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RESEARCH PAPER

Light-harvesting chlorophyll *a*/*b*-binding proteins are required for stomatal response to abscisic acid in *Arabidopsis*

Yan-Hong Xu[1,2,*], Rui Liu[1,2,*], Lu Yan[1,2], Zhi-Qiang Liu[1,2], Shang-Chuan Jiang[2], Yuan-Yue Shen[1,2], Xiao-Fang Wang[2] and Da-Peng Zhang[2,†]

[1] College of Biological Sciences, China Agricultural University, Beijing 100094, China
[2] Bioinformatics and Systems Biology Laboratory of the Ministry of Education, Scholl of Life Sciences, Tsinghua University, Beijing 100084, China

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: zhangdp@tsinghua.edu.cn

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Abstract

The light-harvesting chlorophyll *a*/*b* binding proteins (LHCB) are perhaps the most abundant membrane proteins in nature. It is reported here that the down-regulation or disruption of any member of the LHCB family, LHCB1, LHCB2, LHCB3, LHCB4, LHCB5, or LHCB6, reduces responsiveness of stomatal movement to ABA, and therefore results in a decrease in plant tolerance to drought stress in *Arabidopsis thaliana*. By contrast, over-expression of a LHCB member, LHCB6, enhances stomatal sensitivity to ABA. In addition, the reactive oxygen species (ROS) homeostasis and a set of ABA-responsive genes are altered in the *lhcb* mutants. These data demonstrate that LHCBs play a positive role in guard cell signalling in response to ABA and suggest that they may be involved in ABA signalling partly by modulating ROS homeostasis.

Key words: Abscisic acid signalling, *Arabidopsis thaliana*, light-harvesting chlorophyll *a*/*b* binding protein, reactive oxygen species, stomatal movement.

Introduction

The light-harvesting chlorophyll a/b-binding (LHCB) proteins are the apoproteins of the light-harvesting complex of photosystem II (PSII), which are normally complexed with chlorophyll and xanthophylls and serve as the antenna complex (Jansson, 1994, 1999). As important components of the major light-harvesting complex, the PSII outer antenna proteins LHCBs are perhaps the most abundant membrane proteins in nature. Expression of the *LHCB* genes is regulated by multiple environmental and developmental cues, including mainly light (Silverthorne and Tobin, 1984; Sun and Tobin, 1990; Peer *et al.*, 1996; Millar and Kay, 1996; Weatherwax *et al.*, 1996; Yang *et al.*, 1998; Humbeck and Krupinska, 2003), oxidative stress (for reviews, see Nott *et al.*, 2006; Staneloni *et al.*, 2008), chloroplast retrograde signal (for review, see Nott *et al.*, 2006), circadian clock (Paulsen and Bogorad, 1988; Strayer *et al.*, 2000; Alabadi *et al.*, 2001; Thain *et al.*, 2002; Andronis *et al.*, 2008), and the phytohormone abscisic acid (ABA) (Bartholomew *et al.*, 1991; Chang and Walling, 1991; Weatherwax *et al.*, 1996; Staneloni *et al.*, 2008). Previous studies showed that exogenously-applied ABA down-regulates *LHCB* gene expression in tomato leaves (Bartholomew *et al.*, 1991), *Arabidopsis* seedlings (Staneloni *et al.*, 2008), *Lemna gibba* cells grown on liquid medium (Weatherwax *et al.*, 1996), and developing seeds of soybean (Chang and Walling, 1991), whereas a recent report showed that the treatments of the 6-d-old *Arabidopsis* seedlings with low levels of ABA (from 0.125 to 1 μM) enhanced *LHCB1.2* mRNA levels (Voigt *et al.*, 2010). The regulation of the *LHCB* expression is considered to be one of the

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important mechanisms for plants to modulate chloroplast functions (Nott *et al.*, 2006; De Montaigu *et al.*, 2010; Pruneda-Paz and Kay, 2010; Thines and Harmon, 2010).

ABA is a vital phytohormone to regulate many aspects of plant growth and development, and especially to modulate the plant response to stressful conditions (Finkelstein *et al.*, 2002; Adie *et al.*, 2007). ABA signal transduction has been extensively studied, and numerous signalling components have been identified, which include plasma membrane and intracellular ABA receptors (Shen *et al.*, 2006; Fujii *et al.*, 2009; Ma *et al.*, 2009; Pandey *et al.*, 2009; Park *et al.*, 2009; Wu *et al.*, 2009; Cutler *et al.*, 2010; Shang *et al.*, 2010). Previous reports showed that the members of the LHCB family play an important role in plant adaptation to environmental stresses (Andersson *et al.*, 2001, 2003; Ganeteg *et al.*, 2004; Kovacs *et al.*, 2006), as well as their expression being regulated by ABA (Bartholomew *et al.*, 1991; Chang and Walling, 1991; Weatherwax *et al.*, 1996; Staneloni *et al.*, 2008). However, it remains unknown whether the decline of plant stress tolerance due to a lack of the LHCB proteins is associated with the plant response to ABA under environmental stresses. It is reported here that the *Arabidopsis* LHCBs are positively involved in guard cell signalling in response to ABA, and they may affect ABA signalling partly by modulating ROS homeostasis. These findings help understand the complex mechanism of ABA signalling and the positive role of LHCB proteins in plant stress tolerance.

Materials and methods

Plant materials

Arabidopsis thaliana ecotype Columbia (Col-0) was used in the generation of transgenic plants. The open reading frame (ORF) cDNA of the *LHCB6* gene (At1g15820) was introduced into Col plants as a green fluorescence protein (GFP)-fusion protein to generate *LHCB6*-over-expressing transgenic lines. The cDNA was isolated by polymerase chain reaction (PCR) using the forward primer 5′-GCTCTAGAATGGCGATGGCGGTCTCC-3′ and reverse primer 5′-CGGTCGACTCACAAACCAAGAGCACCGAG-3′. The cauliflower mosaic virus (CaMV) 35S::*LHCB6* chimeric gene construct was generated by ligating the ORF (777 bp) of the *LHCB6* gene into the pCAMBIA1300 vector by *Xba*I and *Sal*I sites. The construct was confirmed by sequencing, and introduced into the GV3101 strain *Agrobacterium tumefaciens* and transformed into plants by floral infiltration. The homozygous T3 seeds of the transgenic plants were used for analysis. More than 20 *LHCB6*-over-expressing transgenic lines were screened, all of which showed ABA hypersensitivity in stomatal movement, and four representative lines have been shown (see Supplementary Fig. S2 at *JXB* online).

The T-DNA insertion mutants *lhcb1.1* (SALK-134810) in the *LHCB1.1* gene (At1g29920; referred to as *LHCB1* and representative of *LHCB1.1*, *LHCB1.2*, *LHCB1.3*, *LHCB1.*, and *LHCB1.5*), *lhcb2.2* (SALK-005614) in the *LHCB2.2* gene (At2g05070; referred to as *LHCB2* and representative of *LHCB2.1*, *LHCB2.2*, *LHCB2.3*, and *LHCB2.4*), *lhcb3* (SALK-036200) in the *LHCB3* gene (At5g54270), *lhcb4.3* (SALK-032779) in the *LHCB4.3* gene (At2g40100; referred to as *LHCB4* and representative of *LHCB4.1*, *LHCB4.2*, and *LHCB4.3*), *lhcb5* (SALK-139667) in the *LHCB5* gene (At4g10340), and *lhcb6* (SALK-074622) in the *LHCB6* gene (At1g15820) were used in this study and the seeds of these mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). The screening for the knockout or knockdown mutants was done following the recommended procedures. The sequences of the primers for the screening are presented in Supplementary Table S1 at *JXB* online. The T-DNA insertion in the mutants was identified by PCR and DNA gel-blot analysis and the exact position was determined by sequencing. The mutants *lhcb1.1* (SALK-134810), *lhcb2.2* (SALK-005614), *lhcb4.3* (SALK-032779), *lhcb5* (SALK-139667), and *lhcb6* (SALK-074622) are also knockdown mutants in their corresponding genes except for the mutant *lhcb3* (SALK-036200) that is a knockout mutant in the *LHCB3* gene. DNA gel-blot analysis showed that all the mutants have one single copy of T-DNA in their genome.

All the double mutants were generated by genetic crosses and identified by PCR genotyping.

Plants were grown in a growth chamber at 19–20 °C on Murashige-Skoog (MS) medium (Sigma) at about 80 μmol photons m^{-2} s^{-1}, or in compost soil at about 120 μmol photons m^{-2} s^{-1} over a 16 h photoperiod.

Complementation of the lhcb mutants

The ABA-related phenotypes of the *lhcb1*, *lhcb2*, *lhcb3*, *lhcb4*, *lhcb5*, and *lhcb6* mutants were complemented by introducing into the mutant plants, respectively, the *LHCB1*, *LHCB2*, *LHCB3*, *LHCB4*, *LHCB5*, and *LHCB6* ORF cDNAs driven by the 35S promoter integrated into the pCAMBIA1300-221 vector. The primers for cloning the *LHCB1*, *LHCB2*, *LHCB3*, *LHCB4*, *LHCB5*, and *LHCB6* ORF cDNAs are listed in Supplementary Table S1 at *JXB* online.

Protein extraction and immunoblotting

The extraction of the *Arabidopsis* total proteins was performed essentially according to procedures proposed by the LHCB-antibody supplier Agrisera. The plant tissues were frozen in liquid N_2, ground in a pre-chilled mortar with a pestle to a fine powder and transferred to a 1.5 ml tube. The extraction buffer consists of 50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v)Triton X-100, 10% (v/v) glycerol, and 5 μg ml^{-1} protein inhibitor cocktail. The extraction buffer was added to the tube (buffer:sample=4:1), which was immediately frozen in liquid N_2. The mixture was carefully subjected to sonication just until the sample was thawed, and was re-frozen immediately in liquid N_2 to avoid heating. The sonication step was repeated three times. The mixture was centrifuged for 3 min at 10 000 *g* to remove insoluble material and unbroken cells and the supernatant was transferred to a new tube for use. The SDS-PAGE and immunoblotting assays were done essentially according to our previously described procedures (Wu *et al.*, 2009; Shang *et al.*, 2010). The specific antibodies against, respectively, LHCB1, LHCB2, LHCB3, LHCB4, LHCB5, and LHCB6 were purchased from Agrisera (Stockholm, Sweden; website:www.agrisera.com; product No. AS08300).

Real-time PCR analysis

Total RNA was isolated from leaves of 2-week-old Arabidopsis seedlings using a Total RNA Rapid Extraction Kit (BioTeke), treated with RNase-free DNase I (TAKARA) at 37 °C for 30 min to degrade genomic DNA and purified by using an RNA Purification Kit (BioTeke). A 2 μg aliquot of RNA was subjected to first-strand cDNA synthesis using M-MLV reverse transcriptase (Promega), and an oligo (dT21) primer. The primers used for real-time PCR are listed in Supplementary Table S1 at *JXB* online. Analysis was performed using the Bio-Rad Real-Time System CFX96TM C1000 Thermal Cycler (Singapore). All experiments were repeated at least three times along with three independent repetitions of the biological experiments.

Chlorophyll measurements

The contents of chlorophyll were assayed essentially by the previously described procedures (Shen *et al.*, 2006).

ROS measurements

ROS detection in whole leaves was conducted by nitroblue tetrazolium (NBT) staining, essentially as described previously by Lee *et al.* (2002). Leaves were sampled from 3-week-old plants and preincubated in a medium composed of 50 mM KCl, 10 mM MES-TRIS (pH 6.15) supplemented with different concentrations of (±)-ABA (as indicated) under light at 200 μmol m^{-2} s^{-1} for 1 h, and then the leaves were vacuum-infiltrated with 0.1 mg ml^{-1} NBT (Amresco, Solon, OH, USA) in 100 mM potassium phosphate buffer, pH 7.6. Samples were incubated at room temperature in the dark for 2 h. To remove chlorophylls, the stained samples were transferred to boiling 80% ethanol for 10 min.

ROS production from guard cells was examined by loading epidermal peels with H_2DCF-DA (Molecular Probes) essentially as previously described by Miao *et al.* (2006). The epidermal strips were preincubated for 2 h under conditions promoting stomatal opening in the MES-TRIS buffer (the same as mentioned above) supplemented with 0 (ethanol, as a control) or 5 μM (±)-ABA, and were incubated in the loading buffer with 50 mM TRIS-KCl (pH 7.2) containing 50 mM H_2DCF-DA in the dark for 20 min, and then the epidermal tissues were washed with the same MES-TRIS preincubation buffer to remove excess dye. Examinations of peel fluorescence were performed using fluorescence microscopy (Olympus, BX51, Japan). Fluorescent optical sections were collected from dye-loaded guard cells with the following settings: excitation, 488 nm; emission, 525 nm.

Stomatal aperture assay

Stomatal aperture was assayed as previously described (Wu *et al.*, 2009; Shang *et al.*, 2010). Leaves sampled from 3-week old plants were used. To observe ABA-induced stomatal closure, leaves were floated in the buffer containing 50 mM KCl and 10 mM MES-TRIS (pH 6.15) under a halogen cold-light source (Colo-Parmer) at 200 μmol m^{-2} s^{-1} for 2.5 h followed by the addition of different concentrations of (±)-ABA. Apertures were recorded on epidermal strips after 2.5 h of further incubation to estimate ABA-induced closure. To study ABA-inhibited stomatal opening, leaves were floated on the same buffer in the dark for 2.5 h before they were transferred to the cold-light for 2.5 h in the presence of ABA, and then apertures were determined.

Accession numbers

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative database under the following accession numbers: At5g13630 (*ABAR/CHLH*), At1g29920 (*LHCB1*), At2g05070 (*LHCB2*), At5g54270 (*LHCB3*), At2g40100 (*LHCB4*), At4g10340 (*LHCB5*), and At1g15820 (*LHCB6*). Germplasm identification numbers for mutant lines and SALK lines: *lhcb1.1* (SALK-134810), *lhcb2.2* (SALK-005614), *lhcb3* (SALK-036200), *lhcb4.4* (SALK-032779), *lhcb5* (SALK-139667), and *lhcb6* (SALK-074622).

Results

Down-regulation or disruption of LHCB *genes reduces, but up-regulation of* LHCB6 *gene enhances, ABA sensitivity in stomatal movement*

AT-DNA insertion mutant for each of the *LHCB* genes was isolated (Fig. 1A–F). The *lhcb1*, *lhcb2*, *lhcb4*, *lhcb5*, and *lhcb6* are knockdown alleles and the *lhcb3* is a knockout allele (Fig. 1G). It was observed that down-regulation of one *LHCB* gene altered the expression of other *LHCB* members (Fig. 1G). This may be due to a feedback effect in the LHCB family, where a decrease or the removal of one protein in a multiple protein complex can result in the decreased stability of the others (Andersson *et al.*, 2001; Ganeteg *et al.*, 2004; Kovacs *et al.*, 2006). The levels of chlorophyll, ABA, and dry substances and the growth of these *lhcb* mutants were not significantly affected in any of these mutants (Fig. 1H; see Supplementary Fig. S1at *JXB* online). It is noteworthy, however, that a knockout *lhcb6* mutation significantly affected seedling growth (Kovacs *et al.*, 2006), while the present *lhcb6* knockdown-mutant allele did not significantly alter plant growth, probably because the LHCB6 level in this knockdown mutant is still sufficient for normal plant growth.

All the T-DNA insertion *lhcb* mutants showed ABA-insensitive phenotypes, but the over-expression lines of the *LHCB6* gene showed ABA-hypersensitive phenotypes in the ABA-induced promotion of stomatal closure and in the inhibition of stomatal opening (Fig. 2A, B; see Supplementary Fig. S2Aat *JXB* online). The stomatal apertures of the mutants were only slightly reduced at 20 μM or 30 μM (±)ABA that reduced dramatically stomatal apertures of the wild-type plants (Fig. 2A, B). The double mutant *lhcb1 lhcb6* showed substantially the same extent of ABA-insensitive phenotypes in stomatal movement (Fig. 2D). This enhanced resistance of stomatal closure to ABA suggests that the *lhcb* mutants should be more susceptible to drought. Indeed, it was also observed that the detached leaves of the *lhcb* mutants lost more water than those of wild-type plants under dehydration conditions (Fig. 3A), and that both young seedlings and mature plants of the *lhcb* mutants had a lower capacity to conserve their water during drought stress compared with wild-type plants (Fig. 3B–D).

A chlorophyll *b*-deficient mutant *ch1-1*, which results in low expression of the *LHCB* genes (Espineda *et al.*, 1999), was used to assess the relationships between chlorophyll-deficiency-caused LHCB decrease and ABA responsiveness. The *ch1-1* mutant did not show any ABA insensitive phenotype (Fig. 2A, B).

The transgenic complementation lines of all the *lhcb* mutants displayed the wild-type ABA phenotypes in the ABA-induced promotion of stomatal closure and the inhibition of stomatal opening (see Supplementary Fig. S3 at *JXB* online), showing that the phenotypes of the *lhcb* mutants did indeed result from the down-regulation or disruption of the *LHCB* genes. The *LHCB*s are expressed ubiquitously in different tissues/organs except for dry seeds and the *LHCB* mRNA is detectable even in roots, although the expression levels are low in roots (see Supplementary Fig. S4 at *JXB* online). This suggests that the LHCB members can function at the whole plant level. It is noteworthy, however, that the function of LHCB members in roots remains to be determined.

Double mutation in LHCB *and* ABAR *genes confers ABA-insensitivity, and over-expression of* LHCB6 *partly restores the ABA sensitivity of the* cch *mutant*

It was previously reported that the Mg-chelatase H subunit (CHLH/ABAR) functions as a chloroplast/plastid ABA receptor (Shen *et al.*, 2006; Wu *et al.*, 2009; Shang *et al.*,

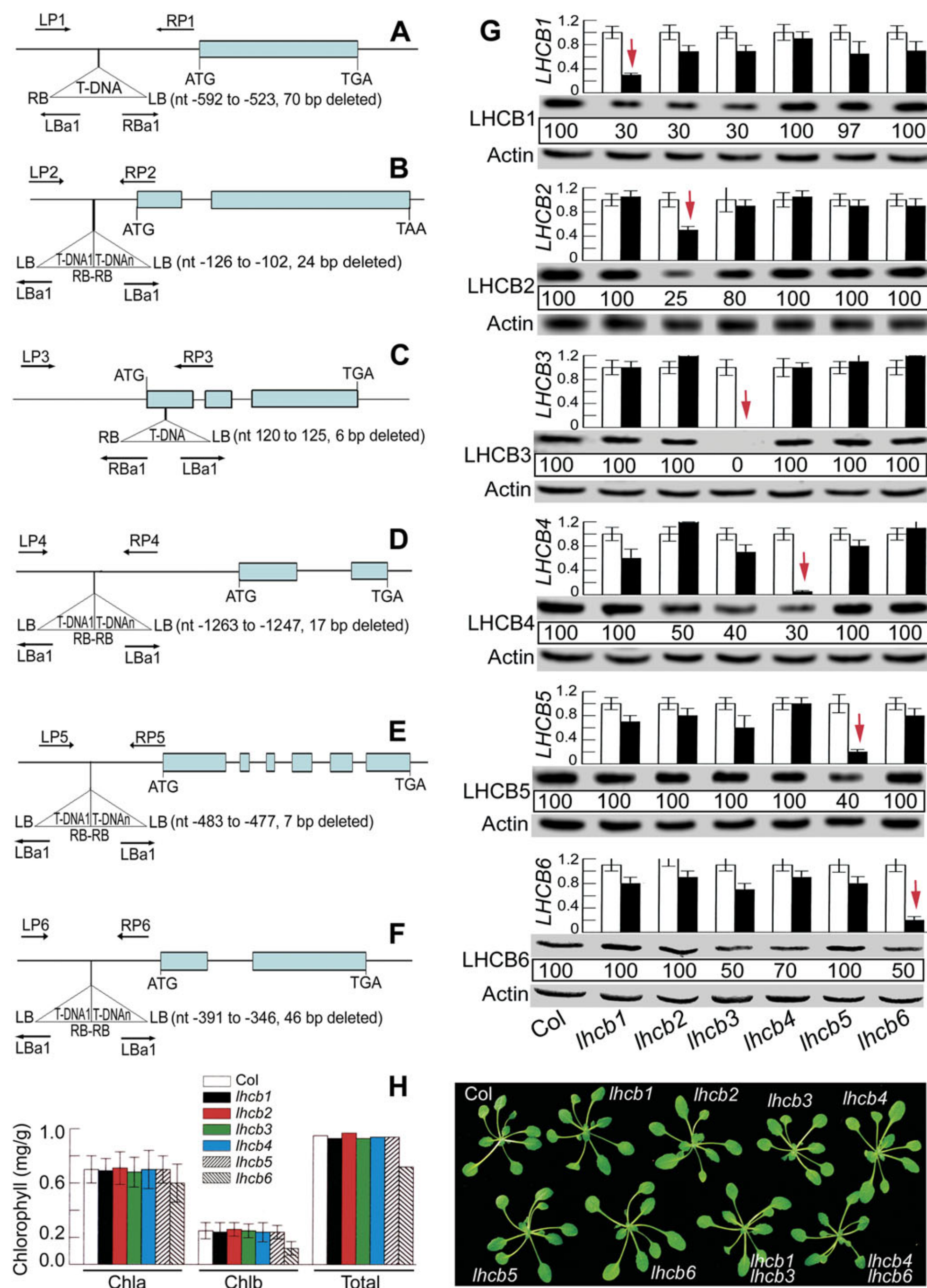

Fig. 1. Molecular and biochemical characterization of the *lhcb* mutants (from *lhcb1* to *lhcb6*). (A–F) T-DNA insertion sites in *lhcb1.1* (SALK_134810) (A), *lhcb2.2* (SALK_005614) (B), *lhcb3* (SALK_036200) (C), *lhcb4.3* (SALK_032779) (D), *lhcb5* (SALK_139667) (E), and *lhcb6* (SALK_074622) (F). LP, left genomic primer, and RP, right genomic primer with the suffix numbers corresponding to the numbers of the *LHCB* genes (1 to 6). LBa1, left border primer, and RBa1, right border primer for the flanking sequences of the T-DNA. Boxes and lines represent exons and introns, respectively. The locations of the primers for the identification of the mutants are indicated by arrows. LB and RB represent the left and right border of the T-DNA insertion, respectively; T-DNA1 and T-DNAn, first and last copy of the inserted T-DNAs, respectively, noting that the two or more than two copies were inserted in an inverted manner. (A) One single copy of the T-DNA was inserted into the promoter region at nt –592 to –523 in the 5′-upstream region of the translation start codon (ATG) of the *LHCB1.1* gene with a 70 bp fragment deleted in the *lhcb1-1* mutant. (B) Tandem T-DNA of two copies (or more than two copies) was inserted into the promoter region in an inverted fashion at the same locus for the *lhcb2* mutant, which generates a 24 bp deletion from nt –126 to –102 in the 5′-upstream region of the translation start codon (ATG) of the *LHCB2.2* gene. (C) One single copy of T-DNA was inserted into the first exon at nt 120 to 125 of the *LHCB3* gene with a 6 bp fragment deleted in the *lhcb3* mutant. (D-F) Tandem T-DNA of two copies (or more than two copies) was inserted into the promoter region in an inverted fashion at the same locus for *lhcb4.3* (D), *lhcb5* (E), and *lhcb6* (F) mutants, which generates a 17 bp deletion from nt –1263 to –1247 for *lhcb4.3*, a 7 bp deletion from nt –483 to

2010). A possible relationship between CHLH/ABAR- and LHCB in guard cell signalling in response to ABA was assessed. Using a strong stomata-ABA-insensitive mutant allele of the CHLH/ABAR gene, *cch* (Shen *et al.*, 2006; Wu *et al.*, 2009; Fig. 2A–D), the double mutant *lhcb6 cch* was generated and it was observed that the *lhcb6 cch* double mutant showed ABA-insensitive phenotypes in the ABA-induced promotion of stomatal closure and the inhibition of stomatal opening, and the strength of the ABA-insensitive phenotypes was comparable with that of the *cch* mutant, stronger than that of the *lhcb6* single mutant and *lhcb1 lhcb6* double mutant (Fig. 2C, D). Interestingly, it was observed that over-expression of the *LHCB6* gene partly restored the wild-type phenotype of the *cch* mutant in the stomatal responses to ABA (Fig. 2C; see Supplementary Fig. S2B at *JXB* online), suggesting that LHCBs function downstream of the ABAR-mediated ABA signalling pathway.

Down-regulation or disruption of LHCB members affects homeostasis of reactive oxygen species

It has been well known that reactive oxygen species (ROS) are involved in ABA signalling (Pei *et al.*, 2000; Murata *et al.*, 2001; Mustilli *et al.*, 2002; Kwak *et al.*, 2006; Miao *et al.*, 2006; Zhang *et al.*, 2009), and chloroplasts are major sites of ROS production (Kwak *et al.*, 2006; Nott *et al.*, 2006; Galvez-Valdivieso and Mullineaux, 2010) where LHCBs play a key role (Jansson, 1994, 1999; Galvez-Valdivieso and Mullineaux, 2010). Thus, ROS production was investigated in the *lhcb* mutants using techniques of NBT-leaf-staining and CFDA-guard-cell staining. The results showed that the ROS levels increased in all the *lhcb* mutants compared with wild-type plants, which was observed in both the whole leaves and in guard cells (Fig. 4A–C). It was found that ABA treatments at relatively low concentrations (1–5 μM) stimulated ROS levels in the wild-type plants (Fig. 4A, B) which is consistent with previous observations (Pei *et al.*, 2000; Murata *et al.*, 2001; Mustilli *et al.*, 2002; Kwak *et al.*, 2006; Miao *et al.*, 2006; Zhang *et al.*, 2009), but high levels of ABA had no significant stimulating effect (10 μM) or, inversely, reduced ROS levels (50 μM) in these wild-type plants (Fig. 4A). In all the *lhcb* mutants, however, ABA treatments at low concentrations reduced ROS levels in both whole leaves (1–10 μM ABA application; Fig. 4A, B) and guard cells (5 μM ABA application; Fig. 4C). The stomatal apertures of the *lhcb* mutants were not significantly affected by the higher levels of ROS in the absence of ABA, but showed resistance to ABA when exogenous ABA was applied (Fig. 2; see Supplementary Fig. 5 at *JXB* online) which, by contrast, reduced ROS levels in these mutants (Fig. 4A–C). In addition, experiments were conducted with 3,5-diaminobenzidine (DAB) staining which detects H_2O_2 production and substantially the same results were obtained as those with NBT staining (see Supplementary Fig. S6 at *JXB* online). These data demonstrate that down-regulation or disruption of the *LHCB* members alters the homeostasis of ROS and ABA responsiveness of ROS in plant cells.

Down-regulation or disruption of *LHCB* genes alters the expression of a set of ABA responsive genes

The expression of the following ABA responsive genes was assayed in the *lhcb* mutants: *ABF1*, *ABF2/AREB1*, *ABF3*, and *ABF4/AREB2* (Choi *et al.*, 2000; Uno *et al.*, 2000), *ABI1* (Leung *et al.*, 1994; Meyer *et al.*, 1994; Gosti *et al.*, 1999), *ABI2* (Leung *et al.*, 1997), *ABI3* (Giraudat *et al.*, 1992), *ABI4* (Finkelstein *et al.*, 1998), *ABI5* (Finkelstein and Lynch, 2000), *ERD10* (Kiyosue *et al.*, 1994), *KIN1* and *KIN2* (Kurkela and Borg-Franck, 1992), *MYB2* and *MYC2* (Abe *et al.*, 2003), *OST1* (Mustilli *et al.*, 2002), *RAB18* (Lang and Palva, 1992), and *RD29A* (Yamaguchi-Shinozaki and Shinozaki, 1994). Expression of ten ABA-positively-responsive genes, *ABI4*, *ABI5*, *ERD10*, *KIN1*, *KIN2*, *MYB2*, *MYC2*, *OST1*, *RAB18*, and *RD29A*, was significantly repressed in all the *lhcb* mutants (from l*hcb1* to *lhcb6*, Fig. 5). Expression of three genes encoding important transcription factors that positively regulate ABA signalling, *ABF1*, *ABF4*, and *ABI3*, was also significantly repressed in the *lhcb* mutants except for *lhcb1*, *lhcb3*, and *lhcb6* (Fig. 5). However, expression of two genes that encode negative regulators of ABA signalling acting directly downstream of the ABA receptor PYR/PYL/RCAR (Fujii *et al.*, 2009), *ABI1* and *AB12*, was not altered in any of the *lhcb* mutants (Fig. 5). By contrast, expression of two genes coding for two transcription factors positively involved in ABA signalling, *ABF2* and *ABF3*, was up-regulated in the *lhcb* mutants except for *lhcb5* and *lhcb6* (Fig. 5). This expression profile of

–477 for *lhcb5*, and a 46–bp deletion from nt –391 to –346 in the 5′-upstream region of the translation start codon (ATG) of the corresponding genes *LHCB4.3*, *LHCB5*, and *LHCB6*, respectively. (G) Quantitative real-time PCR analysis (columns) and immunoblotting (protein bands below the columns) for *LHCB* gene expression in the mutants (from *lhcb1* to *lhcb6*). Actin was used as a loading control for immunoblotting. Relative protein band intensities, normalized relative to the intensity of Col, are indicated by numbers in the box below the bands. Expression of all the six numbers of *LHCB*s (from *LHCB1* to *LHCB6*) was assessed in each mutant, and the red arrow indicates the level of the corresponding mutated *LHCB* gene. Note that *lhcb1-1*, *lhcb2*, *lhcb4*, *lhcb5*, and *lhcb6* are knockdown mutants in their corresponding genes, while *lhcb3* is a knockout mutant in the *LHCB3* gene. The immunoblotting assays were repeated three times with independent biological experiments which gave similar results. Each value for real-time PCR is the mean ±SE of three independent biological determinations. (H) The chlorophyll *a/b* contents are not significantly affected in the mutants (from *lhcb1* to *lhcb6*). Left panel, the concentrations of chloroplast *a* (Chl*a*) and *b* (Chl*b*) and total chlorophyll in the different mutants. Each value is the mean ±SE of three independent biological determinations. Right panel, the status of the seedlings of the different mutants, showing that no chlorophyll-deficient phenotype can be seen for these mutants.

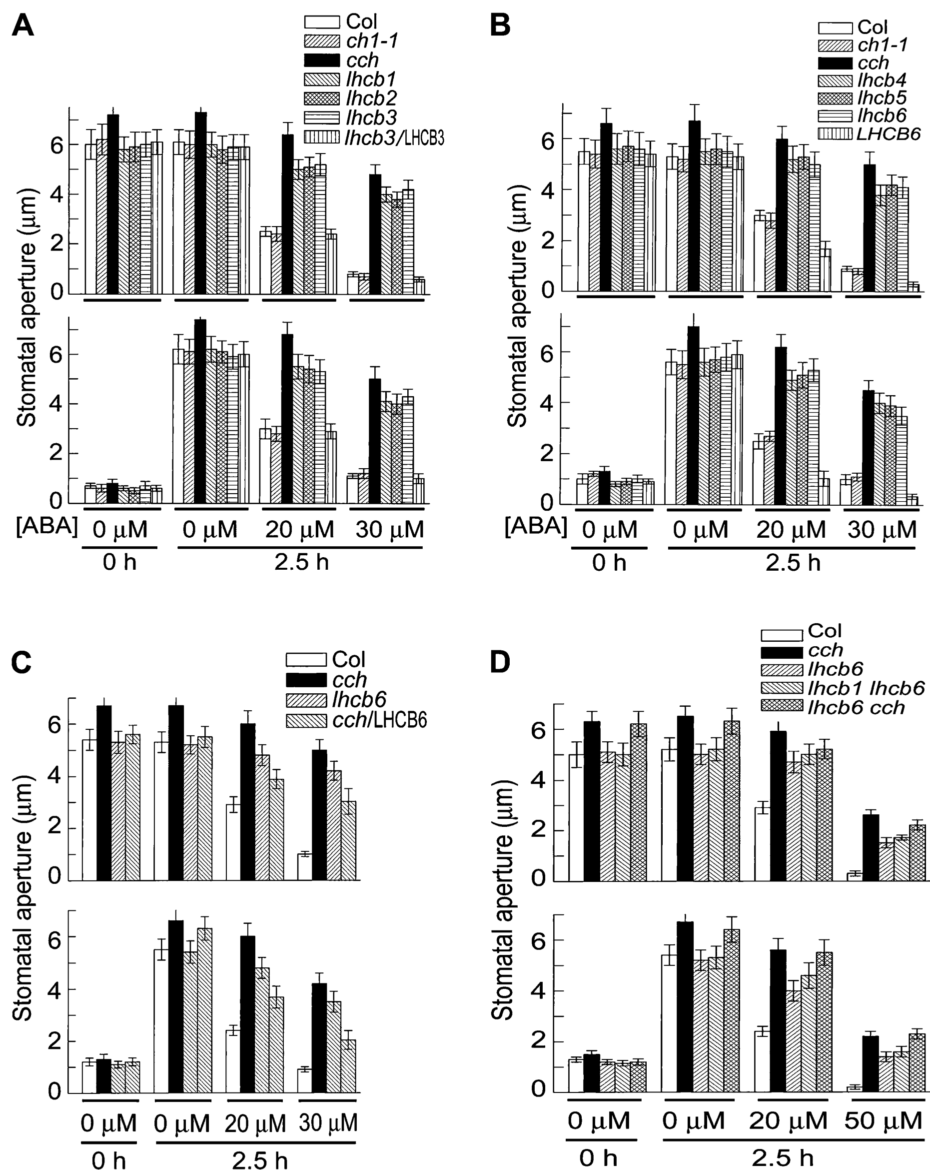

Fig. 2. Down- or up-regulation of members of the *LHCB* family alters ABA sensitivities in stomatal movement. (A) ABA-induced stomatal closure (top) and inhibition of stomatal opening (bottom) in wild-type Col, *ch1-1*, *cch*, *lhcb1*, *lhcb2*, and *lhcb3* mutants and a complemented line of the *lhcb3* mutant (*lhcb3*/LHCB3). (B) ABA-induced stomatal closure (top) and inhibition of stomatal opening (bottom) in wild-type Col, *ch1-1*, *cch*, *lhcb4*, *lhcb5*, and *lhcb6* mutants and a transgenic *LHCB6*-over-expressor (*LHCB6*, line OE5 as described in Supplementary Fig. S2at *JXB* online). (C) ABA-induced stomatal closure (top) and inhibition of stomatal opening (bottom) in wild-type Col, *cch* and *lhcb6* mutants and a transgenic *LHCB6*-over-expressiing line in the *cch* mutant (*cch*/LHCB6). (D) ABA-induced stomatal closure (top) and inhibition of stomatal opening (bottom) in wild-type Col, *cch* and *lhcb6* single mutants, and *lhcb1 lhcb6*, and *lhcb6 cch* double mutants. Values presented in (A) to (D) are the means ±SE from three independent experiments; n=60 apertures per experiment.

the ABA-responsive genes is essentially consistent with the idea that LHCBs are positively involved in ABA signalling but with a complex underlying mechanism.

Gene expression of two major members of the NADPH oxidases was also analysed, termed respiratory burst oxidase homologues (Rbohs), RbohD and RbohF, which are plasma membrane-associated proteins and involved in ABA-induced stomatal closure (Kwak *et al.*, 2003; Bright *et al.*, 2006). The results showed that expression of *RbohD* and *RbohF* genes was not significantly affected in the *lhcb* mutants in comparison with wild-type plants (Fig. 5).

Discussion

LHCB members are positively involved in guard cell signalling in response to ABA

Genetic evidence is provided here that the members of the LHCB family are positively involved in guard cell signalling in response to ABA and so LHCB members have been identified as new players in ABA signalling in stomatal movement. Consistently, previous studies showed that down-regulation of the LHCB members reduced plant

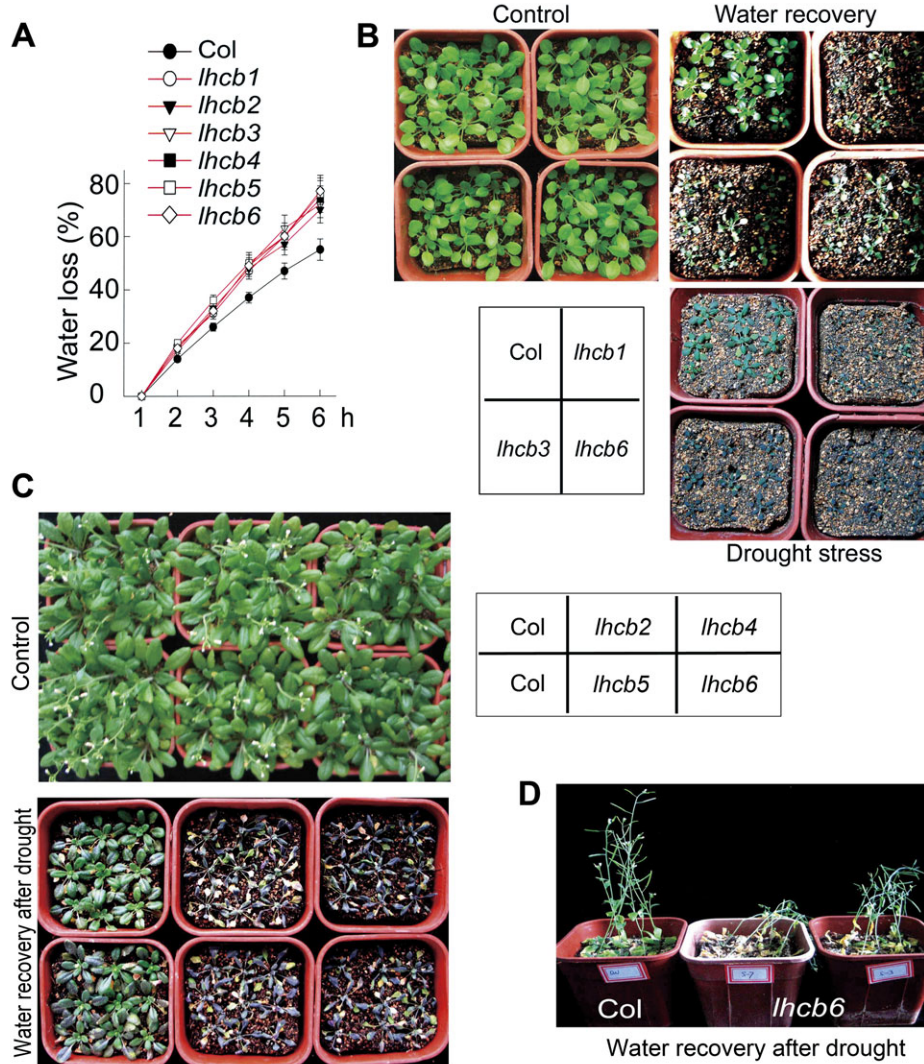

Fig. 3. Down-regulation of members of the *LHCB* family reduces the ability of plants to conserve water. (A) Water loss rates during a 6 h period from the detached leaves of wild-type Col and different *lhcb* mutants. Values are the means ±SE of five individual plants per genotype. (B, C) Water loss assays with young seedlings for wild-type Col, *lhcb1*, *lhcb3*, and *lhcb6* mutants (B) or for wild-type Col, *lhcb2*, *lhcb4*, *lhcb5*, and *lhcb6* mutants (C). Plants were well watered (Control) or drought-stressed by withholding water for 18 d and then the drought-stressed plants were rewatered (Water recovery) and growth status was recorded 2 d later. The entire experiment was replicated three times with similar results. (D) Assays with mature plants for wild-type Col and *lhcb6* mutants. Plants were drought-stressed by withholding water for 21 d and then the plants were rewatered and growth status was recorded 2 d later. The entire experiment was replicated three times with similar results.

tolerance to environmental stresses with lowered seed production (Andersson *et al.*, 2001, 2003; Ganeteg *et al.*, 2004; Kovacs *et al.*, 2006) except for the *lhcb3* mutant that showed comparable seed production with that of wild-type plants (Damkjaer *et al.*, 2009). The differences observed in the *lhcb3* mutants between our present observations and the previously reported data (Damkjaer *et al.*, 2009) may be due to the characteristics of the different *lhcb3* mutants: in the *lhcb3* mutant used here (SALK_036200 or N536200), the protein levels of the other LHCB members were not affected (Fig. 1) while in the *lhcb3* mutant used by Damkjaer *et al* (2009) (N520342 or SALK_020342), the protein levels of LHCB1 and LHCB2 were significantly up-regulated (Damkjaer *et al.*, 2009), which may partly compensate for the disruption of the LHCB3 protein. Nevertheless, the defects in ABA signalling in stomatal movement in the *lhcb* mutants, among other defects in the photosynthesis apparatus (Andersson *et al.*, 2001, 2003; Ganeteg *et al.*, 2004; Kovacs *et al.*, 2006; Damkjaer *et al.*, 2009), are at least partly responsible for the previously-observed decline of the plants' ability to adapt to environmental stresses (Andersson *et al.*, 2001, 2003; Ganeteg *et al.*, 2004; Kovacs *et al.*, 2006).

The concentrations of ABA, dry substances and chlorophyll *a*/*b* were not affected in the *lhcb* mutants used in the present experiments (Fig. 1; see Supplementary Fig. S1 at *JXB* online), which shows that the ABA-insensitive phenotypes of these mutants in stomatal movement were

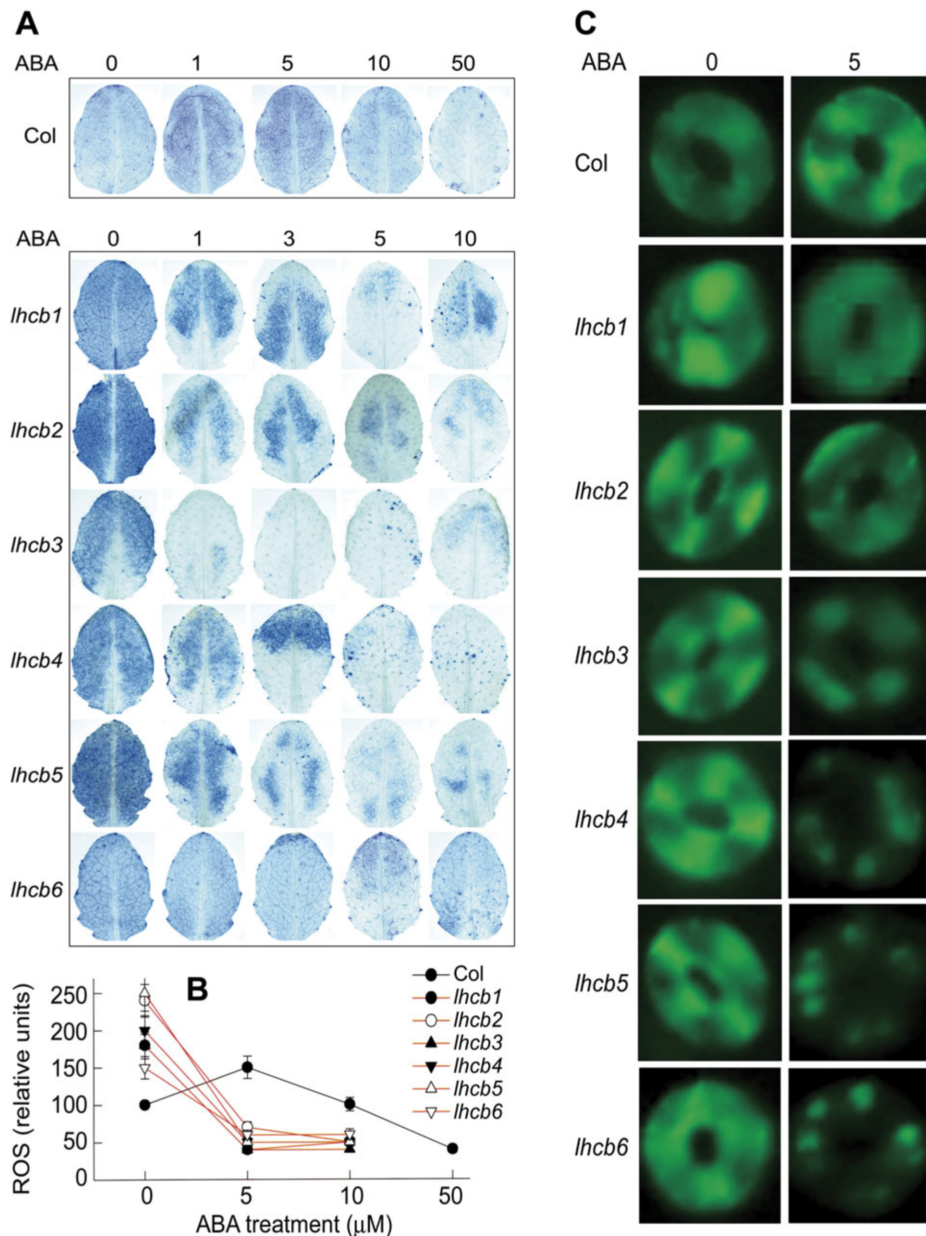

Fig. 4. ROS homeostasis is altered in *lhcb* mutants. (A) ROS production in leaves in response to different concentrations of ABA (from 0–50 μM for Col and 0–10 μM for *lhcb* mutants), detected by nitroblue tetrazolium staining in wild-type Col and different *lhcb* mutants. The entire experiment was replicated five times with similar results. (B) Quantitative estimation of the ROS production described in (A). Relative ROS-staining intensities estimated by scanning the staining profiles, are normalized relative to the ROS-staining intensity of Col (taken as 100%). Each value is the mean ±SE of five independent biological determinations. (C) ROS production from guard cells in response to ABA (5 μM), examined by H_2DCF-DA imaging in wild-type Col and different *lhcb* mutants. The entire experiment was replicated three times with similar results. For the stomatal apertures of the treated plants, see Supplementary Fig. S5 at *JXB* online.

associated neither with ABA biosynthesis nor with photoassimilate accumulation and, in particular, that down-regulation of the LHCB members could affect ABA signalling without altering chlorophyll homeostasis. However, the stability of the LHCB proteins is associated with chlorophyll *a*/*b*, of which the deficiency may result in ac decrease of the LHCB proteins (Adam, 1996; Espineda *et al.*, 1999). The chlorophyll *b*-deficient mutants *ch1-1* and *ch1-2*, which results in low expression of the *LHCB* genes (Espineda *et al.*, 1999), showed slight or no ABA insensitive phenotype in the stomatal response to ABA (Fig. 2; Shen *et al.*, 2006). The possible explanation of this phenomenon is that chlorophyll deficiency may induce more complex consequences than degradation of the LHCB proteins, which may compensate for the effects of the LHCB protein deficiency.

The *lhcb* double mutants showed ABA-insensitive phenotypes similar to the *lhcb* single mutants (Fig. 2), suggesting that a compensatory feed-back mechanism to maintain the LHCB homeostasis may function in the LHCB-related

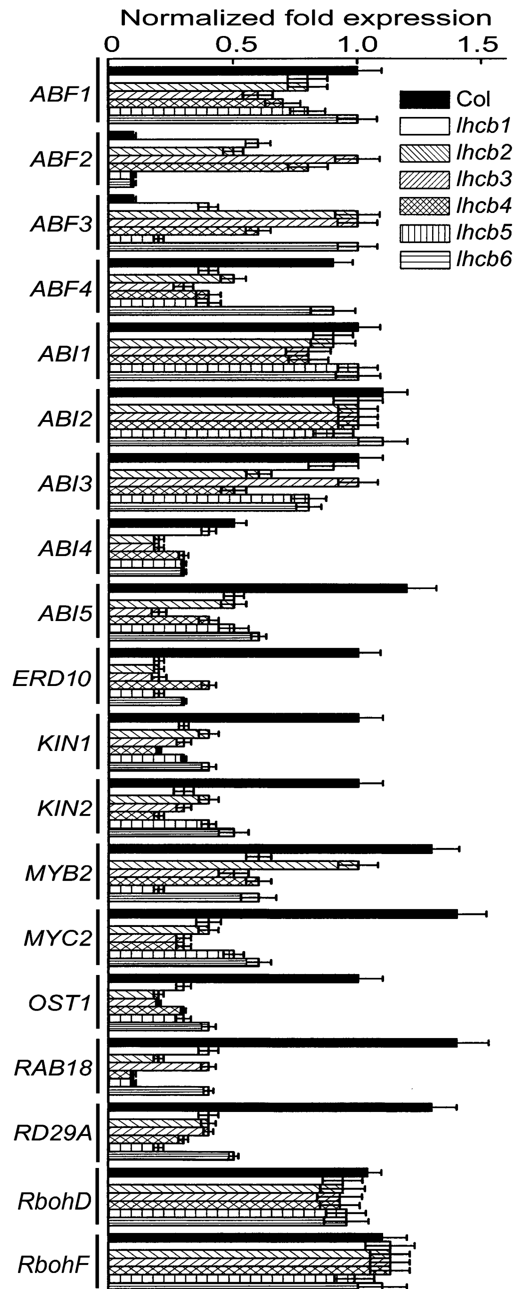

Fig. 5. Expression of a set of ABA-responsive genes is altered in *lhcb* mutants. Gene expression was assayed by real-time PCR. Each value is the mean ±SE of three independent biological determinations.

ABA signalling in guard cells. Each of the six *lhcb* single mutants showed similar ABA-insensitive phenotypes in stomatal movement and similar drought-hypersensitive phenotypes (Figs 2, 3), suggesting that each of the LHCB members is necessary for building the antenna complex and keeping the complex intact, which functions as a whole both in photosynthesis and ABA signalling in guard cells. So, deficiency of any of the LHCB members may damage this core molecular complex of the PSII antenna machinery, and thus affect ABA signalling in stomatal movement. This point of view is consistent with the previous reports, which showed that each member of the LHCB family plays a specific role in the regulation of the photosynthetic machinery (Andersson *et al.*, 2001, 2003; Ganeteg *et al.*, 2004; Kovacs *et al.*, 2006; Damkjaer *et al.*, 2009).

How do the LHCB proteins work in guard cell signalling in response to ABA?

The mechanism by which the LHCB members are involved in ABA signalling in guard cells may be highly complex. The ABA insensitivity of the *cch* mutant in stomatal movement was partly suppressed by *LHCB6*-over-expression, suggesting that CHLH/ABAR may function upstream of the LHCB members. It was observed that down-regulation of the *LHCB* members altered both the ROS homeostasis and the ABA responsiveness of ROS in plant leaves (Fig. 4). Expression of two major plasma membrane-associated NADPH oxidase genes, *RbohD* and *RbohF*, which are involved in ABA-induced stomatal closure (Kwak *et al.*, 2003; Bright *et al.*, 2006), was not affected in the *lhcb* mutants (Fig. 5), suggesting that the alteration in ROS levels in these *lhcb* mutants is mainly caused by a deficiency of the LHCB members, but may not involve the plasma-membrane NADPH oxidases. Under normal conditions, higher amounts of ROS accumulated in the *lhcb* mutants compared with wild-type plants (Fig. 4), which suggests that an imbalanced antenna complex reduces its efficiency, thus leading to ROS accumulation. In the presence of ABA, however, ROS levels in all the mutants decreased compared with the wild type in which ABA stimulates ROS production (Fig. 4), suggesting that ABA probably enhances the already activated ROS-detoxifying systems, thus lowering ROS levels that are abnormally enhanced by *lhcb* mutation. It is possible that LHCBs are involved in ABA signalling in guard cells partly by modulating ROS homeostasis. It will be of importance to elucidate upstream- and downstream-events of LHCBs to understand the complex mechanism of ABA signalling in guard cells and the positive role of LHCB proteins in plant stress tolerance.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Concentrations of endogenous ABA and accumulation of dry substances of the different *lhcb* mutant plants.

Supplementary Fig. S2. Real-time PCR analysis of the *LHCB6*-RNAi and over-expression lines.

Supplementary Fig. S3. Expression of the 35S-promoter-driven *LHCB*s rescues ABA sensitivity of the *lhcb* mutants.

Supplementary Fig. S4. Different members of *LHCB*s are expressed ubiquitously in different tissues/organs except for dry seeds.

Supplementary Fig. S5. ROS homeostasis is altered in *lhcb* mutants.

Supplementary Fig. S6. Stomatal aperture of Col plants and *lhcb* mutants when assaying ROS levels in stomata.

Supplementary Table S1. Primers used in this study.

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