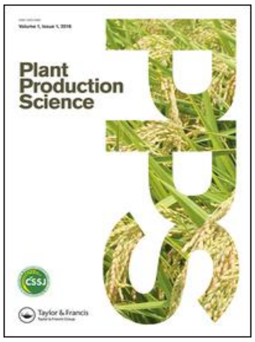

Plant Production Science

ISSN: 1343-943X (Print) 1349-1008 (Online) Journal homepage: https://www.tandfonline.com/loi/tpps20

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To cite this article: Takayuki Yabiku & Osamu Ueno (2017) Variations in physiological, biochemical, and structural traits of photosynthesis and resource use efficiency in maize and teosintes (NADP-ME-type C_4), Plant Production Science, 20:4, 448-458, DOI: 10.1080/1343943X.2017.1398050

To link to this article: https://doi.org/10.1080/1343943X.2017.1398050

PLANT PRODUCTION SCIENCE, 2017
VOL. 20, NO. 4, 448–458
https://doi.org/10.1080/1343943X.2017.1398050

REGULAR PAPER

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Variations in physiological, biochemical, and structural traits of photosynthesis and resource use efficiency in maize and teosintes (NADP-ME-type C_4)

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ABSTRACT

C_4 plants show higher photosynthetic capacity and resource use efficiency than C_3 plants. However, the genetic variations of these traits and their regulatory factors in C_4 plants still remain to be resolved. We investigated physiological, biochemical, and structural traits involved in photosynthesis and photosynthetic water and nitrogen use efficiencies (PWUE and PNUE) in 22 maize lines and four teosinte lines from various regions of the world. Net photosynthetic rate (P_N) ranged from 32.1 to 46.5 μmol m^{-2} s^{-1}. P_N was positively correlated with stomatal conductance, transpiration rate, and chlorophyll, nitrogen and soluble protein contents of leaves, but not with specific leaf weight. P_N was positively correlated with the activities of ribulose-1,5-bisphosphate carboxylase/oxygenase and the C_4-acid decarboxylases, NADP-malic enzyme and phospho*enol*pyruvate carboxykinase, but not with the activity of phospho*enol*pyruvate carboxylase. Leaf structural traits (stomatal parameters, leaf thickness, and interveinal distance) were not correlated with P_N. These data suggest that physiological and biochemical traits are involved in the genetic variation of P_N, but structural traits are not directly involved. PWUE is in the lower class of values reported for C_4 plants, whereas PNUE is in the highest class of values reported for C_4 plants. PNUE was negatively correlated with leaf nitrogen content but not significantly correlated with P_N. PWUE was not correlated with $\delta^{13}C$ values of leaves, indicating difficulty in using $\delta^{13}C$ values as an indicator of PWUE of maize. In general, teosinte lines showed lower P_N but higher PWUE than maize lines.

ARTICLE HISTORY
Received 26 July 2017
Revised 28 September 2017
Accepted 24 October 2017

KEYWORDS
Maize; NADP-malic enzyme type; phospho*enol*pyruvate carboxykinase; photosynthetic nitrogen use efficiency; photosynthetic rate; photosynthetic water use efficiency; teosinte

CLASSIFICATION
Crop Physiology

Introduction

Photosynthetic traits of leaves are one of the most important physiological factors responsible for plant productivity. The improvement of photosynthetic traits promises further increases in plant productivity (Evans, 2013; Zhu et al., 2010). The genetic variation in photosynthetic traits found in both crop and wild species includes a potential to improve crop photosynthesis and ultimately productivity but is largely unexplored (McCouch, 2004; Flood et al., 2011). Photosynthesis is complicatedly regulated by biophysical, biochemical, physiological, and structural traits of leaves. However, our understanding of the regulatory processes is still insufficient (Evans, 2013). The factors that cause the genetic variation in photosynthesis also remain largely unknown (Flood et al., 2011).

It is well known that C_4 plants have higher photosynthetic rate and productivity than C_3 plants (Brown, 1999; Osmond et al., 1982). This is attained by a CO_2 concentrating mechanism (CCM) operating in C_4 plants. In C_4 leaves, mesophyll and bundle-sheath (BS) cells are differentiated and surround vascular bundles. In C_4 photosynthesis, atmospheric CO_2 entering through stomata is fixed by phospho*enol*pyruvate carboxylase (PEPC) in mesophyll cells. The C_4 acids produced are transported to BS cells, where they are decarboxylated by C_4 acid decarboxylase. Released CO_2 is fixed by Rubisco. This biochemical process raises the CO_2 concentration around Rubisco in BS cells and thus reduces photorespiration (Hatch, 1987). The quantitative balance between mesophyll and BS cells is also required to attain the intimate cooperation between C_4 and C_3 cycles (Dengler et al., 1994). In general, C_4 leaves have a denser vascular system than C_3 leaves. It is thought that this structural trait is associated with rapid translocation of photosynthates in C_4 plants (Leonardos & Grodzinski, 2000; Ueno et al., 2006). Because of the complex mechanism of C_4 photosynthesis, the factors determining photosynthetic rate in C_4 plants still remain to be resolved (von Caemmerer & Furbank, 2016). It is suggested that CO_2 delivery process in mesophyll cells, activities and

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Supplementary material for this article can be accessed at https://doi.org/10.1080/1343943X.2017.1398050

properties of C_4 and C_3 photosynthetic enzymes, substrate regeneration in C_4 and C_3 cycles, transport and diffusion of metabolites, electron transport and light capture in chloroplasts, and CO_2 leakiness from BS cells are involved in the regulation of C_4 photosynthesis (reviewed by von Caemmerer & Furbank, 2016). It also appears that biochemical, physiological, and structural traits of leaves are intricately involved in the genetic variation in photosynthetic rate of C_4 plants (Tsutsumi et al., 2017).

Depending on the C_4 acid decarboxylation system in BS cells, the C_4 photosynthetic pathway is classified into three types: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phospho*enol*pyruvate carboxykinase (PCK) (Hatch, 1987). The NADP-ME-type C_4 grasses include crops with high biomass productivity, such as maize, sorghum, and Napier grass (Brown, 1999; Carpita & McCann, 2008). In general, each C_4 species is thought to use only one of the 3 decarboxylation systems (Hatch, 1987). However, some C_4 species may use more than one. Maize (*Zea mays* ssp. mays), a model NADP-ME-type C_4 grass, uses both NADP-ME and PCK (Walker et al., 1997; Wingler et al., 1999). However, the physiological significance of the dual C_4 acid decarboxylation system in C_4 plants is unclear (Furbank, 2011; Koteyeva et al., 2015).

Because C_4 plants have a CCM, their photosynthetic water and nitrogen use efficiencies (PWUE and PNUE) are higher than those of C_3 plants (Brown, 1977; Osmond et al., 1982). These traits of C_4 plants provide advantages for survival in natural habitats and are also useful for sustainable agriculture. PNUE values of NADP-ME-type C_4 grasses are higher than those of NAD-ME-type C_4 grasses, whereas PWUE values are not significantly different (Ghannoum et al., 2001, 2005, 2011). Further extensive studies would be required to clarify the genetic variation in resource use efficiency among C_4 plants.

Table 1. Maize and teosinte lines examined in this study.

Species	Line	Country
Zea mays ssp. *mays*	B73	Iowa, USA
	HP301	Indiana, USA
	IL14H	Illinois, USA
	Ky21	Kentucky, USA
	Mo18W	Missouri, USA
	OH7B	Ohio, USA
	P39	Indiana, USA
	WF9	Indiana, USA
	CM109	Canada
	CML69	Mexico
	Pipoca 4	Brazil
	Pisinga Purpura	Peru
	CB44	Netherlands
	Kuma Mais	Netherlands
	F575	France
	I.C.A.R. 54	Romania
	TZI3	Nigeria
	PI 195114	Ethiopia
	Homedale	South Africa
	Geargian local 1	USSR
	Pakistan	Pakistan
	Ki3	Thailand
Z. mays. ssp. *mexicana*	Ames 8083	Mexico
Z. diploperennis	PI 441930	Mexico
Z. perennis	Ames 21875	Mexico
Z. nicaraguensis	PI 615697	Nicaragua

Maize is important as a grain, forage, and bioenergy crop (Carpita & McCann, 2008). Previous studies have addressed the genetic variation in the photosynthetic rate of maize. Some studies reported a large genetic difference in photosynthetic rate (Crosbie et al., 1977; Heichel & Musgrave, 1969), whereas others found a small difference (Baer & Schrader, 1985; Duncan & Hesketh, 1968). Previous studies suggested that Rubisco and pyruvate, Pi dikinase (PPDK) are rate-limiting enzymes in C_4 photosynthesis in maize (Baer & Schrader, 1985; Usuda, 1984; Usuda et al., 1985; von Caemmerer & Furbank, 2016). However, it is still uncertain whether other physiological, biochemical, and structural factors are involved in the variation in photosynthetic rate of maize.

The aim of this study is to investigate the genetic variations in photosynthetic rate and resource use efficiency in maize lines from various regions of the world. Another aim is to determine which factors in physiological, biochemical, and structural traits of leaves regulate these genetic variations in maize lines. We examined here various traits of leaves such as gas exchange traits, chlorophyll (Chl) and nitrogen (N) contents, carbon isotope ratio, activities of C_3 and C_4 enzymes, stomatal parameters, and interveinal distance (IVD) for these maize lines. On the other hand, because wild lines are valuable as genetic resources to improve physiological traits of crops (Flood et al., 2011), four lines of teosintes (*Z. mays* ssp. *mexicana*, *Zea diploperennis*, *Zea perennis*, *Zea nicaraguensis*) were added for comparison.

Materials and methods

Plant materials and growth conditions

Twenty-two maize lines (*Z. mays* ssp. *mays*) and four teosinte lines (one line per species: *Z. mays* ssp. *mexicana*, *Z. diploperennis*, *Z. perennis*, and *Z. nicaraguensis*) were used in this study (Table 1). These lines were selected from a wide range of countries. Seeds were provided by the Plant Introduction Station, Agricultural Research Service, USDA, and the NARO Genebank, Tsukuba, Japan. They were germinated in nursery boxes filled with loam granules and were grown for 10 days in a greenhouse in an experimental field of Kyushu University (33°35′N, 130°23′E) during summer 2014. Five seedlings per line were transplanted into 5-L pots (one plant per pot) filled with a sandy loam mixed with chemical fertilizer containing 1.0 g each of nitrogen, phosphorus, and potassium. Plants were then grown outdoors at a mean air temperature of 26 °C and midday

PPFD of full sunlight exceeding 2000 μmol m^{-2} s^{-1}. Plants were watered daily to avoid drying of soil. At 2 weeks after transplanting, the chemical fertilizer containing .6 g each of elements was supplied. At 3–4 weeks after transplanting, physiological, biochemical, and structural traits of photosynthesis were examined in fully expanded upper leaves of 3–5 plants per line. At this time, plant height was 80–120 cm in maize lines and 50–80 cm in teosinte lines, and all plants except for CM109 and CB44 were in vegetative stage. However, these two maize lines initiated to develop tassels (Supplemental data 1).

Gas exchange and PWUE

Gas exchange in leaves was measured using a portable photosynthesis system (Li-6400XT; Li-COR, Lincoln, NE, USA). Gas exchange parameters – net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (T_r), and intercellular CO_2 concentration (C_i) – were measured at a PPFD of 2000 μmol m^{-2} s^{-1}, leaf temperature of 30.0 ± .5 °C, relative humidity of 60% ± .5%, and ambient CO_2 concentration (C_a) of 380 μmol mol^{-1}. PWUE was calculated by dividing P_N by T_r.

Chl and N contents, specific leaf weight, and PNUE

Chl content and specific leaf weight (SLW) were measured in the same leaves used for gas exchange measurements. Chl was extracted from the leaves (3.4 cm^2) in 80% acetone, and Chl content was measured spectrophotometrically according to Arnon (1949). Leaves (5.7 cm^2) were air-dried for 2 days at 80 °C and weighed, and SLW was calculated by dividing dry mass by leaf area. Leaf N content was determined in lower leaves next to the ones used for gas exchange measurements. Leaves were air-dried as described above and ground to powder. The N content in .3 g of leaf powder was determined using a micro-Kjeldahl procedure (Tsutsumi et al., 2017). PNUE was calculated by dividing P_N by N content.

Enzyme assays and leaf soluble protein content

Parts of the same leaves used for gas exchange measurements were sampled between 10:00 and 12:00 on a clear day, immediately frozen in liquid nitrogen, and stored at −80 °C. For enzyme assay, leaves (.2 g fresh mass) were ground on ice with a pestle in a mortar containing 1 mL of grinding medium [50 mM HEPES-KOH (pH 7.5), 1 mM EDTA-2Na, 5 mM dithiothreitol, 10 mM $MgCl_2$, and .02% (v/v) Triton X-100] containing .5% (w/v) bovine serum albumin, 5 mg of polyvinylpolypyrrolidone, and .1 g of quartz sand. The homogenates were filtered through two layers of gauze, the filtrates were centrifuged for 5 min at 10,000 × *g* at 4 °C, and the supernatants were used for the enzyme assay. An aliquot of the filtrate was taken for determination of Chl content.

Activities of photosynthetic enzymes were assayed spectrophotometrically in 1-mL reaction mixtures at 30 °C. Activities of PEPC and NADP-ME were assayed as described by Ueno and Sentoku (2006). The activity of PCK was assayed in the carboxylase direction following NADH oxidation according to Sharwood et al. (2014). The total activity of Rubisco was measured as described by Ueno and Sentoku (2006) except that 5 U phosphoglycerate kinase, 5 U glyceraldehyde 3-phosphate dehydrogenase, and 5 U phosphocreatine kinase were used. In the assay of Rubisco activity, the supernatant was preincubated in the presence of 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ at 25 °C for 10 min.

For measurements of leaf soluble protein (LSP) content, leaves (.1 g fresh mass) were ground on ice, and supernatants were obtained as for enzyme assays except that bovine serum albumin was omitted and 1 mM phenylmethylsulfonyl fluoride and .002% (w/v) leupeptin were added to the grinding medium. The LSP content was measured according to Bradford (1976).

Carbon isotope ratio

A part of each leaf used for gas exchange measurement was air-dried at 80 °C and separately ground in a mortar with a pestle. The same amounts of powder from each leaf were thoroughly mixed, and 2 mg of the mixture was used for measurement of ^{12}C and ^{13}C contents as described by Sato and Suzuki (2010). The isotope ratio was expressed in δ notation in parts per million (‰) with respect to the Pee Dee belemnite standard.

Structural traits

The middle portions of leaves used for gas exchange measurements were fixed in formalin–acetic acid–alcohol solution for 1 day and cleared according to Ueno et al. (2006). Stomatal density (SD), guard cell length (GL), and IVD were measured under a light microscope. Stomata were counted on each leaf surface in four .38-mm^2 fields per leaf at 300× magnification. SD was calculated as the sum of the number of stomata on both sides per unit leaf area. GL of 20 cells on each side (40 cells in total) was measured with a micrometer at 600 × magnification. IVD was the mean of 10 measurements of the distance between centers of adjacent small longitudinal veins.

The middle portions of leaves were also fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 1.5 h. After washing with phosphate buffer, they were post-fixed in 2% (w/v) OsO_4

in sodium phosphate buffer (pH 6.8) for 2 h, dehydrated through an acetone series, and embedded in Quetol resin (Nisshin-EM Co. Ltd., Shinjuku, Tokyo, Japan) at 70 °C. The samples were transversely sectioned at 1 μm thickness with a glass knife on an ultramicrotome and stained with 1% toluidine blue O. Leaf thickness was measured in ImageJ software (Schneider et al., 2012) as the mean of 10 points per section.

Statistical analysis

Data were analyzed using BellCurve for Excel (Social Survey Research Information Co., Ltd., Shinjuku, Tokyo, Japan). One-way analysis of variance (ANOVA) was used for all parameters. Pearson's correlation coefficients between the parameters were calculated.

Results

Gas exchange and PWUE

Gas exchange traits differed significantly among the maize and teosinte lines examined ($p < .01$; Supplemental data 2). P_N ranged from 32.1 μmol m^{-2} s^{-1} (teosinte line Ames 21875) to 46.5 μmol m^{-2} s^{-1} (maize line PI 195114) with a mean of 38.9 μmol m^{-2} s^{-1} (Figure 1(A); Supplemental data 2). The three teosinte lines of species other than *Z. mays* (PI 441930, Ames 21875, PI 615697) had lower P_N than most *Z. mays* lines had. However, teosinte line Ames 8083 (*Z. mays* ssp. *mexicana*) had an intermediate P_N value (Figure 1(A)). P_N was positively correlated with g_s (Figure 2(A)) and T_r (Figure 2(B)) but not with C_i/C_a (Table 2). PWUE ranged from 5.25 mmol mol^{-1} (maize line IL14H) to 7.02 mmol mol^{-1} (Ames 21875), with a mean of 6.07 mmol mol^{-1} (Figure 1(B);

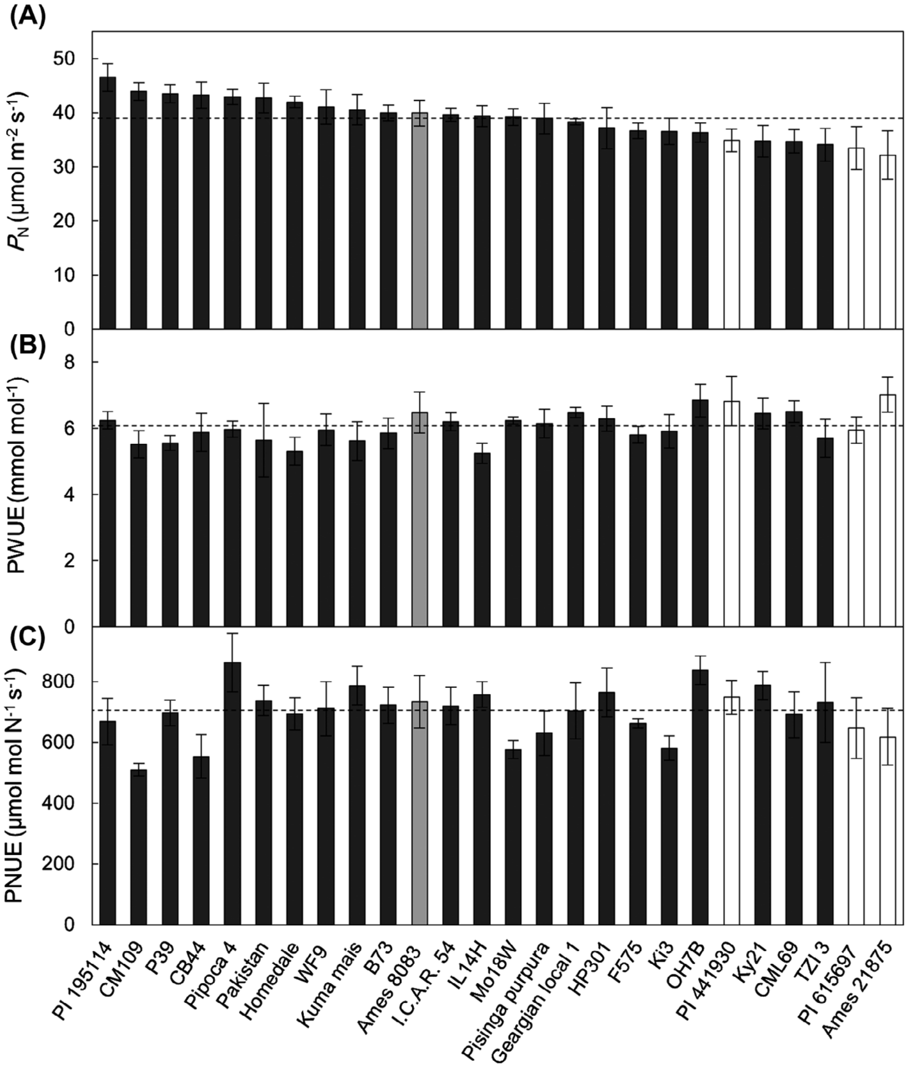

Figure 1. Variations in (A) net photosynthetic rate (P_N), (B) photosynthetic water use efficiency (PWUE), and (C) photosynthetic nitrogen use efficiency (PNUE) in leaves of maize and teosinte lines.

Notes: Means ± SD (n = 3–5). Dashed lines show mean values. Black, maize (*Z. mays* ssp. *mays*); gray, *Z. mays* ssp. *mexicana*; white, *Z. diploperennis*, *Z. perennis*, and *Z. nicaraguensis*.

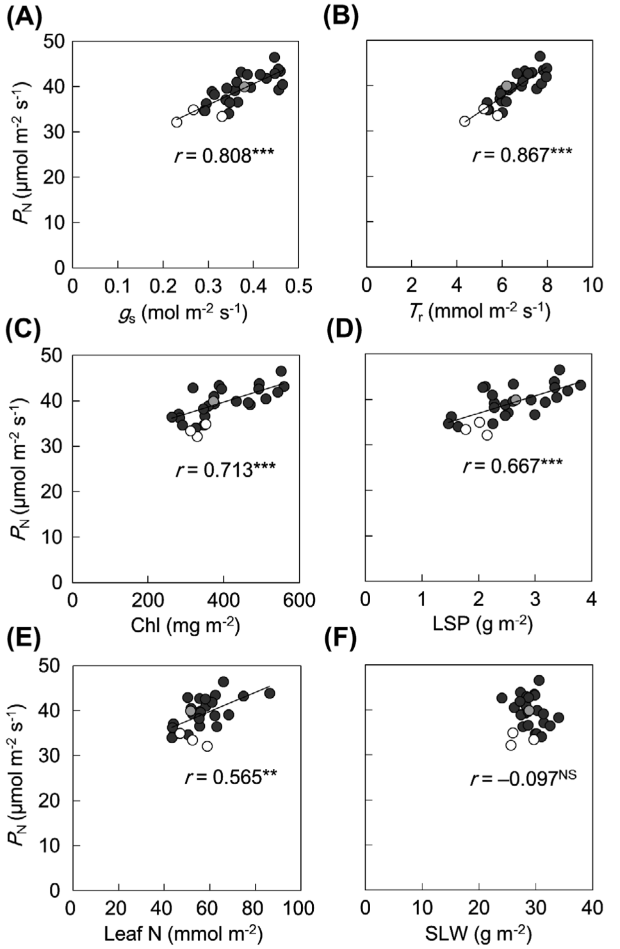

Figure 2. Relationships between P_N and (A) stomatal conductance (g_s), (B) transpiration rate (T_r), (C) chlorophyll (Chl) content, (D) leaf soluble protein (LSP) content, (E) leaf N content, and (F) specific leaf weight (SLW) in leaves of maize and teosinte lines.
Notes: Circles are the mean values of each line ($n = 3$–5): black, maize (*Z. mays* ssp. *mays*); gray, *Z. mays* ssp. *mexicana*; white, *Z. diploperennis*, *Z. perennis*, and *Z. nicaraguensis*. Significant at P: $^{**} < .01$; $^{***} < .001$. ^{NS}not significant.

Supplemental data 2). PWUE was high in all teosinte lines except PI 615697 (Figure 1(B); Supplemental data 2). PWUE was negatively correlated with P_N, g_s, T_r, and C_i/C_a (Table 2).

Other physiological traits

All these traits except δ^{13}C values showed significant differences among lines ($p < .001$; Supplemental data 3). Chl and LSP contents showed large variations among lines, with maximum-to-minimum ratios of 2.1 and 2.6, respectively (Supplemental data 3). The contents of Chl (Figure 2(C)), LSP (Figure 2(D)), and N (Figure 2(E)) were positively correlated with P_N, whereas that of SLW (Figure 2(F)) was not. PNUE ranged from 510 µmol mol N^{-1} s^{-1} (CM109) to 862 µmol mol N^{-1} s^{-1} (Pipoca 4), with a mean of 700 µmol mol N^{-1} s^{-1} (Figure 1(C); Supplemental data 3). Among the four teosinte lines, PNUE was high in PI 441903 and Ames 8083 and low in PI 615697 and Ames 21875. PNUE was not correlated with any gas-exchange or physiological traits except leaf N content (Table 2). The δ^{13}C values were not correlated with any gas exchange or physiological traits (Figure 3; Table 2).

Activities of photosynthetic enzymes

PEPC activity was not significantly correlated with P_N (Figure 4(A)), whereas NADP-ME and Rubisco activities were positively correlated with P_N (Figure 4(B) and (D)). PCK activity varied considerably among the lines (Supplemental data 4) and was high in two lines with high P_N (PI 195114 and CM 109; Supplemental data 4). The sum of NADP-ME and PCK activities (capacity for C_4 acid decarboxylation) varied among lines, with a maximum-to-minimum ratio of 3.0 (Supplemental data 4). PCK activity and the capacity for C_4 acid decarboxylation were positively correlated with P_N (Figure 4(C); Table 2). However, the contribution ratio of PCK activity to the total C_4 acid decarboxylation capacity (PCK ratio) was not correlated with P_N (Table 2). Chl content and LSP were positively correlated with the activities of Rubisco, NADP-ME, and PCK but not with that of PEPC (Table 2).

Structural traits

Variations in leaf thickness, IVD, and GL were small, with maximum-to-minimum ratios of 1.52 for leaf thickness, 1.40 for IVD, and 1.35 for GL (Supplemental data 5). Variations in SD and SD × GL were larger, with maximum-to-minimum ratios of 2.43 for SD and 2.26 for SD × GL (Supplemental data 5). The ratio of adaxial to abaxial GL was 1.04 ± .02 and the ratio of adaxial to abaxial SD was .71 ± .06 (data not shown). There were no correlations between these structural traits and gas-exchange or physiological traits (Supplemental data 6), except that PWUE was weakly negatively correlated with SD ($r = -.412$; $p < .05$) and SD × GL ($r = -.415$; $p < .05$). On the other hand, leaf thickness was positively correlated with IVD, whereas SD was negatively correlated with GL (Table 3).

Discussion

Physiological traits

Our study showed that P_N in maize and teosinte lines ranged from 32.1–46.5 µmol m^{-2} s^{-1}, a factor of 1.45 times. Although some studies have reported a variation of >2 times in P_N of maize genotypes (Crosbie et al., 1977; Heichel & Musgrave, 1969), we found no such large difference. On the other hand, Duncan and Hesketh (1968) reported a variation in P_N among maize cultivars similar

Table 2. Correlation coefficients (*r*) from linear regression analysis and statistical significance of the relationships between physiological and biochemical traits in maize and teosinte lines. P_N, net photosynthetic rate; g_s, stomatal conductance; T_r, transpiration rate; C_i/C_a, intercellular CO_2 to ambient CO_2 concentration; PWUE, photosynthetic water use efficiency; LSP, leaf soluble protein; Chl, chlorophyll; SLW, specific leaf weight; PNUE, photosynthetic nitrogen use efficiency; $\delta^{13}C$, carbon isotope ratio; PEPC, phospho*enol*pyruvate carboxylase; ME, malic enzyme; PCK, phospho*enol*pyruvate carboxykinase; DC, capacity for C_4 acid decarboxylation (=NADP−ME activity + PCK activity); PCK ratio, [=PCK activity/(NADP−ME activity + PCK activity)].

	P_N	g_s	T_r	C_i/C_a	PWUE	LSP content	Chl content	Leaf N content	SLW	PNUE	$\delta^{13}C$	Rubisco activity	PEPC activity	NADP-ME activity	PCK activity	DC	PCK ratio
P_N	1																
g_s	.813***	1															
T_r	.878***	.961***	1														
C_i/C_a	.169^{NS}	.632***	.557**	1													
PWUE	−.510**	−.805***	−.819***	−.835***	1												
LSP content	.718***	.671***	.724***	.276^{NS}	−.592**	1											
Chl content	.726***	.558**	.671***	.129^{NS}	−.433*	.809***	1										
Leaf N content	.581**	.372^{NS}	.487*	.080^{NS}	−.342^{NS}	.576**	.614***	1									
SLW	−.095^{NS}	−.092^{NS}	−.102^{NS}	−.076^{NS}	.069^{NS}	−.233^{NS}	−.279^{NS}	−.030^{NS}	1								
PNUE	−.098^{NS}	.040^{NS}	−.056^{NS}	−.010^{NS}	.135^{NS}	−.287^{NS}	−.311^{NS}	−.832***	−.161^{NS}	1							
$\delta^{13}C$	−.034^{NS}	.095^{NS}	.037^{NS}	.045^{NS}	.022^{NS}	−.026^{NS}	−.268^{NS}	−.247^{NS}	.252^{NS}	.214^{NS}	1						
Rubisco activity	.610***	.652***	.692***	.419*	−.615***	.668***	.667***	.301^{NS}	−.003^{NS}	−.070^{NS}	.176^{NS}	1					
PEPC activity	−.059^{NS}	.013^{NS}	−.055^{NS}	.051^{NS}	.018^{NS}	−.099^{NS}	−.234^{NS}	.114^{NS}	.205^{NS}	−.129^{NS}	−.008^{NS}	−.211^{NS}	1				
NADP-ME activity	.501**	.492*	.519**	.223^{NS}	−.440*	.541**	.389*	.344^{NS}	.212^{NS}	−.141^{NS}	.039^{NS}	.519**	.368^{NS}	1			
PCK activity	.521**	.439*	.469*	.117^{NS}	−.225^{NS}	.438*	.423*	.633***	−.104^{NS}	−.382^{NS}	−.149^{NS}	.188^{NS}	.396*	.437*	1		
DC	.457*	.491*	.509**	.267^{NS}	−.438*	.467*	.326^{NS}	.385^{NS}	.150^{NS}	−.182^{NS}	.012^{NS}	.414*	.486*	.958***	.597**	1	
PCK ratio	.387^{NS}	.186^{NS}	.225^{NS}	−.107^{NS}	.001^{NS}	.344^{NS}	.319^{NS}	.560**	−.088^{NS}	−.400*	−.100^{NS}	.119^{NS}	.313^{NS}	.175^{NS}	.860***	.288^{NS}	1

*Significant at P: < .05;

**< .01;

***< .001;

^{NS}not significant.

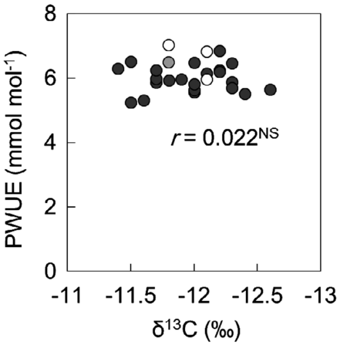

Figure 3. Relationship between photosynthetic water use efficiency (PWUE) and δ^{13}C in leaves of maize and teosinte lines.
Notes: Circles are the mean values of each line (n = 3–5). Circle shading is as in Figure 2. ^{NS}not significant.

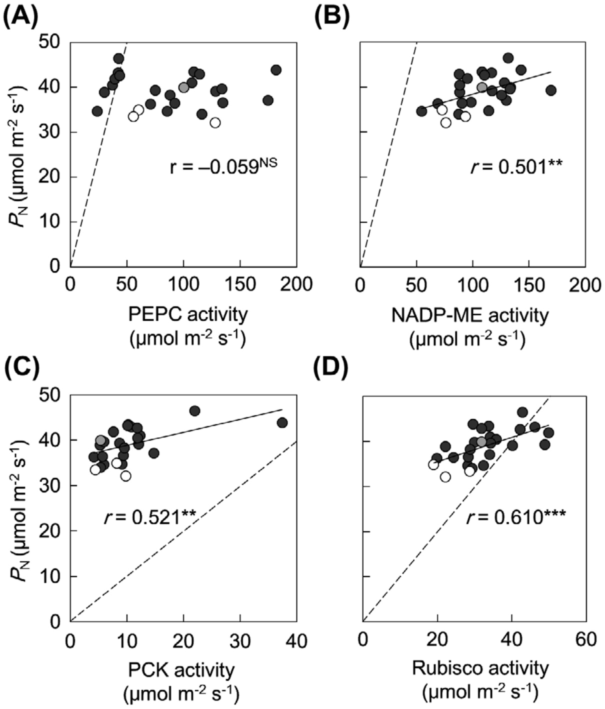

Figure 4. Relationships between P_N and activities of (A) PEP carboxylase (PEPC), (B) NADP-malic enzyme (NADP-ME), (C) PEP carboxykinase (PCK), and (D) Rubisco in leaves of maize and teosinte lines.
Notes: Circles are the mean values of each line (n = 3–5). Circle shading is as in Figure 2. Significant at P: ** < .01; *** < .001. ^{NS}not significant. Broken lines ($y = x$) show enzyme activities that would be required for equal P_N.

to that in our study. It seems likely that the differences in growth condition, plant age, and measurement method of P_N bring about such different results.

We found a positive correlation between P_N and g_s (Figure 2(A)). This relationship has been reported in various C_3 and C_4 species (Fernandez et al., 2015; Tsutsumi et al., 2017; Wong et al., 1985). Because maize has a CCM, CO_2 diffusion in stomata may not be the primary P_N regulator. The P_N of C_4 plants is saturated at the C_i observed at the atmospheric CO_2 concentration, whereas the P_N of C_3 plants is not saturated until a much higher CO_2 concentration (Pearcy & Ehleringer, 1984; Wong et al., 1985). In preliminary measurements of some maize lines, we also confirmed that P_N is saturated at $C_i \approx 100$ μmol mol^{-1}, which is observed at $C_a = 380$ μmol mol^{-1} (data not shown). This suggests that P_N would not greatly increase even though g_s alone increased and thereby higher C_i was attained within the leaf. Therefore, the effect of non-stomatal factors on the genetic variation in P_N of maize and teosinte lines cannot be ruled out. On the other hand, there was a close relationship between P_N and g_s in maize lines. The reason is unknown. However, the coordinated mechanism between mesophyll photosynthesis and stomata (Lawson et al., 2014) may be involved in this relationship. According to this hypothesis, the concentration of CO_2 inside the leaf would help maintain the coordination of the mesophyll photosynthesis with stomatal aperture (Lawson et al., 2014).

The Chl, LSP, and leaf N contents were positively correlated with P_N (Figure 2(C)–(E)), as reported in many plant species, including maize (Wong et al., 1985; Sage & Pearcy, 1987; Tsutsumi et al., 2017), because all these parameters are closely associated with the contents of photosynthetic pigments and enzymes. No significant correlation was detected between P_N and SLW (Figure 2(F)), as in *Saccharum* (Nose et al., 1994) and *Oryza* species (Kiran et al., 2013), although positive correlations were found in leguminous (Pearce et al., 1969), cruciferous (Suresh et al., 1997), and *Amaranthus* species (Tsutsumi et al., 2017). Thus, the relationship between P_N and SLW varies with species.

Biochemical traits

P_N was positively correlated with activities of NADP-ME, PCK, and Rubisco, but not with that of PEPC (Figure 4). A positive correlation between P_N and Rubisco activity was found in C_4 and C_3 species (Ghannoum et al., 2011; Tsutsumi et al., 2017; von Caemmerer et al., 1997). In our study, a considerable number of maize and teosinte lines showed lower Rubisco activities than P_N (Figure 4(D)). Baer and Schrader (1985) reported that, in Rubisco of maize lines, total activities are lower than initial activities. Therefore, this enzymatic trait may be involved in the lower Rubisco activities relative to P_N, because we measured total activities. However, the possibility of deactivation and/or degradation of Rubisco during extraction cannot be ruled out. In NADP-ME-type C_4 grasses, a positive correlation between P_N and NADP-ME activity was also reported (Nose et al., 1994; Usuda, 1984). Using antisense RNA, Pengelly et al. (2012) suggested that NADP-ME is unlikely to be a rate-limiting enzyme in C_4

Table 3. Correlation coefficients (*r*) from linear regression analysis and statistical significance of the relationships between structural traits of maize and teosinte lines. IVD, interveinal distance; SD, stomatal density; GL, guard cell length.

	Leaf thickness	IVD	SD	GL	SD×GL
Leaf thickness	1				
IVD	.645***	1			
SD	−.371^{NS}	−.487*	1		
GL	.257^{NS}	.225^{NS}	−.686***	1	
SD×GL	−.352^{NS}	−.526	.953***	−.439*	1

*Significant at *P*: < .05;
**< .01;
***< .001;
^{NS}not significant.

photosynthesis of *Flaveria bidentis*, an NADP-ME-type C_4 dicot, because P_N of transgenic plants was not significantly reduced until 40% reduction of NADP-ME activity in the wild-type plants. At present, it is unknown whether this relationship between NADP-ME activity and P_N found in the C_4 dicot is applicable to NADP-ME-type C_4 grasses as well. However, these data suggest that the relationship between NADP-ME activity and P_N may more or less differ among species.

Maize has two C_4 acid decarboxylation enzymes, NADP-ME and PCK (Walker et al., 1997; Wingler et al., 1999). Our study has revealed, for the first time, a considerable genetic variation in PCK activity among maize and teosinte lines. The PCK system in maize has been estimated to contribute 10–14% of the carbon in BS (Arrivault et al., 2017). This ratio was within the range found in our study (3.6–19.8%; Supplemental data 4).

The physiological significance of C_4 acid decarboxylation depending on PCK in maize remains to be understood (Furbank, 2011). Sharwood et al. (2014) reported that the ratio of PCK activity to NADP-ME activity changes in maize exposed to shade and salinity. The parallel operation of the two C_4 acid decarboxylation systems may compensate each other under certain environmental conditions (Bellasio & Griffiths, 2014) and may be energetically profitable. The C_4 acid decarboxylation depending on PCK generates phospho*enol*pyruvate (PEP). If PEP is transported to mesophyll cells, ATP needed for the conversion of pyruvate to PEP by PPDK may be saved. We detected no positive correlation between P_N and PCK ratio in maize and teosinte lines (Table 2). Therefore, the difference in P_N among maize and teosite lines would not result from the difference in the contribution ratio of PCK activity to the total C_4 acid decarboxylaton capacity. Further investigation would be required to understand the physiological significance of decarboxylation by PCK in maize.

Our study suggests that biochemical processes from C_4 acid decarboxylation to re-fixation of CO_2 by Rubisco are involved in the genetic variation in P_N. However, we did not examine the activity of PPDK, which is well known to be the rate-limiting factor in C_4 photosynthesis (Baer & Schrader, 1985; Usuda, 1984; von Caemmerer & Furbank, 2016), because a preliminary study on several lines of maize showed that their activities of PPDK were unstable and unreliable. The relationships between P_N and PPDK activity and between PPDK and PCK activities remain to be explored.

Structural traits

P_N was not correlated with leaf thickness or IVD (Supplemental data 6). In C_3 plants, photosynthesis is performed within single mesophyll cells. Therefore, C_3 leaves can change their structure in response to environmental change. The genetic variation in leaf structure of C_4 plants is restricted to a narrower range than that of C_3 plants, because C_4 photosynthesis requires a strict quantitative balance between two types of photosynthetic cells (Dengler et al., 1994; Ghannoum et al., 2011; Tsutsumi et al., 2017). The maximum-to-minimum ratios of leaf thickness and IVD in maize and teosinte lines were generally smaller than those of physiological and biochemical traits. This may conceal the possible relationships between P_N and these structural traits.

We found a negative correlation between SD and GL (Table 3), as reported in other species (Büssis et al., 2006; Lawson & Blatt, 2014; Tsutsumi et al., 2017). The genetic variation in SD (2.43 times) was greater than that in GL (1.35 times; Supplemental data 5), suggesting physical and genetic limitations on the range of alterations in GL, whereas SD is much more flexible (Tsutsumi et al., 2017).

The stomatal parameters were not correlated with P_N, g_s, or T_r (Table 3). It seems that an increase in SD would increase P_N by increasing g_s. Several studies reported positive correlations between SD and P_N in various species, but other studies reported no such correlations (reviewed by Lawson & Blatt, 2014). Importantly, stomatal anatomical features such as SD and GL define the maximum theoretical conductance, whereas P_N is the actual physiological outcome (Dow et al., 2014; Lawson & Blatt, 2014). The relationships between stomatal anatomical features and gas exchange parameters would also be affected by environmental factors such as vapor pressure deficit (Kawamitsu et al., 2002). Therefore, more detailed analyses under different conditions would be needed.

Resource use efficiency

C_4 plants can maintain high CO_2 concentration within BS cells owing to the CCM. This allows C_4 plants to have higher PNUE than that of C_3 plants (Brown, 1977; Ghannoum et al., 2011). A comparative study of C_4 subtypes in grasses showed higher PNUE in the NADP-ME type than in the NAD-ME type, because Rubisco turnover rate is faster in the former than in the latter (Ghannoum et al., 2005). In our study, the maximum-to-minimum PNUE ratio of maize and teosinte lines was 1.69, and the mean was 700 μmol mol N^{-1} s^{-1} (Supplemental data 3), which was far higher than PNUE in C_3 plants and was in the highest class of PNUE values previously reported in C_4 plants (Ghannoum et al., 2005; Taylor et al., 2010; Togawa & Ueno, 2015; Tsutsumi et al., 2017; Vogan & Sage, 2011). Our study suggests that the genetic variation in PNUE in maize and teosinte lines depends on leaf N content but not on P_N (Table 2), because lines with lower leaf N content showed higher PNUE. Wild relatives of cultivated crops often inhabit more severe environment than cultivated conditions and possess useful traits that have been lost in cultivated crops during domestication (Hamaoka et al., 2013; Scafaro et al., 2010). Therefore, we expected that some teosinte lines would have higher PNUE than maize lines, but found no such trend (Figure. 1(C)).

The maximum-to-minimum PWUE ratio of maize and teosinte lines was 1.34 times (Supplemental data 2), which was lower than that of PNUE. The mean PWUE was 6.07 μmol mol^{-1} (Supplemental data 2) and was in the lower class of PWUE values previously reported in C_4 plants (Osmond et al., 1982; Togawa & Ueno, 2015; Tsutsumi et al., 2017).

In C_3 plants, there is a positive correlation between $\delta^{13}C$ values and PWUE, and $\delta^{13}C$ is useful for screening cultivars for high PWUE (Farquhar & Richards, 1984). In this study, the $\delta^{13}C$ values did not vary greatly among maize and teosinte lines (Supplemental data 3) and were not significantly correlated with PWUE (Figure 3). In sorghum (C_4), O'Leary (1988) also found no significant correlation between $\delta^{13}C$ values and PWUE in 120 lines, whereas Henderson et al. (1998) reported a weak but significant correlation in 30 lines. The carbon isotope ratio in plant dry matter reflects carbon isotope discrimination (Δ) during photosynthesis (Cernusak et al., 2013). In C_3 plants, Δ is well correlated with C_i/C_a and hence can be used as an index of PWUE. In C_4 plants, the variation in C_i/C_a is smaller than in C_3 plants and Δ is related to both CO_2 leakiness from BS cells and to C_i/C_a (Cernusak et al., 2013; Ghannoum et al., 2011; Henderson et al., 1998). For these reasons, it would not be easy to use $\delta^{13}C$ values as an indicator of PWUE of C_4 plants.

We found weak negative correlations between PWUE and SD or SD × GL (Table 3). A decrease in SD would reduce water loss from leaves. However, it may also decrease P_N while increasing PWUE, because P_N is negatively correlated with PWUE (Table 2). It is worth noting that all teosinte lines except *Z. nicaraguensis* (PI 615697) showed higher PWUE values than those of cultivated maize lines. *Z. nicaraguensis* is known to grow in wet habitats such as coastal, estuarine, and river environments (Bird, 2000; Iltis & Benz, 2000). The lower PWUE in *Z. nicaraguensis* may reflect these ecological traits.

Conclusion

This study investigated the genetic variations in photosynthetic rate and resource use efficiency in maize and teosinte lines and the regulatory factors involving in these variations. P_N was positively correlated with physiological traits of leaves such as g_s, T_r, and Chl and N contents. However, g_s may not be the primary P_N regulator, because maize has a CCM. P_N was also positively correlated with activities of NADP-ME, PCK, and Rubisco, but not with that of PEPC. These data suggest that biochemical processes from C_4 acid decarboxylation to re-fixation of CO_2 by Rubisco are involved in the genetic variation in P_N. On the other hand, structural traits of leaves such as leaf thickness, IVD, and stomatal parameters were not correlated with P_N. It is suggested that physiological and biochemical traits are involved in the genetic variation of P_N in maize and teosinte lines but structural traits are not directly involved. In maize and teosinte lines, PNUE was in the highest class and PWUE was in lower class of values previously reported in other C_4 plants. It is worth noting that PNUE was negatively correlated with leaf N content. Some teosinte lines showed higher PWUE and have a value as genetic resources. The photosynthetic traits of maize may be also regulated by other factors not examined in this study, such as electron transport and CO_2 leakiness from BS cells. Further studies will be required for our better understanding of the genetic variation in photosynthetic traits in maize.

Acknowledgements

We thank the Plant Introduction Station (Agricultural Research Service, USDA) and the NARO Genebank, Tsukuba, Japan, for their kind gifts of maize and teosinte seeds.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Japan Society for the Promotion of Science KAKENHI [grant number JP 16H04868], [grant number JP24380010] to O.U.

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