolism of abscisic acid in detached leaves of *Phaseolus vulgaris* L. after loss and recovery of turgor. Planta **153**: 156–165

Qin X, Zeevaart JAD (1999) The 9-*cis*-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. Proc Natl Acad Sci USA **96**: 15354–15361

Qiu C, Ethier G, Pepin S, Dubé P, Desjardins Y, Gosselin A (2017) Persistent negative temperature response of mesophyll conductance in red raspberry (*Rubus idaeus* L.) leaves under both high and low vapour pressure deficits: a role for abscisic acid? Plant Cell Environ **40**: 1940–1959

Sack L, John GP, Buckley TN (2018) ABA accumulation in dehydrating leaves is associated with decline in cell volume, not turgor pressure. Plant Physiol **176**: 489–495

Sauter JJ (1980) The Strasburger cells—equivalents of companion cells. Ber Dtsch Bot Ges **93**: 29–42

Sevanto S, McDowell NG, Dickman LT, Pangle R, Pockman WT (2014) How do trees die? A test of the hydraulic failure and carbon starvation hypotheses. Plant Cell Environ **37**: 153–161

Sovonick-Dunford S, Lee DR, Zimmermann MH (1981) Direct and indirect measurements of phloem turgor pressure in white ash. Plant Physiol **68**: 121–126

Sussmilch FC, Brodribb TJ, McAdam SAM (2017) Up-regulation of *NCED3* and ABA biosynthesis occur within minutes of a decrease in leaf turgor but *AHK1* is not required. J Exp Bot **68**: 2913–2918

Sweet KJ, Peak D, Mott KA (2017) Stomatal heterogeneity in responses to humidity and temperature: testing a mechanistic model. Plant Cell Environ **40**: 2771–2779

Takahashi F, Suzuki T, Osakabe Y, Betsuyaku S, Kondo Y, Dohmae N, Fukuda H, Yamaguchi-Shinozaki K, Shinozaki K (2018) A small peptide modulates stomatal control via abscisic acid in long-distance signalling. Nature **556**: 235–238

Takezawa D, Komatsu K, Sakata Y (2011) ABA in bryophytes: how a universal growth regulator in life became a plant hormone? J Plant Res **124**: 437–453

Tan BC, Cline K, McCarty DR (2001) Localization and targeting of the VP14 epoxy-carotenoid dioxygenase to chloroplast membranes. Plant J **27**: 373–382

Tan B-C, Joseph LM, Deng W-T, Liu L, Li Q-B, Cline K, McCarty DR (2003) Molecular characterization of the *Arabidopsis* 9-*cis* epoxycarotenoid dioxygenase gene family. Plant J **35**: 44–56

Taylor HF, Burden RS (1972) Xanthoxin, a recently discovered plant growth inhibitor. Proc R Soc Lond B Biol Sci **180**: 317–346

Taylor HF, Smith TA (1967) Production of plant growth inhibitors from xanthophylls: a possible source of dormin. Nature **215**: 1513–1514

Thompson MV, Holbrook NM (2003) Scaling phloem transport: water potential equilibrium and osmoregulatory flow. Plant Cell Environ **26**: 1561–1577

Truernit E, Sauer N (1995) The promoter of the Arabidopsis thaliana SUC2 sucrose-H+ symporter gene directs expression of beta-glucuronidase to the phloem: evidence for phloem loading and unloading by SUC2. Planta **196**: 564–570

Van Bel AJE (2003) The phloem, a miracle of ingenuity. Plant Cell Environ **26**: 125–149

Yang Y, Costa A, Leonhardt N, Siegel RS, Schroeder JI (2008) Isolation of a strong *Arabidopsis* guard cell promoter and its potential as a research tool. Plant Methods **4**: 6

Zhang J, Schurr U, Davies WJ (1987) Control of stomatal behaviour by abscisic acid which apparently originates in the roots. J Exp Bot **38**: 1174–1181

Zhang Y-J, Rockwell FE, Graham AC, Alexander T, Holbrook NM (2016) Reversible leaf xylem collapse: a potential "circuit breaker" against cavitation. Plant Physiol **172**: 2261–2274