Cloning and Sequence Analysis of Gene Encoding *psbZ* from *Silybum marianum* (L.) Gaertn

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Abstract: *Photosystem II is known as the core of photosynthesis and among of the photosystem II protein complex, PsbZ protein is very important because of its role in connection PSII and LHCII. For identifying and sequencing of the psbZ gene in Silybum marianum plant, seeds were prepared from botanical garden in Tabriz, Iran. DNA was extracted by CTAB method and by using the PCR amplification the fragment length was estimated to be 705 bp. The amplified fragment was cloned in pGEM-T vector and E.coli transformation was carried out. The plasmid was extracted and the sequence was recorded at NCBI with accession number of GQ225868. BLAST of psbZ, glycine tRNA and psbZ-tRNA GLY intergenic spacer showed that each region is highly conserved among species. BLAST of predicted PsbZ protein also represents a severe conservation among species. Because of the importance of PsbZ protein in photosynthesis, it is necessary to create targeted mutations to determine the active sites of proteins.*

Keywords: *Chloroplast, Photosynthesis, PhotosystemII, Sequencing, Transformation, Silybum marianum.*

Introduction

Photosynthesis is a vital process to life on earth. In this process the sunlight transforms chemical energy and carbon dioxide into carbohydrates and this is used as a source of energy and food by heterotrophic organisms. In addition to carbohydrates, oxygen is also produced by photosynthetic organisms (the vascular plants, algae and cyanobacteria) [11]. Photosystem (PS) II complex is located in the thylakoid membranes of photosynthetic organisms and is at the core of the process of photosynthesis. This complex catalyses the splitting of water into dioxygen and reducing equivalents [1]. Photosystem II (PSII) is a giant membrane pigment-binding protein complex (Table 1) which has a molecular mass of almost 1100 kDa. This super-complex does its duties in relation to the three parts: photosystem I with associated light-harvesting antenna (LHCI), cytochrome b6f complex, and adenosine triphosphate (ATP) synthesis [6]. The PsbZ is an important protein in Photosynthesis, encoded by a chloroplast gene *psbZ* and previously denoted *ycf9* and *orf62* genes.This gene is highly conserved among species and is found in all photosynthetic species. In most organisms, the protein is composed of 62 amino acids and the molecular weight of it is about 6.6 kDa. PsbZ is the only low molecular weight subunit which has two transmembrane spans and both ends of the protein are located in the membrane [11, 12]. In other case, using purification and crystallization of

proteins, it was reported that PsbZ is in the vicinity PsbK, Psb30 and CP43 that acts as a connector between PSII and LHCII [15]. Several studies have been conducted to determine the PsbZ function using *psbZ* mutant plants. The results show that mutant plants are capable of photoautotrophic growth. Reducing the amount of chlorophyll in the standard heterotrophic growth conditions, reducing the growth and dwarfing of mutant plants under low light conditions (Fig. 1), reducing the amount of proteins of CP26, CP29, D1, CP43, PsbH, plastid terminal oxidase, increases the amount of phosphorylated LHCII, stimulates the xanthophyll cycle, and highlights the important role of PsbZ in stabilizing the PSII complex with LHCII [11, 12].

Fig. 1 Phenotypes of wild-type and *psbZ* mutant plants [14]:
A) wild type plants grown at high light at 25 °C; B) *psbZ* grown at high light at 25 °C;
C) *psbZ* grown at high light at 20 °C; D) *psbZ* grown at dim light at 20 °C.

Materials and methods

Specific primers design

To design primers, the location of genes including *psbZ*, tRNA glycine and regions between the *psbZ*, tRNA glycine were selected. Sequences of selected locations from several other plants were downloaded in National Center for Biotechnology Information (NCBI). These sequences were aligned by MEGA5 [17] and primer design program (Oligo 7) were initiated from conserved sites [8]. The forward primer was ATCGGGTAGCTGATCG and reverse primer was TGCTACTAGCTGCTG.

Amplification of DNA fragment

Silybum marianum (milk thistle) seeds were prepared from the Botanical Gardens in Tabriz (East-Azarbaijan Research Center for Agriculture & Natural Resources, Tabriz, IRAN) and total DNA extraction was completed with CTAB extraction protocol [9]. The PCR reaction was performed in a final volume of 20 µl mixture containing the DNA template (100 ng), 250 mmol·l^{-1} dNTPs, 3 ng·µl^{-1} of each primer, 50 mmol·l^{-1} $MgCl_2$, 5U µl^{-1} *Taq* DNA polymerase and PCR buffer (1x). After an initial denaturing for 5 min at 94 °C, 35 cycles were performed for 95 °C (1 min), 65 °C (1 min) and 72 °C (2 min) followed by a final extension step of 7 min at 72 °C. The amplified fragment was electrophoresed in a 1% agarose gel in TBA buffer and stained in ethidium bromide.

Table 1. Features of protein subunits of PSII [1, 6, 11, 12]

Subunit name	Gene	Mass (kDa)	Function
D1	*psbA*	39	core RC
CP47	*psbB*	56	core antenna
CP43	*psbC*	50	core antenna
D2	*psbD*	39.5	core RC
Cyt b-559a	*psbE*	9.25	1. PSII assembly at early steps 2. Photoprotection
Cyt b-559b	*psbF*	4.39	Unknown
H	*psbH*	7.63	1. PSII stabilization and assembly 2. Electron transport at acceptor side of PSII 3. Photoprotection, Bicarbonate binding
I	*psbI*	4.19	1. PSII dimerisation/stabilization 2. Maintenance of PSII structure and function under high light
J	*PsbJ*	4.03	1. Assembly of water splitting complex 2. Involved in electron transfer within PSII
K	*PsbK*	428	1. Plastoquinone binding 2. Maintaining PSII dimeric form
L	*PsbL*	4.36	1. Donor side electron transfer 2. Assembly of PSII, 3. Maintaining PSII dimeric form
M	*PsbM*	3.80	Unknown
N	*PsbN*	4.5-5	Might not be a ubiquitous PSII subunit
OEC33	*PsbO*	27	Mn cluster stability, Ca^{2+} and Cl binding
OEC26	*PsbP*	20	Ca^{2+} and Cl binding
Tc	*PsbT*	395	Recovery of photo damaged PSII PSII dimerization/stabilization
U	*PsbU*	12	role in O2 evolution
Cyt c-550	*PsbV*	15	role in O2 evolution
W	*PsbW*	5.89	1. PSII dimerization 2. Photoprotection
Xa Xb	*PsbXa* *PsbXb*	4.06 4.12	Binding or turnover of quinone molecules at Q_B site
Y1 Y2	*PsbY1* *PsbY2*	4.28 4.35	Unknown
Z	*PsbZ*	6.57	Linker between LHCII and PSII core
R	*psbR*	12.8	1. pH-dependent stabilizing protein for PSII 2. Docking protein for PSII extrinsic proteins
Tn	*psbTn*	5	Unknown
Psb27H1	*At1g03600*	11.8	Mn4Ca assembly
Psb27H2	*At1g05385*	15.1	PSII assembly
Psb28	*At4g28660*	15.1	PSII assembly
Psb29	*At2g20890*	26.8	photorepair cycle
OEC23	*psbP1*	23	Unknown
CP22	*psbS*	22	involved in non-photochemical quenching
OEC16	*PsbQ1*	16	calcium ion binding
Psb30	*psb30*	3.3-5	1. Stabilize PSII dimer 2. Prevent Cytb559 from converting to low potential form under high light
Psb31	*psb31*	12.3	Supporting oxygen-evolving
Psb32	*psb32*	32.2	1. Protection oxidative stress 2. Repair PSII
ScpB	*ssl1633*	6	1. Stress-induced 2. Chl stabilizator
ScpC	*ssl2542*	6	
ScpD	*ssr2595*	4	

Construction of the pGEM-T and E. coli transformation

Purification of amplified fragment was carried out using the high pure PCR product purification kit (Catalogue number: K-3034-1, Bioneer Company, Korea). Because the duplicated segment at both ends has adenine base, pGEM-T vector was used for cloning the amplified fragment. This vector has 3 kb size and it has blue-white selection system to identify recombinant colonies. In addition, this vector has an ampicillin resistance gene that used to identify bacteria contained plasmids.The details of the vector and multiple cloning site are depicted in Fig. 2. The resulting fragments of PCR product and pGEM-T were ligated by T4 DNA ligase (Thermo Scientific, USA) and named pGE-PP.

For transformation, the DH5α strain of *E. coli* bacteria was used. In the first step, competent cells were prepared by Chung et al. [3] method. Next, by using thermal shock method, proposed by Sambrook and Russell in [10], the pGE-PP construct was transformed into DH5α and cultured on Luria Bertani (LB) agar with ampicillin antibiotic, IPTG and X gal at 37 °C with 220 rpm (15-16 hours). The plasmid extraction of the white colony was performed according to Sambrook and Russel [10] method and the PCR product of the pGE-PP was sequenced (Macrogen, Korea).

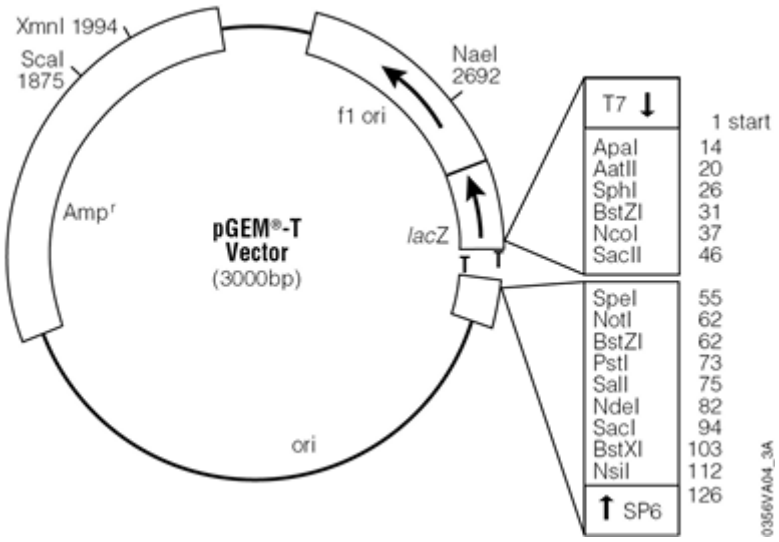

Fig. 2 pGEM-T vector

Results and discussion

Fragment sequence

White colonies showed successful transformation (Fig. 3). The resulting recombinant plasmid was sent to the sequencing and the product of the PCR amplified fragment was 705 bp (Fig. 4).

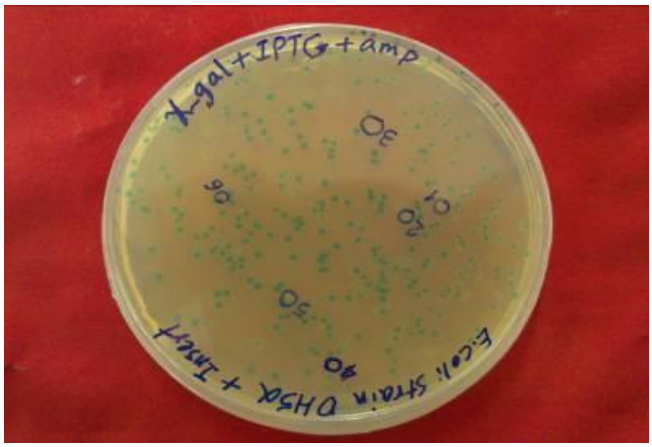

Fig. 3 White colonies observed in transformation process

Fig. 4 A representative of agarose gel for demonstrating the pGE-PP construct

Sequence analysis of psbZ gene

The DNA sequences of the *psbZ* gene from *Silybum marianum* was compared with 13 important plant *psbZ* gene sequences. Similarity between *psbZ* gene with other recorded *psbZ* genes were ranged 75-98% (Table 2). Alignment of the predicted protein sequences of *PsbZ* with other 13 PsbZ revealed that identity ranged 67-98% (Table 3). The minimum identity of the *psbZ* from *Ephedra triandra* was 75%. According to the results, *Ephedra triandra* (Ephedraceae) belongs to Gymnosperms, which has 75% nucleotide similarities with *Silybum marianum*. Our results indicate that it has minimum similarity among examined species [4, 18]. Species of *Oryza nivara*, *Triticum aestivum* and *Hordeum vulgare* (poaceae family and Monocotyledonous Angiosperms) have 89-90% nucleotide similarity with *Silybum marianum* [2, 7]. *Arabidopsis thaliana* (Brassicaceae) and *Gossypium herbaceum* (Malvaceae) and *Glycine max* (Fabaceae) are the Dialipetales Dicotyledonous Angiosperms and have 92-97% nucleotide similarity with *Silybum marianum* [5, 13]. *Artemisia arenaria* and *Tanacetum parthenium* (Asteraceae family) and *Solanum tuberosum*, *Solanum lycopersicum*, *Nicotiana tabacum* and *Datura metel* (Solanaceae family), are Gamopetales Dicotyledonous Angiosperm species that had highest nucleotide similarity (98%) with *Silybum marianum* of Astraceae family [13, 16].

Table 2. The blasting of the *psbZ* gene from *Silybum marianum* with 13 plants

Organism	**Base pairs**	**Query cover, %**	**Identity, %**	**Accession**
Oryza nivara	189	98	88	KM088022.1
Triticum aestivum	189	98	90	KJ592713.1
Solanum tuberosum	189	100	98	KM489056.1
Solanum lycopersicum	189	100	97	NC_007898.3
Hordeum vulgare	189	98	90	EF115541.1
Nicotiana tabacum	189	100	98	NC_001879.2
Arabidopsis thaliana	189	100	93	NC_000932.1
Artemisia arenaria	189	100	98	GQ227399.1
Tanacetum parthenium	189	100	98	GQ227402.1
Datura metel	189	100	98	GQ227401.1
Ephedra triandra	186	95	75	KP788899.1
Gossypium herbaceum	189	100	93	NC_023215.1
Glycine max	189	100	92	NC_007942.1

Table 3. The blasting of the PsbZ protein from *Silybum marianum* with 13 plants

Organism	No. amino acid	Query cover, %	Identity, %	Accession
Oryza nivara	62	100	89	AJC99830.1
Triticum aestivum	62	100	90	AIG61156.1
Solanum tuberosum	62	100	98	AKM22028.1
Solanum lycopersicum	62	100	98	YP_008563085
Hordeum vulgare	62	100	90	ABK79400.1
Nicotiana tabacum	62	100	98	NP_054494.1
Arabidopsis thaliana	62	100	97	NP_051056.1
Artemisia arenaria	62	100	97	ACT20211.1
Tanacetum parthenium	62	100	97	ACT20214.1
Datura metel	62	100	98	ACT20213.1
Ephedra triandra	61	100	67	AKH66162.1
Gossypium herbaceum	62	100	95	YP_008992634.1
Glycine max	62	100	92%	YP_538758.1

Conclusion

Sequencing of *psbZ* gene was carried out for the first time by the present study in *Silybum marianum.* These results will lead to finding similarities and differences in DNA sequence and finally are helpful in classification of Asteraceae family. Through the present study, we introduced a new primer which is able to identify *psbZ* gene and subsequently evaluate the structure and function of plant photosynthesis. In addition, nucleotide similarity observed in the present study was consistent with the classification system based on the morphological traits of the plants.

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