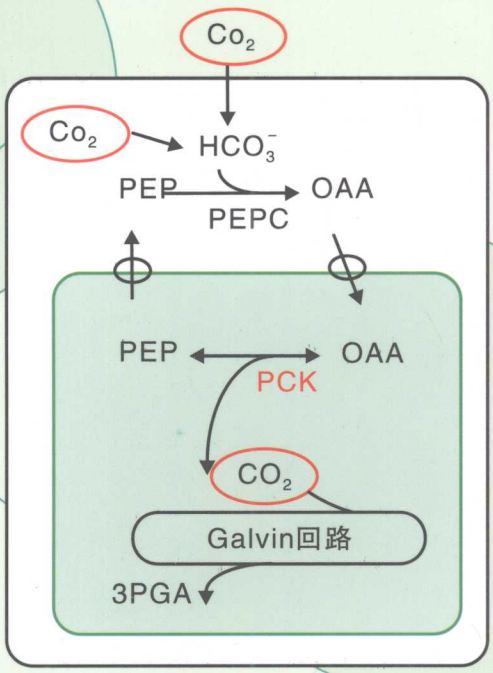
- 昆明理工大学学科建设资金资助项目
- 云南省中青年学术与技术带头人培养费（2004PY01-5）
- 云南省自然科学基金项目（2004C0006R）

Genetic Engineering

of photosynthesis in C_3 plants

Edited by Chen Limei



Yunnan Publishing Group Corporation
Yunnan Science and Technology Press

昆明理工大学学科建设资金资助项目
云南省中青年学术与技术带头人培养费(2004PY01-5)
云南省自然科学基金项目(2004C0006R)

Genetic Engineering of photosynthesis in C_3 plants

Edited by Chen Lanei

Yunnan Publishing Group Corporation
Yunnan Science and Technology Press
· Kunming ·

图书在版编目（CIP）数据

C_3 植物光化学作用遗传工程 - Genetic Engineering of photosynthesis in C_3 plants：英文/陈丽梅编著.
昆明：云南科技出版社，2008.3
ISBN 978-7-5416-2812-2

Ⅰ. C… Ⅱ. 陈… Ⅲ. 光合作用—关系—基因—遗传工程—英文 Ⅳ. Q945.11 Q943.2

中国版本图书馆 CIP 数据核字（2008）第 041852 号

云南出版集团公司
云南科技出版社出版发行
（昆明市环城西路 609 号云南新闻出版大楼 邮政编码：650034）
云南省地矿测绘院印刷厂印刷 全国新华书店经销
开本：850mm×1168mm 1/32 印张：6.25 字数：160 千字
2008 年 4 月第 1 版 2008 年 4 月第 1 次印刷
印数：1～1000 定价：20.00 元

Introduction of Author

Chen Limei: Doctor, Prof, Tutor for doctor degree of Faculty of Life Science and Biotechnology, Kunming University of Science and Technology. She finished her Bachelor degree in Guangxi Normal University, Guangxi Province, China, with major in Biology and obtained master degree in Guangxi University, with major in Molecular Genetics in 1985 and 1991, separately. After graduating from Guangxi University, she became an assistant researcher in the department of Science and Technology in the same university. During the period of 1998 ~ 2002, she studied for Ph. D in Plant Metabolic Engineering in Plant Physiology Lab., Graduate School of Agriculture, Kyoto University, Kyoto, Japan. Since 2002, she had done post - doctor research work for two years in the same lab and engaged in genetic engineering of C_3 photosynthesis and photosynthetic engineering for formaldehyde in plants. From 2004 till now, she has been teaching at Kunming University of Science and Technology and mainly conducting on plant photosynthesis engineering. She has publicized about 35 papers in and abroad (Indexed 10 papers by SCI).

Abbreviations

ACC	1 – aminocyclopropane – 1 – carloxylic acid
ADP	adenosine 5′ – diphosphate
AMP	adenosine 5′ – monophosphate
ASN1	asparagine synthase(1)
ASN2	asparagine synthase(2)
ATP	adenosine 5′ – triphosphate
bp	base pairs
BA	benzyladenine
BSA	bovine serum albumin
Cab	chlorophyll a/b binding protein
CAM	crassulacean acid metabolism
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
Chl	chlorophyll
DTT	dithiothreitol
EDTA	ethlenediaminetetraacetic acid
Ery – 4 – *P*	erythrose 4 – phosphate
Fruc – 1 – *P*	fructose 1 – phosphate
Fruc – 6 – *P*	fructose 6 – phosphate
Fruc – 1,6 – P_2	fructose 1,6 – bisphosphate
Fruc – 2,6 – P_2	fructose 2,6 – bisphosphate
Gdn – HCl	guanidine hydrochloride
Gluc – 1 – *P*	glucose 1 – phosphate

Gluc – 6 – *P*	glucose 6 – phosphate
Gluc – 1,6 – P_2	glucose 1,6 – bissphosphate
GTP	guanosine triphosphate
$I_{0.5}$	concentration of inhibitor required for 50% inhibition
IAA	3 – indoleacetic acid
IPTG	isopropyl – β – D – thiogalactopyranoside
kD	kilo Dalton
Km	kanamycin
LB	Luria – Bertani
MDH	malate dehydrogenase
MerEtOH	2 – mercaptoethanol
NADH	reduced nicotinamide adenine dinucleotide
NADP – ME	NADP – dependent malic enzyme
Nos	nopaline synthase
OAA	oxaloacetate
PAGE	polyacrylamide gel electrophoresis
PCK	PEP carboxykinase
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
3 – PGA	3 – phosphoglycerate
PPDK	pyruvate, orthophosphate dikinase
PPT	PEP/phosphate translocator
PVP	polyvinylpopyrollidone
Rubisco	ribulose – 1,5 – bisphosphate carboxylase/oxygenase

$S_{0.5}$	half – saturation concentration of substrate or metal cofactor
SDS	sodium dodecylsulfate
TK	transketolase
Tris	tris (hydroxylmethyl) aminomethane
V_{max}	maximum velocity
TCA	tricarboxylic acid

Content

Introduction ..... (1)
1 C_3 photosynthesis and C_4 photosynthesis ..... (1)
2 Photosynthesis in Hydrilla verticillata ..... (4)
Chapter 1 Molecular characterization of a phosphoenolpyruvate carboxylase from a thermophilic cyanobacterium, *Synechococcus vulcanus* ..... (16)
1 Introduction ..... (16)
2 Materials and Methods ..... (18)
3 Results ..... (25)
4 Discussion ..... (41)
Chapter 2 Desensitization of *S. vulcanus* phosphoenolpyruvate carboxylase (SvPEPC) to Asp inhibition by site-directed mutagenesis ..... (45)
1 Introduction ..... (45)
2 Materials and Methods ..... (49)
3 Results ..... (52)
4 Discussion ..... (65)
Chapter 3 Expression of SvPEPC in *Arabidopsis* ..... (69)
1 Introduction ..... (69)
2 Materials and Methods ..... (71)

3 Results ..... (75)
4 Discussion ..... (86)
Chapter 4 Effects of expressed SvPEPC on nitrogen and carbon metabolism in *Arabidopsis* ..... (93)
1 Introduction ..... (93)
2 Materials and Methods ..... (97)
3 Results ..... (101)
4 Discussion ..... (124)
Chapter 5 The effects of over – expression of a maize bundle sheath – specific phosphoenolpyruvate carboxykinase on the transgenic tobacco ..... (138)
1 Introduction ..... (138)
2 Materials and Methods ..... (141)
3 Results ..... (147)
4 Discussion ..... (158)
Conclusion and perspectives ..... (167)
References ..... (173)

Introduction

With the rapid growth of world population and increasing demand for food supply, the enhancement of crop productivity is an urgent issue. The yield of crops broadly relates to the amount of light intercepted and the cumulative photosynthesis over a growing season. If photosynthesis could be increased then it would be possible to capitalize on and extend this relationship. For a long time, one of the aims of plant scientists has been to improve photosynthesis to maximize crop productivity to feed burgeoning population (Paul, et al, 2001).

1 C_3 photosynthesis and C_4 photosynthesis

Most of our important crop plants (such as rice, wheat) fix CO_2 by C_3 photosynthesis (Bowes, 1996). Rubisco (ribulose – 1,5 – bisphosphate carboxylase/oxygenase), the key enzyme of C_3 photosynthesis, possesses two activities, acting as a carboxylase and an oxygenase. CO_2 and O_2 compete for the same active site of the enzyme (Ku, et al, 1999). The products of oxygenation have to be salvaged by the photorespiratory cycle (Ogren, 1984). The major inefficiency of this pathway is the loss of one – quarter of the CO_2 already fixed. Moreover, reduced nitrogen is lost during CO_2 refixation and has itself to be refixed. Photorespiratory loss of CO_2 and nitrogen is a particular problem at higher temperatures as the oxyge-

nase activity increases. This can be exacerbated further if the water supply is not optimal, since closure of stomata leads to an increase in the internal O_2/CO_2 ratio (Ku, et al, 1999). Although there was some interest in engineering Rubisco in favor of carboxylase over oxygenase, current chemical evidence suggests it is unlikely that Rubisco can be engineered for increasing carboxylation efficiency (Somerville, 1990; Ku, et al, 1999).

C_4 photosynthesis release CO_2 at high rates in the vicinity of Rubisco and thereby increase the ratio of rubilose $-1,5-P_2$, carboxylation/oxygenation substantially. This strategy prevents major losses of CO_2 by photorespiration and is accompanied by an increase in the water use efficiency compared to C_3 – plants in hot arid climates (Edwards, et al, 1983; Hatch, 1987). The crucial factor for the maintenance of CO_2 assimilation in C_4 plants is the presence of a pumping mechanism (Fig. 1), which elevates the concentration of CO_2 at the carboxylation site from 5 μM to about 70 μM (Fig. 1A). The CO_2 pumping of C_4 metabolism does not rely on the specific function of a membrane transporter but is due to a prefixation of CO_2 (Dai, et al, 1993; Furbank, et al, 1995), after conversion to HCO_3^-, by reaction with phosphoenopyruvate (PEP) to form oxaloacetate (OAA) in the mesophyll cells. After the conversion of this oxaloacetate to malate, the malate diffuses through the plasmodesmata into the bundle sheath cells, where CO_2 is released as a substrate for Rubisco (Heldt, 1997) (Fig. 1B). The reaction of HCO_3^- with PEP is catalyzed by phosphoenolpyruvate carboxylase (PEPC). The reaction is strongly exergonic and irreversible. As the enzyme has a very high affinity for HCO_3^-, micromolar concen-

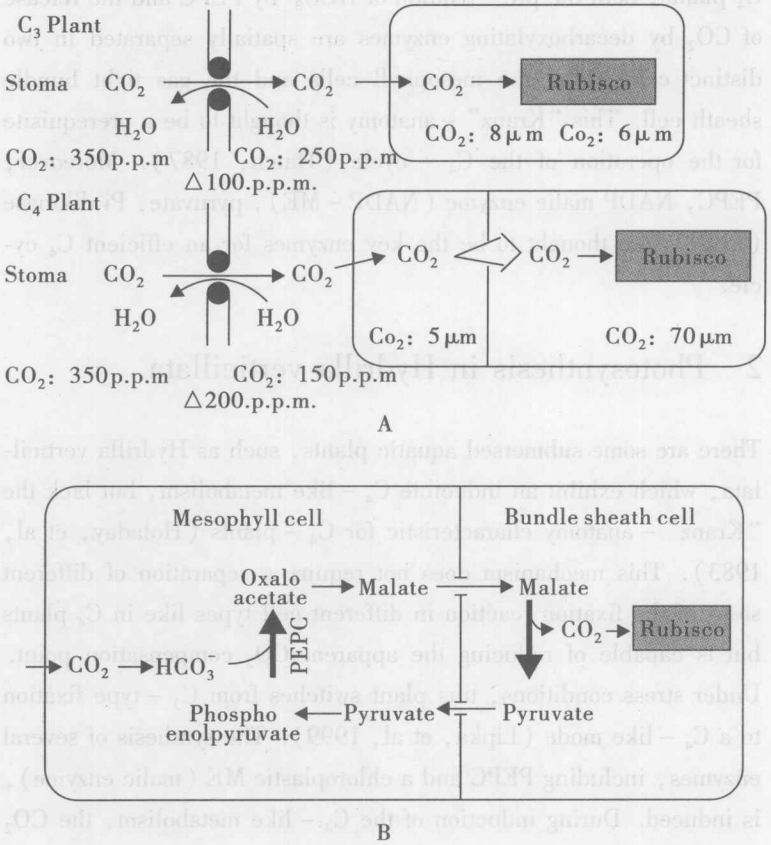


Fig. 1 The scheme for the uptake of CO_2 in C_3 and C_4 plants (A) and the principle of C_4 metabolism (B)

The figures were cited from Heldt (1997).

trations of HCO_3^- are fixed very efficiently (Ku, et al, 1996). In C_4 plants, both the pre - fixation of HCO_3^- by PEPC and the release of CO_2 by decarboxylating enzymes are spatially separated in two distinct cell types, the mesophyll cells and the gas tight bundle sheath cell. This "Kranz" - anatomy is thought to be a prerequisite for the operation of the C_4 - cycle (Hatch, 1987). Moreover, PEPC, NADP malic enzyme (NADP - ME), pyruvate, Pi dikinase (PPDK) are thought to be the key enzymes for an efficient C_4 cycle.

2 Photosynthesis in Hydrilla verticillata

There are some submersed aquatic plants, such as Hydrilla verticillata, which exhibit an inducible C_4 - like metabolism, but lack the "Kranz" - anatomy characteristic for C_4 - plants (Holaday, et al, 1983). This mechanism does not require a separation of different steps of the fixation reaction in different cell types like in C_4 plants but is capable of reducing the apparent CO_2 compensation point. Under stress conditions, this plant switches from C_3 - type fixation to a C_4 - like mode (Lipka, et al, 1999). The synthesis of several enzymes, including PEPC and a chloroplastic ME (malic enzyme), is induced. During induction of the C_4 - like metabolism, the CO_2 compensation point begins to linearly decline. Decrease of the CO_2 compensation point is accompanied by an increase of PEPC, PPDK and NADP - ME (Cooley, et al, 1994). Even in C_3 plants, some tissues display PEPC activities higher than those in leaves. It is believed that some CO_2 respired can be recaptured in this way (Latz-

ko, et al, 1983). These observations may suggest that overproduction of key enzymes for C_4 – metabolism in C_3 plants would affect the efficiency of CO_2 assimilation of C_3 photosynthesis. It would be of great benefit if C_4 – like metabolism could be engineered into C_3 plants.

2.1 Progresses of the photosynthesis engineering in C_3 plants

It has been an active research area in plant biology that evaluation of the potential transfers C_4 traits into C_3 plants to improve their photosynthetic characteristics (Furbank, et al, 1995; Brown, et al, 1993). In addition, enzymes involved in C_4 photosynthesis may also play important roles in plant defense responses to biotic and abiotic stress (Fushimi, et al, 1994; Walter, et al, 1994; Schaaf, et al, 1995). Thus, overexpression of some C_4 enzymes in C_3 plants might confer enhanced tolerance under stress conditions and allow plants to adapt to adverse conditions (Ku, et al, 1999). In the last decade, various attempts have been made to transfer these agronomical important C_4 plant characteristics to C_3 plants either by classical breeding methods or by plant genetic engineering (Ku, et al, 1999). Hybridization between C_3 and C_4 plants by conventional breeding has been successful in only a few plant genera, such as Atriplex (Osmond, et al, 1980), because most of the hybrids exhibit infertility due to irregular chromosome pairing or other genetic barriers (Brown, et al, 1993). Thus, it seems difficult for employing traditional breeding methods to incorporate C_4 traits into C_3 crops (Ku, et al, 1999).

With the development of plant genetic engineering, transfer of foreign genes into crops has become routine, providing a new approach to altering plant traits (Ku, et al, 1999). The application of genetic engineering has made considerable progress in molecular engineering of C_4 photosynthesis, and it enabled us to express enzymes involved in the C_4 photosynthesis pathway at high levels and in desired location in the leaves of C_3 plants (Ku, et al, 1999; Tsuchida, et al, 2001; Fukayama, et al, 2001). After single or double key enzymes for C_4 metabolism was introduced into C_3 plants, some parameter of their photosynthesis appears to be improved. The major progresses are summarized as follows:

The over – expression of PEPC from Corynebacterium glutamicum PEPC in Solanum tuberosum causes an increase in sucrose and starch content in the leaf and a reduction in the CO_2 compensation point in the absence of dark respiration (Hausler, et al, 1999). This would suggest that the CO_2/O_2 ratio increased in the vicinity of Rubisco and resulted in a reduction of photorespiration. It has been shown that the simultanious overexpression of both NADP – malic enzyme and PEPC in potato, led to a reduced electron requirement for CO_2 assimilation (Lipka, et al, 1999).

Pyruvate, orthophosphate dikinase (PPDK) catalyzes the formation of PEP, the initial acceptor of CO_2 in the C_4 photosynthetic pathway. Elevated PPDK activity alters carbon metabolism in C_3 transgenic potatoes expressing a C_4 maize PPDK cDNA. PPDK activity in the leaves of transgenic potatoes was up to 5.4 – fold higher than those of control potato plants. A significant increase in the delta ^{13}C value was observed in the transgenic potato plants, suggesting

a certain contribution of PEP carboxylation as the initial acceptor of atmospheric CO_2. The elevated PPDK activity may lead to a partial operation of C_4 – type carbon metabolism. The altered carbon metabolism had a small effect on the total photosynthetic characteristics of the transgenic plants (Ishimaru, et al, 1998).

When maize intact PPDK gene was introduced into rice plants, the PPDK activity in the leaves of some transgenic lines was greatly increased. In a homozygous line, the PPDK protein accounted for 35% of total leaf – soluble protein or 16% of total leaf nitrogen. In contrast, introduction of a chimeric gene containing the full – length cDNA of the maize PPDK fused to the maize C_4 – Pdk promoter or the rice Cab (chlorophyll a/b apoprotein) promoter only increased PPDK activity and protein level slightly. These observations suggest that the intron(s) or the terminator sequence of the maize gene, or a combination of both, is necessary for high – level expression. The activity of the maize PPDK protein expressed in rice leaves was light/dark regulated as it is in maize (Fukayama, et al, 2001).

Solanum tuberosum was transformed with a chimeric gene consisting of the CaMV (cauliflower mosaic virus) 35S promoter and a potato Rubisco small subunit transit sequence, and the PEP synthetase (a PPDK – like enzyme) gene from *Escherichia coli*. The regenerated trangenic plants produced PEP – synthetase in amount as much as 0.1% of total soluble protein. Most of the protein was located in the chloroplasts and that transit sequence is cleaved off. PEP – synthetase activity was detected in isolated chloroplasts of transgenic plants. Chloroplasts morphology and starch productoin in leaves were affected (Panstruga, et al, 1997).

The photosynthetic carbon flow was changed in transgenic rice expressing C_4 – type PCK (PEP carboxykinase) from *Urochloa panicoides*. Crude extracts prepared from the green leaves of transgenic plants had high PCK activity and the newly expressed PCK was localized in chloroplasts. Further analysis on these transgenic plants indicated that the ectopic expression of PCK in rice chloroplasts was partially able to change the carbon flow in mesophyll cells into a C_4 – like photosynthetic pathway (Suzuki, et al, 2000).

The intact gene of maize PEPC was introduced into C_3 crop rice. Most transgenic rice plants showed high – level expression of the maize gene; the activities of PEPC in leaves of some transgenic plants were two – to three – fold higher than those in maize, and the enzyme accounted for up to 12% of the total leaf soluble protein (Ku, et al, 1999). Over – expression of maize C_4 – form PEPC alone in rice could change carbon isotope discrimination, suggesting that elevated PEPC activity in rice increased the overall CO_2 (HCO_3^-) concentration in the whole leaf cells as well as in the vicinity of the active site of Rubisco (Agarie, et al, 2001).

Taken together, these results suggest that introduction of C_4 photosynthesis enzymes into C_3 plants has a potential to enhance their photosynthetic capacity and yield. However, marked success with many aspects to crop improvements is still far away.

2.2 Functions and regulatory properties of PEPCs

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) catalyzes a reaction that fixes HCO_3^- on phosphoenolpyruvate (PEP) to form