

BRIEF COMMUNICATION

Leaf anatomy and C₄ photosynthetic enzymes in three reed ecotypes

X.-Y. ZHU*, W.-X. XIA and L.-J. CHEN

*School of Life Sciences, Xiamen University, Xiamen 361005, P.R. China***Abstract**

Differences in leaf interveinal distances, chloroplasts distribution in bundle sheath cells (BSC) and activities of C₄ photosynthetic enzymes in the leaves of three ecotypes of *Phragmites communis* Trinius, namely swamp reed (SR), heavy salt meadow reed (HSMR) and dune reed (DR), occurring in the desert region of northwest China were investigated. The two terrestrial ecotypes, DR and HSMR, had denser vascular system, more and longer BSC chloroplasts and higher capacity of CO₂ concentrating mechanism of NAD-ME subtype as compared with the SR ecotype. The enhanced NADP-ME pathway in the HSMR might contribute to its adaptation to the salinity habitat.

Additional key words: chloroplasts, drought, leaf vein frequency, *Phragmites communis*, salinity.

Gradual modification of developmental properties and adjustment of metabolic pathways are involved in the plant adaptation to biotic and abiotic stresses (Ueno *et al.* 1988, Cai *et al.* 2010). The C₄ photosynthetic pathway with CO₂-concentrating mechanisms is regarded as a superior carbon assimilation mode for plants when limitations on carbon acquisition are imposed by a variety of stress conditions (Pyankov and Mokronsov 1993, Sage 2002). Drought and salinity can induce the shift from C₃ to C₄ carbon metabolic pathway (Ueno *et al.* 1988, Reiskind *et al.* 1997, Feldman *et al.* 2008). It is well known that classical C₄ pathway involves not only the special Kranz anatomy but also the higher activities of specific enzymes for releasing and then refixing CO₂, *i.e.*, NAD- and NADP-dependent malic enzymes, (NADP-ME, NAD-ME) and phosphoenolpyruvate carboxylase (PEP-CK). These CO₂-concentrating mechanisms offer great flexibility and adaptability for plant survival in adverse environments. However, which pathway(s) or enzyme(s) involving in the three C₄ subtypes contribute most to adaptation of reed ecotypes is currently not clear.

Vascular architecture is another important feature in evolution of C₄ photosynthetic pathway (Ueno *et al.*

2006). Numerous studies have shown that C₄ plants have a denser vascular system in the leaves than C₃ species (Crookston and Moss 1974, Ueno *et al.* 2006). There are some comparative studies on the leaf anatomy and C₄ enzymes between C₃, C₄ and C₃-C₄ intermediate species within the same genus, such as *Flaveria* (McKown and Dengler 2007), or in the same plant species (*Eleocharis vivipara*) grown under different natural or artificial conditions (Agarie *et al.* 1997).

Phragmites communis is a C₃ hydrophytic species whose typical habitats are the fresh and brackish water areas of swamps, riversides and lakesides. However, in addition to swamp reed (SR), there are two terrestrial ecotypes, heavy salt meadow reed (HSMR) and dune reed (DR), growing in the arid region of northwest China. Stable variations of morphological and physiological characteristics in response to drought and salinity and genetic diversity analysis on these reed ecotypes confirmed that they diverged from a common ancestor (Zhu *et al.* 2003a, Chen *et al.* 2007, Lin *et al.* 2007). The differences observed in their physiological and genetic properties may reflect adaptation to their different habitats. Comparative studies of the leaf anatomy, $\delta^{13}\text{C}$

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Abbreviations: Asp-AT and Ala-AT - aspartate and alanine aminotransferase; NAD-ME and NADP-ME - NAD- and NADP-dependent malic enzyme; NAD-MDH and NADP-MDH - NAD- and NADP-malate dehydrogenase; PEP - phosphoenolpyruvate; PEP-CK - phosphoenolpyruvate carboxylase; PEP-CK - phosphoenolpyruvate carboxykinase; PPDK - pyruvate orthophosphate dikinase; RuBPC - ribulose-1,5-bisphosphate carboxylase.

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* Corresponding author; fax: (+86) 592 2181015, e-mail: zhuxueyi90@xmu.edu.cn

value, PEPC and RuBPC activities in these reed ecotypes suggested a tendency of photosynthetic transition from a C₃-like to C₄-like pathway (Zheng *et al.* 2000). The aim of the present study was to further characterize the ecotype-specific differentiation in photosynthetic carbon assimilation and leaf anatomy.

Three ecotypes of *Phragmites communis* Trinius, SR, DR, and HSMR, were sampled at the end of June 2002 and 2006. The second fully expanded leaves from the apex were collected at midday and frozen in liquid N₂. For anatomical studies, fresh leaves (middle section, 5 × 10 mm) were fixed in Carnoy mixture and then prepared as described in Ueno *et al.* (2006) and observed under a light microscope (*Olympus*, Tokyo, Japan) with CCD camera. The distances between small longitudinal veins (SLV), between large longitudinal veins (LLV) and between transverse veins (TV) were measured with a micrometer. The number of transverse veins per unit area were measured on photomicrographs (40×) as described by Ueno *et al.* (2006). For evaluating the size of the bundle sheet cells (BSC) and observing the characteristics of chloroplasts distribution in the BSCs, fresh leaf pieces (2 × 5 mm) were fixed in 2 % glutaraldehyde buffered with 0.05 M 1,4-piperazine-bis-ethanesulfonic acid (PIPES, pH 7.2) for 14 h. After a standard ethanol dehydrated procedure, the specimens were treated with dimethylbenzene and then embedded in paraffin. Chloroplasts distribution in the BSCs were observed

using a laser scanning confocal microscope (LSCM; *Leica TCS-SP2-SE*, Wetzlar, Germany) and their numbers and sizes were measured and analyzed on confocal photomicrographs by the *Motic Image* advanced 3.0 software of the digital microscope (*DBM-5*, Global Motic Group, Xiemen, China).

Enzymes were extracted from frozen leaves by the method of Sayer and Kennedy (1979). Aliquots of the enzyme extracts were diluted as required with 80 % acetone containing 2.5 mM phosphate buffer (pH 7.8) and then centrifuged at 12 000 g for 20 min. Chlorophyll was determined in the supernatant as described by Sgherri *et al.* (2000). Enzyme assays were carried out according to the methods of Sayer and Kennedy (1979), except for NAD-MDH and NADP-MDH, which were carried out as described by Holtum and Winter (1982). Enzyme activities were related to chlorophyll content. To determine the content of malic acid, 1 g ground frozen leaf powder was homogenized with 0.2 g insoluble polyvinylpyrrolidone (PVP) and 10 cm³ deionized H₂O. After centrifuging (4 000 g, 10 min), the supernatant was used to determine the content of malic acid by HPLC (*HP-1090*, *Hewlett-Packard*, Wilmington, USA) with *Ultrasphe-ODS* C18 reversed-phase column. Data were analyzed by computer employing *Chemstation* (*Agilent Technologies*, USA). Quantification was based on peak area calculations related to a standard (L-malic acid, *Sigma*, St. Louis, USA) curve. The contents of pyruvate

Table 1. BSC chloroplast sizes and numbers, vascular density, levels of the substrates and products of C₄ subtypes and activities of enzymes involved in C₄ decarboxylation mechanisms in the leaves of three ecotypes of reed. Means ± SE; *n* = 5 - 8 different paraffin sections, *n* = 25 - 28 measurements of middle portions of 7 - 10 leaf blades; *n* = 3 for enzyme activities. LSD test at 0.05 level was used. BSC - bundle sheet cell, LLV - large longitudinal vein, TV - transverse vein, SLV - small longitudinal vein.

Parameters	SR	HSMR	DR
Length of BSC chloroplasts [μm]	1.95	2.67	3.29
Number of BSC chloroplasts in a section	26	43	56
Distance between SLVs [μm]	251.54 ± 6.59 ^a	206.25 ± 5.48 ^c	223.48 ± 4.41 ^b
Distance between LLVs [μm]	1408.91 ± 92.79 ^a	860.34 ± 3.35 ^b	840.82 ± 10.08 ^b
Distance between TVs (B) [μm]	1615.50 ± 182.88 ^a	827.62 ± 78.62 ^b	897.34 ± 36.39 ^b
Areolar area (A×B) [×10 ⁻³ mm ²]	401.56 ± 39.93 ^a	176.18 ± 10.60 ^b	209.13 ± 11.28 ^b
Number of TV per unit leaf area [mm ⁻²]	6.74 ± 0.78 ^c	12.11 ± 0.60 ^a	9.46 ± 0.53 ^b
Malate [nmol mg ⁻¹ (Chl)]	23.22 ± 9.34 ^c	59.34 ± 13.73 ^b	96.33 ± 6.31 ^a
Aspartate [nmol mg ⁻¹ (Chl)]	58.17 ± 15.22 ^c	145.49 ± 24.88 ^a	92.85 ± 11.82 ^b
Pyruvate [nmol mg ⁻¹ (Chl)]	334.55 ± 26.97 ^b	501.19 ± 29.85 ^a	544.82 ± 18.34 ^a
PEP [nmol mg ⁻¹ (Chl)]	65.89 ± 30.21 ^b	252.86 ± 15.30 ^a	245.50 ± 39.23 ^a
PPDK [μmol mg ⁻¹ (Chl) min ⁻¹]	0.07 ± 0.02	0.63 ± 0.03	0.40 ± 0.05
PEP-CK [μmol mg ⁻¹ (Chl) min ⁻¹]	0.12 ± 0.01	0.10 ± 0.01	0.24 ± 0.01
Asp-AT [μmol mg ⁻¹ (Chl) min ⁻¹]	5.41 ± 0.52	10.37 ± 1.31	13.56 ± 0.78
Ala-AT [μmol mg ⁻¹ (Chl) min ⁻¹]	4.04 ± 0.55	3.73 ± 0.12	4.69 ± 0.31
NAD-ME [μmol mg ⁻¹ (Chl) min ⁻¹]	1.40 ± 0.20	2.32 ± 0.50	8.20 ± 0.98
NDAP-ME [μmol mg ⁻¹ (Chl) min ⁻¹]	0.53 ± 0.11	3.96 ± 0.36	0.78 ± 0.17
NAD-MDH [μmol mg ⁻¹ (Chl) min ⁻¹]	44.98 ± 5.16	156.41 ± 6.69	137.63 ± 3.99
NADP-MDH [μmol mg ⁻¹ (Chl) min ⁻¹]	6.30 ± 0.72	25.26 ± 2.40	14.10 ± 1.82
Ratio of PEP-CK/ NAD-ME [%]	8.31	4.27	3.05
Ratio of PEP-CK/ NADP-ME [%]	21.46	2.55	29.82

and phosphoenol-pyruvate were determined following the method of Sakae *et al.* (2002). For measurement of aspartate, leaves were extracted three times with a mixture containing methanol:chloroform:water (12:5:3) (v/v/v). The combined aqueous layer was used to estimate amino acid contents following the procedure of Roosens *et al.* (1998) with an amino acid analyzer (Hitachi 853-50, Tokyo, Japan).

The leaf transverse sections from these reed ecotypes showed similar Kranz-like anatomy with mesophyll cells and chloroplast-containing BSCs. However, the two terrestrial ecotypes showed more and bigger chloroplasts in their BSCs and markedly shorter interveinal distances in SLV, LLV, and TV in comparison with the swamp ecotype (Fig. 1, Table 1). The distances between the LLV and TV in the HSMR and DR were only half of that of the corresponding measurements values for SR and were similar to those reported in C_4 species (Ueno *et al.* 2006). Sage (2002) suggested that an increase in the vein density of leaves might be an anatomical pre-conditioning for the evolution of C_4 plants from C_3 plants. The two terrestrial ecotypes exhibited longer and more chloroplasts in their BSCs than the swamp ecotype did. Increased vein densities in the two terrestrial ecotypes could also increase the relative volumes of vein-associated BS tissues and percentages of BSC chloroplasts per unit leaf area, which might therefore contribute to the C_4 -like CO_2 concentration mechanism in BSCs.

The habitat transition from SR *via* HSMR to DR (Zhu *et al.* 2003a) is accompanied by different capacities of mechanisms concentrating CO_2 due to the different activities of enzymes of the C_4 pathway. The key enzyme for PEP regeneration is PPDK and this reaction might be one of the rate-limiting steps of operation of the C_4 pathway. Higher PPDK activities and PEP contents in the two terrestrial ecotypes indicated that the both had higher capacities for regenerating PEP than SR (Table 1). The activities of enzymes involved in the NAD-ME subtype, such as aspartate aminotransferase (Asp-AT), NAD-ME and NAD-MDH, were also markedly higher in the HSMR and especially in DR, than in the SR. The higher contents of the main substrates and products observed in these two ecotypes were also correlated well with their higher activities of enzymes involved in NAD-ME subtype (Table 1). When combined with the previous studies showing the coexistence of mitochondria and chloroplasts in the BSCs of the two terrestrial ecotypes (Zheng *et al.* 1999, Zhu 2003b), our current findings of higher NAD-ME C_4 subtype enzyme activities suggest tendency to NAD-ME C_4 photosynthesis. As for the key enzymes involved in the NADP-ME subtype, the highest NADP-ME activity was observed in the HSMR, while in DR it did not show any significant difference from that in SR (Table 1). It has been suggested that higher NADP-ME gene expression and/or protein activity confer plants enhanced tolerance to salt stress (Liu *et al.* 2007). The highest activities of NADP-ME and NADP-MDH in

the HSMR might indicate a response to long-term natural saline habitat. Meanwhile, coexistence of NAD-ME and NADP-ME subtype pathways for CO_2 -concentration in the HSMR could not be excluded.

Of the three different C_4 decarboxylation reactions, both PEP-CK and NAD-ME C_4 subtypes use aspartate as their decarboxylation substrates, thus it is possible that any differences in activities of PEP-CK and NAD-ME between the three reed ecotypes might reflect their differential preferences for these two carbon metabolism pathways. It was reported that in the PEP-CK subtype pathway, activity of PEP-CK was about 20 times higher than activities of NAD-ME and NADP-ME (Hatch *et al.* 1975). In the present study, however, the PEP-CK

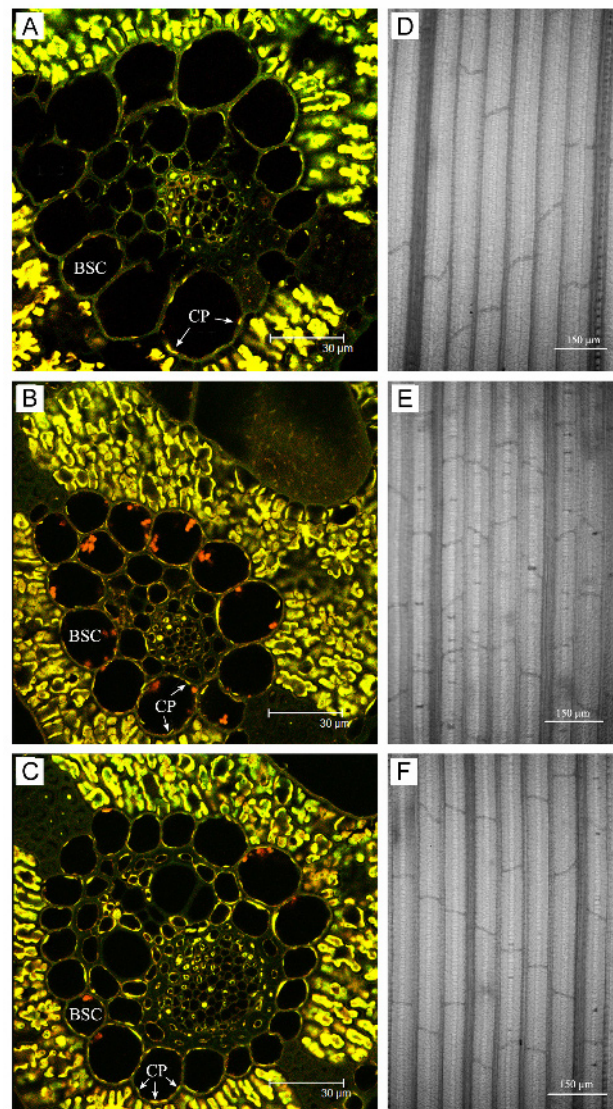


Fig. 1. Laser scanning confocal photomicrographs (A,B and C; bars = 30 μ m) and paradermal view of leaf vascular diversities (D,E and F; bars = 150 μ m) of the three ecotypes of reed. (A,D - SR, B,E - HSMR, C,F - DR. CP - chloroplast, BSC - bundle sheath cell).

activities in the three ecotypes of reed were no more than 30 and 10 % of that of NADP-ME and NAD-ME activities, respectively (Table 1). It seems therefore, that the three ecotypes of reeds are unlikely to conduct PEP-CK subtype pathway.

In summary, when reed, a hydrophyte species, became adapted to long-term drought and salinity habitats, its photosynthetic characteristic shows a general tendency to change from C₃-like to C₄-like (Zheng *et al.* 2000). The two terrestrial reed ecotypes do not possess a typical C₄ Kranz anatomy, but their increased leaf vein densities as well as increased number and size of chloroplasts in BSCs, might be beneficial in facilitating rapid intercellular diffusion of water and photosynthates

between mesophyll and bundle sheath cells. This could also lay the anatomical foundation for conducting effective CO₂ concentration mechanism under arid and saline habitats. Of the three C₄ decarboxylation pathways, the evolution of CO₂ concentration mechanism in *Phragmites communis* induced by habitat transition from swamp *via* salt to dune tends to progress into the NAD-ME subtype. However, the NADP-ME pathway apparently is also of adaptive significance in acclimation of HSMR to saline habitat. The efficiencies of the CO₂ concentrating mechanisms in the two terrestrial ecotypes might not be as high as that in a typical C₄ plants, but could confer them some advantages under natural drought and saline habitats.

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