TISSUE CULTURE OF THE MONOCOT

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ABSTRACT

PsbS is a 22-kDa protein of photosystem II involved in nonphotochemical quenching of chlorophyll fluorescence but the mechanism is still unclear.
We designed and generated transgenic rice plants with significantly reduced PsbS1 protein level using RNA interference (RNAi). Transformation confirmed by vector-specific primers and transformants were screened by RT-PCR for OsPsbS1 transcript levels and PsbS1 protein level. We could identify three PsbS1-RNAi lines.

KEYWORD: Rice (*Oryza sativa*.), PsbS1, RNAi, transformation

INTRODUCTION

Rice (*Oryza sativa*.) is the most important staple food for a large part of the world's human population. It is the grain with the second-highest worldwide production, after maize.
Rice is emerging as a model cereal for molecular biological studies. The main reasons for this are as follows: 1.The complete genome has been sequenced. 2. Tools for functional genomics are available. 3. Production of transgenic plants is relatively easy compared to that of other major cereals.
Light is one of the most important environmental factors that control the growth and development of plants. The synthesis of many component proteins of photosynthesis, which are encoded by nuclear and chloroplast genes, is positively regulated by light at the level of transcription.
PsbS1 is necessary for photo protective thermal dissipation (qE) of excess absorbed light energy in plants, measured as non-photochemical quenching of chlorophyll fluorescence (1). But the mechanism of qE is still unclear.
RNA interference (RNAi) is a post-transcriptional gene-silencing phenomenon induced by double-stranded RNA. It has been widely used as a knockdown technology to analyze gene function in various organisms (2). During the 1990s, a number of gene silencing phenomena that occur at the post-transcriptional level were discovered in plants, fungi, animals and ciliates (3).

MATERIALS AND METHODS

Preparation of callus
Mature seeds of rice (*Oryza sativa* cv.Dongjin) were used as explants material in this experiment. Seed of rice were sterilized with 70% ethanol for 1 min, followed in 4% sodium hypochlorite solution containing 20 ul Tween 20 for 15 min, and then rinsed three times with sterilized distilled water. 16 seeds were plated in Petri dish (90mm in diameter)

containing 25 ml of callus induction medium and sealed with micro pore surgical tape.
Cultures were incubated at 25^0C and 18 hours photoperiod of 45 umol $m^{-2}s^{-1}$ illumination provided by cool white fluorescent lamps. Summary of mediums used in the tissue culture and transformation system of *Oryza sativa* were shown in Table 1.

Table 1

Summary of medium used in the tissue culture and transformation

Type of medium	Composition
Callus induction	N6 salts and vitamins, myo-inositol 100mg/L, 30g/L sucrose, 2mg/L 2,4-D, 3g/L gelrite, pH=5.8
Agrobacterium culture	LB medium supplemented with 50 mg/L hygromycin, pH=7.0
Agrobacterium suspension	AA medium and B5 vitamins, 20g/L sucrose, 2mg/L 2,4-D, 0.2mg/kinetin, pH=5.8, 30mg/L acetosyringone
Co-cultivation	Calcium free N6 and vitamins, 30mg/L sucrose, 10g/L glucose, 2 mg/L 2,4-D, 120 mg/L betaine, 3g/L gelrite, pH=5.2, 30mg/L acetosyringone
Agrobacterium elimination	N6 salts and vitamins, myo-inositol 100mg/L, 30g/L sucrose, 2mg/L 2,4-D, 3g/L gelrite, pH=5.8, cefotaxime 250 mg/L
Shoot induction	MS salts and vitamins medium supplemented with myo-inositol 100mg/L, 15g/L sucrose, 30 g/L sorbitol, 4g/L casamino acid, 0.5 g/L NAA, 2mg/L BA, 0.5 g/L L-proline, 4g/L gelrite, pH=5.8, 250mg/L carbenicillin, 30mg/L hygromycin
Root induction	MS medium supplemented with myo-inositol 100mg/L, 30g/L sucrose, 8g/L agar, pH=5.8, 100mg/L carbenicillin, 50mg/L hygromycin

Plants and growth conditions:

One-month-old seedlings of WT and PsbS1-knockout, PsbS1-RNAi mutant rice (*Oryza sativa* L.) plants were grown in soil in a greenhouse under sunlight or in a culture room at an irradiance of 200 µmol photons $m^{-2}s^{-1}$ white light (16/8 h light – dark cycle) at a temperature of 28 ± 2^0C (28^0C /22^0C temperature cycle). For some experiments, rice seeds were germinated and grown on nutrient Murashige and Skoog (MS) solid agar medium for one week.
Generation of *OsPsbS1*-RNAi Transgenic Rice:
To generate RNA interference vector of *OsPsbS1*, gene fragment of 102 base pair size was amplified by PCR using primers (Forward primer: 5'- ATA GGA TCC CTC GAG CGC GCG GTG TCC GTC AAG AC-3', Reverse primer: 5'- GCG GAA TTC AAG CTT GTC CTC GGT CTT GAA CTT TG-3'), and the fragment was cloned into XhoI-HindIII, BamHI-EcoRI sites of pFGL727 (pBSIIKS-Intron). The SacI-KpnI fragment of the pBSIIKS-Intron-OsPsbS1 was transferred into SacI-KpnI sites of pGA1611. The name of final construct is pFGL702. The OsPsbS1-RNAi plasmid was then transformed into rice using *Agrobacterium* strain LBA4404, as described previously (4).

Reverse transcription polymerase chain reaction (PCR) with an internal standard:

Total cellular RNA was isolated from plant leaves using TRIzol REAGENT® (Life Technologies, USA) according to the manufacturer's instructions. Reverse transcription was performed according to the manufacturer's protocol using RNA samples with RQ1 RNase-free DNase (Promega) and M-MLV (Promega). RNA quality was monitored using the ratio of absorbance at 260 nm and 280 nm and by ethidium bromide staining following gel electrophoresis. The standard PCR conditions involved initial heating at 94°C for 5 min followed thereafter by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 56°C for 30 sec and primer extension at 72°C for 30 sec. The cycles were followed by a final extension at 72°C for 10 min. The final PCR products (10 µl) were analyzed on 1.5% (w/v) agarose gels.
Immunoblotting analysis of the PsbS1 protein:

For RNA extraction and immunoblot analysis, leaves were harvested 3 h into the light period, frozen immediately in liquid nitrogen, and then stored at -80°C. Isolation of thylakoid membranes for immunodetection of proteins was performed as described by Oh et al. (5). Thylakoid membranes

equivalent to 2~10 µg Chl were solubilized in buffer composed of 62.5 mM Tris-HCl, 10% glycerol, 10% SDS, 2.5% β-mercaptoethanol and 6 M urea for 30 min at room temperature. D1 protein analysis was performed according to the procedure of Miyao (6). After SDS-PAGE, proteins were transferred to nitrocellulose membranes for immunoblotting. After washing, they are incubated with HRP-conjugated secondary antibody (Amersham). The ECL detection system (Amersham) was used to visualize the protein bands.

RESULT

Isolation of a rice PsbS1 knock-out line and generation of PsbS1-RNAi transgenic plants

To confirm that the PsbS1-KO phenotype was caused by the insertion in the *OsPsbS1* gene and not by insertion of multiple T-DNAs or other uncontrolled genetic variations including chromosomal rearrangements, inactivation of loci where the T-DNA is inserted, etc, we generated transgenic rice plants with significantly reduced PsbS1 protein level using RNA interference (RNAi). Rice plants were transformed with an RNAi construct consisting of an inverted repeat of a unique 102 bp region of the *OsPsbS1* gene, with a portion of the pBSIIKS vector as a linker, driven by ubiquitin I promoter (Figure1).

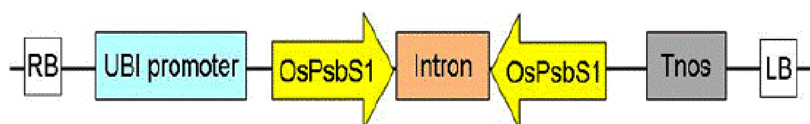

Figure 1. Schematic diagrams of the rice PsbS genes and insertion positions of T-DNA.

RB- right border; UBI promoter- ubiquitin I promoter; *OsPsbS1* an inverted repeat of a unique102 bp of the coding region of PsbS1gene; Intron- a portion of the pBSIIKS vector; Tnos; LB- left border

Transformants (Figure 2) were screened by RT-PCR for *OsPsbS1* transcript levels (Figure 3A) and confirmed by Western blotting (Figure 3B), where we could identify three PsbS1-RNAi lines with varying *OsPsbS1* transcript and PsbS1 protein levels. Among them, the PsbS1-RNAi#2 line produced very little PsbS1.

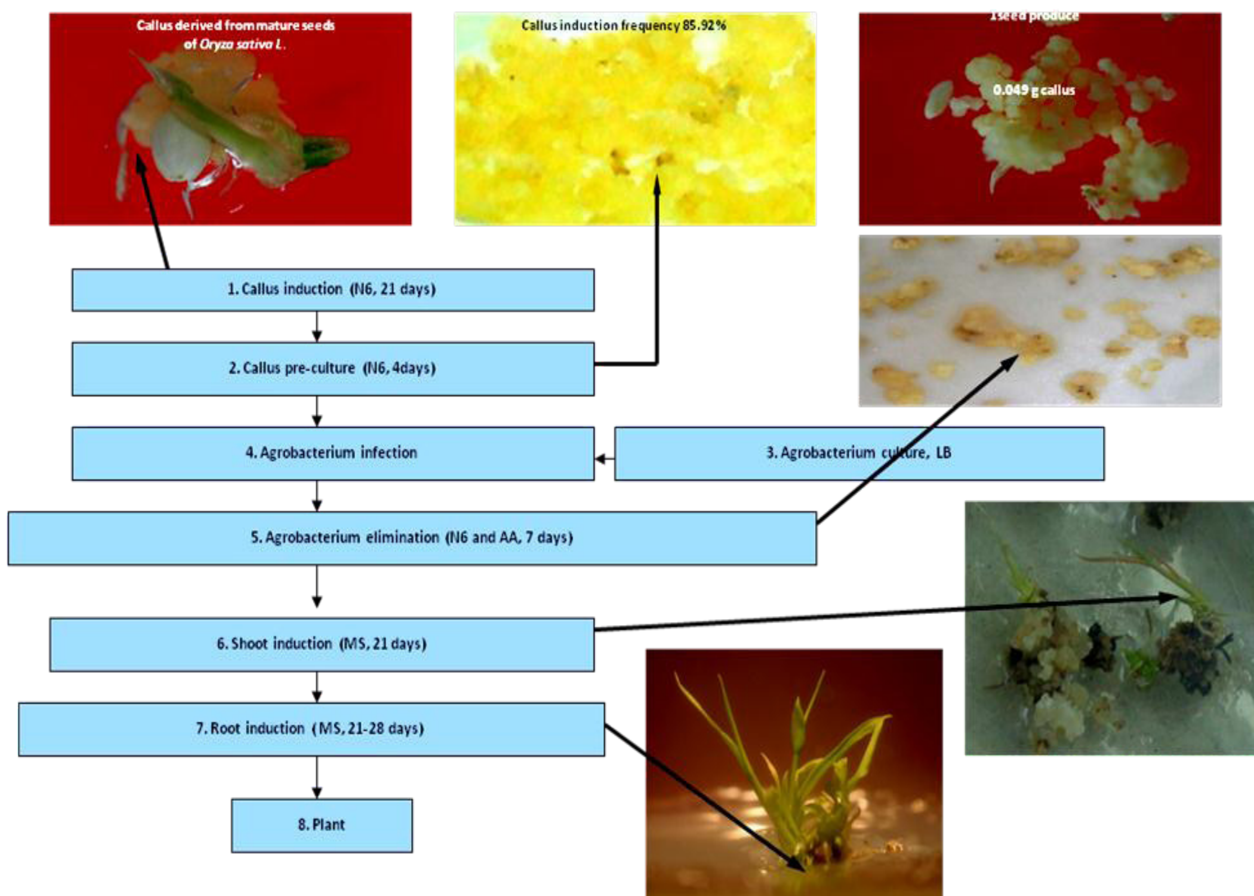

Figure 2. *Agrobacterium tumefacient*-mediated transformation of *Oryza sativa* L

Figure 3. Chracterization of the PsbS1-KO and PsbS1-RNAi rice plants

A. The transcript level of the PsbS1 gene in WT, PsbS1-KO, PsbS1-RNAi construct and Vector only rice plants. The numbers are lines of the PsbS1-RNAi transformants. B. Western blot analysis of the PsbS1-RNAi transformants and vector only rice plants. WT and PsbS1-KO rice plants used as positive and negative control.

SUMMARY

1. We designed and transformed PsbS1-RNAi construct to rice plants.
2. Transformation confirmed by vector-specific primers and by germination of seeds in media with hygromycin.
3. Transformants were screened by RT-PCR for *OsPsbS1* transcript levels, and three PsbS1-RNAi lines without *OsPsbS1* transcript level were identified.

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