DOI: 10.32615/bp.2020.092 BIOLOGIA PLANTARUM 64: 633-641, 2020

Flag leaf vein traits and their correlation with photosynthesis and grain yield in wheat genotypes of differing ploidy

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Abstract

Leaf venation and coupled physiological function of wild plants co-evolve during the natural selection. How artificial selection affects leaf vein traits and coordinated physiological functions of main crops are largely unknown. This study examined the changes of leaf vein traits and their correlation with gas exchange of flag leaves and yield in eight wheat genotypes of differing ploidy under the same growing conditions. The results indicate that flag leaf vein density (VLA), major-vein density (VLA_{major}), and minor-vein density (VLA_{minor}) decreased whereas the proportion of minor-vein length and interveinal distance between small longitudinal veins (IVD) increased during the polyploidization process, and the major advance occurred from the period from diploids to tetraploids. The VLA, VLA_{major}, and VLA_{minor} were closely coordinated with maximum net photosynthetic rate (P_N) and photosynthetic N use efficiency (PNUE), but not with stomatal conductance. The proportion of minor-vein length and IVD were negatively related with P_N and PNUE but positively related with N content per area (N_{area}) during wheat evolution. A higher proportion of minor-vein length and IVD, and a lower VLA_{major} in flag leaves along with a larger N_{area} were largely responsible for the increased yield in modern cultivars. The decreased redundancy of leaf vein density and increased minor-vein proportion in modern cultivars can confer a yield advantage during wheat evolution.

Additional key words: evolution,·gas exchange, leaf N content, stomatal conductance,·*Triticum aestivum*.

Introduction

Leaf veins are an important constituents of leaf structure. They provide mechanical support, transport nutrients and water for photosynthesis and transpiration through the xylem, and transport photosynthates and signal molecules from the mesophyll to the rest of the plant through the phloem (Niklas 1999, Roth-Nebelsick *et al*. 2001). Depending on climatic and environmental conditions during leaf development, leaf vein traits are highly diverse across and within species (Uhl and Mosbrugger 1999, Boyce *et al*. 2008, Sack *et al*. 2012, Sack and Scoffoni 2013). Leaf vein traits have shown repeated evolutionary trajectories across major plant groups. Throughout 380 million years of evolution, angiosperm vein densities (vein length per leaf area, VLA) have reached 8 ~ 25 mm mm^{-2} (about 2 mm mm^{-2} in non-angiosperms) with increasing number of vein orders. High value of leaf VLA enables unparalleled photosynthetic rate and transpiration rate in the angiosperms than their competitors, thus dominate in a range of habitats than other plant lineages (Boyce *et al*. 2009). In the evolution of C_4 within eudicot genera *Cleome* and *Flaveria*, leaf VLA increased from C_3 to C_3/C_4 intermediate and C_4 species (Marshall *et al*. 2007, McKown and Dengler 2007). C_4 grasses have evolved a higher VLA (2.1 times of C_3 grasses), denser transverse veins and denser small longitudinal veins than the C_3 grasses without significant difference in large longitudinal veins (Ueno *et al*. 2006). Increasing VLA may initially have little effect on the performance of an effective CO_2 concentration mechanism, but may enhance the water status of C_4 leaves in hot environments (Sage 2004). Compared to the natural evolution, crop evolution is mainly made by artificial selection. Wheat, as one of the oldest and most important of cereal crops worldwide, began to domesticate ~10 000 years ago (Tanno and Willcox 2006). Modern cultivars of the *Triticum aestivum* hexaploids with large grain yield were domesticated from the more primitive diploids and

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Submitted 23 April 2020, *last revision* 4 June 2020, *accepted* 9 June 2020.

Abbreviations: g_s - stomatal conductance; IVD - interveinal distance between small longitudial veins; LMA - leaf mass per area; N_{area} - N content per area; N_{mass} - N content per mass; NRE - N remobilization efficiency; P_N - net photosynthetic rate; PNUE - photosynthetic N use efficiency; VLA - vein density, VLA_{major} - major-vein density; VLA_{minor} - minor-vein density; WUE_i - intrinsic water use efficiency.

Acknowledgements: This work was funded by the National Science and Technology Supporting Program (2015BAD22B01).

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tetraploids, and polyploidization played an important role during the cultivation and breeding of wheat. How the leaf vein traits changed during this polyploidization process was largely not clear.

Leaf vein traits have been reported to closely associate with leaf physiological function. Generally, high VLA values are positively correlated with higher leaf hydraulic conductance, greater stomatal density and stomatal conductance, and higher rate of gas exchange per unit leaf area (Sack and Frole 2006, Brodribb *et al*. 2007, Brodribb and Jordan 2011, Zhang *et al*. 2012, Sack and Scoffoni 2013). But this is not so under all cases. Negative (Nardini and Salleo 2000, Sack and Scoffoni 2012) and no correlations (Flexas *et al*. 2013, Xiong *et al*. 2015, Gleason *et al*. 2016) have also been reported between leaf VLA and leaf hydraulic conductance. Leaf VLA is negatively correlated with leaf net photosynthetic rate (P_N) and stomatal conductance (g_s) in rice cultivars under different water status (Tabassum *et al*. 2016). Leaf VLA shows no apparent association with stomatal conductance and maximal net CO_2 assimilation across a diverse group of 35 evergreen Australian angiosperms (Gleason *et al*. 2016). These inconsistent results indicate that the evolutionary drivers linking leaf vein traits and hydraulic efficiency or gas exchange are not fully understood. Moreover, leaf VLA was reported to be positively related with N and P resorption efficiencies across 17 dipterocarp tree species (Zhang *et al*. 2015). Another study found that leaf VLA is not correlated with leaf N content per area (N_{area}), but interveinal distance is positively correlated with leaf N_{area} among 11 rice cultivars (Xiong *et al*. 2015). How leaf vein traits are coordinated with these leaf physiological functions during wheat evolutionary process is yet to be known. Knowledge on such comprehensive correlations between leaf vein traits and leaf physiological traits is critical for enhancing our understanding of leaf structural attributes to crop water use and photosynthesis.

Despite the potential importance of leaf vein traits, few studies have examined associations between these traits and whole plant performance. Leaf vein traits are weakly, but significantly related to tree demographic performance (relative growth rate and mortality) along soil environments for 54 co-occurring species in a subtropical forest (Iida *et al*. 2016). Increasing leaf vein vensity *via* mutagenesis in rice results in an enhanced photosynthetic efficiency without increased transpiration (Feldman *et al*. 2017). Leaf VLA is positively correlated with the grain yield in 292 rice landraces (Nawarathna *et al*. 2017), suggesting that the higher VLA can be used in future rice breeding programs. Whether leaf vein traits in wheat have the similar associations with yield potential as in rice remains unknown. Identification of the presence of such leaf vein traits will be helpful to future wheat breeding.

Some morphological and physiological changes have been reported among wheat species differing in ploidy (Evans and Dunstone 1970, Austin *et al*. 1982, Zhang *et al*. 2002, Xiong *et al*. 2006, Huang *et al*. 2007, Li *et al*. 2014, Wang *et al*. 2017). The diploids generally have smaller leaf area, higher stomatal frequencies, and smaller mesophyll cells than the hexaploids, net photosynthetic rate is the highest in diploids, intermediate in tetraploids, and the lowest in hexaploids (Austin *et al*. 1982), but the hexaploids have higher root water uptake capacity, higher yield and water use efficiency, as well as higher drought resistance (longer survival duration and higher yield stability under drought) (Zhang *et al*. 2002, Xiong *et al*. 2006, Wang *et al*. 2017). However, little is known about variation of leaf vein traits and their potential importance in leaf physiological function and yield increment during the evolutionary process. Therefore, the aim of this study was to examine the variation of leaf vein traits, physiological function, yield, and their relationship in eight wheat species of three different ploidy. We assumed that during wheat evolution from diploids to tetraploids and to hexaploids, leaf VLA increased as that directed by natural selection, and leaf VLA is closely coordinated with gas exchange and yield.

Materials and methods

Plants and treatments: Eight species, including two diploids, two tetraploids, and four hexaploids, were used in a field experiment in Yangling, Shaanxi Province in China (Table 1 Suppl.). Soil was silty clay with pH 8.36, bulk density 1.26 g cm^{-3}, organic matter 13.6 g kg^{-1}, total N 0.82 g kg^{-1}, alkaline N 78.4 mg kg^{-1}, Olsen-P 14.2 mg kg^{-1}, and available K 210.2 mg kg^{-1}. Seeds were sown on 11 October 2018 at a spacing of 5 × 15 cm in plots 2 × 3 m^2. There were three replicate plots of each species arranged in randomized blocks. Each plot received 0.022 5 kg(N) m^{-2} and 0.012 kg(P_2O_5) m^{-2}, 70 % of N fertilizer and all P fertilizer were applied before sowing, the left 30 % of N fertilizer was applied with precipitation during the jointing stage. The precipitation during the whole growth season (11. 10. 2018 - 16. 6. 2019) was 252 mm, and 30 mm of irrigation was added in the winter of 2018. During the end of May 2019, the plots were sprayed with chemicals at the manufacturer's recommended rate to prevent aphid. Weeds were pulled out manually.

The flowering date of each species was labeled with tags, and 13 main stems in each plot of each species were labeled. Two tagged flag leaves were used for the measurements of leaf area and leaf vein traits on the fourth day after flowering, two tagged flag leaves were used for the measurement of gas exchange parameters and photosynthetic N use efficiency (PNUE) at the same period, and the left nine flag leaves was used for determination of leaf mass per area (LMA) and N remobilization efficiency.

Measurement of leaf vein traits: Two flag leaves per plot per species were scanned with a scanner at first, and the leaf images were analyzed for leaf area using *imageJ* software (https://imagej.en.softonic.com/), then the two leaves were used to determined leaf vein traits, totally six leaves per species. Two 0.5 cm^2 pieces were quickly excised from the middle portion of each leaf, and preserved in FAA [37 % (m/v) aqueous formaldehyde, 50 % (v/v) ethanol, and 13 % (m/v) glacial acetic acid] until use. The fixed pieces were boiled in 70 % ethanol for about 10~20 min and washed several times in distilled water, the leaf pieces were transferred to boiling 85 % (m/v) lactic acid for 20 min, and then stored in chloral hydrate-saturated ethanol before analysis (Ueno 1995). Four fields per pieces was randomly taken using a light microscope (*CX31*, *Olympus*, Tokyo, Japan) and attached digital camera system (*M-shot*, *Sci-Tech Cor.*, Guangzhou, China), and above mentioned *imageJ* software was used to analyzed vein traits. Wheat vascular bundles can be categorized into four types: midvein (MV), large longitudinal veins (LLVs), small longitudinal veins (SLVs), and transverse veins (TVs) (Nelson and Dengler 1997). In the present study, the major-vein density (VLA_{major}, mm mm^{-2}) was the sum of the densities of the MV and LLVs, and the minor-vein density (VLA_{minor}, mm mm^{-2}) was the sum of the densities of the SLVs and TVs. Vein density and the proportion of minor-vein length [%] were also calculated, and interveinal distance between two neighboring SLVs (IVD) were also measured.

Measurement of gas exchange, intrinsic water use efficiency (WUE_i), and photosynthetic N use efficiency (PNUE): Gas exchange was measured using a portable gas-exchange system (*LI-6400*, *Li-Cor*, Lincoln, NE, USA) from 9:30 to 11:30 on sunny days, and the radiation source was set to a photosynthetic photon flux density (PPFD) of 1 200 µmol m^{-2} s^{-1}. The ambient temperature, relative humidity, and CO_2 concentration were 28.4 - 33.2 °C, 35.5 - 46.8 % and 400 - 412 µmol mol^{-1}, respectively. The leaf enclosed in the chamber of the portable gas-exchange system was marked for identifying the projected areas after each measurement. Then the leaf was cut and taken to the lab for determination of N content per area (N_{area}). Leaf N content was determined using an Auto-Kjeldahl method (*Kjeltec system 2300 Distilling Unit*, Foss, Hoganas, Sweden). The WUE_i was calculated as P_N/g_s, and the PNUE was calculated as P_N/N_{area}.

Measurement of N remobilization efficiency (NRE) and leaf mass per area (LMA) in flag leaves: The NRE was used to represent the phloem transport capacity. N content based on leaf area and dry mass was determined on three dates. Flag leaf N content reached the maximum on the date of flowering for modern wheat cultivars (Cai *et al.* 2008), but we are not sure if this was true in primitive wheat species. So, we sampled on the date of flowering, the fourth day after flowering, and at physiological maturity, and three flag leaves were taken away at each date per plot per species. These leaves were scanned first, then killed at 105 °C for 30 min, later oven-dried at 80 °C to a constant mass. The scanned pictures were used for determining leaf area with *imageJ* software, and leaf mass per area (LMA) was calculated as leaf dry mass/leaf area. The dried leaves were grounded for determination of N content. N content on the fourth day after flower were always larger than those on the day of flowering, so we take N content on the fourth day as the maximum N content (N_{max}). N content based on leaf area and leaf dry matter are strongly correlated ($R^2 = 0.87$, $P < 0.01$, $n = 72$), so we use only N content per dry mass to calculate N remobilization efficiency (NRE) (Zhang *et al.* 2014) as NRE [%] = (N_{max} - $N_{maturity}$) × 100 / N_{max}, where $N_{maturity}$ is the N content per dry matter at maturity.

Grain yield: At physiological maturity of each species, 1 m^2 of each plot was harvested to determine aboveground biomass and grain yield; biomass and grain were weighted after oven-drying at 80 °C for 3 d.

Statistical analyses: Two-level nested analysis of variance (*ANOVA*) was used to assess the significance of leaf vein and physiological parameters across different wheat ploidy. A post-hoc multiple comparison was conducted using the Tukey-Kramer method if the effect of ploidy was significant. A principal component analysis (PCA) was analyzed to examine multivariate associations of leaf traits. The allometric relationships between leaf vein traits and leaf area, LMA were modeled by power functions, and the regressions between leaf vein traits and leaf physiological parameters, biomass and yield were fitted by linear models. The nested *ANOVA* was conducted by a "two-level nested anova" *EXCEL* file provided by Dr. J.H. McDonald on the webpage (http://www.biostathandbook.com/nestedanova.html), all other statistics were conducted using *SPSS Statistics 20.0* software (*IBM Corporate*, Armonk, USA).

Results

Both flag leaf area and LMA in diploids were significantly lower than in tetraploids and hexaploids. Flag leaf area was the lowest in diploids (11.43 cm^2), intermediate in hexaploids (26.19 cm^2), and the largest in tetraploids (31.80 cm^2). The LMA increased from 40.96 g m^{-2} in diploids to 56.72 g m^{-2} in tetraploids, and 53.36 g m^{-2} in hexaploids. For leaf vein traits, diploids had significantly higher VLA, VLA_{major}, and VLA_{minor}, and lower proportion of minor-vein length and IVD than tetraploids and hexaploids. No significant difference of the above vein traits was found between tetraploids and hexaploids except VLA_{major}, which was 14.7 % higher in tetraploids than in hexaploids (Table 1). These results suggested that the evolution of wheat leaf vein traits mainly occurred during the period from diploids to tetraploids. VLA, VLA_{major}, and VLA_{minor} were significantly and positively correlated (VLA *vs.* VLA_{major}: $R^2 = 0.87$, $P < 0.01$; VLA *vs.* VLA_{minor}: $R^2 = 0.96$, $P < 0.001$; VLA_{major} *vs.* VLA_{minor}: $R^2 = 0.72$, $P < 0.01$), and they were negatively correlated with IVD ($R^2 = 0.96$, $P < 0.001$ for VLA; $R^2 = 0.83$, $P < 0.01$ for VLA_{major}; $R^2 = 0.94$, $P < 0.001$ for VLA_{minor}). Proportion of minor-vein length was negatively correlated with VLA_{major} ($R^2 = 0.79$, $P < 0.01$).

VLA, VLA_{major}, and VLA_{minor} across wheat genotypes of differing ploidy decreased with leaf area (pseudo $R^2 = 0.74$, $P < 0.01$ for VLA; pseudo $R^2 = 0.66$, $P < 0.05$ for VLA_{major}, and pseudo $R^2 = 0.76$, $P < 0.05$ for VLA_{minor}) (Fig. 1*A*), while proportion of minor-vein length and IVD increased with leaf area (pseudo $R^2 = 0.46$, $P = 0.06$ for proportion of minor-vein length; pseudo $R^2 = 0.53$, $P < 0.05$ for the

Table 1. Flag leaf area [cm^2], leaf mass per area (LMA) [g m^{-2}], vein density [mm mm^{-2}], proportion of minor-vein length (Proportion) [%], and interveinal distance between two neighboring small longitudinal veins (IVD) [mm] in eight wheat genotypes, including two diploids (D1 and D2), two tetraploids (T1 and T2), and four hexaploids (H1-FC3, H2-ZM101, H3-CW134, and H4-XY6). Means ± SEs, $n = 6$. Different letters indicate significant differences between wheat genotypes of differing ploidy at $P < 0.05$.

Genotype	Leaf area	LMA	Vein density			Proportion	IVD
			major	minor	total		
D1	12.90 ± 1.29	41.93 ± 0.83	1.42 0.03	4.21 ± 0.05	5.63 ± 0.04	74.84 ± 0.54	0.192 ± 0.004
D2	9.96 ± 1.08	39.98 ± 0.79	1.32 ± 0.05	4.28 ± 0.08	5.60 ± 0.09	76.47 ± 0.74	0.205 ± 0.002
Diploid mean	11.43 ± 0.92 c	40.96 ± 0.62 b	1.37 ± 0.03 a	4.25 ± 0.05 a	5.61 ± 0.05 a	75.66 ± 0.50 b	0.198 ± 0.003 b
T1	35.34 ± 1.91	55.72 ± 1.13	0.84 ± 0.04	3.43 ± 0.05	4.26 ± 0.04	80.33 ± 0.87	0.261 ± 0.004
T2	28.26 ± 1.39	57.72 ± 1.72	0.88 ± 0.04	3.15 ± 0.10	4.03 ± 0.11	78.09 ± 0.96	0.283 ± 0.009
Tetraploid mean	31.80 ± 1.55 a	56.72 ± 1.05 a	0.86 ± 0.03 b	3.29 ± 0.07 b	4.15 ± 0.06 b	79.21 ± 0.70 a	0.272 ± 0.006 a
H1-FC3	29.33 ± 2.66	51.07 ± 0.62	0.75 ± 0.03	3.29 ± 0.17	4.03 ± 0.16	81.63 ± 1.13	0.293 ± 0.013
H2-ZM101	19.96 ± 1.62	52.01 ± 1.19	0.74 ± 0.03	3.13 ± 0.07	3.87 ± 0.05	80.89 ± 0.87	0.311 ± 0.005
H3-CW134	24.33 ± 1.85	52.77 ± 1.31	0.78 ± 0.03	3.84 ± 0.10	4.62 ± 0.09	83.09 ± 0.79	0.252 ± 0.007
H4-XY6	31.14 ± 1.62	57.59 ± 1.20	0.73 ± 0.05	2.91 ± 0.10	3.64 ± 0.07	79.84 ± 1.53	0.322 ± 0.011
Hexaploid mean	26.19 ± 1.30 b	53.36 ± 0.74 a	0.75 ± 0.02 c	3.29 ± 0.09 b	4.04 ± 0.09 b	81.36 ± 0.58 a	0.295 ± 0.007 a

Table 2. Flag leaf net photosynthetic rate (P_N) [µmol m^{-2} s^{-1}], stomatal conductance (g_s)[mol m^{-2} s^{-1}], N content per area (N_{area}) [g m^{-2}], intrinsic water use efficiency ($WUE_i = P_N/g_s$) [µmol mmol^{-1}], photosynthetic N use efficiency ($PNUE = P_N/N_{area}$) [µmol g^{-1} s^{-1}], and N remobilization efficiency (NRE) [%] in eight wheat genotypes, including two diploids (D1 and D2), two tetraploids (T1 and T2), and four hexaploids (H1-FC3, H2-ZM101, H3-CW134, and H4-XY6). Means ± SEs, $n = 6$ for P_N, g_s, N_{area}, WUE_i, and PNUE, and $n = 3$ for NRE. Different letters indicate significant differences between wheat genotypes of differing ploidy at $P < 0.05$.

Genotype	P_N	g_s	N_{area}	WUE_i	PNUE	NRE
D1	25.30 ± 0.90	0.38 ± 0.03	1.35 ± 0.10	69.01 ± 6.95	19.28 ± 1.40	80.78 ± 2.36
D2	23.97 ± 0.81	0.44 ± 0.02	1.45 ± 0.05	54.45 ± 3.53	16.65 ± 0.72	86.02 ± 0.66
Diploid mean	24.63 ± 0.61 a	0.41 ±0.02	1.40 ± 0.06 a	61.73 ± 4.32	17.96 ± 0.85 a	83.40 ± 1.61
T1	19.88 ± 0.31	0.38 ± 0.03	2.20 ± 0.09	54.03 ± 4.30	9.12 ± 0.38	86.16 ± 0.26
T2	21.12 ± 0.28	0.47 ± 0.05	2.17 ± 0.07	48.01 ± 5.69	9.76 ± 0.28	80.08 ± 3.43
Tetraploid mean	20.50 ± 0.27 b	0.42 ± 0.03	2.18 ± 0.06 b	51.02 ± 3.52	9.44 ± 0.25 b	83.12 ± 2.05
H1-FC3	21.12 ± 0.70	0.53 ± 0.05	2.15 ± 0.08	41.75 ± 4.53	9.95 ± 0.67	83.57 ± 1.66
H2-ZM101	19.25 ± 0.69	0.26 ± 0.02	1.92 ± 0.11	76.02 ± 6.45	10.10 ± 0.35	84.45 ± 2.12
H3-CW134	19.74 ± 1.12	0.28 ± 0.03	2.09 ± 0.10	72.29 ± 5.35	9.55 ± 0.60	70.51 ± 2.75
H4-XY6	18.53 ± 0.74	0.32 ± 0.04	2.15 ± 0.03	62.97 ± 7.16	8.61 ± 0.31	85.05 ± 0.52
Hexaploid mean	19.66 ± 0.43 b	0.35 ± 0.03	2.08 ± 0.04 b	63.26 ± 3.92	9.55 ± 0.27 b	80.90 ± 2.00

IVD) (Fig. 1*B*,*C*). Due to the strong correlation between flag leaf area and LMA ($R^2 = 0.81$, $P < 0.01$), the variation of leaf vein traits with LMA followed the same trend as with leaf area (Fig. 1 Suppl.). The allometric relationship may partly explain the trend of leaf vein traits during the wheat polyploidization.

The diploids had 20.1 and 25.3 % higher in P_N, 90.2 and 88.1 % higher PNUE, 35.8 and 32.3 % lower in N_{area} than tetraploids and hexaploids, respectively. No difference of these parameters was observed between tetraploids and hexaploids. There was no difference in g_s, WUE_i, and NRE among genotypes with differing ploidy (Table 2). The P_N was not correlated with g_s ($\mathrm{R}^2 = 0.21$, $\mathrm{P} > 0.05$), PNUE was positively correlated with P_N ($R^2 = 0.90$, $P < 0.001$), WUE_i was negatively correlated with g_s ($R^2 = 0.86$, $P < 0.01$), and N_{area} was negatively correlated with P_N ($R^2 = 0.75$, $P < 0.01$) and PNUE ($R^2 = 0.94$, $P < 0.001$) (Fig. 2 Suppl.).

The PCA was employed to evaluate how leaf vein traits and leaf physiological function were associated. The PCA axis 1, accounting for 64.8 3 % of the total variation, showed strong loadings on all five leaf vein traits and some physiological parameters like P_N, PNUE, and N_{area}. PCA axis 2, accounting for 20.6 % of the total variation, showed strong loadings on g_s and WUE_i (Fig. 3 Suppl.). The VLA, VLA_{major}, and VLA_{minor} were significantly and positively correlated with P_N ($R^2 = 0.81$, $P < 0.01$ for VLA; $R^2 = 0.90$, $P < 0.001$ for VLA_{major}, and $R^2 = 0.67$, $P < 0.05$ for VLA_{minor}) and PNUE ($R^2 = 0.84$, $P < 0.01$ for VLA; $R^2 = 0.95$, $P < 0.001$ for VLA_{major}, and $R^2 = 0.71$, $P < 0.01$ for VLA_{minor}) (Fig. 2), but proportion of minor-vein length and IVD were significantly and negatively correlated with P_N ($R^2 = 0.69$, $P < 0.05$ for proportion of minor-vein length and $R^2 = 0.77$, $P < 0.01$ for IVD) (Fig. 2) and PNUE ($R^2 = 0.70$, P 0.05 for proportion of minor-vein length and $R^2 = 0.75$, $P < 0.01$ for IVD) (Fig. 2). The VLA, VLA_{major}, and VLA_{minor} decreased with increased N_{area}, but proportion of minor-vein length and IVD increased with N_{area} (Figs. 2 and 3).

Biomass was positively related to leaf area ($R^2 = 0.63$, $P < 0.05$), proportion of minor-vein length ($R^2 = 0.67$, $P < 0.05$), and N_{area} ($R^2 = 0.58$, $P < 0.05$), but negatively related to VLA_{major} ($R^2 = 0.61$, $P < 0.05$), P_N ($R^2 = 0.55$, $P < 0.05$), and PNUE ($R^2 = 0.58$, $P < 0.05$) (Table 2 Suppl.). Grain yield was positively related with proportion of minor-vein length ($R^2 = 0.88$, $P < 0.01$) , IVD ($R^2 = 0.51$, $P < 0.05$), and N_{area} ($R^2 = 0.49$, $P = 0.053$), but negatively related with VLA ($R^2 = 0.49$, $P = 0.055$), VLA_{major} ($R^2 = 0.75$, $P < 0.01$), P_N (0.62, $P < 0.05$), N_{area} ($R^2 = 0.49$, $P = 0.053$), and PNUE ($R^2 = 0.58$, $P < 0.05$) (Table 2 Suppl.). The above relationship suggested that higher proportion of minor-vein length, larger IVD, and increased N content contribute to the biomass and yield improvement during the process of wheat evolution.

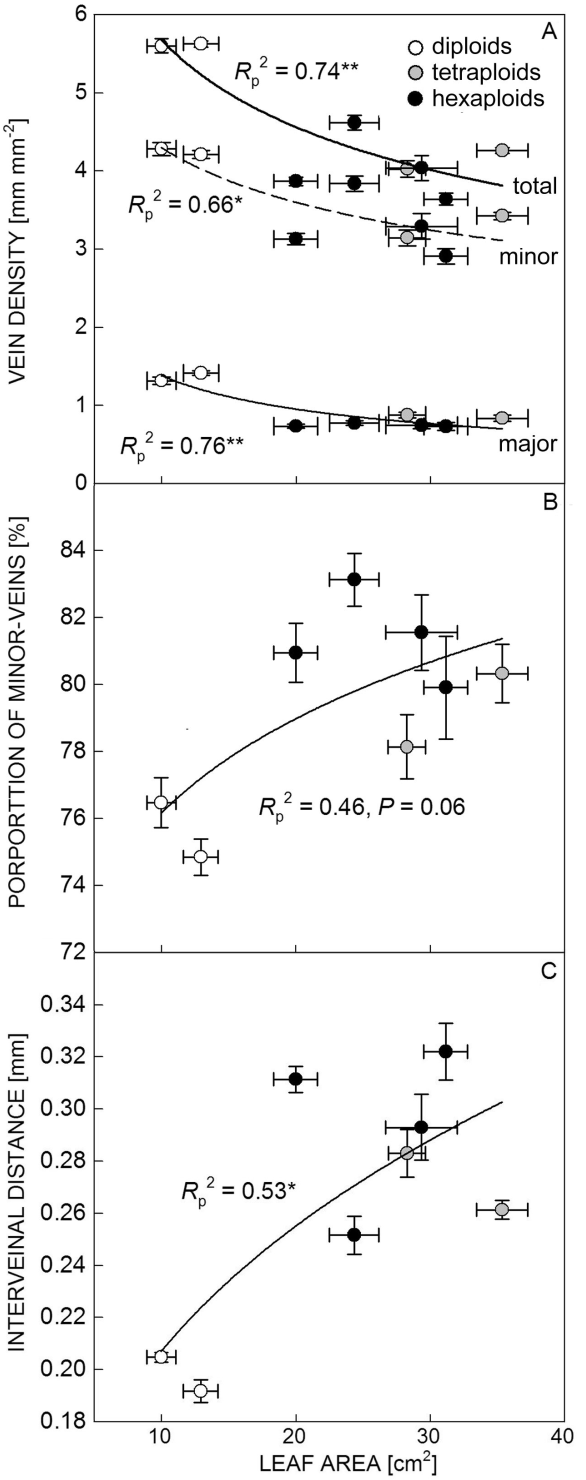

Fig. 1. Allometric relationships between flag leaf vein density (*A*), proportion of minor-veins (*B*) and interveinal distance (*C*), and leaf area across eight wheat genotypes of differing ploidy. Means ± SEs, $n = 6$. The curves are fitted by a power function; R_p^2 - pseudo R^2 of modeling curves. * - $P < 0.05$, ** - $P < 0.01$.

Discussion

During the evolution of wheat, VLA, VLA_{major}, and VLA_{minor} all show the trend to decrease, while proportion of minor-vein length and IVD increased (Table 1). This trend differs from that exhibited in angiosperm and C_4 grasses evolution (Boyce *et al.* 2009, Ueno *et al.* 2006). The variation of wheat leaf vein traits across differing ploidy can be partly explained by the developmental algorithm for vein formation during leaf expansion, showing declined major-vein density with increasing leaf size, and minor-vein density independent of leaf size (Sack *et al.* 2012). Generally, major veins include the first three orders of veins, and vein density of these three orders declines with leaf area (Sack *et al.* 2012). The first two orders of veins (midrib and large longitudinal veins) of wheat leaves are directly connected with leaf sheath, and participate in water transport to leaves, photosynthate export, *etc.* (Altus and Canny 1985). The VLA_{major} in the present study only covered the first two orders of veins and it decreased due to the increased flag leaf area during wheat evolution process. Vein density of the third order of veins (small longitudinal veins) also decreased with increased leaf area and it contributed more than 60 % to VLA and 75 % to VLA_{minor} (data not shown), resulting in decreased VLA and VLA_{minor} during wheat evolutionary process. Meanwhile, we found that proportion of minor-vein length and IVD increased with the increase in leaf area (Fig. 1). Although there is no mechanic association between LMA and vein traits (Sack *et al.* 2013), the changes of vein traits with LMA also showed the same trend as those with flag leaf area during the study since LMA and leaf area are closely related.

Higher VLA in diploid than in tetraploid and hexaploid species may favour their photosynthesis. The earlier studies (Evans and Dunstone 1970, Austin *et al.* 1982) and present study all supported that the more primitive diploids have higher net photosynthetic rate than modern hexaploid cultivars. Except the role of mechanical support, major-vein density has been found to play a role in determining the water transport capacity, in damage tolerance of the vein system, and in leaf drought tolerance (Sack *et al.* 2008, Scoffoni *et al.* 2011). Thus larger major-vein density in diploids provide better water transport and so enable their better survival in harsh environments than tetraploids and hexaploids although some research do not support this (Xiong *et al.* 2006, Wang *et al.* 2017). The minor veins (transverse veins and small longitudinal veins) of wheat leaves play a vital role in lateral transport of photosynthates from the small to the large longitudinal veins (Altus and Canny 1982) because they have large surface area for uploading photosynthates from the surrounding mesophyll. Thus, diploids have acquired a better photosynthate translocation system than the tetraploids and hexaploids to match with their higher photosynthetic capacity. The decreased VLA_{major} in tetraploid and hexaploid species may reduce the carbon cost for construction, and lower vein projected area might potentially enhance mesophyll radiation capture (Sack and Scoffoni 2013), and larger IVD may allow more mesophyll cells occupy (Austin *et al*. 1982).

Past studies showed VLA (especially VLA_{minor}) is closely correlated with g_s, and P_N at intraspecific and interspecific levels (Sack and Frole 2006, Brodribb *et al*. 2007, Brodribb and Jordan 2011, Zhang *et al*. 2012, Sack and Scoffoni 2013). This study also supported the strong positive association between VLA and P_N (Fig. 2), but leaf vein density and g_s was decoupled (Fig. 3 Suppl.). The P_N and g_s also decoupled across wheat genotypes of differing ploidy (Fig. 3 Suppl.). In an early study by Austin *et al*. (1982), the mean g_s of the diploids was only 5 % greater than that of genotypes in this study; g_s in diploids was 17.1 % higher than that of hexaploid cultivars with no statistic difference, indicating leaf internal anatomical features rather than the difference of g_s mainly drive the variation in photosynthetic rate. Anatomically, small leaf size, small mesophyll cell size, and close spacing of veins are correlated with high photosynthetic rate in primitive genotypes (Dunstone and Evans 1974). Water supply capacity of leaves did not constrain water loss and CO_2 diffusion under present ample soil water condition. Although there are reports of increased water and nutrient use efficiency during the wheat evolution process (Zhang *et al*. 2002, Xiong *et al*. 2006, Huang *et al*. 2007), we did not find this at leaf level. Leaf WUE_i did not change and PNUE decreased (Table 2), which were possibly related with the selected genotypes and different expressions in water and nutrient use efficiency at different organ levels. Leaf veins traits were closely associated with PNUE but not related with WUE_i since WUE_i was mainly determined by g_s, and PNUE was mainly determined by P_N (Fig. 3 Suppl.).

Theoretically, leaves acclimated to higher nutrient supplies would have vein traits associated with higher leaf hydraulic conductance and gas exchange rate (Sack and Scoffoni 2013). VLA across rice cultivars was independent of N_{area}, but IVD was positively correlated with N_{area} (Xiong *et al*. 2015). In this study, we found N_{area} was negatively correlated with VLA, VLA_{major}, and VLA_{minor},

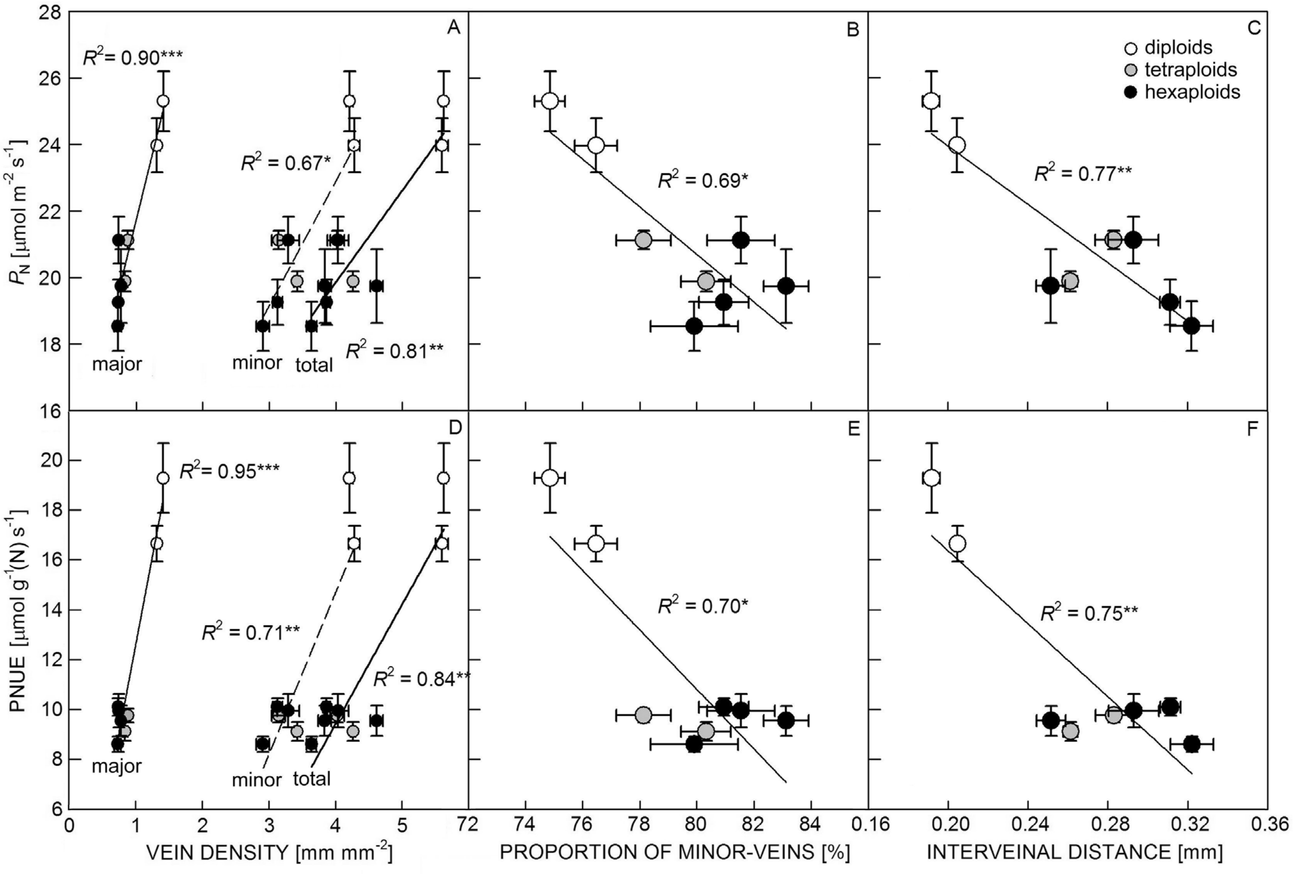

Fig. 2. Relationships between net photosynthetic rate (P_N) or photosynthetic N use efficiency (PNUE), and leaf vein density (*A*,*D*, respectively), proportion of minor-veins (*B*,*E*, respectively) and interveinal distance(*C*,*F*, respectively) ₙin wheat genotypes with differing ploidy. Means ± SEs, *n* = 6. * - *P* < 0.05, ** - *P* < 0.01, *** - *P* < 0.001.

and positively correlated with proportion of minor-vein length as well as IVD (Fig. 3). This can be explained by the developmental algorithm for vein formation (Sack *et al.* 2012) because leaf higher N content is accompanied with larger leaf area ($R^2 = 0.88$, $P < 0.01$). Although higher N content stimulated proportion of minor-vein length, N remobilization efficiency did not change during wheat evolution, the reason may be related to enlarged interveinal distance at higher N content, which increased distance for photosynthate transport to phloem. Leaf phloem transport was a complex process, and N remobilization efficiency may be affected by many factors, such as the distribution of phloem in the vein network, and on species' loading strategies, post-flowering leaf duration period, grain sink size, *etc*. Even tetraploids and hexaploids had higher N_{area}, their P_N was lower than that of diploids, showing high N content in the tetraploids and hexaploids did not increase photosynthesis, contrasted with the studies claiming that leaf N content was closely linked to maximum assimilation across species (Field and Mooney 1986, Harrington *et al.* 1989, Wright *et al.* 2004). The reason may be related to lower N content allocation to the photosynthetic apparatus or high N demand in tetraploids and haxaploids.

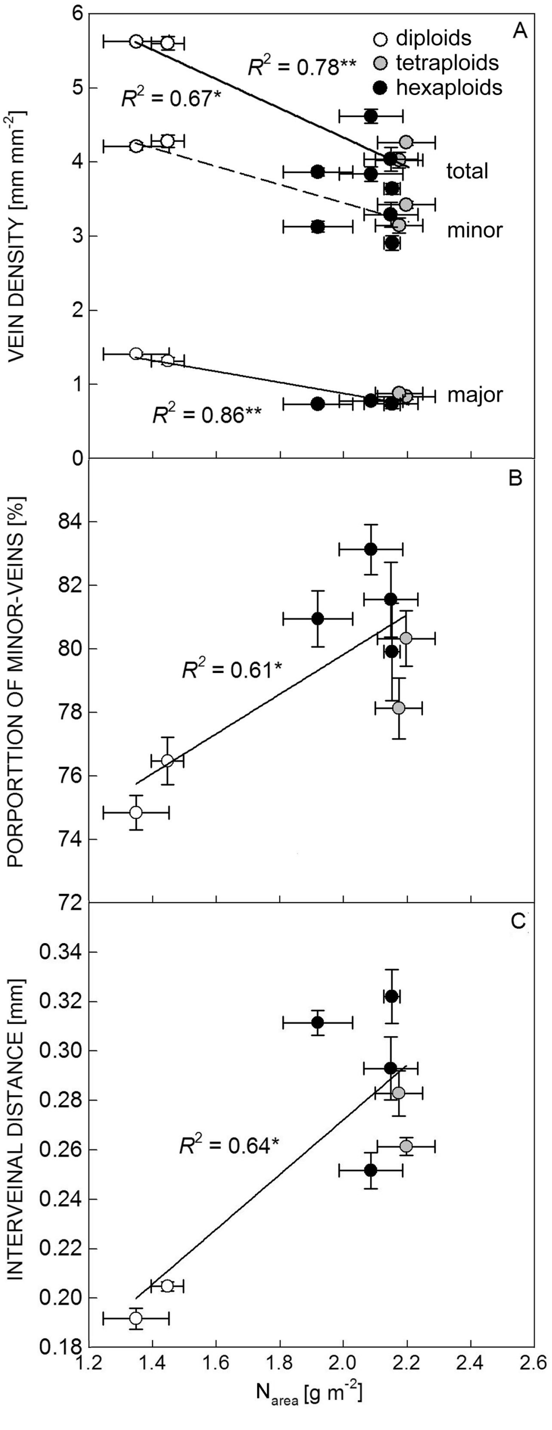

Fig. 3. Relationships between leaf vein density (*A*), proportion of minor-veins (*B*) and interveinal distance (*C*), and N content per area (N_{area}) in wheat genotypes with differing ploidy. Means ± SEs, $n = 6$. * - $P < 0.05$, ** - $P < 0.01$.

The biggest evolution advance of leaf vein and physiological traits occurred during the period from diploids to tetraploids, thereafter they changed little. Tetraploid *Triticum dicoccoides* (AABB) hybridized with *Aegilops squarrosa* (DD) to give rise to the cultivated hexaploid wheats. So, the increased D genome changed the leaf vein traits and physiological function a little and did not increase the photosynthesis. Watanabe *et al.* (1997) found that the increased doses of the D genome reduces photosynthesis, but the depression is dependent on the source of the D genome. This study partly supports their finding. Although there were not much difference in vein traits and physiological function between tetraploids and hexaploids, hexaploids achieved a higher yield than tetraploids, the relevant mechanism needs further study.

We found at leaf level, that larger biomass mainly attributed to the increase in leaf area, proportion of minor-vein length and N_{area}, while high yield was mainly attributed to increased proportion of minor-vein length, IVD, and N_{area} during the process of wheat evolution. It was not possible to determine the relative importance of each trait, but both VLA_{major} and proportion of minor-vein length showed closer association with yield than other leaf traits, and also linked with leaf physiological function like N_{area}, P_N, and PNUE, so they are important leaf attributes for yield increase in modern cultivars. Modern wheat genotypes may improve the carbon allocation proportion to grain production due to artificial selection by lowering carbon construction cost in leaf main veins (decreased VLA_{major}) and/or by optimizing carbon distribution pattern among different orders of leaf vein system (increased proportion of minor-vein length). In contrast to the study in rice, where VLA was positively correlated with the grain yield (Feldman *et al.* 2017, Nawarathna *et al.* 2017), the potential of vein traits in future wheat breeding needs further testing.

Modern cultivars of the hexaploid *Triticum aeativum* produce larger grain yield than the more primitive diploid and tetraploid species despite they have lower P_N (Evans and Dunstone 1970, Austin *et al.* 1982). So, P_N is not the reason of yield increase during wheat domestication, the increase in harvest index, grain, and leaf size, aboveground biomass, the proportion of dry mass mobilized to the grain and water and nutrient use efficiency may be the other major factors in the selection of wheat varieties for increasing yield (Dunstone *et al*. 1973, Zhang *et al*. 2002, Xiong *et al*. 2006, Huang *et al*. 2007, Wang *et al.* 2017). This study showed decreased VLA_{major} and increased proportion of minor-vein length were also closely associated with the yield improvement during wheat evolution. These traits are worthy to be further assessed in future wheat breeding.

Conclusions

The VLA, VLA_{major}, and VLA_{minor} decreased while proportion of minor-vein length and interveinal distance increased during wheat evolution, and the major advance occurred from diploids to tetraploids. VLA, VLA_{major}, and VLA_{minor} were closely correlated with P_N and PNUE but not with g_s. Proportion of minor-vein length and interveinal distance was positively related with N_{area} and negatively with P_N and PNUE, respectively, during wheat evolution. Higher proportion of minor-vein length and interveinal distance, lower VLA_{major} along with increased N_{area} in flag leaves were largely responsible for the increased yield in modern cultivars. This study demonstrates that evolution of leaf vein density in wheat is different from those in wild plants, but a high coordination still exists between leaf vein density and P_N.The study highlights the proportion of minor-vein length, IVD, and VLA_{major} as the potential indexes for future wheat high yield breeding.

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