Variations in physiological, biochemical, and structural traits of photosynthesis and resource use efficiency in maize and teosintes (NADP-ME-type C₄)

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ABSTRACT

C₄ plants show higher photosynthetic capacity and resource use efficiency than C₃ plants. However, the genetic variations of these traits and their regulatory factors in C₄ 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 (Pₙ) ranged from 32.1 to 46.5 μmol m⁻² s⁻¹. Pₙ was positively correlated with stomatal conductance, transpiration rate, and chlorophyll, nitrogen and soluble protein contents of leaves, but not with specific leaf weight. Pₙ was positively correlated with the activities of ribulose-1,5-bisphosphate carboxylase/oxygenase and the C₄ acid decarboxylases, NADP-malic enzyme and phosphoenolpyruvate carboxykinase, but not with the activity of phosphoenolpyruvate carboxylase. Leaf structural traits (stomatal parameters, leaf thickness, and interveinal distance) were not correlated with Pₙ. These data suggest that physiological and biochemical traits are involved in the genetic variation of Pₙ but structural traits are not directly involved. PWUE is in the lower class of values reported for C₄ plants, whereas PNUE is in the highest class of values reported for C₃ plants. PNUE was negatively correlated with leaf nitrogen content but not significantly correlated with Pₙ. PWUE was not correlated with δ¹³C values of leaves, indicating difficulty in using δ¹³C values as an indicator of PWUE of maize. In general, teosinte lines showed lower Pₙ but higher PWUE than maize lines.

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 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 C₄ plants have higher photosynthetic rate and productivity than C₃ plants (Brown, 1999; Osmond et al., 1982). This is attained by a CO₂ concentrating mechanism (CCM) operating in C₄ plants. In C₄ leaves, mesophyll and bundle-sheath (BS) cells are differentiated and surround vascular bundles. In C₄ photosynthesis, atmospheric CO₂ entering through stomata is fixed by phosphoenolpyruvate carboxykinase (PEPC) in mesophyll cells. The C₄ acids produced are transported to BS cells, where they are decarboxylated by C₄ acid decarboxylase. Released CO₂ is fixed by Rubisco. This biochemical process raises the CO₂ 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 C₃ and C₄ cycles (Dengler et al., 1994). In general, C₄ leaves have a denser vascular system than C₃ leaves. It is thought that this structural trait is associated with rapid translocation of photosynthates in C₄ plants (Leonardos & Grodzinski, 2000; Ueno et al., 2006). Because of the complex mechanism of C₄ photosynthesis, the factors determining photosynthetic rate in C₄ plants still remain to be resolved (von Caemmerer & Furbank, 2016). It is suggested that CO₂ delivery process in mesophyll cells, activities and
properties of C₄ and C₃ photosynthetic enzymes, substrate regeneration in C₄ and C₃ cycles, transport and diffusion of metabolites, electron transport and light capture in chloroplasts, and CO₂ leakiness from BS cells are involved in the regulation of C₄ 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 C₄ plants (Tsutsumi et al., 2017).

Depending on the C₄ acid decarboxylation system in BS cells, the C₄ photosynthetic pathway is classified into three types: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PCK) (Hatch, 1987). The NADP-ME-type C₄ grasses include crops with high biomass productivity, such as maize, sorghum, and Napier grass (Brown, 1999; Carpita & McCann, 2008). In general, each C₄ species is thought to use only one of the 3 decarboxylation systems (Hatch, 1987). However, some C₄ species may use more than one. Maize (Zea mays ssp. mays), a model NADP-ME-type C₄ grass, uses both NADP-ME and PCK (Walker et al., 1997; Wingler et al., 1999). However, the physiological significance of the dual C₄ acid decarboxylation system in C₄ plants is unclear (Furbank, 2011; Koteyeva et al., 2015).

Because C₄ plants have a CCM, their photosynthetic water and nitrogen use efficiencies (PWUE and PNUE) are higher than those of C₃ plants (Brown, 1977; Osmond et al., 1982). These traits of C₄ plants provide advantages for survival in natural habitats and are also useful for sustainable agriculture. PNUE values of NADP-ME-type C₄ grasses are higher than those of NAD-ME-type C₄ 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 C₄ plants.

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 C₄ 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 C₃ and C₄ 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

| Species         | Line | Country         |
|-----------------|------|-----------------|
| Z. mays ssp. mays | B73  | Iowa, USA       |
|                 | HP301 | Indiana, USA    |
|                 | IL14H | Illinois, USA   |
|                 | Ky21  | Kentucky, USA   |
| Mo18W          | Missouri, USA |
| OH78           | Ohio, USA         |
| P39            | Indiana, USA      |
| WF9            | Indiana, USA      |
| CM109          | Canada            |
| CML69          | Mexico            |
| Pipoca 4       | Brazil            |
| Pisinga Purpura| Peru              |
| CB44           | Netherlands       |
| Kuma Mais      | Netherlands       |
| FS57           | France            |
| I.C.A.R. 54    | Romania           |
| TZ13           | Nigeria           |
| PI 195114      | Ethiopia          |
| Homedale       | South Africa      |
| Georgian local 1| USSR              |
| Pakistan       | Pakistan          |
| K3             | Thailand          |
| Ames 8083      | Mexico            |
| PI 441930      | Mexico            |
| Ames 21875     | Mexico            |
| PI 615697      | Nicaragua         |
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 cm²) in 80% acetone, and Chl content was measured spectrophotometrically according to Arnon (1949). Leaves (5.7 cm²) 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 MgCl₂, 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 Rubisco 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 NaHCO₃ and 10 mM MgCl₂ 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 13C and 13C 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-mm² 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) OsO₄
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.

### 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](image-url) 

**Figure 1.** Variations in (A) net photosynthetic rate ($P_n$), (B) photosynthetic water use efficiency (PWUE), and (C) photosynthetic nitrogen use efficiency (PNUUE) 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*.

### 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.
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 (\( r = -0.412; p < .05 \)) and SD × GL (\( r = -0.415; p < .05 \)). 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 \( \mu \)mol 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 $\mathrm{CO}_2$ to ambient $\mathrm{CO}_2$ 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, phosphoenolpyruvate carboxylase; ME, malic enzyme; PCK, phosphoenolpyruvate 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     |       |       |           |       |             |             |                |     |      |              |                 |              |                 |              |    |           |
| $g_s$ |       | 0.813*** | 1     |           |       |             |             |                |     |      |              |                 |              |                 |              |    |           |
| $T_r$ |       |       | 0.878*** | 0.961*** | 1     |             |             |                |     |      |              |                 |              |                 |              |    |           |
| $C_i/C_a$ | 0.169** | 0.632*** | 0.557** | 1     |       |             |             |                |     |      |              |                 |              |                 |              |    |           |
| PWUE | $-0.510**$ | $-0.805**$ | $-0.819***$ | $-0.835***$ | 1     | $-0.169nS$ | $-0.576**$ | $0.614***$ |     |      |              |                 |              |                 |              |    |           |
| LSP content | $0.718***$ | $0.671***$ | $0.724***$ | $0.278nS$ | $-0.592**$ | 1     |             |             |                |     |      |              |                 |              |                 |              |    |           |
| Chl content | $0.726***$ | $0.558**$ | $0.671***$ | $0.129nS$ | $-0.033nS$ | 1     | $-0.409***$ | $0.809***$ |     |      |              |                 |              |                 |              |    |           |
| Leaf N content | $0.581**$ | $0.372nS$ | $0.487**$ | $0.080nS$ | $-0.342nS$ | $0.576**$ | $0.614***$ | 1     |      |              |                 |              |                 |              |    |           |
| SLW | $-0.095nS$ | $-0.092nS$ | $-0.102nS$ | $-0.076nS$ | $0.069nS$ | $-0.233nS$ | $-0.279nS$ | $-0.030nS$ | 1     |      |              |                 |              |                 |              |    |           |
| PNUE | $-0.098nS$ | $0.040nS$ | $0.056nS$ | $0.010nS$ | $0.139nS$ | $-0.287nS$ | $-0.311nS$ | $-0.161nS$ | 1     |      |              |                 |              |                 |              |    |           |
| $\delta^{13}C$ | $-0.034nS$ | $0.099nS$ | $0.033nS$ | $0.045nS$ | $0.022nS$ | $-0.026nS$ | $-0.268nS$ | $-0.247nS$ | $-0.252nS$ | $-0.214nS$ | 1     | $-0.107nS$ | $-0.070nS$ | $0.176nS$ | 1     |           |
| Rubisco | $0.610***$ | $0.652***$ | $0.692***$ | $0.419nS$ | $-0.615***$ | $0.668***$ | $0.667***$ | $0.301nS$ | $-0.003nS$ | $-0.070nS$ | $0.176nS$ | 1     | $-0.010nS$ | $-0.003nS$ | $-0.214nS$ | 1     |           |
| Rubisco activity | $-0.059nS$ | $0.013nS$ | $-0.056nS$ | $0.051nS$ | $0.018nS$ | $-0.099nS$ | $-0.234nS$ | $-0.114nS$ | $0.205nS$ | $-0.129nS$ | $-0.008nS$ | $-0.211nS$ | 1     | $-0.010nS$ | $-0.003nS$ | $-0.214nS$ | 1     |           |
| PEPC activity | $0.501**$ | $0.492nS$ | $0.519nS$ | $0.223nS$ | $-0.440nS$ | $0.541nS$ | $0.389nS$ | $0.344nS$ | $0.212nS$ | $-0.141nS$ | $0.039nS$ | $0.519nS$ | $0.368nS$ | 1     |      |           |
| NADP-ME activity | $0.521**$ | $0.439nS$ | $0.469nS$ | $0.117nS$ | $-0.223nS$ | $0.438nS$ | $0.423nS$ | $0.633***$ | $-0.104nS$ | $-0.382nS$ | $-0.149nS$ | $0.186nS$ | $0.396nS$ | $0.437nS$ | 1     |           |
| PCK activity | $0.457nS$ | $0.491nS$ | $0.508nS$ | $0.267nS$ | $-0.438nS$ | $0.467nS$ | $0.326nS$ | $0.385nS$ | $0.150nS$ | $-0.182nS$ | $0.012nS$ | $0.414nS$ | $0.486nS$ | $0.958***$ | $0.597nS$ | 1     |
| PCK ratio | $0.387nS$ | $0.186nS$ | $0.225nS$ | $-0.107nS$ | $0.001nS$ | $0.344nS$ | $0.319nS$ | $0.560nS$ | $-0.088nS$ | $-0.400nS$ | $-0.100nS$ | $0.119nS$ | $0.313nS$ | $0.175nS$ | $0.860***$ | $0.288nS$ | 1     |           |

*Significant at $P < .05$; **$ < .01$; ***$ < .001$; nSnot significant.
the atmospheric CO₂ concentration, whereas the $P_N$ of C₃ plants is not saturated until a much higher CO₂ concentration (Pearcy & Ehleringer, 1984; Wong et al., 1985). In preliminary measurements of some maize lines, we also confirmed that $P_N$ is saturated at $C_i = 100 \mu\text{mol mol}^{-1}$, which is observed at $C_a = 380 \mu\text{mol mol}^{-1}$ (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 CO₂ 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 C₄ and C₃ 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. 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 C₄ 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 C₄ plants.
photosynthesis of *Flaveria bidentis*, an NADP-ME-type C₄ 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 C₄ dicot is applicable to NADP-ME-type C₄ grasses as well. However, these data suggest that the relationship between NADP-ME activity and $P_N$ may differ among species.

Maize has two C₄ 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 6). In C₃ plants, photosynthesis is performed within single mesophyll cells. Therefore, C₃ leaves can change their structure in response to environmental change. The genetic variation in leaf structure of C₃ plants is restricted to a narrower range than that of C₄ plants, because C₄ 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

$P_N$ was not correlated with leaf thickness or IVD (Supplemental data 6). In C₃ plants, photosynthesis is performed within single mesophyll cells. Therefore, C₃ leaves can change their structure in response to environmental change. The genetic variation in leaf structure of C₃ plants is restricted to a narrower range than that of C₄ plants, because C₄ 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.

### Structural traits

$P_N$ was not correlated with leaf thickness or IVD (Supplemental data 6). In C₃ plants, photosynthesis is performed within single mesophyll cells. Therefore, C₃ leaves can change their structure in response to environmental change. The genetic variation in leaf structure of C₃ plants is restricted to a narrower range than that of C₄ plants, because C₄ 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.

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

*Significant at $P < .05$; **< .01; ***< .001; NS not significant.

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.
A comparative study of C₄ 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 C₃ plants and was in the highest class of PNUE values previously reported in C₄ 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 Pₚ (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 C₄ plants (Osmond et al., 1982; Togawa & Ueno, 2015; Tsutsumi et al., 2017).

In C₃ plants, there is a positive correlation between δ¹³C values and PWUE, and δ¹³C is useful for screening cultivars for high PWUE (Farquhar & Richards, 1984). In this study, the δ¹³C values did not vary greatly among maize and teosinte lines (Supplemental data 3) and were not significantly correlated with PWUE (Figure 3). In sorghum (C₄), O’Leary (1988) also found no significant correlation between δ¹³C 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 C₃ plants, Δ is well correlated with C_i/C_a and hence can be used as an index of PWUE. In C₄ plants, the variation in C_i/C_a is smaller than in C₃ plants and Δ is related to both CO₂ 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 δ¹³C values as an indicator of PWUE of C₄ 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 Pₚ while increasing PWUE, because Pₚ 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. Pₚ was positively correlated with physiological traits of leaves such as gₛ, Tₛ, and Chl and N contents. However, gₛ may not be the primary Pₚ regulator, because maize has a CCM. Pₚ 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 C₄ acid decarboxylation to re-fixation of CO₂ by Rubisco are involved in the genetic variation in Pₚ. On the other hand, structural traits of leaves such as leaf thickness, IVD, and stomatal parameters were not correlated with Pₚ. It is suggested that physiological and biochemical traits are involved in the genetic variation of Pₚ in maize and teosinte lines but structural traits are not directly involved. In maize and teosinte lines, PWUE was in the highest class and PWUE was in lower class of values previously reported in other C₄ plants. It is worth noting that PWUE 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 CO₂ leakiness from BS cells. Further studies will be required for our better understanding of the genetic variation in photosynthetic traits in maize.

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