Differences on photosynthetic limitations between leaf margins and leaf centers under potassium deficiency for *Brassica napus* L.

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Analyzing the proportions of stomatal ($S_L$), mesophyll conductance ($MCL$) and biochemical limitations ($BL$) imposed by potassium ($K$) deficit, and evaluating their relationships to leaf K status will be helpful to understand the mechanism underlying the inhibition of K deficiency on photosynthesis ($A$). A quantitative limitation analysis of K deficiency on photosynthesis was performed on leaf margins and centers under K deficiency and sufficient K supply treatments of *Brassica napus* L. Potassium deficiency decreased $A$, stomatal ($g_s$) and mesophyll conductance ($g_m$) of margins, $S_L$, $MCL$ and $BL$ accounted for 23.9%, 33.0% and 43.1% of the total limitations. While for leaf centers, relatively low limitations occurred. Nonlinear curve fitting analysis indicated that each limiting factor generated at same leaf K status (1.07%). Although $MCL$ was the main component of limitations when $A$ began to fall, $BL$ replaced it at a leaf K concentration below 0.78%. Up-regulated $MCL$ was related to lower surface area of chloroplasts exposed to intercellular airspaces ($S_c/S$) and larger cytosol diffusion resistance but not the cell wall thickness. Our results highlighted that photosynthetic limitations appear simultaneously under K deficiency and vary with increasing K deficiency intensity.

Potassium (K), one of the macronutrients essential for plant growth and development, is involved in many physiological processes, such as photosynthesis, enzyme activation, water relations, assimilate transport, and protein synthesis1,2. K deficiency profoundly decreased crop yield3,4, thus strategies of survival and improvement would be important for plant growing under adverse conditions. It is a truism that most of the dry matter is formed by leaf photosynthesis ($A$) which is intimately connected with K status. Multifarious studies have come to a nearly consistent conclusion that leaf $A$ decreases in K-starved plants 3,5,6, therefore an even deep comprehending of mechanism underlying the inhibition of K deficiency on $A$ is necessary2.

During photosynthesis, CO$_2$ moves from external atmosphere to the internal leaf air spaces through the stomata, and from there to carboxylation sites inside the chloroplasts7. It is established that stomatal closure is the foremost limitation to CO$_2$ assimilation due to the vital role of K in stomatal aperture8,9. As a major osmotica, K$^+$ accumulation in vacuole is essential for stomatal opening, which had been verified to be initially dropped under K deficiency10. And because of this, Bednarz et al.5 stated that the most limiting resistance to $A$ of *Gossypium hirsutum* L. came from stomata5. In contrast, K starvation caused a decreased $A$ and stomatal conductance ($g_s$), but an increased intercellular CO$_2$ concentration ($C_i$) of *Carya cathayensis* leaves, suggesting that, in addition to $g_s$, mesophyll conductance ($g_m$) and biochemical limitations might be involved in the depression of photosynthesis in K deficient conditions6. Numerous studies have shown that $g_m$ is relatively low, leading to great draw-down of chloroplastic CO$_2$ concentration from $C_i$, and changed along the variation of water status, nitrogen nutrient, irradiance, temperature and CO$_2$ concentration11–14. Moreover, leaf structures, specific aquaporins, plasma membrane etc. are involved in the determinations of $g_m$15. K starvation might have reduced aquaporin activity16 and increased leaf dry mass per unit area ($M_A$)1,17, therefore, causing a stronger mesophyll diffusion resistance to CO$_2$ delivery18. Besides, K nutrition also known to increase the leaf intercellular air space to enhance $g_m$1.

Additionally, biochemical processes may restrain photosynthesis, particularly under severe and/or long-time K starvation1,5,6. It was reported that Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39)

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activity was decreased under K deficiency, becoming a major limiting factor for photosynthesis in *Oryza sativa* leaves. Chlorophyll synthesis was observed to be significantly impaired under K deficiency in *Eucalyptus grandis* leaves. Moreover, K starvation up-regulated the fraction of electron transport to O₂, resulting in an increased reactive oxygen species (ROS). Carbohydrate accumulation which may feedback regulation of leaf photosynthesis is more easily observed in K starved leaves. Indeed, the relative contributions of these three limiting processes to photosynthesis under K deficiency and the underlying mechanisms have not been fully explored, due to the complicated physiological processes and variation of dominant limiting factors under different K deficiencies. No matter what the primary cause of decrease, the discrepancy between researches was believed to be derived from different physiological K deficiency severities. For this reason, a comprehensive consideration of whole limiting factors and their relationships with leaf K status seems to be important.

In 2005, Grassi and Magnani proposed a method to accurately quantify photosynthetic limitations by separating the relative controls on A resulting from SL, mesophyll conductance (MCL) and biochemical limitations (BL). This method has been successfully applied for evaluating the relative control of leaf A under water stress and during their recovery processes, among inter- and intra-specific. It showed not only great potential for elucidating the magnitude changes of limitations and their dominance in photosynthetic restraints with increasing severity of K deficiency, but also revealing the corresponding critical K concentrations for their transformation.

Winter oilseed rape (*Brassica napus* L.), a model-plant of winter cover crops which needs substantial amount of potassium to growth was used for a deeply aggregate analysis of K deficiency on photosynthetic limitations. This method has been successfully applied for evaluating the relative control of leaf A under water stress and during their recovery processes, among inter- and intra-specific. It showed not only great potential for elucidating the magnitude changes of limitations and their dominance in photosynthetic restraints with increasing severity of K deficiency, but also revealing the corresponding critical K concentrations for their transformation.

### Results

**Plant performance, leaf K concentration and net photosynthesis.** The total dry matter of the –K treatment decreased significantly by 29.9% on average versus the +K treatment (Table 1). The leaf expansion was also restrained, with a 22.1% and 18.0% decline in the individual leaf dry matter and leaf area, respectively. Leaf K concentration was dramatically influenced by potassium supply and leaf position, which was significantly lower in the –K treatment than in the +K treatment. Meanwhile, within an individual leaf, K concentration was remarkably lower in margins than in centers. The mean net photosynthesis (A) in the leaf margins of the –K treatment was 56.9% that of the +K treatment. However, there was no significant difference between leaf margins and centers under the –K treatment, as well as the two positions under the +K treatment.

### Stomatal conductance.

Potassium deficiency led to a significant decline of the mean stomatal conductance (gS) in leaf margins, which was 63.6% that of the +K treatment. However, the mean gS value of the leaf centers was not influenced by K nutrient (Table 2). There was a significantly lower gS in leaf margins than in leaf centers under the –K treatment, whilst the gS values of these two positions were the same under the +K treatment.
well as between the two positions in the K treatment. Potassium nutrient and leaf position did not affect mean + but showed no statistical differences among the other three groups. POD activity increased by 25.5%.

C (concentration. Here a photosynthesis-based concentration threshold with the relative values reaching 95.0% of chloroplastic CO2 compensation point (C*) raised, and the mean Vc,max decreased due to K deficiency, particularly in the leaf margins with a 28.0% decline in the single stomatal pore area of leaf chlorophyll concentration was found in the –K treated leaves, especially in the leaf margins, with a 31.1% decrease. The variation of photosynthetic parameters was verified by chemical analyses (Table 4). A significant decline in these two positions under the same in the leaf centers of the –K treatment and the two positions of the fifth fully expanded leaves.

| Treatment | Position | gm (mol CO2 m⁻² s⁻¹) | Cᵢ (μmol CO2 mol⁻¹) | Cₗ (μmol CO2 mol⁻¹) | Rₛ (μmol CO2 m⁻² s⁻¹) | Γ* (μmol CO2 mol⁻³) |
|-----------|----------|----------------------|---------------------|---------------------|-----------------------|---------------------|
| –K        | margin   | 0.142 ± 0.006b       | 257 ± 6a            | 334.1 ± 7.0a        | 9.11 ± 0.07a           | 2.91 ± 0.02b        | 20.87 ± 0.15b       | 6.97 ± 0.05b       |
|           | center   | 0.231 ± 0.012a       | 240 ± 5a            | 334.7 ± 8.5a        | 9.94 ± 0.04a           | 3.49 ± 0.03a        | 27.30 ± 0.12a       | 9.14 ± 0.04a       |
| +K        | margin   | 0.223 ± 0.017a*      | 238 ± 7a            | 335.7 ± 14.4a       | 10.01 ± 0.05a          | 3.68 ± 0.02a*       | 28.90 ± 0.14a*      | 9.70 ± 0.05a*      |
|           | center   | 0.238 ± 0.011a       | 231 ± 3a            | 338.5 ± 7.9a        | 10.66 ± 0.04a          | 3.54 ± 0.02a        | 29.64 ± 0.15a       | 10.03 ± 0.05a*      |

Table 2. Effects of K deficiency on mesophyll conductance (gm), intercellular CO2 concentrations (Ci), stomatal frequency, length, and width, single stomatal pore area and total stomatal pore area of the two positions in the lower epidermis of the fifth fully expanded leaves. Images were taken at a magnification of ×500 with a scanning electron microscope. Values are mean ± SE of four replications for gm, Ci of 20 replications for stomatal frequencies, and of 300 replications for stomatal lengths, stomatal widths, stomatal pore areas and total stomatal pore areas. 1Different letters in the same column at a given treatment indicate significant differences between positions (P ≤ 0.05). 2*shows significant differences between the two K treatment in same position (P ≤ 0.05).

decrease of the gm value in leaf margins of the –K treatment, the intercellular CO2 concentrations (Ci) value was raised, and the mean Ci were similar to those of other groups.

Potassium supply and leaf position had no effects on stomatal frequency and stomatal length (Table 2). However, stomatal width was significantly decreased in the –K treatment, especially in the leaf margins where the width decreased by 20.9% as compared with the +K treatment. Stomatal pore area was therefore considerably decreased due to K deficiency, particularly in the leaf margins with a 28.0% decline in the single stomatal pore area. Nevertheless, the stomatal length and width as well as the stomatal pore area showed no significant difference in these two positions under the +K treatment.

Mesophyll conductance. Despite a dramatic decrease in the mean mesophyll conductance (gm) in the leaf margins of the –K treatment, the mean chloroplastic CO2 concentrations (Ci) was 9.4% higher than that of the +K treatment (Table 3). The mean gm and Ci values were similar in the leaf centers of different K treatments, as well as between the two positions in the +K treatment. Potassium nutrient and leaf position did not affect mean intercellular CO2 compensation point (Ci*) and mitochondrial respiration rate in the light (Rₛ), and chloroplastic CO2 compensation point (Γ*) in the two positions of the fifth fully expanded leaves. Ci* and Rₛ were measured by Laisk method, Γ* was calculated according to the equation Γ* = Ci* + Rₛ/gm. Values are mean ± SE of four replications for gm, Ci*, and Ci-Cₗ of three replications for Ci*, Rₛ and Γ*. 1Different letters in the same column at a given treatment indicate significant differences between positions (P ≤ 0.05). 2*shows significant differences between the two K treatment in same position (P ≤ 0.05).

Biochemical characteristics. The mean maximum rate of electron transport (Jᵥmax) and maximum rate of carboxylation (Vc,max) in the leaf margins of the –K treatment were the lowest, and minor changes were observed among the other three treatments (Table 4). However, the mean Jᵥmax/Vc,max in leaf margins of the –K treatment was dramatically increased compared with the mean values of the other three groups in the range from 1.42 to 1.46. The variation of photosynthetic parameters was verified by chemical analyses (Table 4). A significant decline of leaf chlorophyll concentration was found in the –K treated leaves, especially in the leaf margins, with a 31.1% decrease. Furthermore, Rubisco activity was dramatically decreased in leaf margins of the –K treatment, but it was the same in the leaf centers of the –K treatment and the two positions of the +K treatment. Potassium deficiency caused severe ROS production in leaf margins where O₂⁻ generation rate increased by 22.8%, and meanwhile, POD activity increased by 25.5%.

The relationship between relative Aᵥ, gm and gm with leaf K concentration. A significant curvilinear relationship between relative Aᵥ, gm, or gm and leaf K concentrations is shown in Fig. 1. The relative values increased with increasing leaf K concentration, and remained stable when the leaf K concentration was beyond a certain concentration. Here a photosynthesis-based concentration threshold with the relative values reaching 95.0% of...
Table 4. Effects of K deficiency on the maximum rate of electron transport ($I_{\text{max}}$), maximum rate of carboxylation ($V_{\text{c,max}}$), ratio between $I_{\text{max}}$ and $V_{\text{c,max}}$ ($I_{\text{max}}/V_{\text{c,max}}$) estimated from A-C curves, chlorophyll concentration, Rubisco activity, $O_2$ generation rate, and POD activity in the two positions of the fifth fully expanded leaves. Values are mean ± SE of four replications. *Different letters in the same column at a given treatment indicate significant differences between positions ($P \leq 0.05$). **Shows significant differences between the two K treatment in same position ($P \leq 0.05$).

| Treatment | Position | $I_{\text{max}}$ (µmol m$^{-2}$ s$^{-1}$) | $V_{\text{c,max}}$ (µmol m$^{-2}$ s$^{-1}$) | $I_{\text{max}}/V_{\text{c,max}}$ | Chlorophyll (g m$^{-2}$) | Rubisco activity (U g$^{-1}$ FW) | $O_2$ generation rate (nmol g$^{-1}$ FW min$^{-1}$) | POD activity (U g$^{-1}$ FW min$^{-1}$) |
|-----------|----------|------------------------------------------|------------------------------------------|---------------------------------|-------------------------|---------------------------------|-------------------------------------------|---------------------------------|
| −K        | margm    | 114.5 ± 9b                              | 70.9 ± 4.8b                              | 1.61 ± 0.12a                    | 0.42 ± 0.03b            | 0.29 ± 0.02b                    | 8.77 ± 0.55a                              | 2868 ± 176a                               |
|           | center   | 166.2 ± 10a                             | 115.9 ± 4.5a                             | 1.46 ± 0.03b                    | 0.54 ± 0.02a            | 0.36 ± 0.01a                    | 7.69 ± 0.15b                              | 2511 ± 74b                               |
| + K       | margm    | 170.6 ± 12a                             | 120.5 ± 3.9a                             | 1.42 ± 0.06a                    | 0.61 ± 0.05a            | 0.33 ± 0.01a                    | 7.18 ± 0.22a                              | 2275 ± 111a                               |
|           | center   | 167.5 ± 9a                              | 118.0 ± 5.6a                             | 1.42 ± 0.13a                    | 0.64 ± 0.04a            | 0.35 ± 0.01a                    | 6.61 ± 0.33a                              | 2085 ± 148a                               |

Discussion

Limitations imposed by K deficiency occur at the same time. In the present study, $A$ in leaf margins were weakened by K deficiency. Generally, the declining $A$ is considered to be limited by stomatal and mesophyll resistances to CO$_2$ diffusion, and biochemical obstacles$^{11,22}$. Here we demonstrated that $g_s$, $g_m$, and biochemical activities were profoundly restricted as $M_j$ down-regulated. Stomatal conductance which determine the vital step of CO$_2$ diffuse from the atmosphere to the interior of leaf was markedly decreased in −K leaf margins, as reported by Laetsch$^1$, Gossypium hirsutum$^1$, and Orzya sativa$^1$. This is mainly because the lack of vacuole K to keep stomatal aperture by providing driving force to promote water impour into the guard cell vacuole$^3$. The declined $A$, to a certain extent, revealed that the K in cytoplasm identified as biochemical functional component was below the critical value$^{10}$. Therefore, malfunction of physiological process could come with limited $A$.

Likewise, $g_m$ was decreased in parallel with $A$. Indeed, $g_m$ might be down-regulated by increasing leaf dry mass per area ($M_A)^{2,23}$, however, in the present study, there was no remarkable difference in $M_i$ between the −K and + K leaves (Table 5; Supplementary Fig. S1). Cell wall thickness ($T_{\text{cell-wall}}$) and surface area of chloroplasts exposed to intercellular airspaces ($S_i/S$) are reported to be the most substantial anatomical traits in determining $g_m$.$^{23,32}$, However, significant differences in mesophyll cell wall surface area exposed to intercellar airspace per leaf area ($S_m/S$) and $S_i/S$, but not $T_{\text{cell-wall}}$ between leaf margins of two K treatments were observed (Table 5). Besides, chloroplast size$^1$ has also been proved to influence $g_m$. In the present study, though the chloroplast length ($L_{\text{chl}}$) decreased under lowest K status, the thickness ($T_{\text{chl}}$) and volume ($V_{\text{chl}}$) of chloroplast were largely increased, however, the $S_{\text{chl}}/V_{\text{chl}}$ was smaller (Fig. 3). The chloroplast enlarging under lowest K concentration was not completely same to that discovered under low nitrogen conditions$^{30,31}$. The increase of $T_{\text{chl}}$ was more likely to be based on the sacrifice of length owing to roughly circular envelope (Fig. 3a,b; Supplementary Fig. S2). Mathematically, ellipsoidal chloroplast, combining with an insectent chloroplast number (see Supplementary Fig. S3) were more probably to have longer length of chloroplasts facing the cell wall than swollen even sphere envelopes. Furthermore, the resistance along diffusion pathway length in cytoplasm (distance of chloroplast from cell wall, $D_{\text{cell-cw}}$) and stroma (taken as half of the chloroplast thickness) account for 10–50% of $g_m$ limitation$^{23}$, which however, reported only up to 22% of liquid phase resistance ($r_{\text{in}}$) by Evans et al. in 1994$^{25}$. Low K status strongly increased $T_{\text{chl}}$ and $D_{\text{cell-cw}}$ (Fig. 3b,f), accordingly, the corresponding resistance would be increased. It is therefore proved that the decreased $g_m$ is primary due to the reduced $S/S$ and larger cytosol diffusion resistance but not $T_{\text{cell-wall}}$. More evidences may seek from the influence of K on plasma membrane and chloroplast envelope conductance$^{32}$, carboonic anhydrase and aquaporins that participated in determination of $g_m$.$^{14,15,23}$

It should be noted that $A$, $g_s$, or $g_m$ started to decline almost at the same time with an extremely similar leaf K status. By another way, the quantitative analysis of limitations indicated that three limiting factors coexist when K concentration below 1.07%. This was similar to the results reported by Grassi and Magnani$^{25}$ and Tezara et al.$^{26}$
in plants suffering from water stress. However, the investigation carried out by Galmés et al.\textsuperscript{13} revealed that B\textsubscript{t} of Hypericum balearicum and Phlomis italica still remained zero under mild water stress even if the total limitation reached 20–30%. The present finding highlights that all photosynthetic limitations simultaneously occur when leaf is in a physiological K-deficiency state.

Figure 1. Relationship between relative photosynthetic parameters and leaf K concentration. Relative (a) photosynthesis rate (\(A\)), (b) stomatal conductance (\(g_s\)), (c) mesophyll conductance (\(g_m\)). The values were the relative proportion of measured values over the mean values of K-sufficient leaf centers. Each point represents one leaf measurement. Open triangles and closed triangles represent the values of leaf margin and leaf center under the –K treatment, while open circles and closed circles represent those of the + K treatment. Equations, regression coefficients, and significance are shown when \(P \leq 0.05\) (*\(P \leq 0.05\); **\(P \leq 0.01\)).
Limitations vary with increasing K deficiency intensity. The leaf K concentration threshold value observed in this study was 1.07%, in consistent with the range of 0.5 to 2.0% reported by Leigh and Wyn Jones. Quantitative limitation analysis gives insight into the contributions of different photosynthetic limitations, revealing that the BL and MC L accounted for the majority of total limitations in K-starved leaf margins.

Figure 2. Photosynthetic limitations and their response to leaf K concentration. (a) Quantitative limitation analysis of photosynthetic CO₂ assimilation in leaf margins and centers under the –K treatment. Values are mean ± SE of four replicates per position. The open, gray, and dark gray bars represent the percentages of stomatal (S L), mesophyll conductance (MC L), and biochemical (BL) limitations, respectively. (b) Relationships between limitations and leaf K concentration. Each point with the same shape represents a single leaf (n = 16). The symbols are as follows: S L, closed squares; MC L, open circles; BL, closed triangles. Solid, dash, and dot lines are regression curves of S L, MC L, and BL, respectively. Equations, regression coefficients, and significance are shown when \( P \leq 0.05 \) (* \( P \leq 0.05 \); ** \( P \leq 0.01 \)).

| Treatment Position | \( T_{\text{leaf}} \) (μm) | \( T_{\text{cell-wall}} \) (μm) | \( S_m/S \) (m² m⁻²) | \( S_c/S \) (m² m⁻²) |
|-------------------|-----------------------------|-----------------------------|---------------------|---------------------|
| –K margin         | 305 ± 27.7a                 | 0.167 ± 0.017a              | 12.9 ± 1.8b         | 8.6 ± 1.1b          |
| –K center         | 310 ± 19.2a                 | 0.170 ± 0.033a              | 17.1 ± 2.4a         | 12.8 ± 0.9a         |
| +K margin         | 309 ± 31.3a                 | 0.172 ± 0.049a              | 17.4 ± 3.3a*        | 13.1 ± 1.6a*        |
| +K center         | 312 ± 22.0a                 | 0.176 ± 0.045a              | 18.2 ± 2.6a         | 13.5 ± 2.1a         |

Table 5. Effects of K deficiency on leaf thickness (\( T_{\text{leaf}} \)), mesophyll cell wall thickness (\( T_{\text{cell-wall}} \)), mesophyll cell wall surface area exposed to intercellular airspace per leaf area (\( S_m/S \)), and surface area of chloroplasts exposed to intercellular airspaces (\( S_c/S \)) in the two positions of the fifth fully expanded leaves. Data are mean ± SE of eight replications for \( S_m/S \) and \( S_c/S \), at least thirty replications for \( T_{\text{leaf}} \) and \( T_{\text{cell-wall}} \). *Different letters in the same column at a given treatment indicate significant differences between positions (\( p \leq 0.05 \)). ** Shows significant differences between the two K treatment in same position (\( p \leq 0.05 \)).
centers, respectively. This is mainly ascribed to the discrepancy of relative severity of K deficiency. As has been stated in the previous studies that some irreversible damages, such as impaired ATP synthesis, depressed Rubisco activity, and cell damage occurred when the limiting A, for the most part, is attributed to BL. Some of which were verified in the present study, such as degraded chloroplast, limited photoassimilate transportation (see Supplementary Fig. S2), and increased O$_2$ generation rate under severe K deficiency. The obstacle of these physiological processes alleviated as K deficient stress mitigating, however, the role of MCL on A began to stand out.

The relationship between relative limitations and leaf K concentration verified that, at a leaf K concentration of less than 1.07%, MCL represented the main component of limitations, but BL replaced it when leaf K concentration below 0.78%. This pattern, to a lesser extent, could be found in plants suffering from water stress which suggested that the variation of limitations depends on the stress intensity and duration. Regrettably, the present study failed to reveal whether or not there is a critical concentration in the shifting process from SL predominance to MCL predominance. Further studies focusing on the photosynthetic limitations of rapeseed leaves subjected to a serial K gradient may help to elucidate this issue.

Figure 3. The relationship between chloroplast characteristics and leaf K concentration. (a) Chloroplast length (L$_{chl}$), (b) thickness (T$_{chl}$), (c) surface area (S$_{chl}$), (d) volume (V$_{chl}$), (e) S$_{chl}$/V$_{chl}$, (f) distance of chloroplast from the cell wall (D$_{chl-cw}$). Values are mean ± SE of four replicates for K concentration and at least thirty replicates for microstructure parameters. Regression coefficients and significance are shown when $P \leq 0.05$ (*$P \leq 0.05$; **$P \leq 0.01$).
500 was 5.9 °C. The total precipitation during oilseed rape cropping season was 660.7 mm, with wintertime account-
ture of the season was 13.8 °C, and the average temperature during winter (from December 2013 to February 2014) was 5.9 °C. The total precipitation during oilseed rape cropping season was 660.7 mm, with wintertime account-
ing for 26.1% of the total. The soil was a sandy loam with pH 5.3, organic matter 30.5 g kg\(^{-1}\), total N 1.7 g kg\(^{-1}\), \(\text{NH}_4\text{OAc}\cdot\text{K} 42.5 \text{mg kg}^{-1}\), Olsen-P 15.7 mg kg\(^{-1}\) and hot-water soluble B 0.78 mg kg\(^{-1}\) in the topsoil layer (0–20 cm). As stated by Zou, the soil belongs to a K-deficient type, which would cause yield reduction without K fertilizer addition.  

### Methods

#### Study site and growth conditions.  
A field experiment was conducted in Wuxue county, Hubei province, central China (30°06′46″N, 115°36′9″E) during the 2013–2014 oilseed rape growing season. The mean temperature of the season was 13.8 °C, and the average temperature during winter (from December 2013 to February 2014) was 5.9 °C. The total precipitation during oilseed rape cropping season was 660.7 mm, with wintertime accounting for 26.1% of the total. The soil was a sandy loam with pH 5.3, organic matter 30.5 g kg\(^{-1}\), total N 1.7 g kg\(^{-1}\), \(\text{NH}_4\text{OAc}\cdot\text{K} 42.5 \text{mg kg}^{-1}\), Olsen-P 15.7 mg kg\(^{-1}\) and hot-water soluble B 0.78 mg kg\(^{-1}\) in the topsoil layer (0–20 cm). As stated by Zou, the soil belongs to a K-deficient type, which would cause yield reduction without K fertilizer addition.

#### Experimental design.  
A complete randomized block design was set up with two treatments and four rep-
licates. The treatments were: (1) Sufficient K supply treatment (+K), with a K fertilizer recommendation rate of 120 kg K\(_2\text{O}\) ha\(^{-1}\) which was tested and well-proved to ensure the optimal growth and yield formation of oilseed rape based on field experiments in this region\(^{39}\). (2) K deficiency treatment (−K), with no K fertilizer applied throughout the growing season.

To ensure that nutrients other than K did not limit plant K uptake, 180 kg N ha\(^{-1}\), 90 kg P\(_2\text{O}_5\) ha\(^{-1}\), and 1.6 kg B ha\(^{-1}\) were applied for these two treatments. The N, P, K, B fertilizers used in the experiment consisted of urea (46% N), superphosphate (12% P\(_2\text{O}_5\)), potassium chloride (60% K\(_2\text{O}\)), and borax (10.8% B). The N fertilizer was applied in three splits: 60% prior to transplanting, i.e., BBCH (Biologische Bundesantalt, Bundessortenamt and Chemische Industrie) 15–16\(^{40}\), 20% at the over-wintering stage (i.e., BBCH 29), and 20% at the initiation of stem elongation (i.e., BBCH 30). Besides, all the P, K, B fertilizers were applied as basal fertilizers. The experimental field was plowed and leveled with a rotary tiller, and basal fertilizers were incorporated during the process. The plot measured 20 m\(^2\) with a length of 10 m and a width of 2 m.

The oilseed rape cultivar was Zhongshuang 11, supplied by Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences. Rapeseeds were sown in prepared seedbeds on 16 September 2013, and then, on 22 October, about 36 d after sowing, oilseed-rape seedlings with five to six leaves (i.e., BBCH 15–16, 3–4 g dry weight plant\(^{-1}\)) were uniformly selected and transplanted by hand in double rows spaced approximately 0.3 m apart with 0.2–0.3 m between plants, corresponding to 112 500 plants ha\(^{-1}\). The oilseed rape was grown under rain-fed conditions. Meanwhile, weeds, pests and disease stresses were controlled by spray herbicides, insecticide and fungicide so that no obvious weeds, insect pests, and diseases infestation occurred during cropping season.

#### Plant and leaf tagging.  
There was an obvious phenotypic difference in plants between the −K and +K treatments 60 d after transplanting. The discrepancy was highlighted in the fifth to ninth fully expanded leaves (with 90% red light and 10% blue light). CO\(_2\) concentration in the leaf chamber (\(C_{\text{a}}\)) was set at 400 \(\mu\text{mol mol}^{-1}\) air, leaf temperature was controlled at 25 ± 0.2 °C, relative humidity was between 50 and 60%, and the flow rate was 500 \(\mu\text{mol s}^{-1}\). In addition to net photosynthesis (\(A\)), stomatal conductance to water vapour (\(g_s\)) and intercellular
CO₂ concentration (Cᵢ), the incorporated fluorometer allowed determination the steady-state fluorescence yield (Fᵣ) under actinic light and maximum fluorescence (F′ᵣ) during light-saturating pulse (0.8 s) of approx. 8000 μmol m⁻² s⁻¹. The relative Aᵢ, gᵢ, and gᵣ values were the relative proportion of measured values over the mean values of K-sufficient leaf centers.

A/Cᵢ curves were measured on the two positions that had been previously acclimated to saturating light conditions for 20 min. The CO₂ concentration (Cᵢ) in the gas exchange chamber was reduced stepwise from 400 to 300, 250, 200, 150, 100, 50 μmol CO₂ mol⁻¹, and then increased from 50 to 400, 600, 800, 1000, 1200, 1500, 1800 μmol CO₂ mol⁻¹ at a constant PPFD of 1200 μmol m⁻² s⁻¹ at 25 ± 0.2 °C, and 50–60% relative humidity. In all cases, the parameters were recorded after the gas exchange rate stabilized at the given Cᵢ. At least four leaves were performed in each treatment.

The actual photochemical efficiency of photosystem II (ΦPSII) was then determined as follows:

\[ \Phi_{PSII} = \frac{F'_m - F_m}{F'_m} \]  

The electron transport rate (J) can be calculated as:

\[ J = \Phi_{PSII} \times PPFD \times \alpha \times \beta \]  

Where α is the leaf absorptance, and β is the fraction of light distributed to PSII. As routinely assumed, α was taken as 0.85⁴²,⁴³ and β was taken as 0.5⁴,⁴⁵. A sensitivity analysis of J biases resulting from rough assumption of α and β on gᵣ variations was also conducted (See Supplementary Table S5).

Mesophyll conductance was estimated according to Harley et al. from combined gas exchange and chlorophyll fluorescence measurements⁴⁶.

\[ \frac{g_m}{C_i} = \frac{A}{C_i - \frac{\Gamma^*}{J - 4(A + R_d)}} \]  

where A, Cᵢ and J were determined as previously described for each treatment, mitochondrial respiration rate in the light (R_d) and the intercellular CO₂ compensation point (Cᵢ) were measured by Laish method, as described by Brooks and Farquhar⁴⁷. Briefly, the A/Cᵢ curves generated with PPFD values of 75, 150, 500 μmol m⁻² s⁻¹, respectively, with each having five different Cᵢ in chamber (i.e. 50, 80, 100, 120 and 150 μmol CO₂ mol⁻¹). A linear regression was then fitted to each A/Cᵢ curve. The x-axis and y-axis of intersection point of three A/Cᵢ curves were defined as Cᵢ and R_d⁴⁶. The Γ* is the chloroplastic CO₂ photocompensation point calculated from Cᵢ and R_d as:

\[ \Gamma^* = C_i + \frac{R_d}{g_m} \]  

For each data point generated, we checked whether it met the range of 10 < dCᵢ/dA < 50⁵⁰. The CO₂ concentration in the chloroplast stroma (C_c) was calculated as:

\[ C_c = C_i + \frac{A}{g_m} \]  

Therefore, A-Cᵢ curves were converted into A-C_c curves. On the basis of C_c, the maximum rate of Rubisco-catalysed carboxylation (V_C,max), and the maximum rate of electron transport (J_max) as defined by Farquhar et al.⁴⁸, were calculated.

Since variable J method is sensitive to many sources of errors, e.g. (1) Γ* and R_d biases; (2) a wrong assumption of p₁ and p₂; (3) biases in the measurements of Cᵢ, A, and J, a sensitivity analysis would be great values to improve the confidence in gᵣ estimates and following limitation calculations⁵¹. Following the method of Harley et al.⁴⁶, we used actual Γ*, R_d and J values calculated in this study and a deviation from the measured values to analyze the effects of Γ*, R_d and J on gᵣ estimates (see Supplementary Table S1, S3, S5). RuBP regeneration can be limited by either insufficient NADPH or ATP, according to Farquhar model, A and J can be linked as follows:

\[ A = \frac{J(\Gamma_c - \Gamma^*)}{p_1C_c + p_2\Gamma^* - R_d} \]  

For insufficient NADPH, p₁ = 4 and p₂ = 8; for insufficient ATP, p₁ = 4.5 and p₂ = 10.5 or p₁ = 4 and p₂ = 9.33. Finally, the sensitivity analysis for photosynthetic limitations was conducted basing on these calculated gᵣ values (see Supplementary Table S2, S4, S6). The analysis showed that the gᵣ was significantly affected by varying Γ* and R_d (see Supplementary Table S1), p₁ and p₂ inputs (see Supplementary Table S3) and J biases (see Supplementary Table S5). However, the gᵣ variation derived from Γ*, R_d, J, p₁ and p₂ biases, did not cause profound effects on photosynthetic limitations (see Supplementary Table S2, S4, S6). In addition, gᵣ appears to be strikingly affected by Cᵢ¹⁴,¹²,¹⁳, nevertheless, the similar Cᵢ in different treatments and positions here seems to have no impact on gᵣ (Table 3). Therefore, the results obtained was unlikely to be altered by these methodological artifacts.

**Plant dry matter, leaf area and dry matter.** Six tagged leaves and six tagged plants in each plot were used to determine the individual leaf area, dry matter, and total dry matter. Each leaf was digitally scanned using an Epson ES-1200C scanner (Epson, Long Beach, CA, USA), and the area determined using ImageJ software.
(National Institutes of Health, Bethesda, Maryland, USA). Individual leaf dry matter and total dry matter were weighed after oven drying at 65 °C for 48 h.

**Biochemical analysis.** Twelve tagged leaves per plot were picked immediately after the determination of photosynthesis. They were divided into two parts along vertical lines (Fig. 4), followed by dissecting the leaf apexes into leaf margins and leaf centers, and removing all the veins. A portion of segments were immersed in liquid N and then stored at −78 °C, and the rest were used for leaf K concentration determination. There were four replications for biochemical determinations.

Leaf segments (2 g) were oven dried at 65 °C for 48 h. After that, about 0.15 g dried leaves were milled and digested with H2SO4–H2O2 as described by Thomas et al., and K concentration in digestion solution was measured by a flame photometer (M-410, Cole-Parmer, Chicago, IL, USA).

The Rubisco extracts were prepared according to Weng et al. with minor modifications. Briefly, leave segments (0.2 g) were ground to a powder using a chilled mortar and pestle with liquid N2 and a small amount of quarsand, followed by homogenization with 4 mL pre-cooled extraction buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM MgCl2, 12.5% (v/v) glycerol, 10 mM (v/v) β-mercaptoethanol and 1% (w/v) PVP-40 (soluble PVP) at 0–4 °C. The homogenate was centrifuged for 15 min at 15 000 g at 4°C, and then the supernatant was immediately used to determine the activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) by an enzyme-linked immunosorbent assay method with a RubPase ELISA kit (CK-E91697P, Shanghai jijin Chemistry and Technology Co., Ltd, China) according to the manufacturer’s instructions. The chlorophyll concentration was determined according to the method of Huang et al.

Superoxide radical O2− production rate was measured by monitoring the nitrite formation from hydroxylamine in the presence of O2− according to Elstner and Heupel. A 0.5 g aliquot of leaf margins and centers was ground and homogenized in 5 mL of 65 mM pre-cooled phosphate buffer (pH 7.8), followed by centrifuging the homogenate at 10,000 g for 15 min at 4 °C and mixing 0.5 mL of the supernatant with phosphate buffer (0.5 mL) and 0.1 mL of 10 mM hydroxylamine hydrochloride. This mixture was incubated at 25 °C for 20 min, followed by the addition of 1 mL of 58 mM sulfanilic acid and 1 mL of α-naphthylamine, and then another 20 min incubation at 25 °C. The as-prepared solution was shaken with equal volume of ether, followed by centrifuging the mixture at 10,000 g for 3 min and measuring the absorbance of the pink water phase at 530 nm. The production of POD (EC 1.11.1.7) was determined using the guaiacol oxidation method.

**Anatomical analysis.** Another six tagged leaves per treatment were collected, and removed all the veins for anatomical analysis. The stomatal size and frequency were measured in six sub-samples either for leaf margin or center. The materials were prepared as described by Meng et al. Briefly, leaf samples (about 1 cm in length and 1 cm in width) were fixed in 2.5% glutaraldehyde (v/v) at 4 °C for 2 h, and washed twice in 0.1 M phosphate buffer (pH 6.8). Next, they were sequentially dehydrated in ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 10 min at each gradient concentration, with 100% ethanol repeated twice. After further drying and spraying with gold, the as-treated leaf samples were observed and photographed with a scanning electron microscope (JSM-5310LV, Jeol Co., Tokyo, Japan). Images were taken of the lower leaf surface for five microscope fields per sub-sample at a magnification of × 500. The number of stomata was counted in each field (a total of 20 measurements of stomatal frequency for each position) as described by Battie-Laclau et al., and the stomatal frequency was calculated by dividing the stomatal count by the area of the field of view. Moreover, the length and width of ten stomata selected at random were measured in each field. Assuming the stomatal pore as an ellipse, the total stomatal pore area was calculated (stomatal frequency × π × 0.25 × stomatal length × stomatal width).

Leaf segments (1–2 mm2) were cut from each part and fixed with 2.5% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.4) for 4 h, followed by washing twice in the same buffer for 30 min and postfixing with 2% osmium tetroxide for 4 h at 4 °C. Next, the samples were dehydrated with an ethanol series (10–100%) and in propylene oxide, followed by embedding them in Epon 812 resin.

For the light microscope observation, they were cut into 1 μm transverse sections by LKB-5 ultramicrotome 359 (LKB Co., Ltd., Uppsala, Sweden), and stained with 0.5% toluidine blue. Micrographs were captured at a magnification of × 400 with a Nikon Eclipse E600 microscope equipped with a Nikon 5 MP digital microscope camera DS-Fi1 (Nikon Corporation, Kyoto, Japan). There were four samples per treatment. For each samples, three cross-sections were chosen to measure their thickness (Tmes), mesophyll cell wall surface area exposed to intercellular airspace per leaf area (Smes/S), and surface area of chloroplasts exposed to intercellular airspaces (Smes/S) according to Tosens et al. (2012):

\[ \frac{S_{\text{mes}}}{S} = \frac{L_{\text{mes}}}{W} \times F \]  
\[ \frac{S_{\text{mes}}}{S} = \frac{L_{\text{mes}}}{L_{\text{mes}}} \times \frac{S_{\text{mes}}}{S} \]

Where \( L_{\text{mes}} \) and \( L_{\text{c}} \) are the length of mesophyll cell wall exposing to intercellular air space and chloroplast surface area touching the intercellular air space. \( W \) is the width of measured cross-section. \( F \) is the curvature correction factor which was obtained as the weight average of palisade and spongy mesophyll.

For the ultrastructural observations, ultrathin sections (90 mm) were examined with a transmission electron microscope 360 microscope (H-7650, Hitachi, Japan) after staining with 2.0% uranacyl acetate (w/v) and lead citrate. Cell wall thickness (Tcell-wal.), chloroplast length (Lcl.) and thickness (Tcl.) were measured from at least 30 chloroplasts. Chloroplasts were assumed as ellipsoids, and chloroplast surface area (Scl.) and volume (Vcl.) were calculated according to Cesaro formula.
\[
S_{\text{chl}} = 4 \times \pi \times \sqrt{a \times b^2}
\]  
(9)
\[
V_{\text{chl}} = \frac{4}{3} \times \pi \times a \times b^2
\]  
(10)

where \(a = 0.5 \times L_{\text{chl}}\), \(b = 0.5 \times T_{\text{chl}}\). Distance of chloroplast from cell wall \(D_{\text{chl-cw}}\) was determined according to Tomás et al.\(^{23}\).

**Quantitative limitation analysis.** The limitations (stomatal limitations, \(S_L\); mesophyll conductance limitations, \(M_{CL}\); biochemical limitations, \(B_t\)) imposed by K deficiency on photosynthesis were investigated by analyzing the leaf margins and centers under two treatments using the quantitative limitation analysis method proposed by Grassi and Magnai\(^{22}\). Relative changes in light-saturated assimilation is expressed in terms of relative changes in stomatal, mesophyll conductance, and biochemical capacity as Equation (11).

\[
\frac{dA}{A} = S_L + M_{CL} + B_t = l_s \cdot \frac{g_{sc}}{g_{sc} + l_m c \cdot \frac{g_{m}}{g_{m} + l_b} \cdot \frac{dV_{c, \text{max}}}{V_{c, \text{max}}}}
\]  
(11)

where \(l_s, l_m c, \) and \(l_b\) are the corresponding relative limitations calculated as Eqsns from (12) to (14), \(g_{sc}\) is stomatal conductance to CO\(_2\) \(g_{sc}/1.6\), and \(V_{c, \text{max}}\) is maximum rate of carboxylation estimated from \(A-C_i\) curve.

\[
l_s = \frac{g_{\text{tot}}}{g_{sc}} \cdot \frac{\partial A}{\partial C_c} \frac{g_{sc}}{g_{sc} + \partial A/\partial C_c} \]  
(12)
\[
l_m = \frac{g_{\text{tot}}}{g_{m}} \cdot \frac{\partial A}{\partial C_c} \frac{g_{m}}{g_{m} + \partial A/\partial C_c} \]  
(13)
\[
l_b = \frac{g_{\text{tot}}}{g_{sc} + \partial A/\partial C_c} \]  
(14)

where \(g_{\text{tot}}\) is the total conductance to CO\(_2\) from leaf surface to carboxylation sites determined as Equation (15). By following Tomás et al.\(^{23}\), \(\partial A/\partial C_c\) was calculated as slope of \(A-C_c\) response curves over a \(C_c\) range of 50–100\(\mu\)mol mol\(^{-1}\).

\[
g_{\text{tot}} = \frac{1}{1/g_{sc} + 1/g_{m}}
\]  
(15)

Then the relative change of \(A, g_{sc}, g_{m}\) and \(V_{c, \text{max}}\) in Equation (11) can be approximated by Chen et al.\(^{59}\).

\[
\frac{dA}{A} \approx \frac{A_{\text{ref}} - A_{\text{max}}}{A_{\text{max}}}
\]  
(16)
\[
\frac{dg_{sc}}{g_{sc}} \approx \frac{g_{sc}^{\text{ref}} - g_{sc}}{g_{sc}^{\text{ref}}}
\]  
(17)
\[
\frac{dg_{m}}{g_{m}} \approx \frac{g_{m}^{\text{ref}} - g_{m}}{g_{m}^{\text{ref}}}
\]  
(18)
\[
\frac{dV_{c, \text{max}}}{V_{c, \text{max}}} \approx \frac{V_{c, \text{max}}^{\text{ref}} - V_{c, \text{max}}}{V_{c, \text{max}}^{\text{ref}}}
\]  
(19)

where \(A_{\text{max}}^{\text{ref}}, g_{sc}^{\text{ref}}, g_{m}^{\text{ref}}\) and \(V_{c, \text{max}}^{\text{ref}}\) are the reference values of net CO\(_2\) assimilation rate, stomatal conductance and mesophyll conductance, and the rate of carboxylation, defined as maximum value measured under light saturation. In the original reference, the authors used the maximum value of seasonal \(A_{\text{max}}\) under light-saturated conditions as a reference to assess the photosynthetic limitations of \(A\) for each determination. In the current study, the maximum \(A\) was generally reached, concomitantly with \(g_{sc}, g_{m}\) and \(V_{c, \text{max}}\) in the leaf centers with the +K treatment, and the mean values of the +K treatments was thus used as a reference, i.e., there was no limitation present in the leaf centers under the +K treatment. Whenever one of the three parameters was higher in either one of the rest treatments than that of the reference, its corresponding limitation was set to zero. In this way, the limitations in the leaf margins and centers under the –K treatment could be quantified. Finally, non-stomatal limitations were defined as the sum of mesophyll conductance limitations and biochemical limitations (\(NS_L = M_{CL} + B_t\)).
Statistical analysis. One-way analysis of variance (ANOVA) was calculated using SPSS 18.0 software (SPSS, Chicago, IL, USA). The mean values were compared using the least significant difference (LSD) test (P < 0.05). Graphics and regression analysis were performed using the OriginPro 8.5 software (OriginLab Corporation, Northampton, MA, USA).

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