

# Carbon isotopes and water use efficiency in C<sub>4</sub> plants

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Drought is a major agricultural problem worldwide. Therefore, selection for increased water use efficiency (WUE) in food and biofuel crop species will be an important trait in plant breeding programs. The leaf carbon isotopic composition ( $\delta^{13}\text{C}_{\text{leaf}}$ ) has been suggested to serve as a rapid and effective high throughput phenotyping method for WUE in both C<sub>3</sub> and C<sub>4</sub> species. This is because WUE, leaf carbon discrimination ( $\Delta^{13}\text{C}_{\text{leaf}}$ ), and  $\delta^{13}\text{C}_{\text{leaf}}$  are correlated through their relationships with intercellular to ambient CO<sub>2</sub> partial pressures ( $C_i/C_a$ ). However, in C<sub>4</sub> plants, changing environmental conditions may influence photosynthetic efficiency (bundle-sheath leakiness) and post-photosynthetic fractionation that will potentially alter the relationship between  $\delta^{13}\text{C}_{\text{leaf}}$  and  $C_i/C_a$ . Here we discuss how these factors influence the relationship between  $\delta^{13}\text{C}_{\text{leaf}}$  and WUE, and the potential of using  $\delta^{13}\text{C}_{\text{leaf}}$  as a meaningful proxy for WUE.

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## Drought — a major, widespread agricultural problem

Drought in both rain-fed and irrigated agricultural land is a worldwide constraint to crop production. Therefore, plant-breeding programs are working to develop new high yielding crop varieties that can cope with these conditions [1,2]. In recent years breeding programs have undergone rapid advancements in molecular and genomics techniques [3,4]; however, high-throughput phenotyping of complex traits related to water use efficiency (WUE) is lagging behind [5,6]. For example, traditional phenotyping for WUE requires laborious and costly measurements that are not feasible on a large numbers of individual plants. Therefore, few crops have been bred for WUE, particularly in C<sub>4</sub> species [7]. However, as outlined below, the carbon isotope signature of leaves ( $\delta^{13}\text{C}_{\text{leaf}}$ ) may be a potential WUE proxy for high-throughput phenotyping of C<sub>4</sub> plants.

## Defining water use efficiency (WUE)

Agricultural WUE is related to the harvestable yield relative to the amount of water used by the crop. Simplistically, this can be demonstrated by the fact that crop yield can be defined by whole plant transpiration efficiency ( $\text{TE}_{\text{plant}}$ ), the total water used by the crop, and the harvest index (HI) as [8]:

$$\text{Yield} = \text{TE}_{\text{plant}} \times \text{Water Use} \times \text{HI} \quad (1)$$

where  $\text{TE}_{\text{plant}}$  is defined as the total harvestable yield for a given amount of transpired water and is an important component of agricultural WUE and yield (see Box 1).  $\text{TE}_{\text{plant}}$  is a complex trait that is partly driven by leaf level TE ( $\text{TE}_{\text{leaf}}$ ), the net rate of CO<sub>2</sub> assimilation ( $A_{\text{net}}$ ) relative to the rate of transpiration ( $E$ ). Where  $A_{\text{net}}$  and  $E$  are linked by stomatal conductance ( $g_s$ ) through

$$A_{\text{net}} = g_{s\text{CO}_2}(C_a - C_i) \quad (2)$$

$$E = g_{s\text{H}_2\text{O}}(e_i - e_a) \quad (3)$$

and

$$\text{TE}_{\text{leaf}} = \frac{A}{E} = \frac{C_a(1 - C_i/C_a)}{1.6(e_i - e_a)} \quad (4)$$

(see Box 2 for term definitions). These simple equations describe how  $A_{\text{net}}$ ,  $g_s$  and the water vapor pressure deficient (VPD) between the leaf and the surrounding air influence  $\text{TE}_{\text{leaf}}$ . However, the intrinsic TE ( $\text{TE}_{\text{intrinsic}}$ )

$$\text{TE}_{\text{intrinsic}} = \frac{A_{\text{net}}}{g_s} = \left(1 - \frac{C_i}{C_a}\right) \quad (5)$$

is a better indicator of genotypic differences in WUE because it is not driven by variation in VPD, which is determined by leaf temperature and the relative humidity of the surrounding air [9]. Values of  $A_{\text{net}}$  and  $g_s$  drive  $\text{TE}_{\text{intrinsic}}$  in opposite directions but both influence  $C_i/C_a$ . For example, greater  $A_{\text{net}}$  can decrease  $C_i/C_a$  through increased CO<sub>2</sub> fixation, while greater  $g_s$  will increase  $C_i/C_a$  by increasing CO<sub>2</sub> supply entering the leaf. From an agricultural perspective it is desirable to take advantage of all water available in a field. Therefore, increasing  $\text{TE}_{\text{plant}}$  by reducing  $g_s$  is not advantageous if the net result is maintaining yield while water remains unused in the field. Instead it is better to increase  $\text{TE}_{\text{plant}}$  by improving  $A_{\text{net}}$  relative to  $g_s$  to obtain higher yields while using all of the available water [9].

## Relationship between $\Delta^{13}\text{C}$ , $\delta^{13}\text{C}_{\text{leaf}}$ and $\text{TE}_{\text{intrinsic}}$

The link between  $\delta^{13}\text{C}_{\text{leaf}}$  and  $C_i/C_a$  is driven by leaf CO<sub>2</sub> discrimination ( $\Delta^{13}\text{C}_{\text{leaf}}$ ), which can be estimated as

$$\Delta^{13}\text{C}_{\text{leaf}} = \frac{(\delta_s - \delta_p)}{(1 + \delta_p)} \quad (6)$$

**Box 1 Measures of WUE across scales.**

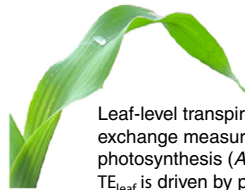
$$WUE_{\text{agricultural}} = \frac{\text{biomass or economic yield}}{\text{evapotranspiration (ET)}}$$

Integrates the total production or harvested yield per volume of water used in the field. Takes into account variation across individual plants and evaporation from the soil and is influenced by several factors such as plant water use, planting scheme (e.g. spacing and density), soil texture, and irrigation design.



$$TE_{\text{plant}} = \frac{\text{total biomass}}{\text{water transpired}}$$

The term transpiration efficiency (TE) is used instead of WUE when the only source of water loss is from transpiration. Plant-level TE ( $TE_{\text{plant}}$ ) is a direct measure of above ground biomass accumulation and transpirational water loss from the entire plant (sometimes referred to as  $WUE_{\text{plant}}$ ). It is independent of all non-physiological factors that affect  $WUE_{\text{agricultural}}$  of which it is a component. It is dependent on environmental conditions that affect growth and transpiration, and does not always correlate well with leaf/genotype-level variation in TE because  $TE_{\text{plant}}$  is dependent on not only  $TE_{\text{leaf}}$  but plant structure.



$$TE_{\text{leaf}} = \frac{A_{\text{net}}}{E} = \left(1 - \frac{C_i}{C_a}\right) * \frac{C_a}{1.6(e_i - e_a)}$$

Leaf-level transpiration efficiency ( $TE_{\text{leaf}}$ ) is calculated by gas exchange measurements of the ratio of  $\text{CO}_2$  fixed through photosynthesis ( $A_{\text{net}}$ ) and  $\text{H}_2\text{O}$  lost through transpiration ( $E$ ).  $TE_{\text{leaf}}$  is driven by plant variation in stomatal conductance ( $g_s$ ) and  $A_{\text{net}}$  but also the vapor pressure deficit (VPD) between the intercellular air space and the air surrounding the leaf ( $e_i - e_a$ ). Therefore,  $TE_{\text{leaf}}$  is in part driven by changes in environmental conditions such as relative humidity in the air and temperature.

$$TE_{\text{intrinsic}} = \frac{A_{\text{net}}}{g_s} = \left(1 - \frac{C_i}{C_a}\right)$$

Intrinsic TE is a genotype-level measure that is specifically determined by rates of  $\text{CO}_2$  and  $\text{H}_2\text{O}$  exchange through the stomata, and is sometimes referred to as  $WUE_{\text{intrinsic}}$  [9]. Both  $A_{\text{net}}$  and  $g_s$  affect  $C_i/C_a$ , but are not directly influenced by VPD.

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where  $\delta_p$  and  $\delta_s$  represent the carbon isotope ratios ( $^{13}\text{C}/^{12}\text{C}$ ) relative to a standard of leaf material and the  $\text{CO}_2$  available to the leaf, respectively. Compared to  $\text{C}_3$  plants, the relationship between  $\Delta^{13}\text{C}_{\text{leaf}}$  and  $C_i/C_a$  is diminished in  $\text{C}_4$  plants and is less variable in response to growth conditions or between genotypes [11<sup>••</sup>, 12<sup>••</sup>].

**Box 2 Definitions of terms.**

$g_{\text{sCO}_2}$  = stomatal conductance to  $\text{CO}_2$

$g_{\text{sH}_2\text{O}}$  = stomatal conductance to  $\text{H}_2\text{O}^*$

$C_i$  = leaf intercellular  $\text{CO}_2$  partial pressure

$C_a$  = ambient  $\text{CO}_2$  partial pressure

$e_i$  = water vapor pressure inside the leaf

$e_a$  = water vapor pressure surrounding the leaf

$a$  = fractionation during diffusion of  $\text{CO}_2$  in air through stomata (4.4‰)

$b_3$  = fractionation by Rubisco (−30‰)

$b_4$  = combined fractionation of PEP carboxylation and preceding isotopic equilibrium during dissolution and hydration of  $\text{CO}_2$  (−5.2‰ at 30 °C)

$\phi$  = leakiness; fraction of  $\text{CO}_2$  released in the bundle sheath cells by the  $\text{C}_4$  cycle and not fixed by Rubisco

$s$  = fractionation during the leakage of  $\text{CO}_2$  out of the bundle sheath cells (1.8‰)

\*The ratio of diffusivity of  $\text{H}_2\text{O}$  vapor to  $\text{CO}_2$  in air is 1.6.

For example,  $\delta^{13}\text{C}_{\text{leaf}}$  of most  $\text{C}_4$  species fall between −12 and −15‰, while  $\delta^{13}\text{C}_{\text{leaf}}$  of most  $\text{C}_3$  plants varies between −23 and −32‰ [12<sup>••</sup>, 13]. However, even in  $\text{C}_4$  plants there are well defined fractionation steps associated with  $\text{CO}_2$  diffusion through the stomata, the biochemistry of  $\text{CO}_2$  fixation by Rubisco,  $\text{HCO}_3^-$  fixation by phosphoenolpyruvate carboxylase (PEPC), mesophyll conductance to  $\text{CO}_2$  ( $g_m$ ), carbonic anhydrase activity, bundle-sheath leakiness ( $\phi$ ), and  $C_i/C_a$  that influence  $\Delta^{13}\text{C}_{\text{leaf}}$  [11<sup>••</sup>, 12<sup>••</sup>, 14<sup>••</sup>]. Therefore, measurements of  $\delta^{13}\text{C}_{\text{leaf}}$  in  $\text{C}_4$  leaves have been used as a proxy to estimate changes in  $\text{C}_4$  photosynthetic efficiency and  $TE_{\text{intrinsic}}$  in response to various growth conditions. However, to be an effective proxy requires detailed understanding of the factors influencing  $\delta^{13}\text{C}_{\text{leaf}}$ . Below we discuss several of the primary mechanisms driving  $\delta^{13}\text{C}_{\text{leaf}}$  in  $\text{C}_4$  plants and the potential use of  $\delta^{13}\text{C}_{\text{leaf}}$  for estimating  $TE_{\text{intrinsic}}$ .

**Leaf  $\text{CO}_2$  discrimination ( $\Delta^{13}\text{C}_{\text{leaf}}$ )**

Several fractionation factors associated with  $\text{CO}_2$  diffusion and fixation by photosynthetic enzymes influence  $\Delta^{13}\text{C}_{\text{leaf}}$ . For example, in  $\text{C}_4$  plants  $\Delta^{13}\text{C}_{\text{leaf}}$  is strongly

**Box 3 On-line (direct)  $\Delta^{13}\text{C}_{\text{leaf}}$  measurements.**

On-line or direct measurements of leaf carbon isotopic discrimination ( $\Delta^{13}\text{C}_{\text{leaf}}$ ) combine measurements of  $\delta^{13}\text{C}$  of  $\text{CO}_2$  entering and leaving a leaf gas exchange chamber. Under these conditions  $\Delta^{13}\text{C}_{\text{leaf}}$  represents the instantaneous fractionation associated with  $\text{CO}_2$  diffusion, photosynthesis, photorespiration, and respiration. Alternatively, the dry matter leaf carbon isotopic composition ( $\delta^{13}\text{C}_{\text{leaf}}$ ) can be used as an indirect measure of  $\Delta^{13}\text{C}_{\text{leaf}}$  that integrates variation in  $\text{CO}_2$  availability ( $C_i/C_a$ ), photosynthetic and post-photosynthetic fractionations over the life of the leaf. Tunable diode laser absorption spectroscopy (TDL) measures concentrations of  $^{13}\text{CO}_2$  and  $^{12}\text{CO}_2$  and allows for continuous measurements of  $\Delta^{13}\text{C}_{\text{leaf}}$  in response to changes in measurement conditions [11\*\*]. However, the TDL measurements provide a snapshot of leaf level  $\text{CO}_2$  isotope exchange and do not necessarily capture all of the integrated fractionation factors that drive variation in  $\delta^{13}\text{C}_{\text{leaf}}$ .

determined by  $C_i/C_a$  and the efficiency of the  $\text{CO}_2$  concentrating mechanism ( $\phi$ ; leakiness) [11\*\*,14\*\*]. At 30 oC  $\Delta^{13}\text{C}_{\text{leaf}}$  forms an inverse relationship with  $C_i/C_a$  when  $\phi$  is below 0.37, but has an opposite relationship if  $\phi$  is higher [11\*\*,12\*\*,14\*\*]. However, as will be discussed in the next section, C<sub>4</sub> photosynthesis is well coordinated through the metabolic flux between the C<sub>4</sub> and C<sub>3</sub> cycles and when  $\phi$  is estimated with direct measurements of photosynthesis and leaf  $\text{CO}_2$  isotope exchange it is relatively constant between species and under various growth conditions (see Box 3; [11\*\*,12\*\*,14\*\*,15–18]). The relationship of  $\Delta^{13}\text{C}_{\text{leaf}}$  in C<sub>4</sub> plants with  $C_i/C_a$  can be explained with a simplified model as:

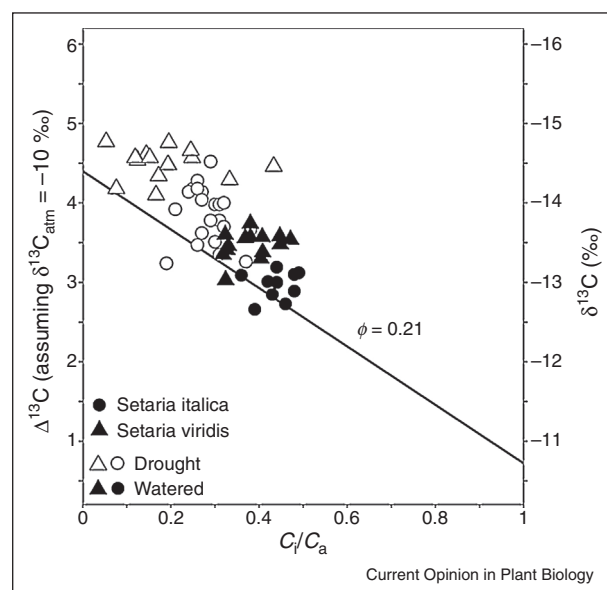
$$\Delta^{13}\text{C}_{\text{leaf}} = a + (b_4 + \phi(b_3 - s) - a)C_i/C_a \quad (7)$$

where  $a$ ,  $b_4$ ,  $b_3$ ,  $\phi$ ,  $s$ ,  $C_i$ , and  $C_a$  are defined in Box 2. By parameterizing these factors under various conditions,  $\Delta^{13}\text{C}_{\text{leaf}}$  can be primarily attributed to variation in  $C_i/C_a$ . Although this equation is a simplified version of the original model described by Farquhar (1983), it incorporates the major fractionation factors influencing  $\delta^{13}\text{C}$  [19].

**Bundle-sheath leakiness**

Bundle-sheath leakiness ( $\phi$ ) describes the efficiency of the  $\text{CO}_2$ -concentrating mechanism by the leak rate of  $\text{CO}_2$  from the bundle sheath relative to the rate of  $\text{CO}_2$  supplied by the C<sub>4</sub> cycle [11\*\*,14\*\*]. Changes in  $\phi$  can have a strong influence on  $\Delta^{13}\text{C}_{\text{leaf}}$  because it controls the extent of Rubisco discrimination, which has a large fractionation factor. Therefore,  $\phi$  increases  $\Delta^{13}\text{C}_{\text{leaf}}$  at a given  $C_i/C_a$ , controlling the relationship between  $\Delta^{13}\text{C}_{\text{leaf}}$  and  $C_i/C_a$  [11\*\*,14\*\*]. Species or environmental-driven differences in  $\phi$ , therefore, will cause differences in  $\Delta^{13}\text{C}_{\text{leaf}}$  independent of changes in  $C_i/C_a$ . Much of the literature that details the response of  $\phi$  to growth conditions was initially tested with spot measurements of  $C_i/C_a$  and  $\delta^{13}\text{C}_{\text{leaf}}$  using Eqs. (6) and (7). However, on-line measurements of  $\Delta^{13}\text{C}_{\text{leaf}}$  coupled with leaf gas exchange have raised questions about the relationship between  $\Delta^{13}\text{C}_{\text{leaf}}$ ,  $\delta^{13}\text{C}_{\text{leaf}}$  and  $\phi$  (see Box 3; [11\*\*,14\*\*]).

For example,  $\Delta^{13}\text{C}_{\text{leaf}}$  calculated from  $\delta^{13}\text{C}_{\text{leaf}}$  was higher in drought- and salt-stressed, and nitrogen-limited plants compared to plants grown under optimal conditions [20–26]. Additionally,  $\Delta^{13}\text{C}_{\text{leaf}}$  calculated from dry matter  $\delta^{13}\text{C}_{\text{leaf}}$  increased when plants were grown under low light [25,27]. These studies suggest from comparisons of spot measurements of  $C_i/C_a$  and  $\Delta^{13}\text{C}_{\text{leaf}}$  estimated from  $\delta^{13}\text{C}_{\text{leaf}}$  that  $\phi$  is affected by growth under low light, limited nitrogen availability, and water and salt stress [20–27]. However, because  $\delta^{13}\text{C}_{\text{leaf}}$  integrates over the life of the leaf, instantaneous measurements of  $\Delta^{13}\text{C}_{\text{leaf}}$  and  $\delta^{13}\text{C}_{\text{leaf}}$  do not always correlate, raising questions to how well  $\delta^{13}\text{C}_{\text{leaf}}$  relates to changes in  $\phi$ . Several studies comparing direct measurements of  $\Delta^{13}\text{C}_{\text{leaf}}$  and  $\Delta^{13}\text{C}_{\text{leaf}}$  calculated from  $\delta^{13}\text{C}_{\text{leaf}}$  have shown significant differences that are not always related to  $\phi$  [28–31]. In general,  $\delta^{13}\text{C}_{\text{leaf}}$  measurements suggest  $\phi$  values are more variable and higher than those calculated from direct  $\Delta^{13}\text{C}_{\text{leaf}}$  measurements ([28–30,32,33], see Figure 1). As a result, dry leaf matter-derived  $\Delta^{13}\text{C}_{\text{leaf}}$  produce  $\phi$  values typically between 0.3 and 0.5, while  $\phi$  values from direct  $\Delta^{13}\text{C}_{\text{leaf}}$  fall between 0.21 and 0.3 [28,30,34]. The disparity between these estimates of  $\phi$  is likely due to differences in time integration of the measurements. For example,  $\delta^{13}\text{C}_{\text{leaf}}$  will be influenced by changes in  $C_i/C_a$  that occur

**Figure 1**

Relationship between  $\Delta^{13}\text{C}_{\text{leaf}}$ ,  $\delta^{13}\text{C}_{\text{leaf}}$  and  $C_i/C_a$  in *Setaria italica* and *S. viridis* grown under well-watered or drought conditions (Ellsworth *et al.*, unpublished data). The y-axes show  $\Delta^{13}\text{C}_{\text{leaf}}$  calculated from  $\delta^{13}\text{C}_{\text{leaf}}$  (Eq. (6)) and the corresponding  $\delta^{13}\text{C}_{\text{leaf}}$ , and the x-axis shows the measured  $C_i/C_a$ . The  $\delta^{13}\text{C}$  of the  $\text{CO}_2$  in the growth chambers was  $-10\text{‰}$ . The line represents the theoretical relationship between  $\Delta^{13}\text{C}_{\text{leaf}}$ ,  $\delta^{13}\text{C}_{\text{leaf}}$  and  $C_i/C_a$  (based on Eq. (7); where  $\phi$  was assumed to be 0.21). The  $\Delta^{13}\text{C}_{\text{leaf}}$  estimated from  $\delta^{13}\text{C}_{\text{leaf}}$  are generally above the theoretical line, consistent with post-photosynthetic fractionation that is not taken into account in Eq. (7) [28–30,32,33].

over the lifetime of the leaf and post-photosynthetic fractionations, which will be discussed below.

Direct measurements of  $\Delta^{13}\text{C}_{\text{leaf}}$  generally find that  $\phi$  is relatively constant under various environmental conditions [11<sup>••</sup>,12<sup>••</sup>,14<sup>••</sup>,15–18,29–32]. An exception is that  $\phi$  has been shown to increase under very low light intensities [27,30,34–39], but much of the increase in  $\phi$  was attributed to oversimplification of  $\Delta^{13}\text{C}_{\text{leaf}}$  model [14<sup>••</sup>,15,17]. In fact, using the full formulation of the  $\Delta^{13}\text{C}_{\text{leaf}}$  model developed by Farquhar (1984) typically indicates little to no change in  $\phi$  under low light [17,34,36,40]. Additionally,  $\phi$  was generally found to acclimate to rapid changes in light quality [41]. Therefore, direct measurements of  $\Delta^{13}\text{C}_{\text{leaf}}$  indicate that  $\text{C}_4$  plants have a remarkable ability to coordinate the metabolic flux through the  $\text{C}_4$  and  $\text{C}_3$  cycles, and that  $\phi$  is relatively constant between species and across different growth conditions. The  $\text{C}_4$  and  $\text{C}_3$  cycles have extensive metabolic and redox regulation between the mesophyll and bundle sheath cells, so that the two cycles are really two parts of one pathway [42].

### Potential post-photosynthetic fractionations

Fractionation that occurs downstream from photosynthesis is less well characterized but will also influence  $\delta^{13}\text{C}_{\text{leaf}}$  [11<sup>••</sup>,43]. Post-photosynthetic fractionations are associated with generating carbon pools for leaf construction, export and import into and out of leaves, and respiration [11<sup>••</sup>,43]. However, there is insufficient research in this area and a better understanding of the fractionations associated with these processes is needed to disentangle the relationship between  $\delta^{13}\text{C}_{\text{leaf}}$  and direct measurements of  $\Delta^{13}\text{C}_{\text{leaf}}$ . To further complicate matters, it is not clear how growth conditions influence post-photosynthetic fractionations.

The preferential use of isotopically distinct carbon pools for respiration or movement between leaves will also affect  $\delta^{13}\text{C}_{\text{leaf}}$  [11<sup>••</sup>]. For example, in both  $\text{C}_3$  and  $\text{C}_4$  plants carbohydrate pools such as sucrose and starch tend to be enriched in  $^{13}\text{C}$  relative to bulk leaf tissue, while lipids, organic acids, and proteins are depleted in  $^{13}\text{C}$  [11<sup>••</sup>,44]. Additionally, sucrose produced from triose-phosphates in the cytosol during the day is depleted in  $^{13}\text{C}$  relative to the leaf, but sucrose produced at night from starch remobilization is enriched in  $^{13}\text{C}$  relative to the leaf [44]. In  $\text{C}_3$  *Ricinus communis*, the isotopic composition of exported sucrose (or imported into sink leaves) depends on the ratio of day/night sucrose synthesis [45]. Unfortunately, the effect that these fractionations have on  $\delta^{13}\text{C}_{\text{leaf}}$  is not well known and further research is needed to understand the fractionations associated with carbon movement between  $\text{C}_4$  leaves, especially under drought.

Respiration may also influence  $\delta^{13}\text{C}_{\text{leaf}}$  by shifting between isotopically different pools of carbon in response to

stresses or other physiological changes. For example, in  $\text{C}_3$  plants consuming triose-phosphates evolved  $\text{CO}_2$  is depleted in  $^{13}\text{C}$ , but the magnitude of this depletion depends on the decarboxylating enzymes involved and the  $\delta^{13}\text{C}$  of carbon atoms in the triose-phosphates [43,46]. For example, when the Krebs cycle is coupled with  $\beta$ -oxidation of fatty acids, evolved  $\text{CO}_2$  is more depleted than when pyruvate dehydrogenation predominates [43]. This likely explains the larger than expected variation in  $\delta^{13}\text{C}$  of nocturnal leaf respiration measured in the  $\text{C}_4$  grass *Sporobolus wrightii* [47]. Furthermore, under stressful growth conditions other carbon sources such as lipids could be used for respiration [43,46]. However, the fractionation associated with the shift in respiratory carbon source would have to be quite large to be responsible for the  $\sim 1\text{‰}$  change in  $\delta^{13}\text{C}_{\text{leaf}}$  that have been measured between well-watered and drought treatments (Ellsworth *et al.* unpublished and in review). In  $\text{C}_3$  plants there were no dramatic shifts in respiratory carbon under drought stress [46,48]; however, this has not been characterized in  $\text{C}_4$  plants. Although respiration is not likely responsible of the entire change in  $\delta^{13}\text{C}_{\text{leaf}}$  measured across water availability, it may have some effect and more research is also needed, particularly in  $\text{C}_4$  plants.

### Drought and $\delta^{13}\text{C}_{\text{leaf}}$

Measurements of  $\delta^{13}\text{C}_{\text{leaf}}$  as a proxy for  $\text{TE}_{\text{intrinsic}}$  in plant breeding programs has long been suggested for both  $\text{C}_3$  and  $\text{C}_4$  crop species [10], but recently has seen more interest because of the need for a high throughput proxy for  $\text{TE}_{\text{intrinsic}}$  [1,2,49,50]. To date,  $\delta^{13}\text{C}_{\text{leaf}}$  has only been successfully used to screen for  $\text{TE}_{\text{intrinsic}}$  in wheat breeding programs in Australia [51]. However, the response of  $\delta^{13}\text{C}_{\text{leaf}}$  to drought has been tested in several  $\text{C}_4$  species such as maize [52–56], sugar cane [20], sorghum [57], green foxtail millet and foxtail millet (Ellsworth *et al.*, in review), pearl millet [54,56], and numerous other grass species covering all three  $\text{C}_4$  biochemical subtypes [24,38,58,59]. Consistently, across these experiments and  $\text{C}_4$  subtypes, the  $\delta^{13}\text{C}$  values are lower in water-limited plants compared to the corresponding well-watered plants, independent of the tissue sampled (leaf, kernel, shoot, silk, or cob). The magnitude of this shift in  $\delta^{13}\text{C}_{\text{leaf}}$  between treatments is generally dependent on the severity of the drought and interactions with other treatments such as nitrogen, phosphorus, or light limitation and elevated  $\text{CO}_2$ . However, plants under drought consistently have lower  $\delta^{13}\text{C}_{\text{leaf}}$  values than the well-watered plants suggesting a robust response of  $\delta^{13}\text{C}_{\text{leaf}}$  to drought in  $\text{C}_4$  plants. Furthermore, studies comparing  $\delta^{13}\text{C}_{\text{leaf}}$  to  $g_s$  and, in some cases,  $\text{TE}_{\text{intrinsic}}$  showed  $\text{C}_4$  plants under drought decreased  $g_s$ , increased  $\text{TE}_{\text{intrinsic}}$ , and had lower  $\delta^{13}\text{C}_{\text{leaf}}$  ([20,24,52,55,57], Ellsworth *et al.*, in review).

These studies demonstrate that decreases in  $C_i/C_a$  under drought conditions consistently influence  $\delta^{13}\text{C}_{\text{leaf}}$ , and with further testing may be an effective proxy for  $\text{TE}_{\text{intrinsic}}$



in C<sub>4</sub> plants (see example in Figure 1). However, the sensitivity to which  $\delta^{13}\text{C}_{\text{leaf}}$  can distinguish variation in WUE still needs to be tested. For example,  $\delta^{13}\text{C}_{\text{leaf}}$  may be sufficient as a first pass to separate genotypes with high and low  $\text{TE}_{\text{intrinsic}}$  but not suitable for fine scale screening to separate genotypes with only slight differences in  $\text{TE}_{\text{intrinsic}}$  [52,53]. Unfortunately, there are few drought experiments where  $\phi$  has been determined using direct measurements of  $\Delta^{13}\text{C}_{\text{leaf}}$  [58]. Therefore, the effect of drought on  $\phi$  and its influence on  $\delta^{13}\text{C}_{\text{leaf}}$  requires further study. However, considering  $\phi$  appears to be relatively constant, the primary driver of  $\delta^{13}\text{C}_{\text{leaf}}$  under drought is likely changes in  $C_i/C_a$ . Of course, the influence of post-photosynthetic fractionations associated with respiration and mobilization of carbon pools within the leaf will potentially limit the sensitivity of using  $\delta^{13}\text{C}_{\text{leaf}}$ .

## Conclusion and future directions

With the current focus on breeding more water use efficient crops and the predicted increase in drought and growing season temperatures, C<sub>4</sub> crops will continue to play an important role in agriculture. In the modern age of marker-assisted plant breeding, techniques to screen for physiological traits such as  $\text{TE}_{\text{intrinsic}}$  are needed to assess phenotypic variation across large numbers of genotypes. Therefore, the development of  $\delta^{13}\text{C}_{\text{leaf}}$  as a tool for estimating  $\text{TE}_{\text{intrinsic}}$  in large-scale plant breeding programs will be beneficial. However, more research is needed to solidify the relationship of  $\text{TE}_{\text{intrinsic}}$ ,  $\delta^{13}\text{C}_{\text{leaf}}$  and  $C_i/C_a$  for more C<sub>4</sub> species under various growth conditions. This will include the elucidation of post-photosynthetic fractionations and their effects on  $\delta^{13}\text{C}_{\text{leaf}}$  to further refine these relationships. Additionally, more experiments are needed to assess  $\delta^{13}\text{C}_{\text{leaf}}$  variation in agriculturally important breeding populations to help identify quantitative trait loci and potential gene candidates influencing  $\delta^{13}\text{C}_{\text{leaf}}$  and  $\text{TE}_{\text{intrinsic}}$ .

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