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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 2

Physiological Traits Contributed to the Recent Increase in Yield Potential of Winter Wheat from Henan Province, China[a]

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10 Chinese genotypes planted in the drip irrigation pots in the Faculty Biology, UB (left), and the spike photosynthesis measurement (right). Photo taken by B. Zhou at Barcelona, 2012, Spain.

Abstract:

To satisfy the future demand for bread wheat in China, it is paramount to study what traits may contribute to higher yield potential among genotypes. This experiment aims to test the traits responsible for the increase in yield potential of winter wheat released in Henan province, China. Seven established cultivars released in the last 20 years and 3 advanced lines were assayed. The results showed that grain yield was positively correlated with HI, kernel number per square meter, and aboveground biomass. In addition, the HI and above ground biomass showed a an increasing trend with the year of release. Positive correlations between grain yield and the $\delta^{13}C$ of the flag leaf, spike and kernel were observed. Therefore we can conclude that bread wheat breeding advances during recent decades in Henan, China, have been achieved through an increase in HI, kernel number per square meter and aboveground biomass. A higher $\delta^{13}C$ seems also to be involved in these advances, which suggests a progressive improvement in constitutive water use efficiency not associated with a trend towards lower stomatal conductance in the most recent genotypes. However, genetic advance does not appear related to changes in photosynthesis rates on area basis when measured in the flag leaf or the spike, but only to a higher, whole-spike photosynthesis. Results also indirectly support the concept that under potential yield conditions, the spike contributed more than the flag leaf to kernel formation.

Keywords: yield potential, grain yield, net carbon exchange, carbon isotopes, nitrogen content

1. Introduction

As the most populous country, answers to the question “Who will feed China?” have been sought by scientist and politicians since the 1990s. The rising Chinese population and the rapid growth of the Chinese economy have resulted in an increasing demand for wheat (*Triticum aestivum L.*) supplies, and this trend will continue for the following decades. The planting area in China is shrinking, because the spring wheat cultivated region is being reduced due to the economic competition with other crops and by the inherent low yield of spring wheat caused by its short growth cycle. Moreover, in spite the high yields achieved in the winter wheat cultivated region, which comprises approximately 94% of China's total wheat production, winter wheat has suffered a slight decrease in planting area (USDA, 2012). Henan province, located in the eastern of Yellow and Huai Valleys Winter-Wheat Zone (YHVWWZ), is the primary winter wheat producer of China. It produces up to 25% of the national production with a planting area of around 5.3 million ha, and a production of ≈ 30.8 million tons (NBS, 2010).

The total wheat production and grain yield per unit area in Henan Province have increased 8.8 and 7.1 times respectively from 1949 to 2007 (Ye et al. 2007). This enhancement in total production and yield of Chinese winter wheat is mainly due to the development of new crop management techniques such as new fertilizers or pesticides, and due to the breeding of new wheat cultivars that have continuously increased the yield potential of wheat (genetic gain) from 1960 to 2010 (Zheng et al. 2011; Xiao et al. 2012).

Worldwide, different breeding strategies have been proposed to speed genetic gains in the next decades. In that context, proper phenotyping is paramount both for conventional breeding as well as to take full advantage of the powerful molecular techniques developed in recent times (Araus et al. 2008; Cabrera-Bosquet et al. 2012). Thus, different phenotyping traits and tools have been proposed, including: (I) plant

agronomic traits associated with the past increase in grain yield (Xiao et al. 2012); (II) high photosynthetic capacity and/or efficiency (Reynolds et al. 2009; Parry et al. 2011), including that of the ear (Araus et al. 1993a); (III) higher efficiency in the use of resources such as water through the stable isotope abundances in plant tissues; and (IV) the optimization of source-sink balance to allow an enhancement of yield potential (Pérez et al. 1989; Schnyder 1993; Zangh et al. 2010, 2012; Aranjuelo et al. 2013).

In the traditional breeding strategy, grain yield components such as kernel number per unit area and/or average individual grain weight have been selected among different genotypes to reach a high yield (Slafer et al. 1996). Meanwhile, some authors have shown that there are other yield components largely associated with the enhancement of yield, such as harvest index (HI), shoot biomass, and kernel number per spike (Yoshida et al. 1972; Rebetzke et al. 2002; Zhou et al. 2007a). High HI was not only the immediate result of the introgression of dwarf *Rht* genes in the middle of the 20th century (Reynolds et al. 2009), it was also due to the continued selection of genotypes with reduced height and increased grain number per unit area in the post-Green Revolution period (Sharma et al. 1985; Zheng et al. 2011). However, Reynolds et al. (2005) showed that the genetic progress of HI in bread wheat has stalled since the mid-1980s. According to Parry et al. (2011) the selection of genotypes with increased biomass and photosynthetic efficiency will go beyond the HI limitation to achieve an increasing harvestable grain number and high yield rates. At the same time, it has been reported for Henan province that breeding since 1970 to the present has not only reduced plant height and increased harvest index but has resulted in increases in spike number per unit area and shoot biomass (Zheng et al. 2011). However, changes in other traits such as the rate of photosynthesis are unclear (Hawkesford et al. 2013). Moreover, breeding changes have not necessarily been uniform across these four decades (Parry and Hawkesford 2010; Zheng et al. 2011; Reynolds et al. 2012; Xiao et al. 2012).

Photosynthesis is one of the most highly integrated and regulated metabolic process to

maximize the use of available light, optimizing the use of limited carbon and nitrogen resources (Paul and Foyer 2001). There is evidence that photosynthesis improvement, simultaneously with well-balanced grain sink strength, was associated with an improvement in yield potential (Fischer et al. 1998; Reynolds et al. 2011). The photosynthetic capacity and efficiency of shoot organs (flag leaf and spike) could be considered as the most important factors among total crop photosynthesis. It has been proposed that increasing leaf net photosynthesis leads to an increase in shoot biomass and hence grain yields (Parry et al. 2011). In particular, under slight stress conditions, a number of authors have reported the positive correlations between grain yield and flag leaf photosynthesis in bread wheat (Shimshi and Ephrat 1975; Richards 2000). In recent years, many studies have revealed that the contribution of spike photosynthesis to grain filling (Araus et al. 1993a; Tambussi et al. 2007) should be considered parallel or even more relevant than the contribution of the flag leaf during the post anthesis period. In that sense, spike traits that contribute to grain filling and ways to increase grain yield need to be further studied by plant biologists and breeders. However, a lack of correlation between photosynthesis and plant yield has been frequently reported among bread wheat genotypes (Makino 2011; Xiao et al. 2012), probably due to the fact that modern cultivars have been bred for photosynthesis. One trait that could be selected to improve the photosynthetic response and then increase yield is the ribulose-1, 5-bisphosphate carboxylase oxygenase (Rubisco) content and/or activity (Parry et al. 2011). During plant growth, especially under stomatal closure, Rubisco abundance and kinetics would be the key limitation to photosynthetic CO_2 assimilation (Parry et al. 2011, 2013).

Based on a good understanding of the physiological and biochemical constraints to yield performance, utilization of key tools that associate wheat physiology traits can provide important information to wheat breeding and agronomy. Carbon stable isotope composition ($\delta^{13}C$) has been proposed as an indirect selection criterion for transpiration efficiency and grain yield in wheat (Araus et al. 2002). The use of $\delta^{13}C$ affords an easy way of screening for grain yield, dry matter, water status and even

photosynthesis (Condon et al. 1990; Araus et al. 1993a). The relationship between $\delta^{13}C$ and grain yield varies from strongly positive (Condon et al. 2002) to strongly negative (Araus et al. 1998) under drought conditions. In many regions such as the Mediterranean Basin, Australia, and China, it was found that $\delta^{13}C$ of the flag leaf and mature kernels correlated negatively with grain yield across genotypes under rain-fed conditions (Araus et al. 1993a, 2002; Rebetzke et al. 2002; Xu et al. 2007). However, other studies have illustrated that the correlation between $\delta^{13}C$ and grain yield is low, in optimal conditions (Fischer et al. 1998; Araus et al. 2003; Monneveux et al. 2005; Serret et al. 2008).

The study of yield potential under field conditions is usually difficult because of natural biotic and abiotic stresses such as drought, plagues etc. that limit the plant yield potential. In order to avoid interference from this stress on yield potential, it is more reasonable to estimate the yield potential of wheat under controlled and favorable conditions. The objective of this study was to study the agronomic and physiological traits responsible for increasing yield potential of winter wheat that have been released in Henan province of China during the last two decades, and to that end, cultivars and advanced lines were included.

2. **Material and Methods**

2.1 Plant material and experimental design

Ten bread wheat genotypes, including 7 milestone cultivars widely cultivated in Henan Province during the past two decades, along with 3 advanced lines bred for high yield potential at Henan, were assayed (Table 1). The selected milestone cultivars had proved their high yield performance in the Yellow and Huai Valleys Winter-Wheat Zone (YHVWWZ), and represent the advancement in wheat breeding in Henan Province from 1995 to 2010. The three advanced lines were selected by

Tianmin Henan Seed Company among ten thousands genotypes for their high yield in a preliminary field assay in Henan province during the 2010/2011 cropping season (www.seedinfo.cn; http://en.tian-min.com).

The experiment was conducted outdoors in the Experimental Field Facilities of the Faculty of Biology, University of Barcelona (Barcelona, Spain), from December 10th, 2011 to June 14th, 2012. Seeds of the ten genotypes were germinated in Petri dishes under 5 ℃ over 15 days. After that, 20 seeds of each genotype were planted in polyvinyl chloride bags filled with an artificial substrate (20 L per bag) following agronomic practices of 400 seeds m^{-2}. The substrate was a mix of gravel (1:5), sand (2:5), and peat (2:5) (v/v) and a slow release fertilizer was added before planting (40 mg of NPK fertilizer for each bag). The experiment was carried out in a randomized complete block design with 3 blocks. Each genotype was assayed with 5 bags (5 replicates) to end up with 150 bags in total (3 blocks x 10 genotypes x 5 replicates = 150 bags). The plants were irrigated with a half-strength Hoagland nutrient solution by drip irrigation to reach the optimum level of nutrition and irrigation. A fungicide (Bupirimate, NIMROD, BRAVOAG, Mexico) was applied, at the flag leaf emergence, spike emergence and the milky stage, to prevent powdery mildew and yellow rust.

2.2 Analysis of total nitrogen content and carbon isotope composition

One week after anthesis, 3 spikes and 3 flag leaves from each plot were randomly collected, and at physiological maturity another 3 spikes were sampled for kernel analysis. All samples were dried at 60 ℃ and the kernels were separated from the spike in order to obtain the samples for the kernel analysis after that all the samples were grounded into a fine powder for the ratio of C^{13}/C^{12} and total N content analyses. For each analysis approximately 1 mg samples of kernels and spikes, and 0.7 mg flag leaves were packed into tin capsules. Measurements were conducted at the Scientific Service Facilities of the University of Barcelona. Total nitrogen concentration was

measured by an elemental gas analyzer (Flash 1112 EA; ThermoFinnigan, Breman, Germany). The same EA coupled with an Isotope Ratio Mass Spectrometer (Delta C IRMS, ThermoFinnigan, Bremen, Germany) operating in continuous flow mode was used to determine the stable carbon ($^{13}C/^{12}C$) . The $^{13}C/^{12}C$ ratios were expressed in δ notation, which was determined according to Farquhar et al. (1989):

$$\delta^{13}C(‰) = [(\frac{R_{sample}}{R_{standard}}) - 1] \quad x \quad 1000$$

Where: sample referred to plant material and standard to the Pee Dee Belemnite calcium carbonate standard. International isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH_7 polyethylene foil, IAEA CH_6 sucrose and USGS 40 L-glutamic acid) were used for calibration with a precision of 0.1‰.

2.3 Gas exchange

Gas exchange parameters were measured on sunny days about one week after anthesis in the central segment of flag leaf blades and the entire spike using a LI-COR 6400 portable photosynthesis system (LICOR biosciences, Lincoln, Nebraska, USA). For each plot the flag leaves and spikes of the main shoots of three plants were measured from 10:00-15:00 (solar time). Net carbon exchange (NCE) was evaluated in ambient CO_2 conditions (approximately 400 µmol mol^{-1} CO_2) with the gas exchange chamber maintained at 25 ℃ and 50% relative humidity (RH). In the case of the flag leaves the standard gas-exchange chamber was used for measuring photosynthesis at light saturation conditions (1800 µmol m^{-2} s^{-1} PPFD), whereas the spikes were placed in a conifer chamber (LICOR biosciences, Lincoln, Nebraska, USA) for measuring photosynthesis under natural light conditions (approximately 1700 µmol m^{-2} s^{-1} PPFD). Following this, dark respiration was assessed by covering the gas exchange chamber with a piece of heavy black cloth until stabilization. Gross Carbon Exchange (GCE) was calculated adding the dark respiration to the net photosynthesis rate . GCE

values were expressed per square meter (μmol CO_2 m^{-2} s^{-1}), per whole organ (nmol CO_2 s^{-1} $organ^{-1}$) or per kernel (nmol CO_2 $kernel^{-1}$ s^{-1}). After gas exchange measurement, the measured flag leaf, was cut and immediately scanned (Canon PIXMA/MP140 scanner, New York, USA) to calculate the area using a commercial software, Digimizer 3.7 (MedCalc Software, Belgium, 2009). Spike area was calculated by measuring the length and width of the four faces of each spike with a millimeter ruler.

2.4 Rubisco activity

Three flag leaf blades and three spikes per plot were harvested one week after anthesis and stored at -80 ℃. Aliquots of 0.5 g of frozen leaves and glumes were ground in a cold mortar using an extraction buffer with 100 mM Bicine-NaOH (pH 7.8), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 2% PVPP (w/v). The extract was clarified by centrifugation at 26,850 g for 10 min at 4 ℃. Enzyme activity was determined by measuring the absorbance at 340 nm with a Cecil CE 7200 spectrophotometer (Cecil Instruments Limited, Cambridge, UK) as described previously by Lilley and Walker (1974).

2.5 Phosphoenol Pyruvate Carboxylase (PEPC) activity

The extraction of PEPC was carried out according to the method of Sayre and Kennedy (1979) with slight modifications. Aliquots of 0.5 g of frozen leaves and glumes collected for the rubisco activity analysis were ground in a cold mortar using an extraction buffer with 100mM Bicine-NaOH (pH 7.8), 10mM$MgCl_2$, 10mM β-mercaptoethanol and 2% PVPP (w/v). The extract was clarified by centrifugation at 26,850 g for 10 min at 4 ℃. Phosphoenol Pyruvate Carboxylase activity was estimated spectrophotometrically (using a Cecil CE 7200 spectrophotometer, Cecil Instruments Limited, Cambridge, UK) coupling the reaction with the oxidation of NADH by malate dehydrogenase (MDH) according to Blanke and Ebert (1992). One

hundred microliters of enzyme extract supernatant were added to a 1ml final reaction solution that contained 50 mmol l^{-1} Tris-HCl (pH7.8), 10 mmol l^{-1} $MgCl_2$, 0.25 mmol l^{-1} EDTA,5.0 mmol l^{-1} $NaHCO_3$,4U of MDH, 0.1 mmol l^{-1} NADH, and 2.0 mmol of phosphoenol pyruvate (PEP).

2.6 Grain yield and yield components

All the plants were harvested at maturity from the three middle bags for measuring the agronomic traits. Before the harvest，plant heights were measured from the soil surface to the tip of the spikes excluding awns. Then the plants were harvested and dried for 48 h at 60℃ to obtain aboveground biomass and grain yield. For estimating other yield components, 5 samples of representative spikes per bag were collected randomly to calculate HI, thousand kernel weight (TKW), kernel number per square meter, spike length, and kernel weight per spike.

2.7 Statistical analysis

The hypothesis of zero difference between means was tested with ANOVA, performed using the general linear model (GLM) procedure to calculate the effect of the different studied genotypes on the measured parameters. Mean separation of genotypes for the measured parameters was done by a Tukey-b multiple comparison test (P <0.05). In order to test the association between grain yield and physiological traits, linear stepwise models across genotypes were constructed, with P = 0.05 as the criterion for variables to be either included or removed from the model. A series matrix of simple coefficient correlations was analyzed based on the yield performance, agronomic and physiological traits of the different genotypes. All the data were analyzed using the SPSS v.16 statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using SigmaPlot 12.0 for Windows (Sysat Software Inc., Point Richmond, CA, USA).

3. Results

The individual traits influencing yield in the set of 10 Chinese bread wheat genotypes from Henan province included in this study were analyzed (Table 1). The analysis of variance (ANOVA) showed that most of the traits were significantly affected by genotype differences (Table 2 and Table 3).

Cultivars/Advanced lines	Year of release	Pedigree
Yumai 35	1995	Mianyang84-27/Neixiang 82C6//Yumai17
Lankao Aizao 6	1998	Lankao(84)184// Wenmai 6
Yumai 66	2000	Yumai 2//Lankao (84)184/Lankao(90)
Zhoumai 18	2004	Neixiang 185/Zhoumai 9
Zhoumai 22	2007	Zhoumai 12/ Wenmai 6//Zhoumai 13
Lankao 198	2009	R81/Bainong 64//Yanzhan 4110
Lankao 298	2010	Lankao Aizao 8// Zhoumai 18
Lankao 0347	2012[a]	Zhengzhou 9023/Yumai 20
Lankao 223	2012[a]	Lankao Aizao 8/Lankao(84)184 // Zhoumai 18
Lankao 282	2012[a]	Hebei 94014//90(6)21-4

[a] under trial seed testing.

Resource: www.seedinfo.cn; http://en.tian-min.com.

Table 1. Name, year of release and pedigree of 10 Chinese winter wheat genotypes from the Henan province of China assayed at the Experimental Fields of the University of Barcelona.

3.1 Grain yield and yield components

Grain yield of different genotypes varied significantly, ranging from 5.55 Mg ha^{-1} for Yumai 35 released in 1995 to 14.84 Mg ha^{-1} for the advanced line Lankao 282. The mean grain yield of all genotypes was 9.12 Mg ha^{-1} (Table 2). Yield was positively correlated with the year of release (r^2 = 0.60, $P \leq 0.01$), with the three advanced lines showing higher grain yield than the varieties already released (Fig. 1A). The HI showed a highly significant difference between genotypes, while aboveground biomass showed a slight, albeit significant increase (Table 2). In fact, the year of release was significantly and positively correlated with grain yield, biomass and HI (Fig. 1A, B, C) as well as with kernel number per square meter (r = 0.79, $p \leq 0.001$)

(data not shown). Spike weight, kernel weight per spike, and thousand kernel weight (TKW) also revealed clear differences between genotypes (Table 2), but they did not show a clear improvement trend according to the year of release (data not shown). Plant height of commercial genotypes showed a decreasing trend from the oldest to the newest genotypes, except for the dwarf genotype Lankao Aizao 6 (Table 2). However, the advanced lines showed a significantly greater height than the commercial genotypes, showing also higher yield as a consequence of a general increase in plant biomass (Table 2).

In order to test which yield components were better related to grain yield, a regression test between grain yield and yield components was performed (Table 4). The highest significant correlation was observed between grain yield and HI ($r = 0.90$, $P \leq 0.001$), followed by kernel number per square meter, aboveground biomass, and kernel number per spike ($r = 0.80$, $P \leq 0.001$; $r = 0.78$, $P \leq 0.001$; $r = 0.71$, $P \leq 0.001$, respectively). The linear relationships of spike length and spike weight with grain yield were also significant, but lower ($r = 0.62$, $P \leq 0.001$; $r = 0.50$, $P \leq 0.01$, respectively). Plant height affected grain yield weakly ($r = 0.44$, $P \leq 0.05$), while TKW did not influence it significantly (Table 4).

Genotype	Grain yield (t ha^{-1})	Biomass (t ha^{-1})	HI	Spike length (cm)	Spike weight (g)	Awn length (cm)	Spike number m^{-2}	Kernel number $spike^{-1}$	Kernel number m^{-2}	Plant height (cm)	TKW (g)
Yumai 35	5.55 c	22.5 ab	0.23 c	7.90 f	1.86 c	1.63 f	363.3 bc	30.5 d	10654 c	82.8 bc	55.15 a
Lankao Aizao6	7.31 bc	20.9 ab	0.31 bc	10.36 de	2.89 bc	3.34 de	335.6 c	52.2 b	14289 bc	72.4 d	47.04 ab
Yumai 66	5.37 c	15.8 b	0.28 bc	11.49 c	2.30 bc	3.38 bcd	284.4 c	51.3 b	10742 c	89.6 ab	40.21 ab
Zhoumai 18	8.35 bc	26.0 ab	0.32 bc	9.39 e	1.89 c	2.88 e	573.3 abc	51.0 b	19553 bc	75.6 cd	40.91 ab
Zhoumai 22	9.45 abc	29.1 ab	0.35 abc	9.50 e	2.47 bc	3.02 e	506.7 abc	36.6 cd	17536 bc	83.3 bc	59.87 a
Lankao 198	7.19 bc	28.3 ab	0.30 bc	9.43 e	1.72 c	4.09 bc	753.3 a	44.1 bc	27617 ab	75.7 cd	30.42 b
Lankao 298	7.63 bc	27.0 ab	0.30 bc	9.88 de	1.87 c	3.57 cd	650.0 ab	35.5 cd	15567 bc	73.5 cd	52.11 a
Lankao 0347	12.51 ab	36.8 a	0.38 ab	10.60 cd	3.58 ab	4.17 b	508.9 abc	53.2 b	24824 ab	87.0 ab	53.50 a
Laokao 223	12.96 ab	33.5 a	0.37 ab	14.20 a	4.91 a	5.60 a	513.3 abc	76.1 a	24448 ab	95.4 a	52.42 a
Lankao282	14.84 a	31.4 ab	0.46 a	12.87 b	2.27 bc	3.94 bc	346.7 c	70.6 a	35032 a	92.2 ab	47.08 ab
Mean	9.12	27.1	0.33	10.56	2.58	3.39	353.6	50.1	20026	82.8	47.87
Replicate	0.94	11.7	0.01	0.52	1.25	0.04	4065	39.1	372 10^5	134.4	71.9
Genotype	287.24***	1042*	0.17***	92.50***	26.73***	28.60**	6.1 10^5***	5783***	1.7 10^9***	1828***	2040***
Error	81.40	603.7	0.02	2.60	6.00	0.82	1.9 10^5	485.4	4.3 10^8	244.7	841.8

Table 2. Mean values and sum of squares type III combined with analysis of variance for grain yield and agronomic yield components of 7 winter wheat cultivars and 3 advanced lines from Henan province, China. Abbreviations used: aboveground biomass (Biomass), harvest index (HI), and thousand kernel weight (TKW). The values are the means for each genotype of 3 replicates per block from a total of three blocks. Means followed by the same letter were not significantly different at $P = 0.05$ by the Tukey-b's test (*, $P \leq 0.05$; **, $P \leq 0.01$ and ***, $P \leq 0.001$).

	Spikes				Flag leaves				Kernels			
	Mean	Range	SE	Genotype	Mean	Range	SE	Genotype	Mean	Range	SE	Genotype
Dry weight (g)	0.75	0.33 – 1.19	0.04	1.21***	0.24	0.13 – 0.40	0.01	0.13***	2.52	1.01 – 4.21	0.15	12.28**
N content (mg g^{-1})	15.22	6.47 – 23.26	0.85	426.08**	11.94	6.79 – 18.08	0.57	220.8***	60.48	25.85 – 103.91	3.91	11241***
$\delta^{13}C$ (‰)	-27.92	-28.69 – -27.00	0.08	3.69**	-28.98	-29.87 – -27.93	0.08	3.96**	-28.48	-29.55 – -27.62	0.08	3.38**
N concentration (%)	2.04	1.75 – 2.33	0.02	0.29**	4.96	4.24 – 5.82	0.06	2.41***	2.54	2.00 – 2.97	0.04	0.42
g_s (mmol m^{-2} s^{-1})	89.62	56.2 – 129.6	3.65	7208***	461.9	319.0 – 652.7	13.37	51442				
GCE m^{-2}(µmol m^{-2} s^{-1})	7.68	4.71 – 10.35	0.24	30.53**	34.25	26.34 – 40.38	0.52	109.38				
GCE $organ^{-1}$ (nmol min^{-1} $organ^{-1}$)	34.8	14.5 – 55.8	1.6	1173	125.7	74.3 – 226.4	6.09	18236**				
GCE $kernel^{-1}$ (nmol min^{-1} $kernel^{-1}$)	7.2	4.3 – 10.9	0.32	63.11***	26.1	15.7 – 42.8	1.23	1185***				
Rubisco activity (nmol s^{-1} g^{-1})	4.29	1.06 – 13.24	0.51	200.1***	23.00	13.42 – 41.10	1.13	749.0**				
PEPC activity (nmol min^{-1} g^{-1})	20.91	8.08 – 34.43	1.32	1329.4***	33.54	16.69 – 55.97	2.28	3457***				

Table 3: Mean value, range, standard error, and sum of squares of different physiological parameters of 7 winter wheat cultivars and 3 advanced lines from Henan province, China. Abbreviations used: carbon isotope composition ($\delta^{13}C$), , stomatal conductance (g_s), gross carbon exchange per square meter (GCE m^{-2}), gross carbon exchange per whole organ (GCE $organ^{-1}$), gross carbon exchange per kernel (GCE $kernel^{-1}$). (*, $P \leq 0.05$; **, $P \leq 0.01$ and ***, $P \leq 0.001$).

	Grain yield	Biomass	HI	Spike length	Spike weight	Awn length	Spike number m^{-2}	Kernel number $spike^{-1}$	Kernel number m^{-2}	Plant height
Biomass	0.78***									
HI	0.90***	0.52**								
Spike length	0.62***	0.32	0.57***							
Spike weight	0.50**	0.46*	0.32	0.66***						
Awn length	0.59**	0.31	0.71***	0.66***	0.18					
Spike number m^{-2}	0.23	0.53**	0.20	-0.27	-0.39*	0.22				
Kernel number $spike^{-1}$	0.71***	0.35	0.67***	0.89***	0.63***	0.60***	-0.22			
Kernel number m^{-2}	0.80***	0.73***	0.72***	0.46**	0.15	0.66***	0.57***	0.58***		
Plant height	0.44*	0.19	0.38*	0.64***	0.45*	0.38*	-0.34	0.55**	0.29	
TKW	0.31	0.19	0.24	-0.02	0.36*	-0.20	-0.28	-0.08	-0.22	0.12

Table 4: Pearson correlation coefficients among grain yield and several agronomic yield components across the set of 10 Chinese winter wheat genotypes. Abbreviations used as in Table 2. (*, $P \leq 0.05$; **, $P \leq 0.01$ and ***, $P \leq 0.001$).

3.2 Grain yield and physiology traits among organs

3.2.1 Organ dry weight and nitrogen content

At anthesis there were significant differences across genotypes for spike and flag leaf dry weight, and total N content and N concentration (Table 3). Total N content of spikes (15.22 mg) was about 30% higher than that of the flag leaf (11.94 mg) in spite of the much lower N percentage in the spike (2.04%) compared with the flag leaf (4.96%). Grain yield was negatively correlated with spike N concentration (r = -0.40, $P \leq 0.05$) (Table 5). However, grain yield was not correlated with the dry weight and N concentration of the flag leaf (Table 6). The kernel N content per spike was highly and positively correlated with the spike N content; however, flag leaf N content was not correlated with kernel or spike N content (Table 5; Table 6).

3.2.2 Carbon isotopes composition

For the set of genotypes assayed, $\delta^{13}C$ values varied significantly among genotypes and organs (Table 3, Supplementary Table 1 and Supplementary Table 2). Significant differences among plant parts were observed for $\delta^{13}C$, with values increasing from the older genotypes to the advanced lines (Table 3, Supplementary Table 1 and Supplementary Table 2). In fact, grain yield was significantly and positively linearly associated with the $\delta^{13}C$ of kernels (r = 0.56, $P \leq 0.001$), spikes (r = 0.52, $P \leq 0.01$) and flag leaves (r = 0.50, $P \leq 0.01$) (Table 5, 6). Moreover, the $\delta^{13}C$ of spikes showed a highly significant and positive correlation with spike dry weight (r = 0.69 $P < 0.001$) (Table 5). In addition, kernel dry weight was positively correlated with $\delta^{13}C$ of kernels (r = 0.54, $P \leq 0.01$) (Table 6). Moreover, kernel $\delta^{13}C$ increased linearly with the year of release (r^2 = 0.55, $P \leq 0.05$) (Figure 1 D). However, flag leaf and spike $\delta^{13}C$ did not show a linear relationship with the year of release (data not shown).

	Grain yield	Biomass	Kernel dry weight spike^{-1}	Kernel N content spike^{-1}	Kernel $\delta^{13}C$	Kernel N concentration	Spike dry weight	Spike N content	Spike $\delta^{13}C$	Spike N concentration	Spike g_s	GCE spike^{-1}	Spike GCE m^{-2}	Spike GCE k^{-1}	Glume Rubisco activity	Glume PEPC activity
Biomass	0.78***															
Kernel dry weight spike^{-1}	0.71***	0.41*														
Kernel N content spike^{-1}	0.77***	0.42*	0.83***													
Kernel $\delta^{13}C$	0.56***	0.44*	0.54**	0.47**												
Kernel N concentration	-0.09	-0.06	-0.23	0.13	0.00											
Spike dry weight	0.56***	0.36	0.48**	0.63***	0.56***	0.12										
Spike N content	0.46**	0.3	0.41*	0.57***	0.49**	0.13	0.98***									
Spike $\delta^{13}C$	0.52**	0.37*	0.56***	0.63***	0.45*	-0.08	0.69***	0.66***								
Spike N concentration	-0.40*	-0.24	-0.36	-0.33	-0.40*	-0.03	-0.32	-0.11	-0.24							
Spike gs	0.17	0.11	-0.04	0.09	0.09	0.24	0.01	0.04	-0.10	0.15						
GCE spike^{-1}	0.40*	0.23	0.41*	0.39*	0.38*	0.08	0.41*	0.41*	0.41*	-0.10	0.48**					
Spike GCE m^{-2}	0.21	0.20	0.04	0.10	0.10	0.13	0.05	0.06	0.05	0.09	0.79***	0.62***				
Spike GCE k^{-1}	-0.29	-0.05	-0.33	-0.47**	-0.20	0.07	-0.41*	-0.33	-0.29	0.46**	0.56***	0.36	0.64***			
Glume Rubisco activity	0.11	0.20	-0.02	-0.16	0.15	-0.18	-0.10	-0.02	0.11	0.47**	0.28	0.10	0.21	0.39*		
Glume PEPC activity	-0.03	0.18	0.08	-0.18	0.15	-0.34	-0.39*	-0.40*	-0.10	0.07	-0.13	-0.19	-0.19	0.14	0.23	
Flag N content	0.25	-0.09	0.34	0.61***	0.38*	0.24	0.51**	0.50**	0.32	-0.18	0.04	0.25	-0.11	-0.45*	-0.31	-0.36*

Table 5: Pearson correlation coefficients among grain yield, aboveground biomass and physiological parameters. Abbreviations used as in Table 3. (*, $P \leq 0.05$; **, $P \leq 0.01$ and ***, $P \leq 0.001$).

	Grain yield	Biomass	Kernel dry weight spike^{-1}	Kernel N content spike^{-1}	Kernel $\delta^{13}C$	Kernel N concentration	Flag dry weight	Flag N content	Flag $\delta^{13}C$	Flag N concentration	Flag g_s	GCE flag^{-1}	Flag GCE m^{-2}	Flag GCE K^{-1}	Flag Rubisco activity
Biomass	0.78***														
Kernel dry weight spike^{-1}	0.71***	0.41*													
Kernel N content spike^{-1}	0.77***	0.42*	0.83***												
Kernel $\delta^{13}C$	0.56***	0.44*	0.54**	0.47**											
Kernel N concentration	-0.09	-0.06	-0.23	0.13	0.00										
Flag dry weight	0.29	-0.10	0.36*	0.61***	0.43*	0.24									
Flag N content	0.25	-0.10	0.34	0.61***	0.38*	0.24	0.98***								
Flag $\delta^{13}C$	0.50**	0.34	0.63***	0.61***	0.43*	-0.16	0.33	0.32							
Flag N concentration	-0.24	0.09	-0.26	-0.37*	-0.45*	-0.19	-0.65***	-0.49**	-0.18						
Flag g_s	-0.25	-0.38*	-0.16	-0.12	-0.04	0.32	0.19	0.09	-0.04	-0.41*					
GCE flag^{-1}	0.01	0.23	0.18	0.37*	0.16	0.25	0.84***	0.80***	0.14	-0.63***	0.47**				
Flag GCE m^{-2}	-0.34	-0.33	-0.28	-0.24	0.01	0.29	0.08	0.02	-0.07	-0.26	0.58***	0.33			
Flag GCE kernel^{-1}	-0.59***	-0.49**	-0.36*	-0.48**	-0.45*	-0.02	-0.19	-0.20	-0.52**	0.06	0.34	0.23	0.55**		
Flag Rubisco activity	-0.20	-0.32	0.02	0.06	-0.23	-0.13	0.21	0.32	0.04	0.30	-0.18	0.19	-0.15	0.02	
Flag PEPc activity	-0.28	-0.08	-0.20	-0.35	-0.16	-0.11	-0.52**	-0.46*	-0.26	0.51**	-0.15	-0.47**	0.22	0.30	0.27

Table 6: Pearson correlation coefficients among grain yield, aboveground biomass and physiological parameters. Abbreviations used as in Table 3. (*, $P \leq 0.05$; **, $P \leq 0.01$ and ***, $P \leq 0.001$).

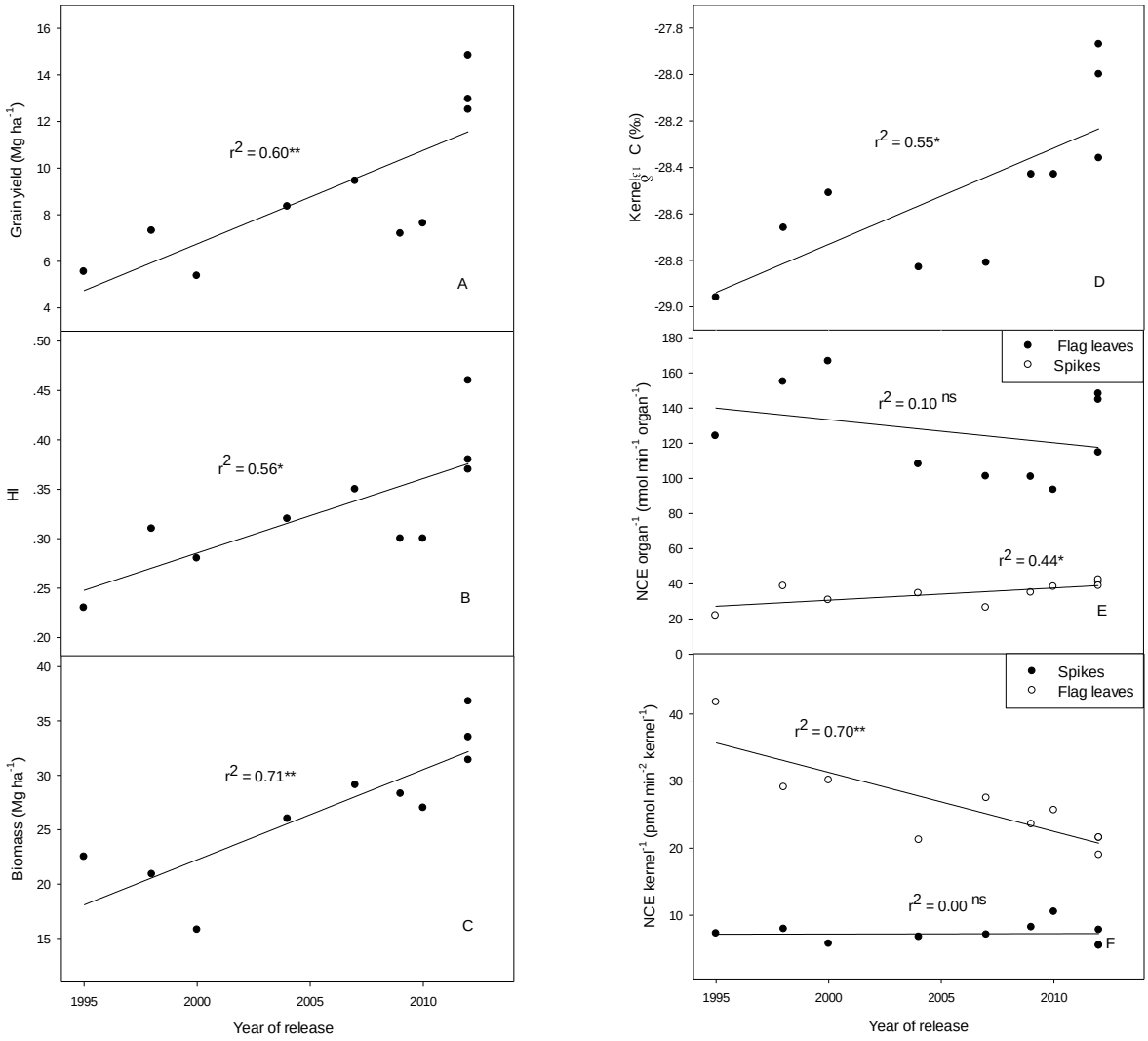

Figure 1. Linear regression on year of release for grain yield (A), harvest index (HI) (B), aboveground biomass (C), kernel isotope composition (D), gross carbon exchange per whole organ (GCE/organ) of spikes and flag leaves (E), and gross carbon exchange per kernel of flag leaves and spikes (F) for the set of 10 Chinese wheat genotypes from Henan province, China, released from 1995 to 2012. The genotypes released in 2012 correspond to advanced lines. Determination coefficient (r^2) and probabilities are given. (ns, no significant; *, $P < 0.05$; **, $P < 0.01$).

3.2.3 Net carbon exchange and stomatal conductance

The stomatal conductance (g_s) of the spike showed a significant difference among genotypes, whereas flag leaf g_s did not. Moreover, gross carbon exchange per unit area (GCE m^{-2}) of the flag leaf did not change among genotypes while spike GCE m^{-2} did (Table 3, Supplementary Table 2). Although the spike and flag leaf g_s did not display a linear tendency with grain yield, positive linear correlations between GCE m^{-2} and g_s in both organs were found ($r = 0.58$, $P \leq 0.001$ for the flag and $r = 0.79$, $P \leq 0.001$, for the spike) (Table 5 and Table 6). Meanwhile, gross carbon exchange per whole organ (GCE $organ^{-1}$) when measured in the flag leaf showed significant differences between genotypes and did not correlate with grain yield (Table 3 and Table 6). In contrast, GCE per whole spike was positively correlated with grain yield in spite of no significant difference among genotypes ($r = 0.40$, $P \leq 0.05$) (Table 3, Table 5). Flag GCE per kernel unit was negatively correlated with grain yield ($r = -0.59$, $P < 0.001$), while spike GCE per kernel unit was not (Table 5). Spike and flag GCE per kernel unit correlated negatively with kernel N content per spike ($r = -0.47$, $P \leq 0.01$; $r = -0.48$, $P \leq 0.01$, respectively).

3.2.4 Phosphoenolpyruvate carboxylase (PEPC) and Rubisco activity

The PEPC activity per unit dry matter of glumes and the flag leaf showed substantial differences among genotypes (Table 3). For almost all genotypes, the flag leaf PEPC activity per dry matter was higher than that in the glumes (Supplementary Table 1 and Supplementary Table 2). In addition PEPC activities of the flag leaf and glumes were negatively correlated with the dry weight of those organs ($r = -0.52$, $P \leq 0.01$ for the flag; $r = -0.39$, $P \leq 0.05$, for the spike). In the flag leaf, the PEPC activity was positively influenced by the N concentration ($r = 0.51$, $P \leq 0.05$); however, this trend was not observed in the spike (Table 5, Table 6). On another aspect, the genotypic effect was significant for Rubisco activity on a dry weight basis of the flag leaf and glumes (Table 3). Rubisco activity per dry matter in glumes showed far lower values than the flag leaves. However, the Rubisco activity of glumes was positively correlated with spike N concentration ($r = 0.47$, $P \leq 0.01$), while flag leaf Rubisco activity was not correlated with flag leaf N concentration (Table 5 and Table 6).

3.3 Overall genotypic difference

A stepwise regression was performed to study which physiological traits influenced grain yield as independent variables for the 10 genotypes. Thus, the independent variables analyzed were $\delta^{13}C$, N concentration, g_s, GCE organ^{-1}, GCE m^{-2}, Rubisco activity per unit dry matter of the flag leaf and the spike, PEPC activity per unit dry matter of the flag leaf and spike (Table 7). The first trait selected was kernel $\delta^{13}C$, while flag GCE m^{-2} was ranked next. The $\delta^{13}C$ of the spike was selected as the last parameter, while flag leaf $\delta^{13}C$ was not chosen.

	Variable chosen			Final stepwise model	r^2	Significance
GY	Kernel $\delta^{13}C$			$GY = 136.23 + 4.46\ \delta^{13}C_{kernel}$	0.31	***
	Kernel $\delta^{13}C$,	Flag GCE m^{-2}		$GY = 151.92 + 4.49\ \delta^{13}C_{kernel} - 0.43$ GCE m$^{-2}_{flag}$	0.43	***
	Kernel $\delta^{13}C$,	Flag GCE m^{-2},	Spike $\delta^{13}C$	$GY = 195.13 + 3.24\ \delta^{13}C_{kernel} - 0.44$ GCE m$^{-2}_{flag} + 2.81\ \delta^{13}C_{spike}$	0.53	***

Table 7: Stepwise analysis for grain yield for the whole set of 10 genotypes of Chinese wheat, the grain yield (GY) as the dependent variable and the independent variables as follows: $\delta^{13}C$ and N concentration of the spike, flag leaf and kernels, g_s, GCE organ^{-1}, GCE m^{-2}, Rubisco activity of the flag leaf leaf and the glumes, PEPC activity, of the flag leaf and glumes as independent variables (***, $P \leq 0.001$).

4. Discussion

Due to an increasing human population and decreasing cropping area, improvement of grain yield is the ongoing primary objective of the Chinese wheat breeding program (Zheng et al. 2011; Xiao et al. 2012). The present study, which focused on recent breeding advances on grain yield of wheats from Henan, showed a generally increasing trend of grain yield with the year of release (Fig. 1A). A genetic advance in yield during the past decades has been reported for different regions of China (Zheng et al. 2011; Xiao et al. 2012) In addition, a positive relationship between grain yield and year of release has been also found in Australia, UK and USA for different cereals (Araus et al. 2008). In our result, the advanced lines developed during the year 2012 showed a remarkably higher grain yield than the older genotypes (Fig. 1A, Table 2),

revealing that the yield potential could be further enhanced by breeding strategies.

The present result indicated that the increase in grain yield depended mainly on HI, kernel number per square meter, and aboveground biomass (Table 4), which agrees with previous reports for wheat in China (Zhou et al. 2007; Xiao et al. 2012). In addition, the HI and aboveground biomass showed an increasing trend with the year of release (Fig. 1B, C). Contrary to this, Zheng et al. (2011) found that grain yield was directly attributed to an increase in thousand kernel weight (TKW), which also contributed to the significant increase in HI. These results were different from ours, which showed that grain yield was closely related to kernel number per square meter but was not related to TKW (Table 4). Such differences observed in traits that had an influence on yield potential could be due to the different strategies related to agronomic and physiological advances employed during the last decades (Hawkesford et al. 2013). However, Foulkes et al. (2011) suggested in a review analyzing the main targets for improving yield potential that the main objectives for breeders should be enhancement of HI through an increase in grain/kernel number per unit area. In the current work, HI showed the highest correlation coefficient with grain yield, reflecting that HI could be the first trait responsible for genetic advances in yield potential. At the same time, kernel number per square meter was the second trait in importance for influencing grain yield (Table 2, Table 4) and it could be the cause of the enhancement of the HI and grain yield, as had been pointed out by a number of authors (Fischer et al. 1998, 2008; Foulkes et al. 2011). Aboveground biomass was the third parameter best correlated with grain yield (Table 3) and showed a significant positive correlation between aboveground biomass and the year of release (Fig. 1C). Therefore, it could be an important aim for future breeding to increase the biomass production while maintaining or even increasing the HI (Foulkes et al. 2007). Although the Chinese breeders have reached a great achievement in their breeding strategy to increase HI (Zhou et al. 2007), the advanced lines assayed in the present study showed higher HI than current cultivars, suggesting that a further increase in plant partitioning towards grain yield might be possible (Table 2, Table 4). In fact, the theoretical maximum HI of 0.62 (Austin, 1980) is still far above the experimental values achieved.

Positive correlations between grain yield and the $\delta^{13}C$ of the flag leaf, spike and kernel were observed (Table 5; Table 6). These correlations indicated that genotypes with higher grain yield (the newest ones) showed an enhancement of ^{13}C isotope content. This result differed from those obtained in other field studies where grain yield was negatively correlated with the $\delta^{13}C$ of upper (penultimate and flag) leaves and kernels (Fischer et al. 1998; Araus et al. 1998; Merah et al. 2001). However, Craufurd et al. (1991) obtained similar results as in this experiment, showing a positive correlation between $\delta^{13}C$ and grain yield in barley grown under well-watered field conditions. According to previous reports, the increase in $\delta^{13}C$ may be due to stomatal closure (low stomatal conductance, and consequently less CO_2 diffusion) due to drought and/or a higher photosynthetic capacity (Farquhar and Sharkey 1982; Ehdaie et al. 1991). In fact, most of the negative relationships between $\delta^{13}C$ and grain yield reported in the literature refer to field studies where the plants suffer some degree of water stress, even if under irrigation, and they grow directly in the soil allowing different genotypes to explore water resources in the soil in a different manner (Condon et al. 1987). In other words, negative relationship between $\delta^{13}C$ and grain yield is probably sustained by genotypic differences in water use instead of water use efficiency (Blum 2009). Nevertheless, as described in the material and methods section, the plants were fully irrigated depending on their needs. Moreover, the estimated yields as well as the values of analyzed $\delta^{13}C$ were well in the range of reports for well-watered genotypes (Craufurd et al. 1991; Zheng et al. 2011; Xiao et al. 2012). In addition, the g_s of the flag leaves (Table 3) ranged similar to that reported in a previous study with Chinese wheat under well watered conditions (Lu et al. 1998). Therefore, the increase in $\delta^{13}C$ of more recent genotypes was not due to the emergence of drought stress. In the same sense the lack of a relationship of the $\delta^{13}C$ of flag leaves and spikes with their g_s, revealed that genotypic variability in $\delta^{13}C$ was due to intrinsic differences in photosynthetic capacity (Farquhar and Richards 1984). In that sense, newer genotypes should exhibit higher intrinsic photosynthetic capacity. This was despite the fact that the newer genotypes exhibited lower N concentration in the photosynthetic organs, which might eventually result in decreasing $\delta^{13}C$ (Shangguan et al. 2000). However, no differences in Rubisco activity were found among genotypes in spite of the lower N concentration of most recent genotypes. In fact, a positive effect on photosynthesis of a larger sink had been reported (Kaschuck

et al. 2010; Aranjuelo et al. 2013) and as the dry weight and size of the spike could be considered as an index of sink strength (Schnyder et al. 2003); therefore, the genotypes with bigger spikes had higher sink strength, displaying a increased photosynthesis that was shown by the positive relationship of GCE per spike with grain yield and spike dry weight (Table 5).

The $\delta^{13}C$ also showed different values among plant organs; spikes had a higher $\delta^{13}C$ than the flag leaves, possibly due to the lower g_s of spikes in comparison to flag leaves. Moreover, glumes can re-fix carbon released from respiration of kernels (Araus et al. 1993a). In fact, for each plot the value of kernel $\delta^{13}C$ was in between the $\delta^{13}C$ of the spike and flag leaf, but usually closer to the value of one of the two organs (Table 3) indicating which photosynthetic organ contributed more to grain filling. However, in an experiment conducted by Aranjuelo et al. (2011) using ^{13}C labeling, it was shown that during the beginning of post-anthesis the C fixed by the flag leaf was stored as structural C compounds, starch, and soluble sugars and then respired. Only a small amount of those soluble sugars arrived to the spike. On the other hand, the C synthesized in the spike was directed towards grain development.

In agreement with previous results (Austin 1994; Fischer et al. 1998; Xiao et al. 2012), flag leaf GCE per unit area did not change significantly among genotypes (Table 3; Supplemental Table 1). However, spike GCE per unit area showed differences between cultivars (Table 4; Supplemental Table 2) possibly due to differences in respiration (data not shown). Moreover, flag leaf and spike GCE per organ changed among genotypes due to the variable leaf area and spike size among different cultivars (Table 2, Supplementary Tables 1 and 2). In this study, GCE per whole organ when measured in the flag leaf was not correlated with grain yield (Table 6; Fig. 1E), suggesting that the GCE of this organ has a minor role in grain filling (Aranjuelo et al. 2011). Nevertheless, the GCE per organ when measured in the spike showed a positive correlation with grain yield, possibly due to the fact that grain yield per spike was largely associated with the size of the spike (Table 3; Table 5; Figure 1E). A larger spike may exhibit not only a higher sink size but also an increased photosynthetic capacity. Thus, spike photosynthesis has an important contribution to grain filling, with the awns being the main photosynthetic organ (Tambussi et al. 2007). As has been explained, grain yield depends on the spike size and/or number of

kernels per spike (sink) and the availability of assimilates fixed by photosynthetic organs (source) to fill these grains (Zhang et al. 2010). Thus, GCE of the flag leaf and of the spike expressed per unit kernel (Flag leaf GCE kernel^{-1} or Spike GCE kernel^{-1}) may represent the balance between source (photosynthesis or GCE) and sink strength (kernel). Flag leaf GCE kernel^{-1} decreased in the newest cultivars and was negatively correlated with grain yield and year of release. However, the spike GCE kernel^{-1} did not show any linear relationship with grain yield or year of release (Table 6, Fig. 1F). This could mean that, in spite of their higher yield, newer genotypes showed a balanced source-sink relationship in terms of spike photosynthesis, while the contribution of the flag leaf assimilates to the kernels had further decreased in more recent genotypes. Beside the above considerations, the increase in grain yield caused by an enhanced sink capacity could be the reason for the decrease in the N concentration in both the spikes and the flag leaves of the newer genotypes, whereas no differences occurred in the N concentration of kernels (Table 3, Supplementary Tables 1 and 2).

In recent years, Zhang et al. (2008, 2012) had shown that PEPC may be involved in protein biosynthesis during grain development and that it could have an important role in regulating C and N metabolism in the spike of wheat. This enzyme synthesizes oxaloacetate by the carboxylation of phosphoenol pyruvate and it is also a key step in the synthesis of aspartate, and malate, used for N accumulation and transport by anaplerotic CO_2 fixation in crops (Smith et al. 1989； Araus et al. 1993b). Therefore, PEPC may have a particular role in the spike, contributing to glume re-fixation of the CO_2 respired by growing grains (Araus et al. 1993b) as well as N partitioning from the spike tissues into the kernels (Mi et al. 2000). In our results, the PEPC activity was significantly and negatively correlated with dry weight of flag leaves and spikes, and positively correlated with N concentration of the flag leaves but not with that of the glumes (Table 5; Table 6). Moreover, in our study the newest genotypes, which had larger spikes and grain yield, did not exhibit higher PEPC activity, possibly due to a dilution phenomenon as had been observed for the N concentration (Table 5). Although the activities of PEPC and Rubisco enzymes showed significant variations between genotypes in different parts of plants, they were, however, unsuitable for assessing genotypic yield performance due to the lack of correlation with grain yield

(Table 5 and Table 6). Moreover, there were no relationships between year of release and activity of either of these enzymes. In the same sense, there were no genotypic differences in the gross carbon exchange rates of the flag leaf on an area basis. By contrast, the carbon stable isotope compositions were more appropriate for genotype differentiation and for higher grain yield prediction. This statement was confirmed by the stepwise analysis of grain yield as dependent of the physiological traits. In this analysis kernel $\delta^{13}C$ and spike $\delta^{13}C$ were the best traits explaining the genotypic differences in grain yield (Table 7). The GCE per square meter when measured in the flag leaf was also included but with a negative sign, meaning that those genotypes with higher yield exhibited lower photosynthetic rates per unit leaf area. In fact, a tendency to lower photosynthetic rates per unit leaf area in the modern cultivars has been reported (Austin RB et al. 1989; Del Blanco et al. 2000). Similar to our study, these studies showed a trend associated with a lower N concentration in photosynthetic tissues due to a dilution effect.

Therefore we can conclude that bread wheat breeding advances during recent decades in Henan, China, have been achieved through an increase in HI, kernel number per square meter and aboveground biomass. A higher $\delta^{13}C$ seems also to be involved in these advances, which suggests a progressive improvement in constitutive water use efficiency not associated with a trend towards lower stomatal conductance in the most recent genotypes. However, genetic advance does not appear related to changes in photosynthesis rates on an area basis when measured in the flag leaf or the spike, but only to a higher, whole-spike photosynthesis. In the same sense, Rubisco and PEPC activities on a dry matter basis did not change with breeding advances. Results also indirectly support the concept that under potential yield conditions the spike contributed more than the flag leaf to kernel formation.

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References

Aranjuelo I, Cabrera-Bosquet L, Morcuende R, Avice JC, Nogués S, Araus JL, Martínez-Carrasco R, Pérez P (2011) Does ear C sink strength contribute to overcoming photosynthetic acclimation of wheat plants exposed to elevated CO_2? **Journal of Experimental Botany** 62, 3957-3969

Aranjuelo I, Sanz-Sáez A, Jauregui I, Irigoyen JJ, Araus JL, Sánchez-Díaz, Erice G (2013) Harvest index, a parameter conditioning responsiveness of wheat plants to elevated CO_2. **Journal of Experimental Botany** doi:10.1093/jxb/ert081

Araus JL, Brown HR, Febrero A, Bort J, Serret MD (1993a) Ear photosynthesis, carbon isotope discrimination and the contribution of respiratory CO_2 to differences in grain mass in durum wheat. **Plant, Cell & Environment** 16, 383–392

Araus JL, Bort J, Brown HR, Bassett C, Cortadellas N **(**1993b) Immunocytochemical localization of phosphoenolpyruvate carboxylase and photosynthetic gas exchange characteristics in ears of *Triticum durum* Desf. **Planta.** 191, 507-514

Araus JL, Amaro T, Casadesus J, Asbati A, Nachit MM (1998) Relationship between ash content, carbon isotope discrimination and yield in durum wheat. **Australian Journal of Plant Physiology** 25, 835–842

Araus JL, Slafer GA, ReynoldsMP, Royo C (2002) Plant breeding and water relations in C3 cereals: what should we breed for? **Annals of Botany-London** 89, 925–940

Araus JL, Villegas D, Aparicio N, del Moral LFG, El Hani S, Rharrabti Y, Ferrio JP, Royo C (2003) Environmental factors determining carbon isotope discrimination and yield in durum wheat under Mediterranean conditions. **Crop Science** 43, 180-190

Araus JL, Slafer GA, Royo C, Serret MD (2008) Breeding for yield potential and stress adaptation in cereals. **Plant Science** 27, 377–412

Austin RB, Bingham J, Blackwell RD, Evans LT, Ford MA, Morgan CL, Taylor M (1980) Genetic improvement in winter wheat yield since 1900 and associated physiological changes. **The Journal of Agricultural Science** 94, 675–689

Austin RB (1989) Genetic variation in photosynthesis. **The Journal of Agricultural Science** 112, 287-294

Austin RB (1994) Plant Breeding opportunities. *In* **International Symposium on Physiology and Determination of Crop Yield**. Boote, KJ; Bennett, JM; Sinclair, TR; et al. Eds. Gainesville, Florida, USA. pp 567-586

Blanke MM and Ebert G (1992) Phosphoenolpyruvate carboxylase and carbon economy of apple seedlings. **Journal of Experimental Botany** 43, 965-968

Blum A (2009) Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop yield improvement under drought stress. **Field Crops Research** 112, 119–123

Cabrera-Bosquet L, Crossa J, von Zitzewitz J; Serret MD, Araus JL (2012) High-throughput Phenotyping and Genomic Selection: The Frontiers of Crop Breeding Converge. **Journal of Integrative Plant Biology** 54, 312-320

Condon AG, Richards RA, Farquhar GD (1987) Carbon isotope discrimination is positively correlated with grain yield and dry matter production in field-grown wheat. **Crop Science** 27, 996–1001

Condon AG, Farquhar GD, Richards RA (1990) Genotypic variation in carbon isotope discrimination and transpiration efficiency in wheat leaf gas exchange and whole plant studies. **Australian Journal of Plant Physiology** 17, 9-22

Condon AG, Richards RA, Rebetzke GJ, Farquhar GD（2002）Improving intrinsic water-use efficiency and crop yield. **Crop Science** 42:122-131

Craufurd PQ, Austina RB, Acevedob E, Hallc MA (1991) Carbon isotope discrimination and grain-yield in barley. **Field Crops Research** 27, 301–313

del Blanco IA, Rajaram S, Kronstad WE, Reynolds MP (2000) Physiological performance of synthetic hexaploid wheat-derived populations. **Crop Science** 40, 1257-1263

Ehdaie B, Hall AE, Farquhar GD, Nguyen HT, Waines JG (1991) Water-use efficiency and carbon isotope discrimination in wheat. **Crop Science** 31, 1282–1288

Farquhar G and Sharkey TD (1982) Stomatal conductance and photosynthesis. **Annual Review of Plant Physiology** 33, 317–345

Farquhar GD and Richards RA (1984) Isotopic Composition of Plant Carbon Correlates With Water-Use Efficiency of Wheat Genotypes. **Australian Journal of Plant Physiology** 11, 539 – 552

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. **Annual Review of Plant Physiology and Plant Molecular Biology** 40, 503-537

Fischer RA, Rees D, Sayre KD, Lu ZM, Condon AG, Larque Saavedra A (1998) Wheat yield progress associated with higher stomatal conductance and photosynthetic rate, and cooler canopies. **Crop Science** 38, 1467-1475

Fischer RA (2008) The importance of grain or kernel number in wheat: A reply to Sinclair and Jamieson. **Field Crops Research** 105, 15–21

Foulkes MJ, Snape JW, Shearman VJ, Reynolds MP, Gaju O and Sylvester-Bradley R (2007) Genetic progress in yield potential in wheat: recent advances and future prospects. **The Journal of Agricultural Science** 145, 17-29

Foulkes MJ, Slafer GA, Davies WJ, Berry PM, Sylvester-Bradley R, Martre P, Calderini DF, Griffiths S, Reynolds MP (2011) Raising yield potential of wheat. III.

Optimizing partitioning to grain while maintaining lodging resistance. **Journal of Experimental Botany** 62, 469-486

Hawkesford MJ, Araus JL, Park R, Calderini D, Miralles D, Shen T, Zhang J, Parry MAJ (2013) Prospects of doubling global wheat yields. **Food and Energy Security** 2, 34-48

Kaschuk G, Hungria M, Leffelaar PA, Giller KE, Kuyper TW (2010) Differences in photosynthetic behaviour and leaf senescence of soybean (Glycine max [L.] Merrill) dependent on N_2 fixation or nitrate supply. **Plant Biology** 12, 60–69

Lilley RM, Walker DA (1974) An improved spectrophotometric assay of ribulose bisphosphate carboxilasa. **Biochimica et Biophysica Acta** 385, 226-229

Lu ZM, Percy RG, Qualset CO and Zeiger Z (1998) Stomatal conductance predicts yields in irrigated Pima cotton and bread wheat grown at high temperatures. **Journal of Experimental Botany** 49, 453-460

Makino A (2011) Photosynthesis, grain yield, and nitrogen utilization in rice and wheat. **Plant Physiology** 155, 125-129

Merah O, Deléens E, Souyris I, Nachit M, Monneveux P (2001) Stability of carbon isotope discrimination and grain yield in durum wheat. **Crop Science** 41, 677–681

Mi GH, Li TG, Zhang FS, Zhang JH (2000) Is nitrogen uptake after anthesis in wheat regulated by sink size? **Field Crops Research** 68, 183–190

Monneveaux P, Reynolds MP, Trethowan R, González-Santoyo H, Peña RJ, Zapata F (2005) Relationship between grain yield and carbon isotope discrimination in bread wheat under four water regimes. **European Journal of Agronomy** 22, 231–242

NBS (2010) National Bureau of Statistics of China. Viewed 30 October, 2012. http://datacenter.cngrain.com/IndexProduce.aspx?Flag=2&PId=10&TId=1

Parry MAJ, Hawkesford MJ (2013) Food security: increasing yield and improving resource use efficiency. **Proceedings of the Nutrition Society** 69, 592–600

Parry MAJ, Reynolds M, Salvucci ME, Raines C, Andralojc PJ, Zhu XG, Price GD, Condon AG, Furbank RT (2011) Raising yield potential of wheat. II. Increasing photosynthetic capacity and efficiency. **Journal of Experimental Botany** 62, 453–467

Parry MAJ, Andralojc PJ, Scales JC, Salvucci ME, Carmo-Silva AE, Alonso H, Whitney SM (2013) Rubisco activity and regulation as targets for crop improvement. **Journal of Experimental Botany** 64, 717-730

Paul MJ, Foyer CH (2001) Sink regulation of photosynthesis. **Journal of Experimental Botany** 52, 1381–1400

Pérez P, Martinez-Carrasco R, Martín Del Molino M, Rojo B, Ulloa M (1989) Nitrogen uptake and accumulation in grains of three winter wheat varieties with altered source-sink ratios. **Journal of Experimental Botany** 40, 707-710

Rebetzke GJ, Condon AG, Richards RA, Farquhar GD (2002) Selection for reduced carbon isotope discrimination increases aerial biomass and grain yield of rainfed bread wheat. **Crop Science** 42, 739–745

Reynolds M, Bonnett D, Chapman SC (2011) Raising yield potential of wheat. I. Overview of a consortium approach and breeding strategies. **Journal of Experimental Botany** 62, 439–452

Reynolds MP, Pellegrineschi A, Skovmand B (2005) Sink-limitation to yield and biomass: a summary of some investigations in spring wheat. **Annals of Applied Biology** 146, 39–49

Reynolds M, Foulkes MJ, Slafer GA, Berry P, Parry MAJ, Snape JW, Angus WJ (2009) Raising yield potential in wheat. **Journal of Experimental Botany** 60, 1899-1918

Reynolds M, Foulkes J, Furbank R, Griffiths S, King J, Murchie E, Parry M, Slafer G (2012) Achieving yield gains in wheat. **Plant, Cell & Environment** 35, 1799-1823

Richards RA (2000) Selectable traits to increase crop photosynthesis and yield of grain crops. **Journal of Experimental Botany** 51, 447-458

Sayre RT, Kennedy RA (1979) Photosynthetic enzyme activities and localization in Mollugo veticillata populations differing in the levels of C_3 and C_4 cycle operation. **Plant Physiology** 64, 293–299

Schnyder H (1993) The role of carbohydrate storage and redistribution in the source-sink relations of wheat and barley during grain filling. **New Physiologist** 123, 233-245

Schnyder H, Schaüfele R, Lötscher M, Gebbing T (2003) Disentangling CO_2 fluxes: direct measurements of mesocosm-scale natural abundance $^{13}CO_2/^{12}CO_2$ gas exchange, ^{13}C discrimination, and labelling of CO_2 exchange flux components in controlled environments. **Plant, Cell & Environment** 26, 1863-1874

Serret MD, Ortiz-Monasterio I, Pardo A, Araus JL (2008) The effects of urea fertilisation and genotype on yield, nitrogen use efficiency, $\delta^{15}N$ and $\delta^{13}C$ in wheat. **Annals of Applied Biology** 153, 243-257

Shangguan ZP, Shao MA, Dyckmans J (2000) Nitrogen nutrition and water stress effects on leaf photosynthetic gas exchange and water use efficiency in winter wheat. **Environmental and Experimental Botany** 44, 141–149

Sharma RC, Smith EL, McNew RW (1985) Stability of harvest index and grain yield in winter wheat. **Crop Science** 27, 104-108

Shimshi D, Ephrat J (1975) Stomatal behavior of wheat cultivars in relation to their transpiration, photosynthesis and yield. **Agronomy Journal** 67, 326-331

Slafer GA, Calderini DF, Miralles DG (1996) Yield components and compensation in wheat: Opportunities for further increasing yield potential. **In Increasing yield potential in wheat: breaking the barriers.** (Eds.) Reynolds MP, Rajaram S, and Mcnab A, CIMMYT, Mexico. pp 101-113

Smith AJ, Rinne RW and Seif RD (1989) Phosphoenolpyruvate carboxylase and pyruvate kinase involvement in protein and oil biosynthesis during soybean seed development. **Crop Science** 29, 349-353

Tambussi EA, Bort J, Araus JL (2007) Water use efficiency in C_3 cereals under Mediterranean conditions: a review of physiological aspects. **Annals of Applied Biology** 150, 307–321

USDA (2012) United States Department of Agriculture Foreign Agricultural Service Viewed 30 October, 2012.
http://www.pecad.fas.usda.gov/highlights/2012/03/china%20wheat/

Xiao YG, Qian ZG, Wu K, Liu JJ, Xia XC, Ji WQ, He ZH (2012) Genetic gains in grain yield and physiological traits of winter wheat in Shandong province, China, from 1969 to 2006. **Crop Science** 52, 44–56

Xu X, Yuan HM, Li SH, Trethowan R and Monneveux P (2007) Relationship between carbon isotope discrimination and grain yield in spring wheat cultivated under different water regimes. **Journal of Integrative Plant Biology** 49, 1497–1507

Ye YL, Yang SQ, Huang YF, Liu F (2007) Development and prospect of wheat production in Henan province. **Chinese Agricultural Science Bulletin** 23, 199-199

Yoshida S (1972) Physiological aspects of grain yield. **Annual Review of Plant Biology** 23, 437–464

Zhang YH, Wang ZM, Huang Q, Shu W (2008) Phosphoenolpyruvate carboxylase activity in ear organs is related to protein concentration in grains of winter wheat. **Journal of Cereal Science** 47, 386–391

Zhang HP, Turner NC, Poole ML (2010) Source–sink balance and manipulating sink–source relations of wheat indicate that the yield potential of wheat is sink-limited in high-rainfall zones. **Crop and Pasture Science** 61, 852-861

Zhang YH, Zhou SL, Huang Q, Leng GH, Xue QW, Stewart BA, Wang ZM (2012) Effects of sucrose and ammonium nitrate on phosphoenolpyruvate carboxylase and ribulose-1, 5-bisphosphate carboxylase activities in wheat ears. **Australian Journal of Crop Science** 6, 822-827

Zheng TC, Zhang XK, Yin GH, Wang LN, Han YL,Chen L, Huang F, Tang JW, Xia XC,He ZH (2011) Genetic gains in grain yield, net photosynthesis and stomatal

conductance achieved in Henan Province of China between 1981 and 2008. **Field Crops Research** 122, 225–233

Zhou Y, He ZH, Sui XX, Xia XC, Zhang XK, Zhang GS（2007）Genetic improvement of grain yield and associated with traits in the northern China winter wheat region from 1960 to 2000. **Crop Science** 47, 245–253

	Kernel dry weight spike^{-1} (g)	Kernel N content spike^{-1} (mg g^{-1})	Flag dry weight (g)	Flag N content (mg g^{-1})	Flag $\delta^{13}C$ (‰)	Flag N concentration (%)	Flag gs (mmol m^{-2} s^{-1})	Flag GCE m^{-2} (µmol m^{-2} s^{-1})	Flag GCE kernel^{-1} (nmol s^{-1} kernel^{-1})	GCE flag^{-1} (nmol s^{-1} flag^{-1})	Flag Rubisco activity (nmol s^{-1} g^{-1}DM)	Flag PEPc activity (nmol min^{-1} g^{-1} DM)
Yumai 35	2.22 bc	41.3 de	0.17 b	8.57 b	-29.57 c	4.94 bc	494.7	36.03	41.77 a	124.15 abc	17.97 b	40.15 ab
Lankao Aizao6	2.50 bc	64.2 bcd	0.29 a	14.43 a	-29.22 bc	4.98 bc	430.1	33.76	29.09 b	155.00 ab	33.02 a	29.91 bcd
Yumai 66	2.06 bc	54.2 cde	0.33 a	15.55 a	-28.81 abc	4.72 cs	552.9	38.67	30.13 b	166.58 a	26.63 ab	35.39 cd
Zhoumai 18	2.13 bc	53.8 cde	0.18 b	9.22 b	-28.65 ab	5.04 bc	497.7	33.26	21.23 cd	108.14 abc	20.44 b	25.61 bcd
Zhoumai 22	2.19 bc	53.2 cde	0.20 b	11.07 ab	-29.22 bc	5.58 a	432.6	32.57	27.48 bc	101.17 bc	26.77 ab	41.36 ab
Lankao 198	1.76 c	33.3 e	0.18 b	9.29 b	-29.37 bc	5.08 bc	434.1	32.98	23.56 bcd	100.95 bc	18.05 b	27.19 bcd
Lankao 298	2.19 bc	44.1 de	0.17 b	8.88 b	-28.89 abc	5.11 b	433.5	35.39	25.66 bcd	93.49 c	24.73 ab	55.02 a
Lankao 0347	2.87 abc	80.5 abc	0.25 ab	12.44 ab	-29.07 abc	5.04 bc	409.7	34.57	21.55 cd	114.74 abc	23.00 ab	41.84 ab
Lankao 223	3.96 a	96.1 a	0.32 a	14.99 a	-28.26 a	4.72 cd	454.8	31.75	18.98 d	144.79 abc	24.18 ab	20.69 cd
Lankao282	3.33 ab	84.0 ab	0.33 a	14.93 a	-28.80 abc	4.46 d	478.8	33.55	21.56 cd	148.10 abc	15.19 b	18.29 d
Mean	2.52	60.48	0.24	11.94	-28.99	4.97	461.88	34.65	26.1	125.71	23.00	33.54
Genotypes	12.39**	11241***	0.13***	220.8***	3.96**	2.41***	51442	109.38	1185***	18236**	749.03**	3457.90***

Supplemental table 1: Mean values and sum of squares type III combined with analysis of variance of different physiological parameters of 7 winter wheat cultivars and 3 advanced lines from Henan province, China. Abbreviations used as in Table 3. All the parameters are the means of 3 replicates of each genotype. Means followed by the same letter were not significantly different at p = 0.05 by the Turkey-b's test. (**, $P \leq 0.01$ and ***, $P \leq 0.001$).

	Kernel $\delta^{13}C$ (‰)	Kernel N concentration (%)	Spike dry weight (g)	Spike N content (mg g^{-1})	Spike $\delta^{13}C$ (‰)	Spike N concentration (%)	Spike g_s (mmol m^{-2} s^{-1})	Spike GCE m^{-2} (µmol $m^{-2}s^{-1}$)	Spike GCE $kernel^{-1}$ (nmol s^{-1} $kernel^{-1}$)	GCE $spike^{-1}$ (nmol s^{-1} $spike^{-1}$)	Glume Rubisco activity (nmol s^{-1} g^{-1} DM)	Glume PEPc activity (nmol min^{-1} g^{-1} DM)
Yumai 35	-28.96 c	2.46	0.41 d	8.35 c	-28.47 b	2.03 abc	75.0 bc	6.41bc	7.27 b	21.89	1.49 d	31.83 a
LankaoAizao6	-28.66 abc	2.61	0.85 abc	17.54 ab	-28.14 b	2.08 abc	104.9 ab	8.31abc	7.92 ab	38.65	2.09 cd	10.48 d
Yumai 66	-28.51 abc	2.64	0.86 abc	18.20 ab	-27.71 ab	2.10 abc	68.4 c	5.88c	5.7 4b	30.77	3.31 bcd	15.43 cd
Zhoumai 18	-28.83 bc	2.62	0.82 abc	15.77 abc	-27.78 ab	1.94 bc	77.2 bc	8.36abc	6.75 b	34.62	2.73 cd	13.16 d
Zhoumai 22	-28.81 bc	2.42	0.46 cd	9.78 bc	-28.15 b	2.15 ab	79.4 abc	7.02abc	7.09 b	26.51	6.15 b	23.26 bcd
Lankao 198	-28.43 abc	2.46	0.65 abc	12.68 abc	-28.30 b	1.95 bc	92.3 abc	7.52abc	8.20 ab	35.06	3.94 bcd	24.19 abc
Lankao 298	-28.43 abc	2.45	0.62 bcd	13.80 abc	-27.82 ab	2.23 a	110.8 a	9.22a	10.50 a	38.28	10.95 a	26.80 ab
Lankao 0347	-28.36 abc	2.81	0.87 abc	17.89 ab	-27.88 ab	2.08 abc	110.4 a	8.88ab	7.80 ab	40.99	3.92 bcd	17.56 cde
Laokao 223	-27.87 a	2.42	1.06 a	20.64 a	-27.15 a	1.95 bc	75.5 bc	7.29abc	5.49 b	42.37	3.04 bcd	28.34 ab
Lankao282	-28.00 ab	2.53	0.94 ab	17.58 ab	-27.82 ab	1.87 c	102.1 ab	7.86abc	5.46 b	38.87	5.31 bc	18.14 cde
Mean	-28.49	2.54	0.75	15.22	-27.92	2.04	89.6	7.68	7.22	34.8	4.29	20.92
Genotypes	3.38**	0.42	1.21***	426.08**	3.69**	0.29**	7208***	30.53**	63.11***	1173	200.06***	1329.45***

Supplemental table 2: Mean values and sum of squares type III combined with analysis of variance of different physiological parameters of 7 winter wheat cultivars and 3 advanced lines from Henan province, China. Abbreviations used as in Table 3. All the parameters are the means of 3 replicates of each genotype. Means followed by the same letter were not significantly different at p = 0.05 by the Turkey-b's test. (*, $P \leq 0.05$; **, $P \leq 0.01$ and ***, $P \leq 0.001$).