Hybrid vigor (heterosis) has been used as a breeding technique for crop improvement to achieve enhanced biomass production, but the physiological mechanisms underlying heterosis remain poorly understood. In this study, to find a clue to the enhancement of biomass production by heterosis, we systematically evaluated the effect of heterosis on the growth rate and photosynthetic efficiency in sorghum hybrid [Sorghum bicolor (L.) Moench cv. Tentaka] and its parental lines (restorer line and maintainer line). The final biomass of Tentaka was 10–14 times greater than that of the parental lines grown in an experimental field, but the relative growth rate during the vegetative growth stage did not differ. Tentaka exhibited a relatively enlarged leaf area with lower leaf nitrogen content per leaf area (N_area). When the plants were grown hydroponically at different N levels, daily CO₂ assimilation per leaf area (A) increased with N_area and the ratio of A to N_area (N-use efficiency) was higher in the plants grown at low N levels but not different between Tentaka and the parental lines. The relationships between the CO₂ assimilation rate, the amounts of photosynthetic enzymes, including ribulose-1,5-bisphosphate carboxylase/oxygenase, phosphoenolpyruvate carboxylase and pyruvate phosphate dikinase, Chl and N_area did not differ between Tentaka and the parental lines. Thus, Tentaka tended to exhibit enlargement of leaf area with lower N content, leading to a higher N-use efficiency for CO₂ assimilation, but the photosynthetic properties did not differ. The greater biomass in Tentaka was mainly due to the prolonged vegetative growth period.

**Keywords:** Biomass • Carbon isotope discrimination • C₄ plant • Heterosis • Hybrid • Sorghum.

**Abbreviations:** A, daily CO₂ assimilation per leaf area; DAS, day after sowing; δ¹³C, carbon isotope discrimination in leaves; Fₚmax, maximum photosynthetic capacity; F₀, steady-state fluorescence level; NPQ, non-photochemical quenching; PEPC, phosphoenolpyruvate carboxylase; PFD, photon flux density; PPDK, pyruvate phosphate dikinase; RGR, relative growth rate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

**Introduction**

Hybrid vigor (heterosis) has been used as a breeding improvement technique and has garnered attention for its potential to enhance biomass and food productivity. In early studies, heterosis was found to result in some advantageous traits such as enhanced biomass, larger leaf area, greater root development and a longer vegetative period (Chen 2010). The greater biomass in F₁ hybrids is thought to be caused by the larger leaf area and the longer vegetative period, but the effects of heterosis on photosynthetic efficiency remain unclear. Fujimoto et al. (2012) subjected an Arabidopsis hybrid (C24 × Col-0) and its parental lines to large-scale transcriptome profiling and reported that genes encoding chloroplast-located proteins involved in Chl biosynthesis and photosynthesis were significantly over-represented and that cell sizes and the number of chloroplasts per cell were larger in the hybrid on the third day after sowing (DAS). These genes, however, were not up-regulated after 3 DAS and the photosynthetic rate per leaf area was not significantly increased. In other reports with super-hybrid rice (LYP9), genes related to nitrogen (N) and carbon assimilation, including Chl a/b-binding proteins and PSI and PSII components, were up-regulated but photorespiratory genes were down-regulated (Bao et al. 2005, Wei et al. 2009). These reports suggest the possibility that the photosynthetic capacity may be greater in hybrid rice. In other lines of super-hybrid rice, such as Liangyou-2186, genes related to carbon fixation in C₄ photosynthesis, i.e. pyruvate phosphate dikinase (PPDK), NADP-malic enzyme, and the starch biosynthesis pathway are up-regulated, indicating the possibility that biomass enhancement is due to greater dry matter production in the hybrid.
enzyme (NADP-ME) and NADP-malate dehydrogenase (NADP-MDH), were up-regulated and the activities of these enzymes and the net photosynthetic rate per leaf area were greater, although it is not known how this up-regulation of C₄ genes affected C₃ photosynthesis (Song et al. 2010). Thus, positive effects of heterosis on photosynthesis have been observed in large-scale transcriptome analyses of hybrids in some plant species, but the effects of heterosis on the photosynthetic efficiency and the growth rate have not been systematically evaluated with respect to N-use efficiency.

In this study, to find a clue to the enhancement of biomass productivity, we systemically evaluated the effect of heterosis on the growth rate and photosynthetic efficiency in a cultivar of high biomass hybrid sorghum, Tentaka. Sorghum is a major crop and widely used for various purposes. For example, the grain is used as food, syrup is obtained from culm, and whole crops are used for silage. Recently, much attention has been paid to sorghum as an alternative to maize as a bioenergy crop, and some projects concerning biorefining using sorghum are ongoing. Because sorghum is a C₄ plant (NADP-ME subtype) with CO₂-concentrating mechanisms and higher N-use efficiency, further improvement in crop biomass is expected.

**Results**

**Growth analysis in field-grown sorghum**

F₁ hybrid Tentaka and its parental lines (maintainer and restorer lines) were grown in an experimental field of the Nagoya University from May through the summer season in 2014. For growth analysis, total above-ground shoots were harvested every week, and biomass, leaf area and physiological traits were examined. Shoot dry weight increased exponentially in Tentaka, but the rate of increase in shoot dry weight gradually decreased, particularly in the parental lines (Fig. 1A). This difference was explained by the difference in flowering time. For the parental lines, flowering was observed at 57 DAS, whereas it was seen at 170 DAS in Tentaka. After flowering, the total leaf area decreased gradually in the parental lines (Fig. 1B).

Growth analysis was carried out between 29 and 43 DAS because it was possible that the growth after 44 DAS was affected by flowering in the parental lines. The relative growth rate (RGR) was not different between Tentaka and the parental lines (Table 2). The leaf area ratio (LAR) was greater in Tentaka than in the parental lines, indicating that leaves developed greatly in Tentaka even during the vegetative growth stage. At 43 DAS, leaf mass per area (LMA) was greater in the restorer line (74LH3213) (Table 1). Because premature young leaves of tillers were included in the calculation of LMA, the lower number of tillers in 74LH3213 contributed to the greater LMA. Similarly, at 43 DAS, the higher N content per leaf area (Nₐₑₑₐₑ) in 74LH3213 was partially explained by the lower number of tillers (Table 1). In 74LH3213, the higher Nₐₑₑₐₑ led to a higher net assimilation rate per leaf area (NAR) (Table 2).

Carbon isotope discrimination in leaves (δ¹³C) did not differ between Tentaka and the maintainer line (MS79B), but was significantly lower in 74LH3213 (Table 1). The lower level of δ¹³C in leaves was observed throughout the vegetative growth stage (Supplementary Fig. S1).

**Photosynthetic properties of sorghum grown at different nitrogen levels**

Sorghum plants were grown hydroponically at different N levels in a temperature-controlled glasshouse. At the flowering time of the parental lines, total dry weight of plants including tillers was significantly greater in Tentaka than in the parental lines grown at 3 and 12 mM N, but it was not different between them at 0.5 mM N (Supplementary Fig. S2). Total dry weight was not significantly different between the 3 and 12 mM N treatments, which indicated that 3 mM N was sufficiently high for plant growth in this experiment.

Highly positive correlations were found for the relationships between CO₂ assimilation and Nₑₑₑₑₑₑ in as well as between the amounts of Chl, C₄ enzymes [phosphoenolpyruvate carboxylase (PEPC) and PPDK], ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and Nₑₑₑₑₑₑ (Fig. 2). In addition, there were no differences between Tentaka and the parental lines in the rate of CO₂ assimilation and the amounts of key photosynthetic components for a given Nₑₑₑₑₑₑ. However, whereas CO₂
assimilation was curvilinearly correlated with \( N_{\text{area}} \) the amounts of the photosynthetic components were linearly correlated.

Diurnal changes of photosynthesis were monitored in the plants at 0.5 and 3.0 mM N under natural sunlight in a glasshouse. A typical example of the diurnal change of photosynthetic parameters such as the quantum yield of PSII (\( \Phi_{\text{II}} \)) and non-photochemical quenching (NPQ) on a sunny day is shown in Supplementary Fig. S3. \( \Phi_{\text{II}} \) decreased and NPQ increased with increasing photon flux density (PFD) in the morning, but there was no difference between Tentaka and the parental lines. At noon, \( \Phi_{\text{II}} \) was higher and NPQ was lower in the 3 mM N treatment than in the 0.5 mM N treatment, but \( \Phi_{\text{II}} \) increased and NPQ decreased to similar levels in the afternoon (Supplementary Fig. S3).

The diurnal pattern of natural sunlight was simulated using red and blue light-emitting diodes (LEDs) (Fig. 3B). and \( \Phi_{\text{II}} \), NPQ and the \( \text{CO}_2 \) assimilation rate were concurrently measured in a laboratory. Responses of \( \Phi_{\text{II}} \) and NPQ to fluctuating light were very similar to those monitored in the glasshouse (Fig. 3E–H). As observed in Supplementary Fig. S3, \( \Phi_{\text{II}} \) was higher and NPQ was lower in the 3 mM N treatment than in the 0.5 mM N treatment under high light. Similarly, the \( \text{CO}_2 \) assimilation rate under high light was greater in the 3 mM N treatment than in the 0.5 mM N treatment (Fig. 3A, B).

### Table 1 Comparisons of final plant biomass at flowering time and leaf properties and tiller number at 43 DAS in sorghum grown in an experimental field in 2014

<table>
<thead>
<tr>
<th></th>
<th>( F_1 ) Tentaka</th>
<th>( \text{♂ MS79B} )</th>
<th>( \text{♀ 74LH3213} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowering time (DAS)</td>
<td>170</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>Shoot dry weight (g)</td>
<td>413.3 ± 40.2a</td>
<td>36.1 ± 3.0b</td>
<td>28.8 ± 1.5b</td>
</tr>
<tr>
<td>Shoot height (m)</td>
<td>4.62 ± 0.08a</td>
<td>1.33 ± 0.02b</td>
<td>1.35 ± 0.02b</td>
</tr>
<tr>
<td>( LMA ) (g m(^{-2})) at 43 DAS</td>
<td>26.2 ± 0.9a</td>
<td>26.6 ± 0.8a</td>
<td>42.8 ± 1.3b</td>
</tr>
<tr>
<td>( N_{\text{area}} ) (mmol m(^{-2})) at 43 DAS</td>
<td>46.1 ± 2.9a</td>
<td>48.4 ± 2.3a</td>
<td>101.9 ± 4.3b</td>
</tr>
<tr>
<td>Leaf ( \delta^{13}\text{C} ) (%) at 43 DAS</td>
<td>-14.14 ± 0.08a</td>
<td>-14.08 ± 0.11a</td>
<td>-14.69 ± 0.11b</td>
</tr>
<tr>
<td>Tiller number at 43 DAS</td>
<td>2.2 ± 0.4a</td>
<td>2.6 ± 0.5a</td>
<td>0.4 ± 0.5b</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (\( n = 5 \)). Different superscript letters denote significant differences between lines by Student’s t-test (\( P < 0.005 \)).

### Table 2 Growth analysis of sorghum grown in an experimental field from 29 to 43 DAS

<table>
<thead>
<tr>
<th></th>
<th>( F_1 ) Tentaka</th>
<th>( \text{♂ MS79B} )</th>
<th>( \text{♀ 74LH3213} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGR (g g(^{-1}) d(^{-1}))</td>
<td>0.171 ± 0.008a</td>
<td>0.175 ± 0.005a</td>
<td>0.181 ± 0.006a</td>
</tr>
<tr>
<td>LAR (m(^2) g(^{-1}) shoot)</td>
<td>0.0251 ± 0.0006a</td>
<td>0.0220 ± 0.0003b</td>
<td>0.0159 ± 0.0007c</td>
</tr>
<tr>
<td>NAR (g m(^{-2}) d(^{-1}))</td>
<td>6.84 ± 0.44a</td>
<td>7.99 ± 0.23b</td>
<td>11.51 ± 0.89f</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (\( n = 5 \)). Different superscript letters denote significant differences between lines by Student’s t-test (\( P < 0.05 \)).

![Fig. 2](https://academic.oup.com/pcp/article-abstract/57/5/944/2223176) Relationships between (A) \( \text{CO}_2 \) assimilation rate, (B) Chls, (C) \( C_4 \) enzymes (PEPC and PPDK) and (D) Rubisco, and total leaf nitrogen contents in sorghum grown hydroponically in a temperature-controlled glasshouse. The \( \text{CO}_2 \) assimilation rate was measured at 400 \( \mu\text{mol mol}^{-1} \) \( \text{CO}_2 \), 29°C and 21% O\(_2\). The relationships were fitted as follows: (A) \( y = 0.697x - 0.003x^2 \) (\( R^2 = 0.66 \)); (B) \( y = 0.0057x \) (\( R^2 = 0.82 \)); (C) \( y = 0.0060x \) (\( R^2 = 0.88 \)); (D) \( y = 0.0178x \) (\( R^2 = 0.89 \)).
Fig. 3 Diurnal changes in photosynthesis under fluctuating light in sorghum grown hydroponically in a temperature-controlled glasshouse. CO₂ assimilation rate (A, B), stomatal conductance (C, D), quantum yield of PSII (ΦII) (E, F) and non-photochemical quenching (NPQ) (G, H) were concurrently measured at 400 μmol mol⁻¹ CO₂ and 29°C using mature leaves during 39–63 DAS. PFD using red and blue LEDs was programmed to imitate a typical example of the diurnal change of natural sunlight (B). Values are means ± SE [n = 3 (74LH3213) or n = 4 (in Tentaka and MS79B)].
From the data in Fig. 3A and B, the total amount of CO₂ assimilation per day \((A)\) was estimated (Table 3). Because the CO₂ assimilation rate under high light increased with increasing \(N_{\text{area}}\) (Fig. 2A), \(A\) was the greatest in 74LH3213 in the plants grown at 3 mM \(N\), but the ratio of \(A\) to \(N_{\text{area}}\) was the lowest (Table 3).

The change in stomatal conductance in response to fluctuating light corresponded to that of the CO₂ assimilation rate (Fig. 3C, D). In 74LH3213, the response of stomatal conductance during the dark to light transition was slower than in Tentaka and MS79B (Supplementary Fig. S4).

**Anatomical traits in sorghum leaves**

Cross-sections were obtained from a fully expanded mature leaf of Tentaka and flag leaves of 74LH3213 and MS79B grown in an experimental field at 49 DAS in 2013. In the flag leaf of 74LH3213, mesophyll cells were densely arranged around bundle sheath cells (Fig. 4). However, this anatomical trait in the flag leaf of 74LH3213 was not observed in the fully expanded mature leaf of 74LH3213 grown hydroponically in a glasshouse (Supplementary Fig. S5). The bundle sheath cell walls were thinner in the mature leaf of Tentaka (0.17 \(\mu m\)) than in the flag leaf of 74LH3213 (0.29 \(\mu m\)) and MS79B (0.28 \(\mu m\)) (Fig. 4D–F).

**Discussion**

**Growth analysis of F₁ Tentaka grown in an experimental field**

In Tentaka, a high biomass hybrid, height extended to approximately 4.6 m in an experimental field (Table 1). Because the

**Table 3** The amount of nitrogen per leaf area and CO₂ assimilation per day in sorghum grown hydroponically in a temperature-controlled glasshouse

<table>
<thead>
<tr>
<th></th>
<th>(F₁) Tentaka</th>
<th>(\oplus) MS79B</th>
<th>(\odot) 74LH3213</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N_{\text{area}}) (mmol (m^{-2}))</td>
<td>47.1 ± 3.9 (a)</td>
<td>75.2 ± 5.4 (bc)</td>
<td>44.6 ± 1.6 (a)</td>
</tr>
<tr>
<td>(A) (mol (m^{-2}) (d^{-1}))</td>
<td>0.60 ± 0.04 (ab)</td>
<td>0.76 ± 0.03 (bc)</td>
<td>0.63 ± 0.02 (ab)</td>
</tr>
<tr>
<td>(A/N_{\text{area}}) (mol CO₂ mol⁻¹ (N) (d^{-1}))</td>
<td>13.0 ± 1.2 (ab)</td>
<td>10.2 ± 0.5 (ab)</td>
<td>14.1 ± 0.8 (b)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE \([n = 3 (74LH3213) or n = 4 (in Tentaka and MS79B)].

Different superscript letters denote significant differences between lines by Tukey-Kramer’s multiple comparison test \((P < 0.05)\).
flowering time was extremely delayed in Tentaka, the final dry weight in Tentaka at flowering time was 11- to 14-fold greater than in the parental lines (Table 1). As observed for other hybrid species such as rice (Zhang et al. 2008) and Arabidopsis (Meyer et al. 2004, Ni et al. 2009, Fujimoto et al. 2012), hybrid Tentaka had a larger leaf area than the parental lines (Fig. 1B). However, because the rate of leaf area expansion gradually decreased in the parental lines by the time of flowering, the larger leaf area in Tentaka may have been caused by the delayed flowering time. In the present study, to minimize the effect of delayed flowering time in the hybrid on plant growth, the RGR was analyzed during the same vegetative period (29–43 DAS). Although a higher RGR was not observed in Tentaka grown in an experimental field, the LAR was greater and the NAR was smaller than those in the parental lines (Table 2). These results indicate that Narea was diluted by the enlarged leaf area in Tentaka, which led to the decrease in the NAR. Because N resources are limited under natural conditions, a larger leaf area would not be compatible with higher Narea.

**Nitrogen use efficiency in Tentaka**

To analyze quantitatively the relationship between Narea and LAR, sorghum plants were grown at different N levels. For the same N treatment, Narea was slightly higher in 74LH3213 than in MS79B and Tentaka (Table 3), which may be caused by the difference in the number of tillers (Supplementary Table S1). The Narea in Tentaka and in MS79B grown in an experimental field was similar to that in 0.5 mM N, but in 74LH3213, it was similar to the Narea of plants grown in 3 mM N (Tables 1, 3). Because the CO2 assimilation rate at saturating light increased with increasing Narea (Fig. 2A), A was the highest in 74LH3213 in 3 mM N (Table 3). Under natural conditions, however, since light intensity greatly fluctuates, plants cannot always attain maximal photosynthetic capacity (Fig. 3). Therefore, N-use efficiency, which is defined as A per Narea, was higher in leaves of the plants grown at 0.5 mM N (Table 3). Thus, the strategy of increased LAR and decreased Narea in Tentaka would be beneficial for high biomass production. Actually, a positive correlation between LAR and RGR has been frequently reported for various species grown by pot culture (Poorter and Remkes 1990, Sugiura et al. 2015), but it has not been observed for sorghum grown in an experimental field (Table 2). The higher leaf area in Tentaka probably has a disadvantageous effect on growth by self-shading in a dense vegetation.

**Diurnal changes of photosynthesis under fluctuating light**

As discussed above, Tentaka leaves had a lower Narea and NAR (Tables 1, 2). It is well known that excess light which is not used for CO2 assimilation is dissipated as heat to diminish photodamage to PS II, which is defined as NPQ. In the present study, NPQ increased with PFD, and the change in ΦII was closely associated with inverse change in NPQ (Fig. 3; Supplementary Fig. S3). These results indicate that excess light energy was efficiently discharged by the dissipation mechanisms associated with NPQ. However, these photosynthetic parameters did not differ between Tentaka and the parental lines for the same N treatments (Fig. 3; Supplementary Fig. S3), which indicates that the efficiency of electron transport and the thermal dissipation mechanisms were not changed by heterosis.

**Physiological and anatomical traits of leaves**

In early reports using super-hybrid rice, LYP9, genes related to enhancement of photosynthesis in leaves and N uptake in roots were up-regulated (Bao et al. 2005), and a greater photosynthetic rate in LYP9 was also observed (Zhang et al. 2008, Zhang et al. 2012). However, in other reports, the higher photosynthetic rates in the hybrids were explained by the delay of leaf senescence (Yang et al. 2007, Zhang et al. 2007). Thus, it is still under debate whether or not photosynthetic enzymes are up-regulated in hybrids. In the present study, to eliminate the effect of different Narea or leaf age on the amounts of photosynthetic enzymes and the CO2 assimilation rate, they were compared for a given Narea. The amounts of Rubisco and enzymes of the C4 cycle (PEPC and PPDK) at the given Narea were not different between Tentaka and the parental lines (Fig. 2).

In an early report using maize seedlings grown under various N levels, the proportion of PEPC and PPDK to soluble protein increased with increasing N, but the proportion of Rubisco decreased with increasing N (Sugiyama et al. 1984). This indicates that maize seedlings preferentially invested N into Rubisco under low N conditions and that the contents of C4 enzymes gradually increased with increasing leaf N. Similar results were also observed in seedlings of an NAD-ME-type C4 plant, *Panicum miliaceum* (Taniguchi et al. 1995). However, such preferential N allocation to Rubisco under low N conditions was not observed in the mature leaves of sorghum in the present study (Fig. 2).

In an early report using hybrid Arabidopsis, chloroplast-targeted genes involved in Chl biosynthesis were up-regulated only in the early developmental stage (3–7 DAS) (Fujimoto et al. 2012), but, in the mature sorghum leaves, the Chl content and Chl α/β ratio did not differ between Tentaka and the parental lines (Supplementary Table S1).

In Arabidopsis hybrids, increases in cell size and number were observed (Fujimoto et al. 2012), but in the present study a larger cell size was not observed in Tentaka (Fig. 4). In 74LH3213, mesophyll cells were tightly arranged around bundle sheath cells, and the intercellular airspace was small. These traits were probably associated with the higher LMA and Narea in 74LH3213 (Supplementary Table S2) and were not observed in the hydroponically grown plants at the same N level (Supplementary Fig. S5), in which LMA and Narea were not significantly greater than in Tentaka (Supplementary Table S1).

It is well known that leaf anatomical traits can affect the photosynthetic efficiency. For example, the thickness of cell walls affects the CO2 conductance during photosynthesis between intercellular airspaces and mesophyll cells (Terashima et al. 2006). In C4 leaves, the bundle sheath cell wall is thick and suberized, which is considered to be a barrier to CO2 leakage from the bundle sheath cells. It has been reported that in
sorghum leaves were not changed by heterosis, but rather by
they also did not differ between Tentaka and the parental
which was explained by the increase in CO2 leakiness under low
natural conditions, leaves were not always exposed to high
light (Tazoe et al. 2006, Tazoe et al. 2008). In the present study, the
value of leaf δ13C was more negative in a C4 dicot (Amaranthus cruentus) grown
under low light conditions than under high light conditions,
which was explained by the increase in CO2 leakiness under low
light (Tazoe et al. 2006, Tazoe et al. 2008). In the present study, the
value of leaf δ13C was more negative in 74LH3213 than in
Tentaka (Table 1; Supplementary Table S2; Supplementary
Fig. S1), which indicates that CO2 leakiness was greater in
74LH3213 than in Tentaka, but the bundle sheath cell walls
were thinner in Tentaka than in 74LH3213 (Fig. 4D–F). Thus, the
difference in the δ13C value between 74LH3213 and
Tentaka was not explained by the difference in anatomical
traits.

Because the δ13C value also reflects the behavior of CO2
diffusion, dissolution and fixation inside a leaf during photosyn-
thesis and respiration, the slower stomatal response
during the dark to light transition in 74LH3213 may affect the
CO2 conductance inside leaves and increase the δ13C value in
74LH3213 (Supplementary Fig. S4). Further quantitative
analysis of the carbon isotope discrimination using an online system of gas exchange and a mass spectrometer
are needed.

Conclusions

The hybrid sorghum, Tentaka, had greater biomass and larger
leaf area than its parental lines, as has also been observed for
other hybrid species such as rice (Zhang et al. 2008) and
Arabidopsis (Ni et al. 2009, Fujimoto et al. 2012). However, the
greater biomass in Tentaka was mainly caused by the pro-
longed vegetative growth period. In the present study, the
effect of the larger leaf area in Tentaka on the photosynthetic
traits and N-use efficiency was investigated in detail. Because
Tentaka had a larger leaf area, Narea was relatively lower than in
74LH3213, but similar to MS79B. Comparing the amounts of
photosynthetic enzymes and Chl in given plants with different
N levels, they were similar between Tentaka and the parental
lines. When the CO2 assimilation rate per leaf area, ΦII and
NPQ were simultaneously measured under fluctuating light,
they also did not differ between Tentaka and the parental
lines for the same N level. Thus, photosynthetic properties in
sorghum leaves were not changed by heterosis, but rather by
Narea.

Narea was lower in Tentaka, which led to a decrease in CO2
assimilation rate per leaf area under high light. However, under
natural conditions, leaves were not always exposed to high
light and could not maximize the photosynthetic ability.

Therefore, the larger leaves with lower Narea in Tentaka
would be effective for the improvement of the N-use efficiency
for photosynthesis in an isolated plant. However, in a dense
vegetation, the higher LAR in Tentaka may give rise to an
adverse effect caused by self-shading. Future experiments
are required to identify which factors determine the enlarge-
ment of leaf area, the elongation of the vegetative growth
period and the increase in tiller number.

Materials and Methods

Plant materials and growth conditions

Tentaka, a high biomass hybrid sorghum [Sorghum bicolor (L.) Moench] culti-
vare, and the maintainer and restorer lines (MS79B and 74LH3213) were grown at
the Togo Field Science and Education Center of Nagoya University (Aichi,
Japan) in 2013 and 2014. Seeds of sorghum were sown in a glasshouse to culture
the seedlings and transplanted to the experimental field 3 weeks later.

The plants were also grown hydroponically in an air-conditioned glasshouse
at Tohoku University for photosynthetic analysis. In the glasshouse, tempera-
tures were controlled at 29.25°C day/night. In autumn and winter, supplemen-
tal light was provided by six metal halide lamps from 05:00 to 07:00 h and from
17:00 to 19:00 h, and the photoperiod was set at 14 h. Sorghum seeds were
germinated in a wet paper towel. After germination, seedlings were transferred
to small pots (Ø6.5 cm × 6.0 cm) filled with large particle soil and grown for 15
d. The seedlings were then transferred to 3.5 liter Wagner pots filled with water
culture medium, containing 0.8 mM KH2PO4, 0.6 mM CaCl2, 0.5 mM MgSO4,
0.05 mM Fe-EDTA, 0.05 mM H3BO4, 0.7 μM MnSO4, 0.3 μM CuSO4, 0.1 μM Na2MoO4 and different concentrations of nitrogen (12, 3 or 0.5
mM N). The NO3/ NH4 ratio was adjusted by mixing NH4NO3 and NaNO3,
and was 3/1, 2/1 and 1/1 in 12, 3 and 0.5 mM N, respectively. The water culture
medium was adjusted to pH 4.9 by adding 5 mM MES to prevent the pH
changes caused by depletion of NH4+ (Bernardo et al. 1984). The water culture
medium was exchanged every fifth day.

Growth analysis

The dry weight of shoots and leaf area (including tillers) were measured every
week until flowering was observed in all samples of the parental lines (57 DAS).
Because the dry weight of shoots and leaf area increased exponentially during
29–43 DAS (Fig. 1), RGR and NAR were given by

\[
RGR = \frac{W_{t} - W_{0}}{t - t_{0}}
\]

and

\[
NAR = \frac{(W_{t} - W_{0})(\ln L_{2} - \ln L_{1})}{(L_{2} - L_{1})(t_{2} - t_{1})}
\]

where RGR, W, L, t and t0 are the leaf area ratio (m² g⁻¹ shoot), shoot dry weight
(g), leaf area (m²) and days of the sampling (t0, 29 DAS; t1, 43 DAS), respectively.
LAR was calculated by Equations (1) and (2).

Measurements of photosynthesis

The CO2 assimilation rate in Fig. 2A and Supplementary Fig. S4 was measured
using young leaves. The middle of a leaf, except for the midrib, was held in a
clamp-on Li-6400 leaf chamber (Li-Cor), in which the PFD, leaf temperature
and reference CO2 partial pressure were set at 2000 μmol quanta m⁻² s⁻¹, 29°C
and 400 μmol mol⁻¹, respectively.

Diurnal changes of Chl fluorescence of leaves under natural sunlight in
Supplementary Fig. S3 were monitored in an air-conditioned glasshouse
using a MONITORING-PAM fluorometer (Heinz Walz GmbH). For monitoring the
Chl fluorescence level, blue measuring light was used. The maximum quantum
yield of photochemistry in PSII (Fv/Fm) was determined by the application
of a saturating pulse for 0.6 s before dawn. The steady-state fluorescence
level (F0) was recorded under natural light. To obtain the maximum fluorescence

level in the presence of NPQ ($F_{m}^{\prime}$), a saturating pulse was applied every 10 min. NPQ was estimated as $(F_{m} - F_{m}^{\prime})/F_{m}$. The photosynthetic rate and Chl fluorescence responding to changing light intensity in Fig. 3 were measured concurrently using a combined system of GFS-3000 and Dual-PAM (Heinz Walz GmbH) in a laboratory. Hydroponically grown mature plants in a glasshouse were transported to a laboratory the night before and the middle part of a young leaf was wrapped in aluminum foil and dark-adapted overnight. Measurements of photosynthesis were started in the morning. CO$_{2}$ partial pressure and leaf temperature were maintained at 400 ppm and 29°C, respectively. PFD was programmed to imitate the diurnal change of natural light (on August 24, 2014; Supplementary Fig. S3). After the measurement of photosynthesis, leaf disks (8 mm diameter) were sampled and stored at −80°C for measurements of Chl and protein contents. Some leaf disks were dried at 80°C and used for N/C analysis. Some leaf disks were dried at 80°C and used for N/C analysis.

**Chl and protein contents**

Frozen leaf disks were homogenized in an extraction buffer containing 50 mM Na-phosphate buffer at pH 7.0, 2 mM iodoacetic acid, the Complete Protease Inhibitor (Roche Applied Science), 5% glycerol and 120 mM β-mercaptoethanol. A 400 μl aliquot of extraction buffer was added to six leaf disks. A part of the homogenate was used for the determination of Chl contents and the Chl a/b ratio by extracting Chls in 80% acetone (Porra et al. 1989). The rest of the homogenate was centrifuged at 10,000 × g for 3 min at 4°C and the supernatant was obtained for determination of soluble protein and SDS–PAGE analysis. Because a part of the soluble proteins could be present in the pellet after the first centrifugation, the pellet was washed with extraction buffer and centrifuged again (10,000 × g for 3 min at 4°C) to increase the collection rate of soluble proteins. These supernatants were mixed with an equal amount of a double-strength loading buffer [5% (w/v) lithium dodecylsulfate, 5% β-mercaptoethanol, 0.05% bromophenol blue, 10% glycerol and 125 mM Tris–HCl at pH 6.8], treated at 95°C for 3 min and analyzed by SDS–PAGE. SDS–PAGE was performed with a 12.5% polyacrylamide gel according to Laemmli (1970). The bands of the Rubisco large subunit, PEPC and PPDK were identified by the molecular masses at 53, 99 and 94 kDa, respectively. Because the bands of PEPC and PPDK were very close and partly overlapped, they were grouped together as C$_{4}$ enzymes (PEPC and PPDK). Small pieces of the gel containing bands of these photosynthetic enzymes were excised with a razor blade and determined using the method of Makino et al. (2003). The intensity in the gel was scanned with a Hickerscanner (Helena Laboratories). The bands of Rubisco large subunit and molecular mass of Rubisco. The authors have no conflicts of interest to declare.

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**Disclosures**

The authors have no conflicts of interest to declare.

**References**


