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Identification and Characterization of *LHCB1* Co-Suppressed Line in *Arabidopsis*

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Abstract

To explore the function of light-harvesting complex protein (LHCP) in *Arabidopsis* growth and development, the Leclere and Bartel seed collection was screened. In this collection randomly cloned cDNAs are expressed under the CaMV35S promoter. A pale green line has been identified and characterized in more details. Analysis of the inserted cDNA in the pale green line showed it encodes LHCB1 proteins. The transcript level of *LHCB1* in the selected line was less than that of the wild type (WT) *Arabidopsis* plant. Characterization of the *LHCB1* co-suppressed line was achieved through its comparison with the WT plants when both were grown under normal irradiance. Then several measurements were performed such as: fresh and dry weight, chl*a*, chl*b*, chl*a/b* ratios and total chlorophyll content, soluble and insoluble sugar contents, total protein level, Hill reaction level and chlorophyll fluorescence. The *LHCB1*co-suppressed plants showed smaller leaf area with pale green coloration. Suppression of the *LHCB1* gene significantly reduced fresh weight, chl*a*, chl*b* content, total chlorophyll, and the rate of Hill reaction. Soluble and insoluble sugars, total protein, and chlorophyll fluorescence did not show significant differences between this line and WT plants. Meanwhile there was not any significant difference in fluorescence parameters between the WT and *LHCB1* co-suppressed line.

Keywords: *LHCB1*, *Arabidopsis*, co-suppressed, photosynthesis, growth.

Introduction

Photosynthesis is the only important biological process that uses the energy of the sun and converts it to chemical energy. The light-harvesting chlorophyll *a/b*-binding proteins of photosystem II (LHCII) are the major components of the photosynthetic machinery in plants which contain more than 60% of plant chlorophyll (Peter and Thornber, 1991). The most important role of LHCII is the collecting of excitation energy and transferring it to the reaction centers of photosystem II (PS II) and photosystem I (PS I) to promote photosynthetic electron transport (Van Amerongen and Dekker, 2003). Meanwhile LHCII organizes the plant photosynthetic system by maintaining the tight appression of thylakoid membranes in chloroplast grana and protects this system from excess energy under light saturated conditions (Allen and Forsberg, 2001; Horton *et al.,* 2008). The LHCII complex is composed of six different proteins (Jansson, 1999). Three minor proteins, CP29, CP26 and CP24 are encoded, respectively, by *LHCB4, LHCB5* and *LHCB6* genes. LHCB1, LHCB2 and LHCB3 are the major pigment-binding proteins which are encoded by *LHCB1, LHCB2* and *LHCB3* genes, respectively (Ruban *et al.,* 1999; Lucinski

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and Jackowski, 2006). It has been reported that the *LHCB* gene expression can be regulated by environmental and developmental changes (Vinit *et al.,* 2005; Aghdasi and Schluepmann, 2009; Staneloni *et al.,* 2008). LHCB1 and LHCB2 are the most abundant proteins in the LHCII complex. LHC polypeptides are able to bind chlorophyll a, chlorophyll b, lutein, neoxanthin and xanthophyll molecules (Ruban *et al.,* 1999). The composition and structure of LHCII complex can be changed by light intensity (Bailey *et al.,* 2001). Plants lacking LHCII have previously been generated by introducing antisense constructs into the *Arabidopsis* genome (Anderson *et al.,* 2003). The antisense plants with lack of LHCB1 and LHCB2 retained LHCB3 activity. This plant showed a pale green phenotype with reduced chlorophyll content and an elevated chl*a/b* ratio. By contrast, overexpression of *LHCB1-2* from pea in tobacco plants led to enhanced cell volume, leaf area, biomass and seed weight when grown under low irradiance levels (Labata *et al.,* 2004). It was also reported that the disruption of any member of the LHCB family results in a decreased tolerance to drought stress in the *Arabidopsis* plant (Xu *et al.,* 2012). The disruption of genes via chemical mutagenesis, irradiation, and insertion of T-DNA or transposable elements has been invaluable for dissecting biological pathways. However, many genes remain difficult to uncover as loss-of-function mutations. LeClere and Bartel have developed a system to co-suppress or over-express cDNA in *Arabidopsis*. They constructed a binary vector containing a complex *Arabidopsis* cDNA library driven by the CaMV35S promoter. The T-DNA in this vector contains a bar-gene cassette for phosphinotricine selection of the transgenic plants and a cassette with a randomly cloned cDNA inserted between the CaM35S promoter and nopaline synthase (NOS) polyadenylation (polyA) sequences (LeClere and Bartel, 2001). The inserted cDNA is likely responsible for the observed phenotype if the phenotype and cDNA co-segregate as a dominant trait. So far, the functional significance of *LHCB1* alone in *Arabidopsis* has not been understood very well. In the current study, we identified the *LHCB*1 co-suppressed line by screening the the Leclere and Bartel seed collection. The selected plant was then further characterized in more detail.

Materials and Methods

Plant materials, growth conditions and screening

The *Arabidopsis thaliana* wild type (WT) plant ecotypes Columbia-0 (COL-0) and *LHCB1*co-suppressed seeds were planted in compost and watered twice per week. Plants were grown in a controlled growth chamber under normal (150 µmol photon $m^{-2}s^{-1}$) irradiances and a 25 °C day/ 20 °C night temperature regime. Seeds from the LeClere and Bartel (2001) collection were obtained from ABRC stock center. Seeds were surface-sterilized by the chlorine gas (Clough and Bent, 1998). Sterilized seeds were plated on ½ Murashige and Skoog (MS) medium solidified with 0.8% agar (Murashigeand Skoog, 1962). Seeds were stratified in darkness at 4 °C for 2 days, before transferring a to growth chamber at 25 °C. A pale green plant was characterized from this collection. The pale green plant was transferred to soil to generate second seed generation (T2). Seeds from T2 generation plants were grown on medium with 12.5 mg/L Phosphinotrice (PPT).

Seedlings resistant to PPT from the secondary screen were transferred to soil along with WT. Upon flowering of the plants, crosses were carried out with the WT plants. The individual siliques were

collected in one bag after ripening (Aghdasi *et al.,* 2012).

Molecular characterization of the pale green line

To determine the presence of the 35S cDNA fragments in the pale green line, PCR was performed with primers 35S-F (CGCAC AATCCCACTATCCTTCCAAG) and Nos-R (GATAATCATCGCAAGACCGGAACA GG) primers. DNA amplification was performed by an initial cycle at 94 °C for 2 min, 35 cycles at 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 2 min. PCR was completed with a final step at 72 °C for 5 min. An aliquot from the PCR product was run on a 1% agarose gel and the remaining amount was purified using a DNA purification kit (Amersham Biosciences, England). The resulting cDNA fragments from the previous step were ligated into the pGEM-T Easy vector (Promega). For this purpose, cDNA was concentrated to a final volume of 3 µl (25 ng) and was then added to 5 µl of 2×ligation buffer, 1 µl of T4 Ligase and 1 µl of pGEM-T easy vector. The ligation mixture was incubated over night at room temperature. An aliquot (100 µl) of competent *E. coli* were taken from the -80 °C freezer and thawed on ice for 20 min. The overnight ligation mixture was added to the cells. The mixture was left on the ice for 20 min. Heat shock was applied for 50 sec at 42 °C, followed by a 5 min cooling period on ice. One ml of medium was added and cells were incubated at 37 °C for 1 h. The LB plates contained 50 µg/ml of ampicillin for selection. Isopropyl-β-D-thiogalactopyranosi-d (IPTG) and X-Galacto pyranoside (X-Gal) were added for screening of blue and white colonies. To check colonies containing the plasmid with the ligated fragment, restriction enzyme analysis was performed. Plasmids were isolated from 5 colonies using a plasmid miniprep kit (Sigma, USA). In the digestion mixture, 2 µl of plasmid, 1 µl of 10 X buffer, 6 µl of milli-Q water and 1 µl of *Eco*R1 were used. Samples were digested at 37 °C for 1.5 hours. The obtained fragments were analyzed by agarose gel electrophoresis.
Sequences obtained from using forward and reverse primers (T7: 5′-TATTTAGGTGAC ACTATAG- 3′and SP6: 5′-TAATACGACT CACTATAGGG- 3′) were aligned and the PCR fragment structure was reconstructed by BLAST (Basic Local Alignment Search Tool) searches in TAIR (http://www. arabidopsis. org/Blast/).

RNA extraction, cDNA synthesis and Q-PCR analysis

Total RNA was extracted from 10 days old *Arabidopsis* plants. Whole plant material was snap frozen in liquid nitrogen and pulverized with glass beads for 2 min at 2800 rpm in a dismembrator (Braun, Melsungen, Germany). Total RNA was isolated with the RNeasy plant mini kit (QIAGEN USA, Valencia, CA). RNA concentration and purity were determined by measuring the absorbance at 260 nm. Following treatment of RNA with DNAase I (DNA-free, Ambion, Austin, USA), first strand c-DNA was synthesized by reverse transcriptase PCR (RT-PCR) using 1 ng of total extracted RNA, 60 U of M-MLV reverse transcriptase (Promega, Madison, WI), 0.5 µg of odT16v (custom oligo from Invitrogen, Carlsbad, CA) and 0.5 µg of random hexamer (Invitrogen, USA). PCR was then performed with reverse and forward primers 5′-CTCAACAATGGC TCTCTCCT- 3′and 5′-AACCCAAGA ACTGAAAATCCAA-3′). Amplification conditions were 94 °C for 2 min followed by 35 cycles of 1 min denaturation at 94 °C, 30 second annealing at 56 °C and 2 min of extension at 72 °C with a final extension time at 72 °C for 10 min. The PCR product was run on 1% agarose gel.

Quantitative PCR (Q-PCR) was carried out using ABI-prism 7700 Sequence Detection System (PE-AppliedBiosystems, Foster City, CA). Each reaction contained 12.5 µl of CYBR green PCR Master Mix (Applied Biosystems, UK) and 2.5 µl of *LHCB1* specific primers. Relative quantitation of gene expression was based on the comparative Ct method (User Bulletin No. 2: ABI PRISM 7700 Sequence Detection system, 1997) using *AtACTIN2* as a calibrator reference. The results are expressed as a target/reference ratio.

Chlorophyll determination

Chlorophyll was extracted with 80% (v/v) acetone and determined as described by the methods of Jeffery and Humphery (1975).

Fluorescence measurement

Chlorophyll fluorescence was measured with a OPTI-Sciences OS-30 fluorometer (Walz, Effeltrich, Germany). The *Arabidopsis* plants were adapted in the dark for 15 minutes before measurement. F0 (the initial fluorescence level of PSII reaction center) was measured in the presence of a 10 µmol photons $m^{-2}s^{-1}$ measuring beam. The maximum fluorescence level in the dark adapted state (*Fm*) was determined by using a 0.8 sec saturating irradiance pulse. The fluorescence parameter *Fv/Fm* was calculated using the DualPAM software.

Chloroplast isolation and determination of Hill reaction rate

The rate of Hill reaction in the chloroplast preparations of WT and *LHCBI* co-suppressed plants was measured according to Trebst (1972). Leaves (0.25 g) were homogenized in a cold mortar in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 0.3 M sucrose, 10 mM EDTA and 5 mM MgCl2 and chloroplasts were isolated. The rate of Hill reaction in the illuminated chloroplast preparations was determined spectrophotometrically by recording the decrease in absorbance at 600 nm due to Dichlorophenol indo-phenol (DCPIP) reduction. The rate of Hill reaction was expressed as the changes in absorbance per milligram chlorophyll per minute (ΔOD. min^{-1} mg chl^{-1}).

Carbohydrate and protein determination

The soluble and insoluble sugars were determined spectrophotometrically by the phenolsulfuric acid method (Chapin and Kennedy, 1987). Soluble and total leaf protein concentrations were determined according to methods of Bradford (1976) and Markwell (1988), respectively.

Statistical analyses

Data from all experiments were processed using the statistical SAS package (version 9). The reported values were means of three replicates. Means were compared for significance using the Duncan's test.

Results

Screening of Leclere and Bartel Seed Collection

Leclere and Bartel (2001) generated a collection of 331 pools of *Arabidopsis* transgenic lines that express a random 35S cDNA together with a Basta resistance gene inserted in the T-DNA of the 35SpBARN vector. In the primary screen, seeds from all 331 pools were screened on soil under normal irradiance condition. Seedlings growth and phenotype was monitored over a period of 5 weeks. A pale green line was identified during primary screen. The pale coloration was uniformly displayed by all leaves throughout the whole life of the mutant (Fig. 1). The selected line were then

transferred to soil and allowed to self-pollinate. Seeds from plants identified during the primary screen were subjected to a secondary screen. The progeny of the T2 plants were tested again for pale green phenotype. Meanwhile seeds fro m the selected line were germinated on MS medium with 12.5 mg/l PPT to analyze segregation of the T-DNA insert carrying the CaMV35 promoter-driven cDNA expression cassette (Fig. 2A). Segregation analysis on PPT showed that the line was homozygous for the T-DNA insertion. Co-segregation of resistance to PPT (flanked to T-DNA) and the pale green phe notype confirmed that the phenotype of the pale green line has co-segregated with T-DNA insertion (data not shown). After this secondary screen, the progeny of the T2 plants, named the 268 line (LeClere and Bartel collection pool number), were selected for further characterization.

Inserted cDNA in the 268 line encodes LHCB1 Protein

The cDNA contained within 268 line was identified by PCR using a forward primer on the CaMV35S promoter and a reverse primer on the nopaline synthase poly-adenylation sequence. Control PCR reactions were on DNA extracted from WT. No fragment was amplified from WT DNA. PCR reactions yielded only one fragment in the 268 line. The fragment size of the amplified PCR product was 850 bp (Fig. 2B).

Sequence of the PCR product of the cDNA fragment was perfectly matched the cDNA of At1g29920, encoding LHCB1 protein. The inserted cDNA was full length, with ATG and TGA and in-sense orientation.

Figure 1. Phenotype of the LHCB1co-suppressed line (co-LHCB1), as compared to the wild type (WT) Columbia-0ecotype.

Expression analysis of *LHCB1*

Expression level of endogenous *LHCB1* was determined in seedlings of WT plants and the 268 line. The expression level of *LHCB1* in the selected line was significantly lower than that of the WT plant and this is consistent with the light green color of the selected plant leaves. This indicates that in the selected line, the pale green phenotype is due to co-suppression of *LHCB1* gene (Fig. 2C). This plant hereafter is named the *LHCB1*co-suppressed line.

Pale green phenotype inherited as a dominant trait in the *LHCB1* co-suppressed line

To find out whether the pale green trait is dominant or recessive, backcrossing between the selected line and WT was performed. Analysis of mode of segregation of resistance to PPT in the selected line revealed that PPT resistance segregates as a single locus. Segregation pattern of pale green phenotype that heterozygotes were also pale green. Segregation of 3:1 in populations from backcrosses (270 pale green plants to 108 green plants; χ^2=2.44, $p<0.05$), indicated that pale phenotype was linked to the inserted T-DNA. Segregation analysis revealed that Pale green trait segregates as a single locus.

LHCB1 suppression affects plant growth and morphology

The *LHCB1*co-suppressed line showed pale green phenotype with smaller leaf area compared to WT when grown on soil under normal irradiances (Fig. 1). There was no significant difference in height and flowering time between *LHCB1*co-suppressed line and WT plants. The height of 4-week old *LHCB1*co-suppressed and WT plants were 13.66±3.16 and 15±1 cm respectively (Table 1).

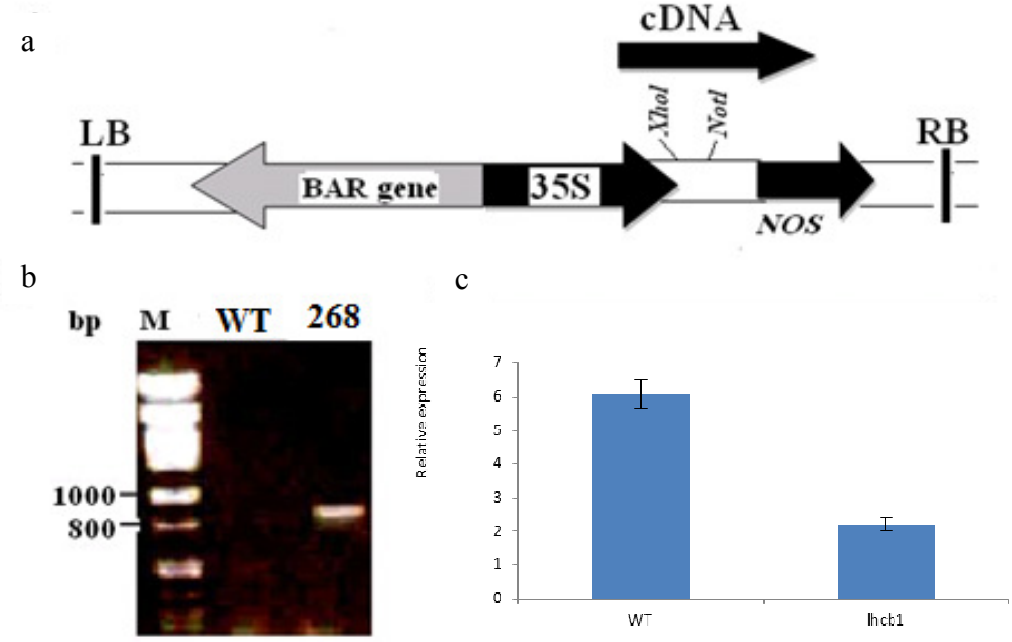

Figure 2. (a) Structure of the T-DNA insert in plants from the LeClere and Bartel collection. Arrows indicate the direction of transcription. The Bar gene is expressed under control of the 1´promoter conferring BASTA resistance to plants transgenic with the T-DNA. The randomly cloned cDNA is expressed under the control of the CaMV35S promoter and mRNA of the cDNA is further stabilized by NOS polyA sequence. (b) PCR amplifiction of the unknown cDNA by 35S and nos primers in WT plant and 268 line, (c) Q-PCR analysis of LHCB1 expression level in WT plant and co-suppressed line. WT: Wild Type, 268: selected line number, M: size markers.

Characterization of *LHCB1*co-suppressed line

The *LHCB1*co-suppressed line was further characterized in more detail by comparing to WT plants when grown on soil under normal irradiances. Compared to WT, dry and fresh weights were significantly reduced in the *LHCB1*co-suppressed line. Dry and fresh weights (FW) were, respectively, 0.087±0.01 and 0.70±0.08g in the WT, while they were 0.035±0.01 and 0.50±0.15g in the *LHCB1*co-suppressed line (Table 1). The measurement of soluble and insoluble sugar content showed that there was no significant difference between the *LHCB1*co-suppressed line and WT plants (Fig. 3A). Similarly, total protein content was similar in both the *LHCB1*co-suppressed line and WT plants (Fig. 3B).

Chlorophyll contents and photosynthesis capacity in the *LHCB1* co-suppressed line

We further examined whether *LHCB1* suppression affected photosynthetic parameters. There was a significant difference in chlorophyll a, chlorophyll b and the total chlorophyll between the *LHCB1* co-suppressed line and WT seedlings. The total chlorophyll content of WT plant leaves was twice that of the

LHCB1 co-suppressed line leaves (Fig. 4A). There was an increase in the ratio of Chl*a/b* in the *LHCB1*co-suppressed line compared to WT plants (Fig. 4B).

The *Fv/Fm* value measurement was performed as an indicator of the intrinsic efficiency of PSII. *LHCB1*co-suppressed line and WT plants showed the similar fluorescence parameters (*Fv/Fm*) when grown under normal irradiances (Fig. 5A). These data indicated that PSII efficiency was not affected by the suppression of *LHCB1*.

In this study we measured the water oxidation capacity of the photosynthetic machinery of both WT and *LHCB1* co-suppressed plants under normal irradiance condition. The water oxidation capacity was significantly decreased in the *LHCB1* co-suppressed line compared to WT plants (Fig. 5B).

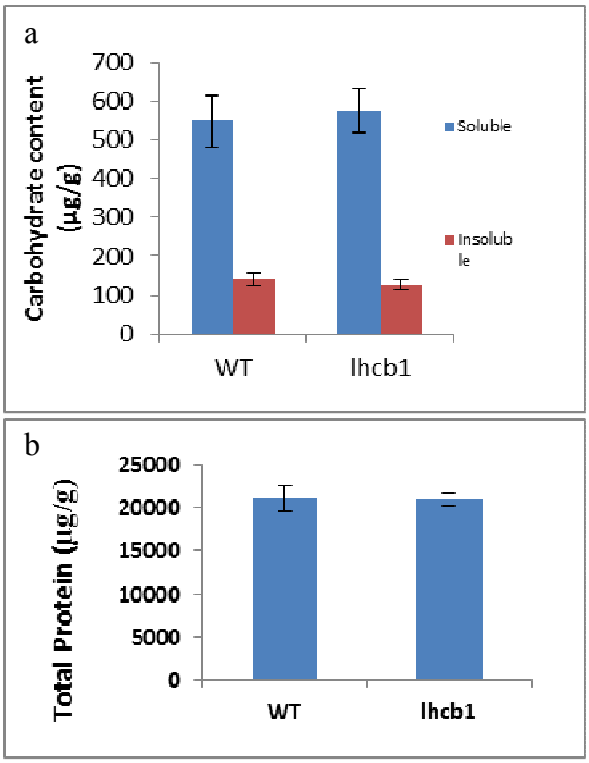

Figure 3. (a) Carbohydrate and (b) total protein contents of Arabidopsis wild type (WT) and LHCB1 co-suppressed (co-LHCB1) plants grown under normal irradiance conditions

Discussion

LeClere and Bartel have designed a system to co-suppress or over-express cDNA in *Arabidopsis*. This method has three major advantages in that 1) the inserted cDNA can be amplified using PCR with primers in the promoter and polyA sequences, 2) random insertion gene disruption and the 3) silencing of the cDNA-corresponding endogenous gene (LeClere and Bartel 2001).

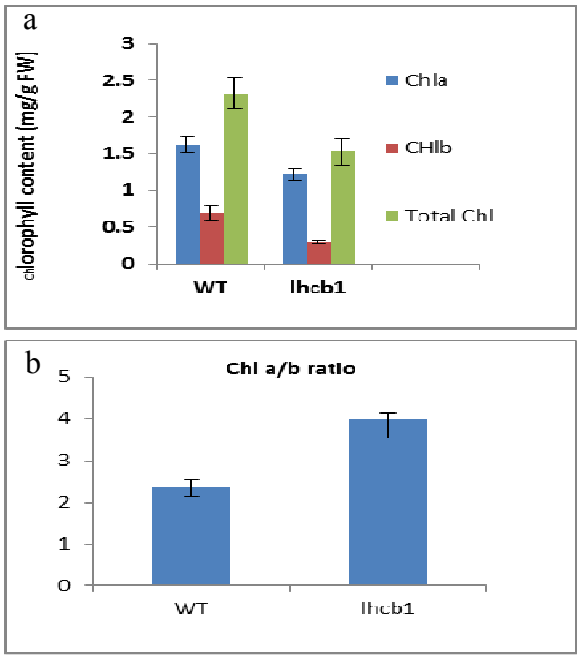

Figure 4. (a) Chlorophyll content (chl) and (b) Chlorophyll ratio (chla/b) of Arabidopsis wild type (WT) and LHCB1 co-suppressed (co-LHCB1) plants grown under normal irradiance conditions.

Here we reported isolation of an *LHCB1* co-suppressed line during screening of 331 pools of T4 seeds from the Leclere and Bartel collection. Definitive confirmation for the correlation between cDNA and the phenotype in plants exhibiting dominant trait, could be obtained following the transformation of cDNA expression cassette into WT plants. Characterization of the *LHCB1* co-suppressed line showed that suppression of the *LHCB1* gene affects dry and fresh weight, chlorophyll content and

water oxidation capacity of the photosynthetic machine.

Plants lacking LHCII protein have previously been generated by mutation of the minor LHC complex (Andrew *et al.,* 1995) or antisense co-suppression of *LHCB1-2* (Andersson *et al.,* 2003) . Studies carried out so far to reveal the functional significance of LHCII protein-chlorophyll complexes in phenotypic alterations of plants have suffered from segregating the specific role played by each individual polypeptide constituting the complex. In this work, we have been successful in producing homozygous *LHCB1*co-suppressed line.

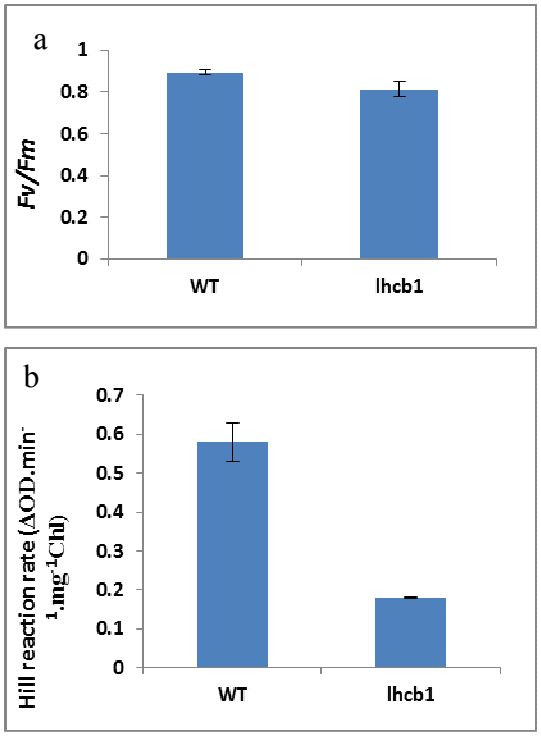

Figure 5. (a) Photosynthetic parameter and (b) water oxidation capacity of WT and LHCB1 co-suppressed (co-LHCB1) plants grown under normal irradiance conditions.

The *LHCB1* co-suppressed plant generated in this research has significantly reduced chlorophyll content compared to WT plants. This was evidenced by their pale green appearance and an elevated in Chl*a/b* ratio. The significant reduction of biomass and leaf area has also been reported for *lhcb1-2* antisense plants (Andersson *et al.,* 2003). These may be because of the significant reduction of water oxidation capacity of the co-suppressed line versus WT plants (Fig. 5b). But suppression of *LHCB1* did not change the quantum efficiency of PSII (Fig. 5a).

Table 1. Data on biomass of Arabidopsis wild type (WT) and *LHCB1* co-suppressed (co-*LHCB1*) plants grown under normal irradiance conditions.

	Plant height (cm)	number of days to flowering	dry weight (g)	fresh weight (g)
WT	15.00±1	38	0.087±0.01	0.70±0.08
co-LHCB1	13.66±3.1	36	0.035±0.01	0.50±0.15

It is expected that *LHCB1* co-suppresses lines show reduced non-photochemical quenching and feedback de-excitation as compared to WT plants. This possibly makes them more susceptible to photoinhibitory conditions which ultimately reduce their fitness.

Considering that LHCB1 is a major target protein for phosphorylation / de-phosphorylation required for state transition (Lunde *et al.,* 2000), its suppression in co-suppressed line might decreases the capacity for state transition, a feature which has been reported for *lhcb1-2* antisense plants. On the other hand, the lack of *LHCB1-2* in *Arabidopsis* mutants is associated with reduced non-photochemical quenching (Anderson *et al.,* 2003).

In conclusion, our findings suggest that the suppression of *LHCB1* can affect *Arabidopsis* growth and development. The functional significance of *LHCB1*in plant growth and development should be confirmed by over-expression of *LHCB1* in WT plants and in the *LHCB1* co-suppressed line.

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