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PHOTOSYNTHETIC CARBON METABOLISM IN PRIMARY AND SECONDARY LEAVES OF *Zea mays* L.

by

GAIL MAN-WAH LAW

A Thesis

Submitted to

The Faculty of Graduate Studies

In Partial Fulfilment

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Department of Botany

Winnipeg, Manitoba

June, 1975

PHOTOSYNTHETIC CARBON METABOLISM

IN

PRIMARY AND SECONDARY LEAVES OF *Zea mays* L.

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GAIL MAN-WAH LAW

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

This thesis is affectionately dedicated to Harold and Helena, my brother and sister-in-law, whose unfailing love, wise counsel, moral and prayer support have enabled me to find myself, and to experience the joy, peace and the liberating power of the Gospel of Jesus Christ.

"In the day of my trouble I will call upon Thee:
For Thou wilt answer me.
Among the gods there is none like unto Thee, O Lord;
Neither are there any works like unto Thy works.
All nations whom Thou has made shall come
and worship before Thee, O Lord;
And shall glorify Thy Name.

For THOU ART GREAT, and DOEST WONDROUS THINGS:
THOU ART GOD ALONE."

Psalm 86: 7 - 10

ABSTRACT

Kinetic experiments of $^{14}CO_2$ incorporation into leaf discs of Zea mays. L. var. Earliking and identification of the metabolic products have shown that the photosynthetic carbon pathway of primary leaves from 6 - 8 day old seedlings differs from that of secondary leaves from 16 - 18 day old seedlings. The kinetic pattern of photosynthetic intermediates in primary leaves indicated that the Calvin cycle was predominant, whereas in secondary leaves the major route of carbon flow was via the C_4-dicarboxylic acid cycle as shown by the early production of malate which decreased with time.

These results were in spite of the fact that phosphoenolpyruvate carboxylase (EC 4.1.1.31), the key enzyme of the C_4 cycle was just as active in the chloroplast and cytoplasmic fractions of both primary and secondary leaves. However, it was observed that the phosphoenolpyruvate carboxylase of primary leaves had a higher apparent Km for its substrate, phosphoenolpyruvate, in comparison to that of the secondary leaves, which may be partly responsible for the differences.

Chloroplasts isolated from both primary and secondary leaves by the 'laceration technique' of Mache and Waygood (FEBS Letters 3: 89 - 92, 1969) were incapable of fixing CO_2 without the addition of a CO_2 acceptor, although some very young, undifferentiated chloroplasts from primary leaves possessed a small but significant capacity for endogenous CO_2 fixation.

Chloroplasts isolated from either primary or secondary leaves

were capable of fixing CO_2 into photosynthetic intermediates in the presence of 3.2 mM of phosphoenolpyruvate (its approximate K_{app}). This capacity was shared by the corresponding cytoplasmic (supernatant) fractions which showed greater activity, but gave a somewhat different pattern of photosynthetic intermediates.

For CO_2 fixation, the apparent Km values for phosphoenolpyruvate, HCO_3^- and Mg^{+2} were respectively: 4.00, 0.56 and 2.00 mM for primary leaf chloroplasts; 7.40, 0.89 and 0.30 mM for primary leaf supernatant; 2.04, 0.67 and 2.22 mM for secondary leaf chloroplasts and 2.44, 0.40 and 0.35 mM for secondary leaf supernatant.

These values corresponded reasonably well with the kinetic parameters in the enzymatic assay of phosphoenolpyruvate carboxylase of the corresponding fractions being respectively: 10.00, 0.43 and 4.00 mM for the enzyme from chloroplasts of primary leaves; 10.00, 0.44 and 1.14 mM for the primary leaf supernatant enzyme; 1.40, 0.27 and 5.71 mM for the enzyme from chloroplasts of secondary leaves and 3.85, 1.33 and 0.21 mM for the secondary leaf supernatant enzyme.

The response to temperature of the CO_2 fixation capacity and the phosphoenolpyruvate carboxylase activity of the chloroplast and supernatant fraction of primary leaves was not severely affected by subnormal temperature conditions (10 - 15^oC) during growth, but these activities were almost completely arrested in secondary leaf chloroplasts and extracts under the same conditions.

From these results it is apparent that phosphoenolpyruvate carboxylase participates in the regulation of CO_2 fixation capacity

of secondary leaf chloroplasts, and their capacity for CO_2 fixation is not merely due to contamination by the phosphoenolpyruvate carboxylase of its corresponding supernatant (cytoplasmic) enzyme.

The distribution of ^{14}C in the products of CO_2 fixation in the presence of 3.2 mM phosphoenolpyruvate in the chloroplast fractions of both leaf types was also different from that obtained in the supernatant fractions of the corresponding leaf types. The former were more heavily labelled in the amino acid and sugar fractions as compared to the latter which favoured incorporation of ^{14}C into the organic acid fraction. More specifically, in the chloroplast fraction of both leaf types the major portion of the radioactivity was recovered in glycerate, dihydroxyacetone and glyceraldehyde. The supernatant fraction showed significant label in glycerate only. Malate was not found as a product of CO_2 fixation in any of the fractions at this concentration (3.2 mM) of phosphoenolpyruvate. The absence of malate and the formation of the three 3-C compounds in chloroplast fractions was taken to be evidence in support of a transcarboxylation reaction resulting from the product of β-carboxylation as first envisaged by Hatch and Slack (Biochem. J. 101: 103-111, 1966) and corroborated biochemically by Pan (Ph. D. Thesis, University of Manitoba, 1974).

Investigations have shown that the 'laceration technique' is superior to the more commonly employed methods of 'grinding' and 'blending' for the isolation of chloroplasts. Although the chloroplast

'pellet' obtained is predominantly mesophyll in origin, the chloroplasts retain more of their in vivo physical and biochemical integrity. Bundle sheath chloroplasts generally account for 10% of the total and they can be separated by differential centrifugation. The method is recommended for the isolation of mesophyll chloroplasts from monocotyledonous plants for studies on CO_2 fixation and enzyme activities.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
DTT	Dithioerythritol
EDTA	Ethylene diamine tetra-acetic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid
MES	2-(N-morpholino) ethanesulfonic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
PEP	Phosphoenolpyruvate
PGA	Phosphoglyceric acid
POPOP	1,4-Bis (2-5) phenyloxazoylbenzene
PPO	2,5-Diphenyloxazole
PVP	Polyvinyl pyrolidone
RuDP	Ribulose diphosphate
TES	N-tris (Hydroxymethyl) Methyl-2-aminoethanesulfonic acid
Tris	Tris (hydroxymethyl) aminomethane

INTRODUCTION

The view has long been held that higher plants assimilate CO_2 through the Calvin cycle via RuDP carboxylase (Benson and Calvin, 1947) in which the initial product is 3-PGA. However, it was shown in the 1960's that in some plants the initial product of CO_2 assimilation was a four-carbon organic acid. For this reason, the former group of plants are referred to as C_3 plants and the latter C_4 plants (Downton and Tregunna, 1968). After the proposal of the new pathway of CO_2 assimilation for C_4 plants by Hatch and Slack (1966), considerable work has been done on many C_4 species (Slack et al., 1969; Bucke and Long, 1971; Latzko et al., 1971) with various newly developed techniques in an attempt to elucidate the details of the pathway. As some areas have been clarified, more problems have arisen and many questions remain unanswered.

Maize is one of the C_4 plants initially studied. While the majority of workers agree that maize is a C_4 plant (Slack et al., 1969; Berry et al., 1970; Samejima and Miyachi, 1971), it has also been demonstrated that RuDP serves as primary CO_2 acceptor in maize leaves (Latzko et al., 1971) which is a characteristic feature of C_3 plants; and the products of CO_2 fixation in vitro by isolated maize chloroplasts are those of the C_3 rather than the C_4-pathway (Gibbs et al., 1970; O'Neal et al., 1971, 1972). The latter group of workers used either primary leaves or whole seedlings of 2 to 4 day old maize plants. But the majority of the other investigators used more mature

plants over two weeks old. It is doubtful that results obtained from very young developing leaf tissue could be representative for mature, fully developed tissue as assumed by some of these authors.

The C_4-pathway as proposed by Hatch and Slack (1966) involves the concerted effort of both mesophyll and bundle sheath chloroplasts. It is believed that the initial site of CO_2 fixation is in the mesophyll chloroplasts resulting in the formation of a C_4-dicarboxylic acid. This initial product has to be either decarboxylated or transcarboxylated before the carbon can be further metabolized by the bundle sheath chloroplasts. If the former takes place, the decarboxylated CO_2 is believed to be refixed by the bundle sheath chloroplasts. If the latter takes place, the carboxyl group is supposed to be 'transferred' onto a carboxyl acceptor with the subsequent formation of regular C_3 photosynthetic intermediates. The enzyme believed to be involved with decarboxylation reaction, NADP-malic enzyme, has been found to be located in the bundle sheath cells (Slack et al., 1969; Hatch and Kagawa, 1973). Bundle sheath cells of maize have also been shown to decarboxylate malate in vitro (Huber et al., 1973). On the other hand, evidence for a transcarboxylation reaction has also been obtained with maize extracts (Pan, 1974). However, decarboxylation and/or transcarboxylation reactions with subsequent formation of regular C_3 photosynthetic intermediates have yet to be demonstrated.

Although isolated chloroplasts from some C_3 plants have been shown to possess the ability of fixing CO_2 without the addition of

substrate (or CO_2 acceptor) (Kalberer et al., 1967), very little work of this nature had been reported on C_4 plants. The only successful reports were all investigations on very young maize seedlings of 2 to 4 day old (Gibbs et al., 1970; Poincelot, 1972). Two reports on CO_2 fixation by chloroplasts isolated from mature C_4 plants were both on studies of chloroplast systems fortified with PEP (Baldry et al., 1969; Waygood et al., 1971). Interestingly enough, this capacity of CO_2 fixation by isolated mesophyll protoplasts of mature maize leaves was also dependent on the addition of PEP (Kanai and Edwards, 1973a). Phosphoenolpyruvate carboxylase is very active in C_4 plants and has been found by most workers to be located in the cytoplasm of the mesophyll cells (Andrews et al., 1971; Lyttleton, 1971). In the light of the activity and location of this enzyme, two questions one would naturally ask are (a) whether the capacity for CO_2 fixation by isolated chloroplasts in the presence of PEP is an artifact due to unavoidable contamination of the inactive chloroplast by the cytoplasm during isolation and (b) if this is the case, what further role the mesophyll chloroplasts are assuming if they are not involved with the β-carboxylation of PEP.

Most work on isolation of C_4 chloroplasts has been criticized on the basis of technical difficulties in eliminating contamination by cytoplasmic enzymes and separation of the two types of chloroplasts (Black, 1973; Laetsch, 1974). Accordingly, whenever a new method is developed the research worker has to be familiar with both the advantages and limitations of the technique and interpret the results