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Leaf microstructure and photosynthetic characteristics of a rice midvein-deficient mutant *dl-14*

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Abstract

Midvein is an important structure of the upright leaf of rice, and its normal development is essential to the formation of a common plant type of rice (*Oryza sativa* L.). To reveal the effect of midvein deficiency on photosynthesis-related characteristics, leaf microstructure, and vein characteristics, the photosynthetic features between the midvein-deficient mutant *dl-14* and wild-type Huanghuazhan plants were analyzed. The results indicated that the midvein area of the *dl-4* mutant lacked large intercellular space and instead it was filled with mesophyll cells. Moreover, the vein density of the *dl-14* mutant was significantly higher than that in cv. Huanghuazhan. Chlorophyll (Chl) *a*, Chl *b*, and carotenoid content were markedly elevated in *dl-14*. In terms of photosynthetic characteristics, we observed that under high irradiance and high CO_2 concentration, the net photosynthetic rate of *dl-14* plants was significantly higher than that of Huanghuazhan plants, but its water use efficiency was significantly lower. In addition, several major photosynthetic parameters, including characteristics of chlorophyll fluorescence (the efficiency of excitation capture of open PS II center, photochemical quenching, effective quantum yield of PS II photochemistry, and electron transfer rate) were significantly higher in *dl-14* plants compared to Huanghuazhan plants, but the nonphotochemical quenching of *dl-14* mutant was significantly lower than that of Huanghuazhan. These findings indicate that the *dl-14* mutant has higher vein density, stronger photon conversion ability, and weaker radiation dissipation ability. This study can provide theoretical support for breeders to use the midvein-deficient mutant.

Keywords: leaf microstructure, midvein-deficient mutant, photosynthetic characteristics, rice.

Introduction

Leaves are plant organs that serve as sites for photosynthesis, water and inorganic salt transport. Veins are important leaf components that provide mechanical support to the leaves. Veins of different orders are intricately arranged during leaf development, thus forming a refined transport network. The mature midvein of rice leaves includes air spaces, parenchyma cells, vascular bundles, and mesophyll cells. A large number of treachery elements are produced in the central region of midveins *via* programmed cell death, thereby forming air spaces that are surrounded by parenchyma cells. The midvein vascular bundle consists of a central vascular bundle and a small vascular bundle on the paraxial surface (Zou *et al.* 2013). The paraxial and abaxial surfaces consist of several layers of mesophyll cells, and the central region is formed by cell proliferation during the early stage of rice

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Abbreviations: AQY - apparent quantum yield; Car - carotenoids; CE - carboxylation efficiency; Chl - chlorophyll; ETR - electron transfer rate; F_0 - minimal initial fluorescence; F_0' - minimal fluorescence yield of the light-adapted state; F_m - maximal fluorescence; F_m' - maximal fluorescence yield of the light-adapted state; F_v/F_m - maximal quantum yield of PS II photochemistry; F_v'/F_m' - efficiency of excitation capture of open PS II center in the light conditions; F_s - fluorescence intensity at any time; NPQ - nonphotochemical quenching coefficient; PAR - photosynthetically active radiation; P_N - net photosynthetic rate; PS - photosystem; q_P - photochemical quenching coefficient; WUE - water use efficiency; Φ_{PSII} - effective quantum yield of PS II photochemistry.

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leaf midvein development. Vein development is a highly complicated physiological process (Carland *et al.* 2009, Ceserani *et al.* 2009, Gardiner *et al.* 2010, Robles *et al.* 2010) that is controlled by multiple genes and regulated by the external conditions. Veins are important structures that affect leaf water utilization and nutrition supply (Sack and Frole 2006, Sack and Scoffoni 2013). The density of leaf veins is a key physiological parameter of water supply for the whole leaf, as well as it reflects the mechanical support provided by the vein system to the leaves. More importantly, leaf vein density also reveals the status of specific fundamental physiological processes such as leaf photosynthetic rate and water transport (Brodribb and Jordan 2011, Sack *et al.* 2012, Pagano and Storchi 2015). Leaves in an arid environment often increase the area and path of water exchange between xylem and surrounding mesophyll cells by having higher leaf vein density to strengthen the "defense" tissue (Sack *et al.* 2013). Leaves can transport water to the mesophyll cells around the cavitation xylem, avoiding the interruption of water transport caused by damaged leaf veins. Under insufficient water supply, the water transport in leaves can be improved, so that the photosynthesis in leaves is not affected and the water use efficiency (WUE) is increased (Nardini *et al*. 2010, Carins-Murphy *et al*. 2014). Previous studies involving sugarcane have shown that leaf vein density is closely associated with net photosynthetic rates (P_N), and high leaf vein density increase drought resistance (Qin *et al.* 2017). In the Chinese willow (*Salix matsudana*), higher vein density is also correlated with higher WUE (Xu *et al.* 2017). However, despite our current understanding of the physiological importance of veins, the effects of midvein deficiency on the photosynthetic characteristics of plants remain unclear. In this research, we used a midvein-deficient mutant *dl-14* from a commonly cultivated rice cultivar Huanghuazhan, which was identified by its leaf drop phenotype. Our aim was to investigate the effects of leaf vein development deficiency on photosynthesis-related characteristics.

Material and methods

Plants and growth conditions: Rice (*Oryza sativa* L.) cv. Huanghuazhan and *dl-14* mutant plants were provided by the College of A&F Engineering and Planning of Tongren University. The plants were seeded on 29 March 2018 and were transplanted into plastic pots (10 seedlings/pot, five pots for each genotype) after 30 d. Each plastic pot was filled with 1000 g of soil (dry mass equivalent). Cultivation was conducted under natural conditions with conventional water and fertilizer management and pest control. After 74 d, 5 plants of each genotype were harvested for analyses of photosynthetic pigment content, leaf vein density, and microstructure of leaf cross-sections.

Photosynthetic pigment content: Photosynthetic pigment content was determined according to the methods of Arnon (1949). Pigments were extracted in 95 % (v/v) ethanol and then the absorbance was determined with the spectrophotometer (*UV-1780*, *Shimadzu*, Kyoto, Japan) at wavelengths of 665, 649, and 470 nm. The equations to determine chlorophyll *a*, chlorophyll *b* and carotenoid content were as follows: Chl $a = 13.95\ A_{665} - 6.88\ A_{649}$; Chl $b = 24.96\ A_{649} - 7.32\ A_{665}$; Car = $(1000\ A_{470} - 2.05\ C_a - 114.8\ C_b)/245$. C_a and C_b are the chlorophyll *a* and chlorophyll *b* concentrations, respectively.

Measuring leaf vein density: To measure vein density, 200-mm^2 middle sections of the leaves were clipped and placed into a test tube containing 5 % (m/v) NaOH. After boiling for 20 - 30 min, mesophyll tissue degradation was monitored. When the veins were clearly visible, the samples were immersed in distilled water for 30 min and then transferred into 5 % (m/v) NaClO for 10 min. Before microscopy observation, the leaves were again soaked in distilled water for 30 min. Leaf vein microstructure was assessed under a *DM 3000* optical microscope (*Leica*, Wetzlar, Germany). For each genotype, at least 30 images were captured. The *ImageJ* software (https://imagej.en.softonic.com/) was used to measure the total length of the veins within the field of view and to calculate the total vein length per unit area (vein density).

Microstructure of cross-section of leaf: Dissected samples or fresh samples were fixed in 2 % (m/v) glutaraldehyde in 100 mM cacodylate buffer, pH 7.2, overnight. Samples were then dehydrated in a series of ethanol, embedded in paraffin, cut and dried onto slides at 37 °C. The sections were stained with 0.1 % (m/v) Toluidine Blue O in 50 mM citrate buffer, pH 4.4, before mounting in epoxy resin for microscopic observation using bright-field optics. Microscopic images were acquired with an optical microscope (*SZX10*, *Olympus*, Tokyo, Japan).

Leaf gas exchange parameters: From 9:00 AM to 11:00 AM, on a sunny day, the net photosynthetic rate of leaves was measured under various irradiances and CO_2 concentrations, using the second leaves from the top. For each genotype, at least three leaves were measured, and the average value was calculated and used in the subsequent analyses. Radiation-response curves were measured using the built-in automatic measuring program of *LI-6400XT* (*Li-Cor*, Lincoln, NE, USA). Before running the program, the samples were exposed to 1 500 μmol(photons) m^{-2} s^{-1} for 5 to 6 min (the common irradiance of the experimental site on a sunny summer morning). When the program started, the temperature was set to 30 °C, and the CO_2 (supplied by a steel cylinder) concentration was set to 400 μmol mol^{-1}. The photosynthetically active radiation (PAR) series, including 2 000, 1 800, 1 600, 1 400, 1 200, 1 000, 800, 600, 400, 300, 200, 100, 50, 25, and 0 μmol m^{-2} s^{-1} (according to the operation manual of *Li-6400XT*), were generated by a red and blue radiation source (*6400-02B LED*) of the instrument. Based on the P_N-PAR curve, we took the linear portion of the curve where the PAR was lower than 250 μmol m^{-2} s^{-1} and then obtained the slope as the apparent quantum yield (AQY) value. Calculations for light saturation point, the light compensation point, and the maximum net photosynthetic rate were performed as described by Bassman and Zwier (1991). The P_N-PAR curve equation, $P_N = P_{max} (1 - C_0 e^{-\Phi PAR/Pmax})$, was fitted using the *SPSS* software. WUE is equal to the ratio of P_N and transpiration rate (E), the WUE-PAR curve was fitted using the *SPSS* software. CO_2 response curves were analyzed using the built-in automatic measuring program of the instrument. Similar to light response curve measurement, the samples were also exposed to an irradiance of 1 500 µmol m^{-2} s^{-1} and a CO_2 concentration of 400 µmol mol^{-1} for 5 to 6 min before running the program. The program conditions included a temperature of 30 °C and irradiance of 1 500 µmol m^{-2} s^{-1}, which was generated by *6400-02B LED* of the instrument. The series of CO_2 concentrations 400, 300, 200, 150, 100, 50, 400, 600, 800, 1 000, 1 200, and 1 400 µmol mol^{-1} were used. CO_2 was provided by a small cylinder, and the P_N-CO_2 curve was fitted using the *SPSS* software. According to the curve, the saturation point was obtained, and for carboxylation efficiency (CE), we first took the linear portion of the curve where the intercellular CO_2 concentration (ci) was lower than 250 µmol mol^{-1}, and then obtained the slope as the CE value (Xu *et al*. 1990). The CO_2 compensation point was also obtained according to the linear equation.

Chl fluorescence kinetics: The minimal initial fluorescence (F_0) and maximal fluorescence (F_m) were measured with *LI-6400XT* fluorometer (*Li-Cor*, Lincoln, NE, USA) after dark adaptation for 20 min. Then minimal fluorescence (F_0') and maximal fluorescence under irradiation (F_m') were measured after adaptation to irradiance of 1 500 µmol m^{-2} s^{-1} for 1 h. The following fluorescence parameters were calculated: $q_P = (F_m'-F_s)/(F_m'-F_0')$; $NPQ = (F_m-F_m')/F_m'$.

Statistical analysis: For parametric data, an analysis of variance (*ANOVA*) was used by *SPSS 22.0* (*SPSS Inc*., Chicago, IL, USA). The *t*-test significance was set at $P < 0.05$ and 0.01 for all tests.

Results

Chl *a*, Chl *b*, and Car content in the leaves of the *dl-14* mutant were significantly higher than in those of cv. Huanghuazhan by 17.1, 15.3, and 13.8 %, respectively ($P < 0.01$), whereas no significant difference in the Chl *a*/*b* ratio was observed (Table 1).

The leaf vein density measured in leaves of *dl-14* was significantly higher than that of Huanghuazhan by 19.5 %

Table 1. Comparison of chlorophyll (Chl) and carotenoids (Car) content [mg g^{-1}(f.m.)] and vein density [mm mm^{-2}] in *dl-14* mutant and cv. Huanghuazhan. Means ± SEs, *n* = 3. ** - differences significant at 0.01 levels (*t*-test).

Plants	Chl *a*	Chl *b*	Car	Chl *a*/*b*	Vein density
Huanghuazhan	3.112 ± 0.122	1.095 ± 0.076	0.609 ± 0.021	2.842 ± 0.115	3.80 ± 0.14
dl-14	3.645 ± 0.223**	1.262 ± 0.008**	0.693 ± 0.011**	2.885 ± 0.074	4.54 ± 0.22**

Table 2. Light compensation point [µmol(photon) m^{-2} s^{-1}], light saturation point [µmol(photon) m^{-2} s^{-1}], apparent quantum yield (AQY), and P_N [µmol(CO_2) m^{-2} s^{-1}] at light saturation of *dl-14* mutant and cv. Huanghuazhan. Means ± SEs, *n* = 3. *,** - differences significant at 0.05 and 0.01 levels, respectively (*t*-test).

Plants	AQY	Light compensation point	Light saturation point	P_N at light saturation point
Huanghuazhan	0.0319 ± 0.0011	11.7 ± 2.2	1846 ± 22	22.5 ± 1.1
dl-14	0.0273 ± 0.0014*	13.5 ± 1.8*	1677 ± 14**	24.1 ± 1.3*

Table 3. Carboxylation efficiency (CE) [mol m^{-2} s^{-1}], CO_2 compensation points [µmol mol^{-1}], and CO_2 saturation point [µmol mol^{-1}] of *dl-14* mutant and cv. Huanghuazhan. Means ± SEs, *n* = 3. *, ** - differences significant at 0.05 and 0.01 levels, respectively (*t*-test).

Plants	CE	CO_2 compensation point	CO_2 saturation point
Huanghuazhan	0.1198 ± 0.0012	43.4 ± 2.1	874 ± 14
dl-14	0.0814 ± 0.0009*	42.8 ± 3.2	1044 ± 16**

Table 4. Comparison of chlorophyll fluorescence parameters between *dl-14* mutant and cv. Huanghuazhan. Means ± SEs, *n* = 3. * - differences significant at 0.05 level (*t*-test). F_v/F_m - maximal quantum yield of PS II photochemistry, F_v'/F_m' - efficiency of excitation capture of open PS II center, q_P - photochemical quenching coefficient, NPQ - nonphotochemical quenching coefficient, Φ_{PSII} - effective quantum yield of PS II photochemistry, ETR - electron transfer rate.

Plants	F_v/F_m	F_v'/F_m'	q_P	Φ_{PSII}	NPQ	ETR
Huanghuazhan	0.811 ± 0.009	0.390 ± 0.023	0.513 ± 0.051	0.201 ± 0.032	2.796 ± 0.152*	105.7 ± 13.6
dl-14	0.814 ± 0.005	0.475 ± 0.034*	0.705 ± 0.017*	0.335 ± 0.031*	2.137 ± 0.132	176.3 ± 14.2*

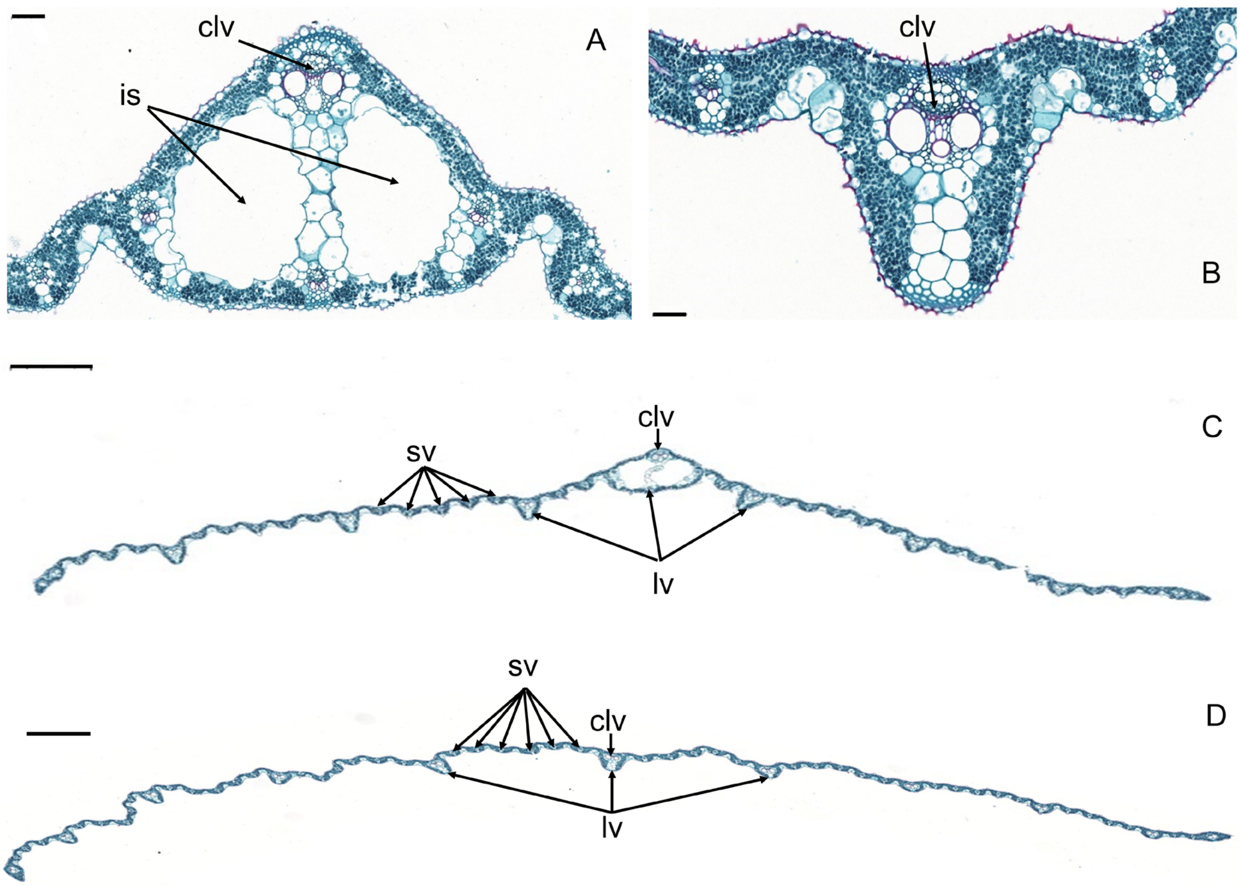

Fig. 1. Microstructure of midvein cross-sections of Huanghuazhan (*A*) and *dl-14* (*B*), and microstructure of leaf cross-sections of Huanghuazhan (*C*) and *dl-14* (*D*). is - intercellular space, clv - central large vascular bundle, lv - large vein, sv - small vein; Scale bars: *A*, *B* = 50 µm, *C*, *D* = 500 µm.

(Table 1). By observing the vein cross-sections, we found that the *dl-14* plants also have a higher number of large leaf veins (Fig. 1*D*), as well as a markedly greater number of small leaf veins between the two large veins. The cross-section of the midvein area of Huanghuazhan leaves had two large cavities called the intercellular space (Fig. 1*A*), which were not observed in the *dl-14* leaves (Fig. 1*B*). There were no air chambers in the midvein area of *dl-14* leaves (Fig. 1*B*). There were central vascular bundle structures in the midvein area of both Huanghuazhan and *dl-14* plants.

The light compensation point and maximum P_N of the *dl-14* mutant were significantly higher than those of wild-type Huanghuazhan by 15.4 and 7.2 % ($P < 0.05$) (Table 2), but the AQY of the mutant was significantly lower than that of Huanghuazhan by ($P < 0.05$). The light saturation point of *dl-14* was also significantly lower than that of Huanghuazhan by 9.2 % ($P < 0.01$). Under irradiance of 600 - 1 600 µmol m^{-2} s^{-1}, the P_N of *dl-14* was significantly higher than that of Huanghuazhan (Fig. 2*A*).

When irradiance was within the range of 200 - 2 000 µmol m^{-2} s^{-1}, the WUE of *dl-14* mutant was significantly lower than that of Huanghuazhan (Fig. 2*B*), indicating that water loss in *dl-14* plants was significantly higher than that in Huanghuazhan.

The CE of the mutant *dl-14* was lower than that of Huanghuazhan by 32.1 % ($P < 0.05$), whereas its CO_2 saturation point was significantly higher by 19.5 % ($P < 0.01$). There was no significant difference in CO_2 compensation points between *dl-14* and Huanghuazhan (Table 3). When CO_2 concentrations were within the range of 800 - 1 400 µmol mol^{-1}, the P_N of *dl-14* was significantly higher than that of Huanghuazhan (Fig. 2*C*).

The NPQ of the mutant *dl-14* was significantly lower than that of Huanghuazhan by 23.6 % ($P < 0.05$) (Table 4); and its F_v'/F_m', q_P, Φ_{PSII}, and ETR were significantly higher than those of Huanghuazhan by 21.8, 37.4, 66.7, and 66.8 %, respectively ($P < 0.05$), suggesting that *dl-14* leaves have greater photon conversion ability. The F_v/F_m ratio of *dl-14* did not significantly differ from that of Huanghuazhan.

Discussion

Leaf anatomical features have important implications for plant physiological functions and specific adaptation to the environment (Sanches and Valio 2008, Tomás *et al.*

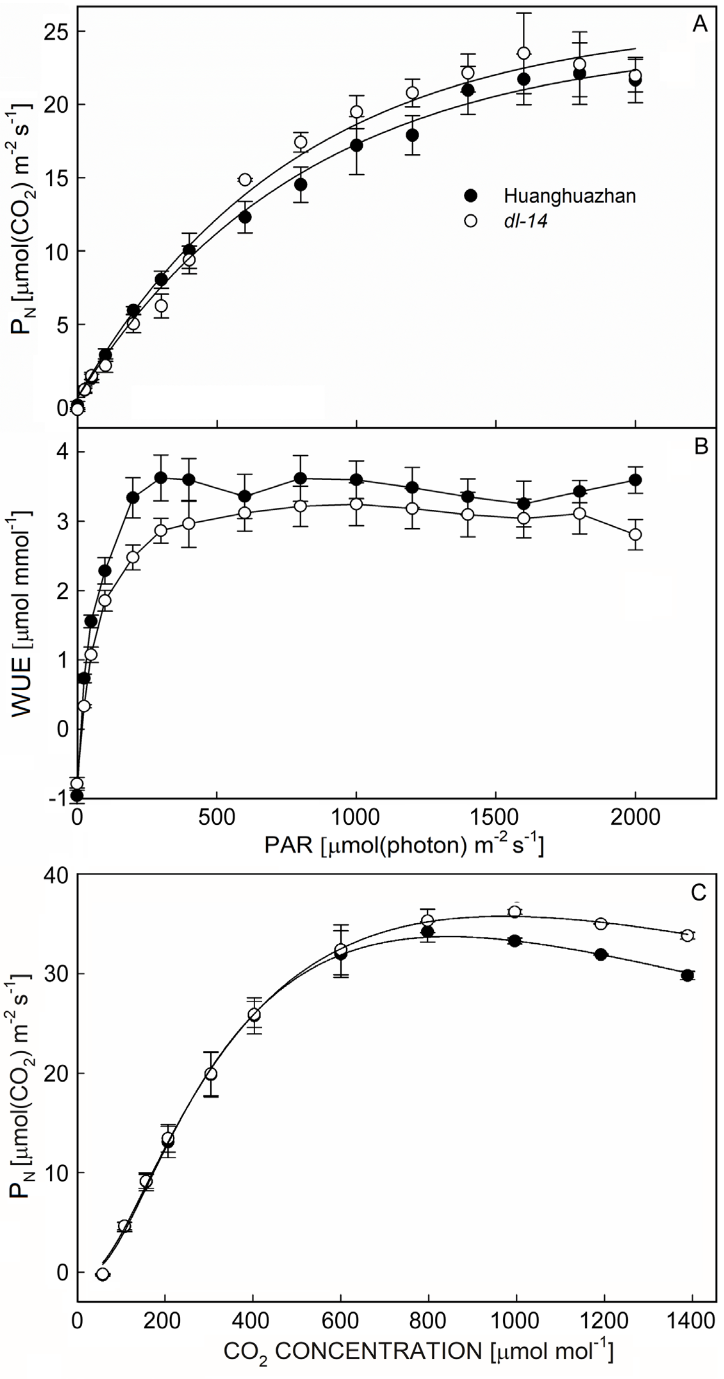

Fig. 2. The net photosynthetic rate (P_N) (*A*) and water use efficiency (WUE) under different irradiance (*B*), and the P_N under different CO_2 concentrations (*C*) in leaves of *dl-14* mutant and cv. Huanghuazhan. Means ± SEs, $n = 3$. PAR - photosynthetically active radiation.

2013). In this study, we found that the midveins of *dl-14* plants have no intercellular spaces, whereas the wild-type Huanghuazhan plants harbor two intercellular spaces. This may be due to the absence of programmed cell death in the same part of *dl-14* leaves. Instead, mesophyll cells are filled in this part, which caused the *dl-14* mutant to have a stronger photosynthetic ability. The leaf vein density and photosynthetic pigment content of *dl-14* were significantly higher than those of Huanghuazhan. Under high irradiance and higher CO_2 concentrations, the P_N in *dl-14* was significantly higher than in Huanghuazhan. This is probably because in *dl-14* plants, the extra mesophyll cells in the intercellular space enhance the P_N under high irradiance and higher CO_2 concentration. Moreover, our results show that *dl-14* plants exhibited higher leaf vein density, indicating improved transport ability of water and nutrients. These observations were consistent with the findings of several previous reports (Song *et al.* 2015, Qin *et al.* 2017, Xu *et al.* 2017). In this study, the mutant *dl-14* had higher values of F_v'/F_m', q_P, Φ_{PSII}, ETR, and lower value of NPQ, indicating that the mutant *dl-14* has stronger photon conversion ability and weaker radiation dissipation ability, which is consistent with the result that the mutant *dl-14* has higher P_N under high irradiance. But it is worth noting that under irradiance of 200 - 2 000 μmol m^{-2} s^{-1}, *dl-14* plants with higher vein density showed a significantly lower WUE than wild-type Huanghuazhan, which is in contrast to the observations of some previous reports (Brodribb *et al.* 2010, McKown and Dengler 2010). To understand the reasons for these different observations, further investigations are needed.

In conclusion, there were no air spaces in the midvein-deficient part of *dl-14*, which was filled with mesophyll cells. This may be that there is no programmed cell death in the same part of *dl-14* leaves, resulting in *dl-14* having a strong photosynthetic ability. Meanwhile, the vein density of *dl-14* was also significantly higher than Huanghuazhan. In terms of photosynthetic characteristics, the mutant *dl-14* has higher P_N under high irradiance and high CO_2 concentrations. In addition, the mutant *dl-14* had higher values of F_v'/F_m', q_P, Φ_{PSII}, ETR, and a lower value of NPQ than Huanghuazhan. These findings indicate that the mutant *dl-14* has higher vein density, stronger photon conversion ability and weaker radiation dissipation ability. We think *dl-14* might be an excellent rice breeding material.

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