Analysis of the activity of promoters from two photosynthesis-related genes \( psaF \) and \( petH \) of spinach in a monocot plant, rice

Amitabh Mohanty, Monendra Grover, Ashok Chaudhury, Quazi Rizwan-ul-Haq, Arun K Sharma, Satish C Maheshwari and Akhilesh K Tyagi

Centre for Plant Molecular Biology and Department of Plant Molecular Biology, University of Delhi, South Campus, Benito Juarez Road, New Delhi 110021, India

Received 27 April 2000; accepted 22 May 2000

The subunit III of photosystem I and ferredoxin-NADP+-oxidoreductase are encoded by nuclear genes, namely \( psaF \) and \( petH \). The activity of their promoters from spinach has been evaluated in transgenic tobacco earlier. Evaluation of the activity of these \( Dicotyledonae\)-specific promoters has been carried out in a monocot system (i.e. rice) by transient gene expression system, based on electroporation-mediated gene delivery into protoplasts from leaves and roots. It has been found that various promoter deletions show higher activity in leaf protoplasts and elements for quantitative response are widely distributed. Transgenic rice has also been produced with a \( petH \) promoter and \( gus \) reporter gene construct. Although \( petH \) promoter is a weak promoter in comparison to the \( 35\S \) promoter, it expresses well in green tissues and could be useful for plant genetic engineering.

In the last few years, isolation of genes for proteins involved in photosynthesis and characterization of their promoters from higher plants has led to a better understanding of the molecular basis of photosynthesis. In most cases, activity of promoters from dicotyledonous as well as monocotyledonous plants has been evaluated in tobacco, a dicot model system or a homologous system. It has been suggested that the mechanism of regulation of monocotyledonous and dicotyledonous genes or the genes from C3 and C4 plants may vary and the regulatory elements may not be common or expressed correctly across the divide. It is, therefore, imperative to investigate these observations further.

In recent years, because of its small genome size, availability of large number of molecular markers, expressed sequenced tags (ESTs) and cDNAs, together with a relatively well established system for transformation, rice has emerged as a model crop plant for investigating regulation of gene expression as well as functional genomics. To investigate the regulation by \( Dicotyledonae\)-specific gene promoters in a monocotyledonous plant, we have employed transient gene expression and transgenic technology of rice plants with photosynthesis-related gene promoters of \( psaF \) and \( petH \) from spinach driving \( gus \) reporter gene.

Materials and Methods

Constructs for transient gene expression

For transient gene expression studies promoter fragments of two photosynthetic genes, \( psaF \) and \( petH \), along with \( gus \) gene, as described in Flieger et al. and Oelmüller et al., respectively, were used. The following deletions of \( psaF \) gene promoter were used: -1074 to +163, -687 to +163, -220 to +163, -178 to +163 and -124 to +163 bp. Similarly, for \( petH \) two deletion constructs (-753 to +231 bp, -118 to +231 bp) were used. In both cases, the constructs include only part of the first exon upstream to ATG codon.

Constructs for rice transformation

To study regulation of heterologous gene promoters in rice, largest fragments from promoters of \( psaF \) and \( petH \) of spinach were cloned as \( SalI/BamHI \) fragments in the cloning site of the promoter cloning vector \( pCAMBIA1391Z \), kindly provided by Dr. R. A. Jefferson, CAMBIA, Australia.
The recombinant plasmids are named as pCAMF and pCAMH, respectively. The gene construct pCAMH was used for stable genetic transformation.

**Transient gene expression**

**Seed material and plant growth**

Dehusked seeds of *O. sativa* var. Basmati 370 were surface-sterilized in 2.5% (w/v) sodium hypochlorite for 30 min and washed several times with sterile distilled water before inoculation on nutrient MS medium. Seedlings were germinated in culture tubes at 26±2°C and ~24 μmol m⁻² s⁻¹ light intensity.

**Protoplast isolation**

Protoplast isolation from leaves and roots was performed as described by Chaudhury et al. Briefly, leaves from 10-day old light-grown seedlings were shredded longitudinally, chopped into small pieces and incubated in an enzyme solution (1 g ml⁻¹) comprising of CPW salts (0.2 mM KH₂PO₄, 1 mM KNO₃, 10 mM CaCl₂, 1 mM MgSO₄, 1 μM KI and 1 μM CuSO₄), 1% Cellulase R-10 and 0.5% Macerozyme R-10 (Yakult Pharmaceuticals, Japan), 0.6 M mannitol and 0.1% MES (pH 5.8) at 30°C for 15 hr without shaking.

Protoplasts from root explants were isolated from 5 to 6-day old dark-grown seedlings. Roots were shredded into small pieces and incubated in an enzyme solution (1 g ml⁻¹) comprising of CPW salts, 2% cellulase RS (Yakult Pharmaceuticals, Japan), 3% Macerozyme R-10, 0.1% Pectolyase Y-23 (Seishin Pharmaceuticals, Japan), 0.5 M mannitol and 0.1% MES (pH 5.8) at 30°C for 15 hr on a shaker (20 rpm).

**Purification of protoplasts**

The protoplast suspension was passed through a sterilized steel mesh (45 μM) to remove undigested tissue and the protoplasts in the filtrate were pelleted in a centrifuge (Clay Adams Co., Inc., USA) by spin at 100 g for five min. Pelleted protoplasts were washed three times with washing solution (CPW salts, 0.6 M mannitol, pH 5.8). Protoplasts were purified by floating on 20% sucrose and centrifugation. Those forming a ring at the interface of 20% sucrose and protoplast suspension medium were finally collected and used for counting the density by a hemocytometer and further experimentation.

**Gene delivery by electroporation**

The plasmid DNA was isolated on large scale using CsCl density gradient and was quantified spectrophotometrically by taking absorbance at 260 nm using a UV 3000 spectrophotometer (Shimadzu, Japan). Its quality was checked by gel electrophoresis after digestion with unique restriction enzymes. Aliquots of solution containing 100 μg DNA were removed in autoclaved 1.5 ml tubes and DNA was precipitated by the addition of 0.1 vol of 3 M sodium acetate (pH 5.2) and 2 vol of ethanol followed by incubation at ~20°C for 15 min. The pellet was obtained by centrifugation at 10,000 rpm and 4°C for 15 min in a microcentrifuge tube and washed in 70% ethanol, air-dried in a laminar flow and dissolved with 100 μl of sterile distilled water.

For gene delivery experiments, protoplasts were resuspended in 1.0 ml of electroporation buffer (0.6 M mannitol, 100 mM MgCl₂, 0.1% MES, pH 5.8), floated on 20% sucrose and centrifuged at 100 g for 5 min. Protoplasts forming a ring at the interface were collected. An electroporator comprising a bank of capacitors was employed to generate pulses. The electroporation chamber consisting of a plexiglass cuvette with stainless steel electrodes (0.5 cm apart) was sterilized in 70% ethanol and dried. The protoplasts suspension (1-2×10⁶ ml⁻¹) in electroporation buffer was incubated on ice for 5 min and then mixed with sterilized plasmid DNA (50 μg ml⁻¹). Protoplasts were given a single electrical pulse (100 μF, 300 V), incubated on ice for 10 min, pelleted at 100 g for three minutes as above and subsequently cultured in R2 medium with 0.6 M glucose added at 25±2°C in diffuse light.

**Agrobacterium-mediated transformation**

**Preparation of Agrobacterium for co-cultivation**

For mobilization of pCAMH into *Agrobacterium tumefaciens* LBA4404, triparental mating was performed with minor modifications. Before plant transformation, a single colony was inoculated into 30 ml liquid AB medium with kanamycin (50 mg l⁻¹) and rifampicin (100 mg l⁻¹) and grown for two days at 28°C. One ml of this bacterial culture was inoculated in fresh medium and grown for another 36-48 hr. Bacteria were pelleted in SS-34 tubes at 4,000 rpm and 4°C for 10 min. The pellet was suspended in 1 ml of AAM liquid medium and an aliquot was diluted to 1 ml to measure absorbance at 600 nm. Bacterial
density was calculated and adjusted to 3.5x10^9 ml^{-1} with AAM medium^{17}.

Callus induction

_Oryza sativa_ var Pusa Basmati 1 seeds, after surface-sterilization, were soaked in sterile water overnight and next day they were blotted dry on a sterile tissue paper and incubated on 3/2 MS medium^{17} at 26±2°C in dark for 21 days. After this period, the proliferating calli derived from scutella were dissected out and sub-cultured on fresh 3/2 MSCA medium^{17} for another 4 days. Fast growing, nodular, healthy looking, embryogenic calli were used for transformation.

Co-cultivation, regeneration and Southern analysis

These procedures were performed as described^{17}. After co-cultivation for four days, calli were washed with sterile water containing cefotaxime (250 mg l^{-1}), blotted on a sterile tissue paper to remove excess moisture and incubated on 3/2 MSCHCA medium^{17} at 26±2°C for three weeks. The hygromycin resistant calli obtained after first round of selection were transferred to plates containing fresh 3/2 MSCHCA medium for two to three weeks. Fresh and healthy looking hygromycin resistant calli were transferred to regeneration medium containing BAP (0.5-1.0 mg l^{-1})^{17} and incubated at 26±2°C under 16 hr photoperiod. The light intensity was 100-125 μmol m^{-2} s^{-1}. Shoots of about 3-4 cm length were transferred to MS basal medium containing hygromycin (50 mg l^{-1}) for root formation. If required, fragile looking plants were conditioned in liquid rice growth medium for a week before transfer to soil. Rooted plantlets were transferred to pots containing a mixture of soillite (Kel Perlite, Bangalore, India) and soil (1:1) and grown to maturity in Growth Chamber (Conviron, Control Environments Limited, Winnipeg, Canada) operating at 24-28°C, 16 hr light at 100-125 μmol m^{-2} s^{-1} and 70-75 % relative humidity. The plants were supplied with rice growth medium^{17}. Southern analysis was performed as described by Mohanty et al^{17}.

Analysis of GUS activity

β-Glucuronidase (GUS) activity was assayed as described earlier^{6,17,24} from electroporated protoplasts and different parts of the plants. As a control, transgenic rice containing _gus_ driven by 35S promoter^{17} was also employed.

All the experiments were carried out at least twice with essentially similar results and data from representative experiments are given.

Results and Discussion

Promoter analysis using transient gene expression

Investigations with heterologous gene expression systems have shown that _Dicotyledoneae_ -specific gene promoters are not efficiently utilized in monocotyledonous plants^{5,9}. In order to study regulation of some of the _Dicotyledoneae_ -specific gene promoters and the regulatory systems in rice, promoters of two photosynthesis-related genes _psaF_ and _perH^{18, 19}_ from spinach were employed in the present study. In these promoters, there is only limited homology at nucleotide sequence level but various elements known to be present in the promoters of photosynthetic genes are present^{7}.

The data from experiments of transient gene expression in leaf protoplasts in terms of GUS specific activity show that for _psaF_ promoter, -1074 to +163 bp construct has maximal activity (Fig. 1a). Except for fragment -687 to +163 bp that showed lower activity, the pattern of expression was similar in rice leaf protoplasts and transgenic tobacco leaves^{18}. The promoter fragment (-220 to +163 bp) resulted in very significant increase in GUS activity as compared to the shorter fragments. It contains extra 42 bp which have been shown to confer similar regulation in transgenic tobacco plants^{18}. However, lower gus expression driven by -687 to +163 bp promoter fragment in rice may reflect differences in regulation in a monocot and a dicot and reveals that sequences present between +220 to +687 bp may act as negative regulatory elements. Further, regulatory elements present between -1074 to -687 bp can nullify repression.

It was reported by Oelmüller et al^{19} that regulatory elements of _perH_ promoter are present in the first 753 bp. Hence this fragment (-753 to +231 bp), together with smaller promoter fragment (-118 to +231 bp), which was reported to be sufficient to confer light regulation, were chosen for transient gene expression in rice. Analysis of this promoter in rice protoplasts revealed a pattern of expression similar to that of transgenic tobacco plants. The larger promoter fragment (-753 to +231 bp) showed higher expression (Fig. 1b)
For both the promoters, the expression in leaf protoplasts is always higher than in root protoplasts and difference in the level of expression increases with larger promoter fragments (Fig. 1). Such organ specificity of photosynthetic gene promoters is well known.

Cloning of petH promoter in pCAMBIA vector

Since pBI101 based vectors do not have introns in gus gene and an efficient selection marker for rice, the need was felt to clone these promoters in another vector where the reporter gene contains intron and hygromycin resistance gene for selection is also present. For this purpose, pCAMBIA vector was used for Agrobacterium-mediated transformation of indica rice. The petH gene promoter fragment (-753 to +231 bp) from spinach was cloned in the multiple cloning site of binary vector pCAMBIA1391Z resulting into pCAMH (Fig. 2). This places intron containing gus gene under the control of the introduced promoter.

Analysis of petH promoter activity in transgenic rice plants

Agrobacterium-mediated transformation of scutella-derived calli was performed with LBA4404 (pCAMH) as per the optimized protocol of Mohanty et al. Out of 172 calli co-cultivated, a total of four hygromycin resistant calli were obtained (transformation efficiency 2.3%). On transfer to regeneration medium, a total of 19 plants were regenerated from these calli. These plantlets were transferred to pots and grown in a growth chamber and only two plants survived. The transformation efficiency obtained was much less than that obtained with the super-binary vectors. However, it was still better than that obtained by direct gene transfer method by us (data not shown).

Genomic DNA was isolated from a putative transgenic plant and digested with BamHI enzyme which makes one cut in the plasmid and another cut in the genome releasing the junction fragments. In this case, the number of hybridizing bands would be equal to the number of copies of the transgene. The filter was probed with hph gene probe which revealed the presence of integrated gene in the genome of rice corresponding to a signal in the undigested genomic DNA. The copy number of the transgene in this particular line was two (Fig. 3a) as evident from the DNA digested by BamHI. No signal was seen in the control.

The activity of the promoter was evaluated by GUS histochemical and fluorometric assays. In comparison to 35S promoter or the activity of petH promoter in tobacco, the activity of petH promoter

Fig. 1—Fluorometric analysis of GUS activity in root and leaf protoplasts of rice after gene delivery by electroporation. [(a) psol-gus constructs with the indicated size of promoter fragments were used. (b) petH-gus constructs with promoter fragment -753 to +231 bp and -118 to +231 bp were used.]

Fig. 2—Schematic representation of the pCAMH vector used for Agrobacterium-mediated transformation of rice.

Presence of the insert was checked by digestion of plasmids and selected clones were mobilized to Agrobacterium tumefaciens LBA4404 for rice transformation.

Analysis of petH promoter activity in transgenic rice plants

Agrobacterium-mediated transformation of scutella-derived calli was performed with LBA4404 (pCAMH) as per the optimized protocol of Mohanty et al.

Out of 172 calli co-cultivated, a total of four hygromycin resistant calli were obtained (transformation efficiency 2.3%). On transfer to regeneration medium, a total of 19 plants were regenerated from these calli. These plantlets were transferred to pots and grown in a growth chamber and only two plants survived. The transformation efficiency obtained was much less than that obtained with the super-binary vectors. However, it was still better than that obtained by direct gene transfer method by us (data not shown).

Genomic DNA was isolated from a putative transgenic plant and digested with BamHI enzyme which makes one cut in the plasmid and another cut in the genome releasing the junction fragments. In this case, the number of hybridizing bands would be equal to the number of copies of the transgene. The filter was probed with hph gene probe which revealed the presence of integrated gene in the genome of rice corresponding to a signal in the undigested genomic DNA. The copy number of the transgene in this particular line was two (Fig. 3a) as evident from the DNA digested by BamHI. No signal was seen in the control.

The activity of the promoter was evaluated by GUS histochemical and fluorometric assays. In comparison to 35S promoter or the activity of petH promoter in tobacco, the activity of petH promoter
in rice was low. The petH promoter also gives much less activity in rice as compared to 35S promoter in rice (Fig. 3b). Histochemical analysis revealed significant GUS activity only in old leaves. However, fluorometric analysis, being more sensitive than histochemical assay, revealed high activity in young leaves and also in young greenish anthers. Apparently, the promoter is active in all green cells of the plant. In roots, the activity was minimal (Fig. 3b). Even 35S promoter shows less activity in rice roots as reported earlier. The low expression of gus by petH promoter of spinach in rice is probably because of the limitations of compatibility of regulatory systems between a dicotyledonous gene promoter and monocotyledonous regulatory mechanisms. Similar observation of low expression of tomato ribulose bisphosphate carboxylase small subunit (rbcS) gene promoter in transgenic rice has been reported earlier. But the expression of gus gene in green tissues and the lack of expression in roots suggest that organ-specific regulation works similarly in dicots and monocots.

The present investigation reveals the organ-specific activity of a dicotyledonous promoter in a monocotyledonous plant which have diverged 150 million years ago, thereby reflecting on conservation of regulatory mechanisms. Further, transient gene expression system can help in identification of monocotyledonous and/or dicotyledonous-specific regulatory elements very quickly and can supplement the transgenic studies.

Acknowledgement

The work was supported by the Department of Biotechnology, Government of India and the Rockefeller Foundation, USA.

References