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RESEARCH PAPER

Overexpression of oligouridylate binding protein 1b results in ABA hypersensitivity

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ABSTRACT

Oligouridylate binding protein 1b (UBP1b), a marker protein of plant stress granules (SGs), plays a role in heat stress tolerance in plants. A previous microarray analysis revealed that the expression of several ABA signaling-related genes is higher in *UBP1b*-overexpressing *Arabidopsis* plants (*UBP1b*-ox) subjected to both non-stressed and heat stress conditions. Root elongation and seed germination assays demonstrated that *UBP1b*-ox exhibited hypersensitivity to ABA. RT-qPCR analysis confirmed that mitogen-activated protein kinase (MAPK) cascade genes, such as *MPK3, MKK4*, and *MKK9* were upregulated in *UBP1b*-ox plants. ABA receptor genes, including *PYL5* and *PYL6*, were also upregulated in *UBP1b*-ox plants. mRNA of *WRKY33* – a downstream gene of MPK3 and an upstream gene of ethylene biosynthesis, exhibited high levels of accumulation, although the level of endogenous ABA was not significantly different between *UBP1b*-ox and control plants. In addition, RNA decay analysis revealed that *WRKY33* was more stable in *UBP1b*-ox plants, indicating that the mRNA of *WRKY33* was protected within UBP1b SGs. Collectively, these data demonstrate that UBP1b plays an important role in plant response to ABA.

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ABA response; ABA sensitivity; RNA stability; UBP1b; UBP1b stress granule

Introduction

Plants have a variable ability to respond to and tolerate environmental stresses, such as drought, heat and high-salinity stress. Understanding the molecular mechanisms responsible for environmental stress tolerance in plants is essential for developing strategies to improve plant productivity under unfavorable environmental conditions. Recent studies have revealed that post-transcriptional regulatory mechanisms, such as the addition of 5′ cap structure,[1] splicing,[2] 3′ poly A addition,[3] and control of mRNA degradation[4] and storage,[5] are involved in stress response and adaptation.

Several papers have also reported that post-transcriptional events are linked to ABA responses. Mutants of genes involved in the post-transcriptional gene regulation exhibited an ABA hypersensitive phenotype. These included: 1) a *Hyponastic leaves 1 (HYL1)* gene encoding a nuclear localized double-stranded RNA (dsRNA) binding protein[6]; 2) genes encoding the mRNA cap binding proteins, ABH1 (ABA hypersensitive Arabidopsis 1), CBP80,[1] and CBP20[7]; 3) a *ABA-hypersensitive germination2 (AHG2)* gene encoding a poly (A)-specific ribonuclease (AtPARN)[3]; and 4) the *Supersensitive to ABA and drought 1 (SAD1)* gene encoding a polypeptide similar to multifunctional Sm-like snRNP protein.[8] The function of the genes related to post-transcriptional gene regulation of ABA signaling and responses, however, is not well understood.

UBP1b, a component of and marker protein of stress granules, protects mRNAs from degradation under abiotic stress conditions by binding to their 3′-UTRs, U-rich introns, and poly(A) tails.[9,10] Our previous research indicated that UBP1b plays an important role in plant heat stress tolerance.[5] *UBP1b*–overexpressing plants exhibited increased heat tolerance, while *ubp1b* mutants exhibited heat sensitivity. Several potential targets of UBP1b, including mRNAs of a DNAJ binding protein and a zinc finger binding protein, were identified.[5] Our microarray analysis of *UBP1b*-ox plants subjected to non-stressed or heat stress conditions revealed higher expression of several ABA signaling-related genes, suggesting that the UBP1b functions in the ABA signaling pathway. Root elongation and seed germination assays demonstrated that *UBP1b*-ox plants exhibit an ABA-hypersensitive phenotype. These data also indicate that UBP1b plays an important role in ABA response.

Results

Several ABA signaling-related genes are upregulated in UBP1b-ox plants

A previous microarray analysis revealed that the expression of 1,103 genes is higher in *UBP1b*-ox plants, relative to

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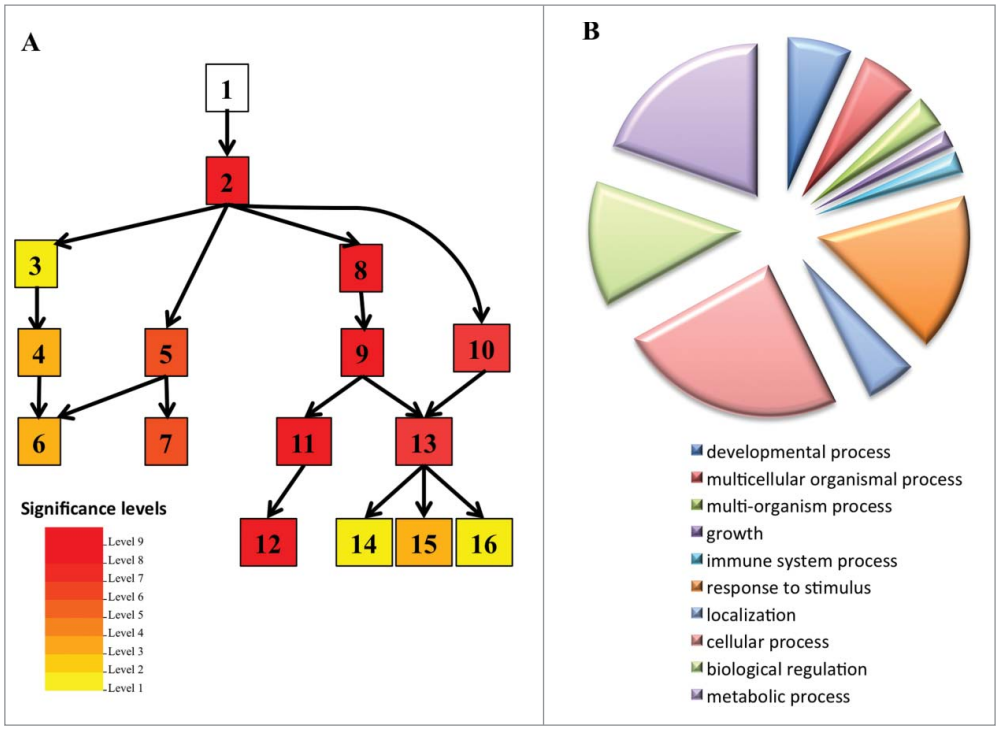

Figure 1. GO analysis of microarray data for biologic process categories. (A) Singular enrichment analysis of the genes with higher expression in *UBP1b*-ox plants under non-stressed and heat stress conditions. Analysis was performed using AgriGO (http://bioinfo.cau.edu.cn/agriGO/). The box colors indicate the level of statistical significance. 1, GO:0008150 biologic process; 2, GO:0050896 response to stimulus ($p = 2.6e^{-28}$); 3, GO:0009628 response to abiotic stimulus ($p = 5.4e^{-6}$); 4, GO:0009266 response to temperature stimulus ($p = 7e^{-8}$); 5, GO:0006950 response to stress ($p = 4.3e^{-14}$); 6, GO:0009409 response to cold ($p = 2.4e^{-8}$); 7, GO:0006952 defense response ($p = 5.1e^{-13}$); 8, GO:0042221 response to chemical stimulus ($p = 8.1e^{-22}$); 9, GO:0010033 response to organic substance ($p = 8.4e^{-26}$); 10, GO:0009719 response to endogenous stimulus ($p = 2.6e^{-13}$); 11, GO:0009743 response to carbohydrate stimulus ($p = 1.5e^{-20}$); 12, GO:0010200 response to chitin ($p = 3.9e^{-21}$); 13, GO:0009725 response to hormone stimulus; 14, GO:009737 response to abscisic acid stimulus ($p = 2.6e^{-13}$); 15, GO:009733 response to auxin stimulus ($p = 5e^{-7}$); 16, GO:0032870 cellular response to hormone stimulus ($p = 2.6e^{-13}$). (B) GO biologic process classification of 1,103 genes with higher expression in *UBP1b*-ox under non-stressed and heat stress conditions. GO-analysis was performed using the Gene Ontology tool (http://pantherdb.org).

non-transformed controls, grown under non-stress or heat stress conditions.[5] Genes that function in ABA signaling and heat stress response-related genes were among the upregulated genes (Fig. 1; Table 1). In particular, expression of the following ABA signaling-related genes was higher in *UBP1b*-ox than in control plants: 1) *Pyrabactin-resistance 5 and 6* (*PYL5, PYL6*) genes encoding 2 members of PYR/PYL/RCAR family proteins, which function as ABA receptors[11]; 2) *MAPK* family genes such as *Mitogen-activated protein kinase 3* (*MPK3*), and *Mitogen-activated protein kinase kinase 4* and *9* (*MKK4* and *MKK9*); all of which are important members of MAPK cascades involved in plant ABA signaling.[12,13]; and; 3) *WRKY DNA-binding protein 33* (*WRKY33*) which is a member of the WRKY transcription factor family. WRKY33 has been reported to be phosphorylated by MPK3/6[14] and is involved in the regulation of ethylene biosynthesis[15] (Table 1). Collectively, these data suggest that UBP1b may affect ABA signaling and the expression of related downstream genes.

Table 1. List of ABA and ethylene-related genes whose expression was higher in *UBP1b*-ox plants than in empty vector, control (*Venus*) plants.

AGI code	Gene name/ Encoded protein	Non-stress		Heat stress		Ratio (ox/*Venus*) under non -stressb)	Ratio (ox/*Venus*) under heat stressc)	Ratio(heat/*non-stress*) in *Venus*d)	Ratio(heat/*non-stress*) in *oxe*)
		Venus controla)	*UBP1b* -oxa)	*Venus* control a)	*UBP1b* -oxa)				
AT5G05440	PYL5	9.2	10.3	8.6	10.5	1.2	1.9	−0.6	0.2
AT2G40330	PYL6	7.7	8.1	5.7	7.3	0.4	1.6	−2	−0.8
AT1G51660	MKK4	9.3	9.9	8.9	9.8	0.6	0.9	−0.4	−0.1
AT1G73500	MKK9	10.7	12.4	9.8	10.4	1.7	0.6	−0.9	−2
AT3G45640	MPK3	11.3	12.4	10.7	11.7	1.1	1	−0.6	−0.7
AT2G38470	WRKY33	10.1	12.2	10.3	12.2	2.1	1.9	0.2	0

a) Average of signal intensity in 3 biologic replicates.
b) Average of the ratio of normalized signal value in *UBP1b*-ox versus *Venus* control under non-stress condition ($p < 0.15$; FDR < 0.0001).
c) Average of the ratio of normalized signal value in *UBP1b*-ox vs. *Venus* control under the heat stress condition ($p < 0.15$, FDR<0.0001).
d) Average of the ratio of normalized signal value in *Venus* control in the heat stress condition vs. non-stress condition vs. ($p < 0.15$, FDR < 0.0001).
e) Average of the ratio of normalized signal value in *UBP1b*-ox in the heat stress condition vs. non-stress condition ($p < 0.15$, FDR < 0.0001).

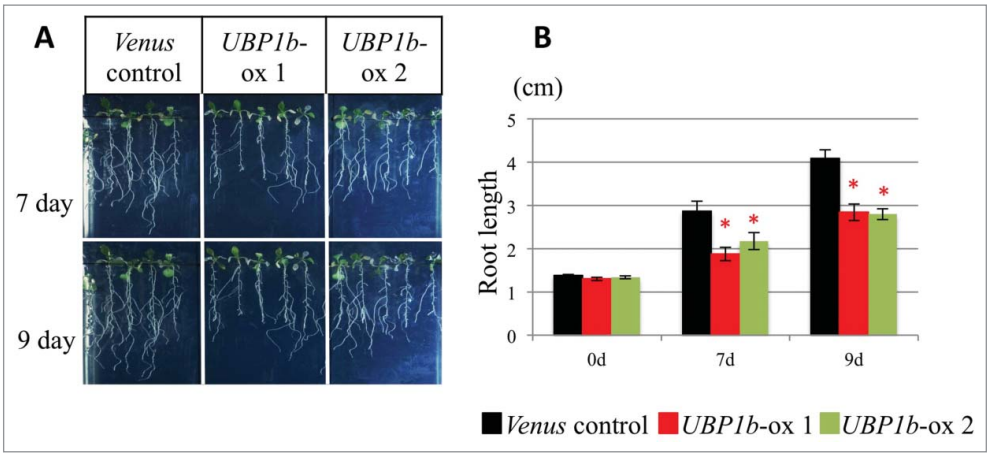

Figure 2. Root elongation assay. (A) Phenotype of *UBP1b*-ox and control plants in response to exogenous application of 10 μM ABA. Eight-day-old plants were transferred to vertical MS plates containing 10 μM ABA and grown in an environmental chamber. Phenotypes were observed after 7 and 9 d. (B) Root length of treated plants at 7 and 9 d. Data represent the mean $\pm$ sd. n = 15. An asterisk indicates a significant difference ($p < 0.05$) between *UBP1b*-ox and control plants as determined by a t-test.

UBP1b-ox plants exhibit an ABA hypersensitive phenotype

The sensitivity of *UBP1b*-ox plants to exogenous ABA treatment was investigated using root elongation and seed germination assays to analyze the function of UBP1b in ABA signaling. Roots in *UBP1b*-ox plants subjected to a 10 μM ABA treatment were shorter than roots in control plants (Fig. 2). Seven days after being treated with ABA, roots in 2 lines of *UBP1b*-ox plants were approximately 2 cm in length, while roots in non-transformed control plants were about 3 cm. After 9 days, *UBP1b*-ox roots were less than 3 cm; while roots in control plants were approximately 4 cm (Fig. 2). Results of the seed germination assay indicated that germination of seeds from control plants was approximately 90% on MS medium, 90% on MS medium amended with 0.3 μM, and 50% on MS medium amended with 0.5 μM ABA (Fig. 3). In contrast, germination rates of *UBP1b*-ox seeds were 50–60% on normal MS medium, and 40% and 10% on MS medium amended with 0.3 μM or 0.5 μM ABA, respectively (Fig. 3). The data on *UBP1b*-ox seeds and roots indicate that *UBP1b*-ox plants are hypersensitive to ABA and that UBP1b may promote ABA response.

Endogenous ABA levels in UBP1b-ox plants is similar to control plants

The level of endogenous ABA was measured in *UBP1b*-ox and control plants to determine the role of UBP1b in ABA biosynthesis and the ABA signal transduction pathway. Results indicated that there was no significant difference in the transgenic

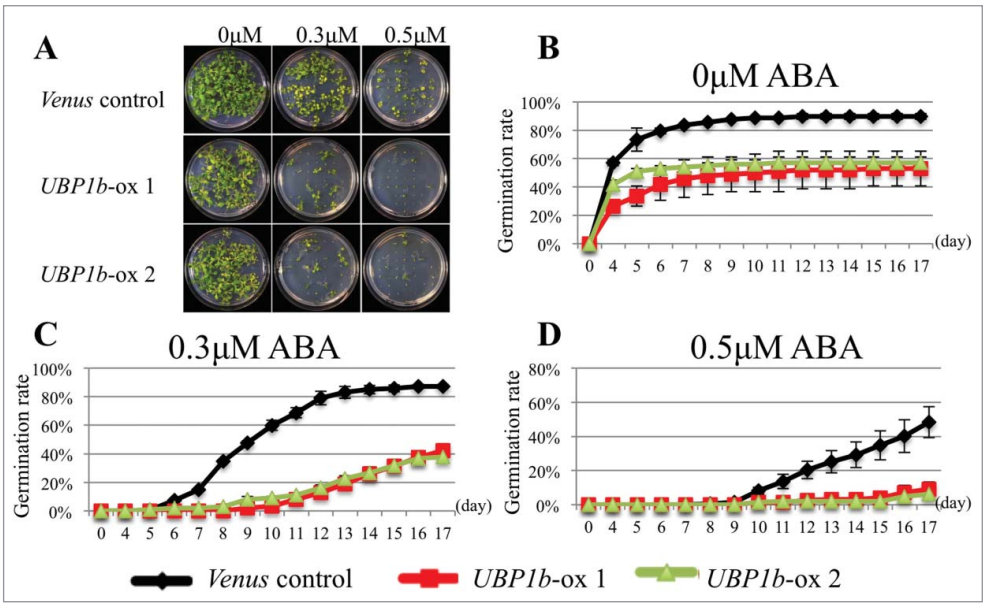

Figure 3. Seed germination assay. (A) Phenotype of 17 day-old seedlings of 2 lines of *UBP1b*-ox (1 and 2) and empty vector (*Venus*) control plants germinated on MS plates amended with ABA. Forty-nine seeds were placed on each MS plate amended with 0.3 μM ABA, or 0.5 μM ABA. (B) Seed germination rate of *UBP1b*-ox and control plants grown on non-amended MS plates. (C) Seed germination rate of *UBP1b*-ox and control plants on MS plates amended with 0.3 μM ABA. (D) Seed germination rate of *UBP1b*-ox and control plants on MS plates amended with 0.5 μM ABA. Data represent the mean $\pm$ sd. n = 3.

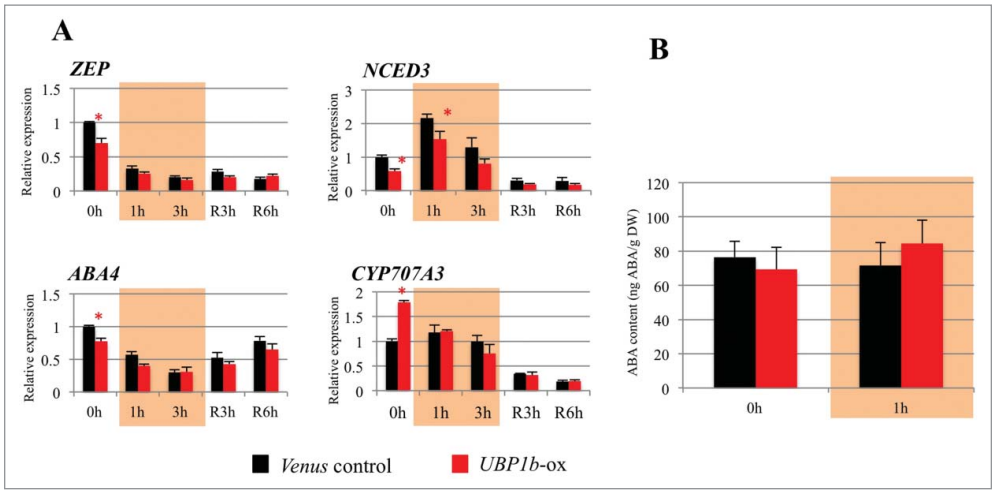

Figure 4. Expression level of ABA biosynthesis- and catabolism-related genes and endogenous ABA level in *UBP1b*-ox plants. (A) RT-qPCR analysis of the expression levels of ABA metabolism-related genes in *UBP1b*-ox and control plants. RT-qPCR analysis of *ZEP, NCED3, ABA4*, and *CYP707A3* expression was performed using the RNA of plant samples collected under non-stress condition (0h), 1 hour and 3 hour treatment with 40°C (1h and 3h), and under recovery at 22°C for 3 hour and 6 hour (R3h and R6h). Data represent the mean ± sd. n = 3. An asterisk indicates a significant difference ($p < 0.05$) between *UBP1b*-ox and control plants as determined by a t-test. (B) Endogenous ABA level in *UBP1b*-ox and control plants under non-stressed and heat stress conditions. Data represent the mean ± sd. n = 6.

and control plants under non-stress or heat stress (40°C for 1 h) conditions (Fig. 4B). Additionally, the expression level of *Zeaxanthinepoxidase*(*ZEP*), *Nine-cis-epoxycarotenoiddioxygenase 3* (*NCED3*), and *ABA-deficient 4 (ABA4)*, which are all ABA biosynthesis-related genes, was a little downregulated in *UBP1b*-ox compared with control (Fig. 4A). In contrast, an ABA catabolism-related gene, *CYP707A3*, was upregulated in non-stress *UBP1b*-ox, but was not different between *UBP1b*-ox and control plants subjected to heat stress (Fig. 4A). It is plausible that the differences of ABA biosynthesis and catabolism genes were not enough to cause the change of endogenous ABA level. These results suggest that UBP1b does not affect endogenous ABA levels but rather regulates ABA signaling-related genes.

mRNA of WRKY33 is a potential target of UBP1b

RT-qPCR analysis indicated that the expression of *PYL5, PYL6, MPK3, MKK9* and *WRKY33* was higher in *UBP1b*-ox plants grown under non-stress condition, while the expression of *MKK4* was higher in *UBP1b*-ox plants subjected to heat stress (Fig. 5). The expression pattern of *WRKY33* was significantly higher in non-stressed *UBP1b*-ox plants and in *UBP1b*-ox plants after 1 h of heat stress (Fig. 5). RNA decay analysis was performed to confirm whether or not those genes are target of UBP1b. If an mRNA interacts with a RNA-binding protein like UBP1b, the degradation of the interacting mRNA will be inhibited. It is possible to measure the rate of mRNA degradation in plants treated with a transcriptional inhibitor. In the present study, cordycepin was used as a transcriptional inhibitor. The degradation rate of mRNAs was measured in *UBP1b*-ox and control plants after they were treated with 0.6 mM cordycepin. Among the ABA-related genes investigated above, the degradation speed of *WRKY33* mRNA was slower in *UBP1b*-ox plants than in control plants. The half-life of *WRKY33* mRNA in *UBP1b*-ox plants was 5.22 h, compared with a degradation rate of 1.62 h in control plants (Fig. 6). The difference of regression coefficient in the linear regression model between overexpressed plants and control plants were calculated using F-test. Among all the target candidates, p-value of the F-test of WRKY33 was smallest and less than 0.1. Other candidates showed high p-values (Fig. 6). Therefore, it is possible that the mRNA of *WRKY33* is protected from degradation by UBP1b, and that *WRKY33* mRNA is a target of UBP1b. The mRNA of other ABA-related genes, however, did not show any differences in the degradation rate of *UBP1b*-ox vs. control plants (Fig. 6).

Ethylene biosynthesis genes are upregulated in UBP1b-ox plants

WRKY33 is an important substrate of MPK3/6 in ABA signaling[14] Under non-stressed conditions, WRKY33 affects plant root growth through the regulation of ethylene biosynthesis.[15,16] WRKY33 binds directly to the W-box of 4 1-amino-cyclopropane-1-carboxylate synthases 2/6 (ACS2/ACS6) and thereby positively mediates the activation of these ethylene biosynthesis genes.[15] High concentrations of ABA and/or ethylene inhibits plant root growth.[16,17] Our microarray data indicated that 4 ACS genes (*ACS6, ACS7, ACS8* and *ACS11*) which are key enzymes in ethylene biosynthesis, are also upregulated in *UBP1b*-ox plants (Table S1). The expression of *ACS2*, however, was not significantly changed in *UBP1b*-ox plants (data not shown). Therefore, the expression level of ACS genes was evaluated by RT-qPCR, to confirm the microarray data. Results indicated that the expression of *ACS6, ACS7*, and *ACS8* was higher in *UBP1b*-ox plants. The expression of *ACS11*, however, was not significantly different in *UBP1b*-ox vs. control plants (Fig. 7).

Microarray data also showed that the mRNA expression of several ethylene treatment-upregulated genes, such as *ethylene-responsive factor (ERF) 1, 2, 4* and *5, Auxin-Regulated Gene involved in Organ Size (ARGOS, AT3G59900),*[18] *ARGOS-like (AT2G44080)*, and *a peroxidase superfamily protein (PER10,*

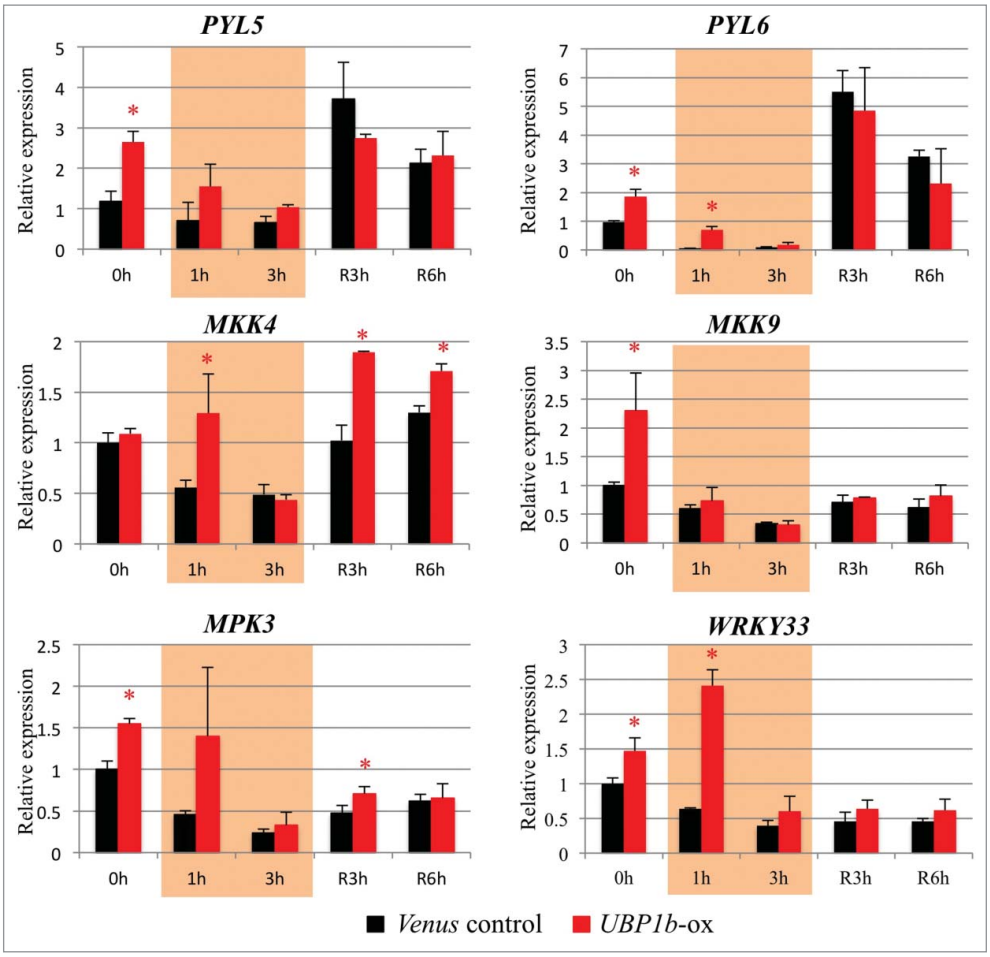

Figure 5. Expression level of UBP1b target candidates. (A) RT-qPCR analysis of target candidate genes was performed in *UBP1b*-ox and control plants under non-stressed and heat stress conditions; *PYL5, PYL6, MKK4, MKK9, MPK3*, and *WRKY33*. Data represent the mean ± sd. n = 3. An asterisk indicates a significant difference ($p < 0.05$) between *UBP1b*-ox and control plants as determined by a t-test.

AT1G49570) was higher in *UBP1b*-ox compared with control plants under normal condition (Fig. S1). On the other hand, the expression of several ethylene treatment-downregulated genes,[18] such as *glycosyl hydrolase 9C2 (GH9C2, AT1G64390), CYP71A16 (AT5G42590)*, and *Expansin B3 (EXPB3, AT4G28250)* was downregulated in *UBP1b*-ox under non-stress condition (Fig. S1). These results indicate the positive regulation of ethylene biosynthesis by UBP1b via the upregulation of ABA signaling. An RNA decay assay was conducted on all of the *ACS* genes, however, none of *ACS* genes exhibited a slower rate of mRNA degradation in *UBP1b*-ox plants compared with control plants. These data indicate that *ACS* genes are not the direct targets of UBP1b (Fig. 6).

Discussion

UBP1b functions as a component of SGs that play a role in protecting mRNAs from degradation. Previously generated microarray data indicated that UBP1b also functions under non-stressed conditions in up-regulating the expression level of various target genes[5] (Fig. 1, Fig. 5, Fig. 7). Many ABA signaling genes, such as ABA receptors *PYL5, PYL6*, and MAPK cascades were upregulated in *UBP1b*-ox plants, suggesting that UBP1b plays a role in regulation in the ABA signaling pathway in plants (Fig. 1, Fig. 5, Fig. 7). Although *UBP1b*-ox exhibited hypersensitivity to exogenous application of ABA, endogenous ABA levels were not significantly different in *UBP1b*-ox plants than they were in vector transformed control plants (Fig. 4). Biosynthetic and metabolic genes expression was slightly changed in transgenic plants, however, the effect was not enough to regulate endogenous ABA (Fig. 4). These data indicate that the ABA hypersensitivity of *UBP1b*-ox plants was not due to high levels of ABA but rather due to the regulation of ABA signaling genes. It is possible that the upregulation of ABA signaling affected ABA biosynthesis and catabolism genes through a feedback regulation mechanism. ABA receptor genes have been previously reported to increase ABA sensitivity.[19,20] Overexpression of MPK3 has also been reported to increase ABA sensitivity in plants,[21] which is consistent with the data generated in the present study. Both germinating seeds and mature seedlings of *UBP1b*-ox exhibited an ABA hypersensitive phenotype in our study, indicating that UBP1b affects ABA signaling at various stages of plant development.

The expression level of *WRKY33* was higher in *UBP1b*-ox plants and RNA decay analysis demonstrated that *WRKY33* transcripts had a slower rate of degradation in *UBP1b*-ox plants than in control plants. These results suggest that *WRKY33*

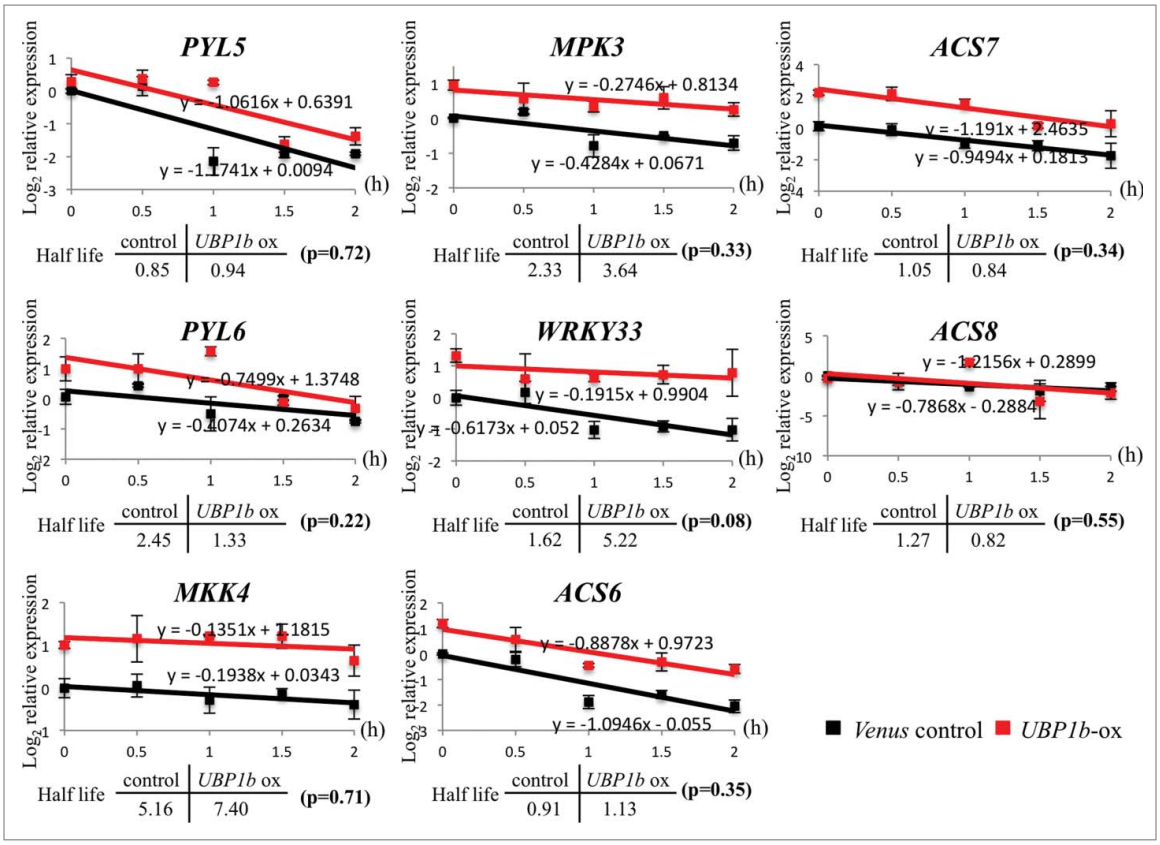

Figure 6. RNA decay assay of UBP1b target candidates. RNA decay rate of target candidate genes were checked in *UBP1b*-ox and control plants; *PYL5, PYL6, MKK4, MPK3, WRKY33, ACS6, ACS7*, and *ACS8*. Data represent the normalized $\log_2$ value of gene expression during cordycepin treatment. Data represent the mean $\pm$ sd. n = 3. F-test was performed to compare the RNA decay rate between parallel linear recognition model and individual linear recognition model and the p-value for each target candidate gene is shown within parenthesis.

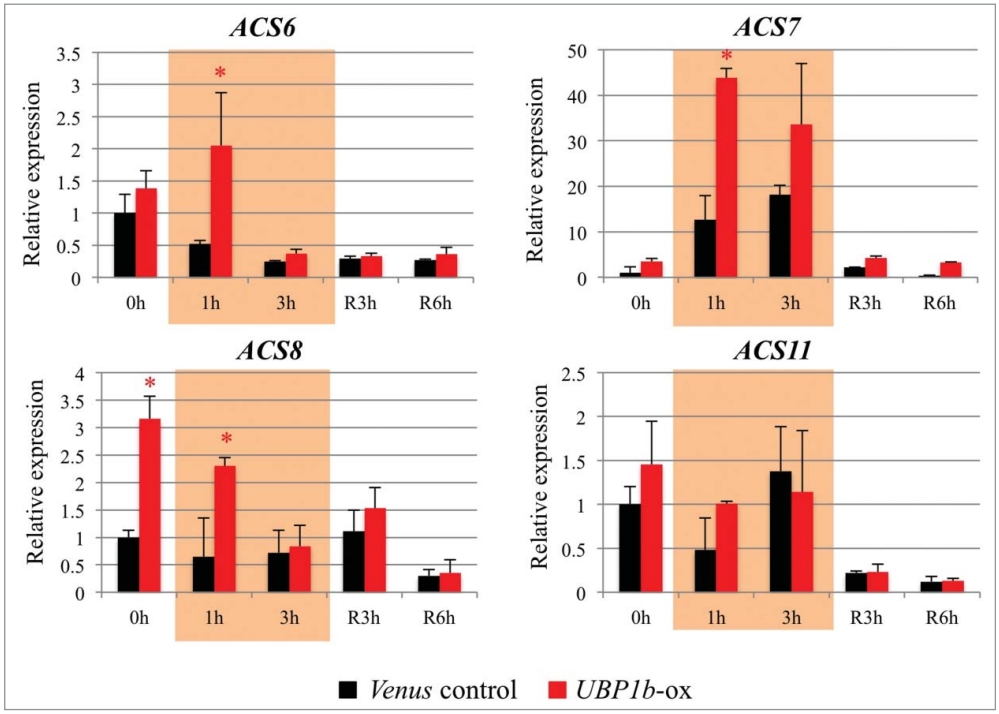

Figure 7. Expression level of ethylene biosynthesis-related genes. RT-qPCR analysis of *ACS6, ACS7, ACS8*, and *ACS11* in *UBP1b*-ox and empty vector, control (*Venus*) plants under non-stressed and heat stress conditions. Data represent the mean $\pm$ sd. n = 3. An asterisk indicates a significant difference ($p < 0.05$) between *UBP1b*-ox and control plants as determined by a t-test.

mRNA is a target of UBP1b. A previous study reported that *WRKY33*-overexpressing plants were sensitive to ABA,[22] which explains the phenotype of *UBP1b*-ox plants that were observed in response to exogenous application of ABA in the present study. Luo et al., 2014 reported that WRKY33 plays a key role in the inhibition of root growth by ABA through its effect on ethylene biosynthesis.[16] Another study also suggested that WRKY33 regulates plant abiotic stress response through an ethylene-dependent process.[23] In our data, ethylene biosynthesis genes, such as *ACS6, ACS7*, and *ACS8*, were upregulated in *UBP1b*-ox plants; and ABA treatment inhibited root growth in *UBP1b*-ox plants. Collectively, our data strongly suggest that UBP1b regulates plant ABA sensitivity by binding and stabilizing mRNAs of *WRKY33*.

Recent studies have reported crosstalk between ABA signaling and ethylene signaling in plants.[15,16,24,25] The ethylene pathway is also important in plant abiotic stress response. Regulation of ethylene biosynthesis genes, as well as ethylene levels, results in changes in the timing of seed germination, stomata closure, and root growth, which in turn affect plant tolerance to salt, heat and osmotic stresses.[16,26-29] ACC synthase (ACS) is a key enzyme in ethylene biosynthesis. There are 9 ACSs in *Arabidopsis*, including ACS1–2, ACS4–9, and ACS11; all of which are involved in the conversion of A-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC).[25,30] ACS2 and ACS6 are phosphorylated by MPK3 and MPK6, which positively regulate ACS protein levels and activity, resulting in the enhancement of ethylene production under stress conditions. [15,24,31,32] ABA treatment activates calcium-dependent protein kinase 4 (CPK4) and 2 (CPK11), which phosphorylate ACS6.[16] Overexpression of *ACS6* resulted in the inhibition of root growth, an increase in the germination rate, and an increase in ethylene production when plants were treated with ABA.[16] ACS7 is the only type 3 ACS protein in *Arabidopsis*, which is characterized by a very short C-terminus and the absence of a phosphorylation site.[33,34] *ACS7* expression is regulated by phytohormones, such as ethylene, ABA, and GA_3; and abiotic stresses such as high light and salt.[25,26] A loss-of-function mutant of *ACS7* exhibited both earlier seed germination and a higher level of growth, as well as enhanced tolerance to salt, heat, and osmotic stresses.[27] WRKY33, a substrate phosphorylated by MPK3/MPK6, regulates the expression of the ethylene biosynthesis genes, *ACS2* and *ACS6*[15] via direct binding to the W-boxes of their promoters. Thus, WRKY33 plays a key role in the activation of ACS2 and ACS6 in response to abiotic stress conditions. [15] WRKY33 has also been reported to increase plant sensitivity to ABA under non-stressed conditions. [22]

There is evidence for a link between ABA sensitivity and WRKY33, as well as between ABA and ethylene signaling. A splicing defect in *WRKY33* was identified in a knockout mutant of *sad1*, which exhibited hypersensitivity to ABA[8] and heat stress,[35] and an activation of alternative splicing.[36] The increased intron retention in WRKY33 transcripts in the *sad1* mutant has been implicated as a factor that contributes to the stress sensitivity of this mutant.[36] In contrast, *WRKY33* expression levels in *aba1–5* and *abi4–1*[37] plants is significantly reduced, indicating that *WRKY33* expression is partially dependent on ABA biosynthesis and the ABA signaling pathway.[22] During seed germination, ethylene acts as a negative regulator of plant ABA response.[38] The impairment in ethylene signaling results in ABA hypersensitivity.[39] After germination, ABA and ethylene signaling pathways exhibit a complex interaction.[37] Both pathways inhibit root growth,[38,39] and a disruption of ethylene signaling results in a decrease in the sensitivity of root growth to ABA.[39] The present research provides new information pertaining to the function of UBP1b that helps to clarify the complex interaction between ABA signaling, ethylene biosynthesis, and WRKY33.

Materials and methods

Plant material and growth conditions

The present study used 35S::*Venus-UBP1b*-overexpressing (*UBP1b*-ox) *A. thaliana* plants (ecotype: Columbia), and empty vector 35S::*Venus* (Venus) *A. thaliana* plants (ecotype: Columbia) as a control.[5] Plants were grown on Murashige and Skoog (MS) agar medium under long day conditions (16 h light/8 h dark) at 22°C in an environmental chamber (TOMY CF-405, Tokyo, Japan) and were used in all of the subsequent experiments.

Microarray analysis

Microarray data were obtained from a previous research study in which 14-day-old *UBP1b*-ox and control plants were sampled under non-stressed and heat stress conditions (40°C for 1h).[5] The resulting microarray data were deposited in and are available on the GEO website (GEO ID: GSE78713). Microarray statistical analysis was performed using Pavlidis method.[40] The genes whose expression level is significantly changed in the experimental conditions were selected as the candidate ones using one-way ANOVA analysis, with FDR <0.0001 as multiple test correction. And then, the gene expression data was subjected to the Student t-test as a post hoc test to find the conditions (*UBP1b*-ox non-stress, *UBP1b*-ox heat stress, control non-stress and control heat-stress) in which the gene expression was changed. The genes whose the expression data satisfies the following criteria were identified as ones with higher expression: $\log_2$ (expression ratio) > 0.7 and p-value of the Student's t-test < 0.15, relative to the control.

Root elongation assay

Eight-day-old *UBP1b*-ox and control plants were transferred to and grown on vertical MS plates containing 10μM ABA for 9 d under long-day conditions. Primary root length was measured on 3 biologic replicates in pictures of taken of the MS plates on 0, 3, and 7 d after plants were transferred to the ABA-containing media. Measurements in the photo were made using ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/download.html) software.

Seed germination rate

Seeds from *UBP1b*-ox and control plants were imbibed in water for 48 hours before being placed on MS plates amended with 0 μM, 0.3 μM, or 0.5 μM ABA. A total of 49 seeds from each *UBP1b*-ox line and control plants were placed on 3 different plates. Seedlings with green cotyledons were counted as germinated seeds. Three biologic replicates were used in each test.

ABA measurements

All plant samples (leaf and stem) were frozen in liquid nitrogen and weighed after lyophilization. Samples were ground and homogenized in 80% (v/v) acetonitrile containing 1% (v/v) acetic acid with defined amounts of D_6-ABA (Icon Isotopes, Summit, NJ, USA) as an internal standard. The solutions were incubated for 12 h at 4°C and then centrifuged at 3,000 g for 20 min at 4°C. The supernatants were dried under a vacuum and then dissolved in 1 mL of water containing 1% (v/v) acetic acid. Oasis WAX 1 mL solid-phase extraction cartridges (Waters) were conditioned with 1 mL acetonitrile followed by 1 mL methanol and 0.5 mL 0.1 M KOH, and then equilibrated with 1 mL of 1% acetic acid (v/v). Samples were loaded onto the cartridges and the cartridges were subsequently washed with 1 mL 1% (v/v) acetic acid and 1 mL acetonitrile. Fractions containing ABA were eluted from the cartridge with 1 mL 80% (v/v) acetonitrile containing 1% (v/v) acetic acid, dried under vacuum, and dissolved in 20 μL of water containing 1% (v/v) acetic acid. The LC–MS/MS system consisting of a quadrupole/time-of-flight tandem mass spectrometer (Triple TOF 5600, AB SCIEX, Toronto, Canada), and a Nexera HPLC system (SHIMADZU, Kyoto, Japan), were used to quantify the ABA concentration in each sample. LC-MS/MS was performed according to procedures as described previously.[41] MultiQuant 2.0, (AB SCIEX, Toronto, Canada) software was used to calculate plant hormone concentrations from the LC–MS/MS data. Four seedlings were collected for each sample, and 6 biologic repeats were used in this experiment.

RT-qPCR

Fourteen-day-old plants were treated with heat (40°C) then recovered at 22°C. Samples were collected at 0 h, heat 1 h, heat 3 h, recovery 3 h and 6 h. Total RNA was extracted using the Plant RNA Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Each of biologic repeat consisted of a pool of 5 seedlings, and 3 biologic replicates were used. Following the manufacturer's protocol, cDNAs were synthesized with a Quantitech cDNA synthesis kit (Qiagen, Venlo, Netherlands) using RNA samples obtained for the microarray analysis and were used for the RT-qPCR analysis. RT-qPCR analyses were performed using Fast SYBR Green MasterMix (Thermo Fisher Scientific) and a StepOne Plus Real Time PCR system (Thermo Fisher Scientific). *YLS8* was used as a reference gene to normalize expression data and RT-qPCR data were analyzed using StepOne Plus software (Thermo Fisher Scientific). Primers used in the RT-qPCR analyses are listed in Table S2.

RNA decay analysis

A total of 25 2-week-old plants were placed in a petri plate containing filter paper and 3 ml of water for 24 h. Subsequently, 2 ml of 1.2 mM cordycepin was added to each Petri dish, creating a final concentration of 0.6 mM cordycepin. The treatment was conducted at 22°C for 2 h and 5 seedlings were collected every 30 minutes (4 biologic replicates were used from a pool of 120 seedlings). Total RNA and cDNA were prepared from cordycepin-treated samples as described above. RT-qPCR was performed and decay rates were calculated based on the $\log_2$ value of the relative expression of the target genes. *SUMO2 (AT5G55160)*, a transcript with a long half-life,[42] was used as a reference gene for the normalization of the expression data. For the test of the difference of 2 regression coefficients, the F-statistic is computed using parallel linear model ($Y1 = \beta0 + \beta1\times1$, $Y2 = \beta2 + \beta1\times2$, where X1 and Y1 indicate time of RNA decay and $\log_2$ copy number in *UBP1b*-ox plants, respectively, and X2 and Y2 indicate those in control plants) and individual linear model ($Y1 = \beta0 + \beta1\times1$, $Y2 = \beta3 + \beta4\times2$). The formula for the F statistic is $F = \{SSrb - (SSr1 + SSr2) / (dF2 - dF1)\} / \{(SSr1 + SSr2) / dF2)\}$, where SSrb is the residual sum of squares for the parallel linear model, SSr1 and SSr2 are the residual sum of squares for the individual linear model of control plants and *UBP1b*-ox plants, respectively. And dF is $N - V$ degrees of freedom, where N is the number of data points and V is the number of parameters being estimated. When the p-value is less than 0.1, we decided that the 2 lines of RNA decay were not parallel.

Disclosure of potential conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

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