

Mechanisms for the relationships between water-use efficiency and carbon isotope composition and specific leaf area of maize (*Zea mays* L.) under water stress

Congzhi Zhang¹ · Jiabao Zhang¹ · Hui Zhang^{1,2} · Jinhua Zhao¹ · Qicong Wu¹ · Zhanhui Zhao¹ · Taiyi Cai¹

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Abstract The quick assessment approaches for accurately measuring water-use efficiency (WUE) in maize under water stress are important to water-saving agriculture. We investigated how and whether carbon isotope composition ($\delta^{13}\text{C}$) and specific leaf area (SLA) could be used to assess WUE for maize as influenced by water stress. A pot experiment was conducted twice during six typical maize (*Zea mays*) growth stages of seedling, jointing, booting, tasseling, filling and maturity, respectively. The ratio between the activities of ribulose 1,5-bisphosphate carboxylase (Rubisco) and phosphoenolpyruvate carboxylase decreased bundle

sheath leakiness (ϕ) under water deficiency, caused more ^{13}C to be assimilated, and resulted in increased $\delta^{13}\text{C}$ in leaves. Water stress increased the fractionation of ^{13}C when assimilates were transported from leaf to stem, indicating that water stress affected leaf expansion and translocation of assimilates from leaf to stem, and resulted in thicker leaves and lower SLA. WUE showed significant positive correlations with leaf $\delta^{13}\text{C}$ and SLA, implying that leaf $\delta^{13}\text{C}$ and SLA could effectively reflect the drought adaptation and high WUE under different water conditions.

Keywords Water-use efficiency · Bundle sheath leakiness · Carbon isotope composition · Specific leaf area · Maize · Water stress

✉ Jiabao Zhang
jbzhang@issas.ac.cn

Congzhi Zhang
czzhang@issas.ac.cn

Hui Zhang
1983hui@sina.com

Jinhua Zhao
zhao.jinhua.123@163.com

Qicong Wu
wuqicong588@163.com

Zhanhui Zhao
zhanhuizhao@126.com

Taiyi Cai
caity2008@issas.ac.cn

¹ State Experimental Station of Agro-Ecosystem in Fengqiu, State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, People's Republic of China

² Institute of Agricultural Resources and Environment, Jiangsu Academy of Agricultural Sciences/Scientific Observing and Experimental Station of Arable Land Conservation of Jiangsu Province, Ministry of Agriculture, Nanjing 210014, People's Republic of China

Introduction

Maize (*Zea mays* L.) is an important crop with the C_4 pathway of photosynthesis, and is widely planted in arid and semi-arid regions around the world. Water shortage is one of the major problems limiting maize productivity in these regions (Yandigeri et al. 2012; Liu et al. 2013). Water-use efficiency (WUE) is defined as the ratio of plant dry matter produced to total water used over the same period (plant WUE) or, defined in physiological terms (transpiration efficiency at the leaf level), as the ratio between the rates of carbon fixation and transpiration (Zhao et al. 2004). Quick assessment approaches to measuring WUE accurately in maize under water stress may be considered as theoretical guides to water saving in agriculture. However, to obtain accurate WUE value for a specific plant cultivar or to compare WUE among various cultivars is time- and labor-consuming in the field, because accurate measurements of crop transpiration and total dry matter

production in the field are difficult (Wright et al. 1994). A series of studies attempting to find a sensitive, quick and exact WUE assessment approach showed that ^{13}C was fractionated by leaves during photosynthesis, and carbon isotope composition ($\delta^{13}\text{C}$) in leaves was positively correlated with WUE in many C_3 species when water was limited, such as cotton (Saranga et al. 1998), upland rice (Zhao et al. 2004) and peanut (Wright et al. 1988). However, most of these studies emphasized C_3 plants and few dealt with C_4 plants.

Isotope composition is usually expressed as the molar ratio of heavier to lighter isotopes (R), and R is generally converted to δ (stable isotope composition) and $\Delta^{13}\text{C}$ (carbon isotope discrimination),

$$\delta(\text{‰}) = [R(\text{sample})/R(\text{standard}) - 1] \times 1000 \quad (1)$$

$$\Delta = \frac{\delta_a - \delta_p}{1 + \delta_p/1000} \quad (2)$$

where $R(\text{sample})$ and $R(\text{standard})$ are the molar ratios of the heavier and lighter isotope of the sample and the standard, respectively. $\delta^{13}\text{C}_p$ and $\delta^{13}\text{C}_a$ are carbon isotope composition of plant and CO_2 in atmosphere, respectively, with $\delta^{13}\text{C}_a$ as a constant of -8.0 ‰ (Farquhar et al. 1989).

In C_3 species, the $\Delta^{13}\text{C}$ during photosynthetic CO_2 fixation is defined as photosynthesis $\Delta^{13}\text{C}$, which is linearly related with the ratio of intercellular and atmospheric concentrations of CO_2 (C_i/C_a) and can be expressed as (Monneveux et al. 2007):

$$\Delta^{13}\text{C} = a + [b_3 - a](C_i/C_a) \quad (3)$$

where a (4.4 ‰) is the discrimination during diffusion of CO_2 in air, b_3 (29 ‰) is the discrimination associated with carboxylation by ribulose 1,5-bisphosphate carboxylase (Rubisco), and C_i/C_a is the ratio of intercellular and atmospheric concentrations of CO_2 . The $\Delta^{13}\text{C}$ and transpiration efficiency are both related to C_i/C_a (Zhao et al. 2004), and WUE positively correlated with $\delta^{13}\text{C}$ and negatively correlated with $\Delta^{13}\text{C}$ for C_3 species under water-limited conditions has been widely reported (Farquhar et al. 1989; Meinzer et al. 1992; Zhao et al. 2004).

In C_4 species, photosynthesis $\Delta^{13}\text{C}$ in leaf tissue is more complex, involving the diffusion of CO_2 through stomata, dissolution and conversion of CO_2 to HCO_3^- , and fixation of CO_2 catalyzed by phosphoenolpyruvate carboxylase (PEPC) in mesophyll and by Rubisco in the bundle sheath (Monneveux et al. 2007). Variation of photosynthesis $\Delta^{13}\text{C}$ in C_4 species depends on both C_i/C_a and bundle sheath leakiness (ϕ) during CO_2 assimilation (Farquhar 1983; Monneveux et al. 2007). ϕ is defined as the proportion of CO_2 released by the decarboxylation of C_4 compounds in the bundle sheath which is not fixed by Rubisco that subsequently leaks back to the mesophyll (Farquhar 1983;

Williams et al. 2001). ϕ may be regarded as a measure of the amount of “overcycling” that occurs in the mesophyll cells in order to raise the partial pressures of CO_2 in the bundle sheath cells (Farquhar 1983), and ϕ is also used as a measure of the extent to which PEPC exceeds Rubisco (Monneveux et al. 2007). The $\Delta^{13}\text{C}$ in C_4 species can be expressed as follows (Farquhar 1983; Henderson et al. 1992):

$$\Delta^{13}\text{C} = a + [b_4 + \phi(b_3 - s) - a](C_i/C_a) \quad (4)$$

where b_4 (-5.7 ‰) is the isotopic discrimination during dissolution of HCO_3^- and fixation by PEPC, and s (1.8 ‰) is the discrimination during leakiness of CO_2 from the bundle sheath to the mesophyll, with other symbols as mentioned above.

Therefore, the correlation between $\Delta^{13}\text{C}$ and C_i/C_a in C_4 species in theory can be either negative or positive, depending on whether ϕ is greater or less than $(a - b_4)/(b_3 - s)$ (Monneveux et al. 2007), which lead to an uncertain relationship between $\delta^{13}\text{C}$ and WUE.

Rubisco discriminates more against ^{13}C than PEPC, and an increase in ϕ results in higher photosynthesis $\Delta^{13}\text{C}$ because b_3 is expressed more as shown in Eq. (4) (von Caemmerer et al. 2014). The variation in ϕ has been widely studied in different biochemical subtypes of C_4 plants, and these studies clarified the effects of physiological characteristics on ϕ , showing that ϕ depended on the tightness and thickness of the walls of bundle sheath cells (Farquhar 1983; Henderson et al. 1992). However, some studies indicated that ϕ was significantly influenced by environmental factors such as water stress and atmospheric CO_2 elevation (Williams et al. 2001; Fravolini et al. 2002), but different or conflicting results were found. For example, Bowman et al. (1989) reported that ϕ varied diurnally and increased with increasing water stress. Williams et al. (2001) found that ϕ was about 0.2 in *Sorghum bicolor* (L.) Moench. cultivated under different irradiances, temperatures and partial pressures of CO_2 . Fravolini et al. (2002) pointed out that ϕ was higher in *Eragrostis lehmanniana* grown with limited water, while in *Aristida glabrata* there was low leakiness under well-watered conditions. Therefore, there are no definitive conclusions about the response of ϕ to environmental change and there is little information on ϕ in maize.

Since the slope of the relationship between photosynthesis $\Delta^{13}\text{C}$ and C_i/C_a in C_4 species (Eq. 4) is lower than in C_3 species (Eq. 3), variations in cultivar and environment in C_i/C_a lead to much smaller changes in $\Delta^{13}\text{C}$ than in C_3 species (Monneveux et al. 2007). And leaf $\delta^{13}\text{C}$ is usually more depleted in ^{13}C compared with estimated made from measurements of photosynthesis $\delta^{13}\text{C}$ (von Caemmerer et al. 2014). Therefore, there is limited information regarding the relationship between $\delta^{13}\text{C}$ and WUE in C_4

crops. Williams et al. (2001) pointed out that the metabolic mechanism of CO₂ in C₄ photosynthesis involved coordinated functioning between mesophyll and bundle sheath cells and two carboxylation pathways. Therefore, we hypothesize that the regulation of activities of PEPC and Rubisco in maize under water stress results in variation of ϕ , and then leads to changes in the relationship between WUE and leaf $\delta^{13}\text{C}$. However, the responses of leaf photosynthesis to water stress significantly affects the fixation of CO₂, distribution of assimilates, and also leaf sizes and shapes. Therefore, specific leaf area (SLA, leaf area per unit dry mass) varies with water conditions during plant growth. Wright et al. (1988, 1994) reported that SLA was closely and negatively correlated with WUE in peanut (C₃ species), and suggested that breeders could use SLA to screen for high WUE among peanut germplasm within particular environments. However, they also indicated that the mechanisms of the relationship between SLA and WUE were unknown and required further investigations. At present, few studies have focused on how water stress influences the relationship between SLA and WUE in C₄ species.

The effects of water stress on leaf $\delta^{13}\text{C}$, ϕ , SLA and WUE in maize are not well documented. Especially, it is still poorly understood how and whether leaf $\delta^{13}\text{C}$ and SLA could be used to assess the influence of water stress on WUE of maize. Therefore, the main objectives of this study were to (1) clarify the mechanisms for the responses of leaf $\delta^{13}\text{C}$, ϕ , SLA and WUE to water stress, and (2) analyze the relationships between leaf $\delta^{13}\text{C}$, SLA and WUE.

Materials and methods

Experimental design

The experiment was conducted from June 9 to September 24, 2011, and repeated again from June 5 to September 20, 2012, in the Agro-ecological Experimental Station of the Chinese Academy of Sciences in Fengqiu, Henan Province, located in the Huang-Huai-Hai Plain of China (35°01'N, 114°24'E). The soil, classified as sandy loam, was collected from the surface layer (at a depth of 0–30 cm). Field moisture capacity and saturated volumetric water content of the soil were 0.275 cm³ cm⁻³ and 0.415 cm³ cm⁻³ respectively.

The maize variety was Zhengdan 958. Plants were grown in steel pots (0.36 m diameter × 0.60 m high), sealed at the bottom with rubber stoppers, which contained 60.00 kg of air-dry soil (equal to 56.15 kg of oven-dry soil). All pots were arranged randomly, and a mobile rainproof canopy was installed over the pots, and only

operated when rainfall events occurred to prevent rain water entering the pots. The position of each pot was changed weekly to reduce edge effects. Before sowing, 5.08 g of urea (with 46 % N), 4.42 g of Ca(H₂PO₄)₂ (with 46 % P₂O₅) and 10.17 g of K₂SO₄ (with 50 % K₂O) were completely mixed with the soil. Additional chemical fertilizer (7.63 g of urea) was applied in each pot at the booting stage when slight N deficiency symptoms were observed. Six maize seeds per pot were sown. Seedlings were thinned to three plants per pot 12 days after planting, and all pots were well watered for 22 days from planting to ensure initially good and uniform seedling growth, when each plant had five leaves. And then the soil surface was covered by two layers of aluminum foil so that almost all water loss could be attributed to plant transpiration. Small slits were opened simultaneously in the aluminum foil for maize growth. After that, the pots were subjected to three water treatments including: W1, with soil moisture kept at field capacity; W2, with soil moisture kept at 75 % field capacity; and W3, with soil moisture kept at 50 % field capacity. Field capacity was measured as the gravimetric water content of saturated soil that was allowed to drain for 6 h in a filter funnel jars with soil equilibrated in darkness (15 days at 25 °C), periodically adjusting soil moisture based on weight loss. The experimental pots were weighted every 3 days and then water was added according to its actual loss. There were 54 pots: three water regimes × six growth stages × three replications. The growth stages of seedling, jointing, booting, tasseling, filling and maturity were observed 12, 44, 57, 75, 89, 107 days after transplanting, respectively.

Measurements

Maize was harvested at seedling, jointing, booting, tasseling, filling and maturity, respectively. At each harvest time, dry matter accumulation, WUE, gas exchange, PEPC and Rubisco activities, stable carbon isotopic component of leaf and stem, leaf area (LA) and SLA were measured, respectively.

Transpiration water loss was measured gravimetrically by weighing the pots every 3 days. Aboveground dry matter of maize in each pot was cut at ground level and roots were washed carefully with tap water. Dry weights of the biomass were determined after drying at 80 °C to a constant weight. WUE was determined by dividing dry weight of the whole plant by the cumulative amount of water transpired during the specific growing stage.

The activities of PEPC and Rubisco were determined on the recently fully-expanded top leaves. PEPC activity was determined spectrophotometrically at 340 nm using an UV-1601 spectrophotometer by coupling the reaction to

the oxidation of NADH from malate dehydrogenase (Blanke and Ebert 1992). Rubisco activity was also measured spectrophotometrically at 340 nm using UV-1601 spectrophotometer according to Camp et al. (1982).

Leaf gas exchanges were measured during each growth stage. Photosynthesis rate (A), leaf stomatal conductance (G), intercellular CO_2 concentration (C_i), atmospheric CO_2 concentration (C_a) and transpiration rate (T) were determined using recently fully-expanded top leaves, by a Portable Photosynthesis System (LI-6400, LI-COR Inc., USA), with a 2 cm \times 3 cm natural light leaf chamber. Measurements of leaf gas exchange were conducted only under cloudless or nearly cloudless conditions, and approximately 25–30 s was required to obtain a steady-state reading for each data measurement. During the measurement processes of all growth stages, CO_2 in the leaf chamber was set to track the ambient concentration. The light intensities, leaf temperatures and relative humidity changed little and their variation ranges were 1600–1800 $\mu\text{mol quanta m}^{-2}$, 26–30 $^\circ\text{C}$ and 78–83 %, respectively. Therefore, leaf gas exchange of all pots was only influenced by water stress and growth stages in this study.

Both recently fully-expanded leaves and stems were collected at the six growth stages for stable carbon isotope analysis. All plant materials were oven-dried at 80 $^\circ\text{C}$ for 48 h and finely ground in a ball mill to powder. The stable carbon isotope composition was determined with an Automated Nitrogen and Carbon Analyzer-Mass spectrometer (Thermo Finnigan America, MAT251). The standard in this study was Pee Dee Belemnite. The analytical precision was 0.1 ‰ and the accuracy was better than 0.1 %.

LA and SLA were measured during the five growth stages except for maturity. LA and SLA were estimated using the following calibration equation based on the measurements of length and maximal width of each leaf,

$$LA = 0.75 \times \sum L_i \times B_i \quad (5)$$

$$SLA = LA/m \quad (6)$$

where L is leaf length (cm), B is maximal leaf width (cm), the subscript of i represents the number of leaves per plant, m is the biomass dry weight of leaves (g).

Estimation of ϕ

The values of ϕ were estimated using the data of gas exchange and photosynthesis $\Delta^{13}\text{C}$ according to Eq. (2), which could be rearranged to yield an expression of ϕ (Farquhar 1983; Henderson et al. 1992),

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

where the symbols are as mentioned above.

Since a , b_4 , b_3 and s were relatively constant, then ϕ could be estimated from $\Delta^{13}\text{C}$ and C_i/C_a . In this study, we used leaf $\Delta^{13}\text{C}$ instead of photosynthesis $\Delta^{13}\text{C}$ to calculate ϕ , and leaf $\Delta^{13}\text{C}$ was calculated by measured value of leaf $\delta^{13}\text{C}$.

Statistical analysis

The regression analyses of $\delta^{13}\text{C}$ between leaf and stem were conducted to determine whether fractionation occurred when photosynthetic carbohydrates transported from leaf to stem under water stress. The statistical model used included sources of variation due to replication, water regime and growth stage. All statistical analyses were performed using SAS V.9.0 (SAS Institute Inc., Cary NC, USA).

Results

Dry matter and WUE

Water stress resulted in marked decrease in biomass accumulation (Table 1). The extent of the decrease was dependent on growth stage and degree of water stress. The differences of dry matter accumulation among the three water regimes varied with growth stages, which were significant from tasseling to maturity, but not from seedling to booting. WUE increased with water stress during different growth stages (Table 1). WUE in the W1 treatment was relatively stable and changed little during the whole growth cycle. WUE in W2 increased gradually from jointing to tasseling, and then decreased at maturity. Moreover, WUE in W3 was greatest among the three water regimes and with a range of 7.69–9.93 g kg^{-1} during the whole growth cycle.

PEPC and Rubisco activities

During each growth stage, the Rubisco and PEPC activities decreased significantly with water stress (Fig. 1A). The ratio between the activities of Rubisco and PEPC increased markedly with water stress (Fig. 1B), indicating that PEPC was more sensitive to water deficiency than Rubisco.

Gas exchange

In the W1 treatment, A increased from seedling to booting, then changed little until the filling stage, and decreased markedly at maturity (Table 2). A similar trend was observed in T during the whole growth cycle; C_a fluctuated

Table 1 Dry matter accumulation, WUE, $\Delta^{13}\text{C}$ in leaf, ϕ , leaf area, and specific leaf area during different growth stages of maize under three water regimes

Growth stages	Water regimes	Dry mater (g plant ⁻¹)	WUE (g kg ⁻¹)	Leaf $\Delta^{13}\text{C}$ (‰)	ϕ (%)	Leaf area (cm ² plant ⁻¹)	Specific leaf area (cm ² g ⁻¹)
Seedling	W1	0.53 (0.07)i	5.38 (0.54)g	5.11 (0.16)a	44.16 (1.13)a	49.01 (7.12)g	141.31 (5.38)d
	W2	0.47 (0.04)i	5.24 (0.43)g	5.10 (0.09)a	44.06 (0.83)a	41.86 (1.53)g	146.16 (6.84)d
	W3	0.42 (0.02)i	5.02 (0.25)g	5.09 (0.10)a	44.20 (1.26)a	40.24 (2.29)g	158.46 (4.01)d
Jointing	W1	9.44 (2.23)h	5.57 (0.60)g	4.71 (0.28)b	39.53 (1.78)b	900.07 (20.79)ef	151.42 (4.39)cd
	W2	5.64 (0.19)h	6.97 (0.45)f	3.97 (0.12)e	33.64 (0.71)fg	491.97 (38.15)fg	138.70 (11.78)e
	W3	4.25 (0.18)h	7.95 (0.62)e	3.54 (0.39)f	30.27 (3.02)h	354.10 (23.38)g	132.05 (3.98)fg
Booting	W1	34.03 (3.85)fg	5.84 (1.20)fg	4.14 (0.17)d	35.42 (1.16)e	3240.75 (297.64)a	190.80 (18.62)a
	W2	25.68 (4.06)g	7.51 (1.32)e	3.95 (0.25)e	34.17 (1.14)f	2292.75 (163.68)b	176.83 (6.84)b
	W3	19.73 (0.53)gh	7.69 (0.83)de	3.75 (0.10)f	31.90 (0.35)g	1361.11 (319.68)d	135.65 (24.48)ef
Taselling	W1	59.58 (3.84)cd	6.54 (0.57)f	4.41 (0.16)c	37.21 (1.06)de	3564.63 (337.60)a	157.42 (8.03)c
	W2	49.61 (4.06)e	9.07 (2.05)bc	4.19 (0.10)d	35.07 (0.81)ef	2345.77 (104.28)b	124.83 (7.57)g
	W3	34.87 (0.53)g	9.61 (0.44)a	3.99 (0.14)e	33.08 (0.98)fg	1526.97 (357.24)cd	115.05 (25.57)g
Filling	W1	86.85 (7.40)b	6.12 (0.36)f	4.74 (0.07)b	39.74 (2.63)b	3453.66 (419.69)a	159.22 (5.45)c
	W2	63.97 (4.86)c	8.40 (1.21)cd	4.72 (0.43)b	40.37 (2.91)b	2144.70 (108.65)bc	134.84 (15.67)f
	W3	44.55 (1.76)ef	9.57 (0.94)ab	4.06 (0.33)de	33.35 (1.09)fg	1113.47 (104.45)de	100.11 (10.65)h
Mature	W1	122.25 (7.64)a	6.24 (0.18)f	4.78 (0.08)b	40.75 (0.44)b	–	–
	W2	92.17 (7.32)b	8.09 (1.19)de	4.52 (0.22)c	38.23 (1.72)c	–	–
	W3	59.61 (10.24)cd	9.93 (1.95)a	4.49 (0.26)c	37.91 (1.78)cd	–	–
Tests of between-subjects effects	Water regimes (W)	**	**	**	**	**	**
	Growth stages (G)	**	**	**	**	**	**

W1, W2 and W3 represent field capacity of 100, 75, and 50 %, respectively. The data in the parentheses refer to the standard deviation. WUE are water use efficiency, $\Delta^{13}\text{C}$ is carbon isotope discrimination, ϕ is bundle sheath leakiness. Different letters on each column are significantly different at $P = 0.05$ according to multiple comparisons by Tukey test

– Presents no data; ** marks significant at $P = 0.01$

within 368.8–380.4 $\mu\text{mol mol}^{-1}$ and remained stable in the different growth stages ($P > 0.05$); the variation in C_i/C_a was consistent with C_i . The same trends also existed in W2 and W3 treatments. The ratio of photosynthesis to transpiration rates was defined as transpiration efficiency (A/T), which represented the instantaneous state at the leaf level, and increased with water stress.

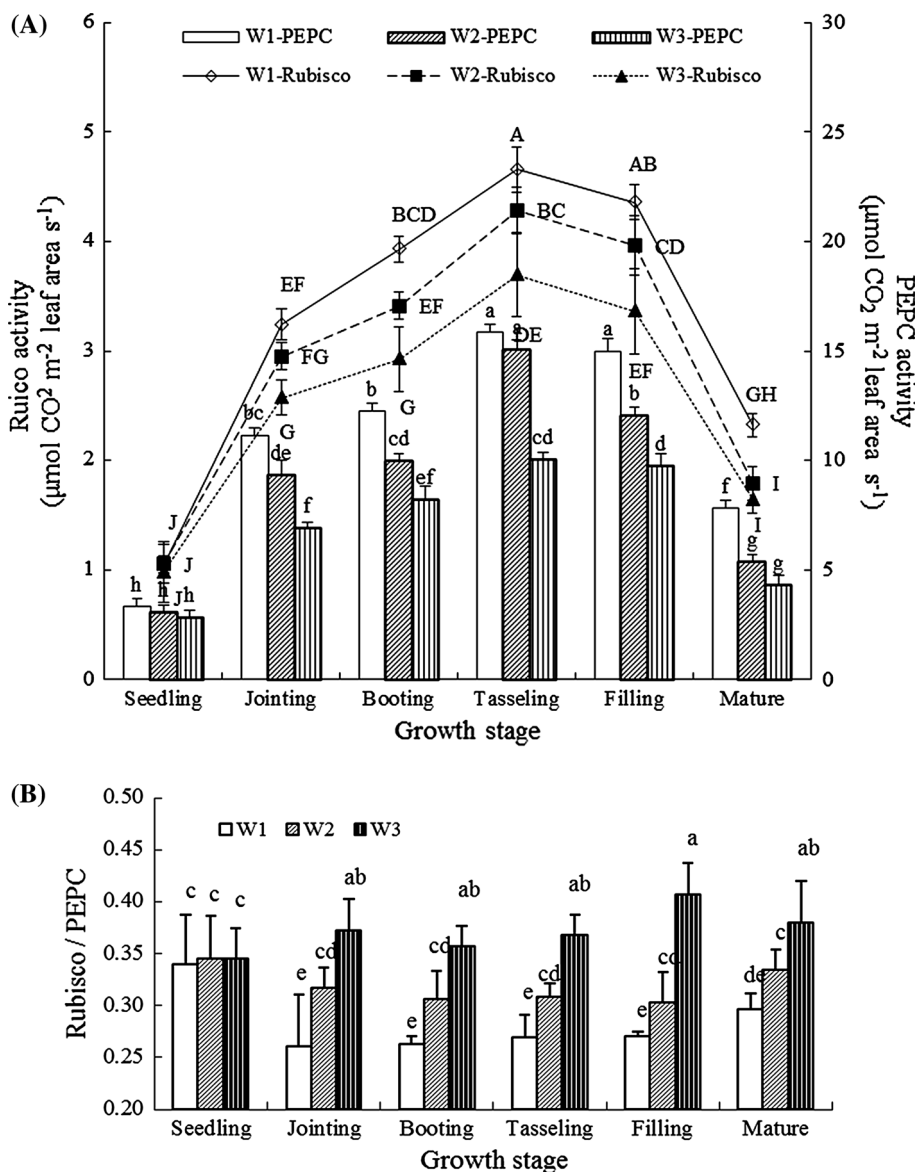
A , G , C_i , T and C_i/C_a showed a general trend of decreasing with water stress (Table 2). At each growth stage, the decrease of G with water stress indicated that leaf stomata closed and stomatal resistance increased when maize grew in drought. However, the variation of C_i/C_a under the three water regimes was less marked (Table 2). This suggested that photosynthesis was not affected by stomatal limitation, but mainly affected by non-stomatal limitation, such as the activities of Rubisco and PEPC (Fig. 1) (Hubbard et al. 2001). A decreased with water stress; however, transpiration efficiency (A/T) increased because the decrease in T was greater than in A with less soil water (Table 2).

Carbon isotope composition and bundle sheath leakiness

All maize was irrigated fully at seedling stage, and the average values of leaf and stem $\delta^{13}\text{C}$ were -13.04 and -12.91 ‰, respectively. During the following growth stages, $\delta^{13}\text{C}$ in both leaf and stem increased with water stress (Fig. 2). Differences in $\delta^{13}\text{C}$ between leaf and stem were observed at each growth stage, and the relationship between leaf and stem $\delta^{13}\text{C}$ under three water regimes was shown in Fig. 3. In the well-watered treatment (W1), leaf $\delta^{13}\text{C}$ showed no significant difference from stem $\delta^{13}\text{C}$, and the regression line was close to a 1:1 line (Fig. 3), indicating no ^{13}C fractionation when photosynthetic carbohydrates were transported from leaf to stem. However, differences in $\delta^{13}\text{C}$ between leaf and stem became greater with water stress.

During the whole growth cycle, ϕ reached highest in seedling. From jointing to mature, ϕ decreased with water stress, and showed an opposite trend to the variation in $\delta^{13}\text{C}$ (Table 1).

Fig. 1 Activities of Rubisco and PEPC (A), and the ratio between the activity of Rubisco and PEPC (B) during different growth stages under three water regimes. W1, W2 and W3 represent field capacity of 100, 75, and 50 %, respectively; Rubisco and PEPC represent ribulose 1, 5-bisphosphate carboxylase and phosphoenolpyruvate carboxylase, respectively. The error bars refer to the standard deviation. Different letters on each error bar are significantly different at $P = 0.05$ according to multiple comparisons by Tukey test



LA and SLA

LA increased rapidly from seedling to booting, then varied slightly from booting to maturity, and then decreased with water stress during each growth stage (Table 1). SLA varied with leaf dry weight and leaf area during different growth stages, which was greatest in booting, and decreased in the following growth stages. SLA decreased with water stress, and its variation trend under the three water treatments was opposite to that of WUE.

Relationship between WUE and leaf $\delta^{13}\text{C}$ and SLA

Leaf WUE in the photosynthetic process is presented as transpiration efficiency. The leaf is the most important organ for photosynthesis, and so should be the most appropriate one for sampling to determine the relationship

between WUE and $\delta^{13}\text{C}$ (Zhao et al. 2004). $\delta^{13}\text{C}$ and WUE changed little in seedlings without water stress (Fig. 4). From jointing to maturity, leaf $\delta^{13}\text{C}$ was significantly positively correlated with WUE. The data of the whole growth cycle under the three water regimes showed a significant positive correlation between WUE and $\delta^{13}\text{C}$. There was a similar trend and significant positive correlation between leaf $\delta^{13}\text{C}$ and SLA.

Discussion

Effects of water stress on PEPC, Rubisco, ϕ , C_i/C_a and leaf $\delta^{13}\text{C}$

Compared with the control pots (W1), the activities of PEPC and Rubisco both decreased in W2 and W3

Table 2 Photosynthetic rate (A), transpiration rate (T), transpiration efficiency as A/T, ratio of intercellular and atmosphere concentration of CO₂ (C_i/C_a), and stomatal conductance (G) measured during different growth stages under three water regimes

Growth stages	Water regimes	Gas exchange parameters				
		A (μmol CO ₂ m ⁻² s ⁻¹)	T (mmol H ₂ O m ⁻² s ⁻¹)	A/T (μmol CO ₂ mol H ₂ O ⁻¹)	C _i /C _a	G (mol H ₂ O m ⁻² s ⁻¹)
Seedling	W1	20.39 (1.20)ghi	2.93 (0.20)cd	6.97 (0.62)ij	0.37 (0.04)def	0.23 (0.01)c
	W2	19.13 (1.59)hi	2.81 (0.23)d	6.82 (0.34)j	0.37 (0.02)def	0.23 (0.01)c
	W3	19.14 (0.59)hi	2.78 (0.27)d	6.88 (0.52)ij	0.36 (0.02)ef	0.22 (0.13)c
Jointing	W1	31.72 (1.39)bc	4.48 (0.21)ab	7.08 (0.43)ij	0.47 (0.05)abc	0.30 (0.02)b
	W2	26.01 (1.29)e	3.06 (0.15)cd	8.53 (0.32)ef	0.45 (0.05)bcde	0.25 (0.02)c
	W3	21.13 (1.13)gh	2.25 (0.18)e	9.41 (0.36)cd	0.44 (0.08)bcd	0.23 (0.03)c
Booting	W1	34.38 (0.92)a	4.70 (0.32)a	7.32 (0.75)ghi	0.56 (0.06)a	0.37 (0.02)a
	W2	28.67 (1.32)d	3.19 (0.16)c	8.98 (0.47)de	0.54 (0.05)a	0.28 (0.01)b
	W3	21.35 (2.30)gh	2.24 (0.18)e	9.54 (0.43)cd	0.46 (0.09)bcde	0.18 (0.01)d
Taselling	W1	34.12 (2.10)ab	4.26 (0.25)b	8.01 (0.38)fg	0.49 (0.03)ab	0.29 (0.01)b
	W2	29.85 (1.50)cd	2.84 (0.24)cd	10.52 (0.42)ab	0.37 (0.03)def	0.24 (0.03)c
	W3	24.50 (2.43)ef	2.17 (0.19)e	11.28 (0.54)a	0.35 (0.03)def	0.13 (0.01)e
Filling	W1	34.20 (2.10)ab	4.49 (0.22)ab	7.62 (0.37)ghi	0.48 (0.07)abc	0.30 (0.02)b
	W2	29.28 (1.08)cd	2.93 (0.14)cd	10.01 (0.46)bc	0.36 (0.02)ef	0.23 (0.02)c
	W3	22.17 (1.96)fg	2.01 (0.21)e	11.01 (0.43)a	0.33 (0.04)f	0.11 (0.02)ef
Mature	W1	17.83 (0.69)i	2.32 (0.13)e	7.68 (0.28)ghi	0.39 (0.05)cdef	0.18 (0.01)d
	W2	13.28 (1.34)j	1.39 (0.21)f	9.56 (0.54)cd	0.40 (0.03)bcdef	0.13 (0.01)e
	W3	9.47 (0.51)k	0.86 (0.16)g	11.05 (0.84)a	0.42 (0.08)bcdef	0.08 (0.01)f

Two-way analysis of variance with *Growth stage* and *Water regime* as treatments

Tests of between-subjects effects	Water regimes (W)	**	**	**	NS	**
	Growth stages (G)	**	**	**	**	**

W1, W2, and W3 represent field capacity of 100, 75, and 50 %, respectively. The data in the parentheses refer to the standard deviation. Different letters on each column are significantly different at $P = 0.05$ according to Tukey test

** Marks significant at $P = 0.01$; NS = no significant

treatments (Fig. 1), and A was lower (Table 2), resulting in a marked decrease in dry matter accumulation during different growth stages (Table 1). However, the functional affinity for CO₂ and the carboxylation efficiency of PEPC are much higher than those of Rubisco (Henderson et al. 1992). The photosynthetic assimilates are finally formed in bundle sheath cells through the C₃ pathway catalyzed by Rubisco. The ratio between the activities of Rubisco and PEPC increased significantly with water stress, implying that PEPC was more sensitive to water stress. At same time, CO₂ in the bundle sheath leaked back to the mesophyll cells (ϕ) decreased with water stress (Table 1) and left more CO₂ in bundle sheath cells to be fixed by Rubisco, and assimilated more ¹³C, and resulted in increased $\delta^{13}\text{C}$ in leaves.

During the photosynthetic process of maize, atmospheric CO₂ is concentrated through PEPC in mesophyll cells, and then released to bundle sheath cells at high

concentrations. The CO₂ in the bundle sheath can partly leak out to the mesophyll cells. In present study, leaf $\Delta^{13}\text{C}$ was used to calculate ϕ by the theoretical model of Eq. (4), which was also conducted by a series of studies (Farquhar 1983; Henderson et al. 1992; Monneveux et al. 2007). However, it was should be photosynthesis $\Delta^{13}\text{C}$ rather than leaf $\delta^{13}\text{C}$ when ϕ was calculated. Generally, leaf $\delta^{13}\text{C}$ is usually different with photosynthesis $\Delta^{13}\text{C}$ due to some post-photosynthetic fractionations (von Caemmerer et al. 2014), respiration typically enriched more ¹³C than the bulk leaf signatures (von Caemmerer et al. 2014) and increased leaf $\delta^{13}\text{C}$, but isotope effects of the biochemical pathways for synthesizing different organic compounds usually deleted more ¹³C compared with estimated made from measurements of photosynthesis $\delta^{13}\text{C}$ (von Caemmerer et al. 2014), which would decreased leaf $\delta^{13}\text{C}$. Because ϕ was positively correlated with $\Delta^{13}\text{C}$ and negatively correlated with $\delta^{13}\text{C}$ from Eqs. (2) and (7). The reduction

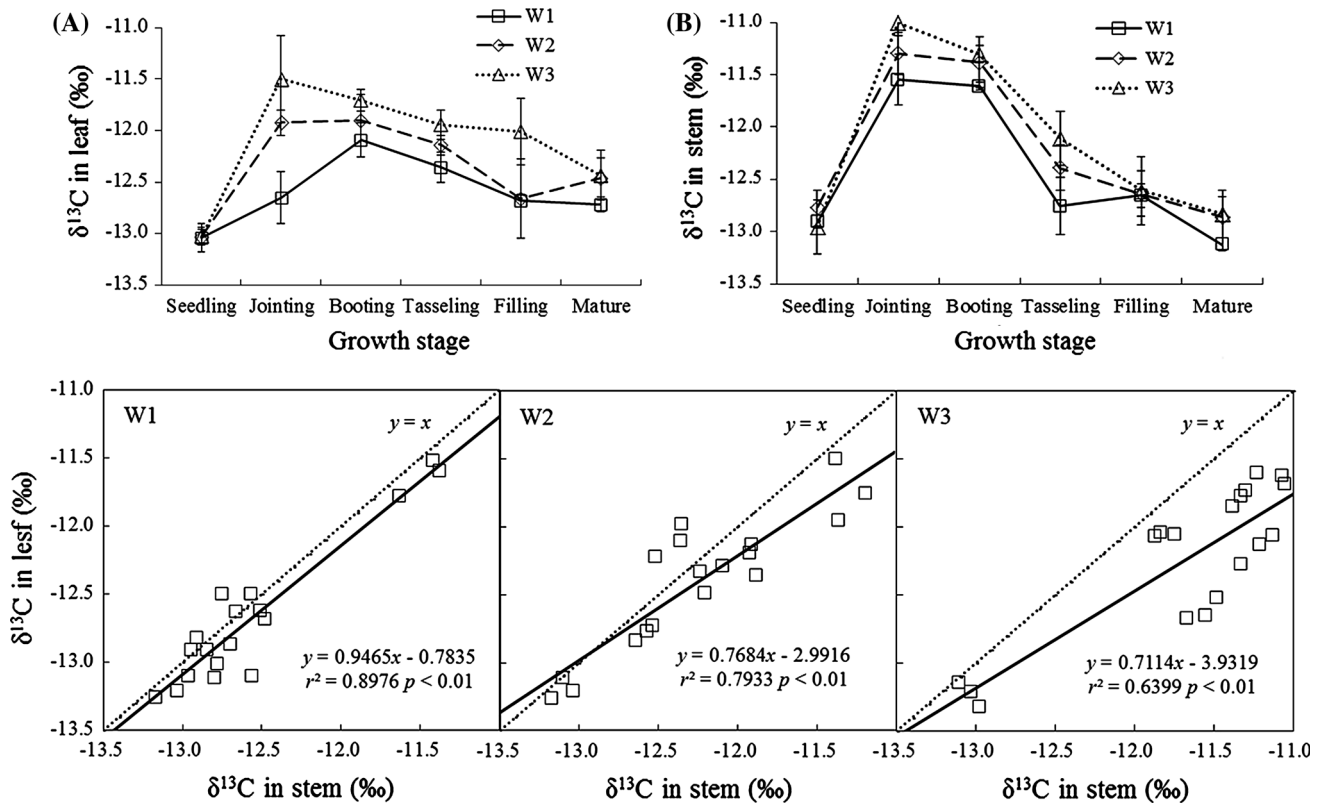


Fig. 2 $\delta^{13}\text{C}$ in maize leaf (A) and stem (B) and their relationship under various water treatments. $\delta^{13}\text{C}$ represents carbon isotope composition. W1, W2 and W3 represent field capacity of 100, 75, and 50 %, respectively. Dashed line was $y = x$

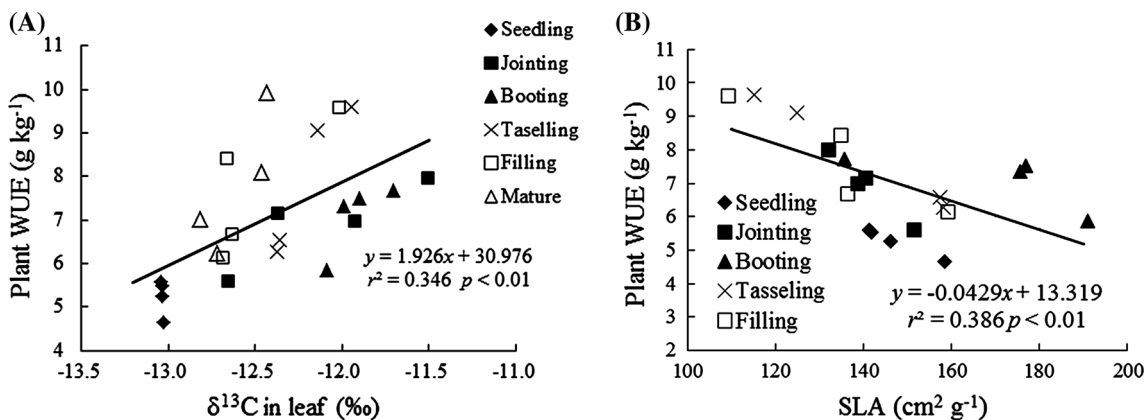


Fig. 3 Relationship between plant WUE and leaf $\delta^{13}\text{C}$ (A) and SLA (B) under three water regimes. Other symbols are the same as mentioned above. $\delta^{13}\text{C}$, represents carbon isotope composition; WUE, represents water use efficiency; SLA, represents specific leaf area

of leaf $\delta^{13}\text{C}$ might overestimate the value of ϕ . The difference between leaf $\delta^{13}\text{C}$ and photosynthesis $\delta^{13}\text{C}$ was not clear. However, all pot experiments in present study were conducted using only a maize variety which avoided the distinction of biochemical subtype, and all plant materials at different growth stages used for stable carbon isotope analysis were recently fully-expanded, which might partially reduce the distinguish brought by respiration.

Therefore, we considered that leaf $\Delta^{13}\text{C}$ used for calculation of ϕ was acceptable when explored the effect of water stress on ϕ .

Leakiness (ϕ) varies with species and growing environment. Henderson et al. (1992) reported that ϕ was about 20 % through the measurement of several C_4 species, while Farquhar et al. (1989) indicated that ϕ should be close to 37 %. Hattersley and Roksandic (1983) measured 31 C_4

species of the Poaceae family and reported that ϕ was mainly affected by biochemical subtypes within the range of 37–53 %. In the present study, ϕ in maize varied with water stress and growth stages, in the range of 30.27–44.17 % (Table 1). Vogel (1993) showed that ϕ was positively correlated with the concentration difference of CO₂ between bundle sheath and mesophyll cells, while negatively correlated with the diffusional resistance during leakiness. In the present study, the variation of ϕ was consistent with that of Rubisco/PEPC (the activity ratio between Rubisco and PEPC; Fig. 1), which increased with water stress. Therefore, the relationship between ϕ and Rubisco/PEPC implied that ϕ in maize varied under different water regimes, possibly due to the regulation of activities of PEPC and Rubisco. According to Eq. (4), the values of leaf $\Delta^{13}\text{C}$ in maize are mainly affected by C_i/C_a and ϕ , and ϕ has a large effect on leaf $\Delta^{13}\text{C}$ at high C_i/C_a , and less at low C_i/C_a . Another interesting calculation result from Eq. (7) is that the value of $\Delta^{13}\text{C}$ should be a constant of 4.4 ‰ when ϕ is 33.6 %.

To determine the extent to which $\Delta^{13}\text{C}$ was influenced by ϕ and C_i/C_a , $\Delta^{13}\text{C}$ was regressed against these two parameters individually (Fig. 4). Leaf $\Delta^{13}\text{C}$ showed an extremely significant positive correlation with ϕ under three water regimes ($P < 0.01$, Fig. 4). However, the negative correlation between leaf $\Delta^{13}\text{C}$ and C_i/C_a was significant in W1 ($P < 0.05$) but not in W2 and W3. The decrease of ϕ and increase of Rubisco/PEPC finally resulted in more fixed ^{13}C , and leaf $\Delta^{13}\text{C}$ decreased due to the increase of leaf $\delta^{13}\text{C}$ (Chaves et al. 2002).

The results of analysis of correlation between leaf $\Delta^{13}\text{C}$ and ϕ , and C_i/C_a showed that the variation of leaf $\Delta^{13}\text{C}$ was mainly affected by ϕ , consistent with the findings of Saliendra et al. (1996). However, Madhavan et al. (1991) reported that the main impact factor on leaf $\Delta^{13}\text{C}$ was C_i/C_a rather than ϕ . Williams et al. (2001) pointed out that the attribution of C_i/C_a and ϕ to leaf $\Delta^{13}\text{C}$ varied with water conditions, and ϕ contributed more in a drought environment. Their findings were inconsistent with our study, in which leaf $\Delta^{13}\text{C}$ in maize was mainly affected by ϕ in all three water regimes. In fact, the reason might be the subtype of NADP-ME in maize, and that leaf $\Delta^{13}\text{C}$ was mainly influenced by ϕ as for Fravolini et al. (2002), who found that ϕ was the main factor in the types of NADP-ME and PCK, while C_i/C_a played a major role in NAD-ME.

Mechanisms for the relationship between WUE and leaf $\delta^{13}\text{C}$

A/T is usually used as the definition of instantaneous WUE at the leaf level. Meinzer et al. (1992) reported that there were two mechanisms in the increase of WUE. The first

was that the degree of water consumed (T) reduced more than that of CO₂ fixed (A), and as a result G was lower when stomata were closed. The second was that photosynthetic capacity enhanced and enabled the increase in A to exceed the increase in T . The effect of water stress on WUE in maize clearly belonged to the first mechanism, and this mechanism has been widely reported in many other species (Chaves et al. 2002), such as spruce (Sun et al. 1996) and cotton (Saranga et al. 1998).

The relationship between transpiration efficiency (WUE in the photosynthetic process at leaf level) and $\Delta^{13}\text{C}$ in C₄ species can be expressed as follows (Wright et al. 1988; Farquhar et al. 1989):

$$WUE = C_a(b_4 + \phi b_3 - \Delta^{13}\text{C}) / [1.6v(b_4 + \phi b_3 - a)] \quad (8)$$

where v is vapor pressure deficiency and the other symbols are as described above.

Based on Eqs. (4) and (8), WUE was positively related to $\delta^{13}\text{C}$ and negatively to $\Delta^{13}\text{C}$ —this has been confirmed in many greenhouse and field experiments, especially under conditions of water deficiency (Fotovat et al. 2007). The same relationship between $\delta^{13}\text{C}$ and WUE has also been observed in cotton (Saranga et al. 1998), upland rice (Zhao et al. 2004) and other crops (Wright et al. 1988). In the present study, WUE was significantly positively correlated with $\delta^{13}\text{C}$, indicating that $\delta^{13}\text{C}$ in leaf could be used as an index for identifying maize WUE in drought conditions. However, some conflicting results have also been reported. For example, Dingkuhn et al. (1991) reported that $\delta^{13}\text{C}$ of upland rice was positively correlated with transpiration efficiency, but was not correlated with WUE in the field. Walley et al. (1999) studied wheat exposed to different tillage systems and pointed out that variations in WUE were not consistent with variations in $\delta^{13}\text{C}$. In general, these previous studies have been conducted under two conditions. The first was that crops were grown under well-watered conditions, or water source was rich around the crop, and the stomata opened and led to high G and C_i/C_a , with less ^{13}C fixed by photosynthesis, and low $\delta^{13}\text{C}$ in leaves. The second was that the studies were conducted in large fields, and the estimation of WUE depended on the calculation methods of water used by crops, with soil evaporation and deep seepage usually included in the crop water consumption.

In the present study, soil evaporation was prevented by the two layers of aluminum foil. The maize transpiration rate reduced more than photosynthetic rate with water stress (Table 2), and thus WUE increased from W1 to W3 treatments (Table 1). However, the leakiness of CO₂ from bundle sheath to mesophyll cells decreased with water stress (Table 1), and left more CO₂ in bundle sheath cells to be catalyzed by Rubisco due to the regulation of Rubisco/PEPC, which increased with water stress (Fig. 1).

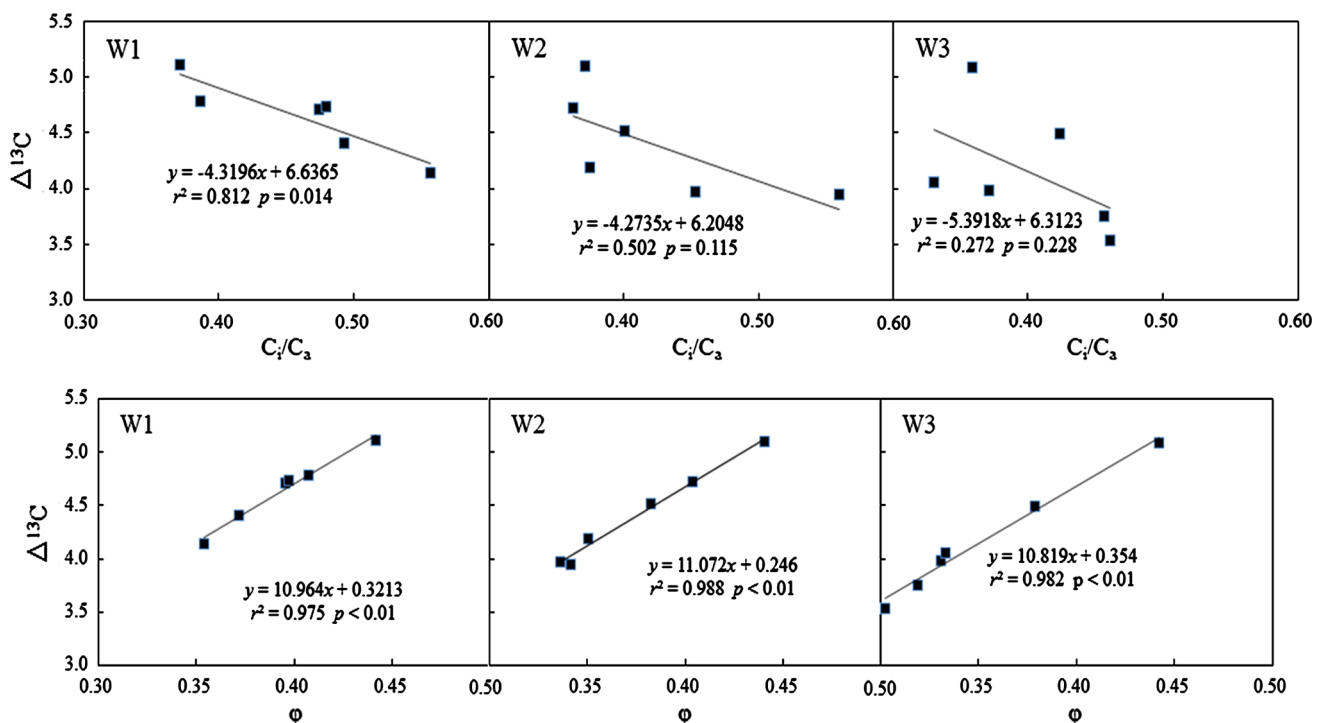


Fig. 4 Relationship between $\Delta^{13}\text{C}$ and C_i/C_a and ϕ under three water regimes. C_i/C_a , represents ratio of CO_2 concentration between intercellular and atmosphere; $\Delta^{13}\text{C}$, represents carbon isotope discrimination; ϕ , represents bundle sheath leakiness

The decrease of ϕ caused more ^{13}C to be assimilated, and then increased $\delta^{13}\text{C}$ in leaves. Therefore, WUE was positively correlated with leaf $\delta^{13}\text{C}$ under water stress.

Mechanisms for the relationship between WUE and SLA

At each growth stage, SLA decreased with the increase in water stress (Table 2), indicating that leaves of maize became thicker in response to water deficiency. Although respiration and other non-photosynthetic processes played a role during these physiological processes, we believe that the main reason for the variation of SLA was that leaf expansion and translocation of assimilates from the leaf were prevented by water stress, resulting in thicker leaves and lower SLA—this was confirmed by the differences of $\Delta^{13}\text{C}$ between leaf and stem (Fig. 3). Compared with stem $\Delta^{13}\text{C}$, leaf $\Delta^{13}\text{C}$ decreased with water stress, indicating that more ^{13}C was enriched in leaves when maize suffered from drought, implying that more assimilates accumulated in leaves rather than being transported to stems under water stress, which resulted in thicker leaves and lower SLA.

Water stress affected leaf expansion and translocation of assimilates from leaves, with less ^{13}C transported to stems and more ^{13}C enriched in leaves. Leaf $\delta^{13}\text{C}$ increased and $\Delta^{13}\text{C}$ decreased simultaneously. In addition, leaves became thicker and SLA decreased. Therefore, SLA was negatively

correlated with WUE (Fig. 4), because SLA was negatively correlated with leaf $\delta^{13}\text{C}$.

Conclusion

Both photosynthetic rate and dry matter accumulation in maize decreased under water stress, due to the decreased activities of Rubisco and PEPC. Additionally, leaf stomata closing decreased the stomatal conductance, and also reduced transpiration rate. Because the transpiration rate was reduced more than the photosynthetic rate, the WUE increased with water stress. The leakiness of CO_2 from bundle sheath to mesophyll cells decreased with water stress, and left more CO_2 in bundle sheath cells to be catalyzed by Rubisco due to the regulation of Rubisco/PEPC. The decrease of ϕ caused more ^{13}C to be assimilated, and then increased $\delta^{13}\text{C}$ in leaves. Therefore, WUE was positively correlated with leaf $\delta^{13}\text{C}$ under water stress. There was no fractionation between leaf and stem when maize was grown in the well-watered condition. However, the fractionation became greater with water stress, indicating that water stress affected leaf expansion and translocation of assimilate from leaves, resulting in thicker leaves and lower SLA. Therefore, WUE was negatively correlated with SLA under water stress. The relationship between WUE and $\delta^{13}\text{C}$ and SLA in maize

implied that leaf $\delta^{13}\text{C}$ and SLA could effectively reflect the drought adaptation and high WUE under different water conditions.

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References

- Blanke MM, Ebert G (1992) Phosphoenolpyruvate carboxylase and carbon economy of apple seedling. *J Exp Bot* 252:965–968
- Bowman WD, Hubick KT, von Caemmerer S, Farquhar GD (1989) Short-term changes in leaf carbon isotope discrimination in salt- and water-stress C_4 grasses. *Plant Physiol* 90:162–166
- Camp PJ, Huber SC, Burke JJ, Moreland DE (1982) Biochemical changes that occur during senescence of wheat leaves. *Plant Physiol* 70:1641–1646
- Chaves MM, Pereira JS, Maroco J, Rodrigues ML, Ricardo CP, Osório ML, Carvalho I, Faria T, Pinheiro C (2002) How plants cope with water stress in the field? Photosynthesis and growth. *Ann Bot* 89:907–916
- Dingkuhn M, Farquhar GD, De Datta SK, O'Toole JC (1991) Discrimination of ^{13}C among upland rice having different water use efficiencies. *Aust J Agric Res* 42:1123–1131
- Farquhar GD (1983) On the nature of carbon isotope discrimination in C_4 species. *Aust J Plant Physiol* 10:205–226
- Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. *Ann Rev Plant Phys Plant Mol Biol* 40:503–537
- Fotovat R, Valizadeh M, Toorchi M (2007) Association between water-use efficiency components and total chlorophyll content (SPAD) in wheat (*Triticum aestivum* L.) under well-watered and drought stress conditions. *J Food Agric Environ* 5:225–227
- Fravolini A, Williams DG, Thompson TL (2002) Carbon isotope discrimination and bundle sheath leakiness in three C_4 subtypes grown under variable nitrogen, water and atmospheric CO_2 supply. *J Exp Bot* 53:2261–2269
- Hattersley PW, Roksandic Z (1983) $\delta^{13}\text{C}$ values of C_3 and C_4 species of Australian Neurachne and its allies (Poaceae). *Aust J Bot* 3:317–321
- Henderson SA, von Caemmerer S, Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C_4 species. *Aust J Plant Physiol* 19:263–285
- Hubbard RM, Ryan MG, Stiller V, Sperry JS (2001) Stomatal conductance and photosynthesis vary linearly with plant hydraulic conductance in ponderosa pine. *Plant Cell Environ* 24:113–121
- Liu C, Li C, Liang D, Ma F, Wang S, Wang P, Wang R (2013) Aquaporin expression in response to water-deficit stress in two *Malus* species: relationship with physiological status and drought tolerance. *Plant Growth Regul* 70:187–197
- Madhavan S, Treichel I, O'Leary MH (1991) Effects of relative humidity on carbon isotope fractionation in plants. *Bot Acta* 104:292–294
- Meinzer FC, Rundel PW, Goldstein G, Sharifi MR (1992) Carbon isotope composition in relation to leaf gas exchange and environmental conditions in Hawaiian *Metrosideros* polymorpha populations. *Oecologia* 91:305–311
- Monneveux P, Sheshshayee MS, Akhter J, Ribaut JM (2007) Using carbon isotope discrimination to select maize (*Zea mays* L.) inbred lines and hybrids for drought tolerance. *Plant Sci* 173:390–396
- Saliendra NZ, Meinzer FC, Perry M, Thom M (1996) Association between partitioning of carboxylase activity and bundle sheath leakiness to CO_2 , carbon isotope discrimination, photosynthesis, and growth in sugarcane. *J Exp Bot* 47:907–914
- Saranga Y, Flash I, Yakir D (1998) Variation in water-use efficiency and its relation to carbon isotope ratio in cotton. *Crop Sci* 38:782–787
- Sun ZJ, Livingston NJ, Guy RD, Ethier GJ (1996) Stable carbon isotopes as indicators of increased water use efficiency and productivity in white spruce (*Picea glauca* (Moench) Voss) seedlings. *Plant Cell Environ* 19:887–894
- Vogel JC (1993) Variability of carbon isotope fractionation during photosynthesis. In: Ehleringer J, Hall A, Farquhar G (eds) Stable isotopes and plant carbon-water relations. Academic Press Inc, California, pp 29–46
- von Caemmerer S, Ghannoum O, Pengelly JLL, Cousins AB (2014) Carbon isotope discrimination as a tool to explore C_4 photosynthesis. *J Exp Bot* 65:3459–3470
- Walley FL, Lafond GP, Matus A, van Kessel C (1999) Water-use efficiency and carbon isotopic composition in reduced tillage systems. *Soil Sci Soc Am J* 63:356–361
- Williams DG, Gempko V, Fravolini A, Leavitt SW, Wall GW, Kimball BA, Pinter PJ Jr, Lamorte R, Ottman M (2001) Carbon isotope discrimination by Sorghum bicolor under CO_2 enrichment and drought. *New Phytol* 150:285–293
- Wright GC, Hubick KT, Farquhar GD (1988) Discrimination in carbon isotopes of leaves correlates with water use efficiency of field grown peanut cultivars. *Aust J Plant Physiol* 15:815–825
- Wright GC, Nageswara RC, Farquhar GD (1994) Water-use efficiency and carbon isotope discrimination in peanut under water deficit conditions. *Crop Sci* 34:92–97
- Yandigeri MS, Meena KK, Singh D, Malviva N, Singh DP, Solanki MK, Yadav AK, Arora DK (2012) Drought-tolerant endophytic actinobacteria promote growth of wheat (*Triticum aestivum*) under water stress conditions. *Plant Growth Regul* 68:411–420
- Zhao BZ, Kondo M, Maeda M, Ozaki Y, Zhang JB (2004) Water-use efficiency and carbon isotope discrimination in two cultivars of upland rice during different developmental stages under three water regimes. *Plant Soil* 261:61–75