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Interspecific differences in how sink–source imbalance causes photosynthetic downregulation among three legume species

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• **Background and Aims** Sink–source imbalance could cause an accumulation of total non-structural carbohydrates (TNC; soluble sugar and starch) in source leaves. We aimed to clarify interspecific differences in how sink–source imbalance and TNC causes the downregulation of photosynthesis among three legume plants. The TNC in source leaves was altered by short-term manipulative treatments, and its effects on photosynthetic characteristics were evaluated.
• **Methods** Soybean, French bean and azuki bean were grown under high nitrogen availability. After primary leaves were fully expanded, they were subjected to additional treatments: defoliation except for two primary leaves; transfer to low nitrogen conditions; transfer to low nitrogen conditions and defoliation; or irradiation by light-emitting diodes. Physiological and anatomical traits such as TNC content, maximum photosynthetic rate, cell wall content and $\delta^{13}C$ values of primary leaves and whole-plant growth were examined.
• **Key Results** Among the three legume plants, the downregulation of maximum photosynthesis and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content was co-ordinated with an increase in TNC only in French bean. Rubisco did not decrease with an increase in TNC in soybean and azuki bean. The defoliation treatment caused an increase in cell wall content especially in soybean, and maximum photosynthesis decreased despite resulting in a higher Rubisco content. This indicates that a decrease in mesophyll conductance could cause photosynthetic downregulation, which was confirmed by an increase in $\delta^{13}C$.
• **Conclusion** The present results suggest that a downregulation of photosynthesis in response to increased levels of TNC in source leaves can result not only from decreases in Rubisco content, but also from anatomical factors, such as an increase in cell wall thickness leading to reduced chloroplast CO_2 concentrations.

Key words: Biomass allocation, cell wall, downregulation of photosynthesis, leaf mass per area, non-structural carbohydrates, sink–source balance, photosynthetic rate, Rubisco.

INTRODUCTION

Climate change, such as global warming and the rise in atmospheric carbon dioxide (CO_2) concentration, threatens food security and ecosystem sustainability. It is therefore important to clarify how these environmental changes affect plant performance. Photosynthesis, which is the most fundamental plant function, is predicted to be strongly affected by future climate conditions. For example, it is generally believed that the photosynthetic rate is downregulated by long-term exposure to elevated CO_2 (Ainsworth and Long, 2005), yet the detailed mechanisms of the downregulation remain unknown. There is an urgent need to clarify the mechanisms of photosynthetic downregulation, and interspecific comparative studies would be vital to this end. In the present study, we compared behaviours of important legume species (Graham and Vance, 2003).

When carbohydrates accumulate in the leaves, expression of genes associated with photosynthesis is suppressed (Sheen, 1994). For example, it was shown that expression of genes such as *rbcS* is suppressed in response to accumulation of hexoses and starch in sugar-fed leaves and cold-girdled source leaves of *Solanum tuberosum* (Krapp *et al.*, 1993) and *Spinacia oleracea* (Krapp and Stitt, 1995). This mechanism could also be responsible for the downregulation of photosynthesis in plants grown at low nitrogen (N) availability or elevated CO_2 (Stitt and Krapp, 1999; Ainsworth and Rogers, 2007). The source activity or the photosynthetic rate of leaves may initially be enhanced by elevated CO_2, and it is ultimately suppressed by longer term exposure to elevated CO_2, especially when plants are grown at low N in small pots (Long *et al.*, 2004). The decrease in source activity may be linked to a decrease in sink activity of growing organs, which is caused by nutrient deficiencies, and rooting volume due to small pot size (Arp, 1991). Therefore, the downregulation of source activity is not severe, especially in field-grown plants with higher sink capacity even at elevated CO_2 (Leakey *et al.*, 2009; Ruiz-Vera *et al.*, 2017). These studies indicate that the decrease in the sink–source ratio causes the decrease in the photosynthetic capacity through accumulation of carbohydrates in the source leaves (Ainsworth *et al.*, 2004; Kasai, 2008). Hereafter, these carbohydrates, such

as soluble sugars and starch, are called total non-structural carbohydrates (TNC).

It is also important to note that various physiological and physical processes are involved in leaf photosynthesis. The CO_2 concentration around ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is determined by stomatal conductance and mesophyll conductance. Mesophyll conductance is greatly influenced by morphological and anatomical characteristics such as cell wall thickness and surface area of chloroplasts exposed to the intercellular airspace (Tholen *et al.*, 2008; Evans *et al.*, 2009). The carboxylation rate is also dependent on the content of active Rubisco, reducing power and ATP. The latter two are produced in the thylakoids and are used for the regeneration of ribulose-1,5-bisphosphate and the activation of Rubisco. Therefore, it is possible that changes in these physiological and morphological characteristics are affected by the accumulation of TNC and cause the downregulation of photosynthesis, and it is necessary to reveal whether and to what extent the accumulation of TNC affects each of these characteristics.

Goldschmidt and Huber (1992) studied effects of TNC accumulation on photosynthetic capacity in heat-girdled leaves of 11 plant species including four legume plants that were grown in a greenhouse. They showed marked differences in the extent of the decrease in the photosynthetic rate in response to accumulation of sugars and starch among the species. However, no clear information about leaf age, experimental period, light intensity and soil nitrogen availability was provided in this study. Kasai (2008) investigated how pod removal affects the photosynthetic rate in *Glycine max* grown under controlled conditions in growth chambers. His study showed that the photosynthetic rate decreased with the decrease in the sink–source ratio at the reproductive stage. The age of source leaves is another important determinant of the downregulation in *Phaseolus vulgaris* (Araya *et al.*, 2006, 2010). It has been pointed out that there are large varietal differences in the effects of temperature and CO_2 conditions on accumulation of TNC and the photosynthetic rate in *Vigna angularis* (Ahmed *et al.*, 1993). Thus, TNC-sensitive changes in photosynthetic characteristics could vary greatly not only varietally but also interspecifically.

Despite numerous studies on the downregulation of photosynthesis, mechanisms and interspecific differences of the downregulation have not yet been fully understood. The studies cited above have investigated changes in physiological traits including gene expression and metabolite levels in detail. However, neither the morphological traits which could affect photosynthesis nor whole-plant growth has been closely studied. In addition, when plants are grown under constant environmental conditions for longer time periods, photosynthetic traits acclimate to the conditions. This makes it difficult to detect and evaluate the downregulation that occurs in the short term.

We previously reported that changes in morphological and anatomical traits are involved in the downregulation of photosynthesis in two reciprocally grafted *Raphanus sativus* with different sink activity (Sugiura *et al.*, 2015, 2017). We showed that the maximum photosynthetic rate was not severely decreased, and the Rubisco content or its activation state was not subject to TNC levels. On the other hand, cell wall mass per leaf area (CMA) and leaf anatomical features such as intercellular air space and mesophyll cell size that affect mesophyll conductance were significantly altered depending on sink–source balance. Thus, it was implied that photosynthetic capacity could be downregulated not only physiologically but also morphologically.

It is known that Rubisco content is selectively decreased under elevated CO_2 conditions in some plant species such as *Brassica napa*, *Chenopodium album* (Sage *et al.*, 1989) and *P. vulgaris* (Nakano *et al.*, 1998). Thus, the selective decrease in Rubisco with the accumulation of TNC may be a good indicator of the physiological downregulation of photosynthesis. The foliar stable carbon isotope ratio ($\delta^{13}C$) is reduced (i.e. the plant is depleted of ^{13}C) due to discrimination against $^{13}CO_2$ during diffusion and during the carboxylation reaction by Rubisco. Since the contribution of the latter process is prominent, foliar $\delta^{13}C$ would increase with a decrease in stomatal conductance and mesophyll conductance. This is because the decrease in mesophyll conductance reduces te chloroplast CO_2 concentration in the same way as a decrease in stomatal conductance, leading to the increase in $\delta^{13}C$ due to the decreased carbon isotope discrimination by Rubisco (Farquhar *et al.*, 1989; Evans *et al.*, 1994). Therefore, $\delta^{13}C$ values can be a useful indicator of the downregulation of photosynthesis by anatomical traits including the cell wall thickness.

In the present study, we aimed at clarifying interspecific differences in the downregulation of photosynthesis among legumes, *Glycine max* (soybean), *P. vulgaris* (French bean) and *V. angularis* (azuki bean). Since all three species develop a pair of simple true leaves (hereafter called primary leaves), it is possible to compare changes in morphological and physiological traits of the primary leaves at the same physiological stage. We mainly investigated short-term responses of the traits to manipulating the sink–source activity ratio. For example, defoliating sink leaves would cause a decrease in the sink–source activity ratio by decreasing sink activity. Transfer to the low nitrogen availability conditions would also decrease the sink activity of each organ, resulting in the accumulation of TNC (Sugiura *et al.* 2017). Meanwhile, the transfer to high light would cause a decrease in the sink–source activity ratio by increasing source activity. These manipulative treatments enabled us to evaluate how and to what extent each photosynthesis-related trait is affected by TNC, which altogether would contribute to uncovering the mechanisms of downregulation of photosynthesis.

Based on analyses of relationships among TNC, CMA, $\delta^{13}C$, nitrogen availability, light intensity and photosynthetic traits in *G. max*, *P. vulgaris* and *V. angularis*, we discuss mechanisms underlying the interspecific differences in the downregulation of photosynthesis.

MATERIALS AND METHODS

Plant materials and initial growth conditions

Abbreviations of parameters and manipulative treatments are listed in Supplementary Data Table S1. We used three legume plants, soybean [*Glycine max* (L.) Merr. 'Tsurunoko'], French bean (*Phaseolus vulgaris* L. 'Yamashiro-kurosando') and azuki bean (*Vigna angularis* L. 'Dainagon'). They were purchased from a commercial supplier (Takii seed, Kyoto, Japan). The seeds were placed on wet paper until they germinated, and

were then planted in plastic pots containing 200 mL of vermiculite. They were grown in a controlled growth room, where light was supplied by a fluorescent lamp during the12 h light period and at a photosynthetically active photon flux density (PFD) of 400 μmol m^{-2} s^{-1}, mean air temperature of 24 °C and relative humidity of about 60 %. A modified Hoagland nutrient solution contained 1 mM NaH_2PO_4, 0.25 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 μM Fe-EDTA, 100 μM $MnSO_4$, 300 μM H_3BO_3, 10 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.25 μM $(NH_4)_6Mo_7O_{24}$ and 1.25 μM $CoCl_2$. Low (0.2 mM N) and high nitrogen (10 mM N) solutions with 5 mM K^+ and Ca^{2+} concentrations were obtained by adding KNO_3, Ca $(NO_3)_2$, KCl and $CaCl_2$. Plants were fertilized every day with 50 mL of the high N solution until the two primary leaves were fully expanded and trifoliate leaves appeared.

Manipulative treatments

Control plants used for initial measurements were called Ct_0. On the day after the initial photosynthesis measurement (see below), short-term manipulative treatments were given to Ct_0 plants to alter the sink–source balance and TNC level in the primary leaves. Ct_0 plants were grown under the same conditions (Ct), under the same conditions but with trifoliate leaves being defoliated (De), transferred to low N conditions (LN), transferred to low N conditions and defoliated (LNDe), or grown under the same conditions but with two primary leaves illuminated with high light from LEDs (light-emitting diodes) (HL). In De plants, trifoliate leaves were defoliated leaving only two primary leaves. In LN, the remaining nutrients in the soil were flushed away using tap water, and a nutrient solution containing 0.2 mM N was applied during the treatment. In HL plants, two primary leaves only were illuminated by LEDs with peak wavelengths at 440 and 550 nm (NSPWR70CS-K1, Nichia, Tokushima, Japan) to increase PPFD to 900 μmol m^{-2} s^{-1} keeping the PPFD on other parts at 400 μmol m^{-2} s^{-1}. Plants were treated for 6 d, and photosynthesis measurements were performed in the morning on the sixth day after the onset of the manipulative treatments (DAT). In soybean, Ct and De plants were grown for another 9 d, and the photosynthesis measurements were performed at 10 and 15 DAT.

Photosynthesis measurements and sampling

Photosynthetic characteristics of the leaves were determined with a portable gas-exchange system (LI-6400; Li-Cor, Lincoln, NE, USA). Measurements of gas exchange for Ct_0 plants were performed at 13, 12 and 15 d after sowing in soybean, French bean and azuki bean, respectively. The measurements for Ct, LN, LNDe and HL plants were performed at 6 DAT in all the legume plants, and those for Ct and De soybean plants were additionally performed at 10 and 15 DAT. The measurements were conducted in 8–12 primary leaves for each species. The rate of photosynthesis under the growth conditions (A_{growth}, μmol m^{-2} s^{-1}) and maximum photosynthetic rate (A_{max}) were measured at a PPFD of 400 μmol m^{-2} s^{-1} at 400 μmol CO_2 mol^{-1} air and at 1500 μmol m^{-2} s^{-1} at 400 μmol CO_2 mol^{-1} air, respectively, after measuring the respiration rate in the dark (R_d). Stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were also recorded. The leaf temperature was maintained at 25 °C and the leaf to air vapour pressure deficit (VPD) was set to 0.9–1.0 kPa. At the end of the light period on the same day, three leaf discs (1 cm in diameter) were carefully sampled from one side of the primary leaves that had been used for the photosynthesis measurements so as not to damage the main veins. They were oven-dried for TNC measurement (see below). On the next day at the end of the dark period, three leaf discs were sampled from the other side of the primary leaves and oven-dried to determine TNC, leaf mass per area (LMA, g m^{-2}) and leaf nitrogen content per area (N_{area}, g N m^{-2}) (see below). Another three leaf discs were sampled from the rest of the primary leaves used for the photosynthesis measurements and stored at –80 °C for measurements of Rubisco and cell wall content (see below). Small lamina segments were excised and fixed in FAA (5 % formaldehyde, 5 % acetic acid and 45 % ethanol) for leaf anatomy (see below). After these samplings, plants were divided into the rest of the primary leaves, trifoliate leaves, stems and roots, and oven-dried at 80 °C to determine the dry mass of each organ.

Rubisco content and cell wall content

Contents of Rubisco and cell wall materials were determined as described previously (Mizokami *et al.*, 2015; Sugiura *et al.*, 2017). The three frozen leaf discs were homogenized in a Tris–HCl buffer (62.5 mM, pH 6.8) with a Multi-beads Shocker (Yasui Kikai, Osaka, Japan) to extract Rubisco. The Rubisco content was determined by measuring absorbance at 595 nm following SDS–PAGE, Coomassie Brilliant Blue staining and formamide extraction. The residual pellet after the extraction was used for the determination of cell wall content. Starch was removed with amyloglucosidase (A-9228, Sigma Aldrich, St. Louis, MO, USA), and cytoplasmic proteins were removed from the pellet with 1 M NaCl. Residual cell wall mass was weighed after drying, and CMA (g m^{-2}) was calculated.

LMA and total non-structural carbohydrates

After weighing three dried leaf discs to determine LMA, they were ground with the Multi-beads Shocker. About 5–10 mg of the ground samples were used to determine the contents of glucose, sucrose and starch following Araya *et al.* (2006). Soluble sugars were extracted with 80 % ethanol, and sucrose was broken down into glucose and fructose with an invertase solution. The precipitate was treated with amyloglucosidase (A-9228) to break down starch into glucose. Finally, glucose and glucose equivalents of sucrose and starch were quantified with the Glucose CII test kit (Wako Chemicals, Tokyo, Japan). TNC were calculated on a leaf area basis, and structural LMA (sLMA, g m^{-2}) was calculated by subtracting TNC from LMA (Bertin *et al.*, 1999).

Nitrogen and $\delta^{13}C$

The nitrogen content (Nmass, g N g^{-1}) and $\delta^{13}C$ of the ground leaf samples were determined with a CN analyser (Vario Micro,

Elementar Analyzensysteme GmbH, Hanau, Germany) connected to an isotopic ratio mass spectrometer (IsoPrime100, IsoPrime, Manchester, UK). N content per area (g N m^{-2}) was calculated as a product of N_{mass} and LMA. $\delta^{13}C$ (‰) was calculated as follows:

$$\delta^{13}C = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000$$

where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratio of the sample and standard (PDB, 0.011180).

Leaf transverse section

The leaf segments fixed in FAA were dehydrated in a series of ethanol solutions and embedded in Technovit 7100 (Heraus Holding, Hanau, Germany). Leaf transverse sections of 1 μm thickness were cut on an ultramicrotome and stained with a staining solution containing 0.1 % (w/v) toluidine blue and 1 % (w/v) sodium borate. Anatomical traits of leaves such as thickness, intercellular air space and average mesophyll size were analysed using Image-J software (Schneider *et al.*, 2012) following Sugiura *et al.* (2017).

Statistical analysis

Statistical tests were performed with Systet13 (Hulinks Inc., Tokyo, Japan). Values of Ct were compared with De, LN, LNDe and HL by Dunnett's test following analysis of variance (ANOVA). Values of Ct and De in soybean at 10 and 15 DAT were compared by Student's *t*-test.

RESULTS

Accumulation of TNC and photosynthetic characteristics of primary leaves

Total non-structural carbohydrates of primary leaves differed markedly among the manipulations in all the legume plants (Fig. 1). TNC decreased by 13–76 % during the dark period depending on the manipulations and the legume species. Compared with Ct plants, TNC was >50 % higher in LN, LNDe and HL plants in soybean (Fig. 1A), and LN and LNDe plants in French bean (Fig. 1B). TNC was not significantly different among the manipulations, except for LNDe plants in azuki bean (Fig. 1C). In soybean, TNC was significantly higher in De plants than in Ct plants at 15 DAT but not at 10 DAT.

Photosynthetic characteristics of primary leaves changed markedly in response to the manipulations in all the legume plants (Fig. 2). Compared with Ct plants, A_{max} and A_{growth} were slightly lower in De, LN and LNDe plants, whereas A_{growth} was slightly higher in HL plants in soybean (Fig. 2A). In French bean, A_{max} and A_{growth} in all the manipulations were significantly lower than those in Ct plants except for A_{growth} in HL plants (Fig. 2B). In azuki bean, A_{max} and A_{growth} in LN and LNDe plants were decreased by about 50 % compared with Ct plants (Fig. 2C). Changes in g_s mostly corresponded to the changes in A_{max} and A_{growth} especially in soybean and azuki bean (Table 1). Compared with Ct plants, R_d was also slightly changed in all the legume plants (Fig. 2D–F). It tended to be higher in De and HL plants and lower in LN and LNDe plants. In soybean, A_{max}, A_{growth}, g_s and C_i did not differ very much between Ct and De plants at 10 and 15 DAT (Fig. 2A; Table 1), whereas R_d was >1.5 times lower in De plants than in Ct plants at 10 and 15 DAT (Fig. 2D).

Nitrogen and Rubisco contents of primary leaves were markedly affected by the manipulations in all the legume plants (Table 1). Compared with Ct plants, N_{area} was 25–50 % higher in De plants and 30–50 % lower in LN plants in all the legume plants. N_{area} was also 20–30 % lower in LNDe plants and 25–40 % higher in HL plants in French bean and azuki bean. Differences in the Rubisco content were mostly consistent with those in N_{area} in all the legume plants. In soybean, N_{area} and Rubisco content were 2–3 times higher in De plants than in Ct plants at 10 and 15 DAT.

Relationships among photosynthetic characteristics, TNC and N_{area}

Although levels of TNC and sucrose at the end of the night were lower than those at the end of the day, the A_{max}–TNC

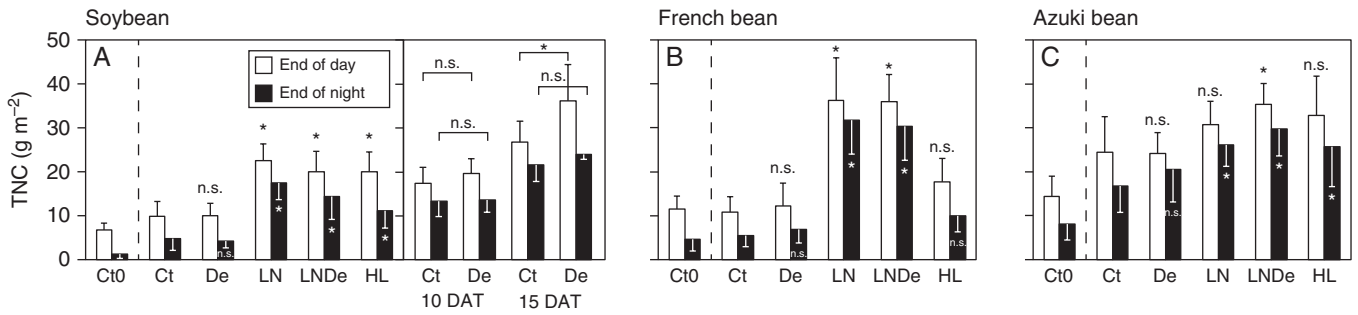

FIG. 1. Total non-structural carbohydrates (TNC) in the leaves of soybean (A), French bean (B) and azuki bean (C) at the end of the day and night before and after the experimental treatments. Control plants before the treatments (Ct_0) were grown under control growth conditions (Ct), defoliated (De), transferred to low N conditions (LN), transferred to low N conditions and defoliated (LNDe), or transferred to high light (HL). They were harvested at 6 days after the treatments (DAT), and soybean plants of Ct and De were also obtained at 10 and 15 DAT. Values of Ct, De, LN, LNDe and HL in all the species were obtained at 6 DAT, and those of Ct and De in soybean were also obtained at 10 and 15 DAT. White bars represent values at the end of the day, and black bars represent values at the end of the night. Values are means ± s.d. (n = 6–12). Values of Ct were compared with those of De, LN, LNDe and HL by Dunnett's test (*P < 0.05, n.s. P > 0.05) following ANOVA. Values of Ct and De in soybean at 10 and 15 DAT were compared by Student's *t*-test (**P < 0.01, *P < 0.05, n.s. P > 0.05).

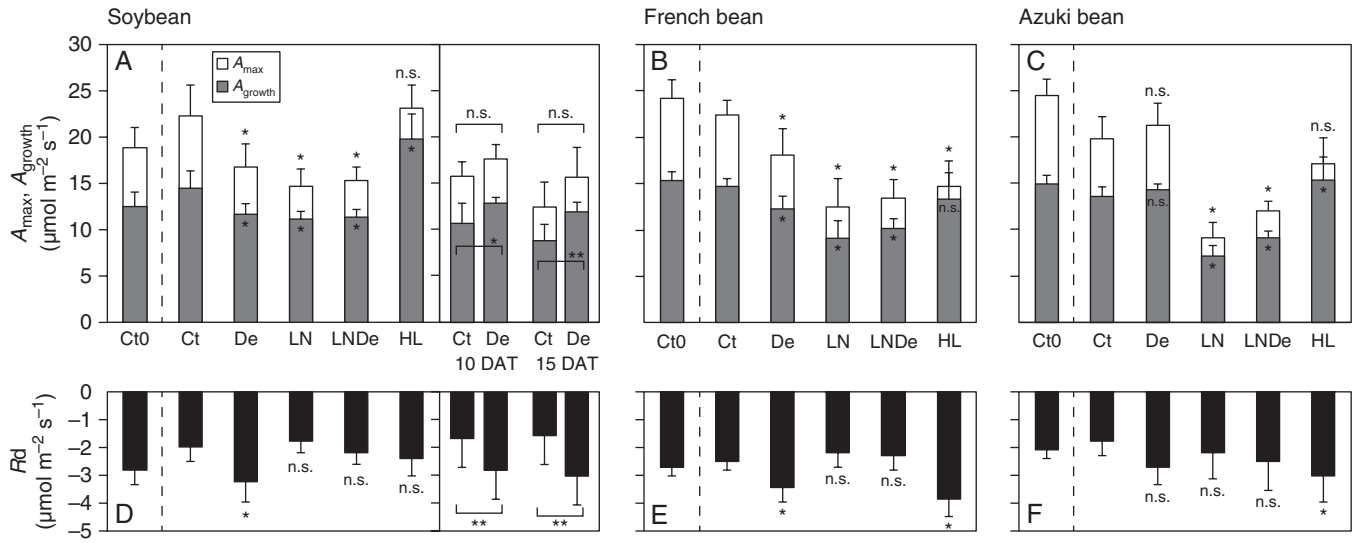

FIG. 2. Maximum photosynthetic rate (A_{max}), photosynthetic rate under growth conditions (A_{growth}) and dark respiration rate (R_d) in the leaves of soybean (A, D), French bean (B, E) and azuki bean (C, F) before and after the experimental treatments. Abbreviations on the *x*-axes are as in Fig. 1. Values of Ct, De, LN, LNDe and HL in all the species were obtained at 6 days after the treatments (DAT), and those of Ct and De in soybean were also obtained at 10 and 15 DAT. Values are means ± s.d. (n = 6–12). Values of Ct were compared with De, LN, LNDe and HL by Dunnett's test (*P < 0.05, n.s. P > 0.05) following ANOVA. Values of Ct and De in soybean at 10 and 15 DAT were compared by Student's t-test (**P < 0.01, *P < 0.05, n.s. P > 0.05).

relationships and A_{max}–sucrose relationships at the end of the night were similar to those at the end of the day (Fig. 3). For the data pooled for all the manipulations, there were weak negative correlations between A_{max} and TNC in soybean (Fig. 3A) and azuki bean (Fig. 3C), whereas, in French bean, a strong negative correlation of A_{max} and TNC was observed (Fig. 3B). Relationships between A_{max} and sucrose were mostly like those between A_{max} and TNC (Fig. 3D–F). We also found a strong negative correlation between Rubisco content and TNC only in French bean (Supplementary Data Fig. S1).

In soybean, A_{max} was positively correlated with the Rubisco content among Ct_0, Ct, LN and HL plants, whereas A_{max} did not increase with the increase in Rubisco content in De or LNDe plants (Fig. 4A). On the other hand, A_{max} was positively correlated with Rubisco content among all the manipulations in French bean (Fig. 4B) and azuki bean (Fig. 4C). Rubisco content was positively correlated with N_{area} among all the manipulations in soybean (Fig. 4D) and azuki bean (Fig. 4F). In French bean, two separate positive correlations between Rubisco content and N_{area} were found for Ct_0 and Ct plants and for LN, De, LND and LNDe plants (Fig. 4E). A_{max} was positively correlated with g_s among the manipulations in soybean and azuki bean (Supplementary Data Fig. S2A, C), whereas A_{max} was less correlated with g_s in French bean among the manipulations (Supplementary Data Fig. S2B). R_d was significantly correlated with N_{area} but not with TNC in soybean (Supplementary Data Fig. S3A, D). Similar trends were observed in French bean (Supplementary Data Fig. S3B, E). On the other hand, R_d was significantly correlated with TNC but not with N_{area} in azuki bean (Supplementary Data Fig. S3C, F).

Biomass allocation and whole-plant growth

At 6 DAT, Ct_0 plants grew differently depending on the species and the manipulations. Total dry masses of De and LNDe plants were 20–30 % lower than that of Ct plants in soybean (Supplementary Data Fig. S4A), whereas total dry masses hardly differed among the manipulations in French bean and azuki bean (Supplementary Data Fig. S4B, C). In soybean, Ct plants grew 2–3 times larger than De plants at 10 and 15 DAT (Supplementary Data Fig. S4A).

Morphological and anatomical traits of primary leaves

The LMA, sLMA and CMA changed markedly in response to the short-term manipulative treatments in all the legume plants (Table 2; Fig. 5). Compared with Ct plants, LMA was 30–55 % higher in all the manipulated plants in soybean, 50–70 % higher in LN, LNDe and HL plants in French bean and 30% higher in HL plants in azuki bean. Similarly, sLMA was also higher in all the manipulated plants in soybean, in De and HL plants in French bean and in HL plants in azuki bean compared with Ct plants. There was a marked difference in CMA among the manipulations in all the legume plants (Fig. 5). CMA was about twice as high in De and LNDe plants than in Ct plants in soybean (Fig. 5A), whereas CMA increased modestly in French bean (Fig. 5B) and azuki bean (Fig. 5C) compared with soybean. In soybean, CMA increased in both Ct and De plants at 10 and 15 DAT, and the value was approx. 4 times higher in De plants than in Ct plants at 15 DAT. CMA was highly correlated with sLMA among all the manipulations in soybean (Supplementary Data Fig. S5A), French bean (Supplementary Data Fig. S5B) and azuki bean (Supplementary Data Fig. S5C). CMA was clearly positively correlated with TNC in azuki bean among all the manipulations (Supplementary Data Fig. S5F), whereas there was no clear correlation between CMA and TNC in French bean (Supplementary Data Fig. S5E). In soybean, CMA in De and LNDe plants was clearly higher than that in Ct, LN and HL plants even though those plants had the same levels of TNC (Supplementary Data Fig. S5D).

TABLE 1. *Physiological traits in the leaves of soybean, French bean and azuki bean before and after the experimental treatments.*

		Ct_0	6 DAT					10 DAT			15 DAT	
			Ct	De	LN	LNDe	HL	Ct	De		Ct	De
g_s (mol m^{-2} s^{-1})	Soybean	0.42 ± 0.07	0.47 ± 0.15*	0.21 ± 0.07*	0.25 ± 0.10*	0.21 ± 0.06n.s.	0.44 ± 0.12n.s.	0.24 ± 0.04	0.27 ± 0.07n.s.		0.23 ± 0.07	0.29 ± 0.19
	French bean	0.51 ± 0.24	0.76 ± 0.15*	0.38 ± 0.08n.s.	0.63 ± 0.31*	0.29 ± 0.08*	0.37 ± 0.08					
	Azuki bean	0.96 ± 0.25	0.66 ± 0.34n.s.	0.80 ± 0.46*	0.21 ± 0.14n.s.	0.30 ± 0.17n.s.	0.56 ± 0.31					
C_i	Soybean	312.9 ± 17.1	296.3 ± 32.2*	251 ± 27.7n.s.	282.6 ± 31.4*	264.0 ± 26.9n.s.	298.9 ± 13.5n.s.	278.6 ± 9.8	280.2 ± 16.4n.s.		298.7 ± 10.8	282.7 ± 46.7
	French bean	296.6 ± 32.1	336.8 ± 9.1*	309.2 ± 14.7n.s.	353.3 ± 13.5*	313.4 ± 18.9n.s.	323.4 ± 14.3					
	Azuki bean	341.6 ± 13.4	329.6 ± 24.2n.s.	331.7 ± 26.4n.s.	296.3 ± 46.9n.s.	307.3 ± 40.8n.s.	324.8 ± 35.7					
N_{area} (g N m^{-2})	Soybean	1.41 ± 0.11	1.59 ± 0.10*	2.51 ± 0.27*	1.07 ± 0.14n.s.	1.64 ± 0.33n.s.	1.80 ± 0.13**	1.37 ± 0.11	3.32 ± 0.27**		1.13 ± 0.11	3.71 ± 0.09
	French bean	1.83 ± 0.13	1.64 ± 0.20*	2.20 ± 0.32*	1.07 ± 0.18*	1.30 ± 0.13*	2.28 ± 0.37					
	Azuki bean	1.64 ± 0.10	1.99 ± 0.16*	2.55 ± 0.19*	0.96 ± 0.10*	1.30 ± 0.16*	2.49 ± 0.21					
Rubisco content (g m^{-2})	Soybean	1.87 ± 0.41	1.90 ± 0.49	2.96 ± 0.45	1.35 ± 0.21*	2.21 ± 0.49n.s.	2.39 ± 0.16*	1.90 ± 0.14	3.28 ± 0.48**		1.18 ± 0.21	3.74 ± 0.55
	French bean	2.64 ± 0.35	2.15 ± 0.17n.s.	1.90 ± 0.46*	1.19 ± 0.57*	1.36 ± 0.17n.s.	1.87 ± 0.53					
	Azuki bean	2.87 ± 0.20	2.39 ± 0.34*	3.15 ± 0.22*	1.12 ± 0.20*	1.73 ± 0.19*	2.94 ± 0.29					

Abbreviations are as in Fig. 1. Stomatal conductance (g_s), leaf nitrogen content per area (N_{area}) and Rubisco content of Ct, De, LN, LNDe and HL in all the species were obtained at 6 days after the treatments (DAT), and those of Ct and De in soybean were also obtained at 10 and 15 DAT.

Values are means ± s.d. (n = 6–12). Values of Ct were compared with those of De, LN, LNDe and HL by Dunnett's test (*P < 0.05, n.s. P > 0.05) following ANOVA. Values of Ct and De in soybean at 10 and 15 DAT were compared by Student's t-test (**P < 0.01, *P < 0.05, n.s. P > 0.05).

Leaf anatomical traits were also affected by the short-term manipulative treatments (Table 2; Supplementary Data Figs S6 and S7). Compared with Ct plants, the primary leaves were 20 % thicker in De plants in soybean, 25 % thinner in LN plants in French bean and 20 % thicker in De plants in azuki bean. The intercellular air space was smaller in De plants in soybean, in LN and HL plants in French bean, and did not differ in azuki bean. The average mesophyll cell size on a cross-sectional area basis was 35 % larger in De plants and 20 % smaller in LN plants in soybean, and 20 % larger in De plants in azuki bean, but the size did not differ in French bean. In soybean, these anatomical traits were significantly different between Ct and De plants at 15 DAT.

δ^{13}C values of the leaves and cell walls that would reflect stomatal and mesophyll conductance differed significantly among the manipulations in soybean, whereas they did not differ significantly among the manipulations in French bean or azuki bean (Fig. 6). Leaf δ^{13}C was significantly higher in De, LNDe and HL plants than in Ct plants in soybean (Fig. 6A). Although cell wall δ^{13}C was not significantly different among the manipulations at 6 DAT, itwas significantly higher in De plants than in Ct plants at 10 and 15 DAT (Fig. 6D). In soybean, the difference between leaf δ^{13}C of Ct plants and De plants was greater at 10 and 15 DAT.

DISCUSSION

Co-ordinated and unco-ordinated changes in leaf traits in response to TNC accumulation

Our present study showed that short-term manipulative treatments can cause marked differences in physiological and morphological traits of the primary leaves in three legume species.

Although we expected negative correlations between A_{max}, Rubisco content and TNC or sucrose content, such strong correlations were found only in French bean (Fig. 3B, E; Supplementary data Fig. S1B). These data indicate that the accumulation of sugars or starch is not a prerequisite for the downregulation of photosynthesis even among legume species having similar leaf morphology. In soybean, the increase in TNC (Fig. 1A) and the decrease in A_{max} (Fig. 2A), N_{area} and Rubisco content (Table 1) in Ct plants at 10 and 15 DAT is likely to be related to leaf senescence due to remobilization of nitrogenous compounds to new leaves (Supplementary Data Fig. S4A) (Ludewig and Sonnewald, 2000). Therefore, the observed decrease in A_{max} cannot be explained by short-term changes in sink–source balance. The clear positive relationships between A_{max} and g_s in soybean and azuki bean (Supplementary Data Fig. S2A, C) suggest that the decrease in the photosynthetic rate was not caused by TNC but rather mainly by stomatal closure and the corresponding decrease in C_i, especially in soybean.

It is reported that sucrose feeding simultaneously caused an increase in TNC and a decrease in the photosynthetic capacity for a short time in French bean (Araya *et al.*, 2006, 2010). Nakano *et al.* (2000) also reported a close relationship between A_{max} and TNC in French bean, where they showed that the photosynthetic capacity was recovered when sucrose and starch were decreased by the shade treatments. Ono *et al.* (2001) also

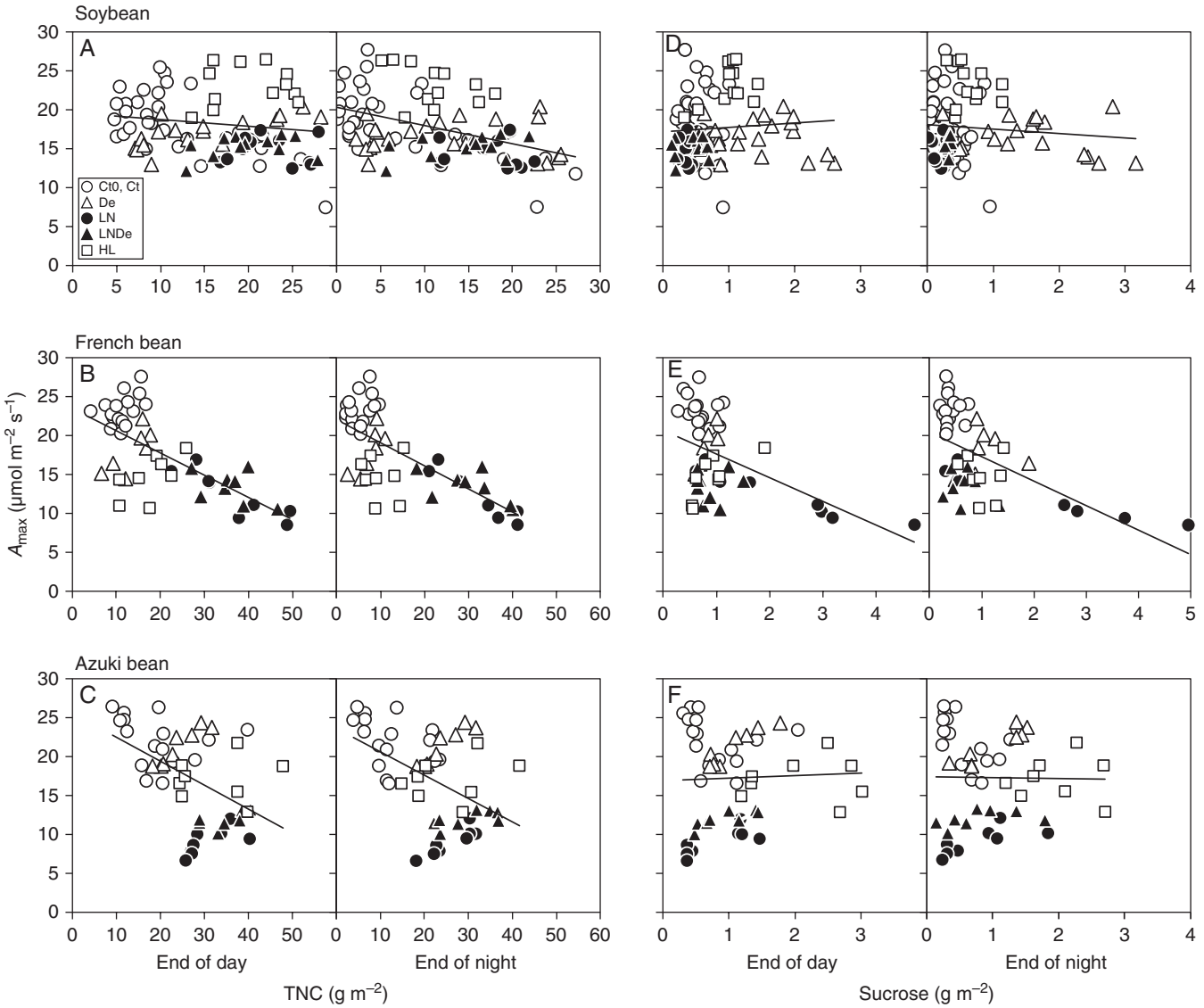

FIG. 3. Relationships between maximum photosynthetic rate (A_{max}) and total non-structural carbohydrates (TNC), and those between A_{max} and sucrose at the end of the day and night in the leaves of soybean (A, D), French bean (B, E) and azuki bean (C, F). Abbreviations are as in Fig. 1. Values of Ct, De, LN, LNDe and HL in all the species were obtained at 6 days after the treatments (DAT), and those of Ct and De in soybean were also obtained at 10 and 15 DAT. Solid lines are regression lines for all the plants. Values of R^2 are (A) 0.03 and 0.20, (B) 0.49 and 0.53, (C) 0.25 and 0.25, (D) 0.01 and 0.01, (E) 0.25 and 0.32, and (F) 0.00 and 0.00.

showed that the Rubisco content was maintained in the first leaves of *Helianthus annuus* in response to a decrease in sucrose content when upper leaves were manipulatively shaded. These results and our present results, where A_{max} and Rubisco content were strongly downregulated with the increase in TNC only in French bean, suggest that French bean is a TNC-sensitive species which regulates photosynthetic characteristics physiologically, whereas soybean and azuki bean seemed to be TNC-insensitive species.

Other previous studies also suggest that the carbohydrate sensitivity varies greatly among plant species. Other TNC-sensitive species include *Chenopodium rubrum*, *Solanum tuberosum*, *Spinacia oleracea* (Krapp *et al.*, 1993; Krapp and Stitt, 1995) and *Saccharum officinale* (Lobo *et al.*, 2015). Meanwhile, TNC-insensitive species are also present. For example, starch excess mutants of *Arabidopsis thaliana* did not show the photosynthetic downregulation even at elevated CO_2 conditions (Y. Mizokami *et al.*, pers. comm.). Large amounts of TNC in the source leaves also did not affect photosynthetic capacity in reciprocally grafted *R. sativus* (Sugiura *et al.*, 2015, 2017).

Differences in physiological changes between TNC-sensitive and TNC-insensitive species

To explain the difference in carbohydrate sensitivity among these species, we carefully investigated physiological changes in response to the manipulative treatments. We found marked interspecific differences in the relationships among A_{max}, N_{area} and Rubisco content (Fig. 4). N_{area} increased in De plants in all the legume plants, because new leaves did not develop, and

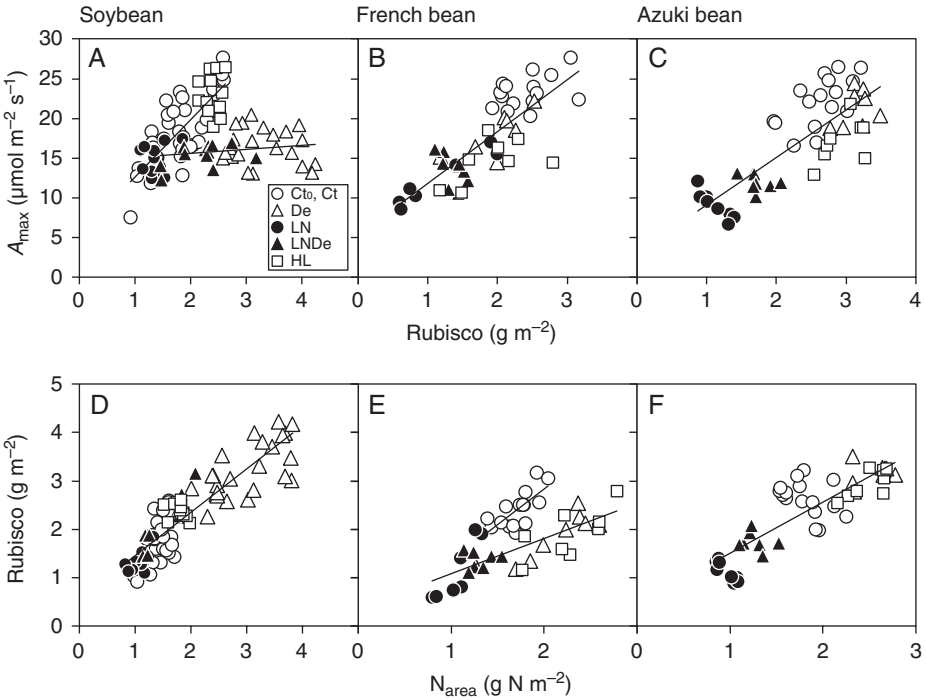

FIG. 4. Relationships between maximum photosynthetic rate (A_{max}) and Rubisco contents, and those between Rubisco content and leaf nitrogen content per area (N_{area}) in the leaves of soybean (A, D), French bean (B, E) and azuki bean (C, F). Abbreviations are as in Fig. 1. Values of Ct, De, LN, LNDe and HL in all the species were obtained at 6 days after the treatments (DAT), and those of Ct and De in soybean were also obtained at 10 and 15 DAT. Solid lines are regression lines for Ct_0, Ct, LN and HL, and for De and LNDe in (A), for all the plants in (B, C, D, F), and for Ct_0 and Ct, and for De, LN, LNDe and HL in (E). Values of R^2 are (A) 0.65 and 0.04, (B) 0.66, (C) 0.64, (D) 0.79, (E) 0.51, 0.64 and (F) 0.71.

because root-derived nitrogen was transported to the primary leaves continuously after defoliation (Table 1). HL plants also showed higher N_{area} compared with Ct plants, implying that root-derived nitrogen was transported to the primary leaves under high light. Nonetheless, in French bean, a strong negative correlation between A_{max} and TNC was found, and the ratio of Rubisco to N_{area} was much lower in the manipulated plants compared with Ct plants (Fig. 4E). Therefore, it is likely that Rubisco content was selectively decreased in responding to the changes in sink–source balance in French bean. A previous study (Nakano *et al.*, 1998) and our present results strongly suggested that such a selective decrease in Rubisco occurs in French bean. On the other hand, neither soybean nor azuki bean showed the selective decrease in Rubisco since the ratio of Rubisco to N_{area} was kept almost constant (Fig. 4D, F). Thus, we propose that TNC-sensitive species actively downregulated photosynthetic capacity in response to sugar accumulation by decreasing the amount of Rubisco, as shown by the clear positive correlation between A_{max} and Rubisco content (Fig. 4B).

Despite the fact that De and LNDe plants showed higher N_{area} and Rubisco content, A_{max} in De and LNDe plants was not positively correlated with Rubisco content in soybean (Fig. 4A). This was partly explained by the lower stomatal conductance, which consequently led to lower C_i (Table 1; Supplementary Data Fig. S2A). Changes in Rubisco activation levels would also contribute to the decrease in A_{max} in those legume species. Kasai (2008) showed thast the sink–source imbalance did not cause the decrease in Rubisco content but rather the decrease in the Rubisco activation level in *G. max*. Although we did not measure the Rubisco activation level in the present study, further studies investigating interspecific differences in the Rubisco activation rate responding to sink–source imbalance are expected.

With the present data, we can further argue that the lower A_{max} was due to a lower chloroplast CO_2 concentration since we found significant changes in morphological traits and $\delta^{13}C$ in these plants (see below). In azuki bean, which also seemed to be a TNC-insensitive species, neither A_{max} nor g_s decreased in De plants, which was in contrast to soybean (Fig. 4C). The different responses between soybean and azuki bean might be related to a higher TNC storage capacity of mesophyll cells in azuki bean (Fig. 1C) than in soybean (Fig. 1A). Instead, A_{max} and A_{growth} were more decreased in LN and LNDe plants in azuki bean than in soybean and French bean (Fig. 2C). This might be because nitrogen and Rubisco were quickly resorbed from source leaves responding to the low N manipulation irrespective of TNC accumulation (Table 1). Thus, it can be argued that azuki bean is the most nitrogen-sensitive species.

Downregulation of photosynthesis by changes in leaf anatomical traits

We also analysed the anatomical characteristics of TNC-insensitive species. It was found that cell wall content significantly increased in all the manipulated plants in soybean and azuki bean (Fig. 5A, C), whereas it did not increase so much in the TNC-sensitive French bean (Fig. 5B). In soybean, since CMA and TNC continued to increase in De

TABLE 2. *Morphological and anatomical traits in the leaves of soybean, French bean and azuki bean before and after the experimental treatments*

		Ct_0	6 DAT					10 DAT		15 DAT	
			Ct	De	LN	LNDe	HL	Ct	De	Ct	De
LMA (g m^{-2})	Soybean	33.8 ± 2.5	42.9 ± 6.0*	61.7 ± 8.5*	57.6 ± 6.7*	66.8 ± 8.5*	63.4 ± 7.7**	53.3 ± 6.4	89.3 ± 4.3**	65.7 ± 7.5	124.1 ± 11.3
	French bean	45.4 ±	4.0	42.3 ± 8.3n.s.	51.8 ± 12.2*	69.9 ± 14.7*	74.6 ± 9.6*	64.0 ± 5.9			
	Azuki bean	44.5 ± 5.7	62.1 ± 13.1n.s.	67.5 ± 7.5n.s.	65.8 ± 8.1n.s.	75.8 ± 8.3*	81.4 ± 15.2				
sLMA (g m^{-2})	Soybean	27.0 ± 1.3	33.0 ± 2.9*	51.7 ± 5.8*	35.0 ± 3.8*	46.8 ± 4.6*	43.3 ± 4.3**	35.9 ± 3.3	69.7 ± 3.3**	38.9 ± 3.1	88.0 ± 5.2
	French bean	33.9 ± 1.8	31.6 ± 5.7*	39.6 ± 7.1n.s.	33.7 ± 5.2n.s.	38.6 ± 6.2*	46.3 ± 3.0				
	Azuki bean	30.2 ± 2.3	37.7 ± 5.3n.s.	43.3 ± 3.3n.s.	35.1 ± 3.0n.s.	40.4 ± 3.8*	48.5 ± 7.0				
Leaf thickness (μm)	Soybean	197 ± 15	283 ± 31*	344 ± 32n.s.	248 ± 27n.s.	296 ± 15n.s.	295 ± 30n.s.	313 ± 47	341 ± 36**	273 ± 21	416 ± 21
	French bean	253 ± 42	379 ± 60n.s.	425 ± 67*	287 ± 28n.s.	326 ± 94n.s.	420 ± 49				
	Azuki bean	230 ± 28	247 ± 37*	301 ± 18n.s.	224 ± 20n.s.	235 ± 25n.s.	253 ± 33				
Intercellular air space (%)	Soybean	33.0 ± 4.1	31.3 ± 3.1*	25.3 ± 2.8n.s.	31.2 ± 6.4n.s.	30.7 ± 3.5n.s.	28.2 ± 5.3n.s.	29.0 ± 5.1	27.4 ± 5.2**	31.5 ± 5.5	25.3 ± 3.6
	French bean	25.6 ± 3.8	30.7 ± 2.8n.s.	31.1 ± 4.1*	24.4 ± 5.5n.s.	28.0 ± 1.9*	22.5 ± 4.8	21.9 ± 2.8			
	Azuki bean	23.0 ± 4.8	24.4 ±	4.6n.s.	25.6 ± 3.6n.s.	22.5 ± 3.0n.s.	20.1 ± 4.6n.s.				
Average mesophyl cell size (μm^2)	Soybean	450 ± 54	671 ± 93*	917 ± 137*	533 ± 82n.s.	688 ± 72n.s.	668 ± 54n.s.	690 ± 211	810 ± 145**	652 ± 88	1178 ± 203
	French bean	503 ± 136	906 ± 156n.s.	1081 ± 212n.s.	716 ± 130n.s.	735 ± 220n.s.	1081 ± 87				
	Azuki bean	446 ± 49	575 ± 35*	699 ± 81n.s.	497 ± 52n.s.	551 ± 58n.s.	585 ± 84				

Abbreviations are as in Fig. 1. Leaf mass per area (LMA), structural LMA (sLMA), leaf thickness, intercellular air space and average mesophyll cell size of Ct, De, LN, LNDe and HL in all the species were obtained at 6 days after the treatments (DAT), and those of Ct and De in soybean were also obtained at 10 and 15 DAT.

Values are means ± s.d. (n = 6–12). Values of Ct were compared with those of De, LN, LNDe and HL by Dunnett's test (*$P < 0.05$, n.s. $P > 0.05$) following ANOVA. Values of Ct and De in soybean at 10 and 15 DAT were compared by Student's t-test (**$P < 0.01$, *$P < 0.05$, n.s. $P > 0.05$).

plants even at 10 and 15 DAT, not only TNC but also structural carbohydrates including cell wall materials accumulated in response to the decrease in the sink–source ratio (Table 2). Positive correlations between CMA and TNC in soybean (Supplementary Data Fig. S5D) and azuki bean (Supplementary Data Fig. S5F) suggest that the increase in CMA caused by TNC accumulation is a characteristic feature of TNC-insensitive species, as observed in radish plants (Sugiura *et al.* 2017).

The increase in CMA in De plants was not due mainly to the increase in leaf lamina thickness but rather to the increase in cell wall thickness. This is because leaf lamina thickness increased by only 20 % (Table 2; Supplementary Data Fig. S7) whereas CMA was almost four times higher in De plants than in Ct plants at 15 DAT. Thicker cell walls could cause a decrease in the mesophyll conductance, which is a measure of CO_2 diffusivity across the cell wall and plasma membrane (Evans *et al.*, 2009). Consequently, the CO_2 concentration in chloroplasts decreased, leading to an increase in $\delta^{13}C$ through the relative decrease in isotope fractionation of CO_2 by Rubisco. Our present study suggests that the increase in cell wall thickness and the decrease in intercellular air space (Table 2) caused the downregulation of photosynthesis by suppressing CO_2 intake in soybean since both the whole-leaf and cell wall $\delta^{13}C$ values were significantly higher in De and LNDe plants only in soybean (Fig. 6A, D). The decrease in intercellular air space might lead to the decrease in the surface area of chloroplasts facing the intercellular airspaces (Sugiura *et al.*, 2017), which would lead to the decrease in mesophyll conductance (Tholen *et al.*, 2008).

Regardless of some increases in CMA in De plants of French bean and azuki bean, $\delta^{13}C$ of De plants changed little (Figs. 5B, C and 6B, C, E, F). The decrease in Rubisco, which is the main factor of CO_2 isotope fractionation, might also contribute to the modest change in $\delta^{13}C$ in French bean. In azuki bean, little change in g_s caused higher C_i (Table 2), which might cause the modest change in $\delta^{13}C$. In soybean, simultaneous decreases in stomatal conductance and mesophyll conductance due to cell wall thickening would result in a significant decrease in chloroplast CO_2 concentration, which would be responsible for the decrease in the isotope fractionation. The decrease in mesophyll conductance could contribute more to the increase in $\delta^{13}C$ than to the decrease in stomatal conductance at 10 and 15 DAT in soybean. This is because g_s was mostly the same in Ct and De plants of soybean (Table 1) whereas CMA was much larger in De plants than in Ct plants of soybean at 10 and 15 DAT (Fig. 5A). The observed increase in the cell wall mass in response to sugar accumulation was also observed in *R. sativus* which is also a TNC-insensitive species (Sugiura *et al.*, 2015, 2017). There were weak negative correlations between A_{max} and TNC, and the ratio of Rubisco to N_{area} was also kept constant in *R. sativus* plants.

These results suggest that there are at least two different ways to downregulate photosynthesis in response to the accumulation of TNC which is dependent on a change in sink–source balance. One is by decreasing Rubisco, the key photosynthetic enzyme, as shown in French bean, and the other is by decreasing stomatal conductance and mesophyll conductance which led to a reduction in CO_2, a substrate for Rubisco, as shown in radish plants and soybean. To what extent mesophyll conductance

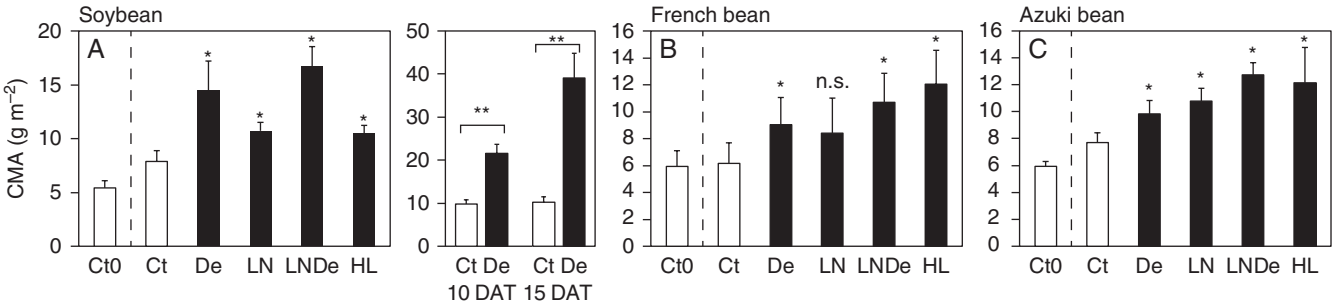

FIG. 5. Cell wall mass per area (CMA) in the leaves of soybean (A), French bean (B) and azuki bean (C) before and after the experimental treatments. Abbreviations on the *x*-axes are as in Fig. 1. Values of Ct, De, LN, LNDe and HL in all the species were obtained at 6 days after the treatments (DAT), and those of Ct and De in soybean were also obtained at 10 and 15 DAT. White bars represent values at the end of the day, and black bars represent values at the end of the night. Values are means ± s.d. (n = 6–12). Values of Ct were compared with those of De, LN, LNDe and HL by Dunnett's test (*P < 0.05, n.s. P > 0.05) following ANOVA. Values of Ct and De in soybean at 10 and 15 DAT were compared by Student's t-test (**P < 0.01, *P < 0.05, n.s. P > 0.05).

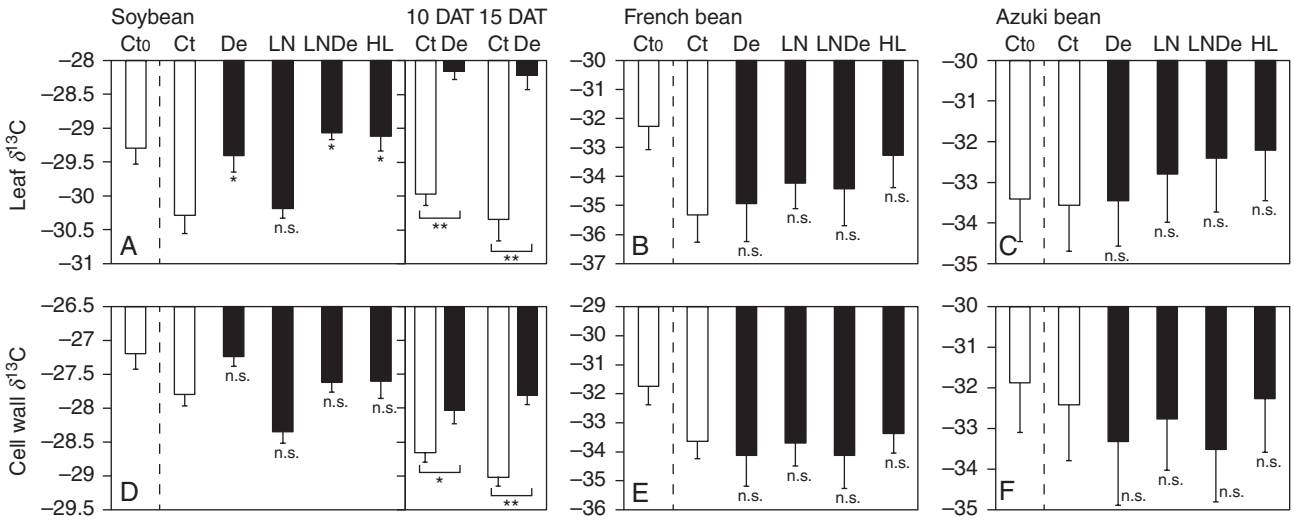

FIG. 6. Leaf δ^{13}C and cell wall δ^{13}C in the leaves of soybean (A), French bean (B) and azuki bean (C) before and after the experimental treatments. Abbreviations on the *x*-axes are as in Fig. 1. Values of Ct, De, LN, LNDe and HL in all the species were obtained at 6 days after the treatments (DAT), and those of Ct and De in soybean were also obtained at 10 and 15 DAT. Values are means ± s.d. (n = 6–12). Values of Ct were compared with those of De, LN, LNDe and HL by Dunnett's test (*P < 0.05, n.s. P > 0.05) following ANOVA. Values of Ct and De in soybean at 10 and 15 DAT were compared by Student's t-test (**P < 0.01, *P < 0.05, n.s. P > 0.05).

changes in response to changes in cell wall thickness caused by sink–source imbalance will be an issue to be addressed in the future. In addition, it is still unclear if these findings were applicable to trifoliate leaves and to other cultivars of the same species. Therefore, leaf developmental stage and intracultivar variation in the observed traits are also important issues to be considered.

In the present experiments, we could not evaluate how the downregulation of photosynthesis influences plant fitness. To show its ecological significance, it is necessary to compare long-term growth and reproductive success in plant species that show or do not show downregulation of photosynthesis. Another way is to create French bean mutants, which do not respond to the accumulation of TNC, and evaluate their growth traits. Krapp and Stitt (1995) showed that the downregulated photosynthetic capacity with an increase in TNC in cold-girdled spinach leaves was upregulated with a decrease in TNC after the cold-girdled system was detached. Thus, it is possible that plastic regulation of photosynthetic capacity in response to abiotic stresses is an important trait especially under natural environments where environmental factors fluctuate greatly.

CONCLUSION

In this study, we investigated the effects of changes in TNC caused by sink–source imbalance on the photosynthetic traits of source leaves and whole-plant growth in soybean, French bean and azuki bean. Only in French bean was a selective decrease in Rubisco content and a downregulation of maximum photosynthesis linked to an increase in TNC. Therefore, we suggest that French bean is a TNC-sensitive species. In contrast, in soybean and azuki bean, maximum photosynthetic rates were not severely downregulated in response to the accumulation of TNC, and N_{area}–Rubisco relationships were constant. In soybean, the defoliation treatment caused the increase in the cell wall content, indicating that not only the decrease in g_s but also the decrease in mesophyll conductance could cause the

downregulation of photosynthesis in TNC-insensitive species. This was supported by the increase in $\delta^{13}C$ in De and LNDe plants of soybean. The present results suggest that the downregulation of photosynthesis is caused not only biochemically, such as by a decrease in photosynthetic proteins, but also morphologically, such as by an increase in cell wall thickness and changes in leaf anatomy.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: abbreviations of parameters and manipulative treatments. Figure S1: relationships between Rubisco content and total non-structural carbohydrates at the end of the night in the leaves of soybean, French bean and azuki bean. Figure S2: relationships between maximum photosynthetic rate and stomatal conductance in the leaves of soybean, French bean and azuki bean. Figure S3: relationships between dark respiration rate and leaf nitrogen content per area, and those between R_d and total non-structural carbohydrates at the end of the night in the leaves of soybean, French bean and azuki bean. Figure S4: dry mass of leaves, stems and roots in soybean, French bean and azuki bean before and after the experimental treatments. Figure S5: relationship between cell wall mass per area (CMA) and structural leaf mass per area, and those between CMA and total non-structural carbohydrates at the end of the night in soybean, French bean and azuki bean. Figure S6: transverse sections of the primary leaves in soybean, French bean and azuki bean at 6 d after the treatments. Figure S7: transverse sections of the primary leaves in soybean at 10 and 15 d after the treatments.

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LITERATURE CITED

Ahmed FE, Hall AE, Madore MA. 1993. Interactive effects of high temperature and elevated carbon dioxide concentration on cowpea [*Vigna unguiculata* (L.) Walp.]. *Plant, Cell & Environment* **16:** 835–842.

Ainsworth EA, Long SP. 2005. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytologist* **165:** 351–372.

Ainsworth EA, Rogers A. 2007. The response of photosynthesis and stomatal conductance to rising [CO_2]: mechanisms and environmental interactions. *Plant, Cell & Environment* **30:** 258–270.

Ainsworth EA, Rogers A, Nelson R, Long SP. 2004. Testing the source–sink hypothesis of down-regulation of photosynthesis in elevated [CO_2] in the field with single gene substitutions in *Glycine max*. *Agricultural and Forest Meteorology* **122:** 85–94.

Araya T, Noguchi K, Terashima I. 2006. Effects of carbohydrate accumulation on photosynthesis differ between sink and source leaves of *Phaseolus vulgaris* L. *Plant & Cell Physiology* **47:** 644–652.

Araya T, Noguchi K, Terashima I. 2010. Effect of nitrogen nutrition on the carbohydrate repression of photosynthesis in leaves of *Phaseolus vulgaris* L. *Journal of Plant Research* **123:** 371–379.

Arp WJ. 1991. Effects of source–sink relations on photosynthetic acclimation to elevated CO_2. *Plant, Cell & Environment* **14:** 869–875.

Bertin N, Tchamitchian M, Baldet P, Devaux C, Brunel B, Gary C. 1999. Contribution of carbohydrate pools to the variations in leaf mass per area within a tomato plant. *New Phytologist* **143:** 53–61.

Evans JR, Caemmerer SV, Setchell BA, Hudson GS. 1994. The relationship between CO_2 transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. *Functional Plant Biology* **21:** 475–495.

Evans JR, Kaldenhoff R, Genty B, Terashima I. 2009. Resistances along the CO_2 diffusion pathway inside leaves. *Journal of Experimental Botany* **60:** 2235–2248.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Biology* **40:** 503–537.

Goldschmidt EE, Huber SC. 1992. Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose, and hexose sugars. *Plant Physiology* **99:** 1443–1448.

Graham PH, Vance CP. 2003. Legumes: importance and constraints to greater use. *Plant Physiology* **131:** 872–877.

Kasai M. 2008. Regulation of leaf photosynthetic rate correlating with leaf carbohydrate status and activation state of Rubisco under a variety of photosynthetic source/sink balances. *Physiologia Plantarum* **134:** 216–226.

Krapp A, Stitt M. 1995. An evaluation of direct and indirect mechanisms for the 'sink-regulation' of photosynthesis in spinach: changes in gas exchange, carbohydrates, metabolites, enzyme activities and steady-state transcript levels after cold-girdling source leaves. *Planta* **195:** 313–323.

Krapp A, Hofmann B, Schäfer C, Stitt M. 1993. Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism for the sink regulation of photosynthesis? *The Plant Journal* **3:** 817–828.

Leakey ADB, Ainsworth EA, Bernacchi CJ, Rogers A, Long SP, Ort DR. 2009. Elevated CO_2 effects on plant carbon, nitrogen, and water relations: six important lessons from FACE. *Journal of Experimental Botany* **60:** 2859–2876.

Lobo AKM, de Oliveira Martins M, Neto MCL, Machado EC, Ribeiro RV, Silveira JAG. 2015. Exogenous sucrose supply changes sugar metabolism and reduces photosynthesis of sugarcane through the down-regulation of Rubisco abundance and activity. *Journal of Plant Physiology* **179:** 113–121.

Long SP, Ainsworth EA, Rogers A, Ort DR. 2004. Rising atmospheric carbon dioxide: plants FACE the future. *Annual Review of Plant Biology* **55:** 591–628.

Ludewig F, Sonnewald U. 2000. High CO_2-mediated down-regulation of photosynthetic gene transcripts is caused by accelerated leaf senescence rather than sugar accumulation. *FEBS Letters* **479:** 19–24.

Mizokami Y, Noguchi K, Kojima M, Sakakibara H, Terashima I. 2015. Mesophyll conductance decreases in the wild type but not in an ABA-deficient mutant (*aba1*) of *Nicotiana plumbaginifolia* under drought conditions. *Plant, Cell & Environment* **38:** 388–398.

Nakano H, Makino A, Mae T. 1998. The responses of Rubisco protein to long-term exposure to elevated CO_2 in rice and bean leaves. *Photosynthesis: Mechanisms and Effects* **5:** 3391–3394.

Nakano H, Muramatsu S, Makino A, Mae T. 2000. Relationship between the suppression of photosynthesis and starch accumulation in the pod-removed bean. *Functional Plant Biology* **27:** 167–173.

Ono K, Nishi Y, Watanabe A, Terashima I. 2001. Possible mechanisms of adaptive leaf senescence. *Plant Biology* **3:** 234–243.

Ruiz-Vera UM, De Souza AP, Long SP, Ort DR. 2017. The role of sink strength and nitrogen availability in the down-regulation of photosynthetic capacity in field-grown *Nicotiana tabacum* L. at elevated CO_2 concentration. *Frontiers in Plant Science* **8:** 998. doi: 10.3389/fpls.2017.00998.

Sage RF, Sharkey TD, Seemann JR. 1989. Acclimation of photosynthesis to elevated CO_2 in five C_3 species. *Plant Physiology* **89:** 590–596.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9:** 671–675.

Sheen J. 1994. Feedback control of gene expression. *Photosynthesis Research* **39:** 427–438.

Stitt M, Krapp A. 1999. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. *Plant, Cell & Environment* **22:** 583–621.

Sugiura D, Betsuyaku E, Terashima I. 2015. Manipulation of the hypocotyl sink activity by reciprocal grafting of two *Raphanus sativus* varieties: its effects on morphological and physiological traits of source leaves and whole-plant growth. *Plant, Cell & Environment* **38:** 2629–2640.

Sugiura D, Watanabe CKA, Betsuyaku E, Terashima I. 2017. Sink–source balance and down-regulation of photosynthesis in *Raphanus sativus*: effects of grafting, N and CO_2. *Plant & Cell Physiology* **58:** 2043–2056.

Tholen D, Boom C, Noguchi K, Ueda S, Katase T, Terashima I. 2008. The chloroplast avoidance response decreases internal conductance to CO_2 diffusion in *Arabidopsis thaliana* leaves. *Plant, Cell & Environment* **31:** 1688–1700.