Biochemical and Cytological Relationships in C₄ Plants

Maria Gutierrez, V. E. Graen, and G. E. Edwards*

Department of Horticulture, University of Wisconsin, Madison, Wisconsin 53706, and
Department of Plant Breeding and Biometry, Cornell University, Ithaca, New York 14850, USA

Received April 1, 1974

Summary. C₄ plants can be divided into three groups based on differences in activities of three decarboxylating enzymes: NADP-malic enzyme, NAD-malic enzyme, and phosphopyruvate carboxykinase.

In the Gramineae the three C₄ groups are distinguished by anatomical and ultrastructural characteristics of bundle-sheath chloroplasts. NADP-malic enzyme species lack well-developed grana in bundle-sheath chloroplasts (grana reduced) and the bundle-sheath chloroplasts are in the centrifugal position. NAD-malic enzyme species have bundle-sheath chloroplasts in the centripetal position and contain grana. Phosphopyruvate carboxykinase species have bundle-sheath chloroplasts in the centrifugal position and they contain grana. NADP-malic enzyme species of the Gramineae have only been found in the subfamilies Aristidoideae and Panicoideae. With the exception of the genera Panicum, and Urochloa, NAD-malic enzyme species and phosphopyruvate carboxykinase species have only been found in the subfamily Eragrostioideae. C₄ species of the genus Panicum are found among all three of the C₄ groups.

The dicotyledonous C₄ species examined fall into two groups: those having high NADP-malic enzyme and those having high NAD-malic enzyme. No phosphopyruvate carboxykinase C₄ species have been found among the dicotyledons. The NADP-malic enzyme C₄ species of the dicotyledons like NADP-malic enzyme species of the Gramineae have bundle-sheath chloroplasts with reduced grana but in contrast to NADP-malic enzyme species of the Gramineae the bundle-sheath chloroplasts are in the centripetal position. The NAD-malic enzyme species of the dicotyledons like the NAD-malic enzyme species of the Gramineae have bundle-sheath chloroplasts in the centripetal position with well developed grana.

The results are discussed in terms of evolutionary and functional diversification of C₄ plants.

Introduction

C₄ plants have a number of common anatomical, physiological and biochemical characteristics, namely, distinct bundle-sheaths consisting of chloroplast-containing cells surrounding the vascular bundles of the leaves (bundle-sheaths), a low CO₂ compensation point, high rates and high temperature optima of photosynthesis, and high activities of phospho-

pyruvate (PEP) carboxylase, the carboxylating enzyme of the C_4 pathway (see Hatch and Slack, 1970; Black, 1973, for reviews). In comparison to species which lack the C_4-dicarboxylic-acid pathway, C_4 plants also have relatively high 13C/12C ratios of leaf dry-matter (Bender, 1968, 1971; Tregunna et al., 1970; Smith and Brown, 1973).

Although C_4 plants have common distinguishing features, it is becoming apparent that there are differences between C_4 species. These differences concern the degree of grana development in the chloroplasts of bundle-sheath cells (Johnson, 1964; Downton, 1970; Black and Mollenhauer, 1971; Laetsch, 1971; Brown and Gracen, 1972); the position of chloroplasts in these cells (Brown, 1960; Brown and Gracen, 1972); and differences in the activities of enzymes related to the pathway of photosynthesis, particularly enzymes thought to function in the decarboxylation of C_4-dicarboxylic acids (Berry et al., 1970; Downton, 1970; Andrews et al., 1971; Edwards et al., 1971; Edwards and Gutierrez, 1972; Huber et al., 1973; Gutierrez et al., 1974; Hatch and Kagawa, 1974). This diversification among C_4 species may reflect differences in the evolution of the C_4 syndrome. In this report, C_4 species of the Gramineae and C_4 species among the dicotyledons have been divided into several groups, based on current evidence for cytological and biochemical differences.

**Materials and Methods**

*Plant Culture.* Plants were grown in a growth chamber in 16/8-h light-dark cycles with a light/dark temperature of 30/20°C. Light was provided by a combination of fluorescent and incandescent lamps giving a quantum flux density, between the wavelengths of 400 and 700 nm, of 40 nE cm^{-2} s^{-1}. For enzyme assays or electron microscopy, leaf samples were taken when the plants were 2-4 weeks of age.

*Enzyme Extraction, Enzyme Assays, and Chlorophyll Determination.* The enzyme extraction medium contained 0.05 M HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) buffer (pH 7.5), 1 mM MgCl_2, 1 mM MnCl_2, 5 mM dithiothreitol and 2% polyvinylpyrrolidone. About 0.5 g of leaves were ground in a mortar with 3 ml of extraction medium and suspended in a final volume of 10 ml. The homogenate was then passed through a French press at 10000 psi. Without the French-press treatment, the bundle-sheath cells of some species were not broken. The extracts were routinely examined by light microscopy to assure that all leaf cells had been broken. The extraction procedure was carried out at 4°C and the crude preparations obtained were used for the enzyme assays.

PEP carboxykinase was assayed in an exchange reaction using NaH^{14}CO_3, oxaloacetate and ATP (Edwards et al., 1971); NADP-malic enzyme, spectrophotometrically following NADP reduction as previously described by Kanai and Edwards (1973b); and NAD-malic enzyme, spectrophotometrically following NAD reduction, according to Hatch and Kagawa (1974). The reaction for NAD-malic enzyme was assayed in a double-beam spectrophotometer and the reaction in the sample cuvette initiated by the addition of manganese. A reference cuvette without manganese was included to subtract any activity due to NAD-malic dehydrogenase. Evidence for pyruvate formation was routinely checked at the end of the assay by addition of 1.5 units of lactic dehydrogenase and following the reoxidation of NADH. All enzymes were assayed at 30°C.