Plant Cell Rep (2015) 34:843–852
DOI 10.1007/s00299-015-1746-4

ORIGINAL PAPER

Overexpression of *LOV KELCH PROTEIN 2* confers dehydration tolerance and is associated with enhanced expression of dehydration-inducible genes in *Arabidopsis thaliana*

Yuji Miyazaki · Hiroshi Abe · Tomoyuki Takase · Masatomo Kobayashi · Tomohiro Kiyosue

Received: 29 October 2014 / Revised: 19 December 2014 / Accepted: 12 January 2015 / Published online: 28 January 2015

Abstract
Key message **The overexpression of *LKP2* confers dehydration tolerance in *Arabidopsis thaliana*; this is likely due to enhanced expression of dehydration-inducible genes and reduced stomatal opening.**
Abstract LOV KELCH PROTEIN 2 (LKP2) modulates the circadian rhythm and flowering time in plants. In this study, we observed that *LKP2* overexpression enhanced dehydration tolerance in Arabidopsis. Microarray analysis demonstrated that expression of water deprivation-responsive genes was higher in the absence of dehydration stress in transgenic Arabidopsis plants expressing *green fluorescent protein–tagged LKP2* (*GFP-LKP2*) than in control transgenic plants expressing *GFP*. After dehydration followed by rehydration, GFP-LKP2 plants developed more leaves and roots and exhibited higher survival rates than control plants. In the absence of dehydration stress, four dehydration-inducible genes, namely *DREB1A*, *DREB1B*, *DREB1C*, and *RD29A*, were expressed in GFP-LKP2 plants, whereas they were not expressed or were expressed at low levels in control plants. Under dehydration stress, the expression of *DREB2B* and *RD29A* peaked faster in the GFP-LKP2 plants than in control plants. The stomatal aperture of GFP-LKP2 plants was smaller than that of control plants. These results suggest that the dehydration tolerance of GFP-LKP2 plants is caused by upregulation of *DREB1A–C/CBF1–3* and their downstream targets; restricted stomatal opening in the absence of dehydration stress also appears to contribute to the phenotype. The rapid and high expression of *DREB2B* and its downstream target genes also likely accounts for some features of the GFP-LKP2 phenotype. Our results suggest that LKP2 can be used for biotechnological applications not only to adjust the flowering time control but also to enhance dehydration tolerance.

Keywords Arabidopsis · Dehydration-responsive element-binding factor (DREB) · Dehydration tolerance · LOV KELCH PROTEIN 2 (LKP2) · Stomatal aperture

Communicated by P. Lakshmanan.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-015-1746-4) contains supplementary material, which is available to authorized users.

Y. Miyazaki · T. Takase · T. Kiyosue (✉)
Department of Life Science, Faculty of Science, Gakushuin University, 1-5-1 Mejiro, Toshima-ku, Tokyo 171-8588, Japan
e-mail: tomohiro.kiyosue@gakushuin.ac.jp

H. Abe · M. Kobayashi
Experimental Plant Division, Department of Biological Systems, RIKEN BioResource Center, 3-1-1 Koyadai, Tsukuba 305-0074, Ibaraki, Japan

Introduction

Plants have evolved various light receptors, such as phytochromes, cryptochromes, and phototropins, to use light as a cue for growth and development (Franklin and Quail 2010; Chen and Chory 2011; Suetsugu and Wasda 2013). LOV KELCH PROTEIN 2 (LKP2), ZEITLUPE (ZTL) or LOV KELCH PROTEIN 1 (LKP1), and FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1) form a small family of photoreceptors in Arabidopsis (Kiyosue and Wada 2000; Nelson et al. 2000; Somers et al. 2000; Jarillo et al. 2001; Schultz et al. 2001) and are involved in the regulation of the circadian clock by ubiquitin-mediated degradation of clock components such as TIMING OF CAB EXPRESSION 1 (TOC1) and

PSEUDO-RESPONSE REGULATOR 5 (PRR5) (Más et al. 2003; Kiba et al. 2007; Baudry et al. 2010; Wang et al. 2010). *ztl* mutants show a long-period phenotype (approximately 27 h; Somers et al. 2004). Both *ztl fkf1* and *ztl fkf1 lkp2* mutants also exhibit a long-period phenotype (approximately 29 h), although the amplitude of the circadian rhythm is weaker in the latter mutant (Baudry et al. 2010). Overexpression of LKP2 or ZTL shortens the period and causes arrhythmicity (Schultz et al. 2001; Somers et al. 2004).

Protein of this family also regulates flowering time. Overexpression of *LKP2* or *ZTL* in Arabidopsis leads to downregulation of the expression of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*), leading to delayed flowering under long-day conditions (Somers et al. 2004; Miyazaki et al. 2011), whereas *ztl* and *lkp2 ztl* mutants exhibit early-flowering phenotypes, although the *lkp2* mutant has a wild-type phenotype (Takase et al. 2011). *fkf1* mutants exhibit a late-flowering phenotype (Nelson et al. 2000; Imaizumi et al. 2003).

The function of LKP2 is postulated to overlap mostly with that of ZTL because they are structurally more closely related to each other than to FKF1, and because overexpression of either gene in Arabidopsis leads to common phenotypes, i.e., arrhythmicity, late flowering, and elongated hypocotyls (Kiyosue and Wada 2000; Somers et al. 2000; Schultz et al. 2001). Furthermore, *ZTL* promoter-driven *LKP2* expression can complement the *ztl* mutant phenotype (Baudry et al. 2010). However, as mentioned above, the phenotypes of *ztl* and *lkp2* knockout mutants are different—the *ztl* mutant shows a longer circadian rhythm and an early-flowering phenotype, whereas the *lkp2* mutant shows wild-type phenotype both in the circadian rhythm and flowering (Somers et al. 2000; Baudry et al. 2010; Takase et al. 2011). Such differences may reflect the differences in the relative expression levels of *LKP2* and *ZTL*. The expression level of *ZTL* is approximately eight times that of *LKP2* under 16 h light/8 h dark and 8 h light/16 h dark conditions (Mockler et al. 2007; Michael et al. 2008), and LKP2 expression is only 4 % of the level observed for ZTL under constant light conditions (Baudry et al. 2010). Overexpression of *ZTL* conferred both strong and weak late-flowering phenotypes, whereas that of *LKP2* conferred only a strong late-flowering phenotype (Kiyosue and Wada 2000, Schultz et al. 2001). Light-dependent signals affect multiple aspects of plant growth, development, and responses to environmental stresses (González et al. 2012; Oh et al. 2014). Thus, LKP2 may have functions beyond its well-studied roles in regulation of the circadian clock and flowering time.

Limited water availability is a major environmental factor affecting productivity of crop plants; recent global warming is predicted leading to further water shortages in most agricultural regions of the world (Xoconostle-Cázares et al. 2010). Many transgenic studies have attempted to improve the drought tolerance of plants by overexpressing genes for dehydration-inducible transcription factors such as dehydration-responsive element-binding factors (DREBs)/C-repeat binding factors (CBFs) or their downstream targets such as *responsive to dehydration* (*RD*)/*cold-regulated* (*COR*) genes (Yamaguchi-Shinozaki and Shinozaki 2006; Saibo et al. 2009; Qin et al. 2011).

In this study, we performed microarray analysis to elucidate unidentified effects of *LKP2* overexpression in plants. We found that LKP2 overexpression confers dehydration tolerance in Arabidopsis, accompanied by the activation of *DREB/CBF* and their downstream target genes.

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* Columbia (Col) accession and the transgenic plants (T5 generation) *35S:GFP-LKP2* line 1 (GFP-LKP2-1), *35S:GFP-LKP2* line 2 (GFP-LKP2-2), and *35S:GFP* (GFP) (Yasuhara et al. 2004; Miyazaki et al. 2011) were used in this study. Plants were grown on plates with 0.8 % (w/v) agar containing 1/2 basal salt Murashige and Skoog medium and 1 % sucrose (GM medium) or in pots with vermiculite supplemented with 0.1 % Hyponex (Hyponex, Tokyo, Japan) under long-day conditions at 22 °C (16 h light/8 h dark, 80–100 μmol m^{-2} s^{-1}).

Microarray analysis

Thirty two-day-old non-stressed GFP-LKP2-1 and GFP plants grown on GM agar plates were used for analysis. Leaves were harvested at zeitgeber time 0 (ZT0). Leaves from three seedlings were pooled before extraction. RNA was prepared using an RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). The microarray experiment was conducted by Miltenyi Biotec K.K. (Tokyo, Japan) using Agilent Whole Arabidopsis Genome Oligo Microarrays 4 × 44 K V4 (G2519F) (Agilent Technologies, Böblingen, Germany). RNA samples (100 ng) were used for the linear T7-based amplification step. To produce Cy3-labeled cRNA, amplified RNA was labeled using an Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies) following the manufacturer's protocol. Yields of cRNA and the dye incorporation rate were measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Hybridization was performed

according to the Agilent 60-mer oligo microarray processing protocol using an Agilent Gene Expression Hybridization Kit (Agilent Technologies). Cy3-labeled fragmented cRNA (1.65 μg) in hybridization buffer was hybridized overnight (17 h, 65 °C) in Agilent's recommended hybridization chamber and oven. The microarrays were then washed once with Agilent Gene Expression Wash Buffer 1 for 1 min at room temperature followed by a second wash with Agilent Gene Expression Wash Buffer 2 (preheated at 37 °C) for 1 min. The last washing step was performed with acetonitrile. Fluorescence signals were detected using Agilent Microarray Scanner System (Agilent Technologies). Agilent Feature Extraction Software (Agilent Technologies) was used to read out and process the microarray image files. Microarray data are accessible through GEO series accession number GSE37197 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37197). Candidate genes with a fold change >2 and $p < 0.01$ were used for pathway analysis with the Gene Functional Classification Tool in DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp). Gene annotations were retrieved from TAIR (http://www.arabidopsis.org/).

Dehydration stress treatment

Eight-day-old seedlings grown on GM agar plates were harvested at ZT4, placed on paper towels (Nippon Paper Crecia, Tokyo, Japan) and allowed to air-dry at 22 °C and 40 % humidity under white light (25 μmol m^{-2} s^{-1}) for various time periods. For bioassay, the seedlings were allowed to air-dry for 75 min, and then returned to GM agar plates for rehydration and growth. Leaf and root numbers of each plant were counted 8 days after the treatment. The plants were then transferred into pots and plant survival rates were determined 3 weeks after the treatment (i.e., 13 days in vermiculite); 20 plants per group were analyzed. The survival experiment was repeated three times.

RT-PCR analysis

Eight-day-old seedlings grown on GM agar plates were harvested or subjected to the dehydration stress, and then immediately frozen in liquid N_2. Total RNA was isolated using Sepasol-RNAI (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. To synthesize cDNA, total RNA (1 μg) was primed with the Oligo d(T) primer and reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed with AmpliTaq Gold (Applied Biosystems) and gene-specific primer sets listed in Table S1. PCR reactions were carried out in a thermal cycler with denaturation at 94 °C for 5 min, followed by a variable number of cycles (Table S1) at 94 °C for 30 s, 50 °C for 20 s, and 72 °C for 1 min (GeneAmp PCR System 2700, Applied Biosystems). PCR products were separated on a 2.0 % (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light. Gel images were captured with a Printgraph video capture device (AE-6911FX; Atto, Tokyo, Japan). Each PCR fragment was sequenced directly to confirm its identity.

Stomatal aperture

Stomatal apertures in the abaxial epidermis of two-week-old rosette plants were measured according to the method described by Kinoshita et al. (2011) using images taken with a BX51 microscope (Olympus, Tokyo, Japan) and Scion Image software (Scion Corporation; http://scion-image.software.informer.com).

Statistical analyses

Data are presented as the mean ± SE. Student–Newman–Keuls test was performed with IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA).

Results

Gene expression profile of GFP-LKP2 plants

Using microarray analysis, we compared the expression profiles of RNAs isolated from leaves of adult GFP-LKP2-1 and GFP (control) plants in the absence of dehydration stress. Only genes with a > twofold change in GFP-LKP2-1 plants were considered (accession numbers GSM913367 and GSM913368; Table S2). A total of 544 genes were upregulated ($p < 0.01$), including the transposable element gene *AT2G27375* (fold change of 23) and *LKP2* (fold change of 21). A total of 739 genes were downregulated ($p < 0.01$), including *ANTHOCYANIN 5-AROMATIC ACYLTRANSFERASE1* (fold change of −100) and *FT* (fold change of −4). The upregulated and downregulated genes were classified into several groups (Table S3). We found that, in GFP-LKP2-1 plants, genes involved in external encapsulating structure organization, response to light stimulus, response to abiotic stimulus, chromatin organization, and secondary metabolic process were upregulated, whereas genes involved in response to organic substances, regulation of transcription, and immune response were downregulated.

RT-PCR analysis of dehydration–related genes detected by microarray analysis

Microarray analysis suggested that many biological processes were altered in GFP-LKP2 plants. We focused on several genes involved in response to dehydration (Cluster 2, response to abiotic stimulus, in Table S3). Among the abiotic stimulus-responsive Cluster 2 genes (Table 1), we selected five dehydration-inducible genes, namely *LTP3*, *DREB1A/CBF3*, *RD29A*, *DEHYDRIN*, and *LTI30*, and performed RT-PCR to evaluate their upregulation in GFP-LKP2-1 and GFP-LKP2-2 plants in comparison with control plants (Col and GFP) using RNA isolated from 8-day-old seedlings (Fig. 1). This experiment confirmed that the expression of *LTP3*, *DREB1A/CBF3*, *RD29A*, and *DEHYDRIN* was higher in both GFP-LKP2-1 and GFP-LKP2-2 plants in comparison with both controls; an ambiguous result was obtained for *LTI30*.

After dehydration stress, GFP-LKP2 plants develop more leaves and roots and have a higher survival rate than control plants

The upregulation of several dehydration-inducible genes in GFP-LKP2 plants suggested that these plants might be more dehydration-tolerant than GFP plants. To assess this possibility, we examined the effect of dehydration stress on leaf and root growth in GFP-LKP2 and control (Col and GFP) plants. Seedlings were dehydrated for 75 min and then grown on GM agar plates. Eight days after release from dehydration stress, GFP-LKP2 plants developed more leaves and roots than treated control plants (Fig. 2a, b). By contrast, in the absence of stress, the number of roots and leaves in GFP-LKP2 plants did not exceed that in control plants (Fig. S1).

Three weeks after release from dehydration stress, almost all GFP-LKP2 plants grew well in pots, while only 12 % of control plants survived (Fig. 2c, d).

Precocious accumulation of DREB1A–C/CBF1–3 and RD29A mRNA in GFP-LKP2 plants under normal conditions is followed by their further increase upon dehydration

Next we examined the expression of four genes, *DREB1A-C/CBF1-3*, *DREB2A*, *B*, and *RD29A*, three of which have been identified by our microarray analysis (Table 1) and three other are known as dehydration-responsive genes involved in stress tolerance (Yamaguchi-Shinozaki and Shinozaki 2006).

In the absence of stress, the levels of *DREB1A–C/CBF1–3* and *RD29A* mRNAs were substantially higher in GFP-LKP2 plants than in control plants (Col and GFP; Fig. 3a). After the onset of dehydration stress, the mRNA levels of all four genes were robustly induced in both GFP-LKP2 and control plants. In control plants, *DREB2B* and *RD29A* peaked at 2 h. However, *DREB2B* peaked at 1 h in both GFP-LKP2 lines, whereas *RD29A* peaked at 40 min in GFP-LKP2-1 and at 1 h in GFP-LKP2-2.

Besides the *DREB/CBF* pathway, three other signaling networks are postulated to be involved in the induction of *RD* or *Early Responsive to Dehydration* (*ERD*) during the response to dehydration (Yamaguchi-Shinozaki and Shinozaki 2006; Saibo et al. 2009), namely (1) myeloblastosis (MYB)–myelocytomatosis (MYC) pathway, (2) the abscisic acid (ABA) pathway, and (3) NAM (no apical meristem)/ATAF (Arabidopsis transcription activation factor)/CUC (cup-shaped cotyledon) (NAC) and zinc finger homeodomain (ZFHD) pathway. We performed RT-PCR to evaluate the contribution of these three pathways to the enhanced dehydration stress tolerance of GFP-LKP2 plants. No obvious upregulation of 13 selected genes was detected in unstressed GFP-LKP2 compared to control plants (Fig. 3b, time 0). After dehydration stress, the expression of only one gene, *ANAC055*, was higher in GFP-LKP2 plants than in both Col and GFP plants (Fig. 3b).

GFP-LKP2 plants exhibit a closed stomata phenotype

We previously reported that a florigen gene *FT* is downregulated in GFP-LKP2 plants, leading to late flowering (Takase et al. 2011). FT positively regulates not only flowering time but also stomatal opening (Kinoshita et al. 2011). Therefore, we speculated that *FT* downregulation might confer a "less open" stomata phenotype in GFP-LKP2 plants. To evaluate this possibility, we examined stomatal apertures microscopically (Fig. 4a). Stomata opened more in the daytime (Fig. 4b) and less at night (Fig. S2) in control plants (Col and GFP), in line with a previous report (Dodd et al. 2005). Stomata were less open in the daytime in GFP-LKP2 plants than in control plants (Fig. 4b). There was no difference in stomata opening at night between the control and GFP-LKP2 plants (Fig. S2). Stomatal density was the same in the control and GFP-LKP2 plants (Fig. 4c). Thus, the "less open" stomata phenotype, which limits water loss by transpiration, may explain the enhanced dehydration stress tolerance of GFP-LKP2 plants.

Discussion

In the present study, using microarray analysis, we characterized the gene expression profile of GFP-LKP2 plants in comparison with GFP plants. *AT2G27375*, a transposable element gene, was most strongly upregulated;

Table 1 Abiotic stress-responsive upregulated genes in GFP-LKP2 plants in comparison with GFP plants identified by the Gene Functional Classification Tool within DAVID Bioinformatics Resources 6.7

AGI code	Primary sequence name	Annotation	Fold change	*P* value
AT1G18330	EPR1	EPR1 (EARLY-PHYTOCHROME-RESPONSIVE1); DNA binding/transcription factor	10.80	1.8E-08
AT4G25470	DREB1C/CBF2	DREB1A (DEHYDRATION RESPONSE ELEMENT B1C)/CBF2 (C-REPEAT BINDING FACTOR 2); DNA binding/transcription activator/transcription factor	9.53	3.7E-09
AT5G11210	GLR2.5	ATGLR2.5 (*Arabidopsis thaliana* glutamate receptor 2.5)	6.83	2.6E-04
AT3G04290	LTL1	ATLTL1/LTL1 (LI-TOLERANT LIPASE 1); carboxylesterase	5.00	4.5E-15
AT5G59320	LTP3	LTP3 (LIPID TRANSFER PROTEIN 3); lipid binding	4.82	9.5E-15
AT2G28190	CSD2	CSD2 (COPPER/ZINC SUPEROXIDE DISMUTASE 2); copper, zinc superoxide dismutase	4.15	1.6E-13
AT5G01600	FER1	[AT5G01600, ATFER1 (FERRETIN 1); ferric iron binding]; [AT5G01595, other RNA]	4.00	7.6E-13
AT4G25480	DREB1A/CBF3	DREB1A (DEHYDRATION RESPONSE ELEMENT B1A)/CBF2 (C-REPEAT BINDING FACTOR 3); DNA binding/transcription activator/transcription factor	3.90	6.2E-13
AT1G75780	TUB1	TUB1 (tubulin beta-1 chain); structural molecule	3.77	1.3E-12
AT1G29920	CAB2	CAB2 (Chlorophyll a/b-binding protein 2); chlorophyll binding	3.71	1.7E-12
AT3G47340	ASN1	ASN1 (DARK INDUCIBLE 6)	3.35	4.5E-11
AT5G20630	GER3	GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding/metal ion binding/ nutrient reservoir	2.98	3.0E-10
AT5G22500	FAR1	acyl CoA reductase, putative/male-sterility protein, putative	2.51	5.8E-08
AT5G52310	RD29A	RESPONSIVE TO DESICCATION 29A	2.46	1.2E-07
AT2G06850	XTH4	EXGT-A1 (ENDO XYLOGLUCAN TRANSFERASE); hydrolase, acting on glycosyl bonds	2.40	7.1E-08
AT2G21660	CCR2	ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding/double-stranded DNA binding/single-stranded DNA binding	2.27	3.2E-07
AT5G13930	TT4	ATCHS/CHS/TT4 (CHALCONE SYNTHASE); naringenin-chalcone synthase	2.24	4.3E-07
AT2G37640	EXP3	ATEXPA3 (*ARABIDOPSIS THALIANA* EXPANSION A3)	2.22	5.7E-07
AT1G08830	CSD1	CSD1 (COPPER/ZINC SUPEROXIDE DISMUTASE 1); copper, zinc superoxide dismutase	2.21	5.8E-07
AT5G17920	ATMS1	ATCIMS (COBALAMIN-INDEPENDENT METHIONINE SYNTHASE); 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase/ methionine synthase	2.17	9.4E-07
AT2G42540	COR15A	COR15A (COLD-REGULATED 15A)	2.17	9.4E-07
AT3G48100	RR5	ARR5 (ARABIDOPSIS RESPONSE REGULATOR 5); transcription regulator/two-component response regulator	2.12	1.7E-06
AT2G21490	DEHYDRIN	Dehydrin family protein	2.12	5.0E-05
AT3G11630		2-cys peroxiredoxin, chloroplast	2.10	2.2E-06
AT3G53420	PIP2A	PIP2A (PLASMA MEMBRANE INTRINSIC PROTEIN 2A); water channel	2.08	3.0E-06
AT3G50970	LTI30	LTI30 (LOW TEMPERATURE-INDUCED 30)	2.01	7.1E-06

however, the biological significance of this upregulation remains unclear. The fact that *LKP2* was the second most upregulated gene validates this microarray analysis. The late-flowering phenotype of LKP2-overexpressing plants is due to downregulation of the florigen gene *FT* (Takase et al. 2011). The fact that *FT* was downregulated in GFP-LKP2 plants also validates this microarray analysis.

Functional classification placed the differentially expressed genes into several groups, such as those involved in cell wall organization, response to light, cell division and growth, and responses to abiotic stimuli. We examined upregulation of several dehydration responsive genes, as this process is one of the most fundamental in both basic and applied plant biology. The enhanced dehydration tolerance of the GFP-LKP2 plants is underscored by the following observations. First, the leaf and root numbers in the GFP-LKP2 plants were higher than in the control plants after the recovery from dehydration. Second, after dehydration stress the survival rate of GFP-LKP2 plants was higher than that of controls.

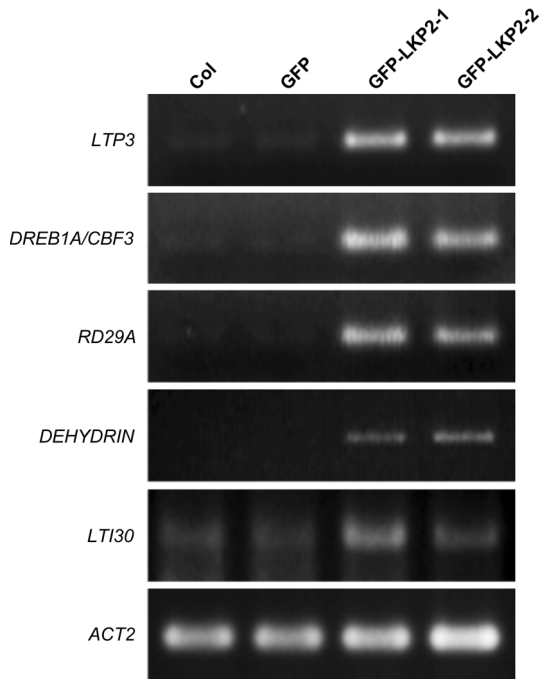

Fig. 1 Confirmation of microarray data using RT-PCR. Expression of genes identified by microarray analysis as upregulated in LKP2-GFP plants and annotated as dehydration-inducible genes in 8-day-old GFP-LKP2-1, GFP-LKP2-2, and control (Col and GFP) seedlings in the absence of stress. Actin gene (*ACT2)* was used as a control

DREB1A–C/CBF1–3 play a central role in cold stress responses in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki 2006). These genes encode transcription factors that belong to a small group within the AP2/ERF superfamily, whereas *DREB2A* and *DREB2B* encode transcription factors that belong to another subgroup of the same superfamily (Liu et al. 1998; Qin et al. 2011). *DREB1A–C/CBF1–3* are induced by cold stress, whereas *DREB2A* and *DREB2B* are induced following dehydration (Qin et al. 2011). Since DREB1A–C/CBF1–3, DREB2A, and DREB2B recognize the DRE *cis*-acting element, which contains the core motif A/GCCGAC, they activate many drought- and cold-inducible genes (including *RD29A*) whose promoters contain the DRE element (Fowler and Thomashow 2002; Sakuma et al. 2006; Qin et al. 2011). The cold-inducible genes function not only in cold stress, but also in dehydration tolerance (Yamaguchi-Shinozaki and Shinozaki 2006). Indeed, transgenic plants overexpressing *DREB1A* and those overexpressing constitutively active *DREB2A* exhibit dehydration tolerance (Liu et al. 1998; Sakuma et al. 2006). Thus, both the enhanced expression of *DREB1A–C/CBF1–3* in the absence of stress and accelerated accumulation of *DREB2B* mRNA in response to dehydration stress could explain the enhanced dehydration tolerance of GFP-LKP2 plants.

The transcript levels of approximately 1/3 of expressed *A. thaliana* genes, including genes responsive to abiotic or biotic stresses, are regulated in a circadian manner (Covington et al. 2008). *DREB1A–C/CBF1–3* have circadian expression that peaks at midday (Dong et al. 2011). This higher *DREB1A–C/CBF1–3* expression before night may prepare plants for the daily temperature changes by providing a certain level of preformed cold tolerance (Nakamichi et al. 2009). PRR5 (PSEUDO-RESPONSE REGULATOR 5), a clock component, represses *DREB1A–C/CBF1–3* expression via binding to their promoter regions (Nakamichi et al. 2009, 2012). LKP2 is reported to degrade PRR5 (Baudry et al. 2010). Thus, the reduction in the PRR5 level due to LKP2 overexpression may engender higher expression of *DREB1A–C/CBF1–3* and their target genes (direct, indirect, or both) in GFP-LKP2 plants.

The MYB–MYC pathway, ABA pathway, and NAC and ZFHD pathway are postulated to be involved in dehydration tolerance in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki 2006; Saibo et al. 2009). Using RT-PCR, we analyzed the expression of seven genes (*MYB2*, *MYC2*, *RD22*, *AREB1*, *AREB2*, *ABF3*, and *RD29B*) that belong to these pathways. Under dehydration stress, ABA accumulation induces ABA-responsive genes that encode proteins required for dehydration tolerance (Saibo et al. 2009). The expression of *MYB2* and *MYC2* genes is induced by ABA (Urao et al. 1993; Abe et al. 1997) and in its turn induces *RD22* (Abe et al. 2003). Most ABA-responsive genes contain *cis*-acting elements called ABRE (ABA-responsive elements), which are recognized by a group of bZIP-type transcription factors named AREBs (ABRE-binding proteins), or ABRE-binding factors (ABFs) (Yamaguchi-Shinozaki and Shinozaki 2006). Expression of *AREB1*, *AREB2*, and *ABF3* is also upregulated by ABA (Uno et al. 2000; Fujita et al. 2005; Yoshida et al. 2010). *RD29B* is a downstream target of *AREB1*, *AREB2*, and *ABF3* (Yoshida et al. 2010). As the expression levels of *MYB2*, *MYC2*, *RD22*, *AREB1*, *AREB2*, *ABF3*, and *RD29B* were not apparently different in GFP-LKP2 and control plants under both conditions (Fig. 3b), we infer that the MYB2–MYC2 and ABA pathways are unlikely to be involved in dehydration tolerance of *LKP2*-overexpressing plants.

In the NAC and ZFHD pathway, NAC and ZFHD transcription factors modulate the expression of early response genes upon dehydration stress (Fujita et al. 2004; Tran et al. 2004). Upon dehydration, only the level of *ANAC055* was apparently higher in the GFP-LKP2 plants than in the controls (Fig. 3b). Transgenic plants overexpressing *ANAC055* have increased drought tolerance (Tran et al. 2004); thus, ANAC055 protein may protect LKP2-overexpressing plants against dehydration stress.

We do not know how overproduction of GFP-LKP2 enhances accumulation of *DREB2B* and *ANAC055*

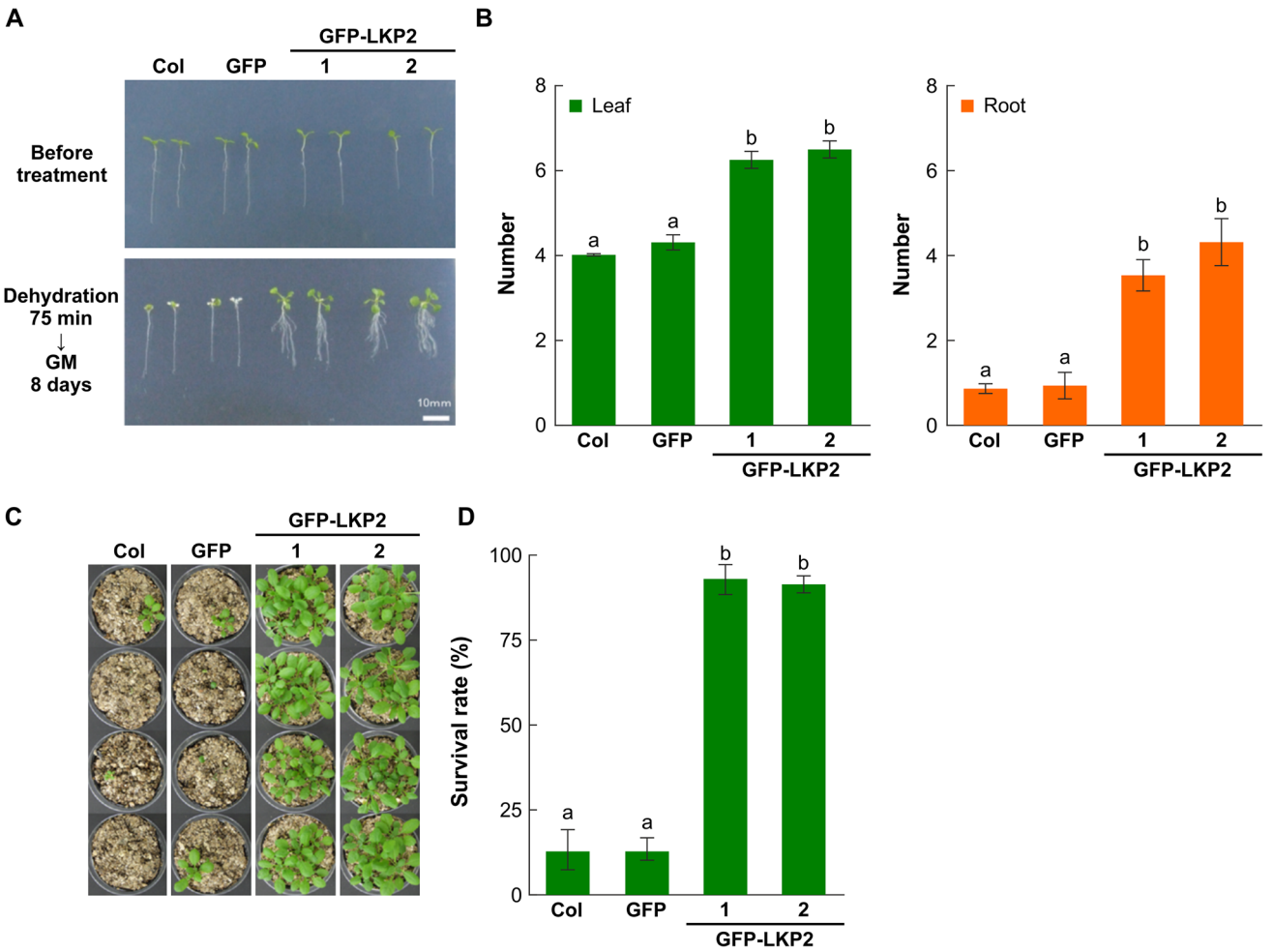

Fig. 2 Dehydration tolerance of GFP-LKP2 plants. **a** GFP-LKP2 (*lines 1* and *2*) and control (Col and GFP) seedlings on GM agar plates before and after dehydration stress. **b** Leaf and root numbers of GFP-LKP2 and control plants grown for 8 days on GM agar medium after dehydration stress for 75 min ($n = 20$; mean ± SE). **c** GFP-LKP2 and control plants grown for 3 weeks in pots with vermiculite after exposure to dehydration stress. **d** Survival rates of the GFP-LKP2 and control plants 3 weeks after dehydration stress. Twenty plants from each group were used in one experiment ($n = 3$; mean ± SE). *Different letters* indicate statistical difference ($p < 0.05$, Student–Newman–Keuls test)

transcripts. Overexpression of *LKP2* causes circadian disorder and late flowering partially by degradation of PRR5 and inhibition of FKF1 by its exclusion from the nuclei; it probably also causes polyubiquitination and subsequent degradation of unidentified LKP2 target proteins by 26S proteasome (Schultz et al. 2001; Takase et al. 2011; Miyazaki et al. 2011). One or more of these or some unidentified mechanisms may underlie the elevated sensitivity of GFP-LKP2 plants to dehydration stress.

FT is a florigen gene expressed in vascular tissues; in the apical meristem, FT interacts with the bZIP transcription factor FD (Abe et al. 2005). In the guard cells, FT positively regulates stomatal opening. Ectopically expressed *FT* in guard cells confers a continuously wide-open stomata phenotype, whereas a loss-of-function *ft* mutant exhibits a continuously narrowly opened stomata phenotype (Kinoshita et al. 2011). LKP2 overexpression reduces *FT* expression in leaves (Takase et al. 2011). This may explain the continuously narrowly opened stomata phenotype in GFP-LKP2 plants (Fig. 4, S2). Although in the guard cells, CO and GIGANTEA are involved in *FT* expression (Ando et al. 2013), no such data are available for the LKP2/ZTL/FKF1 family photoreceptors.

The loss-of-function *lkp2* mutants do not exhibit a dehydration-tolerant phenotype (data not shown). This is not surprising, because the *lkp2* mutants also retain normal circadian rhythm and flowering time (Baudry et al. 2010; Takase et al. 2011). The reason why *lkp2* mutant seedlings do not show enhanced dehydration tolerance may be that the expression level of LKP2 is much lower than that of ZTL. *ZTL* expression level is about eight times that of *LKP2* under 16 h light/8 h dark conditions (Mockler et al.

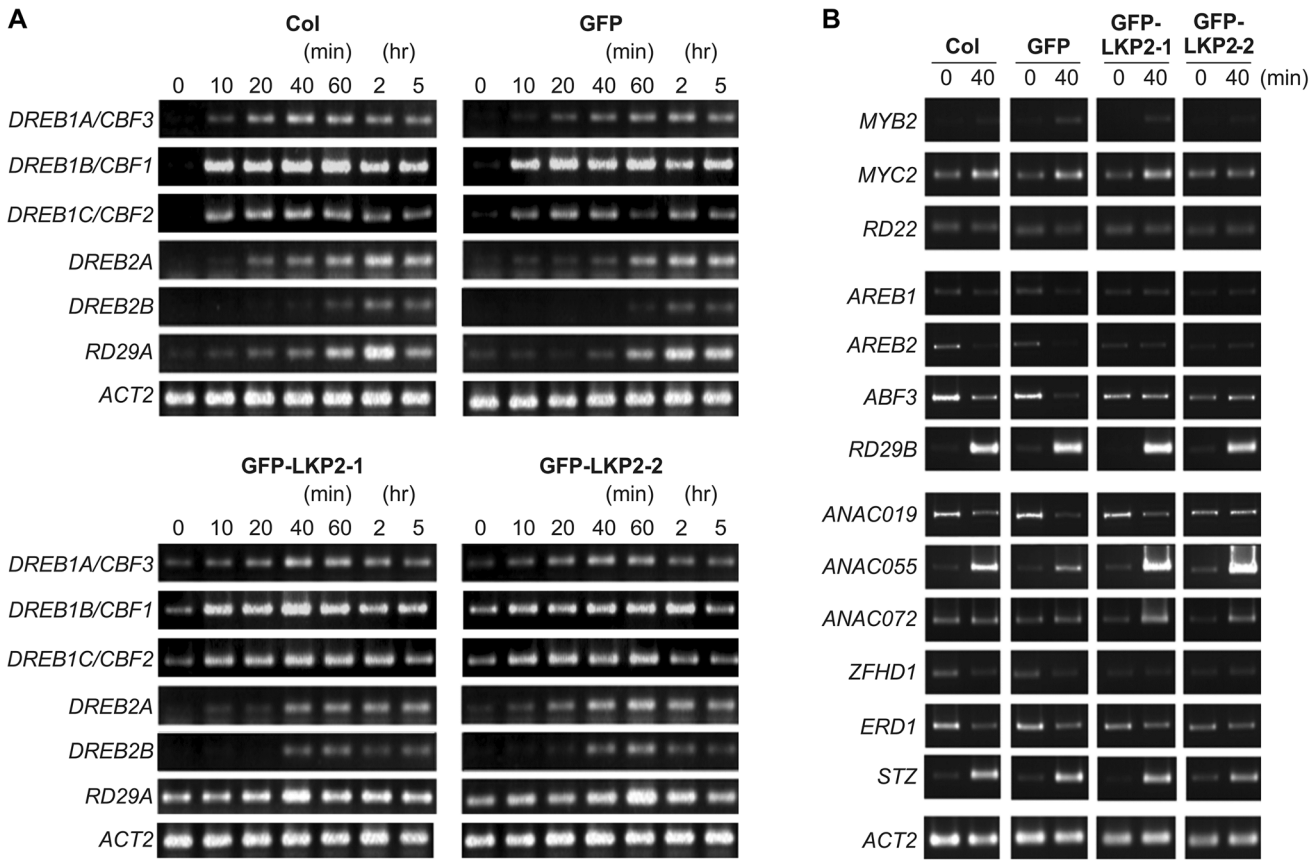

Fig. 3 RT-PCR analysis of stress-responsive transcripts during and after dehydration stress in GFP-LKP2 (*lines 1* and *2*) and control (Col and GFP) 8-day-old seedlings. **a** *DREB1A/CBF3*, *DREB1B/CBF1*, *DREB1C/CBF2*, *DREB2A*, *DREB2B*, and *RD29A*. **b** *MYB2*, *MYC2*, *RD22*, *AREB1*, *AREB2*, ABF3, *RD29B*, *ANAC019*, *ANAC055*, *ANAC072*, *ZFHD1*, *ERD1*, and *STZ*. *ACT2* was used as a control. Time 0 = untreated

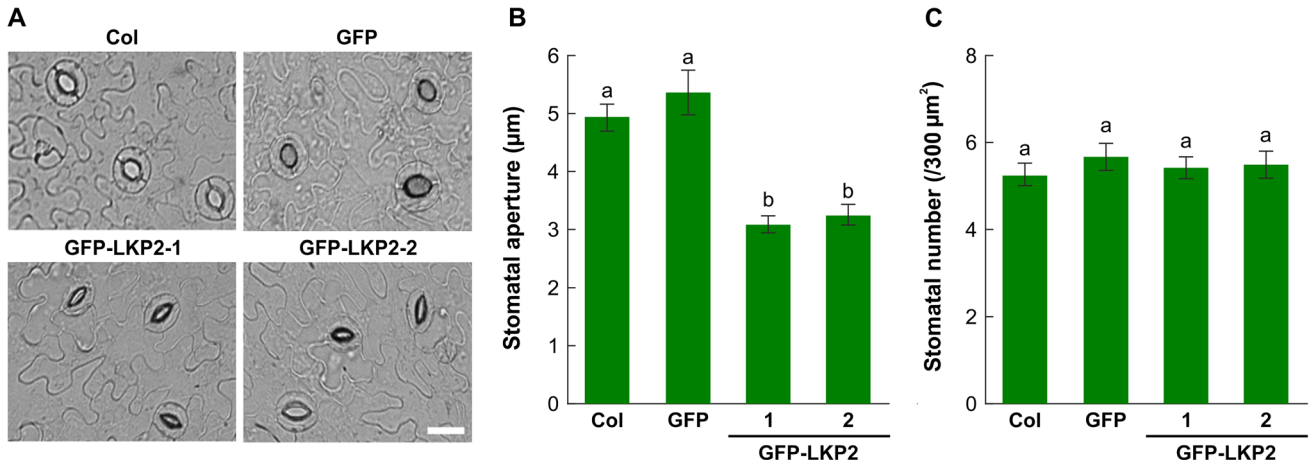

Fig. 4 Stomatal aperture and density in GFP-LKP2 (*lines 1* and *2*) and control (Col and GFP) plants. **a** Stomata at zeitgeber time 4 (ZT4). *Bar* 10 μm. **b** Stomatal aperture under long-day (16 h light/8 h dark) conditions ($n = 20$; mean ± SE). **c** Stomatal density ($n = 20$; mean ± SE). *Different letters* indicate statistical difference ($p < 0.05$, Student–Newman–Keuls test)

2007; Michael et al. 2008). Thus, ZTL, FKF1, or both might be sufficient in the absence of LKP2, thereby compensating for any defects in response to dehydration.

We cannot exclude the possibility that the GFP-fusion protein has an artificial effect, though overexpression of either GFP-LKP2 or LKP2 conferred elongated hypocotyls,

a circadian rhythm defect, and late flowering in Arabidopsis (Schultz et al. 2001; Miyazaki et al. 2011). Regardless of whether or not GFP-LKP2 has an artificial effect, it would be useful for biotechnological applications since overproduction of GFP-LKP2 enhances dehydration tolerance.

Plants that overexpress *LKP2* show pleiotropic phenotypes, such as late flowering (Schultz et al. 2001; Takase et al. 2011), hypocotyl elongation (Schultz et al. 2001; Miyazaki et al. 2011), circadian rhythm defects (Schultz et al. 2001; Miyazaki et al. 2011), and dehydration tolerance (this study). This suggests the involvement of LKP2 in the basic processes of plant development, growth, and stress responses. Further studies of LKP2 will increase our understanding of the functions of LKP2/FKF1/ZTL photoreceptor family. Furthermore, our results suggest that, in addition to its well-documented activity in the regulation of flowering time, this photoreceptor family gene can be exploited for future biotechnological applications, such as the enhancement of dehydration tolerance.

Author contribution statement Conceived and designed the experiments: YM, HA, TK. Performed the experiments: YM, HA. Analyzed the data: YM, HA, TT. Contributed reagents, materials, and analysis tools: YM, HA, MK. Wrote the paper: YM, TK.

Acknowledgments We thank Dr. K. Shimazaki (Kyushu University) and Dr. T. Kinoshita (Nagoya University) for their suggestions on the measurement of stomatal aperture. This research was partially supported by a fund from Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest The authors declare no conflict of interest.

References

Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K (1997) Role of arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. Plant Cell 9:1859–1868

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15:63–78

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science 309:1052–1056

Ando E, Ohnishi M, Wang Y, Matsushita T, Watanabe A, Hayashi Y, Fujii M, Ma JF, Inoue S, Kinoshita T (2013) TWIN SISTER OF FT, GIGANTEA, and CONSTANS have a positive but indirect effect on blue light-induced stomatal opening in Arabidopsis. Plant Physiol 162:1529–1538

Baudry A, Ito S, Song YH, Strait AA, Kiba T, Lu S, Henriques R, Pruneda-Paz JL, Chua NH, Tobin EM, Kay SA, Imaizumi T (2010) F-box proteins FKF1 and LKP2 act in concert with ZEITLUPE to control Arabidopsis clock progression. Plant Cell 22:606–622

Chen M, Chory J (2011) Phytochrome signaling mechanisms and the control of plant development. Trends Cell Biol 21:664–671

Covington MF, Maloof JN, Straume M, Kay SA, Harmer SL (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol 9:R130

Dodd AN, Salathia N, Hall A, Kévei E, Tóth R, Nagy F, Hibberd JM, Millar AJ, Webb AA (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309:630–633

Dong MA, Farré EM, Thomashow MF (2011) Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway in Arabidopsis. Proc Natl Acad Sci USA 108:7241–7246

Fowler S, Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14:1675–1690

Franklin K, Quail PH (2010) Phytochrome functions in Arabidopsis development. J Exp Bot 61:11–24

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LS, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J 39:863–876

Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. Plant Cell 17:3470–3488

González CV, Ibarra SE, Piccoli PN, Botto JF, Boccalandro HE (2012) Phytochrome B increases drought tolerance by enhancing ABA sensitivity in *Arabidopsis thaliana*. Plant, Cell Environ 35:1958–1968

Imaizumi T, Tran HG, Swartz TE, Briggs WR, Kay SA (2003) FKF1 is essential for photoperiodic-specific light signalling in Arabidopsis. Nature 426:302–306

Jarillo JA, Capel J, Tang RH, Yang HQ, Alonso JM, Ecker JR, Cashmore AR (2001) An Arabidopsis circadian clock component interacts with both CRY1 and phyB. Nature 410:487–490

Kiba T, Henriques R, Sakakibara H, Chua NH (2007) Targeted degradation of PSEUDO-RESPONSE REGULATOR$_5$ by an SCF^{ZTL} complex regulates clock function and photomorphogenesis in *Arabidopsis thaliana*. Plant Cell 19:2516–2530

Kinoshita T, Ono N, Hayashi Y, Morimoto S, Nakamura S, Soda M, Kato Y, Ohnishi M, Nakano T, Inoue S, Shimazaki K (2011) FLOWERING LOCUS T regulates stomatal opening. Curr Biol 21:1232–1238

Kiyosue T, Wada M (2000) LKP1 (LOV kelch protein 1): a factor involved in the regulation of flowering time in Arabidopsis. Plant J 23:807–815

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell 10:1391–1406

Más P, Kim WY, Somers DE, Kay SA (2003) Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. Nature 426:567–570

Michael TP, Breton G, Hazen SP, Priest H, Mockler TC, Kay SA, Chory J (2008) A morning-specific phytohormone gene expression program underlying rhythmic plant growth. PLoS Biol 6:e225

Miyazaki Y, Yoshizumi T, Takase T, Matsui M, Kiyosue T (2011) Overexpression of LOV KELCH PROTEIN 2 enhances cell elongation and increases cell number and ploidy in the hypocotyl of *Arabidopsis thaliana*. Plant Biotech 28:267–272

Mockler TC, Michael TP, Priest HD, Shen R, Sullivan CM, Givan SA, McEntee C, Kay SA, Chory J (2007) The DIURNAL project: DIURNAL and circadian expression profiling, model-based pattern matching, and promoter analysis. Cold Spring Harb Symp Quant Biol 72:353–363

Nakamichi N, Kusano M, Fukushima A, Kita M, Ito S, Yamashino T, Saito K, Sakakibara H, Mizuno T (2009) Transcript profiling of an Arabidopsis PSEUDO RESPONSE REGULATOR arrhythmic triple mutant reveals a role for the circadian clock in cold stress response. Plant Cell Physiol 50:447–462

Nakamichi N, Kiba T, Kamioka M, Suzuki T, Yamashino T, Higashiyama T, Sakakibara H, Mizuno T (2012) Transcriptional repressor PRR5 directly regulates clock-output pathways. Proc Natl Acad Sci USA 109:17123–17128

Nelson DC, Lasswell J, Rogg LE, Cohen MA, Bartel B (2000) FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. Cell 101:331–340

Oh S, Warnasooriya SN, Montgomery BL (2014) Mesophyll-localized phytochromes gate stress- and light-inducible anthocyanin accumulation in *Arabidopsis thaliana*. Plant Signal Behav 9:e28013

Qin F, Shinozaki K, Yamaguchi-Shinozaki K (2011) Achievements and challenges in understanding plant abiotic stress responses and tolerance. Plant Cell Physiol 52:1569–1582

Saibo NJ, Lourenço T, Oliveira MM (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Ann Bot 103:609–623

Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression. Plant Cell 18:1292–1309

Schultz TF, Kiyosue T, Yanovsky M, Wada M, Kay SA (2001) A role for LKP2 in the circadian clock of Arabidopsis. Plant Cell 13:2659–2670

Somers DE, Schultz TF, Milnamow M, Kay SA (2000) *ZEITLUPE* encodes a novel clock-associated PAS protein from *Arabidopsis*. Cell 101:319–329

Somers DE, Kim WY, Geng R (2004) The F-box protein ZEITLUPE confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. Plant Cell 16:769–782

Suetsugu N, Wasda M (2013) Evolution of three LOV blue light receptor families in green plants and photosynthetic stramenopiles: Phototropin, ZTL/FKF1/LKP2 and aureochrome. Plant Cell Physiol 54:8–23

Takase T, Nishiyama Y, Tanihigashi H, Ogura Y, Miyazaki Y, Yamada Y, Kiyosue T (2011) *LOV KELCH PROTEIN2* and *ZEITLUPE* repress Arabidopsis photoperiodic flowering under non-inductive conditions, dependent on *FLAVIN-BINDING KELCH REPEAT F-BOX1*. Plant J 67:608–621

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the *early responsive to dehydration stress 1* promoter. Plant Cell 16:2481–2498

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc Natl Acad Sci USA 97:11632–11637

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis *myb* homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5:1529–1539

Wang L, Fujiwara S, Somers DE (2010) PRR5 regulates phosphorylation, nuclear import and subnuclear localization of TOC1 in the Arabidopsis circadian clock. EMBO J 29:1903–1915

Xoconostle-Cázares B, Ramírez-Ortega FA, Flores-Elenes L, Ruiz-Medrano R (2010) Drought tolerance in crop plants. American J Plant Physiol 5:241–256

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803

Yasuhara M, Mitsui S, Hirano H, Takanabe R, Tokioka Y, Ihara N, Komatsu A, Seki M, Shinozaki K, Kiyosue T (2004) Identification of ASK and clock-associated proteins as molecular partners of LKP2 (LOV kelch protein 2) in *Arabidopsis*. J Exp Bot 55:2015–2027

Yoshida T, Fujita Y, Sayama H, Kidokoro S, Maruyama K, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2010) AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. Plant J 61:672–685