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Involvement of Bacterial Pyrroloquinoline in Plant Growth Promotion: A Novel Discovery

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Abstract: There have been very few studies of the functional role of pyrroloquinoline (PQQ) in plant growth. CMG 860 is a native bacterial isolate having multidimensional biofertilizing abilities. CMG 860 has been found to have pqq operon in two fragments A_D and E whose sequences are homologous to those of earlier reports. To investigate the role of pqq as a main plant growth promoting factors in CMG860, mutants were produced which had mutation in pqq gene; this was confirmed by HPLC analysis. These mutants when compared with wild type showed a 22-25 % decrease in plant growth activity. However when pqq was introduced in the mutants they regained their ability of plant growth promotion perhaps in the similar way as it promotes growth of mammals.

Key word: Pyrroloquinoline · Role · Plant · Growth promotion

INTRODUCTION

Pyrroloquinoline quinone [4, 5-dihydro-4, 5-dioxo-1H-pyrrolo-[2, 3-f] quinoline-2, 7, 9-tricarboxylic acid is an aromatic, tricyclic ortho quinone that serves as the redox cofactor for several bacterial dehydrogenases. PQQ is water soluble, heat stable and has the ability to carry out redox cycles [1]. The holo enzyme is composed of a single polypeptide apoenzyme, the redox cofactor PQQ linking the oxidation of many different compounds to the respiratory chain in Gram negative bacteria. It has been reported that PQQ acts as a reactive oxygen species (ROS) scavenger by directly neutralizing reactive species in *Escherichia coli* [2].

PQQ was the first of the class of quinone cofactors that have been discovered in the last 18 years and make up the prosthetic group of quinoproteins [3,6,7]. Although plants and animals do not produce PQQ themselves, PQQ has invoked considerable interest because of its presence in human milk and its remarkable antioxidant properties. Recently, the first potential eukaryotic PQQ dependent enzyme [(aminoadipic 6-semialdehyde-dehydrogenase (AASDH; U26)] has been identified, Rather soon after the discovery of PQQ, a matter of debate was its wideness of distribution and its significance as a biofactor or vitamin. PQQ was the vitamin identified after 50 years in 2003 [6-8]. After it had been established that PQQ occurs in several bacterial enzymes, a logical next question was whether it also occurs in higher organisms. Perhaps stimulated by the reports [9] that PQQ occurs at high levels in certain body fluids and tissues of mammalian milk and in citrus fruits, several reports followed in which beneficial effects were ascribed to its administration, e.g. that a diet supplemented with PQQ improved the "health" of mice substantially or prevented the outbreak of certain diseases [3]. In mammals, pyrroloquinoline quinone (PQQ) functions as a potent growth factor, enhances DNA synthesis activity and displays nerve growth factor inducing activity. Although biological functions of PQQ are not fully understood [10] PQQ has attracted considerable interest because of its presence in a wide variety of foods and its remarkable antioxidant properties [11, 12, 10].

There have been few studies of the functional roles of PQQ in plants. It is known that PQQ stimulates pollen germination in vitro in the plant species Lilium, Tulipa and Camellia [13, 14] but the mechanisms are unclear. PQQ is at least 100 times more efficient than ascorbic acid, isoflavonoids and poly phenolic compounds in assays assessing redox cycling potentials [1]. In addition to scavenging superoxide, PQQ could also scavenge other toxic free radicals, as do vitamin E, b-carotene and carotenoids, vitaminC, flavonoids, conjugated linoleic acid and phenolic compounds [15] PQQ is found in plant and animal tissues in the nanogram to gram range even though plants and animals do not produce PQQ themselves [11, 12].

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MATERIALS AND METHODS

Amplification of Pqq Gene: the pqqa-d gene of cmg860 (eu037096) was amplified by using primer pair of af_2s (forward) and drs (backward) primers. cmg860 (eu037096) produced single pcr product of expected size (2.2kb) with those primers (Figure1). sequencing was done commercially by using abi prism 377 automated dna sequencer. sequences data obtained were analyzed by blastaligothirm (www.ncbi.nlm.nih.gov /blast/cgi)

Reaction Mixture and Cycling Condition: PCR amplification of pqq gene of CMG860 was carried out with thermocycler (Eppendorf) by using Epicentre PCR core kit catalog no. amp 4670. Polymerease chain reaction was carried out in total volume of 50 µl in 0.5 ml microfuge tubes. The PCR mixture consisted of the following components H2O: 56.5µl; Master Amp Taq 10X PCR Buffer:10µl; 25 mM $MgCl_2$ Solution: 5µl; dNTP mix: 8µl; Master Amp 10X PCR Enhancer: 15µl; P1: 2.5µl;P2:2.5µl; Master Amp Taq DNA polymerase0.5µl. Colony PCR was performed by initially denaturing the DNA at 96°C for 10 minutes followed by 30 cycles at 95°C for 30 seconds. Annealing temperature was set at 52°C -62°C (gradient was applied)

Mutation in Pqq Gene: To identify genes that confer plant growth promotion, mutation experiments were conducted. A stock solution of acridine orange (5mg/25ml) as a mutagenic agent was prepared. An aliquot of 10 µl overnight grown culture was inoculated in test tubes containing variable concentrations of acridine orange (10µl, 50µl, 100 µl, 200 µl and 400 µl). These test tubes were incubated at 37°C for 24 hours at 100 rpm. A100µl culture from 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread over nutrient agar plates and incubated for 24 hrs at 37°C to get isolated colonies. About 100 colonies were randomly selected and tooth picked on to a control (nutrient agar plate) and test plate (tris minimal agar plate having 5mM zinc phosphate). The colonies which lost phosphate solubilization activity were initially selected and designated as CMG860 mutant colonies CMG860 M2 and CMG860 M4.

Auxin Production Activity: Auxin production activity of the wild type cmg860 and mutants cmg860m2 and cmg860m4 were carried out by using salkovski reagent method [6].

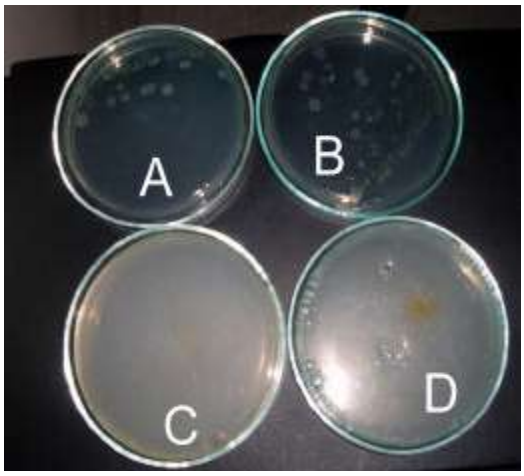

Fig. 1: Following Random Mutagenesis of Pseudomonas aeruginosa CMG860 with Acridine Orange, Mutant Strains CMG860M2 and CMG860M4 Were Isolated Primarily on the Basis of Their Failure in Halos Formation
Keys A=Growth of M2on nutrient agar plate
B= Growth of M4on nutrient agar plate
C= Growth of M2on Tris agar plate
D= Growth of M4on Tris agar plate

Isolation of PQQ Mutants: Mutant strains m2 and m4 were studied for the growth promotion of the Mung beans seedlings. Briefly, the surface sterilized seeds were incubated with late log phase cultures of wild type cmg860 and mutant strains cmg860m2 and cmg860m4 by incubating them with 10ml of respective bacterial suspension (10^{-7}cfu per seed) in a sterile 15ml test tube. Non inoculated control seeds were incubated with 15 ml of nutrient broth. The tubes were then gently shaken on an orbital shaker for 2 hrs. After 2hrs the seeds were planted (10 seeds per pot) at a depth of 2cm. in plastic pots having 200gm autoclaved soil. The plants were rendered for 10 days in a green house under a 16 hrs light and 8 hrs dark. The mutant strains cmg860m2 and cmg860m4 were then finally selected on the basis of their demolished plant growth promotion activities.

Revival of Plant Growth Promotion Activities of Mutants in Pots: Mutant strains cmg860m2 and cmg860m4 were analyzed by the method of Choi *et al.* 2008 [1] with slight modification for the revival of their plant growth promotion activities via addition of synthetic pqq. Mung beans seeds were surface sterilized and placed in petri plates lined with moistened filter paper to germinate at room temperature. Following treatments were made.

- Set A contained: O/N grown culture of CMG860 wild type
- Set B contained: O/N grown culture of CMG860M2/CMG860M4
- Set C contained: synthetic PQQ +O/N grown culture of CMG860M2 /CMG860M4

Two days old mung beans seedlings were immersed in 10 ml O/N culture of wild type CMG860 (Set A) , mutants CMG860M2 / CMG860M4 (Set B) and in 10 ml of 100nM of synthetic PQQ (Set C) for 1 hr and then transferred into the plastic pots contained 200gm autoclave sand and the surplus synthetic PQQ solution that remained after treatment was poured into the pot of Set C. Pots were placed in a green house and set to a 16-h light/8-h-dark cycle with a relative humidity of 60%. Shoot length and root length of the plants were recorded after 10 days.

Analysis of PQQ: To analyze pqq production of wild type and mutant strains a method of Choi *et al.,* 2008 was applied. Wild type cmg860 and mutant strains cmg860m2 and cmg860m4 were grown for 48 hrs at 20°c in tris minimal medium containing 1% glucose. One volume of cell culture was diluted with nine volumes of methanol and the precipitated materials were removed by centrifugation. After evaporation of the methanol, a sep-pak c18 cartridge (waters) was washed with 10 ml of methanol and subsequently with 10 ml of water. The sample was acidified with HCl to ph 2.0 and loaded onto the cartridge. After washing with 10 ml of 2mm HCl, pqq was eluted with 70% methanol. To identify the peak of pqq, 200 ml of the sample were mixed with 100 ml of 0.2 m $na_2b_4o_7$ buffer and adjusted to ph 8.0 with hcl and 90 ml of 0.5% (v/v) acetone. Rp-hplc was performed using a shimadzu lc-6a hplc system as described previously [17] with a fluorescence detector. Fluorescence was monitored at e_x 5=360 and e_m 5=480 nm. A c18 column (150 mm 3x4.6 mm i.d., 5-mm particle size; phenomenex) was used for analytical separation. Fractions corresponding to the acetone adduct (5-acetonyl-pqq) were analyzed using esi-ms (jeol).

RESULTS

Sequence Homology of Amplified PCR Product (PqqA-D) of CMG 860: The PCR product was purified using quick PCR purification kit. The ABI373 DNA sequencing system was used for sequence pqqA -D operon. A total of 2212 bases were determined which showed 95% homology with pqqBC gene of *Pseudomonas aeruginosa* LESB58 and 97% homology to pqqAB gene of *Pseudomonas aeruginosa*PAO1 and 77% of pqqA_Dgene of *Pseudomonas fluorescens* strain B16.

Sequence Homology of Amplified PCR Product (PqqE) of CMG860: The purified pcr product of pqqe was sequenced. A total of 1026 bases were determined which showed 98% homology with pqqe gene of *pseudomonas aeruginosa* pao1 and 98% *pseudomonas aeruginosa* lesb58 and 98% of *pseudomonas aeruginosa* ucbpp- pa14.

Data Analysis of Sequences: Nucleotide analysis was performed by blastn and all overlapping sequences were removed. it was observed that all the sequences of pqqa_d and pqqe gene of cmg860 (eu037096) were 99% homologous to *pseudomonas aeruginosa* pao1. Gene sequences of pqqa-d and pqqe were submitted to genbank, their accession no are given in Table 1.

Isolation of PQQ Mutants: Following random mutagenesis of *pseudomonas aeruginosa* cmg860 with acridine orange, mutant strains cmg860m2 and cmg860m4 were isolated primarily on the basis of their failure in halos formation (Figure 1).

Table 1:

CMG Code	Gene Name	No. of Nucleotide Sequenced	Accession No.	Percentage Similarity
CMG 860	pqqA-d	2212	EU072017	*95% homology with pqqBC gene of *Pseudomonas aeruginosa* LESB58 *97% homology to pqqAB gene of *Pseudomonas aeruginosa*PAO1 *77% of pqqA_Dgene of *Pseudomonas fluorescens* strain B16.
CMG 860	pqqe	1026	EU072016	*98% homology with pqqE gene of *Pseudomonas aeruginosa* PAO1 *98% *Pseudomonas aeruginosa* LESB58 *98% of *Pseudomonas aeruginosa* UCBPP- PA14

Table 2:

CMG Code	Pqq Production	Auxin Production	Halos Fromation	Plant Growth Promtion
CMG 860 (wild type)	+	+	+	+
CMG 860M2 (Mutant)	-	+	_	_
CMG 860M4 (Mutant)	-	+	_	_

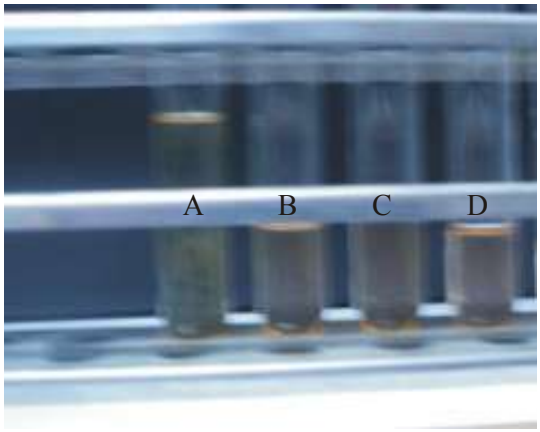

Fig. 2: Auxin Production Activity of wild type CMG860 and mutants; Keys: A = Control, B = CMG860, C = CMG860M2, D= CMG860M4

Auxin Production Activity: The auxin production activity of wild type and mutants was performed by using salkovski assay reagent method. Both mutant strains (cmg860m2 and cmg860m4) and wild type cmg860 showed auxin production activity (Figure 2).

Growth Promotion of Mung Been Seedling: Effect of wild type and mutant strains were checked on the growth of mung beans seedlings. Heights of the plants were measured every 3 days up to 10 days after the treatments. Mutant strains cmg860m2 and cmg860m4 were failed to promote growth of mung beans seedlings while revival of plant growth promotion activities was observed (Figures 3&4) when synthetic pqq was added in o/n culture of mutant strains (cmg860m^2 and cmg860m^4). The height of mung beans plants treated with wild type cmg860 was increased by approximately 25% at 10 days after treatment (Figures 3&4).

Identification and Quantification of PQQ via RP-HPLC in CMG860 Wild Type and Mutants: Production of pqq from wild type cmg860 and mutant stains cmg860m2 and cmg860m4 were confirmed via rp- hplc. The retention time of standard pqq is 1.8. Quantification of pqq in wild type cmg 860 was also performed via hplc by the integration of peak heights. Cmg60 wild type produced 2.44 mg/ml of pqq . No pqq production was detected in mutant strains cmg860m2 and cmg860m4 (Table 2).

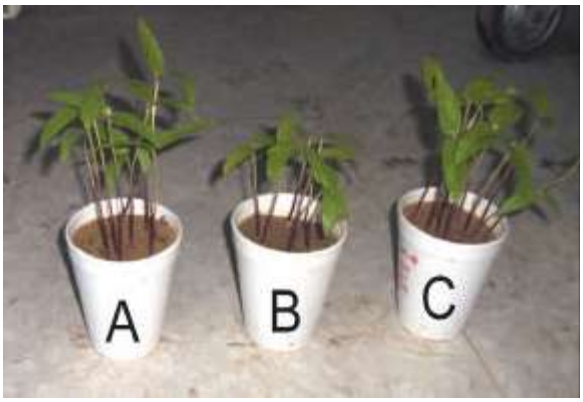

Fig. 3: Mutant Strains M2 and M4 Were Studied for the Growth Promotion of the Mung Beans Seedlings; Keys; A = Wild Type 860, B = Mutant M4, C = Mutant Supplemented with Synthetic pqq

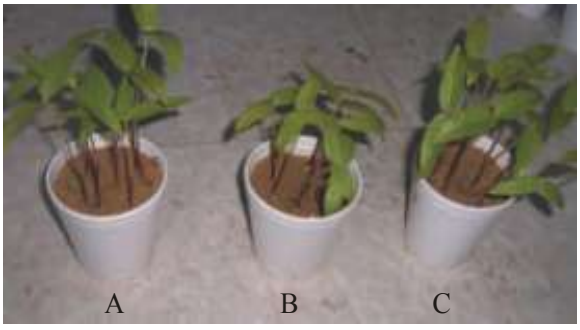

Fig. 4: Mutant Strains M2 and M4 Were Studied for the Growth Promotion of the Mung Beans Seedlings. A = Wild Type 860, B = Mutant M4, C = Mutant Supplemented with Synthetic pqq

DISCUSSION

Gene encoding PQQ cofactor was detected in CMG860 by PCR (Figure1). The purified PCR product of CMG860pqqA-D (EU72017) was sequenced. A Total of 2212 bases were determined which showed 95% homology with pqqBC gene of *Pseudomonas aeruginosa* LESB58 and 97% homology to pqqAB gene of *Pseudomonas aeruginosa*PAO1 and 77% of pqqA_D gene of *Pseudomonas fluorescens* strain B16. The purified PCR product of CMG860pqqE (EU72016) was also sequenced. A total of 1026 bases were determined which showed 98% homology with pqqE gene of *Pseudomonas aeruginosa* PAO1 and 98% *Pseudomonas aeruginosa*

LESB58 and 98% of *Pseudomonas aeruginosa* UCBPP-PA14.

There have been few studies of the functional roles of PQQ in plants. It is known that PQQ stimulates pollen germination in vitro in the plant species Lilium, Tulipa and Camellia [13,14]but the mechanisms are unclear. To investigate the role of PQQ as a main plant growth promoting factor in CMG860, mutagenesis experiments were performed. Results of mutagenesis clearly indicated that wild type CMG860 showed better plant growth as compared to mutants (Figure 3-4). Wild type CMG860 markedly increased the shoot length of mung beans seedlings up to 114 % and 87 % respectively over mutants M2 and M4 while wild typeCMG860 increased the root length of mung beans seedlings up to 200% over mutants. Cultural analysis of the mutant and wild type CMG860 via HPLC clearly indicated the production of PQQ as main agent responsible for plant growth promotion activities (Figure 3-4). The revival of plant growth in presence of synthetic PQQ clearly indicated that PQQ is a plant growth promoter. Therefore it was assumed that the biochemical basis of plant growth promotion mediated by PQQ is more or less similar to that of its growth promotion in mammals. [8] It was likely to propose here that many PGPR produce PQQ, which would illuminate previously unknown plant growth promotion mechanisms. As in mammals, PQQ has great potential to be used as a growth promoting factor in plants. Plant growth promotion by PGPR has received attention for academic and practical reasons because beneficial interactions between PGPR and plants offer tremendous potential for field applications. This result led us to identify a new plant growth promotion factor, PQQ, from *Pseudomonas aeruginosa* CMG860 which could be used as potential biofertilizer.

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