

ABA Accumulation in Dehydrating Leaves Is Associated with Decline in Cell Volume, Not Turgor Pressure^{1[OPEN]}

Lawren Sack,^{a,2} Grace P. John,^a and Thomas N. Buckley^b

^aDepartment of Ecology and Evolutionary Biology, University of California, Los Angeles, Los Angeles, California, 90095

^bDepartment of Plant Sciences, University of California, Davis, Davis, California 95616

ORCID IDs: 0000-0002-7009-7202 (L.S.); 0000-0002-8045-5982 (G.P.J.).

Identifying the mechanisms for cell responses as plants dehydrate is crucial for analyzing and predicting crop and ecosystem responses to climate change (Blum, 1996, 2017; Bartlett et al., 2016), for isolating the proteins and the genes underlying the responses (Christmann et al., 2013), and for the design of model plants and crops with increased water use efficiency and/or drought tolerance (e.g. Nemhauser and Torii, 2016; Yang et al., 2016). The dehydration-sensing mechanisms involved in driving the accumulation of the hormone abscisic acid (ABA; see symbols in Table I) are of special importance as it is implicated in stomatal closure during drought (Rodríguez-Domínguez et al., 2016) or increasing vapor pressure deficit (McAdam et al., 2016), and may contribute to the decline of leaf hydraulic conductance (Shatil-Cohen et al., 2011; Pantin et al., 2013). Cellular ABA accumulation during dehydration may occur due to modulation of transport from cellular or apoplastic stores, de novo synthesis, and/or turnover (Finkelstein, 2013). However, disentangling the factors that leaf cells sense during dehydration is difficult as many changes typically occur in tandem: turgor is lost, solute concentrations increase, relative water content (RWC) decreases, and cell membranes shrink, altering interactions with the cytoskeleton and cell wall (Haswell and Verslues, 2015). Two recent articles (McAdam and Brodribb, 2016; Sussmilch et al., 2017) have argued based on applying external pressure to leaves that turgor loss provides the endogenous signal triggering ABA accumulation and that species differ greatly in the turgor loss threshold that triggers ABA accumulation. We derived new equations from plant water relations theory enabling the calculation of turgor, solute potential, and RWC for the experimental leaves in those studies. These calculations establish that

the accumulation of ABA in these artificially dehydrated leaves was not due to decline of turgor pressure but instead was associated with the decline of RWC. These analyses further show that the RWC loss associated with ABA accumulation varied by approximately 10% across the diverse angiosperm species, indicating functional convergence in cellular drought sensing and providing clues for identification of the components of the signaling pathway.

The debate on the precise determinants of ABA accumulation began decades ago. In a dehydrating leaf, cell volume, turgor, osmotic potential, and leaf water potential decline together, and making a distinction among these may seem at first semantic. However, it is critical to distinguish exactly which of these or related physical properties is ultimately sensed and leads to ABA accumulation. For example, changes in cell volume independently of turgor may affect sensors of cytoskeletal properties, ion concentrations or ion transport rates, or cell membrane interactions with the cell wall, whereas sensing of membrane tension might be affected by volume and/or turgor. The idea that turgor loss was the driver for ABA accumulation arose from early experiments showing the hormone levels increased in drying leaves as leaf water potential (Ψ_{leaf}) declined (e.g. Zabadal, 1974; Beardsell and Cohen, 1975; Wright, 1977), and was later further supported circumstantially by the finding that in many species, stomatal closure, known to be driven by ABA levels, apparently coincides roughly, on average, with turgor loss point (global data recently synthesized in Bartlett et al., 2016). Subsequent experiments took the necessary next step by dehydrating leaves of several species on the bench top and measuring ABA accumulation, and used pressure volume curves to estimate solute and pressure potentials from leaf water potentials (Pierce and Raschke, 1980). These calculations showed that increases in ABA accumulation correlated more closely with the decline of turgor pressure (Ψ_p) than with the declines of either osmotic potential (Ψ_s) or Ψ_{leaf} . Yet, those studies did not consider the decline of RWC as a potential driver.

Subsequent experiments confirmed that Ψ_s did not drive ABA accumulation: leaf sections of spinach (*Spinacia oleracea*) and maize (*Zea mays*) accumulated ABA if incubated in mannitol or polyethylene glycol, which

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² Address correspondence to lawrensack@ucla.edu.

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Table 1. Symbols used in the text

Symbol	Term	Unit
ABA	Absciscic acid	n/a
Ψ_{leaf}	Bulk leaf water potential	MPa
Ψ_S	Osmotic potential, a.k.a. solute potential	MPa
Ψ_P	Pressure potential, a.k.a. turgor pressure	MPa
$\Psi_{S,o}$	Solute potential at full turgor	MPa
$\Psi_{S,tlp}$	Solute potential at turgor loss point	MPa
a_t	Apoplastic water fraction at full hydration	%
Ψ_x	Apoplast (and xylem) water potential	MPa
P_x	Apoplast (and xylem) pressure potential	MPa
Total RWC	Total relative water content of apoplast and symplast	MPa
Symplastic RWC	Relative water content in the symplast (cellular compartment)	%
RWC _{tlp}	Relative water content at turgor loss point	%
c	Solute concentration	mol L ⁻¹

dehydrated the leaf, but not when incubated with ethylene glycol, which penetrates the cell membrane and thus decreases Ψ_S with only transient changes in Ψ_P or RWC (Creelman and Zeevaart, 1985; Jia et al., 2001). Additionally, osmotic adjustment (i.e. the decrease of Ψ_S) generally enhances or sustains gas exchange during drought, whereas if decreased Ψ_S per se enhanced ABA accumulation, one would expect the opposite response (Turner et al., 1978). In subsequent years, with the increasing recognition of the importance of xylem negative pressure (tension) in driving cavitation and the importance of water potential and xylem pressure gradients as driving forces for water movement in the soil-plant-atmosphere continuum (Kramer, 1988; Tyree and Zimmermann, 2002), Ψ_{leaf} and leaf Ψ_P have eclipsed changes in cell volume or RWC as indicator variables for predicting plant function during drought.

Subsequent studies, however, suggested that Ψ_P decline is not in fact the primary determinant of ABA production. When cotton (*Gossypium hirsutum*) or maize leaves were dehydrated under sustained pressure in a pressure chamber, such that the leaves lost water by extrusion through the petiole, ABA accumulated (Ackerson and Radin, 1983; Jia et al., 2001). The authors argued that pressurizing the leaves during dehydration maintained cell turgor, and thus that ABA accumulation was driven instead by cellular volume shrinkage or relaxation of the cell wall, i.e. corresponding to a decline in RWC or volume, independently of Ψ_P . An important control showed that pressurizing leaves entirely enclosed within the pressure chamber—without the petiole protruding and thus without leaf water loss—only led to minimal stimulation of ABA accumulation, indicating that increases in Ψ_P alone were not the stimulus (Ackerson and Radin, 1983). This same approach was revived in recent articles (McAdam and Brodribb, 2016; Sussmilch et al., 2017), though these authors argued that the application of external pressure would reduce leaf turgor and that this reduction of turgor triggered ABA accumulation. In these experiments, leaves were treated in a pressure chamber with petiole protruding, and subjected to a range of

pressures (in 0.5 MPa intervals from 0 to 3.5 MPa) for 20 or 60 min, after which they were immediately snap-frozen and analyzed for ABA concentration (McAdam and Brodribb, 2016). In a subsequent study, this approach was applied to Arabidopsis (*Arabidopsis thaliana*) leaves pressurized at 1.5 MPa for 1, 5, 10, or 20 min (Sussmilch et al., 2017). Leaves of angiosperm species subjected to sufficient pressures for a long enough time showed increased ABA accumulation, whereas the three conifer species, two fern species, and one lycophyte species tested did not, and the authors concluded that turgor reduction was responsible for triggering ABA accumulation in angiosperms. This conclusion was based on the assumption that turgor pressure declined in the treated leaves from its value in fully hydrated leaves (determined from pressure volume curves) by an amount equal to the applied external pressure, and plots of [ABA] increase against reduction of turgor calculated in this way apparently showed threshold responses, which varied strongly across the four angiosperm species (supplemental figure S1 of McAdam and Brodribb, 2016). A major advance of these experiments is that they used modern analytical methods to show ABA accumulation occurred at lesser levels and durations of dehydration than previously thought based on earlier work. The authors argued that subtle decreases of turgor would drive ABA production in angiosperms, thus triggering stomatal closure. They argued further that the turgor threshold for ABA production varied strongly across species and was closely related to their turgor loss points (see differences in thresholds required in Table II).

We show here that applying external pressure to the leaf increases cell turgor throughout the leaf, and thus that decrease in Ψ_P is not itself the stimulus for ABA accumulation. Indeed, the external pressure dehydration treatment (Ackerson and Radin, 1983; Jia et al., 2001; McAdam and Brodribb, 2016; Sussmilch et al., 2017) enables the independent resolution of Ψ_P decline from RWC decline, whereas in vivo these typically occur together. The method thus provides a powerful tool to distinguish these as drivers of physiological responses. We derived new theory from the pressure chamber equations to

Table II. New analyses of cell water relation parameters during external pressurization experiments that simulated ABA accumulation (McAdam and Brodribb, 2016; Sussmilch et al., 2017)

Pressure volume curve parameters are provided (based on methods of Sack et al., 2010): osmotic potential at full turgor ($\Psi_{s,0}$) and at turgor loss point ($\Psi_{s,lp}$), the relative water content (RWC) at turgor loss point (RWC_{lp}), and the apoplastic fraction (a_i); see Appendix and Supplemental Data S1. The external pressure threshold for ABA production and the minimum tested time necessary for the pressure treatment to induce ABA production are provided, as well as calculated values for the water relations of the leaves using the equations provided in the Appendix: total and symplastic RWC, osmotic and turgor potentials (Ψ_s and Ψ_p , respectively), and the % increase in Ψ_p , the % decrease in Ψ_s , the % increase in solute concentration c (estimated given that $\Psi_s = -RTc$, where R is the ideal gas constant and T is temperature), and the declines in total and symplastic RWC relative to turgid leaves. While the four first rows are based on data from McAdam and Brodribb (2016), the last row, marked with an asterisk, is from Sussmilch et al. (2017), and the external pressure was not a threshold for ABA accumulation but chosen to exceed turgor loss point.

Species	During External Pressure Treatment														
	$\Psi_{s,0}$	$\Psi_{s,lp}$	RWC _{lp}	a_i	External Pressure Threshold	Shortest Time Req'd	Total RWC	Symplastic RWC	Ψ_p	Ψ_s	Ψ_p Increase	Ψ_s Decrease	c Increase	Total RWC Decline	Symplastic RWC Decline
	MPa	MPa	%	%	MPa	min					%	MPa	%	%	%
<i>Arabidopsis thaliana</i>	−0.684	−0.807	87.7	19.4	0.5	20	0.923	0.905	0.76	−0.76	10.5	−0.072	10.5	7.66	9.51
<i>Pisum sativum</i>	−0.927	−1.29	80.2	29.6	1	60	0.843	0.777	1.19	−1.19	28.7	−0.266	28.7	15.7	22.3
<i>Nothofagus cunninghamii</i>	−0.955	−1.24	96.0	82.8	1	20	0.967	0.810	1.18	−1.18	23.4	−0.223	23.4	3.25	18.9
<i>Olea oleaster</i>	−1.91	−2.34	91.6	53.6	1.5	60	0.945	0.882	2.17	−2.17	13.4	−0.256	13.4	5.48	11.8
<i>Arabidopsis thaliana</i> *	−0.684	−0.807	87.7	19.4	1.5*	5	0.562	0.456	1.50	−1.50	119	−0.816	119	43.8	54.4

quantitatively determine their changes within the treated leaves in the recent high-resolution studies (McAdam and Brodribb, 2016; Sussmilch et al., 2017).

For a leaf being dehydrated at a given applied pressure in the pressure chamber, once water is extruded from the petiole and has stopped flowing at that pressure, and the leaf equilibrates at the new balance pressure, the xylem is at equilibrium with the atmosphere and thus

$$P_x = 0 \text{ MPa},$$

where P_x is the pressure in the xylem and 0 is gauge pressure (relative to atmospheric pressure). Assuming the solute potential of the xylem and apoplast is negligible (i.e. less negative than -0.05 MPa; Scoffoni et al., 2012), then

$$\Psi_x \cong 0 \text{ MPa}.$$

In a leaf held at balance pressure until water ceases to exude from the petiole and equilibrates among cells and tissues, the bulk cell water potential will equal that of the xylem and apoplast surrounding the cells, so it follows that

$$\Psi_{\text{leaf}} \cong 0 \text{ MPa}$$

because $\Psi_{\text{leaf}} = \Psi_p + \Psi_s$, at balance pressure,

$$\Psi_p \cong -\Psi_s.$$

During these experiments, in which the leaf is subjected to pressure in the pressure chamber, given that water has been squeezed out of cells, Ψ_s has become more negative relative to its value in a leaf at full turgor. Therefore, Ψ_p will have increased, not decreased as assumed by McAdam and Brodribb (2016) and Sussmilch et al. (2017).

Our new analysis of the pressure volume equations (see Appendix) enables quantification of the RWC, Ψ_p and Ψ_s for the treated leaves in those authors' experiments, and shows that the increase of Ψ_p was substantial: at the threshold pressures that corresponded to ABA accumulation, Ψ_p had increased by 11% to 119% (Table II). Our analysis also establishes that declining Ψ_{leaf} itself was not the driver, because it was equal across treatments and held at $\cong 0$ MPa. Assuming that ABA accumulation was not driven by the increase of Ψ_p (Ackerson and Radin, 1983), nor by the reduction of Ψ_s associated with the increase in cellular solute concentrations (Creelman and Zeveaart, 1985), the decline of cell volume and its associated processes would be responsible (Table III). We note that a strong but very brief reduction of Ψ_p would have been triggered when the external pressure was released and the leaf removed from the chamber before snap freezing for ABA analysis. However, ABA accumulation was closely related to the duration of the pressure chamber treatment (figures 1 and 2 of McAdam and Brodribb, 2016), which indicates that it was not this brief exposure to a low Ψ_p that drove ABA accumulation.

Table III. Summary of evidence for and against the declines in leaf water potential (Ψ_{leaf}), turgor potential (Ψ_p), solute potential (Ψ_s), or RWC or cell volume as drivers of ABA accumulation in previous studies, and whether the putative driver is supported as important for ABA accumulation in studies using externally applied pressure, as analyzed in Table II

Cell Behavior during Dehydration Potentially Driving ABA Accumulation	Correlative Evidence for a Role in Driving ABA Accumulation	Evidence against a Role in Driving ABA Accumulation	Supported by Observed Effect of External Pressure on ABA Accumulation?
1. Leaf water potential decline	Correlation with ABA production in dehydrating leaves ^a	Weak relation with ABA production in dehydrating leaves ^a	No: Ψ_{leaf} is 0 for the treated leaves
2. Turgor pressure decline	Correlation with ABA production in dehydrating leaves ^a	–	No: Ψ_p increased in the treatment ^{b,c} ; this itself should not cause ABA accumulation ^c
3. Solute potential decline	–	Weak relation with ABA production in dehydrating leaves ^a No relation to ABA production in leaf samples floated on solute solutions ^d	Potentially: Ψ_s declined in the treatment ^b
4. Relative water content or cell volume decline	Correlation with ABA production in dehydrating leaves ^a	–	Yes: Increase of ABA coincided with RWC decline in four studies ^{b,c}

^aPierce and Raschke (1980) and references therein. ^bOur analysis of data of McAdam and Brodribb (2016); Sussmilch et al. (2017; Table I).
^cAckerson and Radin (1983); Jia et al. (2001). ^dCreelman and Zeevaart (1985); Jia et al. (2001).

The decline of RWC during dehydration would correspond to reduction of cell volumes within the leaf. While a substantial portion of the leaf water is apoplastic (i.e. within cell walls or xylem), this apoplastic water would be “bound” by surface tension in the cell wall pores or xylem conduits, until very strong tissue dehydration would trigger embolism and drain xylem conduits. Thus, under mild dehydration above turgor loss point, whether naturally or using the pressure chamber, the loss of leaf water would be virtually all cellular, and would necessitate volume shrinkage of cells, such that declining RWC would correspond to declining cell volumes.

The idea that turgor loss drove ABA accumulation led to the conclusion that angiosperm species showed striking differences in their water status thresholds for rapid increases in ABA levels, coinciding with their strong differences in turgor loss point (Table II; McAdam and Brodribb, 2016). However, our finding of the importance of cell shrinkage instead emphasizes potential convergence, not diversity, in these thresholds across the tested species. The decline of RWC associated with ABA accumulation was 3% to 16% across the four species tested (McAdam and Brodribb, 2016), corresponding to symplastic RWC declines of 10% to 22% (Table II), although we note that calculating symplastic RWC entails estimating apoplastic water fraction by extrapolating pressure-volume curves, which contributes a level of uncertainty (Andersen et al., 1991; Wardlaw, 2005). Further, the intervals of external pressure applied in the experiments to determine the thresholds for ABA accumulation were rather wide, and higher resolution studies may show the range of RWC decline associated with ABA accumulation across diverse angiosperms to be yet narrower.

Given the association of ABA accumulation with RWC decline in these experiments, our findings lend support to a role for RWC decline in ABA accumulation during rapid changes in vapor pressure deficit. Further, the finding that volume loss rather than turgor loss is associated with ABA accumulation is consistent with additional experiments, e.g. showing that ABA accumulated in leaf disks floated on saline solutions (McAdam and Brodribb, 2016), as the cells would have reduced RWC as well as Ψ_p .

Why sense cell volume rather than turgor? Declining cell volume may trigger signals via sensors within the cytoskeleton, or the cell membrane (i.e. sensors of membrane tension, of membrane protein distances, of the increase of specific ions or metabolites), or at the interface of cell membrane and cell wall (Christmann et al., 2013; Kumar et al., 2013; Haswell and Verslues, 2015; Pandey, 2017). These proteins may directly sense negative effects on processes or structures threatened by cell volume shrinkage, such as the cytoskeleton, membrane-cell wall contacts, or ion transport, even before detrimental biochemical effects arise (Oliver, 1996; Zhang et al., 2001; Pandey 2017). By contrast, a direct detrimental impact of declining turgor on cell processes, independent of cell volume, has not been demonstrated to our knowledge in mesophyll cells, though key functions of specialized, often semi-isolated tissues do depend on the maintenance of critical positive pressures—e.g. growth, phloem translocation, guard cell opening, and plant movements.

Identification of dehydration sensors thus depends on knowing whether the decline of Ψ_p or RWC is important, as this will inform screens of ecotypes and mutants, and application of genetic association studies

to find key genes (Wohlbach et al., 2008; Haswell and Verslues, 2015; Gupta et al., 2016). Notably, in vivo, dehydration may initiate different processes throughout the mesophyll or surrounding tissues (i.e. epidermis, bundle sheath, vasculature) that may differ from the bulk leaf in RWC, Ψ_p , Ψ_s , and/or Ψ_{leaf} (Bennett et al., 1987). It is thus both a strength and a weakness that the external pressure experiment (Ackerson and Radin, 1983; Jia et al., 2001; McAdam and Brodribb, 2016; Susmilch et al., 2017) imposes an equal and simultaneous decline in water status in all leaf cells: this ensures the sensing cells' status is reflected in bulk leaf variables while precluding the determination of those cells' identity. New tools are needed to measure cell volumes and turgor, osmolyte movement, ion flux, and organelle and cell membrane tension cells in different tissues in dehydrating leaves. Such work will enable resolution of the most important thresholds for declines in function and triggers for active processes such as ABA accumulation, osmotic adjustment, and stomatal closure (Haswell and Verslues, 2015).

Supplemental Data

The following supplemental materials are available.

Supplemental Data S1. Spreadsheet tool.

Supplemental Data S2. Supplemental information.

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APPENDIX

DETERMINING RELATIVE WATER CONTENT, SOLUTE POTENTIAL, AND PRESSURE POTENTIAL FOR LEAVES OF KNOWN LEAF WATER POTENTIAL

See Supplemental Data S1 for Spreadsheet Tool with Calculations

When a leaf is dehydrated in the pressure chamber until equilibrated at a given applied pressure P , its relative water content (RWC) can be determined based on pressure-volume equations given its known leaf water potential upon release of the applied pressure ($\Psi_{\text{leaf}} = -P$). For that leaf, Ψ_{leaf} is the sum of the pressure potential (Ψ_P) and the solute potential (Ψ_S),

$$\Psi_{\text{leaf}} = \Psi_S + \Psi_P. \quad (1)$$

When considering a leaf dehydrated below the total RWC at which turgor loss occurs (RWC_{tlp}), $\Psi_P = 0$. When considering a leaf dehydrated more mildly than RWC_{tlp} , Ψ_P can be calculated (Bartlett et al., 2012) as

$$\Psi_P = \begin{cases} -\Psi_{S,o} \cdot \left(\frac{\text{RWC} - \text{RWC}_{\text{tlp}}}{1 - \text{RWC}_{\text{tlp}}} \right), & \text{if } \text{RWC} > \text{RWC}_{\text{tlp}} \\ 0, & \text{otherwise} \end{cases}, \quad (2)$$

where $-\Psi_{S,o}$ is the osmotic potential at full turgor. Note that this formulation considers the slope of Ψ_P versus RWC to be linear above turgor loss point, consistent with empirical studies (Koide et al., 2000); nonlinearity in the interval between full and zero turgor would not affect our findings. Further, we note that such a constant slope represents an “absolute” version of the elastic modulus and its invariance above turgor loss point is not inconsistent with reports of a declining “relative” elastic modulus above turgor loss point, when this is defined as the slope of Ψ_P versus RWC multiplied by RWC for each leaf at its stage of dehydration (Robichaux, 1984).

The solute potential can be calculated based on the linear relationship of $1/\Psi_S$ to RWC:

$$-\frac{1}{\Psi_S} = m(1 - \text{RWC}) + b, \quad (3)$$

where the slope, m , is

$$m = \left(\frac{1}{\Psi_{S,o}} - \frac{1}{\Psi_{\text{tlp}}} \right) / (1 - \text{RWC}_{\text{tlp}}), \quad (4)$$

where Ψ_{tlp} is the water potential (and osmotic potential) at turgor loss point, and the intercept, b , is

$$b = -1/\Psi_{S,o}. \quad (5)$$

Combining Equations 3 to 5 and rearranging the terms gives

$$\Psi_S = \frac{\Psi_{S,o} \Psi_{\text{tlp}} (1 - \text{RWC}_{\text{tlp}})}{\Psi_{S,o} (1 - \text{RWC}) + \Psi_{\text{tlp}} (\text{RWC} - \text{RWC}_{\text{tlp}})}. \quad (6)$$

Ψ_{leaf} is then given by combining Equations 1, 2, and 6. If $\text{RWC} \leq \text{RWC}_{\text{tlp}}$, Ψ_{leaf} is simply equal to Ψ_S as given by Equation 6. If $\text{RWC} > \text{RWC}_{\text{tlp}}$, Ψ_{leaf} is given by Equation 7:

$$\Psi = \Psi_{S,o} \cdot \left(\frac{\text{RWC}_{\text{tlp}} - \text{RWC}}{1 - \text{RWC}_{\text{tlp}}} + \frac{\Psi_{\text{tlp}} (1 - \text{RWC}_{\text{tlp}})}{\Psi_{S,o} (1 - \text{RWC}) + \Psi_{\text{tlp}} (\text{RWC} - \text{RWC}_{\text{tlp}})} \right) \quad (7)$$

Equations 6 and 7 can be rearranged to calculate RWC from Ψ_{leaf} and pressure volume curve parameters. If $\Psi_{\text{leaf}} > \Psi_{\text{tlp}}$, then RWC is given by

$$\text{RWC} = \frac{1}{2} \left[-x - y + \sqrt{(x + y)^2 - 4(xy - z)} \right], \quad (8)$$

where

$$x = \frac{\Psi}{\Psi_{S,o}} (1 - \text{RWC}_{\text{tlp}}) - \text{RWC}_{\text{tlp}} \quad (8a)$$

$$y = \frac{\Psi_{S,o} - \Psi_{\text{tlp}} \text{RWC}_{\text{tlp}}}{\Psi_{\text{tlp}} - \Psi_{S,o}} \quad (8b)$$

$$z = \frac{\Psi_{\text{tlp}} (1 - \text{RWC}_{\text{tlp}})^2}{\Psi_{\text{tlp}} - \Psi_{S,o}}. \quad (8c)$$

If $\Psi_{\text{leaf}} \leq \Psi_{\text{tlp}}$, then RWC is given by

$$\text{RWC} = \frac{z}{x + \text{RWC}_{\text{tlp}}} - y. \quad (9)$$

See the Supplemental Data S2 for derivations of Equations 6 to 9.

The symplastic RWC ($\text{RWC}_{\text{symplastic}}$) can be calculated as:

$$\text{RWC}_{\text{symplastic}} = (\text{RWC} - a_f) / (1 - a_f), \quad (10)$$

where a_f is the apoplastic fraction of water content at full turgor.