SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067, INDIA

CERTIFICATE

The research work embodied in this thesis entitled **"Physiological significance of different isoforms of pyruvate orthophosphate dikinase (PPDK) in green plants"** has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. This research work is original and has not been submitted so far, in part or in full for any other degree or diploma of any University.

Ramesh Kumar Kushwaha
(Candidate)

Prof. Baishnab.C.Tripathy
(Supervisor)

Prof. B. N. Mallick
(Dean)

Physiological significance of different isoforms of pyruvate orthophosphate dikinase (PPDK) in green plants

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RAMESH KUMAR KUSHWAHA

School of Life Science
Jawaharlal Nehru University
New Delhi-110067
INDIA

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DEDICATED TO MY BELOVED PARENTS & FAMILY

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ABBREVIATIONS

APS	Ammonium persulphate
ATP	Adenosine triphosphate
BAP	6-benzyl aminopurine
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumine
□-ME	□-mercapto ethanol
CAM	Crassulacean Acid Metabolism
CaMV	Cauliflower mosaic virus
Chl	Chlorophyll
Cab	Chlorophyll a/b binding promoter
CBB	Coomassie brilliant blue
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
DCMU	(3-(3,4-dichlorophenyl)-1,1-dimethylurea)
DEPC	Diethyl pyrocarbonate
DDT	Dithiotreitol
DDW	Double distilled water
2,4-D	2,4-dichloro phenoxy acetic acid
EDTA	Ethylenediaminetetraacetic acid
EtBR	Ethidium bromide
ETR	Electron transport rate
F_0	Minimum fluorescence, when all reaction centers are open
Fm	Maximum fluorescence, when all reaction centers are open
Fv	Variabe fluorescence (Fm-F0)
FW	Fresh weight
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IAA	Indol acetic acid
µg	Microgram
□l	Microliter
ml	Mililiter
min	Minute
MES	2-(N-morpholino) ethane sullfonic acid
MOPS	3-(N-morpholino) propane sulfonic acid
NAA	Nephathalene acetic acid
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form Nicotinamide adenine dinucleotide phosphate

NBT	Nitroblue tetrazolium
OAA	Oxaloacetic acid
PAGE	Polyacylamide gèl electrophoresis
PPDK	Pyruvate phosphate dikinase
PEPC	phosphoenolpyruvate carboxylase
PEP	phosphoenolpyruvate
PS I	Photosystem I
PS II	Photosystem II
qP	Photochemical quenching
qN	Non-photochemical quenching
rbcS	Rubisco small subunit protein promoter
RT	Room temperature
Rubisco	Ribulose-1, 5-biphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TBST	Tris buffer saline with Tween 20
TRNS	Transgenic
Tris	Tris (Hydroxymethyl) amino methane
WT	wild-type
wt	Weight

Table of Contents

CERTIFICATE
DEDICATION
ACKNOWLEDGMENT
ABBREVIATION
1 INTRODUCTION **1**

2 REVIEW OF LITERATURE **3**

2.1 Photosynthesis: an overview 3

2.1.1 C_3 photosynthesis 3

2.1.2 C_4 photosynthesis 4

2.2 Single-cell C_4 photosynthetic plants in terrestrial environment 10

2.3 Single-cell C_4 photosynthetic plants in aquatic environment 11

2.4 Pyruvate Orthophosphate Dikinase 12

2.4.1 Structure of pyruvate orthophosphate dikinase 14

2.4.2 Isoform of pyruvate orthophosphate dikinase 16

2.4.3 Regulation of PPDK 19

2.4.4 Effect of pH on PPDK 20

2.4.5 Affinity of PPDK for pyruvate and PEP 20

2.4.6 Effect of ADP on PPDK 21

2.4.7 Effect of Environmental factor on PPDK 21

2.4.8 Transgenic plants over expressing PPDK 22

3 **MATERIALS AND METHODS** 24

3.1 Plant material as rice seed 24

3.2 Suface sterilization 24

3.3 Hormones were used 24

3.4 Media were used 24

3.5 General sterilization procedures 26

3.6 Plant material and growth conditions for *Arabidopsis* 26

3.7 Nutrient media for *Arabidopsis* ... 26

3.8 Salt treatment of Arabidopsis plant ... 29

3.9 Analysis of physiology of *Arabidopsis* plant ... 29

3.9.1 Chlorophyll and carotenoid estimation after 10 days of salt treatment ... 29

3.9.2 Protein estimation ... 29

3.9.3 Proline estimation ... 30

3.9.4 Pulse amplitude modulation (PAM) measurements after 10 days of salt treatment ... 30

3.10 Polyacrylamide gel electrophoresis of proteins (SDS-PAGE) ... 31

3.10.1 Staining with Coomassie brilliant blue R 250 (CBB R 250) ... 33

3.11 Western blot ... 33

3.12 Fresh weight and dry weight after 12 days of salt treatment ... 35

3.13 Agrobacterium strain and gene cloned in suitable vector ... 35

3.14 Setting up a restriction digest ... 35

3.15 Purification of DNA fragment from agarose gel ... 36

3.16 Qiaquick/ GFX gel extraction column ... 36

3.17 Preparation of competent cells and transformation ... 36

3.18 Sense orientation of PPDK ... 37

3.19 Isolation and purification of plasmid DNA ... 37

3.20 Spectrophotometric estimation of nucleic acid ... 38

3.21 *Agrobacterium* competent cells ... 38

3.22 Transformation of *Agrobacterium* ... 38

3.23 Preparation of *Agrobacterium* culture ... 39

3.24 Transformation of rice callus ... 39

3.25 Co-cultivation and selection of transformed calli ... 39

3.26 Regeneration of transformed macrocalli ... 40

3.27 Preparation of plant genomic DNA for PCR analysis ... 40

4 *ARABIDOPSIS* RESULTS 42

4.1 External morphology of plants 42

4.2 Chlorophyll content 42

4.3 Total protein 42

4.4 Proline content 43

4.5 Fresh weight and dry weight 43

4.6 Pulse amplitude modulated (PAM) chlorophyll *a* fluorescence measurements 43

4.6.1 Electron transport rate (ETR) 43

4.6.2 Quantum yield of photosystem II (ΦPSII) 44

4.6.3 Photochemical quenching (qP) 44

4.6.4 Non-Photochemical quenching (qN) 44

4.7 **RICE RESULTS** 44

4.7.1 Cloning of maize PPDK in pCAMBIA vector 44

4.7.2 Transformation of *Agrobacterium* with pCAMBIA-PPDK 45

4.7.3 Embryogenic callus development from rice 45

4.7.4 Preparation of *Agrobacterium* culture 46

4.7.5 Transformation of rice callus 46

4.7.6 Callus on selection media 46

4.7.7 Regeneration of plant on regeneration media 47

4.7.8 Screening of transgenic plant through PCR 47

5 DISCUSSION 48

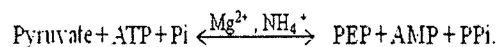
6 REFERENCES 50

INTRODUCTION

1 INTRODUCTION

With increase of the world population the demand for food, fodder and biofuel is constantly rising. It has come to our realization that another "green revolution" is needed for enhanced crop yields to full fill the ever increasing demands. One of the potential solutions is to introduce a higher capacity photosynthetic mechanism (i.e. C_4 photosynthetic pathway) into rice. Following the concept of single cell C_4 photosynthetic plant *Bienertia sinuspersici* (Edward et al., 2004, Offermann et al., 2011) and algae *Hydrila verticillata* (Magnin et al., 1997), generation of single cell C4 transgenic plants could be a reality.

In C_4 photosynthesis, Pyruvate orthophosphate dikinase (PPDK) is a key enzyme that synthesizes primary CO_2 acceptor phosphoenol pyruvate (PEP) in mesophyll cells of C_4 plant and CAM plants. This is not only present in CAM and C_4 plants but also present in C_3 plants (Aoyagi and Bassham 1984), bacteria (Pocalyko et al.,1990 ; Herzberg et al., 1996), green algae (Chastain and Chollet, 2003, Xu J et al, 2012), fungi (Marshall et al., 2001) and protozoa (Bringaud et al., 1998). PPDK catalyses the regeneration of the CO_2 -acceptor PEPC from pyruvate according to the equation



$$\text{Pyruvate} + \text{ATP} + \text{Pi} \xleftrightarrow{Mg^{2+},\ NH_4^+} \text{PEP} + \text{AMP} + \text{PPi}.$$

The products of this reaction are involved in C_4 photosynthetic pathway as well as in non-photosynthetic metabolism. In C_4 plant leaves it is more abundant than in C_3 plants, its contribution in C_4 plant leaves is up to 10% of the soluble protein fraction (Edwards et al., 1985; Chastain, 2011). The catalytic activity of PPDK is regulated under dark and light through dephosphorylation by a single regulatory protein. In active phase, it forms homotetramer complex of approx 95 kDa subunits while in inactive phase as in the dimeric and monomeric forms and it requires Mg^{2+} for oligomerization and NH_4^+ as a cofactor for optimal catalysis.

In C_3 and C_4 plants, PPDK is located in both cytoplasmic and plastid compartments (Chastain and Chollet, 2003). The localization of PPDK in cytoplasm or chloroplast is encoded by two promoters in single gene or two separate genes. In eudicots, PPDK is encoded by a single gene locus (Huang et al., 2008), but contains two promoters in the 5'-region to produce cytoplasmic or plastid targeted transcripts

(Parsley and Hibberd, 2006). In monocot species, the gene is encoded at two loci (Sheen, 1991; Huang et al., 2008), one of which expresses an isoform exclusive to the cytoplasm and the other loci configured as a two promoter gene to generate plastid and cytoplasmic. In C_3 plants, it is a very low abundance enzyme (Chastain and Chollet, 2003) while cytoplasmic form shows an increased level in developing cereal seeds (Kang et al., 2005; Chastain et al., 2006; Mechin et al., 2007; Hennen-Bierwagen et al., 2009), senescencing *Arabidopsis* leaves (Taylor et al., 2010), sperm cells of maturing *Arabidopsis* pollen (Hruz et al., 2008), and water stressed roots of rice (Moons et al.; 1998). In the leaves of *Arabidopsis*, cytosolic PPDK is up-regulated to facilitate remobilization of nitrogen into glutamine (Taylor et al., 2010) while in maize and rice during grain development help in starch and protein metabolism. In rice during hypoxic conditions, flood conditions, ABA treatment, salt stress there is an enhanced PPDK expression in roots (Moons et al.,1998).

Objectives of the present study are:

1. Cloning of maize PPDK and transformation of rice to produce ppdk overexpressing transgenic plants.

2. Tolerance to salt-stress by transgenic *Arabidopsis thaliana* overexpressing the maize *ppdk* in the cytosol.

REVIEW OF LITERATURE

2 REVIEW OF LITERATURE

2.1 Photosynthesis: an overview

Photosynthesis is the fundamental process used by phototrophic organisms to convert solar light energy into chemical energy in the form of sucrose. Photosynthetic organisms include plants, algae, some protists, cyanobateria and photosynthetic bacteria. The light energy is absorbed by the photosynthetic pigments carotenids, chlorophyll, and phycobillins. In higher plants chloroplast is the photosynthetic organelle that is composed of stack of internal membranous structure called grana (unit membranous structure called thylakoid) and remaining space called stroma. Two reactions are involved in photosynthesis: light reaction in grana which produces O_2, ATP and NADPH (Arnon, 1971) and other is dark reaction in stroma which utilises ATP, NADPH and reduces CO_2 to sucrose (also called Calvin- Benson cycle). Generally in pathway of CO_2 fixation most of photosynthetic organisms produce first stable three carbon compound 3-phosphoglycerate (3-PGA) but in some photosynthetic organism a four carbon compound oxaloacetic acid (OAA). So, on the basis of pathway of CO_2 fixation first stable carbon compound, the plants are divided into C_3 photosynthetic, C_4 photosynthetic and CAM plants. CAM plants are also a type of C_4 photosynthetic plant in which there is temporal separation of C_3 and C_4 pathway.

2.1.1 C_3 photosynthesis

C_3 photosynthesis is the ancestral pathway for carbon fi xation and occurs in all photosynthetic organisms. C_3 photosynthesis is a multi-step process; caboxylation, reduction and regeneration, which occurs in chloroplastic stroma of mesophyll cells of plants where the CO_2 is fi xed into stable organic products. In caboxylation steps, CO_2 first combined with RuBP (5-C compound) in the presence of ribulose bisphosphate carboxylaseoxygenase (Rubisco) and form two molecules of phosphoglycerate (3-C compound) which used in further steps. Overall in this multistep Calvin cycle 3ATP and 2NADPH are required for reduction of single molecule of CO_2. Rubisco enzyme is catalyses carboxylase activity as well as oxygenase activity and this activity depends on the temperature of environment and ratio of concentration of CO_2 and O_2. The carboxylase activity leads to the formation of two molecules of 3-PGA where CO_2 is the substrate and the oxygenase activity results into one molecule each of 3-PGA and

phosphoglycolate (2-C compound) where oxygen (O_2) is the substrate. Phosphoglycolate has no known metabolic purpose and in higher concentrations it is toxic for the plant (Anderson, 1971) and its formation metabolic pathway called photorespiration (C_2-cycle). Both carboxylation and oxygenation are interlocked to each other, so the competition between these cycles decreased the efficiency of C_3 photosynthesis (Taiz & Zeiger book).

2.1.2 C_4 photosynthesis

To increase the efficiency of photosynthesis, some photosynthetic organisms have developed an extra pathway in addition to C_3 pathway and plants that posses this pathway called C_4 plants. This pathway evolved as an adaptation to high light intensities, high temperatures, and drought. That's why, mostly C_4 plants dominate on grassland floras and biomass production in the warmer climates of the tropical and subtropical regions (Edwards et al., 2010). C_4 photosynthesis evolved through the enormous change in gene expression in C_3 plants. The transcriptomes analysis of mature leaves of the C_4 plant *Cleome gynåndra* and the closely related its C_3 species *Cleome spinosa* (Brautigam et al., 2011) has been compared quantitatively by a RNA-Seq-based digital gene expression approach and found C_4 evolved from C_3. Anatomically, the leaf structures of C_4 photosynthetic plant were modified from C_3 plant so as to form the inner compartment where Rubisco is localized and CO_2 can be concentrated (Dengler & Nelson, 1999). In most C_4 plants the mesophyll cells form a wreath-like cell arrangement around the vascular bundle sheath cell, termed as the Kranz anatomy. The plants with Kranz anatomy showed the differential expression of gene pattern in mesophyll and bundle sheath cells and it was found that approximately 18% of the genes in maize are differentially expressed between mesophyll and bundle sheath cells (Sawers et al., 2007). C_4 photosynthesis is also a series of biochemical reactions which occurs in mesophyll cells of Kranz anatomy plants which was discovered by Hatch & Slack (1966) (Fig. 1) in the leaves of sugarcane. In sugarcane's it was found two site of CO_2 fixation, primary carboxylation in the mesophyll cells by phosphoenol Pyuvate carboxylase (PEPC) (Chollet et al.,1996) and secondary carboxylation in the bundle sheath cells by Rubisco. During the primary carboxylation forms a four-carbon compound OAA, which further changes into another four carbon compound malate or aspartate.in presence of NADP malate dehydrogenase (MDH) or aspartate aminotransferase (AST) respectively. This malate/aspartate moves to the site

where Rubisco is localized (Hatch, 1987; Kanai & Edwards, 1999). Here, CO_2 is released by the decarboxylation of the four carbon acid, and its concentration rises to a level that nearly saturates the Rubisco active site (von Caemmerer, 2000). The decarboxylation reaction also produces pyruvic acid (3-carbon compound) which diffuses back to the compartment where PEP carboxylase is located and if needed, it may be converted into PEP in presence of enzyme pyruvate orthophosphate dikinase (PPDK) or it might remain as it is. In all C4 photosynthetic plants PEP carboxylase is common for the initial carboxylation reaction to yield OAA. For the decarboxylation reaction three types of enzymes have been identified as; NADP-malic enzyme, NAD-malic enzyme and PEP carboxykinase (PEPCK).

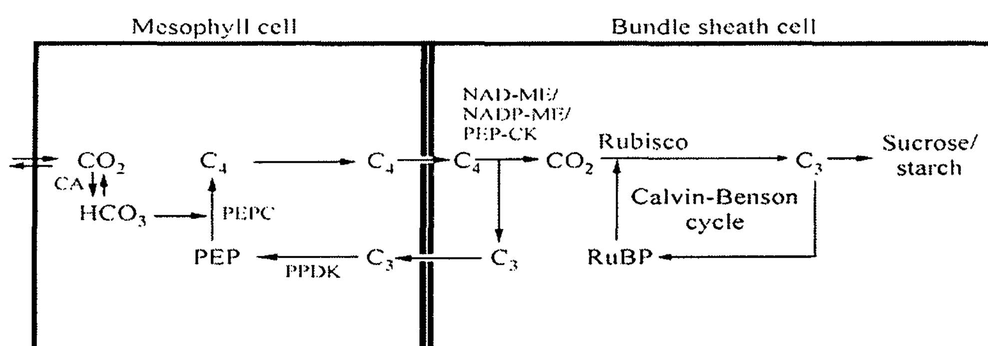


Figure:-1 Simplified schematic diagrams of the C_4 photosynthesis in Kranz anatomic plant (LUNG et al., 2012).

On the basis of their relative abundance, the C_4 photosynthesis pathway is divided into three biochemical subtypes (Figure.2): (a) If NADP-malic enzyme is used, OAA is converted to malate which then diffuses at site of Rubisco and dissociates into pyruvate and CO_2. (b) If NAD-ME is used then OAA is transaminated to asparatate then it diffuses at Rubisco site and dissociate into pyruvate and CO_2. In case of NAD-ME decarboxylation reaction, pyruvate is formed but this is transaminated to alanine, which then returns at the PEPC site where it is converted to pyruvate. (c) In PEP carboxykinase-type plants, during the decarboxylation reaction directly PEP is formed instead of pyruvate and then it returns to PEPC site (Leegood & Walker, 1999). Overall in C_4 plants including C_3 and C_4 phtosynthesis 5ATP and 2NADPH are required for reduction of single molecule of CO_2.

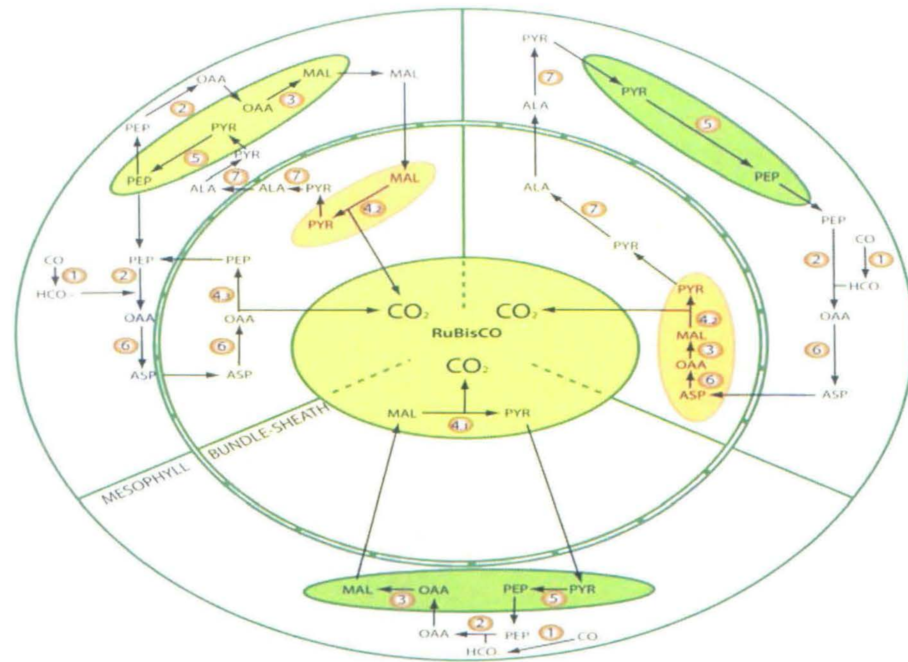


Figure.2 Schematic representation of C4-related reactions in the three known subtypes. 1. Carbonic anhydrases. 2. Phosphoenolpyruvate carboxylase, 3.NAD/P-Malate dehydrogenase, 4 Decarboxylases, 5. Pyruvate,orthophosphate dikinase, 6. Aspartate amino acids transferases. 7.Alanine amino acids transferases. (Aubry et al., 2011).

A third photosynthetic pathway, known as crassulacean acid metabolism (CAM) (Fig-3) exists in succulents such as cactus and other desert plants. In CAM plants stomata open at night, a unique character as Kranz anatomy in C_4 plant. These plants have same two carbon fixing pathway as in C_4 plant but both pathways occur within same cell. Also the biochemical pathway is same like C_4 plants but primary fixation of CO_2 at night and the product is different in charge and it is referred to malic acid instead of malate. This malic acid is stored in vacuole and during day it is transported to chloroplast where it dissociates into pyruvate and CO_2.

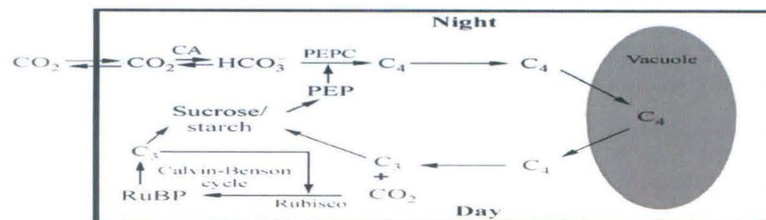


Figure. 3 Schematic diagram of CAM photosynthetic pathway. (LUNG et al., 2012)

At the cost of two extra ATP per C_4 pathway, C_4 plants concentrate the CO_2 at Rubisco site and suppress the photorespiration that results in an enhancement of photosynthetic efficiency. C4 plants have high photosynthetic capacities at warmer temperatures as compared to C_3 plants, and also water use efficiency (WUE) and nitrogen use efficiency (NUE) are greater than C_3 plants (Long, 1999; Sage and Pearcy, 2000; Kocacinar et al., 2008; Ghannoum et al., 2011). C_4 and CAM plants represent evolutionary advancements over the ancestral C_3 pathway that results in superior CO_2 fixating capacities under particular environmental conditions (Osmond et al. 1982; Monson 1989; Ehleringer and Monson 1993).

Eleocharis vivipara, E. baldwinii and *Eleocharis acicularis* are amphibious plants which show intermediate biochemistry between C_3 and C_4 photosynthetic plants because of the localization of Rubisco enzyme which is present in both mesophyll and bundle sheath chloroplasts and it suggests that segregation of the two carboxylases is incomplete (Brown and Bouton, 1993; Uchino et al., 1995; Ueno, 1996). It dramatically changes the photosynthetic and anatomical character with changes in its environment condition (Ueno et al., 1988, 1996; Sakae et al., 1997). This plant develops the C_4 character with Kranz anatomy when the environment is terrestrial and C_3 character with non-Kranz anatomy when in submerged aquatic environmental conditions. Therefore this species was considered as a useful model plant for studies of genetic and developmental aspects of C_3 and C_4 photosynthesis. In *Eleocharis acicularis* species there is an absence of the leaf blade formation and only clum is responsible for both photosynthetic functions (Uneo et al., 1988; 1996). The environmental conditions determine the development of new culms in the plant with either the C_3 mode or the C_4 mode of photosynthesis (Ueno 1988).When the submerged form is exposed to air, the old culms die due to rapid desiccation and within few days, plant starts to develop new culms, which already have Kranz anatomy and the C_4 biochemical traits. It may be possible that the C_4-like character in the aerial leaves is triggered by water stress and 6esides entire enzymes and metabolite transporters which are involved in C_4 photosynthesis are also reported in C_3 plants in different tissue in low activity and in different function (Table 1).

Table:-1 Reported functions of C_4 cycle enzymes and transporters in C_3 plants (Hausler et al., 2002 Aubry et al., 2011)

Enzyme	**Location**	**Function**
PEPC	Non green tissue	Recapture of respired CO_2
	Leaves	Anaplerotic suppply of carbon skeletons for amino acid biosynthesis. Buffering of cytosolic OH^- formation during nitrate reduction by malic acid formation
	Stomatal guard cells	Formation of malic acid during opening of stomata
NADP-ME	Fruits	De-acidification of vacuole, provision of reducing equivalents and carbon skeletons for gluconeogenesis, with PEPC involve in pH stat, with NAD-MDH involved in NADH/NADPH conversion
	Seeds	Provide reducing equivalents and carbon skeleton for fatty acid biosynthesis.
	Leaves	Provide reducing equivalent and carbon skeleton for lignin biosynthesis. Stress response
NAD-ME	Leaves	With PEPC involved in anaplerotic provision of carbon skeletons of amino acid biosynthesis
NADP-MDH	Leaves	Reduction of OAA in chloroplast
PEPCK	Non green tissue	Gluconeogenetic PEP production from OAA
	Trichome	Involvement of secondary metabolite
PPDK	Stomatal guard cell Leaves	PEP production for shikimate pathway, gluconeogenetic PEP production from pyruvate
Transporters		
Pyruvate	Leaves	Fatty acid and branched amino acid biosynthesis
Malate /OAA	Leaves	Malate valve
PPT	Leaves	Provision for PEP for the shikimate pathway inside the chloroplast

PPT-Phosphoenol pyruvate/phosphate translocator

The role of PEPC in C_3 plants to supply the carbon skeleton for the amino acid biosynthesis after nitrogen assimilation (Andrew, 1986; Melzeir and O'Leary, 1987). During nitrate reduction OH^- generated in cytosol and so the cytosolic PH balance by malic acid formation via OAA. Indirectly PEPC also play important role in stomata opening by formation of malic acid via OAA.

NADP-ME play role in many metabolic pathway in C_3 plant found in both green and non-green tissue (Edwards and Andreo, 1992). In the leaves of potato and tobacco showed good activities of NADP-ME in cytosol (Knee et al., 1996). In fruit it involved in ripening and in de-deacidification of vacuole and also involved in reducing equivalent and carbon skeleton in sucrose biosynthesis via gluconeogenesis. The expression of NADP-ME also increased severly in stress responses (Casati et al., 1999) and wounding with glutathionine treatment (Schaaf et al., 1995). NAD-ME is present in mitochondria of C_3 and it involved in anapleorotic carbon supply for amino acid biosynthesis.

NADP-MDH in the leaves of C_3 plant plays an important role in the suttling excessive redox equivalents from chloroplast into cytosol (Scheibe, 1987). It operates malate valve at high light intensity, when the NADPH and ETR generation more than demand for CO_2 assimilation and leads to prevent Mehler peroxide reaction (Polle, 1996).

PEPCK is a cytosolic enzyme which involved in ATP dependent gluconeogenetic PEP production from OAA (Review Leegood et al., 1999). In C_3 plant, it was found increased expression during oil seed germination, during fruit ripening and in developing seeds. Also detected in trichome of tobacco and cucumber leaves so it may involved in secondary metabolite production (Review Hä usler et al., 2002)

In C_4 photosynthesis, some metabolite transport across chloroplast and cytosol through transporter, particularly through inner chloroplast envelope. In mesophyll cells pyruvate enters chloroplast through pyruvate H^+ symporter which act as substrate for PPDK (Flü gge et al., 1985). While PEP generated in chloroplast exported in cytosol through PEP/phosphate translocator (PPT).Before 1960, it was generally accepted that Kranz anatomy is essential character for C_4 photosynthesis. Now so many aquatic and

terrestrial plants have been discovered which perform C_4 photosynthesis without Kranz anatomy.

2.2 Single-cell C_4 photosynthetic plants in terrestrial environment

Chenopodiaceae family contains around 1300 species including vegetable crops like spinach and beets, and desert plants such as Atriplex (saltbush) (Edwards et al 2011) Currently it has been reported that four chenopod species have C_4 photosynthesis *Bienertia cycloptera* (Voznesenskaya et al., 2002, 2005), *B. sinuspersici* (Akhani et al., 2005), *B. kavirense* (Akhani et al., 2012), and *Suaeda aralocaspica* (also called *Borszczowia aralocaspica*; Freitag and Stichler, 2000; Voznesenskaya et al., 2001). *B. cycloptera*, *B. sinuspersici*, and *B. kavirense* exhibit temporal expression of enzymes and spatial partitioning of organelles into two distinct intracellular compartments within individual photosynthetic cells as equivalent to Kranz anatomy. These species have two types of chloroplast i.e. dimorphic chloroplast which are separated in cytoplasmic compartments within the cell (Voznesenskayaet al., 2001, 2002, 2005; Chuong et al., 2006). Towards the peripheral region chloroplasts contain PPDK but Rubisco is absent and in central cytoplasmic region there are Rubisco-containing chloroplasts and it surrounds a large number of mitochondria containing NAD-ME (Voznesenskaya et al., 2002; Chuong et al., 2006). Both cytoplasmic and central chloroplasic regions are connected by thin cytoplasmic channels, which allow for the exchange of metabolic products between the two chloroplasts.

Another chenopod species *S. aralocaspica* shows a different mode of C_4 photosynthesis than that of above three species. This species contains elongated palisade chlorenchyma cells, in which Rubisco containing chloroplasts and NAD-ME-containing mitochondria are concentrated at the proximal end of the cell which are closer to the vascular tissues. However, PPDK-containing chloroplasts are uniformly distributed at the distal end of the cell, which are away from the vascular tissues (Voznesenskaya *et al.*, 2001; Chuong *et al.*, 2006 ;). This type of chloroplast arrangement is analogous to plants having the Kranz anatomy of the mesophyll and bundle sheath arrangement without the intervening cell walls. The distribution of chloroplast at opposite ends of the chlorenchyma cell and differential expression of enzymes in the organelles at the two regions appear during maturation chlorenchyma cells.

2.3 Single-cell C_4 photosynthetic plants in aquatic environment

In water CO_2 is present in the form of bicarbonate ion and its concentration is very low as compared to atmosphere. There are so many plants present in aquatic condition but some of those plants perform C_4 photosynthesis within single cell (Fig.4) without Kranz anatomy. Plants like submerged monocots *Hydrilla verticillata, Egeria densa,* and *Lagarosiphon major* (Hydrocharitaceae) (Brown et al.,1974) lack the stomata and its lamina thickness is composed of only two photosynthetic cells and thus lack the chlorenchymatous mesophyll and bundle sheath cells as in terrestrial C_4 species (Bowes et al., 2002). In above species low CO_2 induces the biochemical changes in the leaves from C_3 to single-cell C_4 photosynthesis and that is why it is called facultative C_4 photosynthesis. In *Hydrilla* it was found that environmental conditions favour toward C_4 photosynthesis when the CO_2 concentration decreases and then there is an up regulation of C_4 cycle genes without formation of new leaves (Holaday and Bowes, 1980; Spencer et al., 1994; Rao et al., 2006a) which results in an enhancement of the photosynthetic efficiency and also increases the nitrogen use efficiency. In cytoplasm PEPC combines with HCO_3^- and forms the four carbon compound malate which is transported to chloroplast where it is decarboxylated into pyruvate and CO_2 in presence of NADP-ME and finally CO_2 enters into Calvin cycle.

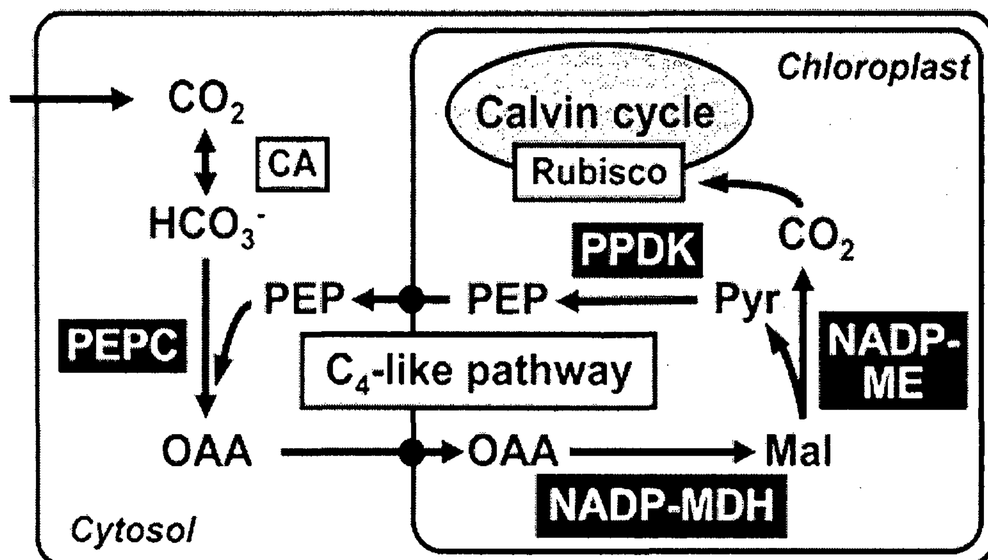


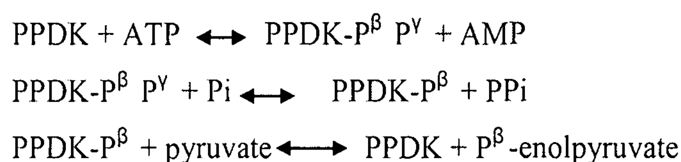
Figure:-4 The C4 pathway of *H. verticillata*, consisting of PEPC, PPDK, NADP-MDH, and NADP-ME. (Miyao et al., 2011)

Many plants have two types of leaves: one in free air and other in submerged condition and these submerged leaves perform single-cell C_4 photosynthesis. Plants which are submerged monocot *Sagittaria subulata* (Alismataceae) (Keeley, 1998a), the grasses *Orcuttia californica* and *O. viscida* (Poaceae), and the sedge *Eleocharis acicularis.* There are also reports of existence of single cell C_4 photosynthesis in marine macroalga (*Udotea flabellum*, Chlorophyta) (Reiskind et al.1988; Reiskind and Bowes 1991) and a diatom (*Thalassiosira weissflogii*) (Reinfelder et al., 2001, 2004). In these submerged leaves primary fixation of ·CO_2 occurs in cytosol in presence of PEPC and decarboxylation by NADP malic enzyme in the chloroplasts produces a chloroplastic CO_2 concentrating mechanism (CCM). It is also reported that the photorespiration of submerged plants might be less than that of terrestrial plants in air (Hough and Wetzel; 1972; Hough, 1974). *Sagittaria subulata* (Alismataceae family) is a submerged monocot which shows single cell C_4 photosynthesis and has been reported initialliy it was a CAM plant, on the basis of day-night (diel) fluctuations in titratable acidity and malate level (Keeley, 1998a). But the fluctuation was small when compared with the true submerged CAM species *Isoetes howellii* and the C_4 *Hydrilla* leaves (Holaday and Bowes, 1980; Keeley and Bowes, 1982; Keeley, 1999). *Sagittaria* species contains not only C_4 and CAM plant but also an intermediate between C_4 and CAM: a species *S. latifolia* contains ten folds more Rubisco activity than PEPC and diel malate fluctuations are negligible (Bowes et al., 2002). Poaceae family contains some amphibious plants which show the C_4 photosynthetic character like *Neostapfia colusana*, *Tuctoria greenei*, *Orcuttia californica* and *O. viscid* (Keeley, 1998b). *Neostapfia colusana* and *Tuctoria greenei* show the Kranz anatomy in both aerial and submerged leaves and thus it lacks single cell C_4 photosynthesis. While *Orcuttia californica* and *O. viscid* show Kranz anatomy only in aerial leaves yet the submerged leaves also show the C_4 photosynthesis.

In both above terrestrial and aquatic single-cell C_4 systems, have the partition between the biochemically different chloroplast which results into increase CO_2 concentration around the Rubisco-containing chloroplasts, which results into inhibits the oxygenase activity of Rubisco and photorespiration.

2.4 Pyruvate Orthophosphate Dikinase

Pyruvate orthophosphate dikinase is an enzyme that catalyzes the reversible reaction as below:



$$PPDK + ATP \leftrightarrow PPDK\text{-}P^{\beta} P^{\gamma} + AMP$$

$$PPDK\text{-}P^{\beta} P^{\gamma} + Pi \leftrightarrow PPDK\text{-}P^{\beta} + PPi$$

$$PPDK\text{-}P^{\beta} + pyruvate \leftrightarrow PPDK + P^{\beta}\text{-enolpyruvate}$$

This enzyme is absent in mammals but found in organisms that range from bacteria (Cooper and Kornberg 1967; Pocalyko et al. 1990) and protozoa (Bruderer et al. 1996), to plants (Hatch and Slack 1968). PPDK catalyses the reversible three-step reaction in which the conversion of ATP, Pi and pyruvate to AMP, PPi and phosphoenol pyruvate (PEP) respectively (Wood et al. 1977; Carroll et al. 1990). In the first step is the transfer of the γ - and β -phosphates from ATP to a conserved histidine residue in the active site of the enzyme, the second step is the transfer of the γ -phosphate to orthophosphate, and the third the transfer of the remaining β -phosphate to pyruvate. In the C4 pathway, PPDK catalyses the regeneration of the primary CO_2 acceptor PEP in the stroma of leaf-mesophyll cell chloroplasts, CAM plant and single cell C_4 photosynthetic plant. It is active in homotetramer of around 95 kDa subunits and is inactive in the dimeric and monomeric forms. It requires Mg^{2+} for oligomerization and NH_4^+ as a cofactor for optimal catalysis.

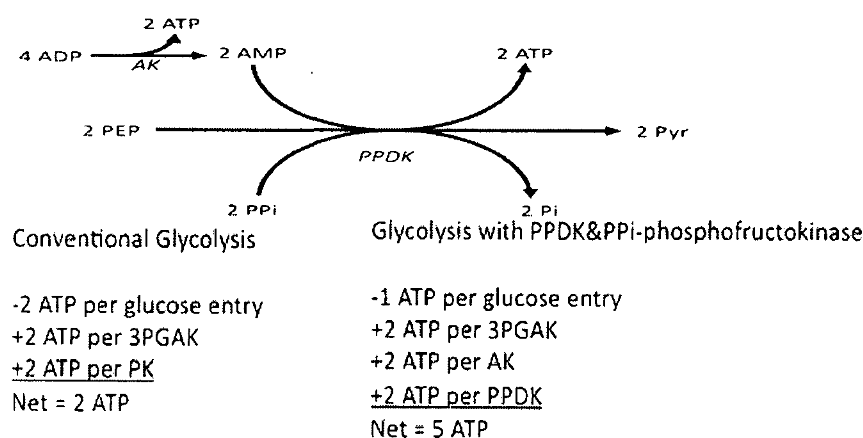


Figure:-5 Glycolytic ATP production for PPDK/PPi-dependent glycolysis versus conventional PK-based glycolysis. adenylate kinase (AK), pyruvate kinase (PK) (Chastain C.J. et al., 2011)

In the non-plant organisms like Archaea, eubacteria, and protists, PPDK enzyme is either a primary or secondary enzyme of glycolysis. The use of PPDK for generation of glycolytically derived ATP by the formation of pyruvate from PEP and also consume high bond energy from pyrophosphate (Huang et al., 2008). Ultimately, the yield of glycolytically derived ATP is increasing by two ATPs per glucose oxidized as in

conventional PK-dependent glycolysis and results into five ATPs per molecule of glucose oxidation (Fig. 5). In bacteria, PPDK is used in the generation of ATP by forming pyruvate from PEP during anaerobic condition and also in case of lack of pyruvate kinase (PK) (Reeves et al., 1968; Pocalyko et al., 1990). In protozoa use of PPDK is for ATP synthesis as well as in anaerobic or low oxygen environments and also in lack of mitochondria (Bringaud et al., 1998; Varela-Gomez et al., 2004; Feng et al., 2008).

2.4.1 Structure of pyruvate orthophosphate dikinase

Initially the primary structure of the PPDK protein was first reported through deduced amino acid sequence from the analysis of a maize cDNA clone (Matsuoka et al. 1989). The structural aspects of the PPDK catalytic mechanism although originate from studies of crystallized PPDK homodimer from the bacterium *Clostridium symbiosum* (Pocalyko et al., 1990 ; Herzberg et al., 1996 ; Lin et al., 2006 ; Lim et al., 2007). The structural model of PPDK in *C. symbiosum* is considered to be homologous to that of the plant PPDK as indicted by a high degree of conserved primary structure between plant and bacterial PPDKs (Pocalyko et al., 1990), and also havening the identical reaction mechanism (Carroll et al., 1990). The maize PPDK cDNA encodes a length of 947 amino acid residues with a molecular weight of 102,673Da. The comparison of the N-terminal amino acid sequence of the purified enzyme with that deduced from the nucleotide sequence of cDNA encoding PPDK reveals that the deduced sequence contains extra 71 amino acid residues. The extra 71 amino acids are considered as transit peptide which is responsible for the transport of the precursor protein from the cytosol into chloroplasts where the mature PPDK protein functions as an enzyme in the C_4 photosynthetic pathway. Thus, the mature form of PPDK in maize chloroplasts contains 876 amino acid residues.

The cDNA of PPDK have also been obtained from various organisms and compared against C_4 plants (Rosche and Westhoff 1990, Ohta et al. 1994), CAM plants (Fisslthaler et al. 1995) and C_3 plants (Imaizumi et al. 1997, Rosche et al. 1994) plants, protozoa (Bruchhaus et al., 1993; Saavedra-Lira and Perez-Montfort, 1994) and bacteria (Pocalyko et al. 1990). It was found that bacterial enzyme is a homodimer (Milner et al., 1975) while the C_4 enzyme is a homotetramer (Sugiyama, 1973). The primary structures are very similar to each other and while the plants PPDKs contain transit peptide sequences, the PPDK from protozoa and bacteria do not contain transit

peptide. Such a high degree of homology among the primary structures of PPDKs suggests that an ancestral *ppdk* gene might have existed before the divergence of prokaryotes and eukaryotes. In bacteria (*B. symbiosus*) reported GGMTSHAAWA amino acid sequence around the catalytic site of PPDK (Goss et al., 1980). The same amino acid sequence in catalytic site is found in all plant PPDKs (Fisslthaler et al., 1995) with histidine as the catalytic residue. In bacteria histidine residue in catalytic sequence accept phosphoryl of PEP for formation of ATP while in plant catalytic histidine residue accepts the pyrophosphoryl group from ATP for formation of PEP (Roeske and Chollet, 1987; Roeske et al., 1988). In protozoan PPDK catalytic sequence does not contain the same sequence as in plant and bacteria but contains a very similar sequence namely as GGKTSHAAWA. Two residues upstream from catalytic histidine residue a threonine residue present, which is conserve in bacteria, protozoa and plant PPDK (Table-2). In plants the deactivation and activation of PPDK takes place by the phosphorylation and dephosphorylation of conserve threonine residue by the bifunctional PDRP (Edwards et al. 1985).

Plants	456 458
Maize (C_4)	GMTSHAAVVAR
F. trinervia (C_4)	GMTSHAAVVAR
F. brownii (C_4-like)	GMTSHAAVVAR
E. vivipara (C_3-C_4)	GMTSHAAVVAR
M. crystallinum (CAM)	GMTSHAAVVAG
Arabidopsis (C_3)	GMTSHAAVVAR
F. pringlei (C_3)	GMTSHAAVVAR
Rice (C_3)	GMTSHAAVVAR
Protozoa	
E. histolytica	GKTSHAAVVAR
G. intestinalis	GQTSHAAVVAR
Bacteria	
R. prowazekii	GMTSHAAVVAR
S. coelicolor	GKTSHAAVVAR
C. symbiosum	GMTSHAAVVAR

Table:-2 Aligned active-site sequences of representative green plant, protozoan and

bacterial PPDKs showing the strict conservation of the catalytically essential His and plant regulatory Thr residues in all groups (Chastain *et al.*, 2003).

In all PPDK proteins, the surrounding region of the catalytic site is predicted to form a short a-helix, and this helical structure may be terminated by two adjacent reverse-turn structures that contain helix-breaking amino acid residues like glycine and arginine (Matsuoka et al. 1989). Two adjacent glycine residues are considered to inhibit the secondary structure formation and results into increase the flexibility of the peptide chain. Thus, it may be important for the PPDK activity that the a-helical region with the catalytic histidine residue rotates easily to afford a dynamic active site.

2.4.2 Isoform of pyruvate orthophosphate dikinase

In all C_4 plants and in some C_3 plants, it is reported that a single gene having two promoters is able to generate two alternates of transcript that encode either the cytosolic or chloroplastic isoforms of PPDK (Sheen, 1991)(Imaizumi et al., 1997; Parsley and Hibberd, 2006). In maize three PPDK genes have been found, one for the C_4 chloroplast PPDK gene (C4ppdkZm1) and two genes for cytoplasmic PPDK (cyppdkZm1 and cyppdkZm2)(Glackin and Grula, 1990; Sheen, 1991). In C_3 plant rice also reported three PPDK genes which are organized in two loci. Two genes for cytoplasmic PPDK are as follows: OsPPDKA and cytOsPPDKB which encode a cytosolic isoform and another one is C_4-type chloroplastic PPDK (chOsPPDKB) (Imaizumi et al., 1997; Moons et al., 1998). In *Arabidopsis* PPDK1 and PPDK2 are two transcripts which are transcribed from single gene containing two promoters (Parsley and Hibberd, 2006). In all plants it is believed that the chloroplastic isoform is created from cytoplsmic isoform by adding an extra exon, which codes for the chloroplast targeting transit peptide such as C4ppdkZm1derived from cyppdkZm1(Sheen, 1991). The chloroplastic isoform of PPDK is generated from a promoter that directs transcription of an exon that acts as a chloroplast targeting peptide when translated. The first intron of this long form of the gene contains a promoter that is responsible for transcription from a second start site. In this case the sequences encoding the transit peptide are lacking, and so this protein remains in cytosolic (Glackin and Grula, 1990; Parsley and Hibberd, 2006). In the vein of *Arabidopsis*, abundant transcript of both PPDK were found and that implies two pools for generation of PPDK and it is proposed that it is used in metabolism to generate aromatic amino acid and for lignin biosynthesis (Hibberd and Quick, 2002). Cytosolic PPDK transcripts also increase

during dark-induced senescence in leaves, in cotyledons during early seedling growth, in cauline leaves during seed development of *A. thaliana* (Lin and Wu, 2004; Parsley and Hibberd, 2006) and in roots of rice (Oryza sativa) during anoxia (Moons et al., 1998). Due to the presence of transcripts for PPDK in cotyledons of *Arabidopsis*, it was proposed that in cotyledons PPDK is used in the formation of pyruvate which is generated from amino acids to PEP and then it is used in gluconeogenesis (Parsley and Hibberd, 2006). During the dark induced senescence in *Arabidopsis* leaves the abundance of transcripts of cytosolic PPDK and other proteins that help in of transport of amino acids leads to the nitrogen remobilization from leaves (Fig. 6)(Lin and Wu, 2004). However, the transcript level is detected in both dark induced senescence and natural senescence (Buchanan-Wollaston et al., 2005), so it is unclear whether PPDK plays an important role in nitrogen remobilization during natural senescence. In rice the expression of PPDK was found at a greater level in kernel during milky stage (Fig. 6) (Imaizumi et al., 1997) and in other organ leaf, sheath, glume, rachis, stem and roots a very little amount is present (Fukayama et al., 2001). The chloroplastic isoform of PPDK in rice is also for PEP generation and its main role is in stromal shikmic pathway which leads to aromatic amino acid biosynthesis (Coruzzi et al., 2000).

In maize the cyPPDKZm1 codes for the cytosolic PPDK and its expression is detected during the grain filling in kernel. It is used in the metabolism of amino acid synthesis, protein storage and also provides carbon skeleton in endosperm (Fig.7) (Unger et al., 1993; Wang and Larkins, 2001). Also it has been reported that the expression of PPDK during endosperm development is regulated by Opaque-2 (O2) gene which encodes a leucine-zipper transcription factor (Maddaloni et al., 1996).

Still many of the functions of PPDK in C_3 plants are yet to be known and also how its expression is regulated still remains unclear.

Figure:-6 Scheme of the PPDK-dependent pathway that allows carbon skeletons in pyruvate to be supplied for production of the transport amino acid glutamine. Pyr, pyruvate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; Glu, glutamate; Gln, glutamine; Ser, serine; Gly, glycine; Fred, reduced ferredoxin; Fdox, oxidized ferredoxin.(Taylor et al., 2010)

Figure:-7 Possible metabolic role of cytosolic PPDK during rice kernel filling. Solid arrows indicate metabolic pathways; dashed line, regulation; solid circle, sucrose transporter. PKc, cytosolic pyruvate kinase (Kang et al., 2005).

2.4.3 Regulation of PPDK

The enzyme activity of PPDK in C_4 photosynthesis is controlled as inactivation in dark and activation in the light (Burnell and Hatch, 1983). For the detection of regulation of PPDK, the approach was based on the isolation of crude leaf extract from light and dark adopted tissue and lysate from isolated chloroplast (Edwards et al., 1985, Yamamoto et al., 1974). A bifunctional protein having both kinase as well as phosphatase activity, which is responsible for phosphorylation and dephosphorylation of PPDK (Fig.8). This PPDK regulatory protein (PDRP) catalyses the light-dependent regulation by reversible phosphorylation of Thr456 residue at active-site in maize (Burnell and Hatch, 1985)(Burnell and Hatch, 1985; Roeske and Chollet, 1987; Chastain, 2011)(Burnell and Chastain, 2006). The regulatory protein PDRP also plays the same role in light dependent regulation of non-photosynthetic PPDK in C_3 plants such as *Oryza sativa, Spinacia oleracea, Flaveria pringlei,* and *Vicia faba* (Chastain et al., 2002; Chastain et al., 2008)(Chastain and Chollet, 2003). *Arabidopsis* contains two isoforms of regulatory protein (RP) RP1 and RP2 which are encoded by AtRP1 and AtRP2 respectively. RP1 has both kinase and phophotase activity and RP2 has only kinase activity which regulates the non-photosynthetic PPDK (Chastain et al., 2008; Astley et al., 2011). RP1 protein is found in the chloroplast while RP2 is found in the cytoplasm and also RP2 differs from the plastid isoform RP1 by lacking a Pi-dependent, PPi-forming PPDK phosphotransferase activity (Chastain et al., 2008). From bacterial genomic databases it was found that bacteria have the homology with plant PDRP and are referred to as domain of unknown function (DUF 299) gene which is present in all PPDK-containing bacteria (Burnell, 2010). This homology of PDRP is also found in those bacterial species which lack PPDK (Burnell, 2010) and its function is the phospho-regulation of PEP synthetase. In *E.coli* the homology of PDRP is referred to as DUF 299 proteins function in regulate the PEP synthetase (PEPS) activity via reversible phosphorylation of Thr residue of PEPS at the active site (Burnell, 2010). So the main purpose in bacteria is to regulate the terminal steps of glycolysis through on/off regulation of PPDK and/or PEPS (Burnell, 2010; Chastin et al., 2011).

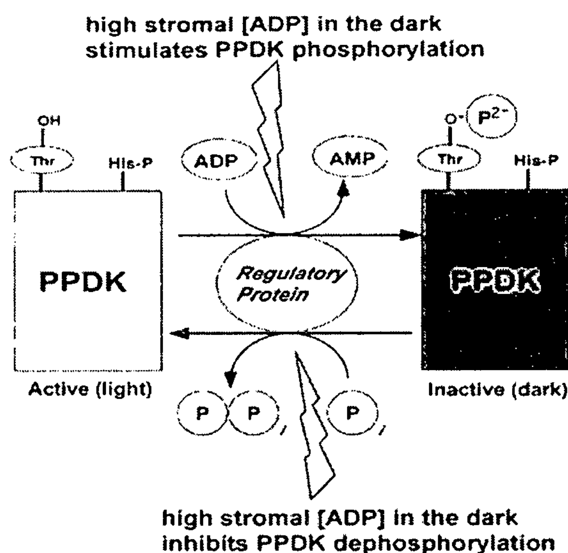


Figure:-8 Reversible phosphorylation of PPDK by PDRP. Inactivation of PPDK by PDRP proceeds by phosphorylation of an active-site Thr residue (Thr 456 in maize). Only the E-His-P intermediate enzyme form, as indicated by the His-P residue (His-458 in maize), is amenable to PDRP phosphorylation. Reactivation of PPDK is catalysed by Pi dependent dephosphorylation of this same (Chastain et al., 2011)

2.4.4 Effect of pH on PPDK

pH is a very important factor for stability, conformation and activity of any enzyme in the cells. Thus there is a change or an inhibition in the function of enzyme when there is a fluctuation in pH. PPDK enzyme is found in both cytoplasm and in chloroplasts of C_3 plants as well as C_4 plants. In cytoplasm, the cytoplasmic isoform of PPDK at pH 7.0 favors pyruvate formation from PEP in both C_3 and C_4 plant (Jenkins and Hatch, 1985) and its activity is similar in both plants (Chastain et al.,2011). At pH 8.3 it favours the reaction in PEP formation direction from pyruvate in both C_3 and C_4 plants and its activity is also found to be nearly equivalent (Chastain et al., 2008).

2.4.5 Affinity of PPDK for pyruvate and PEP

The PPDK enzymes have three-domain a swiveling central catalytic phospho-transfer domain interacts with remote N and C terminal domain. N-terminal have ATP binding domain while C-terminal have Pyr/PEP substrate binding domains (Herzberg et al., 1996; Lin et al., 2006; Lim et al., 2007). The affinity of PPDK for pyruvate and PEP is found to be different in C_3 and C_4 plants and it is calculated by Michalis Mentone constant (Edwards et al., 1985; Carroll et al., 1990). In *Arabidopsis* at pH 7.0 the Km value for pyruvate was found to be 17μM and in maize Km value for pyruvate was 178μM (Chastain et al., 2011). By comparing between both C_3 isoforms PPDK have

10-fold lower Km for pyruvate than maize C_4 PPDK and in reverse, maize PPDK have higher affinity for PEP(Km¼194 μM) than C_3 isoform PPDK (Km¼294 μM) (Chastain et al., 2011).

2.4.6 Effect of ADP on PPDK

PPDK is regulated by bifunctional PDRP whose kinase and phosphatase activity depends on the concentration of ADP inside chloroplast. So, the direction of PDRP catalysis depends on its kinase substrate ADP and also ADP act as a potent competitive inhibitor of Regulatory protein phosphatase activity (Burnell, 1984; Roeske and Chollet, 1987). The concentration of ADP in chloroplast act as adenylate energy charge (ACE) [AEC= (ATP+0.5ADP)/(ATP+ADP+AMP)] and its concentration fluctuates up and down, with change in rate of photophosphorylation. It is reported that, the decrease in illumination also decrease photophosphorylation that transiently causes an increase in ADP concentration and leads to PDRP catalysis in direction of phosphorylation of Thr residue at active site of PPDK (inactivation) (Burnell and Hatch, 1985a, b). There are other studies on the changes in ADP concentration when there was an application of PSII electron-transport inhibitor, DCMU and an uncoupler of photophosphorylation, CCCP in maize mesophyll protoplast and its effect on PPDK activity (Nakamoto and Edwards, 1986)(Nakamoto and Young, 1990).

2.4.7 Effect of Environmental factor on PPDK

Environmental stresses like biotic and abiotic factors, effect on the physiology and biochemistry of plants. Generally in *invitro* C_4 PPDK subjected to cold temperatures (e.g., ≤ 12°C) it dissociates into inactive dimers and monomers when (Shirahashi et al., 1978). One exception, the C_4 -like NADP-ME dicot species *Flaveria brownii* where its cold stable PPDK retains tetrameric structure at temperatures down to 0°C in vitro (Burnell, 1990). Later *invitro* it was found three hydrophobic amino acid residue in C-terminal of PPDK are responsible to maintain tetramer at cold temperature (Ohta et al., 1997). In C_4 plants *Miscanthus X giganteus* is an enhanced expression of PPDK in response to cold condition (Naidu and Long, 2004; Ohta et al., 2004; Wang et al., 2008) and also in response to UV-B irradiation (Casati and Walbot, 2004). In rice coleoptiles, enhancement of PPDK expression occurs during prolonged anoxia condition (Huang et al., 2005). Abscisic acid, polyethylene glycol, low oxygen stress, all type of water stress (drought, high salt, and mannitol treatment) and submerged conditions markedly induce the expression of PPDK in rice roots (Moons et al., 1998).

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2.4.8 Transgenic plants over expressing PPDK

Till now four transgenic C_3 plant have been successfully developed such as tobacco (Sheriff et al., 1998), potato (Ishimaru et al., 1998), *Arabidopsis* (Ishimaru et al., 1997) and rice (Nomura et al., 2000, Fukuyama et al., 2001). Transgenic potato with over expression of maize PPDK resulted in 5 times increase in PPDK activity and decrease in pyruvate and finally slightly increased in PEP content. Also, the malate level was considerably higher, presumably due to higher fluxes through PEPC which results in malic acid formation (Ishimaru et al., 1998). This elevated PPDK in potato led to partial operation of C_4 metabolism and it was found that there is no change in CO_2 compensation point.

In rice, full gene of maize PPDK including intron and exon with its own promoter and terminator sequence was expressed and there was a drastic increase in PPDK activity than 35S, *Cab* and *rbcS* promotor with maize cDNA (Fukuyama et al., 2001). Ultimately the result was found that there was a lighter leaf color and lower rates of growth and germination than wild-type plants. Also it lacked a visible phenotype or change in growth or fertility and the photosynthetic activity of transgenic rice was not appreciable (Fukuyama et al., 2001). Transgenic rice plant expressing two genes: PPDK with PEPC under 35S promoter was found to have a 35% increase in photosynthesis and 22% increase in grain yield (Ku et al., 2001). Pyruvate orthosphate dikinase gene of *Echinochloa* have introduced into H65, a upland rice variety through *Agrobacterium* mediated system. The expression of *Echinochloa* C_4 type PPDK in upland rice upto 1 – 11 folds enzyme activity than WT. However, no appreciable change in carbon assimilation was found in the transgenic upland rice and also increased photoinhibition under high light intensity (Wang and Li 2008).

Transgenic tobacco plant which expresses chloroplastic CAM PPDK from *M. crystallinum* under 35S promoter and it was found that a 40% increase in number of seed per capsule and 20% increase in weight per seed; also there is a 1.5 fold increase in PPDK acivity in leaves (Sheriff et al. 1998). However, when the transit peptide sequence was removed from PPDK enzyme and expressed under 35S promoter, transgenic tobacco produced less number and weight per seed than wild type (Sheriff et al. 1998). The important point is that this transgenic plant with both construct under control of root specific promoter B33 (potato tuber) has significant positive effects to

stimulate citrate synthesis and making plant more resistant to Al stress (Trejo-Téllez et al., 2010).

Transgenic *Arabidopsis* plants which expressed maize PPDK gene with 35S promoter and there is a 2-3 fold increase in PPDK in transgenic plant as compare to the untransformed *Arabidopsis* (Ishimaru et al., 1997). This transgenic plant did not show any change in morphology of plant and photosynthetic efficiency or CO_2 compensation point (Ishimaru et al., 1997).

MATERIALS AND METHODS

3 MATERIALS AND METHODS

3.1 Plant material as rice seed

Mature seeds of indica variety of *oryza sativa* IR-64 were used, which were taken from CRRI Cuttack. Manually seed were dehasked and then surface sterilized (discussed below) the dehasked seed. Surface sterilized seed were platted over rice callus induction media (RCIM) containing plate in laminar hood then it incubated at dark in growth chamber at 28^{0}C. After two weeks embryogenic calli were developed which were used in transformation or it maintained on same RCIM.

3.2 Suface sterilization

Dehasked seed were sterilized in hood with 70% ethanol and 2% sodium hypochlorite. In first step seed were taken in sterilize 250 ml conical flask then add 10ml of 70% ethanol for 60-90 second followed by washing five times with autoclaved double distilled water. After washing, add 30 ml of 2% sodium hypochlorite and were leave it for 30-40 minute at 110 rpm shaker then wash with autoclaved double distilled water (DDW) as above. After that seeds were spread on autoclaved Whatman paper to soak the extra water from seed surface. Twelve to fifteen seeds were inoculated in each petriplate containing RCIM.

3.3 Hormones were used

Hormone	Stock
2, 4-dichlorophenoxy acetic acid	10 mg/ml
1-Naphthaleneacetic acid	10 mg/ml
6-benzyleaminopurine	10 mg/ml
Kinetin	10 mg/ml

All above hormone were dissolved in 0.1N NaOH

3.4 Media were used

1. Rice callus induction media (RCIM):-

MS basal salts	4.4 g/l
Tryptone	0.3 g/l
Maltose	3%
2, 4-D	2.5 mg/l
BAP	0.1 mg/l

Phytagel	3 g/l
pH	5.8

2. Yeast extract peptone media(YEP)

Yeast extracts	10 g/l
Bactopeptone	10 g/l
Sodium chloride	5 g/l
pH	7.0

3. Resuspension media (RSM)

MS basal salt	4.4 g/l
Sucrose	3%
pH	5.2

4. Co-cultivation media (CCM)

MS basal salts	4.4 g/l
Maltose	0.3%
Glucose	1.0%
2, 4-D	2.5 mg/l
BAP	0.1 mg/l
Phytagel	3 g/l
Acetosyringone	100µM
pH	5.2

5. Selection media (SM)

MS basal salt	4.4 g/l
Maltose	3%
Tryptone	0.3 g/l
2, 4-D	2.5 mg/l
BAP	0.1 mg/l
Phytagel	3 g/l
pH	5.8
Cefotaxime	250 mg/l
Hygromycine	50 mg/l

6. Selection media-II

Same as SM-I

7. Selection media-III:

Same as SM-II

8. Regeneration media(RM)-

MS basal salt	4.4 g/l
6-bezylaminopurine	2.5 mg/l
Kinetin	1.0 mg/l
NAA	0.5 mg/l
Hygromycine	30 mg/l
Agarose	1.0%
pH	5.8

9. Regeneration media (RM)-II

Same as RM-I but agarose 0.8%

10. Root Induction media (RIM)

MS basal salt	2.2g/l
Sucrose	3%
Phytagel	0.3%
Hygromycin	30 mg/l
pH	5.8

3.5 General sterilization procedures

Culture media, glassware and tissue culture tools were sterilized by autoclaving at 121^0C and 15 lb/inch^2 for 15 min. Antibiotics and other heat-labile components were filter-sterilized using a syringe filtration unit fitted with an autoclaved cellulose nitrate membrane filter of 0.22 μm pore size (Mdi, India).

3.6 Plant material and growth conditions for *Arabidopsis*

Homozygous transgenic *Arabidopsis thaliana* ecotypes Columbia-0 (Col-0) were already developed in laboratry, which were expressing chloroplastic maize PPDK (*mPPDK*) without transit peptide under control of 35S promoter. Seeds were surface sterilized with 10 ml of sterilization solution (2% Sodium hypochlorite with 1μl/ml of 20% triton X-100) and gently mixed by inversion for 12 min and then washed with autoclaved distilled water at least 5 times for 3 min each and were grown under cool-white-fluorescent light (100 μmoles photons m^{-2} s^{-1}) at 22^0C.

3.7 Nutrient media for *Arabidopsis*

The growth medium (Gamborg *et al.*, 1968) consists of 0.5X MS basal medium (Sigma) containing the macro- and micronutrients, vitamins, 1% sucrose (Table-3). The

medium was prepared and the pH was adjusted to 5.7 using 1M KOH prior to the addition of agar and then autoclaved. The filter-sterilized solution of heat labile antibiotics was added to the autoclaved medium pre-cooled to 45^0C and poured into sterile petridishes on a clean bench. The Petridishes containing the medium were kept open for 10-15 min inside the hood under laminar flow to cool and dry the medium.

Table-3. Composition of the GM medium (Gamborg *et al.*, 1968)

Constituents	Concentration
0.5X MS salt with macro-and micronutrients, Vitamins (Sigma)	2.2 g/L
1% Sucrose	10 g/L

Adjusted to pH 5.7 with 1 M KOH, 0.7 - 0.8 % Difco Bacto Agar

MS media:-

MS basal salt	2.2 g/l
Sucrose	3%
Agar	0.8%
pH	5.6

Salt concentration media:-

MS basal salt 2.2 g/l

Sucrose 3%

Salt concentration (I) 150 mM

(II) 200 mM

Agar 0.8%

pH 5.6

Acid ninhydrin reagent:-

Ninhyrin (2.5%) 1.25gm

Glacial acetic acid	30 ml
Phosphoric acid (6M)	20 ml

Table-4. Primers used

Name	**Sequence**	**Tm (^{0}C)**	**Annealing temperature used (^{0}C)**
Maize PPDK	F: 5'ggggtaccatggcggcatcggtttcca 3' R:5'ggggtacctcagacaagcacctgagct 3'	71 69.5	59 59
35S Internal	F: 5' ccc act atc ctt cgc aag ac 3'	59.4	58
Rice actin	F:5'tcgagcatggtatcgtcagc 3' R:5'gcatacagagagaggacggc 3'	66.3 63.9	58 58
Rice PPDK Int.	F:5'aagtacagaagcattaaccagat 3' R:5'cagtcctcaccagaatgacagat 3'	57.6 64.4	58 58
Maize int-PPDK	F:5'tcaacgacgaagtggccgcc 3' R:5'cgctgagcggggctcaacaa 3'	69.52 69.67	58 58
Oligo dT	5'ttt ttt ttt ttt ttt ttt ttt ttt 3'	39.1	37
Hyg	F:5'atgaaaaagcctgaactcacc 3' R:5'ctatttctttgccctcggac 3'	56.8 57.3	58

Hyg=Hygromycin, int=Internal

3.8 Salt treatment of Arabidopsis plant

After 10 days of seed plating on growth media plant were four leaves stage. These plants were transfer on petridishes containing different salt concentration media and simple MS media as control and incubated at above growth condition.

3.9 Analysis of physiology of *Arabidopsis* plant

3.9.1 Chlorophyll and carotenoid estimation after 10 days of salt treatment

The extraction of Chlorophylls & carotenoids from tissues was done under a dim, green safe light. Leaf tissues (also seedlings) were homogenized in 90% chilled ammonical acetone (10 ml) in a pre-chilled mortar and pestle. For preparing 90% ammonical acetone, 1 N ammonia solution (7.48 ml in 100 ml distilled water) was prepared and then diluted ten times. This 0.1N ammonia solution was taken and acetone was added so as to obtain 90% ammonical solution. Three replicates were taken for each batch. Homogenate was centrifuged at 10,000 rpm for 10 min at 4^0C. Supernatant was taken for estimating Chlorophyll and carotenoids. Absorbance was taken at 663 nm, 645 nm and 470 nm. Reference cuvette contained 90% ammonical acetone. Chl was calculated as described by Porra *et al.*, 1989 and carotenoids were calculated as described by Welburn and Lichenthaler, 1984.

Chl a = $(14.21 \times OD_{663} - 3.01 \times OD_{645})$ V/W

Chl b = $(25.23 \times OD_{645} - 5.16 \times OD_{663})$ V/W

Chl (a+b) = $(9.05 \times OD_{663} + 22.2 \times OD_{645})$ V/W

Carotenoids = $(1000 \times OD_{470} - \{3.27 \times \text{Chl a} - 1.04 \times \text{Chl b}\}/5)$ V/227 x W

3.9.2 Protein estimation

During the chlorophyll isolation pellet were resuspended in 0.1 KOH solutions leave it for 30 min at ice. Estimation of protein concentration in various extracts was determined according to Bradford's method (Bradford, 1976). Bradford's reagent was prepared by dissolving Coomassie brilliant blue G-250 in 40 ml of 95% ethanol. The solution was mixed gently with 40 ml 85% phosphoric acid with constant stirring. The final volume was adjusted to 480ml with distilled water. Bradford's reagent was stored in brown bottles. Bradford microassay was performed by adding 1 ml of reagent to 2-10 μl of protein sample and absorbance was taken at 595 nm within 5 min. BSA was used as test protein for plotting a standard curve for each time.

3.9.3 Proline estimation

Proline was estimated a/c to Bates 1973 appoximatly 500 mg of leaves tissue was homogenise into 10 ml of 3% sulphosalicylic acid then filtered through Whatman paper #2. 2 ml ot this filtrate was react with 2 ml of acid ninhydrin reagent and 2 ml of glacial acetic acid in 15 ml falcon tube at 100°C for 1 h and reaction terminated in ice. In this reaction mixture 4 ml of toulene was added and was vigorously mixed for 15-20 sec. Upper layer of toulene containing chromophore used to take the absorbance at 520 nm and toulene taken as blank. The proline concentration was determined from standerd curve and calculared on fresh weight as follow:-

μmoles proline/gm fresh weight =

$$\frac{[(\mu g \text{ proline/ml} \times \text{ml toulene})/115.5 \mu g/\mu mole]}{[(g \text{ sample}/5)]}$$

3.9.4 Pulse amplitude modulation (PAM) measurements after 10 days of salt treatment

All measurements of chlorophyll fluorescence were performed with a portable PAM-2100 fluorometer (Walz, Effelteich, Germany). Before each measurement, the sample leaf was dark-adapted for 20 min with leaf-clips provided by the Walz Company. The angle and distance from the leaf surface to the end of the optic fiber cable were kept constant during the experiments. Chla fluorescence was detected by a photodiode (BPY 12; Siemens, Munich, Germany) that was shielded by a long-pass far-red filter (RG9; Southbridge, MA, USA) and a heat filter. To determine the initial fluorescence, F_0, the weak measuring light was turned on and F_0 was recorded. Then the leaf sample was exposed to a 0.8s saturation flash of approximately 3000 μmol photons m^{-2} s^{-1} to obtain the maximal fluorescence, Fm. Optimum quantum efficiency of PSII was calculated as Fv/Fm = (Fm-F_0)/Fm and the quantum yield of PSII as Fv′ /Fm′ = (Fm′ - F)/Fm′ where Fm′ and F are maximum fluorescence yield reached in a pulse of saturating light when the sample is pre-illuminated and measured fluorescence yield at any given time respectively (Genty et al,. 1989). The electron transport rate (ETR), expressed in μmoles electrons m^{-2} s^{-1}, was calculated on the basis of the measured value of Yield and PAR using the equation ETR = yield x PAR x 0.5 x 0.84 where PAR corresponds to the flux density of the incident photosynthetically active radiation, measured in μmoles photon m^{-2} s^{-1}. The coefficient of photochemical fluorescence

quenching (qP) and non-photochemical fluorescence quenching (qN) were calculated using the following equations, $qP = (Fm' - F)/(Fm' - F_0)$ and $qN = (Fm - Fm')/(Fm - F_0)$. All measurements of F_0 were performed with the measuring beam set to a frequency of 0.6 KHz, whereas all measurements of Fm were performed with the saturation flash automatically switching to 20 KHz.

3.10 Polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli, 1970. The chemicals and solutions used for SDS-PAGE:

Acrylamide (30%): Acrylamide 58.4g

Bis-acrylamide 1.6g

Distilled water added to make vol. 200ml

This stock solution was filtered and stored at 4^0C in an amber bottle.

Separating gel buffer (4X): Tris (1.5M) 36.3g

Final vol. made to 200ml with distilled water after adjusting pH to 8.8 with HCl. Stored at 4^0C.

Stacking gel buffer (4X): Tris (0.5M) 6.0g

Final vol. made to 100ml with distilled water after adjusting pH to 6.8 with HCl. Stored at 4^0C.

SDS (10%): SDS 10g

Vol. made to 100ml with distilled water and stored at room temperature.

Sample buffer (2X): Tris Cl (0.125M, pH 6.8) 2.5ml of stacking gel buffer

SDS (4%) 4ml of 10% SDS

β -mercaptoethanol (10%) 1ml

Glycerol (20%) 2ml

Distilled water added to make up the vol. to 10ml.

Tank buffer (4X):

Tris (0.025M)	15g
Glycine (0.192M)	72g
SDS (0.1%)	40ml of 10% SDS

Distilled water added to make up the vol. to 1L.

APS 10%: Always prepared fresh.

TEMED from Sigma Company

β -mercaptoethanol from Sigma company

Recipes	**12.5% Separating gel**	**4.5% Stacking gel**
Monomer stock solution	10.0ml	1.7ml
Buffer	6.0ml (pH 8.8)	2.5ml (pH 6.8)
SDS (10%)	0.24ml	1.0ml
Water	7.6ml	4.8ml
TEMED	15μl	5μl
APS (10%)	400μl	150μl
Total volume	**24ml**	**10ml**

SDS-PAGE was carried out in a vertical gel electrophoresis apparatus (regular size) (ATTO Corp., Japan) according to Laemmli, 1970. Gels were prepared and electrophoresed under reducing and denaturing conditions in presence of β-ME and SDS. Protein samples (30 μg) were prepared by mixing with ½ volumes of 2X sample buffer (constituents of sample buffer are mentioned above). The samples were boiled for 3 min in a water bath and centrifuged at 13000 rpm for 2 min at room temperature. The supernatant was loaded on the stacking gel. Gels were run either at a constant voltage (100V) or a constant current (20 mA). After electrophoresis, the gels were

stained with Coomassie brilliant blue R 250 (CBB R 250) for visualization of the proteins.

3.10.1 Staining with Coomassie brilliant blue R 250 (CBB R 250)

Fixing solution: 40% methanol, 10% acetic acid in double distilled water

Staining solution: CBB (0.05%) in 50% methanol and 5% acetic acid

CBB was dissolved in methanol. Acetic acid was added, followed by Distilled water to make the volume 1L.

Destaining solution: 5% methanol, 7.5% acetic acid in double distilled water

The gel, after electrophoresis, was incubated in 10 volumes of fixing solution for 20 min on a gyratory shaker. The gel was stained in 5 vol. of CBB 4 h to overnight on the shaker. After staining, stain was removed; gel was rinsed with double distilled water and was then left in 20 vol. of destaining solution for 2-3 h. The destaining solution was replaced 2-3 times at 1h interval, until the background was clear. The gel was preserved in 7% acetic acid in distilled water.

3.11 Western blot

Western blot analysis was done according to Towbin *et al.*, 1979. The chemicals and solutions used for western analysis:

TBS (Tris buffered saline):

TBS (Tris buffered saline):	Tris (25mM)	3.03g (pH 7.4)
	NaCl (136mM)	7.95g
	KCl	0.2g

Distilled water added to make up the vol. to 1L.

TBST buffer: TBS + 0.05% Tween 20

Blocking solution: TBST + 4% BSA

Transfer buffer:	Tris (0.1M)	6.05g
	Glycine (0.192M)	7.2g
	5% Methanol (v/v)	25ml

Distilled water added to make up the vol to 500ml.

Alkaline phosphatase (AP) buffer: TrisHCl(100mM,pH 9.5) 1.211g

NaCl (100mM) 0.5844g

MgCl2 (5mM) 0.10165g

Distilled water added to make up the vol. to 100ml.

AP colour development solution: AP buffer 10 ml

NBT sol^n 66μl

BCIP 33μl

Ponceau S: 0.1% (w/v) in 1% acetic acid (v/v)

Transfer of proteins from polyacrylamide gels onto nitrocellulose (NC) membranes was carried out in a semi-dry Transblot apparatus (ATTO Corp., Japan), as per the manufacturer's instructions. Protein (20 μg) loaded on SDS-PAGE was run in ATTO gel electrophoresis apparatus as described above. After the run, gel was first equilibrated in transfer buffer for 15 min. NC membrane and Whatman papers (3mm) were also soaked in the transfer buffer. For transfer, 4-6 pieces of 3mm Whatman paper were placed on the platform of the apparatus, on the top of which membrane was placed followed by gel and 4-6 layers of Whatman paper. Air bubbles trapped were removed. Constant current equal to twice the area of gel was applied (e.g. if the gel area was 50 sq. cm, 100mA current was applied) during transfer. Handling of membrane was done wearing gloves. After the transfer over nitrocellulose membrane, gel-facing side of the membrane was marked and the membrane was stained in Ponceau S (0.1% Ponceau S in 1% acetic acid). Markers were marked with a ballpoint pen and the membrane was destained in water. Membrane was then kept in blocking solution containing 3% BSA in TBST, at room temperature for 2 h. After this the membrane was washed in TBST thrice (5 min each) with constant shaking. The membrane was incubated for 1 h at room temperature with primary antibody at the appropriate dilution. Dilution was made in TBST containing 0.1% BSA. After the incubation, membrane was washed in TBST thrice (5 min each) with constant shaking and then incubated with alkaline phosphatase-conjugated secondary antibody (1:7500 dilutions) for 1 h at room

temperature. Membrane was again washed in TBST thrice (5 min each) with constant shaking. The NC membrane was then stained using substrate for alkaline phosphatase. 16μl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 33μl of nitroblue tetrazolium (NBT) were added to 5 ml of AP buffer. Stock solution of BCIP was prepared by dissolving 0.5 g BCIP disodium salt in 10 ml of 100% dimethyl formamide and stored at -20°C and of NBT was prepared by dissolving 0.5 g in 10 ml of 70% dimethylformamide and stored at -20°C. The blot was developed till purple-blue bands appeared at the site of antibody binding (Towbin *et al.*, 1979).

3.12 Fresh weight and dry weight after 12 days of salt treatment

After 12 days of salt treatment total weight and shoot weight of 15 plants of each grown on MS, 150 mM and 200 mM were weighted separately on Metller weighing machine. 15 plants divided into three groups contain 5 plants in each in Aluminum foil and was kept in dry oven for 5 days at 60°C. After five days dry weight were calculated as:-

Dry weight = (total wt with Al foil) before dry-(total wt with Al foil) after dry

3.13 Agrobacterium strain and gene cloned in suitable vector

Agrobacterium strain LBA 4404 was taken from ICGEB New Delhi, which was used in this experiment. In this experiment pyruvate orthophosphate dikinase (PPDK) gene was used, which was prepared from maize cDNA library by using kpn1 restriction site attached forward and reverse primer of maize PPDK (GenBank accession no.NM_001112268.1). Amplified PPDK was cloned in pGEMT-easy cloning vector (Promega, USA) and transformed E.coli (DH5α) with this construct and was screened blue white colony (this work had already done in lab). There after PPDK was cloned in binary plant transformation vector pCAMBIA1304 at kpn1 site. pCAMBIA 1304 vector has nptII (neomycin phosphotransferase) and hptII (hygromycin phosphotransferase) genes as the selectable markers for bacteria and plants respectively. It has both uidA (for GUS) and green fluorescent protein (mGFP) as the reporter genes.

3.14 Setting up a restriction digest

Restriction digestion was set according to Promega protocols. To 3 μl of 10X restriction enzyme buffer, 0.2 μl of BSA, 1 μg DNA, 0.5 μl of restriction enzyme (10u/μl of DNA) was added and the final volume was made up to 30 μl with sterile,

deionised water. The reaction mixture was incubated at 37^0C for 3 h and then fractionated and visualized on 1.2% agarose gel.

3.15 Purification of DNA fragment from agarose gel

Restriction enzyme digested plasmid was electrophoresed and visualized on 1.2% agarose gel (Agarose, MB, Pharmacia). The desired fragment was identified using standard molecular weight marker (1 kb ladder) and purified using the following technique.

3.16 Qiaquick/ GFX gel extraction column

To the excised pieces of agarose gel containing DNA fragment, 500 μl of capture buffer (GFX gel extraction kit, GE health care) was added and dissolved by heating to 55^0C for 10 min. The mixture was loaded onto GFX spin column and spun briefly in a microfuge (maximum speed, 30 sec). The flow through was discarded and 500 μl of wash buffer was added to the spun column. The column was again centrifuged at maximum speed for 30 sec. Purified DNA fragment was eluted with 50 μl of 10 mM Tris- HCl, pH 8.0.

3.17 Preparation of competent cells and transformation

DH5α cells were made competent by following the protocol of Hanahan, (1983). A single colony of DH5α was picked up and inoculated into 5 ml of LB medium (10g NaCl, 10g tryptone, 5g yeast extract per 1L) and grown overnight at 37^0C. One ml of overnight grown culture was inoculated freshly into 100 ml of LB and grown at 37^0C till $O.D_{600}$ of 0.4 - 0.5 was reached (2 – 3 hrs). The cells were harvested by centrifugation at 3000g for 10 min. The pellet was resuspended in 40 ml of ice cold 100 mM $CaCl_2$ solution, incubated on ice for 1 hr, centrifuged and the pellet was re-suspended in 4 ml of ice cold 100 mM $CaCl_2$ containing 15 % glycerol solution. The cell suspension (0.1ml) was aliquoted into autoclaved un-opened Eppendorf tubes and stored at - 80^0C.

Kpn1 digested PPDK fragments were ligated with same R.E digested binary plant transformation vectors by using T4 DNA ligase (Promega, USA) overnight at 16^0C. The ligation mixture was added to the competent cells and mixed by tapping and then incubated for 30 min at 4^0C. All steps were carried out in a laminar hood under sterile conditions. The cells were subjected to heat shock by incubating at 42^0C for 90

sec, allowed to stand for 2 – 5 min on ice followed by addition of 0.9 ml of LB and then grown at 37^0C with gentle shaking (185 rpm) for 1h. Aliquots of these transformed competent cells were plated onto LB plate (10g NaCl, 10g tryptone, 5g yeast extract and 15g agar per 1L) containing appropriate antibiotic.

3.18 Sense orientation of PPDK

Transformed DH 5α colonies were appear after 24 h and analysed the presence of PPDK by colonies PCR. Colonies PCR was done in by adding bacterial colony in each 20 μl volume 15 pmoles of each primer (internal forward and reverse), 200μM of dNTPs, 4 units of Taq DNA polymerase in buffer containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 15 mM $MgCl_2$. PCR amplification was done with a program having 94^0C for 4 minutes (initial denaturation) followed by 30 cycles of 94^0C (denaturation) for 30 seconds, 58^0C (annealing) for 30 seconds, 72^0C (extension) for 45 seconds in a program thermal cycler (USA). PCR products was analyse by 1% agarose gel run with standard 1kb ladder (fig 10.B-C). The positive colonies were further checked for the orientation of gene either in sense or antisense. Next colonies PCR was done same as above but here used the forward primer of 35S and PPDK gene reverse primer. Amplified product analyse by 0.8% agarose gel run with 1kb standard ladder. The sense colonies were inoculated in autoclaved 5 ml LB media in test tube and incubated at 37°C 220 rpm for overnight.

3.19 Isolation and purification of plasmid DNA

Five ml of overnight grown culture of bacterial cells were harvested by centrifugation at a maximum speed for 1 min in a microfuge. The pellet was re-suspended in 100 μl of ice-cold solution I (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 μg/ ml RNase A) and vortexed vigorously. Then cells were lysed by adding 200 μl freshly prepared solution II (200 mM NaOH, 1% SDS) and gently mixed by inverting the tube five times and then kept on ice for 5 min. Following this, 150 μl of chilled solution III (3.0 M potassium acetate, pH 5.5) was added and mixed by inversion. This mixture was incubated on ice for 3-5 min and centrifuged at maximum speed in a microfuge for 5 min at 4^0C. The supernatant was extracted with equal volumes of Phenol: Chloroform: Iso amyl alcohol (25:24:1) at maximum speed for 2 min at 4^0C. The DNA precipitation was carried out by adding 1/10 volume of 3M sodium acetate (pH 5.2) and two volumes of ethanol (room temperature). The mixture was incubated at room

temperature for 2 min and centrifuged at maximum speed for 5 min to precipitate DNA. The plasmid DNA was washed with 70 % ethanol, vacuum dried and dissolved in minimal volume of water or TE buffer.

3.20 Spectrophotometric estimation of nucleic acid

The quantity and quality of the nucleic acid was determined by measuring the absorbance at 260 nm and 280 nm. The amount was calculated taking 1.0 A_{260} = 50 μg/ml for DNA and 1.0 A_{260} = 40 μg for RNA. The purity of the nucleic acid was determined by calculating the ratio A_{260}/A_{280} for each sample.

3.21 *Agrobacterium* competent cells

Agrobacterium was inoculated in primary 5ml of liquid YEP medium (1% yeast extract, 1% peptone, 0.5% NaCl) with the help of sterilized loop from 3 to 5 days old grown culture on LB plate (25mg/l streptomycin, 10mg/l refampicin) and incubated at 28°C 220 rpm for 24 h. For secondary culture 0.4% volume of primary culture was inoculated in autoclaved 50 ml of YEP media in 250 ml conical adding with same antibiotics and incubated as above for 12-16 h to reach OD_{600} 0.4-0.6 for making competent cells. When OD_{600} reached 0.4-0.6 culture was taken in sterilized SS-34 tube and centrifuge at 4°C 6000 rpm for 10 min. Pellet was resuspended in 40 ml autoclaved 20 mM $CaCl_2$ and kept it for 2 h on ice and then again centrifuge as above. The pellet was re-suspended in 1 ml of 15% ice cold $CaCl_2$ (20 mM). Aliquots (0.1 ml) were dispensed in pre-chilled Eppendorf tubes (autoclaved) and stored at - 80 °C

3.22 Transformation of *Agrobacterium*

Recombinant plasmid construct (pCAMBIA 1304-PPDK) was transformed into *Agrobacterium* by freeze thaw method. Aliquoted competent cells (~100μl) in 15% glycerol were used for transformation. 10μg of gene construct recombinant plasmid was added in aliquoted competent cells and kept on ice for 30 min and then given temperature shock with 2 min liquid N_2 followed by 5 min at 37°C. There after added 900 μl YEP media and incubated at 28°C 220 rpm for 2-3 h and centrifuged at 4°C at 6000 rpm for 2 min. 900μl supernatant was removed and resuspended in remaining, this resuspended culture platting on YEP plate (with antibiotics 25 mg/l Streptomycin, 10 mg/l Rifampicin, 50 mg/l Kanamycin) and incubated at 28°C for 48 h. Transformed colonies that appeared after 2 days were analyzed by PCR.

3.23 Preparation of *Agrobacterium* culture

Primary culture of *Agrobacterium* contains desire construct was prepared by the inoculation of bacteria with the help of sterile loop from 3 to 5 days old grown bacterial colony in test tube contains 5ml liquid YEP media supplemented with 25 mg/l streptomycin, 10 mg/l Rifampicin, 50 mg/l Kanamycin. The culture tube was then kept for 16 to 20 h on rotatory shaker in dark at 220 rpm and 28^0C temperature. The secondary culture was prepared in 500 ml of conical flask containing 100 ml liquid YEP media (supplemented with same antibiotic as in primary culture) by adding 0.4% of primary culture and kept it (as in primary culture condition) for 12 h. When the OD at 600 reaches ~1, then *Agrobacterium* cells were pelleted in SS-34 tube by centrifugation at 4^0C temperature and at 6000 rpm for 10 minutes. The supernatant was discarded and pellet was resuspended in resuspension media (RSM), till to reaches the OD_{600} 0.6. Just before to agroinfection of callus, the resuspended culture was supplemented with 100 mM acetosyringone (3, 5-Dimethoxy-4-hydroxyacetophenone).

3.24 Transformation of rice callus

Embryogenic calli were developed from seed after two week on RCIM. There after the only the embryogenic calli were cut into 3-4 approximately equal part and were subculture on fresh RCIM containing plate for 4 days at 28°C before agroinfection. After 4 days the globular calli were developed and it were choosen for transformation or May subculture on RCIM for further agroinfection. The globular calli were collected in sterilized conical flask. The resuspended *Agrobacterium* supplemented with acetosyringone were added in conical flask containing calli and were incubated in shaker at 28°C at 110 rpm for 18-20 minute.

3.25 Co-cultivation and selection of transformed calli

The infected calli from *Agrobacteria* resuspension culture flask were transferred on the autoclaved Whatman papper for dried. After drying, the calli were spread on co-cultivation media (CCM) containing plate and incubated at 28°C for 48 h in dark condition. After two days enough *Agrobacterium* growth was observed on the co-cultivation media. The infected calli were washed 5-8 times with autoclaved double distilled water in a sterilized conical flask. After that the calli were incubated in autoclaved double distilled water with 250 mg/l Cefotaxime for 15 minute, then dried on Whatman papper. The dried calli were transferred on selection media (SM-I) containing plate and incubated in dark at 28°C for 15 days. After 15 days on selection

media, the black and brown calli were removed and only creamish color calli were transfer on SM-II for second selection. On the SM-II, after 8-10 days the microcalli were started to develop from transformed calli and it become white and granular. Only these microcalli were transferred for third selection on SM-III in incubated for next 10-15 days at previous selection condition.

3.26 Regeneration of transformed macrocalli

After 3rd selection, black/brown microcalli were discarded and only white granular macrocalli were transferred on the petriplate containing regeneration media (RM-1) for regeneration. The macrocalli on the regeneration media were kept at 28°C in dark condition for 8-10 days. After 8-10 days, hairy structures on the calli were developed, and then this plate was transferred in exposure of light at 28°C. The duration of light exposure was 16h light and 8 h dark, during this period callus were stared to green just after 3-days. All the greenish calli were transferred on next regeneration media (RM-II) and incubated as like at RM-I. When the growing shoot were touched the cover plate, then it were transferred in rooting media (RIM) and kept under 16 h light conditions at 28°C for 15 days. The rootlet plat were transferred to pot containing vermiculite and covered with polythene bag for 7 days for hardening. After 7 days, polythene bag were removed and kept another 7 days at same condition after that it transferred to soil in green house.

3.27 Preparation of plant genomic DNA for PCR analysis

Small piece of leaf tissue (1cm X 1cm) was ground with 400 μl of genomic DNA extraction buffer (2% CTAB, 1.4M NaCl, 20 mM EDTA, 100 mM Tris-Cl and 0.1% β-ME) at room temperature. To the homogenate additional 400 μl of genomic DNA extraction buffer was added and kept at 60°C in a water bath for 30 min. To this 650 μl of phenol: chloroform: isoamyl alcohol (24:24:1) was added and mixed by vortexing for 30 sec followed by centrifugation at maximum speed for 5 min in a microfuge at room temperature. The aqueous layer was transferred to another fresh tube and once again extracted with 650 μl of phenol: chloroform: isoamyl alcohol (24:24:1) in Eppendorf tube. To the final aqueous phase 2/3 volume of isopropanol was added, mixed properly and kept at room temperature for 5-10 min to precipitate the genomic DNA. After precipitation of genomic DNA samples were centrifuged at maximum speed for 5 min at room temperature in a microfuge. The pellet was washed 3 times

with 70% ethanol, dried and dissolved in 30 μl sterile water and incubated at 37^0C for 30 min.

RESULTS

4 *ARABIDOPSIS* RESULTS

4.1 External morphology of plants

Sterilized seeds of *Arabidopsis* wild-type (WT) and *mPPDKx* were grown in MS medium for 9 days and subsequently transferred to different concentrations of NaCl (150 mM and 200 mM) and MS media for another 10 days. Plants were grown at 22^0C under 14h L/ 10h D photoperiod in cool white fluorescent light (100 µmoles photons m^{-2} s^{-1}) and their morphology was observed (Fig. 14). On the MS media *mPPDK* plants were more greenish as compared to WT. At 150 mM and 200 mM salt concentrations *mPPDKx* and WT plants were almost similar morphological features (Fig.14A-D).

4.2 Chlorophyll content

WT and *mPPDKx* plants were grown in MS medium for 9 days and subsequently transferred to MS plates having different concentrations of NaCl i.e., 0 mM, 150 mM and 200 mM for another 11 days. Plants were grown at 22^0C under 14h L / 10h D photoperiod in cool-white-fluorescent light (100 µmoles photons m^{-2} s^{-1}). Their total chlorophyll was measured as described in "Material & Methods". On MS media Chl a, Chl b and Chl a+b content in *mPPDKx* plants had 12%, 23% and 18% higher in than that of WT respectively. In *mPPDKx* plants, at 150 mM salt concentration Chl a, Chl b and Chl-a+b content were less than that in WT. At 200mM salt concentration *mPPDKx* plants had reduced Chl a, Chl b and Chl-a+b content i.e., 30%, 27% and 30% respectively as compared to that of WT (Fig.9 A, B, D). The Chl a/Chl b ratio was more or less equal in both WT and *mPPDK* plant at each salt concentration (Fig.9C).

4.3 Total protein

WT and *mPPDK* plants were grown in MS medium for 9 days and subsequently transferred to different concentrations of NaCl (150 mM and 200 mM) and MS media for another 10 days. Plants were grown at 22^0C under 14h L / 10h D photoperiod in cool-white-fluorescent light (100 µmoles photons m^{-2} s^{-1}). Their total proteins were extracted and quantified as described in "Materials & Methods". On the MS media total proteins level was 10% higher in WT as compared to *mPPDKx* plants (Fig.10 A). At 150 mM and 200 mM salt concentration total protein were 18% and 16% higher in WT than that of *mPPDKx* plants respectively. At 150 mM the protein level in transgenic plants was slightly higher than in WT (Fig.10 A).

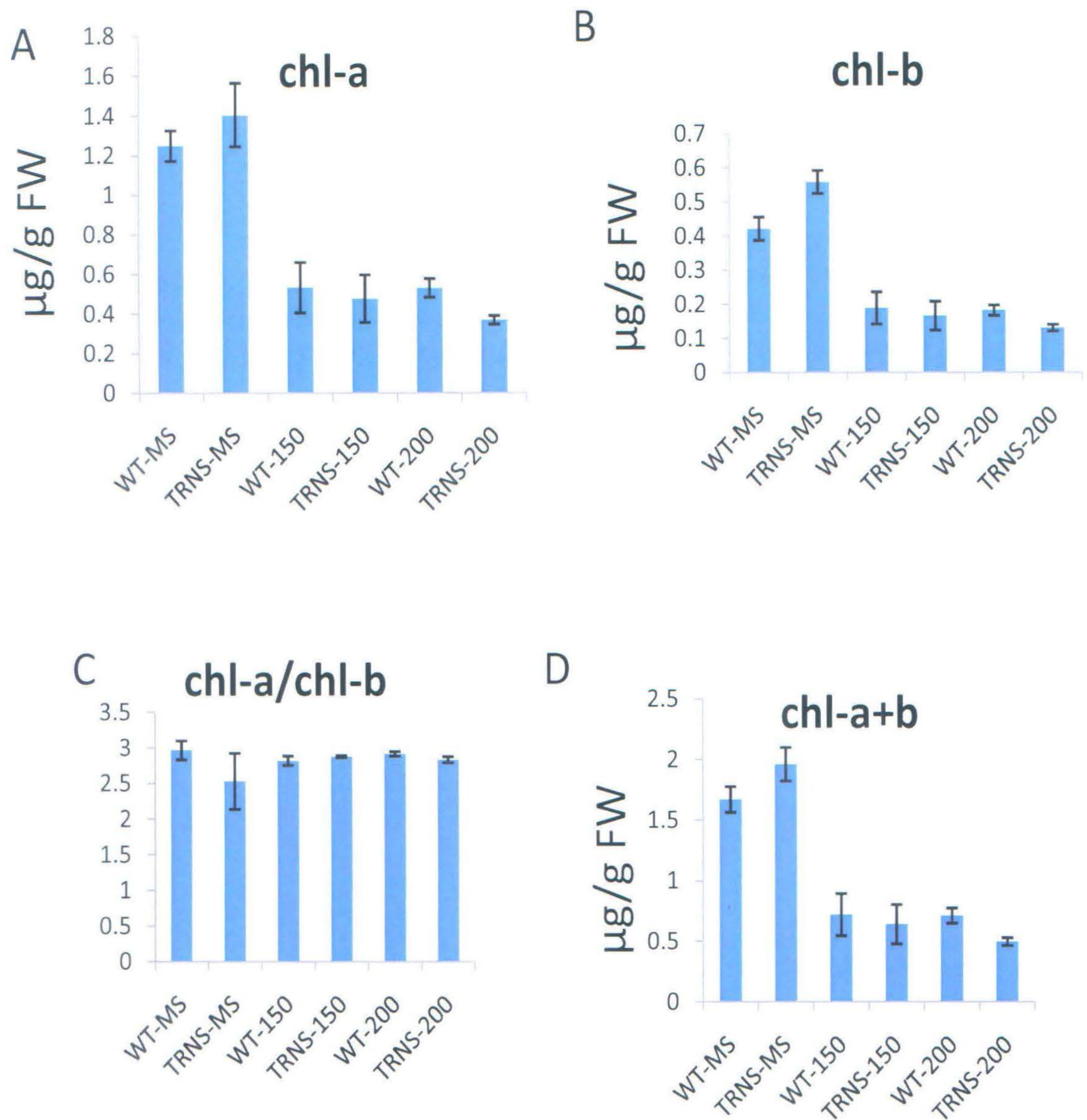


Figure 9. Pigment content of WT and PPDK Transgenic arabidopsis plants. Estimation of chlorophyll done in WT and PPDK plants after 11 days of salt treatment.. WT and PPDK overexpressing plants were grown at 22^{0}C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m^{-2} s^{-1}) for ten days in MS medium and then transferred to plates at different salt concentrations in MS medium and their (A) Chl a, (B), (B) Chl b, (C) Chla/b and (D) Chla+b were measured as described in "Material & Methods". Each data point is an average of three replicates. The error bar represents SD.

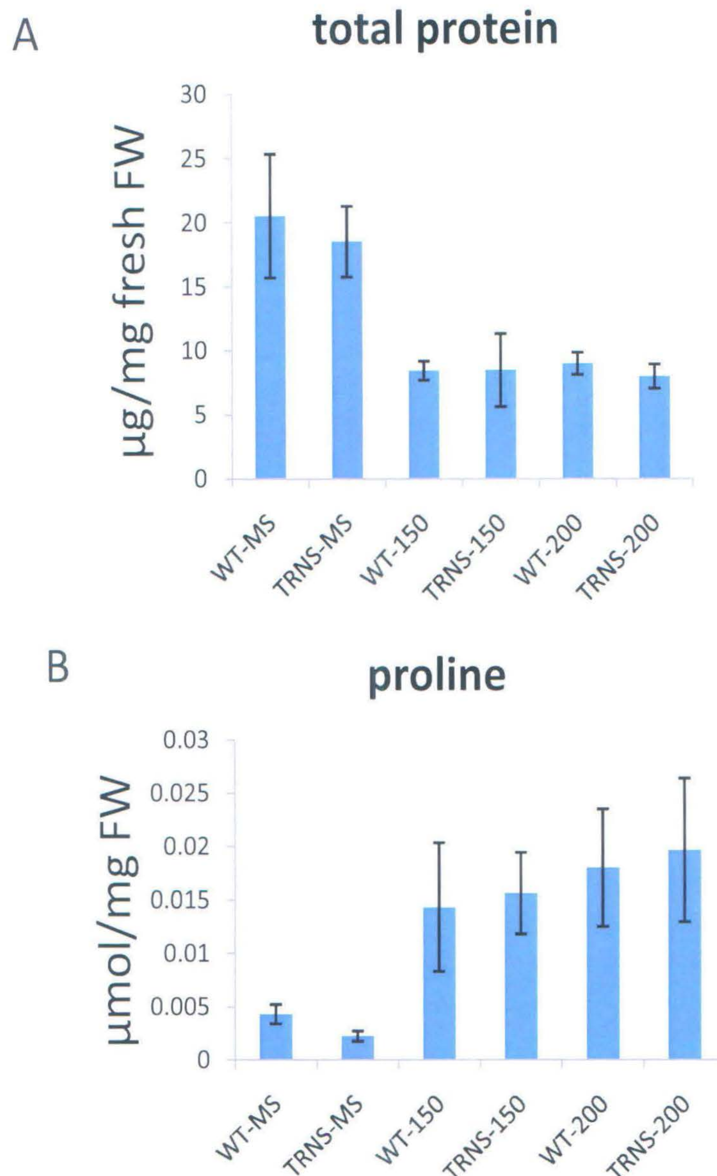


Figure 10. Total protein and proline content of WT and PPDK overexpressing transgenic plants Estimation of total protein and proline in WT and PPDK plant done after 10 days of salt treatment.. WT and PPDK overexpressing plants were grown at 22^0C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m^{-2} s^{-1}) for ten days in MS medium and then transferred to plates at different salt concentrations in MS medium for 10 days and protein isolation and proline content determination done as described in "Material & Methods". Each data point is an average of three replicates. The error bar represents SD

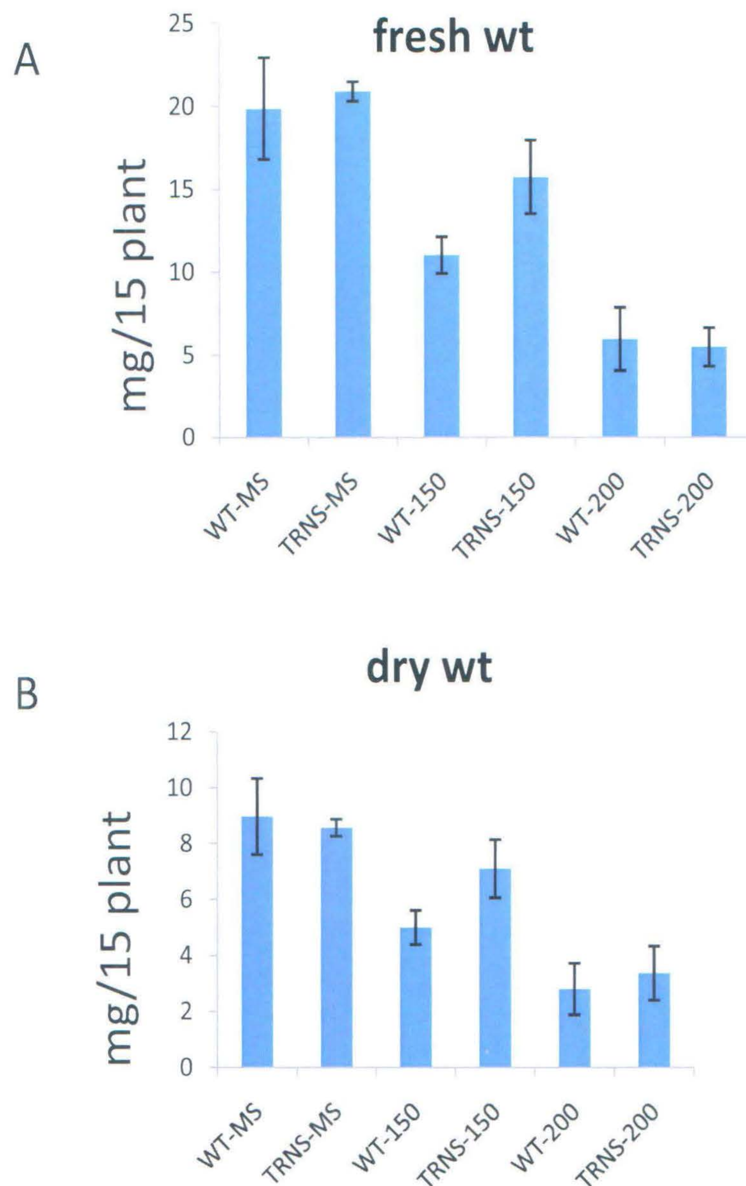


Figure 11. Fresh weight and dry weight of WT and PPDK plant after 12 days of salt treatment. WT and PPDK overexpressing plants were grown at 22^{0}C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m^{-2} s^{-1}) for ten days in MS medium and then transferred to plates at different salt concentrations in MS medium for 12 days and their fresh weight and dry weight were measured as described in "Material & Methods". Each data point is an average of three replicates. The error bar represents SD.

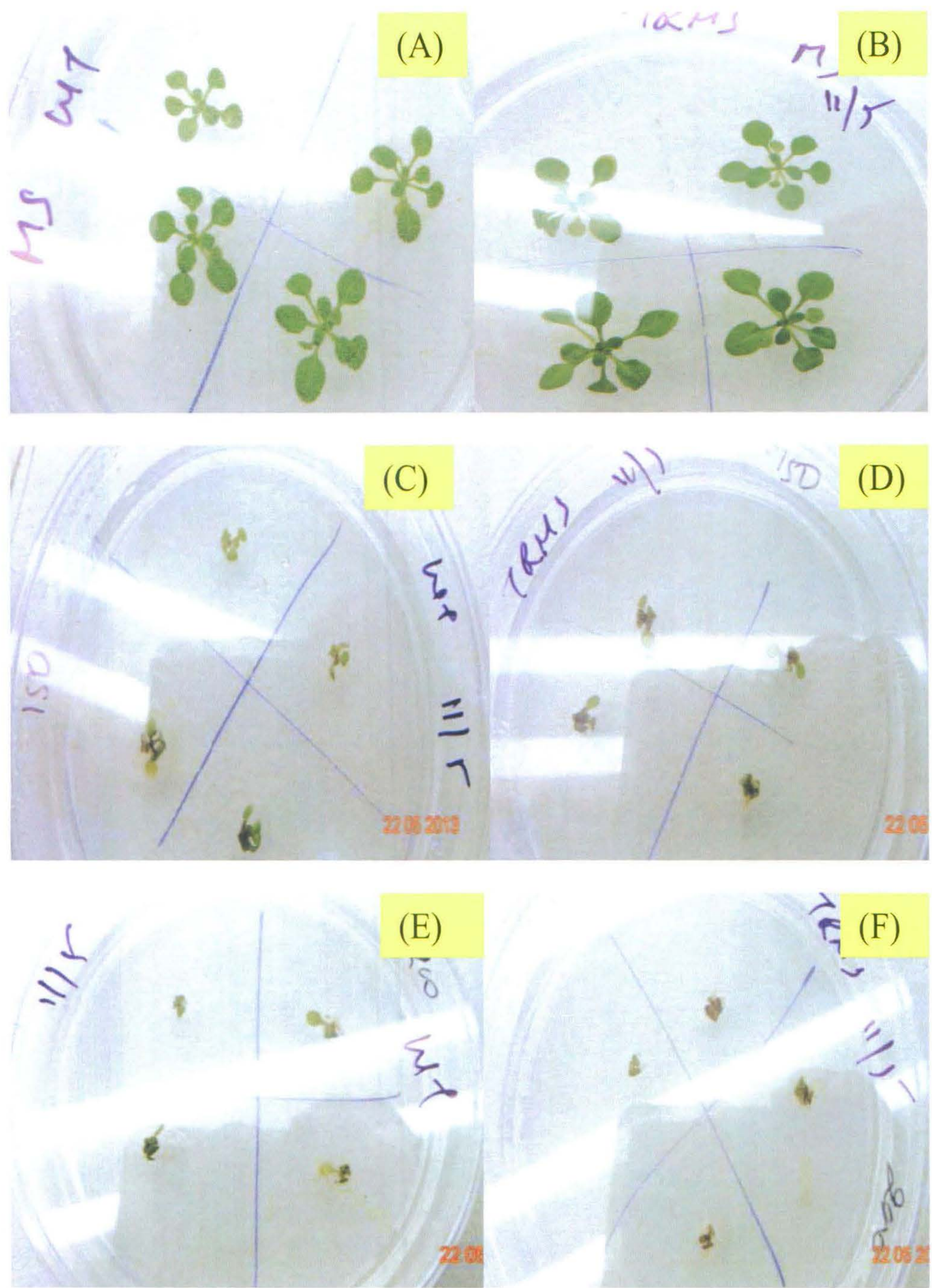


Figure 14. External morphology of WT and PPDK transgenic arabidopsis plant after 10 days grown on MS and salt media. (A) WT on MS (B) transgenic on MS (C) WT on 150mM (D) transgenic on 150mM (E) WT on 200mM (F) transgenic on 200mM.

4.4 Proline content

WT and *mPPDKx* plants were grown in MS medium for 9 days and subsequently transferred to different concentrations of NaCl (150 mM and 200 mM) and MS media for another 11 days. Plants were grown at 22^0C under 14h L / 10h D photoperiod in cool-white-fluorescent light (100 µmoles photons m^{-2} s^{-1}). Leaf tissue was taken for proline extraction and estimation as described in "Material and Method". The proline concentration increased with increasingsalt concentration both in *mPPDK* and WT plants (Fig.10 B).

4.5 Fresh weight and dry weight

WT and *mPPDKx* plants were grown in MS medium for 9 days and subsequently transferred to MS plates having different concentrations of NaCl i.e., 0 mM, 150 mM and 200 mM for another 12 days and were grown at 22^0C under 14h L / 10h D photoperiod in cool-white-fluorescent light (100 µmoles photons m^{-2} s^{-1}). Their fresh weight and dry weight was measured. Increase salt concentrations decreased the fresh weight and dry weight in both WT and *mPPDKx* plants. On MS media Fresh weight of *mPPDK* plants was 5% higher than that of WT. At 150 mM salt concentration *mPPDKx* plants had 25% higher fresh weight and 40% higher dry weight than that of WT (Fig.11A-B).

4.6 Pulse amplitude modulated (PAM) chlorophyll *a* fluorescence measurements

To study further, Chl fluorescence was monitored as a signature of photosynthesis (Govinjee, 1995) in WT and *mPPDKx* transgenic *Arabidopsis* plants.

Chl a fluorescence was measured in WT and *mPPDKx* plants grown in MS medium for 9 days and subsequently transferred to different concentrations of NaCl (150 mM and 200 mM) and MS media for another 10 days. Plants were grown at 22^0C under 14h L / 10h D photoperiod in cool-white-fluorescent light (100 µmoles photons m^{-2} s^{-1}). Various Chlorophyll a fluorescence parameters i.e., F_0, Fm, Fv/Fm, electron transport rate (ETR), quantum yield of PSII (ØPSII), photochemical quenching (qP) and non-photochemical quenching (qN) were studied as described in "Materials & Methods".

4.6.1 Electron transport rate (ETR)

The ETR (µmoles electrons m^{-2} s^{-1}) increased in response to photosynthetic active radiation (PAR) (µmoles photons m^{-2} s^{-1}) (Fig.12A). ETR value in both *mPPDKx* and

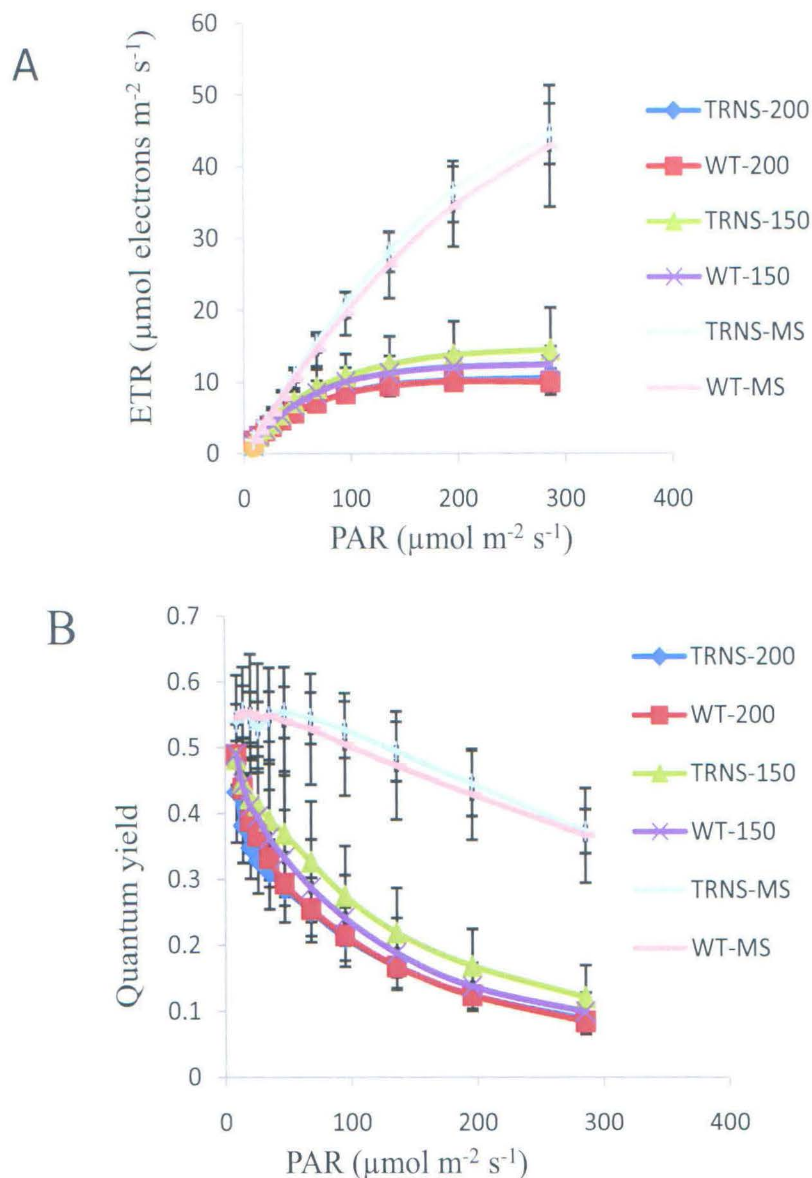


Figure 12. Pulse amplitude modulated (PAM) Chlorophyll a fluorescence measurements of WT and *PPDK* overexpressing plants. ETR and Yield of WT and PPDK plant after 10 days of salt treatment. WT and PPDK overexpressing plants were grown at 22^{0}C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 µmoles photons m^{-2} s^{-1}) for ten days in MS medium and then transferred to plates at different salt concentrations of 150, 200mM in MS medium for ten days and were used for studying various parameters of Chlorophyll a fluorescence measurement including **(A)** electron transport rate (ETR), **(B)** Quantum Yield as described in "Material & Methods". .Each data point is an average of six replicates. Error bar represent SD.

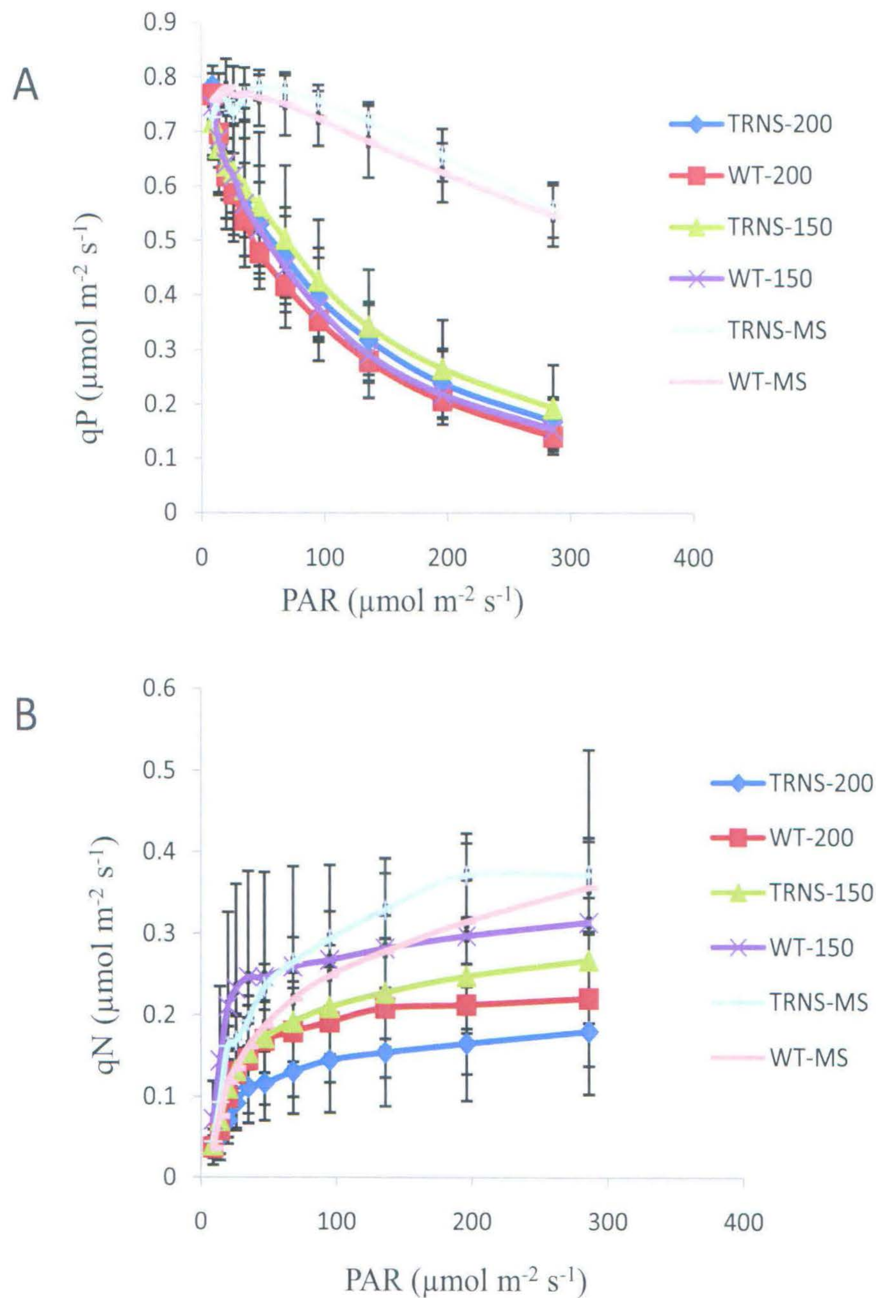


Figure 13. Pulse amplitude modulated (PAM) Chlorophyll a fluorescence measurements of WT and *PPDK* overexpressing plants. qP and qN of WT and PPDK plant after 10 days of salt treatment. WT and PPDK overexpressing plants were grown at 22^0C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 µmoles photons m^{-2} s^{-1}) for ten days in MS medium and then transferred to plates at different salt concentrations of 150, 200mM in MS medium for ten days and were used for studying various parameters of Chlorophyll a fluorescence measurement including **(A)**qP and **(B)**qN as described in "Material & Methods". .Each data point is an average of six replicates. Error bar represent SD.

WT plants increased with increasing light intensity. On MS media at saturating light intensity the ETR of WT and *mPPDK* plants were nearly similar. In 150 mM and 200 mM salt-treated plants the ETR of *mPPDK* was higher than WT by 16.9% and 5% respectively (Fig.12A).

4.6.2 Quantum yield of photosystem II (ΦPSII)

It measures the overall efficiency of PSII reaction centre in light. The quantum yield of PSII decreased in response to photosynthetic active radiation (PAR) (μmoles photons $m^{-2} s^{-1}$) (Fig.12B). On MS media quantum yield in *mPPDKx* plants was similar to WT. However, at 150mM and 200mM salt concentration *mPPDKx* plants was 23% and 3.5% higher than WT respectively (Fig.12B).

4.6.3 Photochemical quenching (qP)

It is the quenching parameter that represents coefficient of photochemical quenching. It is a measure of the fraction of still open PSII reaction centers. On MS media qP in *mPPDKx* plant was almost similar to that of WT. Due to 150mM and 200mM salt treatment the qP of *mPPDK*x plants was 26% and 19% higher than WT (Fig.13A).

4.6.4 Non-Photochemical quenching (qN)

The qN is a measure of heat dissipation and a combined total for the combination of photo-protective mechanisms, state 1 and state 2 transitions quenching, and photo-inhibition and photo-damage. The qN increased in response to higher light intensities (Fig.13B). Due to 150mM and 200mM salt treatment qN of mPPDKx plants was 17.2% and 22% lower than WT respectively (Fig.13B).

4.7 RICE RESULTS

4.7.1 Cloning of maize PPDK in pCAMBIA vector

Chloroplastic isoform of maize *ppdk* gene was amplified from cDNA preparation of *zea maize* by polymerase chain reaction (PCR) using gene specific forward and reverse primers. PCR fragment was cloned in pGEM-T Easy vector by AT ligation using T4 DNA ligase. pGEM-T Easy-*Zmppdk* recombinant vector was transformed into DH5α competent cells. Colony PCR was done to select positive colonies. All the above work had done previously in the laboratory.

pCAMBIA 1304 plant transformation vector containing hygromycin (*hpt*) gene and CaMV 35S-Ω-enhancer cassette was used for *Agrobacterium*-mediated plant

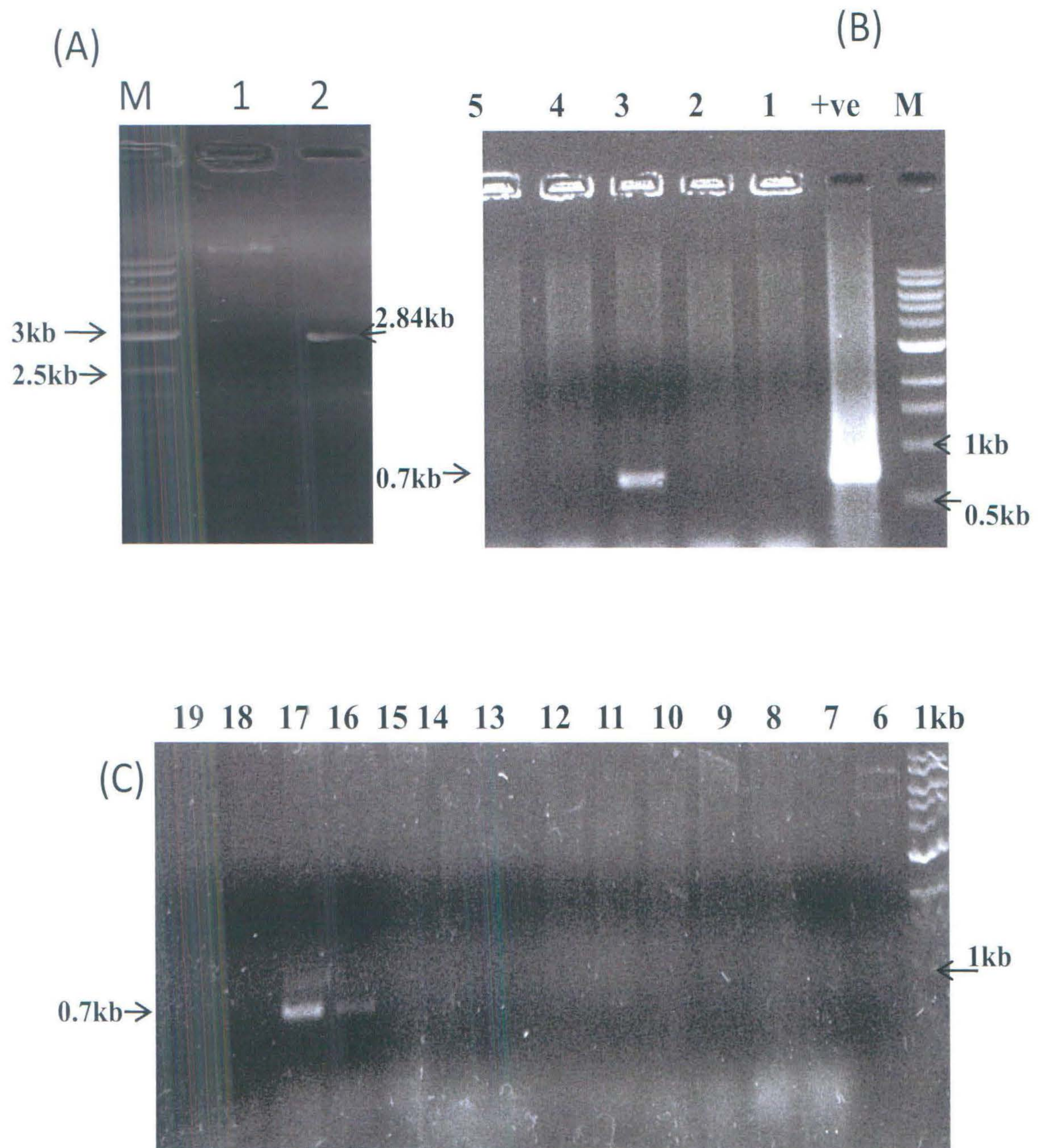


Figure 15. Cloning of *Zmppdk* to modified pCAMBIA 1304 vector having CaMV 35S-Ω-poly A promoter cassette and *hpt* gene. (A) *Kpn1* digestion of pGEM-T Easy having *Zmppdk* gene and modified pCAMBIA 1304 vector having CaMV 35S-Ω-polyA cassette and *hpt* gene.The digestion resulted in excision of *Zmppdk* gene from pGEM-T Easy vector. Lane1-*Kpn1* digested linear modified pCAMBIA 1304, lane 2-*Kpn1* digested ZmPPDK (B) & (C) PCR amplification of *ZmPPDK* gene from colonies of *E.coli* transformed with modified pCAMBIA 1304 vector with gene specific internal forward and reverse primers.

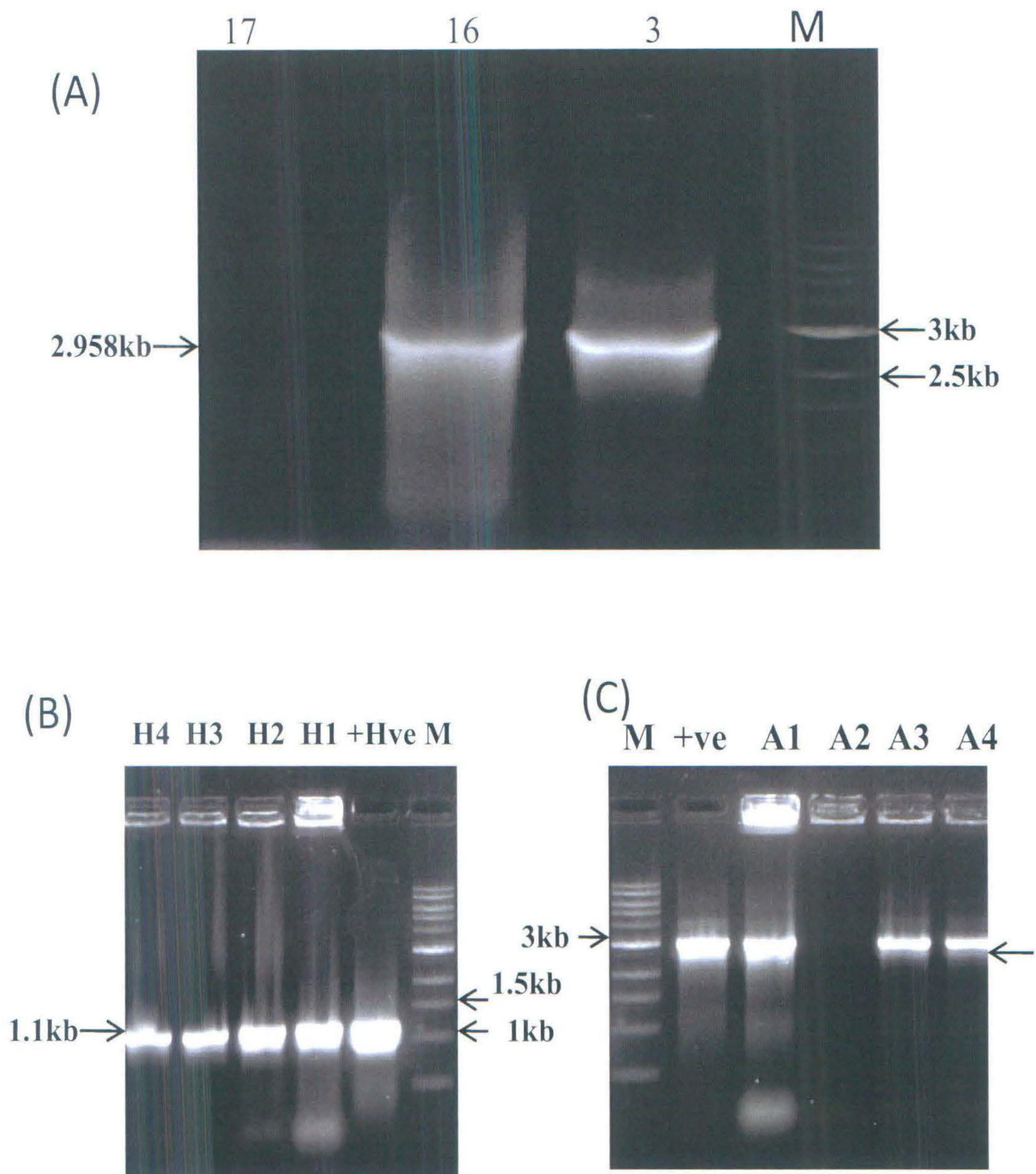


Figure 16. Confirmation of sense orientation of *ZmPPDK* in binary vector pCAMBIA 1304 and *Agrobacterium* colony PCR. (A) orientation check of *ZmPPDK* in pCAMBIA 1304 in *E.coli* by colony PCR with 35 S Internal forward and gene specific reverse primer (B) *hpt* gene confirmation in *Agrobacterium* by colony PCR with 35 S Internal forward and *hpt* specific reverse primer.(C) *ZmPPDK* Colony PCR confirmation of *ZmPPDK* sense construct in *Agrobacterium tumefacience.*

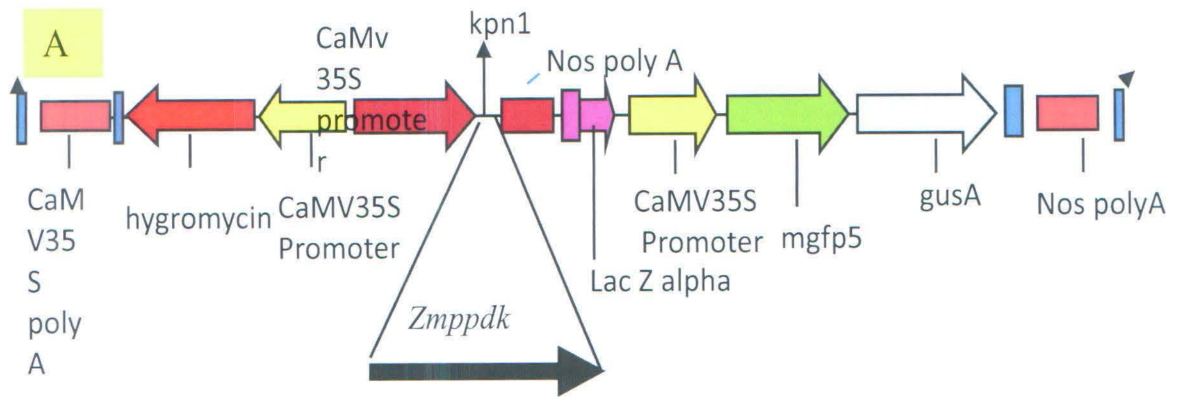
transformation. Cloning of *Zmppdk* cDNA in pCAMBIA 1304 was done by taking out the *PPDK* fragment form recombinant pGEM-T Easy vector (pGEM-T Easy-*Zmppdk*) by digesting with the *kpn1* restriction enzyme and ligating it with *kpn1* digested linearized pCAMBIA 1304 plant transformation vector (Fig.15A). Recombinant pCAMBIA 1304-*Zmppdk* was transformed into DH5α competent cells. Positive colonies were screened by colony PCR using *Zmppdk* gene specific internal primers (Fig. 15B-C). It was a non-directional cloning; both sense and antisense constructs were obtained after transformation. Orientation check for cloning of *Zmppdk* in pCAMBIA 1304 sense construct was done by PCR using 35S internal forward primer and gene specific reverse primer (Fig. 16A). In Figure 16A, the orientation of *Zmppdk* with respect to 35S promoter and sense positive colonies were used for plasmid isolation.

4.7.2 Transformation of *Agrobacterium* with pCAMBIA-PPDK

Transformation of competent *Agrobacterium* (LBA4404) with pCAMBIA-*Zmppdk* plasmid construct was carried out by mixing 10 μg of DNA followed by immediate freezing in liquid nitrogen. Subsequently, cells were thawed by incubating the eppendorf tubes at 37^0C for 5 min. One ml of YEP medium was added to the tube and incubated at 28^0C for 3 h. Cells were spread on a YEP agar plate supplemented with 50 μg/ml Kanamycin, 10 μg/ml Rifampicin and 25 μg/ml streptomycin and incubated at 28^0C. Transformed colonies that appeared in 2 days were analyzed by colony PCR. In colony PCR, *Zmppdk* was confirmed by using 35S internal primer and PPDK gene reverse primer(Fig.16C), while hygromycin was confirmed by using 35S internal primer and hygromycin gene reverse primer (Fig. 16B).

4.7.3 Embryogenic callus development from rice

Mature seeds of Indica rice cultivar (IR-64) were dehusked manually. Dehusked seeds were sterilized inside the laminar air flow hood as described in "Material and Method". Sterilized seeds were spreaded on autoclaved Whatman paper to soak extra water from seed. 14-15 seeds were inoculated in petriplate containing rice callus induction media (RCIM) (Fig.17B) and kept in dark inside culture room at 27-28^0C. After 14 days highly embryogenic calli were developed on RCIM plate (Fig.17C). Only embrygenic calli were selected and further cut into three equal parts and transferred onto RCIM media for next 4 days before transformation with *Agrobacterium*. During this period



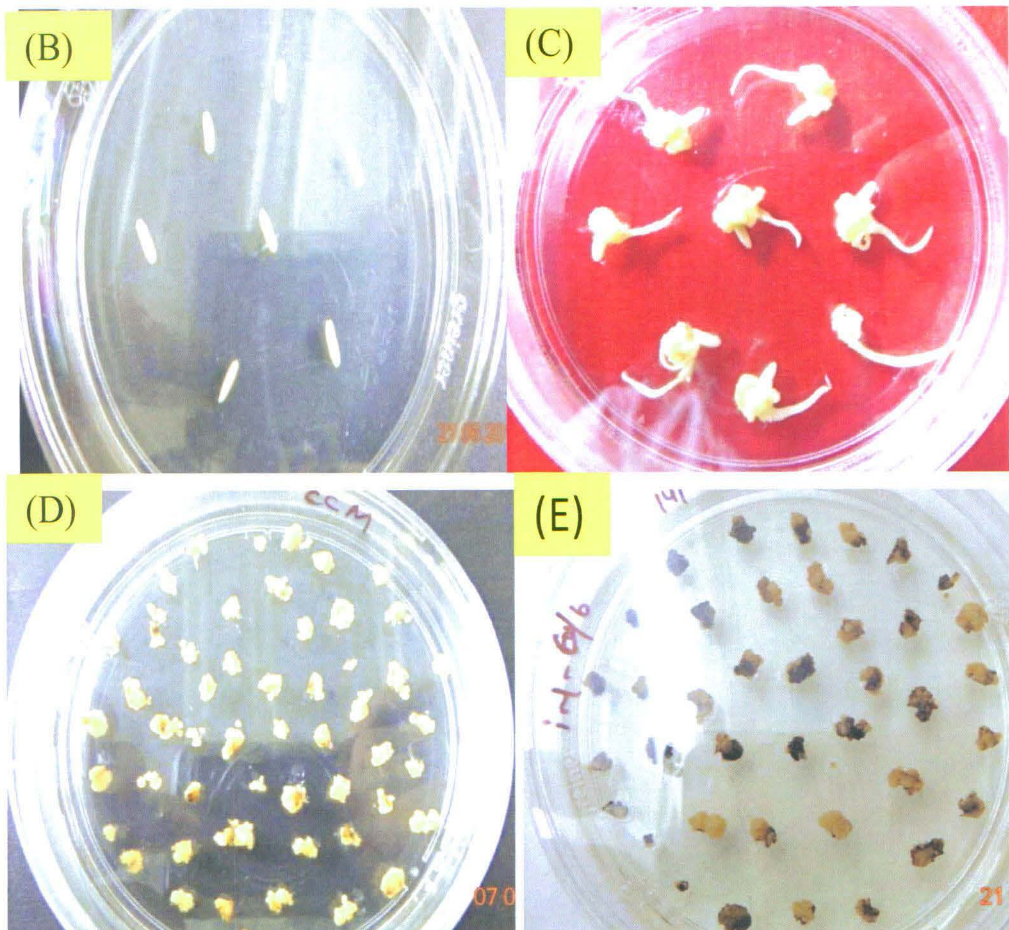


Figure 17. Generation of *Zmppdk* overexpressing *Oryza sativa* cv IR64 (*Zmppdkx*) plants. (A) Schematic representation of pCAMBIA 1304 vector with *zmPPDK* gene in sense orientation (B) Rice plating was done on Rice callus induction media (RCIM). The plates were kept in dark for fifteen days at 26ºC for the embryogenic calli to appear.. (C) Embryonic callus developing from rice embryo (D) Transformed callus on Co-cultivation media. The calli were kept in dark for two days at 26ºC as described in 'Materials & Methods' (E) Transformed callus on selection media I (SM-I). The media preparation and transformation of the calli was done as described in 'Materials & Methods' .

globular embryos were developed and calli appeard friable, which were choosen for transformation with *Agrobacterium tumefacian* LBA4404 having the transgene.

4.7.4 Preparation of *Agrobacterium* culture

Primary culture of *Agrobacterium tumefacian* (LBA4404) containing desired construct was prepared by inoculating bacteria with the help of sterilized loop from 3-4 days old bacteria culture plate in 5 ml autoclaved liquid YEP supplemented 50 μg/ml Kanamycin, 10 μg/ml Rifampicin and 25 μg/ml Streptomycin in a sterile glass tube. The culture tube was kept for 16-20 h on rotatry shekar at 220 rpm in dark at 28^0C. The secondary culture was prepared in 100 ml liquid YEP in 500ml conical flask (supplemented with same antibiotic as in primary culture) by adding 0.4% of primary culture and grown for 12 h at similar condition. When OD_{600} of secondary culture reached 0.6-0.8, then were centrifuged and resuspended in resuspention media to adjust OD_{600} 0.6 as described in "Material and Method".

4.7.5 Transformation of rice callus

Highly embryogenic calli were collected from RCIM plate in sterilized conical flask. Callus transformation was carried out by transferring the resuspended *Agrobacterium* culture in flask containg calli for 18-20 min in a shaker at 110 rpm. Infected calli were transferred on an autoclaved Whatman paper and dried. After drying, infected calli were transferred on co-cultivation media (CCM) and incubated at 28°C in dark for 48 h (Fig.17D). After 2 days on CCM, *Agrobacterium* growth was appeared in the periphery of callus.

4.7.6 Callus on selection media

After 2 days on CCM enough growth of *Agrobacteria* were appeared around periphery of calli. The infected calli were washed 6-8 times with autoclaved double distilled water (DDW) followed by washed with 250mg/l cefotoxim for 15 min. Washed calli were dried on autoclaved Whatman paper and were transferred on selection media-I (SM-I) for 10 days in dark at 28^0C (Fig.17E). After first selection media some of callus or parts of callus had become blacked, blacked part/callus was removed and transferred on SM-II for next 10 days and incubated as SM-I (Fig.18A). After 2nd selection brown/black calli were removed and only creamish color microcalli were transferred on SM-III for next 10 days in dark at 28^0C to allow to proliferation (Fig. 18B).

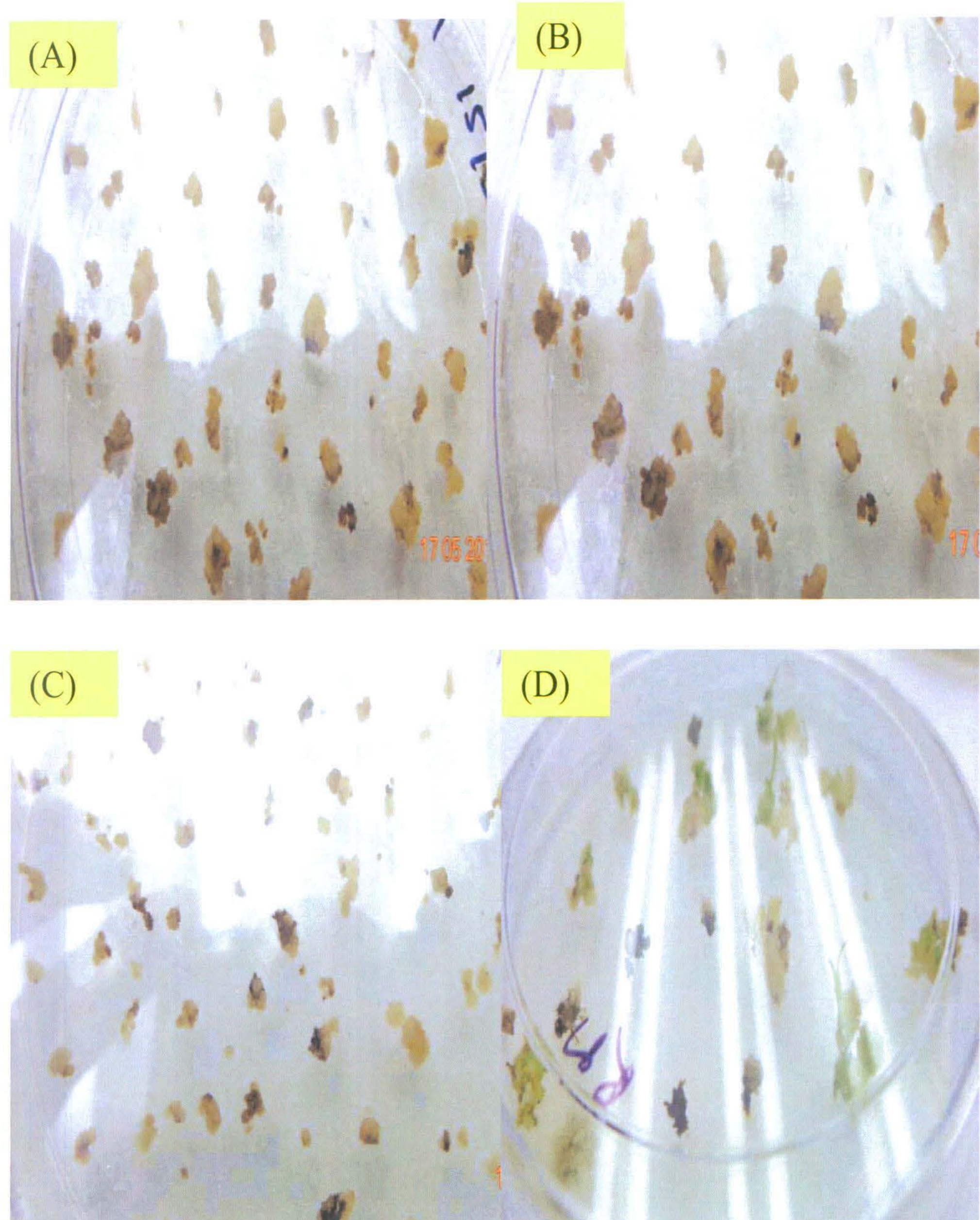


Figure 18. Generation of *Zmppdk* overexpressing *Oryza sativa* cv IR64 (*Zmppdkx)* plants contd... The calli on Selection media and Regeneration media (A) The calli on Selection media (SM-II) were kept in dark at 26°C for ten days.(B) The calli on Selection media (SM-III) were kept in dark at 26°C for ten days.(C) The calli on Regeneration media I (RM-I)(D) The plantlets regenerated on RM-II. The media preparation and transfer of the calli was done as described in 'Materials & Methods'.

4.7.7 Regeneration of plant on regeneration media

After 3rd selection brown/black microcalli were removed and only white and granular macrocalli were transferred on first regeneration media (RM-I) and incubated in culture room in dark at 28^0C for 8-10 days (Fig. 18C). After 8-10 days on RM-I hairy structure were developed on macrocalli then transferred in light condition as 16 h L/8 h D at 28^oC. After 2-3 days it appeared greenish color in macrocalli. The greenish calli were again transferred on RM-II at same light and temperature condition up to development of shoot from callus (Fig. 18D). After 4 weeks shoot growth touched the cover of plate, these were transferred to rooting media (RRM) (Fig. 19A). For hardening the rooted plants were transferred in pots containing vermiculite and covered with poly bag for 7 days at 28^oC 16 h L and 8 h D. After 7 days poly bag were removed and kept in green house for next 7-days in normal growth condition (Fig. 19B). After hardening plants were transferred in soil pots and kept at normal growth condition.

4.7.8 Screening of transgenic plant through PCR

Hygromycin resistant *PPDK* over-expressing rice transgenic lines were taken and genomic DNA was isolated. Using the 35S internal forward primer and gene specific reverse primer, the genomic DNA from 7 individual lines of T0 generation was amplified by PCR. The PCR condition was denaturation at 94^0C for 30 sec, annealing at 59^0C for 30 sec and extension at 72^0C for 3 min for each cycle and continued for 32 cycles. PCR products were analyzed by 0.8 % agarose gel run with standard 1 kb ladder and get 2.958 kb amplification size of sense orientation of *Zmppdk* from 3 transgenic lines (Fig. 20A). For hygromycin using 35S forward primer and hygromycin reverse primer and PCR condition for amplification was denaturation at 94^0C for 30 sec, annealing at 58^0C for 30 sec and extension at 72^0C for 60 sec for each cycle and continued for 32 cycles. PCR product analyzed by 0.8 % agarose gel run with standard 1 kb ladder and get 1.14 kb amplification size of sense orientation of hygromycin from 3 transgenic lines (Fig.20B). pCAMBIA recombinant plasmid containing the *Zmppdk* cDNA and hygromycin was used as positive control and the genomic DNA of WT plants as negative control.

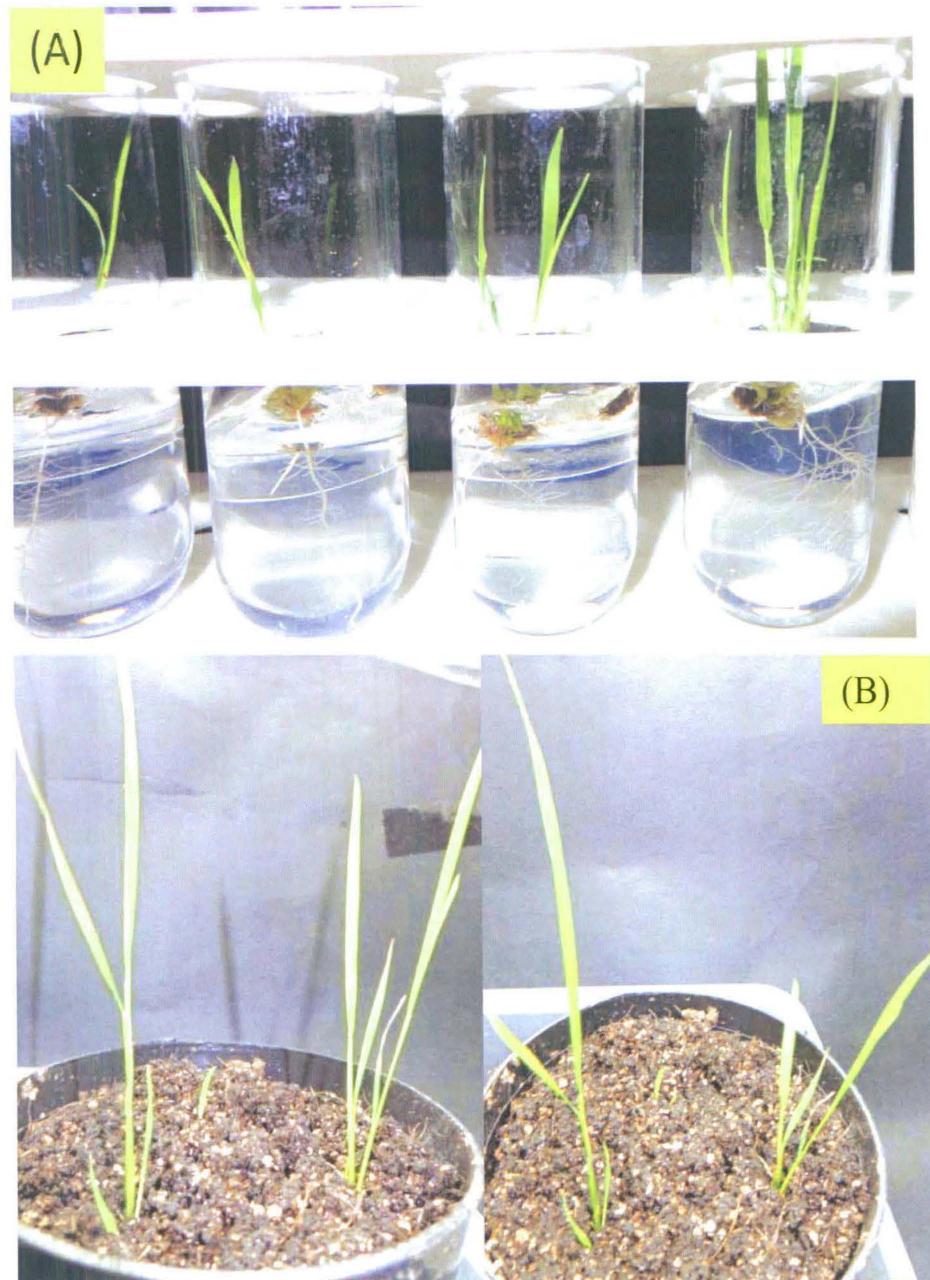


Figure 19. Generation of *Zmppdk* overexpressing *Oryza sativa* cv IR64 (*Zmppdkx)* plants contd…Root development on Regeneration media after transfer from RM-II and plantlet on agropet soil.(A) The plantlet on Root regeneration media (RRM) (B) T0 generation *Zmppdk Oryza sativa* transgenic plants on agropeat soil.The plants were kept for hardening on the soil for one week under under 16h L/8h D photoperiod in cool-white-fluorescent light (100 μmoles photons m^{-2} s^{-1}) and subsequently transferred to clay soil in the greenhouse for furthur growth and analysis.

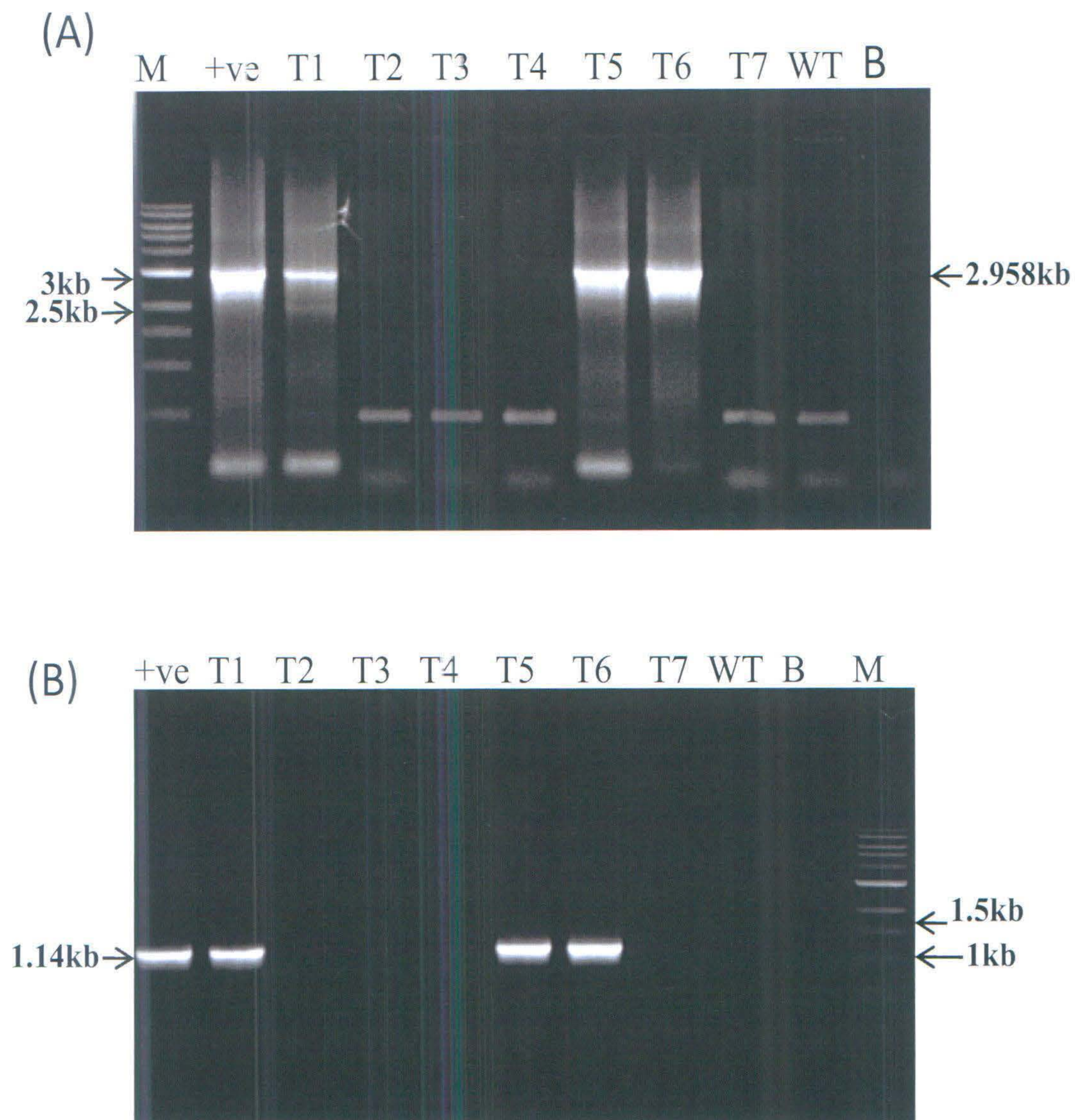


Figure 20. Confirmation of *Zmppdk containing* transgenic rice (sense) plants.

(A) PCR reaction was performed with genomic DNA isolated from different sense transgenic lines of T0 generation plants by using 35 S internal forward primer and gene specific reverse primer that yielded a fragment size of 2.958 kb integrated in sense orientation (T1-T7).

(B) Genomic DNA of WT and T0 generation *Zmppdk* sense plants, having resistance to Hygromycin were used as template and PCR of *hpt* gene was done by using Hygromycin (*hpt*) primers. A fragment of 1.14 kb corresponding to *hpt* was amplified that confirmed the integration of the T-DNA cassette with the host *Oryza sativa* genome.

Modified pCAMBIA 1304 vector containing the gene *Zmppdk* was taken as positive control.

DISCUSSION

5 DISCUSSION

Pyruvate orthophosphate dikinase (PPDK) is a key enzyme forming primary CO_2 acceptor phosphoenol pyruvate (PEP) for C_4 photosynthesis in mesophyll cells of C_4 plant and CAM plant. This is found not only in CAM and C_4 plants but also present in C_3 plants, green algae bacteria, fungi and protozoa. PPDK catalyzes the reaction of ATP, Pi and pyruvate to synthesize AMP, PPi and phosphoenol pyruvate (PEP). In C_3 and C_4 plants it has two isoforms which present in cytoplasm and chloroplast. In C_4 plants chloroplastic isoform plays a very important role in C4 photosynthesis by regenerating PEP from pyruvate. The cytosolic isoforms of PPDK in C_3 and C_4 plants are involved in non-photosynthetic metabolism such as starch accumulation in seed, nitrogen assimilation in leaves during senescence and in shikimate pathway and ATP supply.

The cytosolic form of PPDK expression increases in response to salt in roots (Moons et al., 1998). Therefore, it is of interest to ascertain if increased PPDK expression in the cytosol could protect plants from salinity stress. The transit peptide of maize *ppdk* was removed and overexpressed in *Arabidopsis thaliana* for targeting the protein to cytosol instead of chloroplast. Plants overexpressing maize chloroplastic *ppdk* targeted to cytosol were taken to study if these plants would be tolerant to salt stress.

Generally, salinity stress results in reduction of turgor pressure, reduction in photosynthetic activity and its accumulation in cell affect the metabolic activities, molecular responses and oxidative stress (Younis et al. 2003; Hassan and Nemat Alla 2005; Duan et al. ity and 2007; Zushi and Matsuzoe 2009; Yang et al. 2009). The results demonstrate that the salt affect plant growth in both WT and transgenic *Arabidopsis* plants overexpressing maize chloroplastic *ppdk* in the cytosol. However, in the presence of 150-200 mM NaCl, transgenic plants had higher dry weight as compared to WT. This was due to increased photosynthetic performance of transgenic plants treated with salt. The ØPSII, the electron transport rate (ETR) were higher in salt-treated *mPPDKx* transgenics than in WT (Fig.12A-B). In the same vein the non-photochemical quenching of salt-treated transgenic plants were lower than that in WT (Fig.13A-B). These demonstrate that the plants overexpressing maize chloroplastic ppdk in the cytosol were more tolerant to salt stress. In salt stress proline is most common osmolyte produced in plants and protects sub-cellular structures and the

macromolecule (Kishor et al., 2005). Proline act as a molecular chaperone and protects protein integrity and enhance the activities of different enzymes. Proline also protects the photosystem I and II through capturing of ROS and singlet oxygen (Alia et al., 1997). The higher proline content of salt-treated transgenic plants could protect them from salinity stress. The plastidic form of pyuvate orthophosphate dikinase is known for its role in C_4 photosynthesis. However, its role in cytosol is not studied. Our results suggest that increased expression maize *ppdk* in the cytosol of Arabidopsis could protect plants from environmental stress.

For increased plant productivity and grain yield, there is a global initiative to generate C4 rice. The C4 photosynthetic genes need to be engineered in rice to convert the C3 rice to a C4 plant. In the present study chloroplastic full length maize *ppdk* hooked to 35S promoter was overexpressed in rice towards raising C4 rice.

Results demonstrate that rice plants (IR 64) are successfully transformed with chloroplastic full length maize PPDK under the control of 35S promoter. Transgenic plants were confirmed by PCR of rice genomic DNA with primer sets of 35S forward and *ppdk* gene reverse that amplified and yielded 2.958 kb PCR product. The integration of the transgene to rice genome remains to be confirmed by Southern blot.

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