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Physiological traits associated with recent advances in yield of Chinese wheat

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(Rasgos fisiológicos asociados con los recientes avances en el rendimiento del trigo chino)

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CHAPTER 4

Wheat ears: an active carbon/nitrogen sink-source organ[a]

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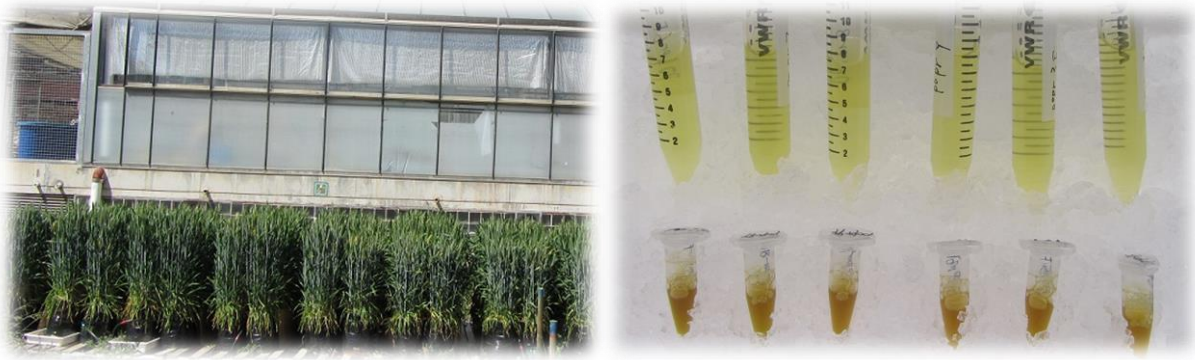

5 Chinese genotypes labeled by ^{15}N planted in pots in Faculty Biology, UB (left), and Rubisco fractionation samples (right). Photo taken by B. Zhou at Barcelona and Navarra, respectively, 2013, Spain.

Abstract:

Despite increasing evidence in wheat that ears are an important source of carbon photoassimilates during grain filling, their role as a source of N has barely been studied. Five wheat genotypes were labeled with ^{15}N enriched (0.5 %) nutrient solution during the pre/post-anthesis periods. Ear and flag leaf relative carbon photoassimilate contributions were analyzed via gas exchange. N remobilization and absorption were estimated via the nitrogen isotope composition ($\delta^{15}N$) of total organic matter and Rubisco. Gas exchange analyses showed that although C lost through respiration was high in ears, CO_2 fixed by ears represented a relevant source of C to sustain grain filling. ^{15}N labeling revealed that while Rubisco-derived N remobilization was lower in flag leaves than in glumes in low grain-yielding genotypes, in high yielding genotypes the opposite trend was observed. Therefore, the ears of high yielding genotypes had the highest N remobilization capacity, highlighting the active role of ears in grain filling. Such findings underscore the significance of characterizing spike physiology and the importance it may have for research supporting wheat breeding programs.

Keywords: carbon accumulation, ear, grain yield, nitrogen accumulation, Rubisco.

1. **Introduction**

The world's nitrogen fertilizer demand is expected to increase from a total of 105.3 million tonnes in 2011 to 112.9 million tonnes in 2015 with an annual growth of 1.7 percent (Food and Agriculture Organization, FAO 2011). Moreover, it is expected that by 2050, fertilizer application will increase to 240 million tonnes (Masclaux-Daubresse et al. 2008). In the case of China, modern winter wheat (*Triticum aestivum* L) cultivars are being currently fertilized with a large amount of N; for example, in central and north China more than 300 kg ha^{-1} of N is normally applied on-farm, and the trend for increased fertilizer input will be maintained in the future (Cui et al. 2008; Cui et al. 2010). However, despite its importance, N use efficiency (NUE) in developed economies is estimated to be only around 33% (Raun and Johnson 1999). Besides its economic impact on farmers' economies, low NUE has a dramatic consequence on the environment and population health (Duan et al. 2014). Therefore, within this context, optimization of NUE is one of the main challenges that breeders are working on. Selection of genotypes that absorb and/or metabolize available N resources more efficiently represents a major goal in plant breeding programs (Kichey et al. 2007).

Improving wheat performance and NUE requires improved knowledge of yield determining factors (Masclaux-Daubresse et al. 2008, 2010). Grain filling, and by extension grain yield, are mainly sustained by assimilation and management of C and N compounds. The selection of genotypes with optimized C/N assimilation and further remobilization to sustain grain filling has been linked to enhanced crop yield (Richards 2000). Leaf carbohydrate accumulation is determined by the C source (photosynthesis) and sink balance (i.e. growth, respiration, and partitioning to other organs) (Aranjuelo et al. 2011). Carbon required to sustain grain filling is mostly provided by flag leaf photosynthesis (Evans 1983), remobilization of C assimilated before anthesis (mainly stored in the internodes; Gebbing and Schnyder, 1999) and ear photosynthesis (Tambussi et al. 2007). Differences in the ear contribution to grain

filling have been explained as being caused by environmental and genetic factors (Araus et al. 2003; Tambussi et al. 2005; Sánchez-Bragado et al. 2014a,b). Classic approaches for analyzing the contribution of different wheat organs towards grain filling are based on preventing photosynthesis in different organs (ear, flag leaf, shoot) via a number of methods (Aggarwal et al. 1990; Ahmadi et al. 2009). These methods have been subjected to criticism because such manipulation might also alter the rate of photosynthesis in the "unaltered" organs (Eyles et al. 2013). Moreover, as observed by Plaut et al. (2004), remobilization of pre-anthesis photoassimilates is also affected by these artificial methods. Consequently, information derived from such studies should be regarded with caution.

Photosynthetic improvement has been described as one of the main avenues for enhancing yield potential (Parry et al. 2011; Reynolds et al. 2011). Although traditionally the flag leaf has been assigned as the main photosynthetic organ supporting grain filling, it is also true that several studies conducted in the past (Richards 2000; Xiao et al. 2012) have not detected any correlation between flag leaf photosynthetic rates and grain yield. Moreover, recent findings suggest that ear photosynthetic activity also represents a key source of photoassimilates that sustain grain filling (Tambussi et al. 2005; 2007; Zhou et al. 2014; Sánchez-Bragado et al. 2014a, b).

The quantity of nitrogen present in grains is determined using several steps including uptake, assimilation, translocation, recycling and remobilization (Masclaux-Daubresse et al. 2010). Kernel N content is largely conditioned by the amount of N remobilized from pre-anthesis (N_{rem}) reserves (accumulated in the shoot), which is supplemented by the amount of N taken up during post-anthesis (N_{abs}) (Kichey et al. 2007). Leaves are classically considered as the main N contributor to kernels due to their large protein content. The fact that Rubisco might represent up to 50 % of the total soluble protein (TSP) and 25 % of the nitrogen (N) content in leaves (Parry et al. 2003, Aranjuelo et al. 2013a) implies that it can be considered as a major

N storage form (Millard 1988). Although Rubisco is mostly known as a key enzyme involved in CO_2 assimilation during the Calvin cycle, comparatively little is known about its role as a pool of nitrogen storage in leaves (Masclaux-Daubresse et al. 2008).

Remobilization of N from pre-anthesis reserves and assimilation of N during the post-anthesis period are generally estimated by the 'apparent remobilization' method; a method that is subject to large experimental errors (Masclaux-Daubresse et al. 2008). This method is based on the determination of differences in N content during the pre/post-anthesis period in different plant organs. Although useful in the past, subsequent studies have concluded that this method does not enable the identification of N sources such as N uptake from soil and remobilization from senescent organs (Masclaux-Daubresse et al. 2008). Moreover, N volatilization through stomata and root exudation are not considered. Labeling with the stable ^{15}N isotope has been proposed as a method to analyze N remobilization (Cliquet et al. 1990). Previous studies (Rossato et al. 2002; Malagoli et al. 2005) conducted with ^{15}N as a tracer in oilseed rape and legumes have revealed that N uptake and N_2 fixation decline during seed filling as plants mature. Moreover, the same authors observed that because newly acquired N is insufficient to sustain seed requirements, previously assimilated N must be remobilized. In a different study, Kichey et al. (2007) described that both N_{abs} and N_{rem} can be estimated with great accuracy and reproducibility. Although a large majority of these studies have determined ^{15}N at the total organic matter (TOM) level, it should be noted that development of new protocols during recent decades has enabled the purification and subsequent isotopic analysis of individual metabolites like amino acids (Gauthier et al. 2013, Molero et al. 2011, 2014), soluble proteins (Aranjuelo et al. 2011, Makino et al. 2011, Gauthier et al. 2013), DNA (Gauthier et al. 2013) and organic acids (Gauthier et al. 2010). However, despite its importance, no direct analyses of the contribution of Rubisco-derived N remobilization towards sustaining grain filling have been undertaken via ^{15}N labeling. Moreover, the role of glumes as an additional source of N for kernel filling has not been considered.

The aim of this study was to determine the role of the ear and the flag leaf during beginning of the pre-anthesis and two weeks post-anthesis stages as major contributors to C and N accumulation in kernels. The C sources that feed grain filling were elucidated through *in vivo* photosynthetic measurements of ears and flag leaves. N accumulation was assessed through ^{15}N labeling from sowing to anthesis (pre-anthesis) and anthesis to maturity (post-anthesis), respectively, and N remobilization was characterized by analyzing TOM and Rubisco $\delta^{15}N$ in the flag leaf, glumes and kernels in whole pre/post-anthesis ^{15}N labeled plants.

2. **Material and Methods**

2.1 Growth conditions

Five commercial Chinese high yielding bread wheat (*Triticum aestivum* L.) cultivars (Yumai 2, Lankao 198, Yumai 20, Zhoumai 22, Zhoumai 18), which are cultivated in Henan Province (China), were selected for our study. Plants were assayed outdoors in the Experimental Field Facilities of the Faculty of Biology, University of Barcelona (Barcelona, Spain), from December 4th, 2012 to June 8th, 2013. Seeds were germinated in Petri dishes for approximately two weeks at 4 ℃ in a cold chamber. After germination, the plants were transferred to specially designed 30 L polyvinyl chloride pots. These pots were filled with a mixed gravel (1:5), sand (2:5) and peat (2:5) (v/v) artificial substrate. Twenty seeds of each genotype were sown (at a density of 400 seeds m^{-2}). The experiment was conducted in a randomized complete block design with 3 blocks in ambient conditions. Each genotype was assayed with 6 pots per block to end up with 90 pots in total (3 blocks x 5 genotypes x 6 pots = 90 pots). The seedlings were drip irrigated with a whole-strength Hoagland solution (Hoagland and Arnon 1950). Approximately 50 L of solution was applied to each pot during the whole planting season. During the weeks of booting, heading, anthesis, and the milk stage, the mean ambient temperatures were 13.2 ℃, 15.5 ℃, 14.2 ℃, and 17.0 ℃, and

the integrated solar irradiation levels were 16.6 MJ m^2, 22.2 MJ m^2, 22.0 MJ m^2, and 21.0 MJ m^2, respectively.

2.2 Pre/post-anthesis ^{15}N labeling procedure and sampling

In order to determine the source of N (N absorbed during pre-anthesis/post-anthesis) that sustained grain filling, ^{15}N labeling was conducted at two different periods: i) from sowing until anthesis and ii) from anthesis until kernel maturity. In both cases, ^{15}N labeling was conducted by replacing N in the Hoagland solution with ^{15}N enriched $K^{15}NO_3$ (10%) and $^{15}NH_4Cl$ (99%). In plants labeled from sowing to anthesis, once the labeling period finished, the substrate was washed with abundant water in order to remove all the ^{15}N remaining in the substrate. Further, these plants were then watered with Hoagland solution until maturity.

Flag leaf and ear samples of plants labeled from plating until the anthesis period were harvested (1) the last day of labeling (pre-anthesis), (2) 14 days after the end of labeling (post-anthesis) and further kernels were harvested at maturity. In the case of plants labeled from anthesis until maturity, harvests were conducted 14 days after anthesis (post-anthesis) and further kernels were collected at maturity.

2.3 Grain yield and yield components

Grain yield determinations correspond to plants harvested at maturity. Before harvest, plant height was measured (length from ground surface to the ear top, excluding awns). Harvested plants were dried in an oven for 48 h at 60 ℃ to determine total aerial biomass. Yield components were determined in 5 plants where harvest index (HI), thousand kernel weight (TKW), ears and kernels per pot, kernels per ear and ear length were determined.

2.4 Gas exchange parameters

Gas exchange determinations were conducted, at the pre-anthesis/post-anthesis stages, in the central segment of flag leaf blades and the entire ears by using a LI-COR 6400 portable photosynthesis system (LICOR Biosciences, Lincoln, Nebraska, USA). Analyses were carried out in fully expanded flag leaves on sunny days, from 10:00-15:00. The lag leaf net assimilation rate (Pn) determinations were conducted with a LICOR 6400-40 leaf chamber connected to a portable infrared gas analyzer. Determinations were conducted in the following conditions: 400 µmol mol^{-1} CO_2, 25 ℃, 50 % relative humidity (RH) and 1200 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The whole ear Pn was measured in a conifer chamber at 1200 µmol m^{-2} s^{-1} PPFD, 25 ℃ and 50% RH. The ear dark respiration rate (Rdark) was measured in the same samples where total ear Pn was determined. For this purpose, before Rdark determinations, plants were dark adapted for 45 min. by covering all the plants with a black sheet until the gas exchange data was stable. Once gas exchange analyses were conducted, the flag leaves were cut and immediately scanned to calculate the leaf area with Digimizer 3.7 (MedCalc Software, Belgium, 2009) image analysis software. Ear area was expressed as the area of the four faces of each ear (excluding awns) calculated by multiplying the length by width of each of the four faces of each ear measured with a ruler. The gross assimilation rate (GAR) was calculated as the Pn plus Rdark. GAR rates were expressed per unit area (µmol CO_2 m^{-2} s^{-1}), and per whole organ (nmol CO_2 s^{-1} ear^{-1}/$flag^{-1}$).

2.5 Rubisco isolation, fractionation and quantification

Four plants were randomly selected from labeled plants during pre-anthesis and also from two weeks after anthesis, and stored at -80 ℃. Aliquots of 1.5 g of frozen leaves and 2.5g of frozen glumes for each sample were ground in a cold mortar in liquid nitrogen. The frozen sample powder was slowly added to 10 mL of 0.1 M sodium phosphate buffer (pH 7.5) with continuous stirring over a period of 15 to 20 min, during which time the temperature was kept below 0 ℃. All sub-sequent steps were carried out at 4 ℃. The extract was filtered through 4 layers of muslin into a small

cold beaker and the filtrates were precipitated with 30% ammonium sulfate prior to centrifugation at 12000 rpm for 10 min. The supernatant was separated and further precipitated with 50% ammonium sulfate under centrifugation at 12000 rpm for another 10 min. The precipitate was dissolved by 0.1 M sodium phosphate buffer and fractionated on an Ultrogel AcA-34 (Pharmacia LKB, France) gel filtration column (10ml, 32 cm length), which was previously equilibrated with the same buffer at 4 ℃. The flow rate of the elution was fixed at 6 ml h^{-1} using 0.1 M sodium phosphate buffer. For each sample, 500 µL fractions were collected by using 16 Eppendorf tubes. The protein content in each fraction was screened by SDS-PAGE (12.5 % polyacrylamide) to confirm the Rubisco fraction. 150 µl of solution was transferred from the Eppendorf containing fraction 8 aliquot into tin capsules and oven-dried at 60^{o}C until the capsules' weights were constant. Capsules were stored until nitrogen isotope signature analysis was carried out.

Following the methods of Makino et al. (1986), Rubisco protein content was determined in the first extract by the Bradford assay (Bio-Rad) and SDS-PAGE (12.5 % polyacrylamide) Gel images were scanned and analyzed using the Typhoon^{TM} Trio Imager (GE Healthcare) densitometer.

2.6 Nitrogen isotope composition ($\delta^{15}N$) and concentration in TOM

The nitrogen ($\delta^{15}N$) isotope compositions and total N content were analyzed on flag leaf and ear samples harvested at pre/post-anthesis as well as in mature kernels. One mg (for kernels and ears) and 0.7 mg (for leaves) of dried ground sample were used for each determination. The total N concentration together with the $^{15}N/^{14}N$ ratios (*R*) of plant material were determined using an elemental analyzer (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen Germany) operating in continuous flow mode at the Scientific Service Facilities of the University of Barcelona (Spain). The amount of ^{15}N atoms in labeling samples was expressed by atom% abundances in

A notation (Robinson, 2001): $A = 100 * {}^{15}\mathrm{N}/({}^{15}\mathrm{N} + {}^{14}\mathrm{N})$, where ^{15}N and ^{14}N are the numbers of ^{15}N and ^{14}N atoms present in a sample, respectively; *A* was used to calculate the N accumulation and remobilization equations. This equation is used in the calculation when *A* exceeds ~0.5 atom%, which applies to practically all studies in which ^{15}N is used as a tracer. The δ^{15}N values were calculated by using the δ^{15}N(‰) = [(Rsample/Rstandard)-1] x 1000 (Farquhar et al. 1989), and R is the ^{15}N/^{14}N ratio of samples from both labeled and unlabeled (i.e. natural abundance) results; the standard referred to N_2 in air. Atropine was used as a system check in the elemental analyses of nitrogen. Isotope secondary standards of known ^{15}N/^{14}N ratios (IAEA N_1 and IAEA N_2 ammonium sulfate and IAEA NO_3 potassium nitrate) were used for calibration of δ^{15}N to a precision of 0.2‰.

2.7 Assessment of N remobilization and uptake

In this work, ATOM% ^{15}N excess was calculated as the difference between *A* (^{15}N atoms ATOM% abundance) of labeled plant samples and the corresponding *A* of non ^{15}N-labeled plants. The N_{abs} and N_{rem} of ears and flag leaves were estimated by the following equations (1) and (2) modified from Kichey et al. (2007):

$$N_{abs} = \frac{\left[N_{final} * \left(E_{final} - E_{flo}\right)\right] - \left[N_{flo} * \left(E_{flo} - E_{rem}\right)\right]}{E_{rem} - E_{abs}} \quad (1)$$

$$N_{rem} = \frac{\left[N_{flo} * \left(E_{abs} - E_{flo}\right)\right] - \left[N_{final} * \left(E_{abs} - E_{final}\right)\right]}{E_{rem} - E_{abs}} \quad (2)$$

where N_{abs} (g m^2) represented the organ total N absorbed from anthesis until two weeks after anthesis, and N_{rem} was the total N remobilized from anthesis until two weeks after anthesis, with both N_{abs} and N_{rem} being calculated from the pre-anthesis labeled plants.; N_{final} and E_{final} (%) represented, respectively, the total N and ^{15}N excess two weeks after anthesis; N_{flo} and E_{flo} represented the total N and ^{15}N excess at

the flowering stage; E_{abs} is the ^{15}N excess of N absorbed after flowering, which was considered to correspond to the ATOM% ^{15}N of the substrate water solution; and E_{rem} is the ^{15}N excess of N assimilated during anthesis, which was considered to be equal to ^{15}N excess at flowering of the ears and flag leaves combined together during calculation.

For the mature kernel accumulated N originating from N_{abs} during the period from anthesis to maturity and the N_{rem} derived from pre-anthesis, N assimilation was calculated as follows:

$$N_{abs} = \frac{N_{kernel} * E_{kernel}}{E_{abs}} \quad (3)$$

$$N_{rem} = \frac{N_{kernel} * E_{kernel}}{a * E_{rem\ ear} + (1 - a) * E_{rem\ flag}} \quad (4)$$

$$N_{rem} = N_{kernel} * \left(1 - \frac{N_{abs}}{N_{kernel}}\right) \quad (5)$$

where N_{kernel} (g m^{-2}) was considered as the total N content per kernel; N_{abs} was the N derived from absorption during the reproductive stages, and N_{rem} was the N derived from remobilization during the vegetative stages; E_{kernel} was considered as the ^{15}N excess in labeled plant kernels; 'a' was the ear contribution to N_{rem}; $E_{rem\ ear}$ and $E_{rem\ flag}$ were considered to be equal to the mean values of ^{15}N excess in the ears and the flag leaves for all the growth stages (including pre/post anthesis), and E_{abs} referred to the corresponding substrate water soluble ^{15}N excess; and the N_{abs} of kernels was calculated with the post-anthesis ^{15}N labeling of plants from equation (3). Here we assumed that each genotype had the same N_{rem} capacity in the same plot, therefore, the N_{rem} of kernels was calculated with the pre-anthesis ^{15}N labeling of plants from equations (4) and (5).

Flag leaf and ear N_{rem} were assessed through equations (4) and (5). In equation (4),

the contribution ratio of the ear and flag leaf in sustaining grain filling (E_{rem}) was considered because they are the main N remobilizing organs. On the other hand, the N_{rem} was also examined by equation (5), which was set up based on the assumption that the N_{rem} of the total grain storage was equal to the remainder of the N_{abs} amount in the total grains. For equation (4), the best fit was achieved when the N_{rem} of the ear was assigned a relative contribution of 90% to the kernel, which exhibited a higher linear correlation with grain yield than equation (5). Therefore, in the current work we adopted N_{rem} from equation (4), which indicated that the ear contributes 90% of the total N in the grains. Moreover, the nitrogen use efficiency (NUE) was assessed by the total N accumulated in the grains divided by the total N applied during the whole crop cycle.

2.8 Statistical analyses

In order to analyze the effects of the different genotypes on the agronomical and physiological parameters related to C and N assimilation, a hypothesis of zero difference between means was carried out with analysis of variance (ANOVA) performed by using the general linear model (GLM) procedure. Mean separation of genotypes for the measured traits was done by a Tukey's-b multiple comparison test ($P < 0.05$). Matrices of simple Pearson coefficient correlations were performed based on yield, agronomical and physiological traits of the different genotypes. All the data were analyzed using the SPSS v.16 statistical package (SPSS Inc., Chicago, IL, USA).

3. **Results**

3.1 Grain yield and yield components

The grain yield of different genotypes varied significantly (ranging from 8.3 g $plant^{-1}$ to 11.8 g $plant^{-1}$) with the genotypes "Zhoumai 18" and "Zhoumai 22" showing the highest values, and Yumai 2 the lowest (Table 1). The aerial plant biomass and the

plant height also showed significant differences across genotypes with a pattern similar to the grain yield. The ears and kernels per plant also exhibited a pattern across genotypes similar to that of grain yield, but without reaching significant genotypic difference. Grain yield was highly and positively correlated to biomass, HI and ears per plant and to a lesser extent to the kernels per plant and plant height (Supplementary table 1). The greatest biomass was correlated to taller plants with the largest number of ears and kernels per plant. Other traits such as kernels per ear, TKW and ear length also exhibited significant genotypic differences but these traits did not correlate with grain yield.

	Grain yield (g plant^{-1})	Biomass (g plant^{-1})	HI	Kernels (ear^{-1})	TKW (g)	Ears (plant^{-1})	Kernel No. (plant^{-1})	Ear length (cm)	Plant height (cm)
Yumai 2	8.3 b	20.6 b	0.41 a	48.8 b	59.0 ab	1.6 a	79 a	8.9 b	70 b
Lankao 198	9.6 ab	24.7 a	0.39 a	44.2 b	67.8 a	1.8 a	82 a	9.9 ab	72 b
Yumai 20	9.9 ab	25.3 a	0.38 a	57.3 a	55.4 b	1.7 a	99 a	10.6 a	83 a
Zhoumai 22	10.8 a	27.2 a	0.41 a	46.7 b	62.2 ab	2.1 a	96 a	9.9 ab	84 a
Zhoumai 18	11.8 a	26.7 a	0.44 a	56.7 a	59.1 ab	1.9 a	106 a	9.2 b	80 a
Mean	10.1	24.9	0.41	50.7	60.7	1.8	92.6	9.7	77.7
Genotypes	20.7*	84.5***	0.01	427.9***	256.1*	0.96	5232	6.0*	530.7***
Replicates	0.9	5.7	0.00	88.9	33.1	0.37	1639	2.2	32.4

Table 1. Mean values and sum of squares type III combined with analysis of variance (ANOVA) for grain yield and agronomic yield components of 5 winter wheat genotypes assayed under natural ambient conditions. The parameters recorded are: grain yield, above-ground biomass (biomass), harvest index (HI), kernels per ear (kernels), thousand kernel weight (TKW), ears per plant (ears), kernel number per plant (kernel No.), ear length and plant height. Values are the means of 3 replicates of each genotype. Means followed by the same letter are not significantly different at P = 0.05 by the Tukey's-b test. (*, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).

3.2 Flag leaf and ear photosynthesis and respiration

The flag leaf net assimilation rates (Pn, on a per area basis), which differed between genotypes at two weeks after anthesis, showed higher values during the pre-anthesis stage (Table 2). Similarly, the ear gross assimilation rate (GAR, on area basis) displayed a lower value at two weeks post-anthesis than at pre-anthesis, with a

negative linear relationship to grain yield (r = -0.56, P < 0.05) (Table 2 and Figure 1A). While the GAR of the whole ear varied significantly among genotypes in both phenological stages, the whole ear Pn at two weeks post-anthesis positively correlated with grain yield (r = 0.50, P < 0.05) (Figure 1B). The dark respiration rate (Rdark) on a total ear basis showed a highly significant difference between genotypes during the two weeks post-anthesis (Table 2).

		Flag Pn m^{-2} (μmol m^{-2} s^{-1})	Pn $flag^{-1}$ (nmol $flag^{-1}$ s^{-1})	Ear GAR m^{-2} (μmol m^{-2} s^{-1})	Pn ear^{-1} (nmol s^{-1} ear^{-1})	Rdark ear^{-1} (nmol s^{-1} ear^{-1})	GAR ear^{-1} (nmol s^{-1} ear^{-1})
Pre	Yumai 2	28.9 a	137.8 a	8.7 ab	18.3 b	13.3 a	31.6 b
	Lankao 198	25.2 a	102.1 a	9.2 a	24.8 a	14.4 a	39.2 a
	Yumai 20	26.9 a	131.8 a	8.8 ab	21.2 ab	15.6 a	36.8 ab
	Zhoumai 22	27.2 a	114.3 a	8.5 ab	22.0 ab	15.1 a	32.1 ab
	Zhoumai 18	26.9 a	113.7 a	7.7 b	18.7 b	14.1 a	36.2 ab
	Mean	27.0	119.9	8.6	20.1	14.5	35.2
	Genotypes	20.0	2536.6	3.58*	85.5*	10.2	127.8*
Post	Yumai 2	25.8 ab	122.5 a	3.7 a	11.3 a	11.8 b	23.4 b
	Lankao 198	24.3 b	98.9 a	5.3 a	14.4 a	13.6 b	28.0 ab
	Yumai 20	23.2 b	113.3 a	4.8 a	14.6 a	14.3 ab	28.9 ab
	Zhoumai 22	28.6 a	120.3 a	5.1 a	15.1 a	11.4 b	26.6 ab
	Zhoumai 18	23.5 b	101.2 a	6.6 a	15.5 a	17.8 a	33.3 a
	Mean	25.1	111.2	5.1	14.2	13.8	28.0
	Genotypes	58.0*	1401.4	12.6	34.0	77.6**	165.9*

Table 2. Mean values combined with analysis of variance for physiological traits during pre-anthesis (Pre) and two weeks post-anthesis (Post) for 5 winter wheat genotypes assayed under natural ambient conditions. The parameters recorded are: flag leaf net CO_2 assimilation rate per unit area (flag Pn m^{-2}), Pn per total flag leaf (Pn $flag^{-1}$), ear gross CO_2 assimilation rate per unit area (ear GAR m^{-2}), Pn and dark respiration rate per total ear (Pn ear^{-1}, Rdark ear^{-1}). Values are the means of three replicates (6 pots) of each genotype. Means followed by the same letter are not significantly different at P = 0.05 by the Tukey's-b test. (*, P < 0.05; **, P < 0.01 and ***, P < 0.001).

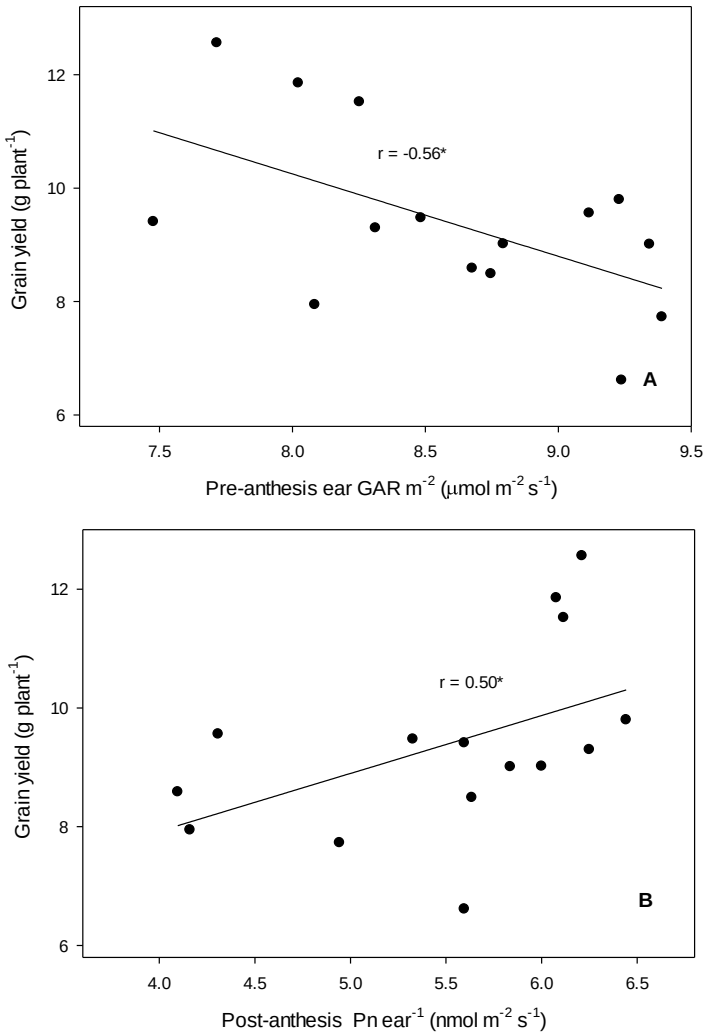

Figure 1. Linear correlations between grain yield and the pre-anthesis ear gross net assimilation rate per unit area (GAR m^{-2}) (A), and the two weeks post-anthesis net assimilation rate per whole ear (Pn ear^{-1})(B) for Chinese wheat grown in a natural ambient environment. The three replicates and 5 genotypes are plotted together. Determination coefficient (*r*) and probabilities are given. *, $P<0.05$.

3.3 Nitrogen isotope composition change in TOM and Rubisco

$\delta^{15}N$ analyses conducted in whole pre-anthesis labeled plants showed that the $\delta^{15}N$ of the total organic matter (TOM) of the ear exhibited significant differences across genotypes through all growth stages (Table 3). The $\delta^{15}N$ of glume Rubisco did not show genotypic differences, regardless of the growth stage. However, the two

genotypes with high grain yield had the lowest ear TOM $\delta^{15}N$ (in pre-anthesis labeled plants). In contrast, both flag leaf TOM and Rubisco $\delta^{15}N$ were not significantly different between genotypes. Moreover, correlation analyses revealed that in plants labeled during heading, the grain yield was not associated with the glume Rubisco $\delta^{15}N$ during the pre-anthesis stage (Figure 2A). On the other hand, in contrast, the glume Rubisco $\delta^{15}N$ significantly and negatively correlated with the grain yield during post-anthesis (in pre-anthesis labeleld plants) (r = -0.61, $P < 0.05$) (Figure 2A). Interestingly, grain yield *versus* Rubisco content (in glumes) showed a similar trend (Figure 2B). N use efficiency (NUE) differed significantly between genotypes and showed a similar pattern to grain yield (Table 4). Flag Rubisco $\delta^{15}N$ was not correlated with NUE during pre-anthesis, but a high and significant linear relationship was found two weeks post-anthesis (r = 0.60, $P < 0.05$) (Figure 2C). Although the relationship between the post-anthesis glume Rubisco $\delta^{15}N$ and NUE was negative, the coefficient did not reach statistical significance, which indicated that high NUE genotypes could maintain more $\delta^{15}N$ in the flag leaf Rubisco than in the ear in the post-anthesis stage (Table S2). On the other hand, although the Rubisco content in glumes did not significantly correlate with NUE during the post-anthesis period, a positive correlation was detected between NUE and glume Rubisco content during pre-anthesis (Figure 2D). In contrast, no correlation was detected between grain yield and flag leaf Rubisco $\delta^{15}N$ (Table S2). Our study also showed that the $\delta^{15}N$ of glume Rubisco exhibited a positive linear correlation with the $\delta^{15}N$ of flag leaf Rubisco during pre-anthesis (r = 0.53, $P < 0.05$), but this relationship disappeared in the post-anthesis period (Table S2). Labeling conducted during the post-anthesis period showed significant differences between genotypes for $\delta^{15}N$ of TOM and $\delta^{15}N$ of flag Rubisco (Table 3). The highest $\delta^{15}N$ values were detected in the kernel TOM, followed by the flag and ear TOM, and glume and flag leaf Rubisco.

		Pre ear $\delta^{15}N$ (‰)	Pre glume Rubisco $\delta^{15}N$ (‰)	Pre flag $\delta^{15}N$ (‰)	Pre flag Rubisco $\delta^{15}N$ (‰)	Post ear $\delta^{15}N$ (‰)	Post glume Rubisco $\delta^{15}N$ (‰)	Post flag $\delta^{15}N$ (‰)	Post flag Rubisco $\delta^{15}N$ (‰)	Kernel $\delta^{15}N$ (‰)
Pre-anthesis labeled plants	Yumai 2	1189.6 b	1074.6 a	1205.7 a	1199.4 a	1099.0 ab	1111.8 a	1122.9 a	1131.0 a	829.3 a
	Lankao 198	1226.0 ab	1240.1 a	1230.2 a	1291.6 a	1130.5 a	1133.0 a	1131.9 a	1167.4 a	761.2 a
	Yumai 20	1246.6 a	1179.8 a	1248.3 a	1225.5 a	1105.3 ab	1181.3 a	1128.1 a	1185.7 a	703.1 a
	Zhoumai 22	1186.2 b	1248.9 a	1184.9 a	1199.7 a	1079.5 b	1059.8 a	1104.8 a	1173.3 a	717.6 a
	Zhoumai 18	1172.8 b	1183.7 a	1183.3 a	1221.2 a	1089.8 ab	1083.0 a	1127.2 a	1179.2 a	864.9 a
	Mean	1204.3	1185.4	1210.5	1227.5	1100.8	1113.8	1123.0	1167.3	775.2
	Genotypes	8586.7**	57974.9	9705.4	17128.0	4641.3*	26414.2	1896.2	5509.3	59045.1
Post-anthesis labeled plants	Yumai 2					68.7 b	50.6 a	75.3 b	52.2 ab	443.2 b
	Lankao 198					57.8 b	66.0 a	63.9 b	43.9 b	454.5 b
	Yumai 20					64.3 b	66.4 a	76.5 b	49.4 ab	524.3 ab
	Zhoumai 22					96.0 a	102.2 a	105.3 a	66.5 a	572.5 a
	Zhoumai 18					60.4 b	58.0 a	63.2 b	39.4 b	502.4 ab
	Mean					69.4	63.8	76.8	51.6	499.4
	Genotypes					2847.3**	4731.5	3495.0***	1279.3**	33391.5*

Table 3. Mean values combined with analysis of variance for nitrogen isotope composition ($\delta^{15}N$) of total organic matter (TOM) and Rubisco for five genotypes of winter wheat labeled with ^{15}N from sowing to anthesis (pre-anthesis labeled plants) and ^{15}N labeling from anthesis to maturity (post-anthesis labeled plants). The specific parameters are: $\delta^{15}N$ of ear TOM, flag leaf TOM, ear Rubisco and flag leaf Rubisco (Pre, sampled in pre-anthesis); $\delta^{15}N$ of ear TOM, flag leaf TOM, ear Rubisco and flag leaf Rubisco (Post, sampled in two weeks post-anthesis); $\delta^{15}N$ of kernel (sampled in maturity). Values are the means of 3 replicates (6 pots) for each genotype. (*, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).

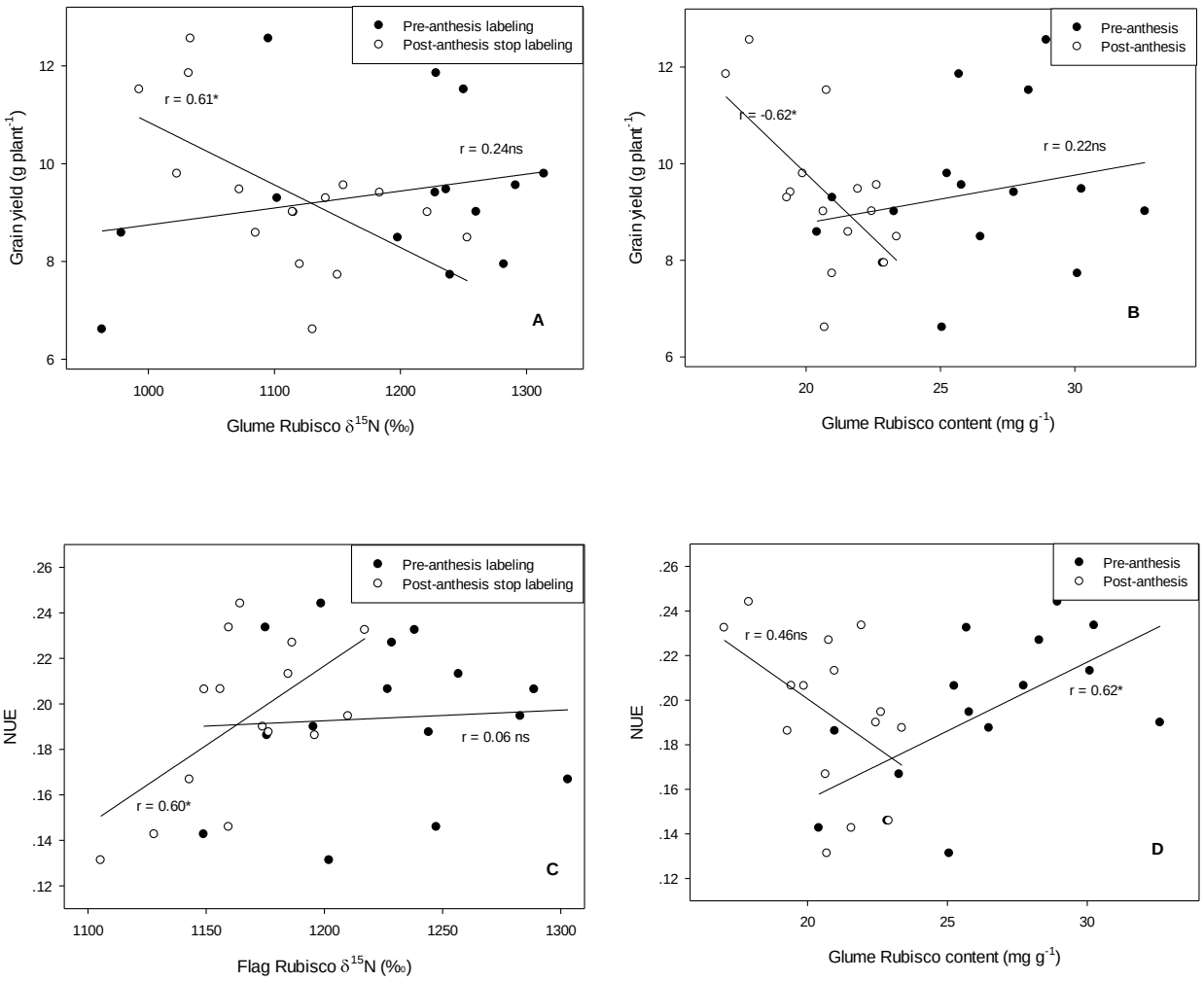

Figure 2. Linear correlations between grain yield and glume Rubisco $\delta^{15}N$ (A), grain yield and glume Rubisco content (B), nitrogen use efficiency (NUE) and flag leaf Rubisco content $\delta^{15}N$ (C) and NUE and glume Rubisco content (D) in the pre-anthesis ^{15}N labeling stage and the two weeks post-anthesis ^{15}N labeling stage for Chinese wheat grown in a natural ambient environment. The three replicates and the 5 genotypes are plotted together. Determination coefficient (*r*) and probabilities are given. ns, not significant. *, $P < 0.05$.

3.4 Nitrogen concentration among organs

N content data revealed genotypic differences in the N concentration of ears (at both harvest stages), and flag leaves during post-anthesis (Table 4). Interestingly, we also observed that as plant phenology advanced, the N content in ears diminished more than in flag leaves. Moreover, correlation analyses highlighted the fact that the flag leaf N at pre-anthesis was negatively associated with the flag leaf TOM $\delta^{15}N$ in the same period, and flag leaf Rubisco $\delta^{15}N$ and ear N concentration during post-anthesis (r = -0.74, $P < 0.05$; r = -0.52, $P < 0.05$; r = -0.52, $P < 0.05$, respectively), while the flag leaf N concentration two weeks post-anthesis exhibited negative correlations with the $\delta^{15}N$ of the flag leaf and ear in the same period (r = -0.75, $P < 0.01$; r = -0.53, $P < 0.05$, respectively) (Table S2).

3.5 Rubisco content of the flag leaves and glumes

The Rubisco content of glumes and flag leaves was analyzed in both the pre-anthesis and two weeks post-anthesis stages. Regardless of the phenological stage, significant genotypic differences were found in glumes but not in the flag leaf (Table 4). The Rubisco content of both glumes and flag leaves tended to decline in the period from pre-anthesis to post-anthesis. The Rubisco content also revealed that at pre-anthesis the two high grain-yielding genotypes had more Rubisco in the glumes than the low yielding ones. However, the data also showed that in the high yielding genotypes the decrease in the amount of glume Rubisco from heading to two weeks post-anthesis was greater than in the low yielding genotypes. Moreover, at post-anthesis the flag leaf Rubisco content was positively correlated with flag leaf Pn per unit area (r = 0.71, $P < 0.01$), whereas during the pre-anthesis stage there was no correlation (Figure 3A). By contrast, the glume Rubisco content at two weeks post-anthesis negatively correlated with ear GAR per unit area (r = -0.70, $P < 0.01$), while no correlation was detected during the pre-anthesis stage (Figure 3B). Pre-anthesis glume Rubisco content were positively correlated to kernel N content, while it did not find in post-anthesis Rubisco content (r = 0.65, $P < 0.01$) (Figure 4).

		Flag Rubisco content (mg g^{-1})	Glume Rubisco content (mg g^{-1})	Flag N (%)	Ear N (%)	Kernel N (%)	NUE	Ear N_{abs} (g m^{-2})	Ear N_{rem} (g m^{-2})	Flag N_{abs} (g m^{-2})	Flag N_{rem} (g m^{-2})	Kernel N_{abs} (g m^{-2})	Kernel N_{rem} (g m^{-2})
	Yumai 2	34.7 a	22.8 b	5.8 a	2.2 a								
	Lankao 198	36.4 a	24.8 b	5.5 a	2.2 a								
	Yumai 20	40.4 a	25.9 b	5.3 a	2.1 a								
Pre	Zhoumai 22	39.5 a	30.4 a	5.8 a	2.1 a								
	Zhoumai 18	34.2 a	27.4 ab	5.6 a	2.0 b								
	Mean	37.0	26.2	5.6	2.1								
	Genotypes	94.2	99.0**	0.4	0.1**								
	Yumai 2	34.8 a	21.7 a	5.5 b	1.8 a	2.2 b	0.15 c	3.7 b	4.0 a	4.0 b	0.9 a	7.2 c	17.0 b
	Lankao 198	36.6 a	21.0 a	5.3 c	2.0 a	2.5 ab	0.20 b	3.2 b	3.8 a	3.2 b	1.0 a	10.1 bc	20.4 b
	Yumai 20	25.5 a	21.2 a	5.1 c	1.8 b	2.7 a	0.22 ab	6.3 a	6.9 a	6.5 a	3.1 a	12.0 ab	19.1 b
Post	Zhoumai 22	35.2 a	21.7 a	6.0 a	1.9 ab	2.7 a	0.24 a	3.9 b	4.2 a	5.3 b	4.0 a	14.5 a	22.6 ab
	Zhoumai 18	26.3 a	18.1 b	5.2 c	1.8 b	2.5 a	0.24 a	3.4 b	6.0 a	2.4 b	2.3 a	13.4 a	26.4 a
	Mean	31.7	20.8	5.4	1.8	2.5	0.21	4.1	5.0	4.3	2.3	11.4	21.1
	Genotypes	339.2	27.3*	1.4***	0.1**	0.5**	0.02***	19.0*	22.0*	30.7*	21.3	99.0***	183.6**

Table 4. Mean values combined with analysis of variance for: Rubisco content of flag leaves and glumes, and nitrogen content per unit total organic matter (N,%) of flag leaves, ears and mature kernels (Pre, sampled at pre-anthesis; Post, sampled at two weeks post-anthesis); nitrogen use efficiency (NUE), nitrogen absorption (N_{abs}) from nitrogen uptake in Post, and nitrogen remobilization (N_{rem}) from nitrogen accumulated in Pre for ears and flag leaves from pre-anthesis to two weeks post-anthesis; and total kernel nitrogen N_{rem} derived from the whole pre-anthesis accumulation and N_{abs} for the whole post-anthesis for five winter wheats under natural ambient conditions. Values are the means of 3 replicates (6 pots) for each genotype. (*, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).

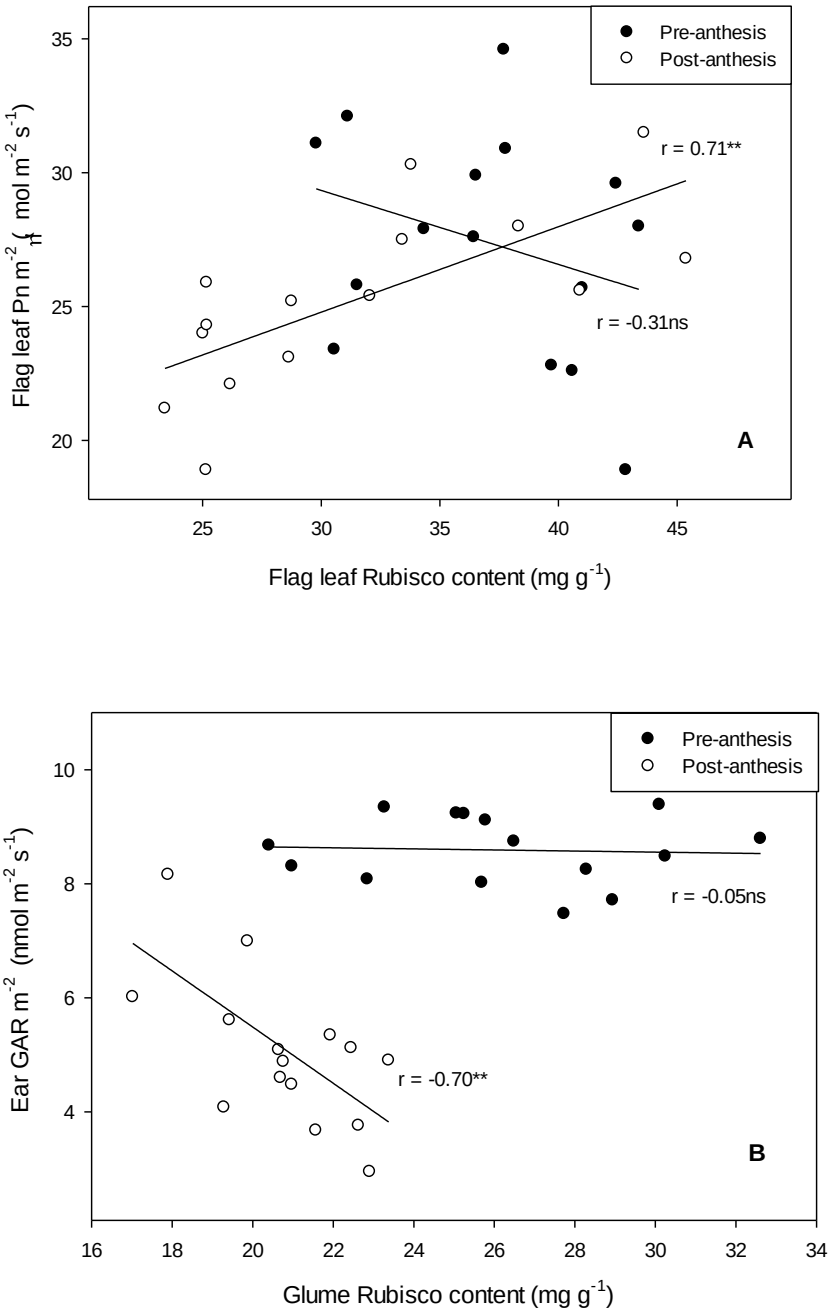

Figure 3. Linear correlations between the flag leaf net photosynthesis rate per unit area (Pn) and the flag leaf Rubisco content (A), and the ear gross assimilation rate (GAR) and glume Rubisco content (B) in pre-anthesis and two weeks post-anthesis for Chinese wheat grown in a natural ambient environment. The three replicates and the 5 genotypes are plotted together. Determination coefficient (*r*) and probabilities are given. **, $P < 0.01$.

3.6 N absorption (N_{abs}) during post-anthesis and remobilization (N_{rem}) from N accumulated during pre-anthesis

Significant genotypic differences were found for the whole pre-anthesis N_{abs} of the ears, the flag leaves and the mature kernels (Table 4). The N_{rem} of kernels also showed genotypic differences. The total N absorbed from anthesis to maturity accounted for 30% ─ 40% of kernel total N accumulation. Meanwhile, 60% ─ 70% of N present in the kernel came from remobilization of N assimilated during pre-anthesis. The proportion of remobilized N was larger in the high yielding than in the low yielding genotypes. It is also noteworthy that while the ear had a similar amount of N_{abs} and N_{rem}, in the flag leaves at the post-anthesis stage N_{abs} was nearly double the N_{rem}; therefore, the N_{rem} of flag leaves represented the smallest proportion of all the N derivation sources. Grain yield was revealed as more correlated with total kernel N_{rem} from pre-anthesis than the N_{abs} from post-anthesis (r = 0.86, P < 0.001; r = 0.74, P < 0.01, respectively) (Table S3).

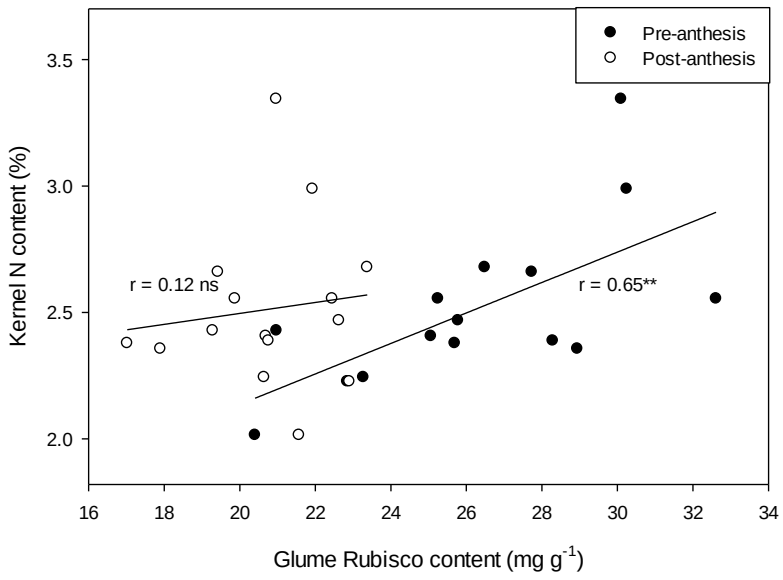

Figure 4. Linear regression between the kernel nitrogen content proportion and the glume Rubisco content in the pre-anthesis and two weeks post-anthesis stages for Chinese wheat grown in a natural ambient environment. The three replicates and the 5 genotypes are plotted together. Determination coefficient (r) and probabilities are given. ns, not significant. **, P < 0.01.

4. Discussion

4.1 Biomass and grain yield

Our study showed that genotypic differences in grain yield were mainly associated with plant biomass, HI and plant height (Xiao et al. 2012; Zhou et al. 2014). Data also revealed that high grain-yielding genotypes exhibited more ears and kernels (Fischer 2008). However, in these genotypes yield was not conditioned by TKW, kernels per ear, and ear length. Moreover, the study also showed that the number of ears per plant had a greater impact on grain yield than the number of kernels per ear. Such different contributions indicated that tillering capacity was the main factor determining grain yield and biomass. In a broad sense, the resource accumulation prior to anthesis was critical to determining kernel numbers (Fischer 2008; Sinclair and Jamieson 2008).

4.2 C photoassimilate contribution of the ear and the flag leaf during grain filling

As mentioned before, the role of the ear as one of the main photosynthetic organs supporting grain filling is matter of recent discussion (Sánchez-Bragado et al. 2014a, b). During the last decade a number of studies have highlighted the fact that the contribution of ear photosynthesis towards grain filling is higher than expected (Tambussi et al. 2005; 2007; Zhou et al. 2014; Sánchez-Bragado et al. 2014a,b). Although genotypic differences were detected for flag leaf Pn (at two weeks post-anthesis stage), no correlation between flag leaf Pn (expressed either on a per area basis or per whole organ) and yield was detected in any harvest (Lawlor 1995; Richards 2000; Xiao et al. 2012). Even if such apparent discrepancies suggest that the flag leaf was not the main photosynthetic contributor to growing grains (Tambussi et al. 2007), the fact that Pn represents the plant's physiological activity at a specific moment should be taken into account. By contrast, the ear photosynthetic rates (determined per total organ) positively correlated with grain yield.

Changes in the content and activity of Rubisco have been described as target processes that condition photosynthetic performance (Long et al. 2006). In this sense, the correlation between flag leaf and ear Rubisco content and their corresponding photosynthetic activities highlighted that genotypic differences in Rubisco availability were the main factor conditioning photosynthetic rates. The positive correlation between grain yield and total ear Pn, together with the absence of a relationship between grain yield and total flag leaf Pn at

two weeks after anthesis, supported the idea that the ear photosynthetic rate contributed substantially to grain filling, which is in agreement with previous studies (Abbad et al. 2004; Tambussi et al. 2007; Zhou et al. 2014). The fact that the Rubisco content in glumes was lower than in flag leaves revealed that the differences in photosynthetic activity were not explained by protein availability. The positive correlation (during post-anthesis) between flag leaf photosynthesis and Rubisco content showed that the flag leaf Pn (per unit area) was limited by the lower Rubisco availability. However, during pre-anthesis, as a consequence of the larger Rubisco content, the plants were capable of sustaining higher flag leaf photosynthetic rates (Makino et al. 1992). Our study also indicated that ear behavior was different from that described for flag leaves. Glume Rubisco content correlated negatively with ear GAR (per unit area) when determined at two weeks post-anthesis. Such results show that the flag leaves were capable of fixing more CO_2 with less Rubisco content (Pn/Rubisco) than ears. This finding discards Rubisco availability as the only parameter conditioning ear GAR. When analyzing the ear photosynthetic contribution to grain filling it must be considered that this organ may respire up to 60 % of the recently fixed C, probably in association with grain filling (Aranjuelo et al. 2011; 2013a). This implies that photosynthesis was greatly underestimated in the ear because of the high rate of respiration, which diminished the GAR. In the flag leaf, the lost C that was derived from respiration was comparatively low (Araus et al. 1993; Zhou et al. 2014). Moreover, the fact that positive correlations between Pn (on a whole organ basis) and grain yield were only detected in the ear two weeks post-anthesis further supports the concept that the relative photosynthetic contribution of the ear to the final grain yield represents a significant source of C sustaining grain filling.

4.3 Pre/post-anthesis N contribution to grain filling: N remobilization and uptake

Grain N content is conditioned by the amount of N remobilization during pre-anthesis and the N taken up after anthesis (Dupont and Altenbach, 2003). Genotypic NUE showed that the high grain-yielding genotypes (Zhoumai 18 and 22) had the highest kernel N accumulation capacity, whereas the lowest NUE values were detected in the low yielding Yumai 2. As mentioned before, both N assimilation and remobilization become critical sources of N that sustain kernel development. Leaves and shoots have been described as acting as the main sources of N (Kant et al. 2011). The ^{15}N labeling revealed that N absorbed during the post-anthesis stage provided 30-40 % of the N present in the kernel, whereas the remaining 70-60 % was derived from remobilization of N that was assimilated prior to anthesis. During

grain filling (from anthesis to physiological maturity) N remobilization was larger in high yielding genotypes than in the low ones. In this sense, enhanced N_{rem} and N_{abs} were factors that explained the larger N uptake efficiency of high yielding genotypes (Papakosta and Gagianas 1991). Our study also showed that N_{rem} was larger in ears than in flag leaves during the post-anthesis stage, even though ears and flag leaves showed similar N_{abs} rates. Therefore, the capacity of N remobilization in ears was higher than that of flag leaves. Such findings showed that the ear contribution to sustaining kernel N demand (during the beginning of grain filling) was more important than expected (Lopes et al. 2006). This contrasts with most of the available literature, which assigns to the flag leaf blade a main role of supplying N to the growing grains (Evans 1983, 1989; Drouet and Bonhomme 1999; Bertheloot et al. 2008).

4.4 Pre/post-anthesis N contribution to grain filling: TOM versus Rubisco

Although the contribution of pre-anthesis and post-anthesis N has been studied in the past (Palta and Fillery 1995; Lopes et al. 2006; Dupont and Altenbach 2003; Bernard et al. 2008), the real contribution to grain filling of the flag leaf Rubisco-derived N remobilization has not been fully elucidated. Traditionally it has been assumed that the Rubisco from the flag leaf blade is the main source of N that feeds the kernels (Drouet and Bonhomme 1999; Bertheloot et al. 2008). Such an assumption is mostly based on the temporal coincidence between the fast N accumulation in the grain and leaf senescence during the last part of grain filling (Bertheloot et al. 2008). However, direct evidence of the contribution of the Rubisco N from the flag leaf N is scarce (Farooq et al. 2014). This aspect is especially important because Rubisco can represent up to 50% of the total soluble protein (Parry et al. 2003, Aranjuelo et al. 2013a). N remobilization efficiency has been described as being subjected to genetic variability (Masclaux-Daubresse et al. 2008). As plant phenology advances and gets closer to the senescence period, the N and C assimilation pathways are altered and the expression of proteases increases (Millard 1988; Masclaux-Daubresse et al. 2008). As a result of protein hydrolysis, the resulting amino acids from the ear and the flag leaf are exported to grains (Lopes et al. 2006; Feller et al. 2008). Previous studies (Lopes et al. 2006; Bernard et al. 2008) suggest that re-assimilation of ammonium derived from protein degradation is conditioned by glutamine synthetase (GS) protein. Moreover, as observed by Bernard et al. (2008), GS activity and Rubisco content are tightly coordinated during the grain filling period. In order to characterize the role of TOM, and more specifically Rubisco as an N source sustaining grain N requirements, $\delta^{15}N$ was analyzed in flag leaves and glumes. Our study revealed that during the beginning of the post-anthesis period, N remobilization in flag leaves and glumes

occurred differently in low versus high grain-yielding genotypes. While TOM N_{rem} (understood as the diminishment of $\delta^{15}N$) was similar in the 5 genotypes, Rubisco derived N_{rem} showed a different pattern. In low yielding genotypes N_{rem} was derived, mostly, from the Rubisco present in the flag leaf. Moreover, the positive correlation between flag leaf $\delta^{15}N$ during pre-anthesis and the post-anthesis ear $\delta^{15}N$ highlighted the fact that the N present in flag leaves during pre-anthesis was transferred to the ear (during post-anthesis). Our data also suggest that this remobilized flow of N was derived from degradation of flag leaf Rubisco (Millard 1988; Gregersen et al. 2008). On the other hand, in the high grain-yielding genotypes the opposite effect was observed. At the beginning of grain filling, remobilization from glume Rubisco was much more marked than in flag leaves. Interestingly, the labeling conducted from anthesis until maturity revealed that 80 % of the N absorbed at this stage was directly translocated to the kernel. The remaining 20 % was localized, in similar proportions, in the flag leaves and glumes. The large kernel N demand was more marked in high yielding genotypes than in the low yielding ones.

In this work it was concluded that genotypic differences in grain yield were determined by the above-ground biomass, HI, plant height and ear number. Gas exchange analyses indicated that the relative photosynthetic contribution of ears to grain yield was highly relevant. At the same time our results confirmed ^{15}N labeling as a powerful tool to evaluate N remobilization and N uptake during pre-anthesis and post-anthesis. N_{rem} represented the most important N source to the kernel, while flag leaf and glume $\delta^{15}N$ revealed different patterns in high and low grain-yielding genotypes. In low yielding genotypes, flag leaf Rubisco-derived N represented a major N source, while in the high yielding ones Rubisco-derived N_{rem} from the ear was more relevant. In summary, our study highlighted the role of the ear as a source of C and a contributor of N, especially in high grain-yielding genotypes.

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	Grain yield	Biomass	HI	Kernels ear^{-1}	TKW	Ears plant^{-1}	Kernels plant^{-1}	Ear length
Biomass	0.87***							
HI	0.65**	0.20						
Kernels ear^{-1}	0.12	0.06	0.08					
TKW	0.21	0.21	0.2	-0.50				
Ears plant^{-1}	0.77***	0.79**	0.36	-0.14	0.22			
Kernels plant^{-1}	0.59*	0.56*	0.29	0.78***	-0.29	0.51		
Ear length	0.08	0.21	-0.14	0.28	-0.01	0.24	0.36	
Plant height	0.61*	0.68**	0.10	0.38	-0.36	0.50	0.61*	0.50

Supplementary table 1. Pearson correlation coefficients of grain yield, above ground biomass (biomass), harvest index (HI), kernels per ear, thousand kernel weight (TKW), ears per plant, kernels per plant, ear length and plant height across the set of 5 winter wheat genotypes assayed under natural ambient conditions. (n = 15; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).

	Grain yield	NUE	Pre flag Rubisco content	Post flag Rubisco content	Pre glume Rubisco content	Post glume Rubisco content	Kernel N	Pre flag N	Post flag N	Pre ear N	Post ear N	Kernel $\delta^{15}N$	Pre flag $\delta^{15}N$	Post flag $\delta^{15}N$	Pre ear $\delta^{15}N$	Post ear $\delta^{15}N$	Pre flag Rubisco $\delta^{15}N$	Post flag Rubisco $\delta^{15}N$	Pre glume Rubisco $\delta^{15}N$
NUE	0.78***																		
Pre flag Rubisco content	-0.24	0.12																	
Post flag Rubisco content	-0.10	-0.14	0.10																
Pre glume Rubisco content	0.22	0.62*	0.14	0.03															
Post glume Rubisco content	-0.62*	-0.46	0.19	0.18	0.06														
Kernel N	-0.19	0.46	0.47	-0.11	0.65**	0.12													
Pre flag N	-0.34	-0.24	0.07	0.52*	0.30	0.24	0.08												
Post flag N	0.21	0.27	0.23	-0.17	0.33	0.09	0.11	0.11											
Pre ear N	0.14	-0.06	-0.15	0.21	-0.45	0.11	-0.28	-0.30	0.10										
Post ear N	0.32	0.15	0.00	-0.09	-0.30	-0.15	-0.23	-0.52*	-0.09	0.65**									
Kernel $\delta^{15}N$	0.23	-0.22	-0.58*	-0.21	-0.25	-0.22	-0.67**	-0.16	-0.10	-0.26	-0.10								
Pre flag $\delta^{15}N$	-0.05	-0.18	0.09	-0.28	-0.64*	0.13	-0.19	-0.74**	-0.20	0.60*	0.58*	-0.12							
Post flag $\delta^{15}N$	0.14	0.01	-0.33	-0.04	-0.34	-0.15	-0.16	-0.27	-0.75**	0.32	0.42	0.05	0.39						
Pre ear $\delta^{15}N$	-0.23	-0.16	0.48	-0.19	-0.38	0.27	0.10	-0.47	-0.04	0.38	0.25	-0.44	0.72**	0.14					
Post ear $\delta^{15}N$	-0.07	-0.15	-0.18	0.06	-0.43	0.08	-0.13	-0.28	-0.53*	0.38	0.35	-0.16	0.47	0.62*	0.50				
Pre flag Rubisco $\delta^{15}N$	0.00	0.06	0.04	0.07	0.00	0.06	0.12	-0.23	-0.43	0.06	0.30	-0.04	0.18	0.43	0.37	0.59*			
Post flag Rubisco $\delta^{15}N$	0.51	0.60*	-0.04	-0.21	0.22	-0.16	0.24	-0.52*	0.17	0.42	0.46	-0.06	0.23	0.29	0.08	-0.07	0.20		
Pre glume Rubisco $\delta^{15}N$	0.24	0.50	0.37	0.10	0.45	0.19	0.42	-0.16	0.19	0.02	0.26	-0.12	0.00	-0.03	0.05	-0.10	0.53*	0.59*	
Post glume Rubisco $\delta^{15}N$	-0.61*	-0.44	0.11	-0.45	-0.15	0.45	0.17	-0.25	-0.24	-0.20	-0.10	-0.04	0.23	0.06	0.47	0.27	0.26	-0.10	-0.12

Table 2. Relationship of grain yield, nitrogen use efficiency (NUE), and physiological traits during pre-anthesis (Pre, heading) and post anthesis (Post, two weeks after anthesis) with Rubisco content of glumes and flag leaves, N content (N, %) of ears, flag leaves and kernels, N isotope composition ($\delta^{15}N$) of ear and flag leaf total organic matter (TOM), Rubisco and mature kernel dry matter for 5 winter wheat genotypes labeled by ^{15}N from sowing to anthesis assayed under natural ambient conditions. (n = 15; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).

	Grain yield	Kernel N_{abs}	Ear N_{abs}	Flag N_{abs}	Kernel N_{rem}	Ear N_{rem}
Kernel N_{abs}	0.74**					
Ear N_{abs}	0.11	-0.17				
Flag N_{abs}	0.08	-0.01	0.52*			
Kernel N_{rem}	0.86***	0.66**	0.23	0.36		
Ear N_{rem}	0.06	0.29	-0.76**	-0.29	0.16	
Flag N_{rem}	0.23	0.41	-0.61*	-0.80***	0.03	0.51

Supplementary table 3. Pearson correlation coefficients of grain yield, nitrogen absorption (N_{abs}) from post-anthesis (Post) N uptake, remobilized (N_{rem}) from pre-anthesis (Pre) N assimilate of ear and flag leaf, and Nabs and Nrem accumulated in mature kernel for 5 winter wheat genotypes labeled with ^{15}N, which were assayed from sowing to anthesis under natural ambient conditions. (n = 15; *, P < 0.05; **, P < 0.01 and ***, P < 0.001).