IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 2, Issue 7 (Nov. – Dec. 2016), PP 64-72
www.iosrjournals.org

Overexpression of ProtochlorophyllideOxidoreductase C (Por C) in Oryza Sativa Var. IR64

Ritu Kumari Singh
Jawaharlal Nehru University, New Delhi, India

Abstract: *India accounts for a total 20% of world rice production. However a significant amount of rice crop is damaged by high incoming irradiance. Thus photo-protective responses are induced in the plant even under otherwise ideal conditions. Further the stress response can greatly escalate if other abiotic factors such as water and nutrients are also limiting. Thus Quantum yield of photosynthesis is greatly reduced under prolonged exposure (hours or days) to high irradiance. It causes damage to the primary photosynthetic reactions and subsequently a reduction in the photosynthetic rate of the crop. It has been observed in rice plants in the dry season even under well-watered, optimal conditions.Therefore the main objective to engineer the transgenic Protochlorophyllide overexpressing rice plants is not only to protect the plant from excessive irradiation but also to maintain the chlorophyll content during the high light conditions. This would help to improve both the food quality, yield and recover the decreased chlorophyll molecules in rice induced by exposure to high light intensity.*

Keywords:*Chlorophyll molecules, irradiation, photo-protective, transgenic protochlorophyllide, quantum yield*

I. Introduction

Rice is the staple food for half of the world's population and 90% of the world's rice is grown and consumed in Asia. This rice production is important to the food security of the world. The world's population is projected by FAO to rise from the current 6.5 billion to 9 billion in the next 40-50 years, with most of the population increases in rise consuming countries. Thus, rice yield must beincreasedbyatleast 50% over the next 40 years to preventmalnutritionfor the 700 million Asianthat currently rely on rice form ore than 60% of their daily calorificintake. After the successful application of the hybridrice technology in recent years, rice yields have been sub stantiallyimprovedandt he wideadoption of the technology will further increasethe global rice production. However, hybrid rice technology alone cannot meet the demands in the long run.Furthermore, traditionalbreedingapproachistime-consumingandineffectiveinsolvingthefoodshortageproblems.Incontrast, molecularbreeding, atechnology combining geneticengineeringandtraditionalbreeding, willbea power ful and effective approach to overcome the food productionproblems.Theoreticalanalysishassuggested that they ieldincreasesrequired to match the projectedpopulationgrowthcanonlybeachievedbyincreasingthe efficiency withwhich photos ynthesisusessolarenergy.

NADPH: protochlorophyllideoxidoreductase (POR) catalyses the light-dependent reduction of protochlorophyllide to chlorophyllide, a key regulatory reaction in the chlorophyll biosynthetic pathway. POR from the cyanobacteriumSynechocystis has been overproduced in Escherichia coli with a hexahistidine tag at the N-terminus. This enzyme (His6-POR) has been purified to homogeneity and a preliminary characterization of its kinetic and substrate binding properties is presented. Chemical modification experiments have been used to demonstrate inhibition of POR activity by the thiol-specific reagent N-ethyl maleimide. Substrate protection experiments reveal that the modified Cys residues are involved in either substrate binding or catalysis (Derren et al., 2000).

NADPH:protochlorophyllideoxidoreductase (POR) catalyzes the light-dependent reduction of protochlorophyllide. To elucidate the physiological function of three differentially regulated POR isoforms (PORA, PORB and PORC) in *Arabidopsis thaliana*, we isolated T-DNA tagged null mutants of *porB*and*porC*. The mature seedlings of the mutants had normal photosynthetic competencies, showing that PORB and PORC are interchangeable and functionally redundant in developed plants. In etiolated seedlings, only *porB*showed a reduction in the photoactive protochlorophyllide and the size of prolamellar bodies (PLBs), indicating that PORB, as well as PORA, functioned in PLB assembly and photoactive protochlorophyllide formation in etiolated seedlings. When illuminated, the etiolated *porB* seedling was able to green to a similar extent as the wild type, whereas the greening was significantly reduced under low light conditions. During greening, high light irradiation increased the level of PORC protein, and the greening of *porC*was repressed under high light conditions. The *porB*, but not *porC*, etiolated seedling was more sensitive to the far-red block of greening than the wild type, which is caused by depletion of endogenous POR proteins resulting in photo-oxidative damage. These results suggest that, at the onset of greening, PLBs are important for efficient capture of light energy for photoconversion under various light conditions, and PORC, which is induced by high light irradiation,

contributes to photoprotection during greening of the etiolated seedlings (Masuda et al. 2003).A homology model of NADPH: protochlorophyllide (Pchlide) oxidoreductase A (POR; E.C. 1.3.33.1) of barley is developed and verified by site-directed mutagenesis. PORA is considered a globular protein consisting of nine -helices and seven _-strands. The model predicts the presence of two functionally distinctive Pchlide binding sites where the pigment is coordinated by cystein residues. The pigment bound to the first, high-affinity Pchlide binding site is used for the formation of the photoactive state of the enzyme. The pigment bound to the second, low-affinity Pchlide binding site is involved in the PORA: PORB interaction, allowing for resonance energy transfer between the neighboring PORs in the complex. In the *in vitro* reconstituted light-harvesting POR:Pchlide complex (LHPP), light absorbed by PORA-bound Pchlide*b*is transferred to PORB-bound Pchlide*a*. That induces the conversion of Pchlide*a*tochlorophyllide (Chlide) *a*. This energy transfer eliminates the possibility of Pchlide*b*photoreduction and prevents that excited triplet states of either Pchlides*a*or*b* accumulate and provoke singlet oxygen production. Together, our results provide a photoprotective role of PORA during greening (Buhr et al. 2008).To understand the impact of water stress on the greening process, water stress was applied to 6-day-old etiolated seedlings of a drought-sensitive cultivar of rice (*Oryzasativa*), Pusa Basmati-1 by immersing their roots in 40 mM polyethylene glycol (PEG) 6000 (-0.69 MPa) or 50 mM PEG 6000 (-1.03 MPa) dissolved in half-strength Murashige and Skoog (MS)-nutrient-solution, 16 h prior to transfer to cool-white-fluorescent + incandescent light. Chlorophyll (Chl) accumulation substantially declined in developing water-stressed seedlings. Reduced Chl synthesis was due to decreased accumulation of chlorophyll biosynthetic intermediates, that is, glutamate-1-semialdehyde (GSA), 5-aminolevulinic acid, Mg-protoporphyrin IX monomethylester and protochlorophyllide. Although 5-aminolevulinic acid synthesis decreased, the gene expression and protein abundance of the enzyme responsible for its synthesis,GSA aminotransferase, increased, suggesting its crucial role in the greening process in stressful environment. The biochemical activities of Chl biosynthetic enzymes, that is, 5-aminolevulinic acid dehydratase, porphobilinogendeaminase, coproporphyrinogen III oxidase, porphyrinogen IX oxidase,Mg-chelatase and protochlorophyllideoxidoreductase, were down-regulated due to their reduced protein abundance/gene expression in water-stressed seedlings. Down-regulation of protochlorophyllideoxidoreductase resulted in impaired Shibata shift. Our results demonstrate that reduced synthesis of early intermediates, that is, GSA and 5-aminolevulinic acid, could modulate the gene expression of later enzymes of Chl biosynthesis pathway.(Dalal and Tripathy, 2012).

NADPH:protochlorophyllideoxidoreductase (POR) catalyzes photoreduction of protochlorophyllide (Pchlide) to chlorophyllide in chlorophyll (Chl) synthesis, and is required for prolamellar body (PLB) formation in etioplasts. Rice faded green leaf (fgl) mutants develop yellow/white leaf variegation and necrotic lesions during leaf elongation in field-grown plants. Map-based cloning revealed that FGL encodes OsPORB, one of two rice POR isoforms. In fgl, etiolated seedlings contained smaller PLBs in etioplasts, and lower levels of total and photoactive Pchlide. Under constant or high light (HL) conditions, newly emerging green leaves rapidly turned yellow and formed lesions. Increased levels of non-photoactive Pchlide, which acts as a photosensitizer, may cause reactive oxygen accumulation and lesion formation. OsPORA expression is repressed by light and OsPORB expression is regulated in a circadian rhythm in short-day conditions. OsPORA was expressed at high levels in developing leaves and decreased dramatically in fully mature leaves, whereas OsPORB expression was relatively constant throughout leaf development, similar to expression patterns of AtPORA and AtPORB in Arabidopsis. However, OsPORB expression is rapidly upregulated by HL treatment, similar to the fluence rate-dependent regulation of AtPORC. This suggests that OsPORB function is equivalent to both AtPORB and AtPORC functions. Our results demonstrate that OsPORB is essential for maintaining light-dependent Chl synthesis throughout leaf development, especially under HL conditions, whereas OsPORA mainly functsions in the early stages of leaf development. Developmentally and physiologically distinct roles of monocot OsPORs are discussed by comparing with those of dicot AtPORs (Sakuraba et al. 2013).

Geranylgeranylreductase (CHL P) catalyzes the reduction of geranylgeranyldiphosphate to phytyldiphosphate, and provides phytol for both Chlorophyll (Chl) and tocopherol synthesis. In this study, we isolated a yellow-green leaf mutant, 502ys, in rice (Oryza sativa). The mutant exhibited reduced level of Chls, arrested development of chloroplasts, and retarded growth rate. The phenotype of the 502ys mutant was controlled by a recessive mutation in a nuclear gene on the long arm of rice chromosome 2. Map-based cloning of the mutant resulted in the identification of an OsChl P gene (LOC_Os02g51080). In the 502ys mutant, a single base pair mutation was detected at residue 1279 in DNA sequence of the gene, resulting in an amino acid change (Gly-206 to Ser) in the encoded protein. HPLC analysis of Chls indicated that the majority of Chl molecules are conjugated with an unsaturated geranylgeraniol side chain, in addition to small amount of normal Chls in the mutant. Furthermore, the mutant phenotype was complemented by transformation with the wild-type gene. Therefore, this study has confirmed the 502ys mutant resulted from a single base pair mutation in OsChl P gene (Wang et al. 2014).

Gao et al. (2015) studied, a mutant which was identified and named as *ygl3* (*yellow green leaf3*) as the leaves of the mutant are yellowish green at seedling stage and turning to yellow/white from the leaf-tip area

during the late vegetative stages. The gene *YGL3* encoding OsPORB, a chloroplast protein, was isolated through map-based cloning and used to complement the *ygl3* mutation successfully. The expression pattern and the relationship between *OsPORA*and*OsPORB*, two *PORs* existed in rice, were then investigated. It was found from RT-PCR that expression of *OsPORB*was constitutive while the high level expression of *OsPORA*was occurred only in neonatal stems, leaves and spikes. The analysis of subcellular localization provided evidence that both OsPORA and OsPORB are chloroplast protein. The mutated phenotype of *ygl3* could be complemented by *OsPORA*driven by the 35S promoter. The inhibition for the expression of *OsPORA*was then conducted through RNAi for both wild type and *ygl3* plants, the same phenotypic characteristics was observed from the transgenic plants of wild type but not the ones of *ygl3*, illustrating that OsPORA and OsPORB are essential for chlorophyll synthesis. These results indicated that the function of OsPORA and OsPORB are redundant, OsPORB is more important, and the OsPORs are conservative during chlorophyll synthesis.

II. Material and Method

Chemicals and experiment

Various specific experimental materials, used in this study are given in Table All other chemicals used were of analytical grade. The antibiotics and their concentrations used are given in the Table.

Table 2. Concentrations of various antibiotics used:-

	MATERIAL	SOURCE
Plants	RICE Oryza Sativa var., IR64	IARI pusa
Plasmids	pGEM-TEasy, pCAMBIA 1304,	Promega, CAMBIA
Bacterial strains	Escherichiacoli(E.coli) DH5α, LBA4404	Novagen,
Markers	1kb ladder, λ Hind III	NEB,Fermentas,bANGALOREGenei
Restriction Enzymes	Enzymes in general use	Promega, NEB, Fermentas

- Ampicillin (Amp) -100µg/ml (working), (100mg/ml stock)
- Kanamycin (Km) - 50µg/ml (working), (50mg/ml stock)
- Rifampicine (Rf) - 25µg/ml (working), (25mg/ml stock)

Plant Material And Growth Conditionriceis Plants were grown on agropeat soil at 28'C under 100 µmol photon m^{-2} s^{-1} for 16h light/8h dark for 7 days after transfer from rooting media.General sterilization proceduresCulture media, glassware and tissue culture tools were sterilized by autoclaving at 121ºC and 151 p/inch2 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 □ m pore size (Mdi, India).

RNA extraction

Materials

- Chloroform(CHCl)
- Isopropanol
- Ethanol 70%
- TRI-reagent (trizole)

Method

Total RNA was isolated as described by Chomczynski and Sacchi (1987). All the glassware used during RNA isolation were baked overnight at 200°C, the plastic ware were treated with DEPC and then autoclaved to avoid the possible contamination of RNase.Leaves (100 mg) were taken and homogenized in liquid N_2 in mortar and pestle. 1ml trizole was added to the powdered leaves while the leaf powder was in liquid nitrogen. It was allowed to stand for 5min at room temperature. The trizole containing leaf powder was then transferred to polypropylene centrifuge tube. 0.2 ml of chloroform was added and vortexed(shake vigorously). It was allowed to stand for 5-15min at room temp .The mixture was centrifuged at 12,000 rpm for 15 minutes at 4°C. To the supernatant 0.5 ml volume of isopropanol was added and mixed properly and kept at room temperature for 5-10 minutes.This was then centrifuged at 12,000 rpm for 10 minutes at 4°C and the pellet was obtained. Then 1 ml of pellet was taken and washed with 75% ethanol. It was centrifuged at 7,500 rpm for 5min. RNA pellet was resuspended by dissolving it in 20-30µl (55-60◦C) DEPC treated water for 5min. It was stored at -80◦C.

CDNA Synthesis

Materials
- dNTP mixture
- RNase inhibitor
- OligodT
- Ribonuclease free water RNA
- RT-buffer
- RNA sample
- AMV reverse transcriptase

Method
Total RNA was extracted from leaf tissue. Sterile nuclease free microfuge tube was taken and 3µg (0.4µl) RNA, 1µl (conc.5pm /µl for a 50µl reaction) oligodT primer was added.
The following were added in the order given below:

RT-buffer (5X)	10 µl
dNTP mixture	2µl (10mM)
RNase inhibitor	0.5 µl (40 unit)
AMV reverse transcriptase	1.0 µl (30 unit)
Ribonuclease free water	50 µl (To make up)

(The above was incubated for 60 min at 37 ºC).
Termination step: The mixture was kept at 70ºC-72ºC for 10 min. Then it was kept on ice for 5-10 min. Finally it was checked on 0.8% agarose gel.

Polymerase chain reaction
Materials (with Taq polymerase)
- Taq Buffer10X (stock),1X(working)
- Taq polymerase5000U/ml(company),1-1.5U/rxn(used)
- dNTP100mM(company),1mM(stock),0.1mM/rxn(used)
- Primer F'& R'100pmole(stock),5pmole(working)

Method
The following component was added to an autoclaved micro centrifuge tube:

Taq Buffer	2µl
Taq polymerase	0.3µl
dNTP	3µl
Primer F'& R'	3µl (each)
Water(dH_2O)	10.7µl
DNA template	>1µl

The total mixture was made to 20µl reaction. The contents present in the tube were mixed well.
The micro- centrifuge tube was placed in the PCR machine under the following condition:

Step-1	Initial denaturation temp	95ºC (5min)
Step-2	Denaturation	95ºC (30-45sec)
Step-3	Primer annealing	55-60ºC (30-45sec)
Step-4	Extension	72ºC (Gene length dependent 500bp/30sec)
31 cycle from the Step-2		
Final extension		72ºC (5min)

The reaction was maintained at 4ºC after cycling. The sample can be stored at -20ºC until use. The product was analyzed by running it on 0.8% agarose gel & visualizing it by ethidium bromide staining.

Purification of DNA fragment from agarose gel
PCR amplified products or restriction enzyme digested plasmid was electrophoresed on 0.8 % agarose gel. The desired fragment was identified using standard molecular weight marker (1 kb ladder) and purified using the following techniques:
Materials
- Agarose
- Capture buffer
- 70%Ethanol (wash buffer)
- Gel loading dye (6X)-Bromophenol blue 0.25%
- Xylene0.25%
- Wash buffer (Amersham)

- Column

Method

0.8% agarose gel in 100ml of 1X TAE buffer was prepared and 4µl of ethidium bromide was added to it. The sample was loaded onto the wells and the gel was then run. The gel was observed on a UV-transilluminator. The gel piece containing DNA fragment was excised from the gel with a clean blade and weighed. Capture buffer was added to agarose gel according to the weight (For 100 mg of gel slice, 100 µl of capture buffer was added). This was incubated at 60°C for 15 min until the gel slice gets dissolved completely. The sample was applied to the quick column and 0.5ml of wash buffer (70%ethanol) was added to it. To elute DNA, it was then centrifuged (Hitachi) for 1 minute in Beckman microfuge for 13,000rpm. Then double distilled autoclaved water was poured into the column and the column was centrifuged (Hitachi) at 13000rpm for 1 minute. The flow through contained the DNA. The DNA sample was stored at -20^0C.

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 this 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 3000 X g for 10 min. The pellet was resuspended in 40 ml of ice cold 100 mM CaCl2 solution, incubated on ice for 1 h, centrifuged and the pellet was resuspended in 4 ml of ice cold 100 mM CaCl2 containing 15 % glycerol solution. The cell suspension (0.1ml) was aliquoted into autoclaved un-opened eppendorf tubes and stored at - 800℃. DNA fragments were ligated to the appropriate vectors by using T4 DNA ligase (Promega, USA) overnight at 4℃ or 16℃. The ligation mixture was added to the competent cells and mixed by tapping and then incubated for 30 min at 4℃. All steps were carried out in a laminar hood under sterile conditions. The cells were subjected to heat shock by incubating at 42℃ 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℃ with gentle shaking (185 rpm) for 1h. Different aliquots of these transformed competent cells were plated onto LB plate (10g NaCl, 10g Tryptone, 5 g yeast extract and 15g agar per 1L) containing appropriate antibiotic. The LB medium-containing agar was autoclaved. The filter-sterilized solution of heat labile antibiotics was added to the autoclaved medium pre- cooled to 45℃ and poured into sterile petridishes in a clean bench. For blue white selection, 10 µg IPTG and 1 µg X- gal per plate were spread prior to plating the cells. The plates were incubated overnight at 37℃. Transformed cells containing recombinant plasmid were confirmed by colony PCR and restriction digestion.

Isolation and purification of plasmid DNA

Materials

1. Solution

50 mM glucose
25 mMtrisHCl (pH 8.0)
10 mM EDTA (pH 8.0)

2. Solution 2(freshly prepared)

0.1M NaOH
1% SDS

3. Solution 3

60 mL 5 M potassium acetate (final concentration 3M)
1.5 mL Glacial acetic acid (Final concentration 5M)
28.5 mL dH_2O

Alkaline Lysis Method

Single colony was picked up using a sterile tip and cultured in 5ml LB medium containing antibiotic ampicillin (100 µg / ml) at 37°C for 16 hours. The 2 ml of culture was taken in microfuge tube. Cells were pulsed down (10,000rpm) for 5min. To the pellet, 150µl of ice-cold solution-1 was added and the solution was vortexed for 1-2min.Then 250µl of freshly made solution-2 was added. It was mixed by gentle inverting and rolling the tube to make sure all the bacterial slurry along the sides and cap are mixed with solution 2, incubated on ice until translucent. Then 200µl of solution-3 was added. Immediately vortexed and incubated on ice for 5-10 minutes. It was spun at 13000rpm for 12 minutes. The supernatant was taken using a sterile micro tip. Then 600µl isopropanol (ice chilled) was added to the supernatant. It was mixed by inverting several times and incubated on ice for 30 minutes. Then it was centrifuged at 13000 rpm for 12 minutes. After decanting the

supernatant the pellet was washed with 0.5ml 70% ice chilled ethanol .It was spun at 13000rpm for 10 minutes. The pellet was dried and was dissolved in 25-30 microlitres autoclaved water. Plasmid quality was checked with 0.8% agarose gel.

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 A260 = 50 µg/ml for DNA and 1.0 A260 = 40 µg for RNA. The purity of the nucleic acid was determined by calculating the ratio A260/ A280 for each sample.

Check Plasmid with gel electrophoresis

Materials
Agarose- 0.8%
TAE buffer-50X(stock),1X(used)
For 50X- (242gm tris base,57.1ml glacial acetic acid,100ml of 0.5M EDTA in 1lit.)
Gel loading dye - 6X(Bromophenol blue 0.25%w/v, Xylene cyanol,0.25 w/v, Glycerol 30%w
Ethidium bromide-10mg/ml(stock)
Method
0.8% agarose gel in 100 ml 1X TAE was prepared and 2µl of ethidium bromide was added to it. The sample was loaded onto the wells and the gel was run. The gel was observed on a UV-transilluminator.
RESTRICTION DIGESTION

Enzyme kpnI	0.2 µL(10,000U/ml)
NEB buffer	2µl
BSA	0.5µl
Plasmid	7µl
Water	10.3µl20µl

The solution mixture is then kept at 37º for 2½ hours for restriction digestion .The restriction digestion was checked on agarose gel.

Method

0.8% agarose gel in 100ml of 1X TAE was prepared and 3µl of ethidium bromide was added to it. The sample was loaded onto the wells and the gel was then run. After the run was over, the gel was observed on a UV-transilluminator.Pink fluorescence bands on the gel was observed under UV-transilluminator.

Amplification of AtPORCand its ligation to pGEM-T Easy

For the amplification of aAtPORCcDNA fragment (915bp), cDNA library of A thaliana was used. PCR was amplified with a pair of primers: forward primer 5'-cg ggatcctctagaatggcagcaaccttacctcta -3' and reverse primer 5'-gcgtcgactctagactaatatccgaa act taaata -3' (XbaI restriction sites were introduced at the 5'-end of the forward primer and the reverse primer), designed based on the cDNA sequence (Accession no. AT5G04490) available in the Gene Bank. PCR was done in 20 µl volume containing 20 ng of template DNA, 15 pmoles of each primer, 200 µM of dNTPs, 4 units of taq polymerase in buffer containing 10 mMTris-HCl pH 8.3, 50 mMKCl, 15 mM MgCl2. PCR amplification was done with a program having 940C for 2 minutes (initial denaturation) followed by 30 cycles of 940C (denaturation) for 30 seconds, 560C (annealing) for 30 seconds, 720C (extension) for 1 minute in a Perkin-Elmer (USA) thermal cycler. The resulting amplification products were gel purified and the purified fragment was ligated to pGEM-T Easy (Promega, USA) vector. The recombinant plasmid (pGEM-T Easy-PORC) was transformed into competent E.coli(DH5α) cells. Plasmid DNA was prepared and the nucleotide sequence of the AtPORCwas confirmed by sequencing using standard procedure.

Cloning of AtPORCin modified plant transformation vector

Plasmid of the modified pCAMBIA 1304 containing the kanamycin (npt) gene and CaMV 35S omega enhancer cassette was digested by XbaI and dephosphorylated. The AtPORCcDNA was taken out from pGEM-T Easy cloned AtPORC(pGEM-T Easy-AtPORC) recombinant plasmid. The recombinant plasmids were digested with XbaI enzyme that resulted in the removal of the AtPORCcDNA from pGEM-T Easy vector. AtPORCcDNA was ligated to the above dephosphorylated modified pCAMBIA 1304 vector containing the kanamycin (npt) gene and CaMV 35S omega enhancer cassette. After ligation and transformation, plasmids were isolated from the recombinant colonies and were digested by XbaI enzyme to check integration of the AtPORCcDNA in to modifiedpCAMBIA vector.

Transformation and Regeneration of Indica Rice Ir64

Rcim Plates Preparation
gm/l

MS	4.42
Proline	0.6
Casein	0.3
Maltose	30
2-4,D	250µl
PH	5.8
Phytagel	4

The medium was prepared and the PH was adjusted to 5.8 using 1M NaOH prior to the addition of phytagel and then autoclaved. Medium was precooled at 45ºC and poured into sterile petridishes into 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.

Yep Preparation
gm/l

Peptone	10
Yeast Extract	10
NaCl	5
PH	7.0

Ccm Preparation
gm/l

MS salt	4.4
Maltose	3
Glucose	10
2,4-D	250µl
BAP	10µl
PH	5.2
Phytagel	3

Protocol for transformation

- Healthy, mature seeds were dehusked.
- Seeds were surface sterilized with double distilled water and then tween20 was added just one drop. Ten seeds were washed with double distilled water by shaking and then washed with 70% ethanol for 2min in swinging bucket style, 4% NaOCl used to washed seeds for 5min and then washed with HgCl2 for 15min and then seeds were washed with double distilled water 7-8 times to remove all chemicals properly and placed on whatsman paper to dry the seed for 1h.All the above process was done inside the laminar hood.
- Seeds were inoculate on RCIM plates for callus induction.
- After 14days callus were grown, cut into small pieces and subculture on RCIM.
- After 4 days callus were infected by Agrobacterium for 20-25min.(Primary culture of Agrobacterium in 5ml YEP. After 24 h secondary culture of Agrobacterium in 100ml YEP. After 8-10h culture pellet down at 8000Xg for 10min and resuspend in MS liquid medium)
- After infection of 20-25min callus were inoculated on CCM.
- After 2days callus were washed with cefotaxime.

And then transfer for selection, regeneration and to soil for hard

III. Conclusion

The Total genomic DNA from various independent transgenic lines would be extracted analyzed by PCR using AtPORC forward and reverse primers. The PCR products would be analyzed on 1% agarose gel containing ethidium bromide and visualized under UV light. Finally the PCR-positive plants would be analyzed by Southern blot analysis for the integration of At*PORC*gene in the rice genome. Various transformation protocols are used to introduce foreign gene into rice to generate the transgenic plants. These include particle gun delivery, direct gene transfer by chemical methods, electroporation and *Agrobacterium* mediated transfer. Agrobacterium mediated transfer could be through *inplanta* transformation or callus mediated routes. In the present study Agrobacterium mediated transformation of *Oryza sativa IR64*calli was used to generate *Oryza sativa IR*64 transgenic lines. Various studies have shown that rice varieties like japonica are easy to transform and regenerate. Howerev IR64 is a recalcitrant variety which is very difficult to regenerate. At times from a single transformation experiment with 500 calli I could obtain only one or two positive transformants which may or maynot survive the regeneration phase. It took about 6 months from the initiation of callus to the transfer of regenerated plants to soil. Although different methods are available for the agrobacterium mediated transformation in rice.Th e but agrobacterium mediated transformation by Pareek at al is the best protocol till date to generate stable transgenics in rice. Using the above protocol transgenic rice plants were generated in a

very short time with a transformation frequency of 30–50%. Rice seeds were used as the explants as they are available throughout the year and require no special storage conditions.There are a number of critical factors necessary for successful transformation by Agrobacterium especially in rice varities like IR64. Firstly it is imperative to choose actively growing, embryogeniccalli for transformation. Though all calli are sources of cells for the production of transgenic rice slow grownngcalli are not easily transformed and have a very poor regeneration rate. Thus only healthy and actively growingcalli should be selected (based on their appearance) for transformation. Transformation efficiencies were dramatically different with different batches of calli in many transformation experiments in my studies (ranging from 10% to 40%). Another factor that affects the transformation efficiency is the temperature during the co-cultivarionphase. The optimum temperature should be between 26°C to 22°C during the cocultivation phase for successful transformation efficiency.Regeneration of the transformed calli was a major obstacle in transformation of IR64. It took 25 to 30 days for the callus to generate the shoots . The shoots took 15 to 25 days to regenerate in the rooting medium. Though several laboratories around the globe are making a serious effort towards breaking this obstacle still there is need for improvisation in this area. The concentration and the type of gelling agent used is also an important factor during the regeneration process. The best regeneration frequency of shoots was obtained using 4% clerigel. Another important factors that were of great importance was using the appropriate proportion of growth regulators and the time period of dark incubation .Here we could regenerate about 10 of the transformed plants. During the selection period black or brown microcalli were discarded and only few granular 'macrocalli could be transferred to the regeneration media (10 to 15%) . However when the selected calli were transferred to the regeneration medium a high regeneration frequency (70%) was observed for the transformed calli.

Thus the present study shows that the protocol followed in the experiment is feasible for large-scale production of transgenic plants in *Oryza sativa IR64*. Other potential areas that are important in enhancing the regeneration capability of IR64 included a precise and effective control of callus quality and an optimal time of infection. Various studies have shown that numerous factors including genotype of plants, types and ages of tissues inoculated, kind of vectors, strains of Agrobacterium, selection methods, and various conditions of tissue culture including pH of the media and co-cultivation temperature, are important factors in the Agrobacterium-mediated transformation of rice. The most critical factors necessary for successful transformation by Agrobacterium are (i) the activation and proper expression of vir genes and (ii) the active division of the infected cells. Although vir gene induction is maximal at approximately 25 to 27°C (Turk et al., 1991; Jin et al., 1993), the pilus of some Agrobacterium strains is most stable at lower temperatures (approximately 18 to 20°C) (Fullner and Nester, 1996; Lai et al., 2000; Baron et al., 2001)Another important consideration is using the appropriate concentration of the vir gene inducer acetosyringone. The pre-induction of Agrobacterium strain LBA4404 with 150 µM acetosyringone prior to co-cultivation with immature embryos is important in rice transformation.

Acknowledgement

In the journey of writing this dissertation, I assume that such a research work would not have been possible without the help of the almighty God and many other people who helped me out in the completion of this work. I would like to extend my sincere thanks to all those who supported during this endeavor.

I would like to take this opportunity to express my greatest sense of gratitude towards Prof. B.C. Tripathy for giving me a space in his lab to do my work without any difficulty, which culminated into gaining a valuable research experience under his guidance. His ebullient encouragement, informative discussions and measured appreciation made my research skills better. I am so grateful to Ms. PratisthaVerma for her patient guidance, constant encouragement and being with me and guiding my whole work with valuable suggestions right from the beginning of the project. The dissertation could have never been successful without her insightful suggestions and ungrudging support.

I am also thankful to my lab seniors including Miss Kamal Ruhil, Miss DeepikaKandoi, , Ms. Bernali, Ms.GarimaMsGarima and MsPooja Ms. Kanchan, Ms.SampurnaGarai, MrsShilalipi.Mrs.Ranjanafor their constructive suggestions and their moral support in the lab. I am extremely thankful to my dearest and nearest friends and classmates ShephaliBajpai, AbhaSaxena, JyotsanaDhama, NupurMongre, and ChhaviBansal for their unconditional love and support; it was a great fun to be there along with them during the entire my six month dissertation program.I would very much thankful to Dr. Shailesh Marker Director Research Sam Higginbottom Institute of Agriculture, Technology & Sciences Allahabad, UP. for his valuable suggestion.

I would like to thank lab attendant Mr. Naresh for their regular cleaning of lab instruments, washing of glass ware and maintenance of lab.Last but not the least, my deepest gratitude goes to my parents for their unflagging love and emotional support throughout my life. My beloved father ShriNarendra Kumar Singh and Mother Smt. Maya Devi have been a source of great strength and inspiration for me, without whose moral support this work would have remained incomplete. I also feel proud of having my loving elder brother Neeraj, elder sister Mausam and Brother in Law Amit Singh for their unconditional support.

References

[1]. *Affek HP and YakirD(2002) Protection by isoprene against singlet oxygen in leaves. Plant Physiol 129: 269—277*
[2]. *Armstrong GA, Runge S, Frick G, Sperling U and Apel K (1995) Identification NADPH:protochlorophyllideoxidoreductase A and B: a branched pathway for light-dependent chlorophybiosynthesis in Arabidopsis thaliana. Plant Physiol 108: 1505-151*
[3]. *Aronsson H, Sundqvist C, Timko MP and Dahlin C (2001a) The importance of the C-terminal region and Cys residues for the membrane association of the NADPH: protochlorophyllideoxidoreductase in pea. FEBS Lett 502: 11--15*
[4]. *Begley TP and Young H. (1989) Protochlorophyllidereductase. 1. Determination of the regiochemistry and the stereochemistry of the reduction of protochlorophyllide to chlorophyllide. J Am ChemSoc 111: 3095--3096*
[5]. *Benli M, Schulz R and ApelK (1991) Effect of light on the NADPH-Pchlideoxidoreductase (POR) of A thaliana. Plant MolBiol 16: 615--625*
[6]. *Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D (2003) The molecular analysis of leaf senescence--a genomics approach. Plant Biotechnol J 1: 3-22*
[7]. *Dahlin C, Aronsson H, Almkvist J and Sundqvist C (2000) Protochlorophyllide-independent import of two NADPH:Pchlideoxidoreductase proteins (PORA and PORB) from barley into isolated plastids. Physiol Plant 109: 298--303*
[8]. *Davison PA, Schubert HL, Reid JD, Iorg CD, Heroux A, Hill CP, Hunter CN (2005) Structural and biochemical characterization of Gun4 suggests a mechanism for its role in chlorophyll biosynthesis. Biochemistry 44: 7603-7612*
[9]. *Eckhardt U, Grimm B, Hortensteiner S (2004) Recent advances in chlorophyll biosynthesis and breakdown in higher plants. Plant MolBiol56: 1-14*
[10]. *Eggink LL, LoBrutto R, Brune DC, Brusslan J, Yamasato A, Tanaka A, Hoober JK (2004) Synthesis of chlorophyll b: localization of chlorophyllide a oxygenase and discovery of a stable radical in the catalytic subunit. BMC Plant Biol4: 5*
[11]. *Franck F, Sperling U, Frick G, Pochert B, van Cleve B, Apel K and Armstrong GA (2000) Regulation of etioplast pigment– protein complexes, inner membrane architecture, and protochlorophyllide a chemical heterogeneity by light-dependent NADPH:protochlorophyllideoxidoreductases A and B. Plant Physiol 124: 1678--1696*
[12]. *Fusada N, Masuda T, Kuroda H, Shimada H, Ohta H and Takamiya K (2005) Identification of a novel cis-element exhibiting cytokinin-dependent protein binding in vitro in the 5'-region of NADPH-protochlorophyllideoxidoreductase gene in cucumber. Plant MolBiol 59: 631-64*
[13]. *Fusada N, Masuda T, Kuroda H, Shiraishi T, Shimada H, Ohta H and Takamiya K (2000) NADPH–protochlorophyllideoxidoreductase in cucumber is encoded by a single gene and its expression is transcriptionally enhanced by illumination. Photosynth Res 147-15464:*
[14]. *Goslings D, Meskauskiene R, Kim C, Lee KP, Nater M, Apel K (2004) Concurrent interactions of heme and FLU with GlutRNAreductase (HEMA1), the target of metabolic feedback inhibition of tetrapyrrole biosynthesis, in dark- and light-grown Arabidopsis plants. Plant J 40: 957-967*
[15]. *Gupta I and Tripathy BC.(1999) Oxidative Stress in Cucumber (CucumissativusL.) Seedlings Treated with Acifluorfen. Z. Naturforsch. 54C: 771-781.*
[16]. *Gupta I and Tripathy BC.(2000) Oxidative stress in cucumber (Cucumissativus L) seedlings treated with acifluorfen. Indian J BiochemBiophys. 37: 498-505. Govindjee(1995) Sixty-three years since Kautsky: chlorophyll a fluorescence. Aust J Plant Physiol 22: 131--160*
[17]. *Govindjee(2004) Chlorophyll a fluorescence: a bit of basics and history, in: G.C. Papageorgiou, Govindjee (Eds.), Chlorophyll a Fluorescence a Signature of Photosynthesis, Springer, Dordrecht, pp. 1--41*
[18]. *Govindjee(2005) Chlorophyll a fluoroscence: a bit of basics and history. In GC Papageorgiou, Govindjee, eds, Chlorophyll a Fluoroscence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19. Springer, Dordrecht, The Netherlands, pp 1–42*
[19]. *Maathuis FJ (2009) Physiological functions of mineral macronutrients. CurrOpin Plant Biol12: 250-258*
[20]. *Mapleston RE and Griffiths WT (1980) Light modulation of the activity of protochlorophyllidereductase.Biochem J 189: 125--133*
[21]. *Masuda T, Fusada N, Oosawa N, Takamatsu K, Yamamoto YY, Ohto M, Nakamura K, Goto K, Shibata D, Shirano Y, Hayashi H, Kato T, Tabata S, Shimada H, Ohta H and Takamiya K (2003) Functional analysis of isoforms of NADPH:protochlorophyllideoxidoreductase (POR), PORB and PORC, in Arabidopsis thaliana. Plant Cell Physiol 44: 963-974*
[22]. *Masuda T, Fusada N, Shiraishi T, Kuroda H, Awai K, Shimada H, Ohta H and Takamiya K (2002) Identification of two differentially regulated isoforms of protochlorophyllideoxidoreductase (POR) from tobacco revealed a wide variety of lightand development-dependent regulations of POR gene expression among angiosperms. Photosynth Res 74: 165-172*
[23]. *Masuda T, Fujita Y. (2008) Regulation and evolution of chlorophyll metabolism.Photochem*
[24]. *McCormac AC, Fischer A, Kumar AM, Soll D, Terry MJ (2001) Regulation of HEMA1*
[25]. *Rudiger W, Benz J, Guthoff C (1980) Detection and partial characterization of activity of chlorophyll synthetase in etioplast membranes. Eur J Biochem109: 193-200*
[26]. *Sakuraba Y, Rahman L Md, Cho Hwan Sung, Kim Sol Ye, Koh Jong Hee (2013) Rice faded green leaf locus encodes OsPORB and is essential for chlorophyll synthesis under high light condition. The Plant Journal 74:122-133*
[27]. *Schoefs B and Franck F (2003) Protochlorophyllide reduction: mechanism and evolution. PhotochemPhotobiol 78: 543--557*
[28]. *Schoefs B, Garnir HP and Bertrand M (1994) Comparison of the photoreductionprotochlorophyllide to chlorophyllide in leaves and cotyledons from dark-grown beans as a function of age. Photosynth Res 41: 405--417*
[29]. *Tripathy BC, Rebeiz CA (1985) Chloroplast biogenesis: quantitative determination of monovinyl and divinyl Mg-protoporphyrins and protochlorophyll(ides) by spectrofluorometry. Anal Biochem149: 43-61*
[30]. *Tripathy BC, Rebeiz CA (1986) Chloroplast biogenesis. Demonstration of the monovinyl and divinyl monocarboxylic routes of chlorophyll biosynthesis in higher plants. J BiolChem261: 13556-13564*
[31]. *Tripathy BC, Sherameti I, Oelmuller R Siroheme: an essential component for life on earth. Plant Signal Behav5: 14-20*
[32]. *Tripathy BC and Singhal GS (1999) Oxidative stress in photodynamic herbicidal action of 5-aminolevulinic acid. In Concepts in Photobiology Photosynthesis and Photomorphogenesis. (G. S. Singhal, G. Renger, S. K. Sopory, K-D Irrgang and Govindjeeeds.) Narosa Publishing house New Delhi pp 668--688*
[33]. *Tripathy BC, Mohapatra A and Gupta I (2007) Impairment of the photosynthetic apparatus by oxidative stress induced by photosensitization reaction of protoporphyrinIX.BiochimBiophysActa 1767: 860--868*
[34]. *Tripathy BC, Mohapatra A and Pattanayak GK (2004) Subplastidic distribution of chlorophyll biosynthetic intermediates and characterization of protochlorophyllideoxidoreductase C. in Agricultural applications in green chemistry (W M. Nelson ed, ACS ss 887.) American Chemical Society, Washington DC pp 117--128*