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Characterization of an *Arabidopsis thaliana* mutant with impaired *psb*O, one of two genes encoding extrinsic 33-kDa proteins in photosystem II

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Abstract A 33-kDa protein component of the oxygen-evolving complex in photosystem II is essential for photosynthesis, and it has been believed that mutants with deletion of this 33-kDa protein are not found in higher plants. We report here the first isolation of an *Arabidopsis thaliana* mutant with a defect in one of the genes for the 33-kDa proteins, *psb*O, and an intact gene (*psb*O2). This mutant showed considerable growth retardation, suggesting that there is a functional difference between *psb*O and *psb*O2.

Key words: Chlorophyll fluorescence; Oxygen-evolving complex; Photosystem II; *psb*O; *psb*O2

1. Introduction

Photosystem II (PSII) is a pigment–protein complex found in thylakoid membranes that drives light-induced electron transfer from water to plastoquinone with a concomitant evolution of molecular oxygen. An oxygen-evolving complex consisting of three extrinsic proteins of 33 kDa, 23 kDa and 16 kDa is located on the lumen side of thylakoid membranes, where the oxidation of water occurs [1,2]. Among these extrinsic proteins, the 33-kDa protein is present in all oxygen-evolving organisms and plays a crucial role in the water-splitting reaction. The cells of green algae and thylakoid membranes isolated from higher plants have been reported to be unable to evolve oxygen without this 33-kDa protein [3,4]. This 33-kDa protein is also important for stabilizing the PSII core center [5–8].

In *Arabidopsis thaliana*, the 33-kDa proteins are encoded by two genes, *psb*O [9,10] and *psb*O2 [11,12]. Since each PSII complex contains a single molecule of the 33-kDa protein, this duplication of the *psb*O genes suggests that there may be a functional difference between their products.

To isolate *Arabidopsis* mutants with defects in the 33-kDa proteins, we used a chlorophyll fluorescence analysis. While the energy absorbed by chlorophyll pigments in chloroplasts is primarily used for photosynthetic electron transport under light-limited conditions, excess light energy that is not used in photosynthesis is released as heat and fluorescence. Various endogenous and exogenous factors involved in photosynthesis affect the yield of chlorophyll fluorescence emitted from PSII. Therefore, plants with defects in the photosynthetic apparatus or machinery that regulates the use of light energy may exhibit high chlorophyll fluorescence [13,14].

We previously classified the high fluorescence mutants into three groups [13]: photochemical mutants, which had defects in energy utilization, non-photochemical mutants, which had defects in dissipating excessive energy, and finally high-F_o (minimum fluorescence yield at closed PSII centers) mutants, which may have a defect in the oxidizing side of P680 [15]. Plants with any of these defects cannot drive electron transfer efficiently, and the resulting shortage of electrons for photosynthesis activates the expression of genes for PSII components. However, these defects also make the PSII core protein, D1, unstable [7]. Acclimation probably increases the amount of chlorophyll per PSII core, resulting in an increase in F_o. Mutation that affect the overall gene expression in plastids also show high F_o [16], since defects in chloroplast gene expression generally lead to failure of the formation of a functional photosynthetic apparatus, which results in photoinhibition of PSII.

We focused on high-F_o mutants to isolate mutants that have mutations in *psb*O or *psb*O2. We screened high-F_o mutants in which one of the 33-kDa protein genes was lost or was defective, as indicated by protein analysis. By screening 20 high-F_o mutants, we identified a mutant, LE18–30, with a low accumulation of 33-kDa proteins. Molecular characterization of this mutant, which showed retarded growth, indicated that it had a mutation in *psb*O.

2. Materials and methods

2.1. Plant materials

Seeds of wild-type *Arabidopsis* (Landsberg *erecta*) and LE18–30 were sown in soil after cold treatment for 24 h at 4°C. They were grown at 23 ± 0.5°C under white light (50–100 μmol photons m^{-2} s^{-1}) with a light/dark cycle of 9/15 h.

2.2. Molecular characterization

We prepared genomic DNA from both the wild-type and the mu-

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Abbreviations: ΔF, difference between F'_m and steady-state fluorescence yield during illumination; F_o, minimum fluorescence yield at open PSII centers; F_m, maximum fluorescence yield induced by a saturation pulse in the dark-adapted state; F'_m, maximum fluorescence yield during illumination; F_v, $F_m - F_o$; SDS, sodium dodecylsulfate; PSII, photosystem II

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tant according to the method of Shure et al. [17]. We amplified the *psb*O and *psb*O2 sequences by polymerase chain reaction (PCR) with primers designed based on the genomic sequences (the accession numbers for *psb*O are P23321 and O81917 and that for *psb*O2 is Q9S841). Amplified DNA fragments were subcloned into pBluescript (Stratagene), and the nucleotide sequences were determined in both directions with a DNA sequencer (Shimadzu, Kyoto, Japan) using a Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Bioscience).

The similarity and relationships among the 33-kDa proteins of higher plants were evaluated using the CLUSTAL W program [18–20]: accession numbers: *Nicotiana tabacum*, Q40459; *Solanum tuberosum*, P26320; *Pisum sativum*, P4226; *Fritillaria agrestis*, O49079; *Spinacia oleracea*, P12359; *Triticum aestivum*, P27665; *Lycopersicon esculentum*, P23322. The alignment parameters used were: protein weight matrix: BLOSUM series, gap opening penalty: 10.0, gap extension penalty: 0.05 and delay divergent sequences: 40%.

The CLUSTAL W program was used to calculate the phylogenetic tree using the neighbor-joining method.

2.3. Isolation of thylakoid membranes

Thylakoid membranes were isolated from wild-type and mutant leaves about 90 days after germination according to the method of Endo et al. [21]. The chlorophyll concentration was measured by spectrophotometry in 80% acetone [22]. All procedures were performed at 4°C.

2.4. Immunoblot analysis

Thylakoid membrane proteins were separated in a 15% sodium dodecylsulfate (SDS)–polyacrylamide gel [23]. Proteins of 10 μg chlorophyll equivalent were loaded in each lane. The 33-kDa proteins in polyacrylamide gel were transferred to a polyvinylidene difluoride membrane using a semidry blotting system, and detected with rabbit antiserum against the 33-kDa protein of spinach [24], kindly provided by the late Dr. A. Watanabe of the University of Tokyo. Thylakoid proteins of 1 μg chlorophyll equivalent were applied to each gel lane.

2.5. Measurements of chlorophyll fluorescence and oxygen evolution

The patterns of induction of chlorophyll fluorescence in mature rosette leaves were measured under low (30 μmol photons m^{-2} s^{-1}) and high (1000 μmol photons m^{-2} s^{-1}) light conditions with a PAM2000 chlorophyll fluorometer (Waltz, Effeltrich, Germany). F_m and F'_m were measured by applying a 1-s pulse of saturating white light.

Oxygen evolution from thylakoid membranes was measured at 25°C with a Clark-type oxygen electrode (Hanzatech, UK) in the presence of 0.5 mM $K_3Fe(CN)_6$ as an electron acceptor for PSII under red saturation light with an R-60 red long-pass filter (Kenko, Tokyo, Japan).

3. Results

3.1. Identification of psbO mutant

The level of chlorophyll fluorescence emitted from PSII reflects the state of the photosynthetic electron flow. Therefore, we expected that mutants for the 33-kDa protein might exhibit high F_o. Twenty high-F_o mutants were identified by chlorophyll fluorescence imaging [13] and further characterized by immunoblot analysis using antibodies against the 33-kDa protein. A mutant line (LE18–30) obtained by mutagenesis with ethyl methanesulfonate, whose ecotype was Landsberg *erecta*, exhibited a considerable reduction in the amount of the 33-kDa protein (Fig. 1B), but contained a nearly normal amount of total thylakoid proteins per unit amount of chlorophyll and showed a nearly normal protein electrophoretic profile as compared to the wild-type (Fig. 1A).

To characterize LE18–30 genetically, we crossed it with the wild-type Landsberg *erecta*. The F1 generation showed the wild-type phenotype. F2 plants consisted of 28 with the wild-type phenotype and nine with the mutant phenotype.

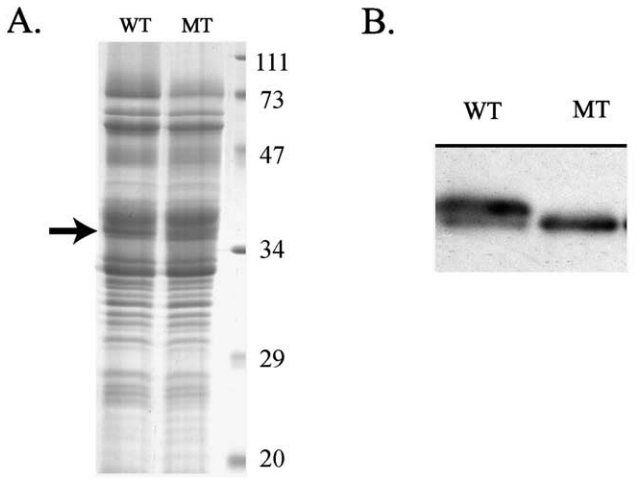

Fig. 1. Coomassie brilliant blue staining of thylakoid proteins (A) and immunoblot analysis with polyclonal antibodies against the 33-kDa protein from spinach (B). The arrow in A shows the 33-kDa proteins. WT, wild-type (Landsberg *erecta*); MT, LE18–30.

This approximately 3:1 ratio of wild-type and mutant phenotypes in the F2 generation (χ^2 value 0.009) suggested that LE18–30 is a recessive mutant with a defect in a single gene.

Genomic analysis of *A. thaliana* showed the presence of two genes, *psb*O and *psb*O2, for the 33-kDa proteins. Immunoblot analysis detected two signals with slightly different molecular weights, suggesting that both genes were expressed in the wild-type plants (Fig. 1B, lane WT). The best separation of the two bands was achieved using 15% polyacrylamide gel. Amino-terminal sequences of these bands were identical up to the fifth residue and were consistent with the common amino-terminal of the deduced sequences from the two genes for the 33-kDa protein. In LE18–30, the signal with a higher molecular weight was missing (Fig. 1B, lane MT), which suggested that one of the genes for the 33-kDa protein is defective in LE18–30. To examine this possibility, *psb*O and *psb*O2 genes were amplified by PCR from the genomic DNA of the wild-type Landsberg *erecta* and from LE18–30. Although there were eight differences between the Landsberg *erecta* nucleotide sequence determined in this study and the sequence reported for the Columbia *psb*O gene, these differences did not result in amino acid sequence alterations. However, the *psb*O gene of LE18–30 had a nucleotide substitution from C to T, which resulted in a nonsense mutation at Gln159 (Fig. 2). Since LE18–30 lacks mature OEC33 to 33-kDa protein, this mutation is at a null allele of *psb*O.

On the other hand, no difference in *psb*O2 was found between the wild-type Landsberg *erecta* and LE18–30. The 29th amino acid in Landsberg *erecta* was Leu (TTA), while it was Ser (TCA) in Columbia.

3.2. Evolutionary relationship between psbO and psbO2

Other than *Arabidopsis*, only pea has been reported to have two genes for the 33-kDa protein [25,26]. Since *psb*O2 of *Arabidopsis* was more similar to *psb*O of *Arabidopsis* than to the genes of pea and other plants (Fig. 3), it is likely that *psb*O2 diverged from *psb*O recently. Whether there are generally two genes for the 33-kDa protein in higher plants is still unclear. However, a detailed protein analysis in spinach revealed only one PsbO polypeptide [12].

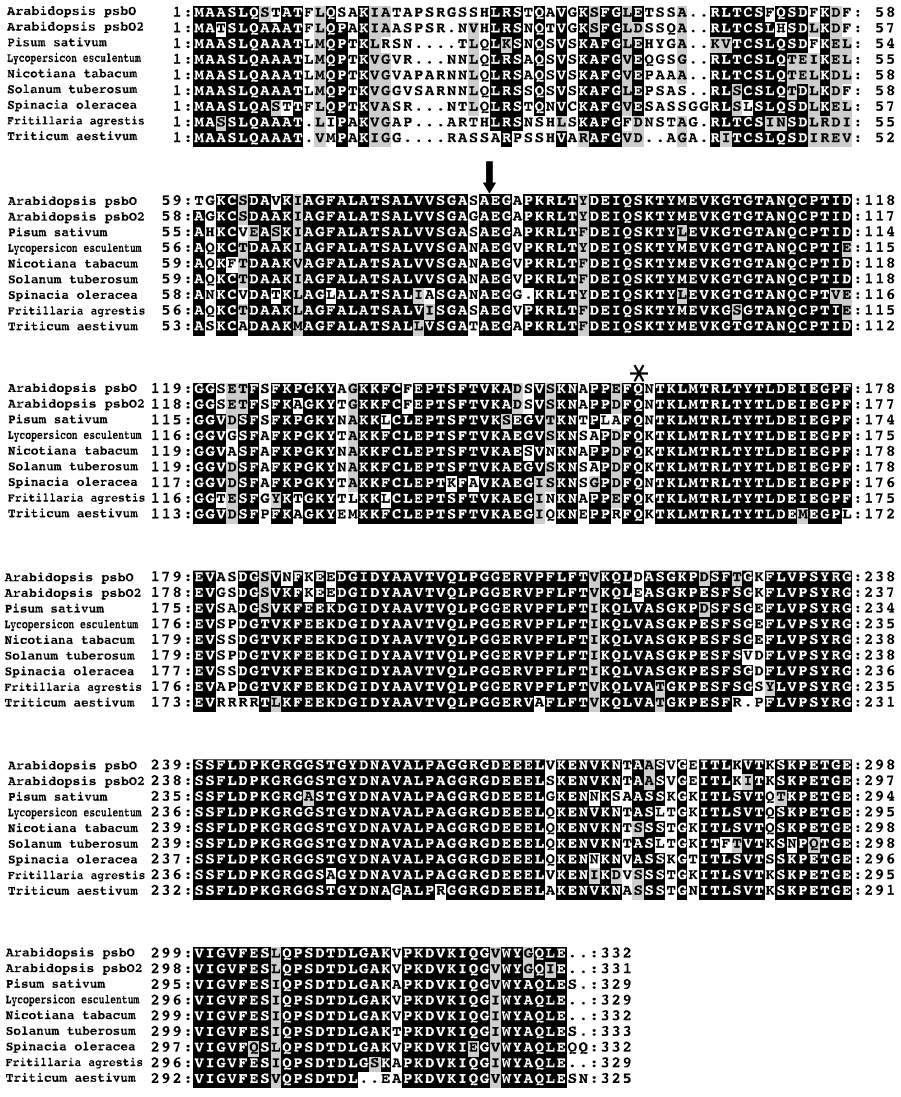

Fig. 2. The amino acid sequences of the 33-kDa proteins synthesized from the *psb*O and *psb*O2 genes in *A. thaliana* and other higher plants. The N-terminal 85 amino acids of *psb*O and 84 amino acids of *psb*O2 are transit peptides. The arrow indicates the start of mature protein. The asterisk shows the mutation point in LE18–30, which results in the formation of a stop codon.

3.3. *Patterns of induction of chlorophyll fluorescence and oxygen evolution*

LE18–30 was first identified by its high-F_o phenotype [13]. We further characterized the patterns of induction of chlorophyll fluorescence using a chlorophyll fluorometer. Chlorophyll fluorescence analysis showed that the potential quantum yield of PSII, expressed as F_v/F_m, was much lower in the mutant (0.5–0.6) than in the wild-type (about 0.8) (Fig. 4). The low F_v/F_m value in the mutant was apparently due to a high F_o. The actual quantum yield of PSII ($\Delta F/F'_m$) in both low and high light was also lower in the mutant than in the wild-type. Oxygen evolution under saturating light in thylakoid membranes from the wild-type and LE18–30 were 67 ± 8.1 μmol O_2 h^{-1} (mg chlorophyll)$^{-1}$ and 28 ± 7.9 μmol O_2 h^{-1} (mg chlorophyll)$^{-1}$, respectively. The lower PSII activities in the mutant might directly result in the slower growth. The fluorescence drop below F_o, observed in LE18–30 with high light, has also been reported in other high-F_o mutants with diverse genetic backgrounds [13,27,28]. This peculiar phenomenon might suggest a light-induced conformational change in PSII which may occur only in partly inactivated PSII. Alternatively, a light-induced state 1-to-2 transition might be markedly stimulated in these mutants.

4. Discussion

We report here the first mutant of one of two 33-kDa proteins in the oxygen-evolving complex in *Arabidopsis*. Immunoblot analysis indicated that the responsible mutation in *psb*O abolished the accumulation of the 33-kDa protein with less mobility on SDS–PAGE in LE18–30 (Fig. 1). The differences in mobility of the two forms of the 33-kDa protein on SDS–PAGE may be explained by differences in p*I* between the two proteins (the mature product of *psb*O is predicted to have p*I* 4.68 and molecular mass (MM) 26 536 Da, while that of *psb*O2 is predicted to have p*I* 4.78 and MM 26 542 Da). Two-dimensional electrophoresis also showed that the product of *psb*O migrated more slowly on SDS–PAGE and was more acidic than that of *psb*O2 [11,12].

LE18–30 showed a severe defect in photosynthetic activity

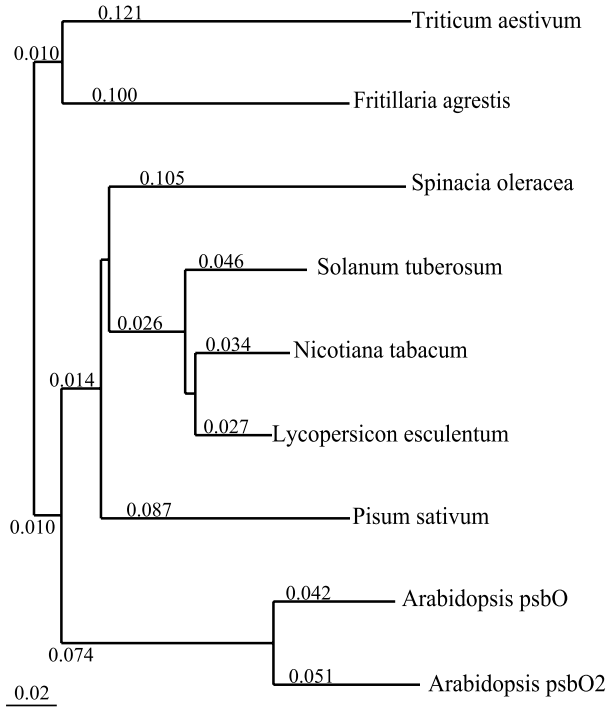

Fig. 3. The similarity among the 33-kDa proteins in *Arabidopsis* and other higher plants. The branch length is proportional to the estimated divergence distance of each protein. The scale-bar (0.02) corresponds to a 2% change.

due to a lack of one of two genes for the 33-kDa protein. However, the total amount of the 33-kDa protein in the mutant was comparable to that in the wild-type, due to an increased accumulation of PsbO2 polypeptide upon deletion of PsbO (see Fig. 1B). These findings suggested a functional difference between the two polypeptides: PsbO might have a higher affinity for the PSII core or support a greater yield of oxygen evolution than PsbO2. Although the mature protein products of these genes have quite similar primary structures, they differ with regard to 10 amino acids (Fig. 2). Surprisingly, there are no differences in the chemical properties of the substituted amino acids, although the negative and positive charges of amino acids have been reported to be important for the interaction between the 33-kDa protein and the PSII complex [2,22,23]. In addition, Cys (amino acids 114 and 137 in the *psb*O product), which is important for forming disulfide bridges [24,29], and Val (amino acid 233 in the *psb*O product), which is important for β-sheet formation, are also conserved [30]. Thus, at present, an important amino acid difference between the two polypeptides that may be responsible for the alteration of protein function cannot be determined. Further analysis of LE18–30 might give us insight to resolve this problem.

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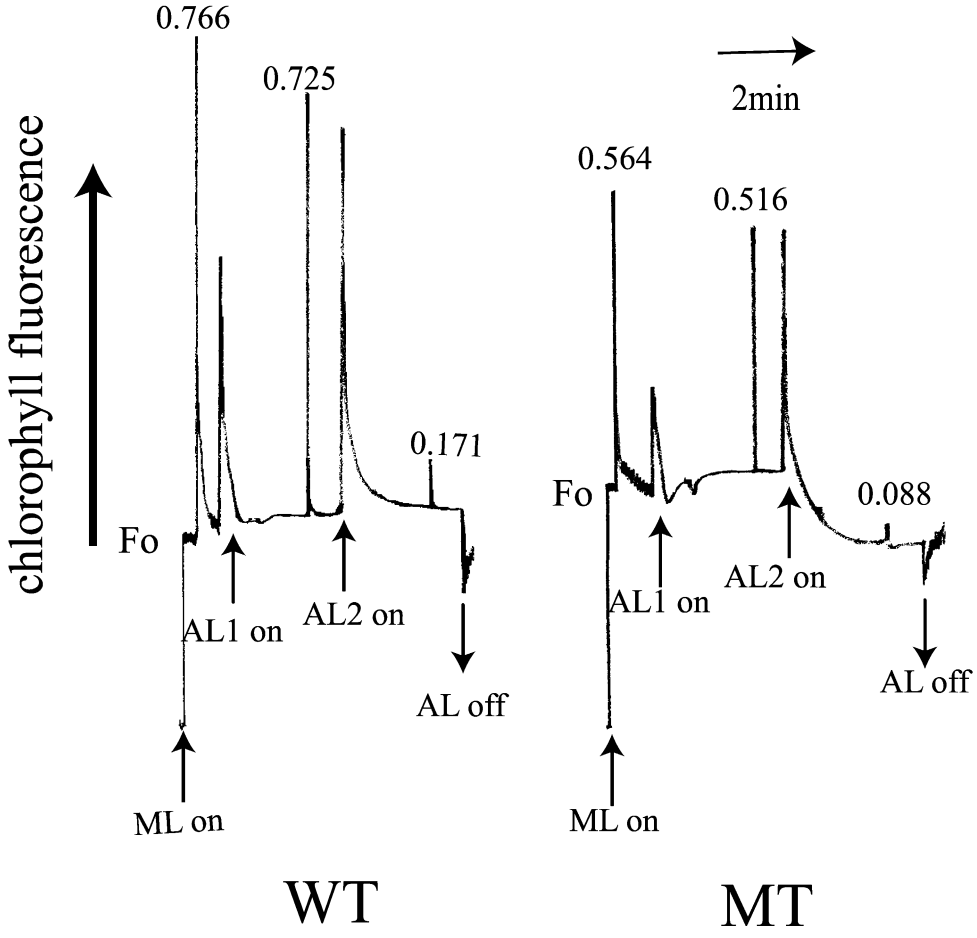

Fig. 4. Chlorophyll fluorescence induction in wild-type and LE18–30. Values show the quantum yield of PSII. AL1, actinic light, 30 μmol photons m^{-2} s^{-1}; AL2, actinic light, 1000 μmol photons m^{-2} s^{-1}; ML, measuring light; WT, wild-type (Landsberg *erecta*); MT, LE18–30.

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