RESEARCH PAPER

# *Major latex protein-like protein 43* (*MLP43*) functions as a positive regulator during abscisic acid responses and confers drought tolerance in *Arabidopsis thaliana*

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## Abstract

**Drought stress is one of the disadvantageous environmental conditions for plant growth and reproduction. Given the importance of abscisic acid (ABA) to plant growth and abiotic stress responses, identification of novel components involved in ABA signalling transduction is critical. In this study, we screened numerous *Arabidopsis thaliana* mutants by seed germination assay and identified a mutant *mlp43* (major latex protein-like 43) with decreased ABA sensitivity in seed germination. The *mlp43* mutant was sensitive to drought stress while the *MLP43*-overexpressed transgenic plants were drought tolerant. The tissue-specific expression pattern analysis showed that *MLP43* was predominantly expressed in cotyledons, primary roots and apical meristems, and a subcellular localization study indicated that *MLP43* was localized in the nucleus and cytoplasm. Physiological and biochemical analyses indicated that *MLP43* functioned as a positive regulator in ABA- and drought-stress responses in *Arabidopsis* through regulating water loss efficiency, electrolyte leakage, ROS levels, and as well as ABA-responsive gene expression. Moreover, metabolite profiling analysis indicated that *MLP43* could modulate the production of primary metabolites under drought stress conditions. Reconstitution of ABA signalling components in *Arabidopsis* protoplasts indicated that *MLP43* was involved in ABA signalling transduction and acted upstream of SnRK2s by directly interacting with SnRK2.6 and ABF1 in a yeast two-hybrid assay. Moreover, ABA and drought stress down-regulated *MLP43* expression as a negative feedback loop regulation to the performance of *MLP43* in ABA and drought stress responses. Therefore, this study provided new insights for interpretation of physiological and molecular mechanisms of *Arabidopsis MLP43* mediating ABA signalling transduction and drought stress responses.**

**Key words:** ABA signal reconstitution, abscisic acid, drought stress, metabolite profile, MLP43, reactive oxygen species, SnRK2.6.

## Introduction

Abiotic stresses greatly affect plant growth and crop production. To date, plants have evolved many mechanisms to adapt and survive against these stresses, which include developmental, morphological, physiological and biochemical strategies. Plant hormones play essential roles in promoting and mediating these defence responses (Peleg and Blumwald, 2011). Abscisic acid (ABA) is regarded as a key signal involved in regulating the response of plants to various stresses, and particularly in regulating drought stress responses when plants experience water deficit (Cutler *et al.*, 2010; Lee and Luan, 2012). ABA production increases radically under drought stress conditions and stimulates stomatal closure, changing the expression of various osmotic stress-responsive genes (Kim *et al.*, 2010). In recent years, significant research progress has been made in studies using plants bearing gene mutations involved in hormone-biosynthetic and signalling transduction pathways, and to the identification of the ABA receptors PYR/PYL through chemical genetic approaches (Park *et al.*, 2009; Ma *et al.*, 2009; Hubbard *et al.*, 2010). Given the importance of ABA to plant physiology and development, identification of novel components involved in ABA signalling transduction is critical.

Major latex protein (MLP) was first identified from the latex of the opium poppy (*Papaver somniferum*) (Nessler *et al.*, 1990; Nessler and Burnett, 1992). The orthologues of *MLP*, called *MLP*-like proteins (*MLP*), were later found in *Arabidopsis*, soybean and tobacco (Aggelis *et al.*, 1997; Wu *et al.*, 2008). MLP proteins belong to the Bet v 1 family, also known as the pathogenesis related 10 (PR10)-like protein family, which contains proteins with low sequence similarity, but shares a similar three-dimensional structure (Radauer *et al.*, 2008). The Bet v 1 family is divided into 11 subfamilies, including MLP, PR10, cytokinin receptors and plant polyketide cyclase-like subfamilies (Radauer *et al.*, 2008). Most of the Bet v 1 proteins function through binding ligands, such as cytokinins, brassinolides and secondary metabolites and trigger downstream signal transduction (Koistinen *et al.*, 2005; Radauer *et al.*, 2008). In *Arabidopsis*, the ABA receptor RCAR/PYR/PYL family of START proteins share amino acid and structural similarity with Bet v 1 proteins (Ma *et al.*, 2009; Park *et al.*, 2009). Previous studies indicated that *MLPs* are down-regulated by blue light in the *cry1* loss-of-function mutant (Phee *et al.*, 2007). Two At*MLPs* (At2g01520 and At2g01530) could be induced by cis-cinnamic acid and promotes vegetative growth and delays flowering (Guo *et al.*, 2011). One report showed that *MLP*s were related to the translocation of hydrophobic compounds in the Cucurbitaceae family, which could contribute to decreasing the hydrophobic contamination of fruit (Inui *et al.*, 2013). Three *MLP*s, existing as duplicated gene pairs, were significantly down-regulated by oxidative stress through whole-genome DNA microarrays analysis, indicating that *MLP* might be involved in plants stress responses (Kim *et al.*, 2005). Interestingly, a study by Chen and Dai (2010) indicated that overexpression of *MLP* in *Arabidopsis* leads to salt stress insensitivity. The homologues of the *MLP* gene family are differentially expressed in various tissues; however, the molecular mechanism of MLPs in abiotic stress response is still elusive.

In this study, we screened numerous *Arabidopsis* T-DNA insertion mutants through seed germination assay, and identified the mutant *mlp43* with decreased ABA sensitivity in seed germination. The drought stress responses of the *mlp43* mutant and *MLP43*-overexpressed transgenic plants were also examined. The tissue-specific expression patterns and subcellular localization of *MLP43* were detected. Physiological and biochemical analysis indicated that *MLP43* was involved in ABA- and drought-stress responses in *Arabidopsis* through modulating primary metabolite profiling and gene expression. Reconstitution analysis of ABA signalling component assay in *Arabidopsis* protoplasts and direct interaction assay indicated that *MLP43* functions as a positive regulator in ABA signalling transduction and could directly interact with SnRK2.6 and ABF1 in yeast.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used in this study and all transgenic plants were generated in a Col-0 background. The line of *mlp43* mutant (Salk_109337 and Salk_033347) was obtained from the Arabidopsis Biological Resource Center (ABRC; http://arabidopsis.org/abrc/) and identified by PCR. Primers used are listed in Supplementary Table S1 at *JXB* online. The mutants of *ost1-1* (Mustilli *et al.*, 2002), *aba1-1* (Koornneef *et al.*, 1982) and *abi1-1* (Koornneef *et al.*, 1984) are derived from the *Arabidopsis* accession Landsberg *erecta* (Ler). The *pyr1/pyl1/4* triple mutant (Park *et al.*, 2009) is derived from the *Arabidopsis* accession Col-0. Seeds were surface sterilized and sown on Murashige and Skoog (MS) agar plates containing full-strength MS salts, 0.8% (w/v) agar, and 1% (w/v) sucrose. Germination assay was performed with or without ABA on MS plates (Sigma, A1049). The seeds were stratified at 4ºC for 4 d in darkness and then transferred to growth chamber with 16 h/8 h light/dark cycle at 23ºC, or were directly sown in soil after stratification under the same conditions.

### Plasmids construction and transgenic plant generation

MLP43 cDNA was amplified using the primers MLP43-F and MLP43-R and cloned into Pro35S::GFP to generate Pro35S::MLP43-GFP fusion construct. The fused protein was then digested by XhoI/SacI and inserted into a binary vector pBA to generate pBA-MLP43-GFP construct. Promoter fragment of MLP43 (-1769 bp before start code ATG) was amplified using primers ProMLP43-F and ProMLP43-R and subsequently cloned into the pBI101 to generate ProMLP43::GUS construct. The plasmids used in reconstituted ABA signaling pathway have been described by Lu *et al.* (2013). The detail information of plasmids used in yeast two hybrid (Y2H) assay is listed in Supplementary Table S2. The primers used for plasmids construction are listed in Supplementary Table S3. The mutated ABI5 was generated previously through site mutation (Wang *et al.*, 2013). All transgenic plants were generated by introducing *Agrobacterium tumefaciens* (strain GV3101) carrying the corresponding plasmids through floral dip-mediated infiltration into Col-0 background (Clough and Bent, 1998). Complementary transgenic plants of *Com-3* were generated by introducing a pBA-MLP43-GFP construct into *mlp43* mutant background.

### GUS histochemical analysis and subcellular localization

GUS signals were detected according to the method described by Jefferson *et al.* (1987). Plants were pretreated with or without 100 μM ABA for 3 h, and then immersed in 90% acetone for 30 min. After incubation in the GUS staining solution (0.5 mg/ml X-Gluc, 50 mM

PBS, pH 7.0; 5.0 mM potassium ferricyanide, 5.0 mM potassium ferrocyanide, 0.1% Triton X-100) at 37ºC over night, the stained plants were washed with 70% ethanol overnight. Images were taken with an inverted microscope (SMZ1500, Nikon). For subcellular localization of *MLP43*, we transformed the plasmids into *Arabidopsis* rosette leaves (Col-0) by bombardment. Images were taken with an inverted microscope (TE2000 U, Nikon) equipped with cool CCD (CoolSNAP HQ2, Roper Scientific). GFP fluorescence was acquired with 488 nm excitation, and the chloroplast auto-fluorescence was acquired with 543 nm excitation.

### Drought treatment, water loss analysis, and stomatal aperture measurement

For measurement of drought tolerance, water was withheld from 14-day-old plants comparable in size and growing in pots. After 21 d of drought treatment, the plants were re-watered. Survival rates were determined and the plants were photographed 2 d after rewatering. For measurement of water loss from detached leaves, the rosette leaves were detached from four-week-old plants and weighed at the indicated times. To analyse stomatal apertures, we incubated rosette leaves in a solution containing 50 mM KCl, 10 mM CaCl$_2$ and 10 mM MES (pH 6.15) for 3 h under light condition. ABA was then added to the solution to a final concentration of 50 μM. Stomatal apertures were then measured after 30 min and 1 h of ABA treatment, respectively. For each experimental repeat, at least 50 stomata were counted and measured by photoshop software. The values of stomatal width to length ratios (V) were defined as: V≥0.5, open; 0.5 >V≥0.25, partially open; and V<0.25, closed.

### Comparison of EL, ROS contents and antioxidant enzymes activities

EL was determined from the detached aerial parts of drought-stressed plants with the indicated time points. The detailed procedure was performed as described by Wang *et al.* (2013). Superoxide radicals (O$^-$) and hydrogen peroxide (H$_2$O$_2$) were detected by nitroblue tetrazolium chloride (NBT) staining and 3,3'-diaminobenzidine (DAB) staining, respectively, as described previously (Ramel *et al.*, 2009). Two-week-old seedlings were grown in soil, and then withheld water for the number of days indicated. Quantification of H$_2$O$_2$ content was determined using the method described by Hu *et al.* (2012). The activities of antioxidant enzymes were measured after drought treatment application followed by the procedure described by Wang *et al.* (2013).

### Gene expression analysis by real-time quantitative RT-PCR

Two-week-old plants were treated as indicated phytohormones or abiotic stresses. Total RNA was extracted using a plant RNA purification kit (Tiangen, Beijing, China). Equal amounts of RNA were used for reverse transcription with ReverTra Ace-α-TM (TOYOBO, Tokyo, Japan) according to the manufacturer's instructions. The primers used in qRT-PCR were designed using web tool GenScript (https://www.genscript.com/ssl-bin/app/primer). The primers used for qRT-PCR experiment are listed in Supplementary Table S4.

### Metabolite profiling by gas chromatograph time-of-flight mass spectrometry

Plant samples for metabolite profiling were withheld water for two weeks from the 15th day after sowing in the growth chamber, and then all seedlings were harvested and immediately frozen in liquid nitrogen. The experimental procedure for extract preparation was performed as described previously (Lisec *et al.*, 2006). The total extracts were quantified by performing chromatography on GC-TOF MS (Agilent 7890A/5975C, USA). The detailed procedure has been described by Shi *et al.* (2014), with the data representing the mean values of three independent experimental repeats. The results were analysed using the Cluster 3.0 program (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and visualized using Java Treeview (http://jtreeview.sourceforge.net/).

### Transient expression assay

Mesophyll protoplasts were isolated from four-week-old Col-0 plants according to the methods described previously (Yoo *et al.*, 2007). All of the plasmids used in this assay were prepared through purification with caesium chloride/ethidium bromide (Sambrook *et al.*, 1989). For the luciferase assay, protoplasts were harvested after 12-h incubation under light conditions at 23ºC with or without stimuli (50 μM ABA). The activities of LUC and GUS were measured with the GloMax-Multi Jr Single Tube Multimode Reader (Promega, USA). All experiments were repeated at least three times.

### Yeast two hybrid (Y2H) assay

The Y2H assay was carried out according to the instructions for the Matchmaker GAL4-based two-hybrid system (Clontech). The full-length sequence of *MLP43* was cloned and inserted into pGADT and pGBKT, respectively. Another two homologous genes, *MLP34* and *MLP168*, were cloned and inserted into pGBKT. We cloned *ABI5*, *SnRK2.2*, *SnRK2.3*, *PYL1/2/5/9/13* and *ABI1* and fused them into the pGADT vector, respectively. The genes of *SnRK2.6*, *SnRK2.8*, *SnRK2.10*, *PYR1*, *ABF1* and *ABF3* were cloned and fused into the pGBKT vector, respectively. The restriction sites and primer sequences for the plasmids used in this assay have been provided in Supplementary Tables S2 and S3, respectively.

## Results

### The mlp43 mutant was insensitive to ABA during seed germination

To discover other novel regulators and to expand ABA signalling networks during seed germination and abiotic drought stresses, we screened various T-DNA insertion mutants purchased from the ABRC (http://www.arabidopsis.org/) on MS medium containing 1.0 μM ABA during seed germination. The mutant *mlp43-1* (Salk_109337) showing insensitivity to ABA was selected for further anlysis. T-DNA was inserted into the 5'-UTR of *MLP43* (Fig. 1A). Transgenic plants of overexpressed *MLP43* (*OE-2* and *OE-4*) was generated by introducing Pro35S::MLP43-GFP plasmids into Col-0 plants. The relative expression level of *MLP43* was verified by qRT-PCR (Fig. 1B). The expression levels of MLP43-GFP fusion protein was detected using anti-GFP antibody through western-blot assay (Supplementary Fig. S1A). In the absence of ABA, no obvious differences were observed in germination rates between Col-0 and the *mlp43-1* mutant (Fig. 1C, D). However, the *mlp43* mutant showed significantly decreased ABA sensitivity on the 1.0 μM ABA plate, while overexpressed *MLP43* enhanced ABA sensitivity during seed germination (Fig. 1C, D). Moreover, the kinetics of germination time of Col-0, *mlp43-1*, and *MLP43 OE* were compared in the presence of 1.0 μM ABA by analysing the percentages of emerged radicles and open green cotyledons (Fig. 1E, F). On the 4th day after stratification, about 80% and 25% of Col-0 emerged with radicles and green cotyledons, respectively. However, the corresponding percentages of *mlp43-1* were more than 95% and 50%. Overexpressed *MLP43* transgenic plants showed much lower germination rates than

Col-0 plants (Fig. 1E, F). We also examined the ABA sensitivity with another T-DNA insertion Salk line *mlp43-2* (Salk_033347) in the seed germination assay. As indicated in Supplementary Fig. S1, this mutant line harbours almost null expression levels of *MLP43* and was insensitive to ABA in seed germination. The ABA sensitivity of root growth was also examined after ABA treatment and the results indicated that no significant differences in primary root growth and lateral root number were observed among Col-0, *mlp43-1*, and *MLP43 OE* seedlings (Supplementary Fig. S2A, B). Taken together, *MLP43* might function as a positive regulator in ABA response during seed germination.

### Modulation of MLP43 expression by ABA and abiotic stress treatments

Based on publicly available microarray data (http://bar.uto-ronto.ca/efp/cgi-bin/efpWeb.cgi; Winter *et al.*, 2007), the *MLP43* transcript was inhibited by ABA (30 μM) after 24 h of treatment in seeds, but induced by gibberellic acid 3 (GA$_3$, 5 μM) after 3 and 6 h of treatment, respectively (Fig. 2A, B).

The effects of abiotic stresses on *MLP43* transcripts were further analysed. Interestingly, cold (4ºC), osmotic stress (300 mM d-mannitol), salt (150 mM NaCl) and drought (air dry) negatively regulated *MLP43* expression (Fig. 2C). To further confirm the effects of phytohormones and abiotic stresses on *MLP43* expression, qRT-PCR was applied to examine relative expression levels of *MLP43*. The results indicated that *MLP43* was actually inhibited by ABA (50 μM, 3 h), drought (water withheld for two weeks) and salt (NaCl, 100 mM for 3 h) treatment, respectively. No significant changes were observed after cytokinin (6-BA), auxin (IAA) or ethylene (ACC) treatment except that GA$_3$ treatment slightly up-regulated *MLP43* expression (Fig. 2D). These results showed that ABA and drought treatment decreased *MLP43* transcription, which were consistent with the public microarray data.

### Overexpressed MLP43 confers enhanced drought tolerance

As the *mlp43* mutant showed significantly decreased ABA sensitivity in seed germination, and drought treatment inhibited
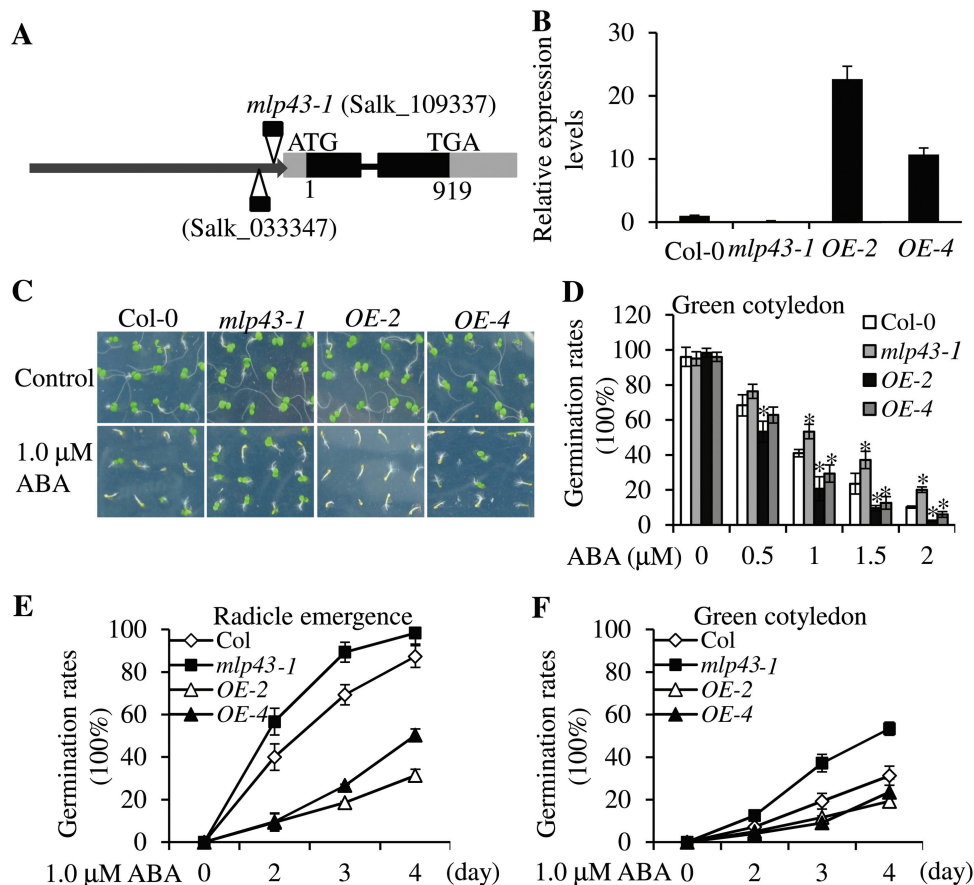


**Fig. 1.** *MLP43* was involved in ABA responses in seed germination assay. Asterisk symbols (*) indicate *P*<0.05 (Student's *t*-test). (A) Diagram showing of the T-DNA insertion site of *mlp43-1* mutant. (B) Relative expression levels of *MLP43* in the *mlp43-1* mutant and overexpressed transgenic plants. Expression levels of β-*ACTIN8* represent the internal control. Data represent the means ±SEs of three replicated experiments. (C) Seeds growing on MS medium with or without 1.0 μM ABA for 5 d after stratification. Both overexpressed transgenic plants (*OE-2* and *OE-4*) showed a hypersensitive response to ABA. Photographs were taken to document the phenotypes. (D) Germination rates of green cotyledons with the indicated ABA application. Germination rates (%) were scored 5 d after stratification. Data represent means ±SEs of three replicated experiments (*n*>60 for each experiment). (E) Germination rates of radicle emergence in 1.0 μM ABA plates at the indicated time points. Data represent the means ±SEs of three replicated experiments (*n*>60 for each experiment). (F) Germination rates of green cotyledons in 1.0 μM ABA plates at the indicated time points. Data represent the means ±SEs of three replicated experiments (*n*>60 for each experiment). (This figure is available in colour at *JXB* online.)
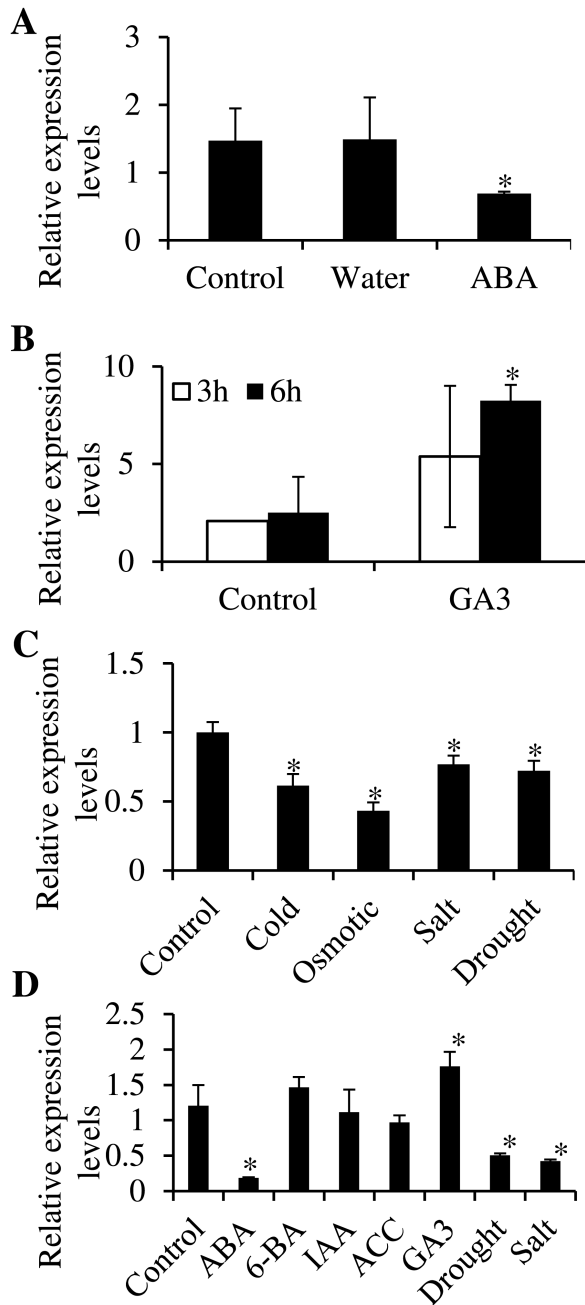
**Fig. 2.** ABA and abiotic stresses affected *MLP43* expression. Data represent the means ±SEs of three replicated experiments. Asterisk symbols (*) indicate *P*<0.05 (Student's *t*-test). (A) Publicly available microarray data illustrating the expression level of *MLP43* was inhibited by 30 μM ABA treatment for 24 h (http://www.bar.utoronto.ca/NASCArrays/index.php?ExpID=183), but was (B) induced by gibberellic acid 3 in seeds (GA₃, 5 μM, treated for different time courses) (http://www.bar.utoronto.ca/NASCArrays/index.php?ExpID=184). (C) Publicly available microarray data illustrating the effect of abiotic stress treatment (cold, osmotic, salt and drought) on expression level of *MLP43* (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).(D) Effects of hormones and abiotic stresses on *MLP43* expression using qRT-PCR. ABA (10 μM, 3 h), 6-BA (1 μM, 3 h), IAA (5 μM, 3 h), ACC (5 μM, 3 h), GA₃ (10 μM, 3 h), drought (water withheld for two weeks), salt (NaCl, 100 mM, 3 h). The values of gene expression levels of Col-0 seedlings without any treatment were taken as '1'. Expression levels of β-*ACTIN8* represent the internal control.

*MLP43* expression (Figs 1, 2), the responses to drought stress of the *mlp43-1* mutant and *MLP43*-overexpressed plants were further examined. Two-week-old plants grown under normal conditions were withheld water for three weeks, and then rewatered for 2 d. The results indicated that only 20% of *mlp43-1* mutants but more than 80% of *MLP43 OE* plants recovered from wilting after rehydration, while the survival rate of Col-0 was about 58% (Fig. 3A, B). The EL test showed that the *mlp43-1* mutant exhibited a significantly higher EL percentage than Col-0, but *MLP43 OE* plants showed a lower EL percentage relative to Col-0 (Fig. 3C). Transpirational water loss from detached leaves of 4-week-old plants were further compared at room temperature with a humidity of ~50–60%. Much higher water loss rates were detected in the *mlp43-1* mutant compared with Col-0, while *MLP43 OE* plants showed lower water loss rates compared to Col-0 (Fig. 3D).

ABA-induced stomatal closure is usually responsible for plant adaptation to drought stress. We defined the stomatal phases as open, partially open and closed, according to the ratios of width to length of stomatal aperture (Fig. 3E). Under normal conditions, the rates of different stomatal phases were comparable among wildtype (Col-0), *mlp43-1* and *MLP43 OE*. However, after ABA treatment for 1 h, the rates of closed stomata were significantly increased in Col-0, especially in *MLP43 OE*. In contrast, the *mlp43-1* mutant exhibited fewer closed stomata and more partially closed stomata than those in Col-0 and *MLP43 OE* (Fig. 3E). Together, these results suggested that the improved drought tolerance of *MLP43 OE* was associated with an increased sensitivity to ABA-induced stomatal closure in *MLP43 OE*. The complementary overexpressed transgenic plants generated by introducing Pro35S::MLP43-GFP into *mlp43-1* were further assessed for drought resistance. The results indicated that the hypersensitivity of *mlp43-1* mutant to drought stress could be restored by *MLP43* overexpression (Supplementary Fig. S3).

*Tissue-specific expression patterns and subcellular localization of MLP43*

Tissue-specific expression patterns of *MLP43* were firstly analysed based on the microarray data available in public resources (Fig. 4A; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter *et al.*, 2007). The results indicated that *MLP43* preferentially expressed in cotyledon and hypocotyls in *Arabidopsis,* while lower expression levels could be detected in roots, shoots, stems and vegetative rosette leaves (Fig. 4A). Almost no expression patterns could be detected either in dry seed or in imbibed seeds (Fig. 4A). To further confirm the specific expression patterns of *MLP43* in *Arabidopsis*, the promoter sequence of *MLP43* (−1769 bps upstream of start code ATG) was cloned and a transgenic plant carrying plasmid ProMLP43::GUS was generated. GUS histochemical signals were analysed in various tissues (Fig. 4B–I). There were predominant GUS signals in cotyledons, primary roots and apical meristems (Fig. 4B–D, H, I). GUS signals also could be detected in rosette leaves, flowers and the abscission zone (Fig. 4E–G) whereas no GUS signals had been examined in hypocotyls, which was inconsistent with the microarray data. We transiently expressed the Pro35S::MLP43-GFP fusion construct into epidermal cells of *Arabidopsis* rosette leaves by bombardment and observed the subcellular localization
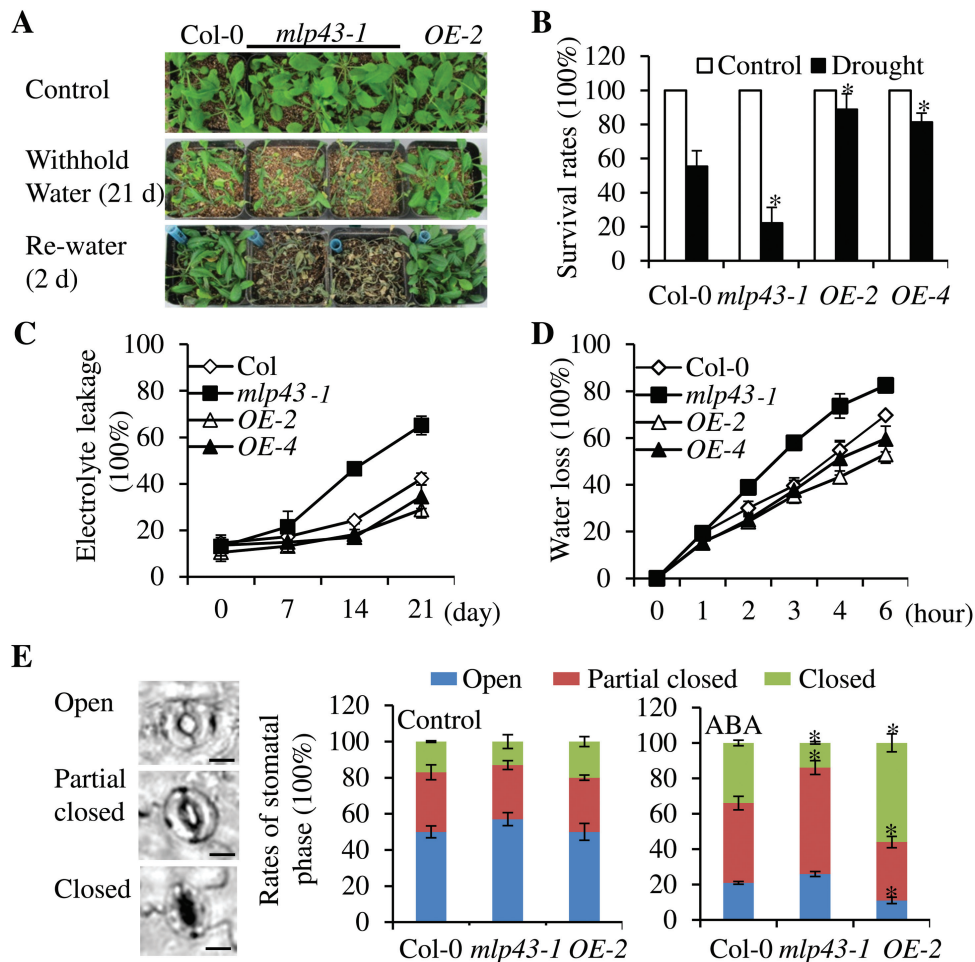
**Fig. 3.** *MLP43* involvement in drought stress responses. (A) Two-week-old plants were well watered (control) or deprived of water for 21 d and then rewatered. The photos were taken two days after rewatering. (B) Survival rates after the drought treatment in panel A. Data represent the means ±SEs of three replicated experiments (*n*>20 for each genotype, *P*<0.05). (C) Electrolyte leakage comparison at the indicated time points after drought treatment applied. Data represent the means ±SEs of three replicated experiments (*n*=15 for each genotype). (D) Transpirational water loss from well-watered detached leaves of four-week-old plants. Water loss rates are indicated as the percentage of the initial fresh weight (% FW). Results are shown as the means ±SEs of three replicated experiments (*n*=15 for each genotype). (E) Stomatal aperture under ABA treatment. The stomata phases are defined as shown in left panel. Scale bar, 5 μm. The rates of different stomata phases are calculated with or without ABA (30 μM) treatment for 30 min. The data represent the means of four replicated experiments (*n*>50, *P*<0.05). (This figure is available in colour at *JXB* online.)

of the MLP43-GFP fusion protein under a confocal fluorescence microscope. The results indicated that MLP43 were localized in nucleus and cytoplasm in *Arabidopsis* epidermal cells (Fig. 4J–L).

*Mutation of MLP43 increased ROS production and modulated primary metabolites after drought treatment*

ABA and drought stress trigger ROS accumulation, and a ROS detoxification mechanism is necessary to enable plant survival under drought stress (Smirnoff, 1993). To characterize whether ROS accumulation was altered in the *mlp43-1* mutant, we determined $H_2O_2$ and $O^-$ production by DAB and NBT staining, respectively. The *mlp43-1* mutant exhibited a dark colour relative to Col-0 with both DAB and NBT staining after air drought stress treatment, indicating more accumulation of $H_2O_2$ and $O^-$ in the *mlp43-1* mutant (Fig. 5A). We also measured the $H_2O_2$ content in the young seedlings, and found that drought stress induced rapid accumulation

of $H_2O_2$ after 14 d of drought treatment in all genotypes. However, the $H_2O_2$ level was significantly higher in the *mlp43-1* mutant plants, but lower in *MLP43 OE* when compared with Col-0 plants (Fig. 5B). Next, the enzymatic activities of SOD and CAT were measured under the same drought conditions. The results indicated that no significant differences of CAT activity were observed among three genotypes after drought treatment for 7 d, however, SOD activities were 1.5-fold higher in *MLP43 OE* plants than wildtype (Col-0) and the *mlp43-1* mutant, respectively (Fig. 5C, D). Moreover, both SOD and CAT activities showed a significant decrease in *mlp43-1* and increase in *MLP43 OE* relative to Col-0 plants after drought treatment for 14 d and 21 d, respectively.

To elucidate the metabolic responses of *MLP43* to drought stress treatment, GC-TOF-MS was applied to test whether the primary metabolite profiling was modified by *MLP43* overexpression. The results indicated that 34 metabolites including 9 carbohydrates, 9 amino acids, 9 organic acids and 7 other derivatives were reproducibly identified (Fig. 6;
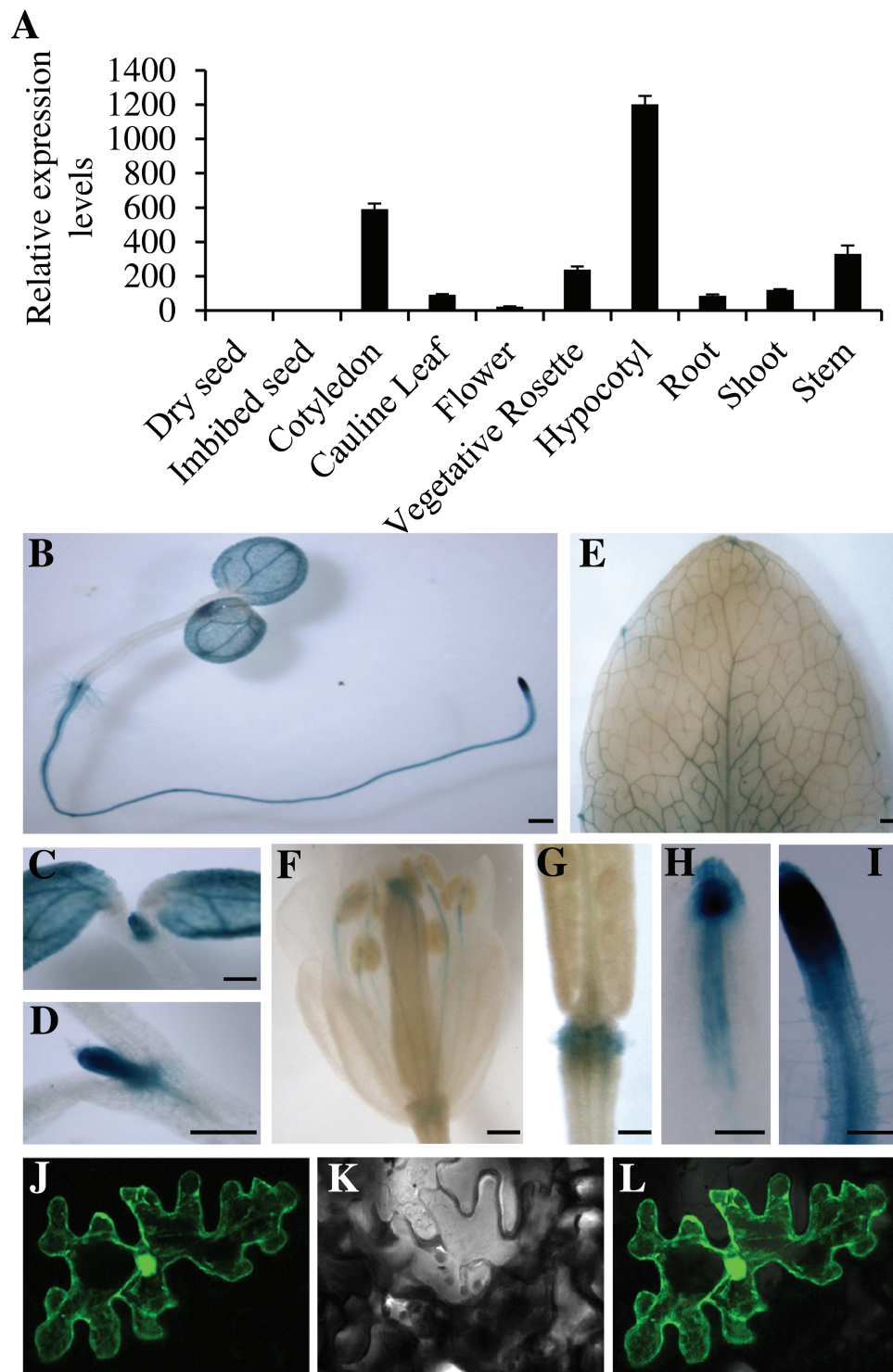
**Fig. 4.** Tissue-specific expression patterns of *MLP43*. (A) Publicly available microarray data illustrating the tissue specific expression levels of *MLP43* (*http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi*). (B)–(I) Analysis of tissue-specific expression patterns by GUS histochemical staining. Scale bar, 0.5 mm. (J)–(L) Subcellular localization of MLP43-GFP fusion protein drived by the 35S promoter. Bar, 10 μM. Photos taken under cofocal microscope J, GFP; K, bright field; L, overlay. (This figure is available in colour at *JXB* online.)

Supplementary Table S5). Compared with wildtype (Col-0), most of the detected metabolites showed <2-fold differences in *MLP43* ectopic expression lines without drought treatment. However, the contents of most of the examined carbohydrates and amino acids were significantly decreased in the *mlp43-1* mutant, but increased in *MLP43 OE* transgenic plants after drought treatment (Fig. 6). Except D-(+)-galactose, all carbohydrates, including maltose, sorbose, cellobiose, turanose, glucose, psicose and galactose, decreased in the *mlp43-1* mutant but increased in *MLP43 OE* when compared to Col-0 under the withheld water condition (Fig. 6). A similar change was observed for citrulline, threonine, valine, serine, proline, alanine and lysine, with the exception of glutamine and glycine (Fig. 6). In addition, hexadecanoic acid, galactinol,
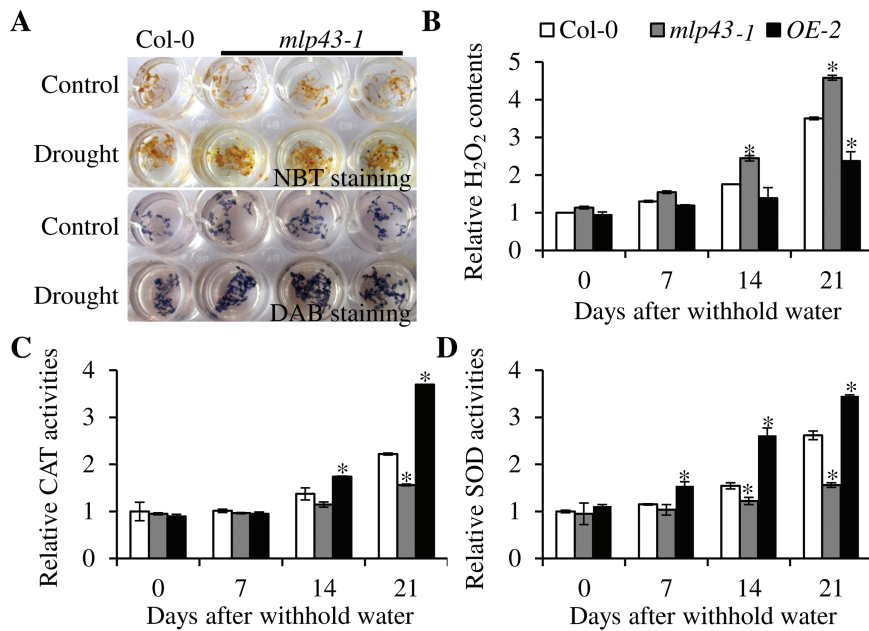
**Fig. 5.** *MLP43* enhanced drought stress tolerance through inhibiting ROS production and promoting antioxidant enzyme activities. (A) Visualization of superoxide radical and hydrogen peroxide detected by NBT and DAB staining using two-week-old MS-grown plants subjected to subsequent treatment with or without air dry for 30 min. Comparison of (B) $H_2O_2$ content, (C) CAT and (D) SOD activity in Col-0, *mlp43* mutant and overexpressed transgenic plant *MLP43 OE*. Two-week-old plants were well watered and then withheld water for the indicated number of days. The relative contents of $H_2O_2$ and the activity of CAT and SOD were calculated as fold-changes compared with the Col-0 control. Data represent the means ±SEs of three replicated experiments (*n*=10; *P*<0.01). (This figure is available in colour at *JXB* online.)

myo-inositol and androst-2-en-17-amine increased, while cinnamic acid, propanoic acid and 2-butenedioic acid decreased either in *mlp43-1* mutants or in *MLP43* transgenic plants under drought treatment conditions.

### Transcriptional alterations of ABA- and drought-responsive genes by MLP43

To further determine the role of *MLP43* in the ABA signalling pathway and drought stress responses, we assessed the expression patterns of genes involved in ABA- and drought-responsive processes. Two-week-old seedlings were pretreated with 50 μM ABA for 3 h or air dried for 6 h, respectively, after which gene expression levels were analysed by qRT-PCR. We compared the expression levels of several ABA- and/or abiotic stress-responsive genes, including *DREB2A*, *KIN2*, *RAB18*, *RD20*, *RD29A* and *RD29B*. All of them showed extensively increased transcriptional levels after ABA and air-dry treatments in all three genotypes (Fig. 7). However, the fold changes of *DREB2A*, *KIN2*, *RAB18* and *RD29A* were significantly lower in the *mlp43-1* mutant, but higher in the *MLP43 OE* plants compared to those in Col-0. ABA treatment significantly enhanced the transcription of above ABA- and drought-responsive genes in *MLP43 OE* plants, which was consistent with the ABA hypersensitivity of *MLP43 OE* in seed germination and stomatal aperture responses. Dehydration stress usually promotes ABA production and actives ABA signal transduction (Chan, 2012), and our results substantiate this, with ABA and air-dry treatments inducing similar expression patterns of the above detected genes (Fig. 6). In addition, we also examined expression level changes of three ABA biosynthesis genes, *ABA1*, *ABA2* and

*NCED3*. The results indicated that there were no significant differences among the three genotypes after ABA or air-dry treatments. As MLP43 modulated ROS production, we also examined the relative expression levels of *RbohD* and *RbohE* encoding NADPH oxidases which promote ROS production especially after ABA and drought treatment (Torres *et al.*, 2002; Miller *et al.*, 2010). Results revealed no significant differences except that *RbohE* showed a relatively higher expression level in the presence of ABA (Fig. 7, last panel). Based on the above results, we concluded that the overexpression of *MLP43* enhanced plant drought stress adaptation through an ABA-dependent manner.

### MLP43 positively modulated ABA responses in reconstituted ABA signalling pathway

To elucidate how *MLP43* modulated the expression of downstream targets of the ABA signalling pathway, we performed a transient expression assay in *Arabidopsis* mesophyll protoplasts extracted from Col-0 and *snrk2.2/2.3/2.6*, respectively, using ProRD29A::LUC as reporter and ProUBQ::GUS as internal control, and Pro35S::GFP as empty vector control. The results indicated that transient overexpression of *MLP43* significantly enhanced the transcriptional activity of RD29A promoter after ABA treatment in Col-0 but not in *snrk2.2/2.3/2.6* (Fig. 8A). We then checked expression patterns of *MLP43* in mutants of primary ABA signalling components, including the *pyr1/pyl1/pyl4* triple mutant *(pyrtm)*, *ost1-1*, *abi1-1* and the ABA-deficient mutant *aba1-1*. When compared with wildtype (Col-0 and Ler), the expression level of *MLP43* was up-regulated in all the examined mutants, especially in the ABA-deficient mutant *aba1-1* with ~3-fold increases at the transcriptional level (Fig. 8B).
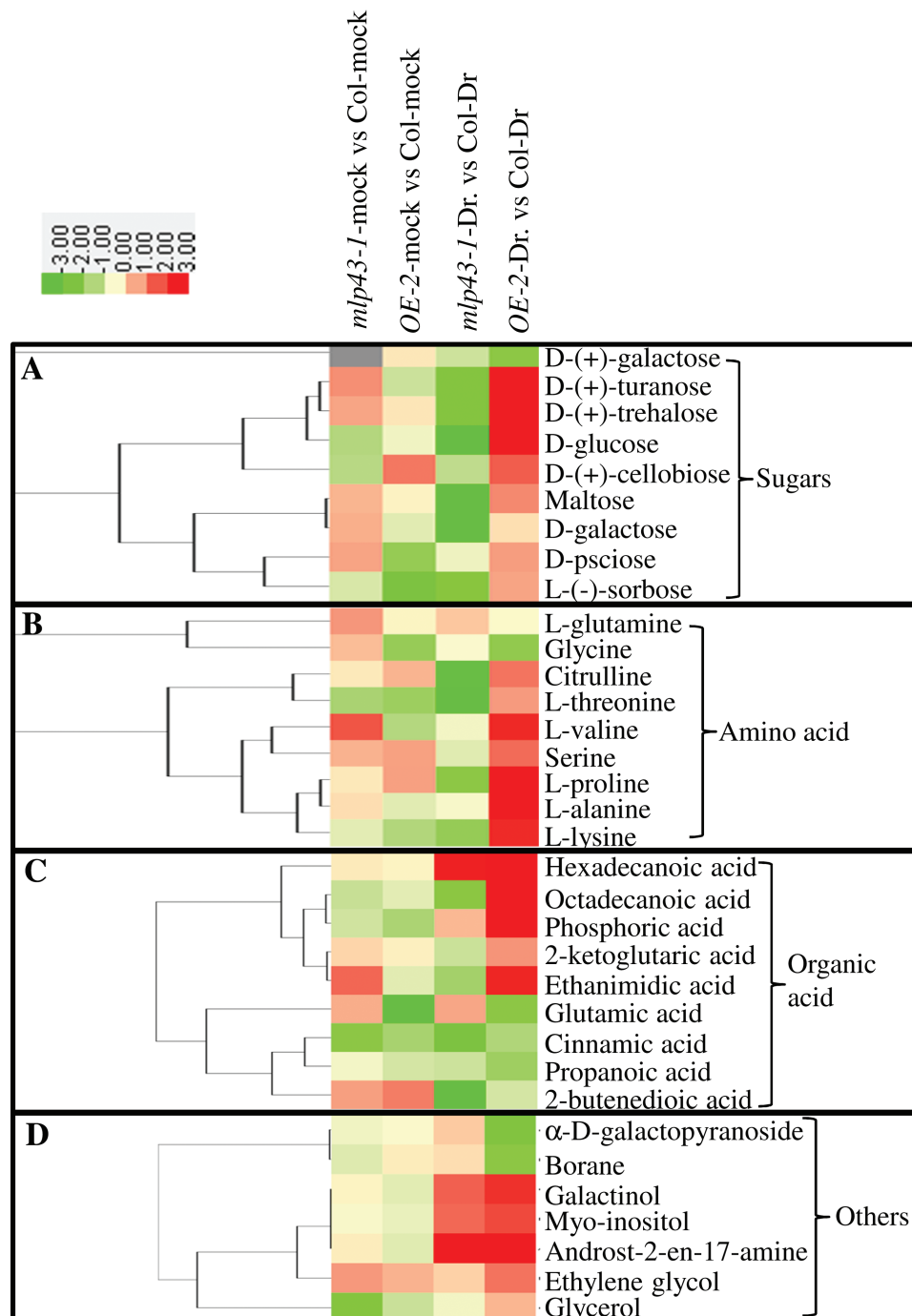
**Fig. 6.** Comparisons of metabolite profiling under control and drought treatment in Col-0, *mlp43-1* and *MLP43 OE* plants. Thirty-four compounds that could reproducibly be detected by GC-TOF MS in all three genotypic plants were analyzed by hierarchical clustering. The resulting tree was analyzed using CLUSTER 3.0 and shown using Java Treeview. (This figure is available in colour at *JXB* online.)

In order to gain more clues about the effect of *MLP43* in the ABA signalling pathway, we reconstituted the ABA signalling pathway in wildtype (Col-0) protoplasts (Fig. 8C). As expected, co-transformation of ABF2 and SnRK2.6 could extensively activate RD29A::LUC activity and PYR1 inhibited ABI1 activity and then enabled expression of the ABA-dependent transcription of RD29A::LUC (Fig. 8C). However, in the presence of *MLP43* overexpression, the activity of RD29A::LUC significantly increased, especially after ABA treatment when compared with empty vector control (Fig. 8C).

We further examined the interactions between *MLP43* and the key ABA signal components. First, the phylogenetic relationships among nine *MLP* homologous genes were analysed based on the full length amino acid sequences (Supplementary Fig. S4). Another two *MLPs* (*MLP34* and *MLP168*), which shared differential homology with *MLP43*, were selected for the yeast two-hybrid assay together with *MLP43*. The results indicated that MLP43 could interact with SnRK2.6 and ABF1 in the yeast two-hybrid assay independent of ABA (Fig. 8D; Supplementary Fig. S5). However, we did not
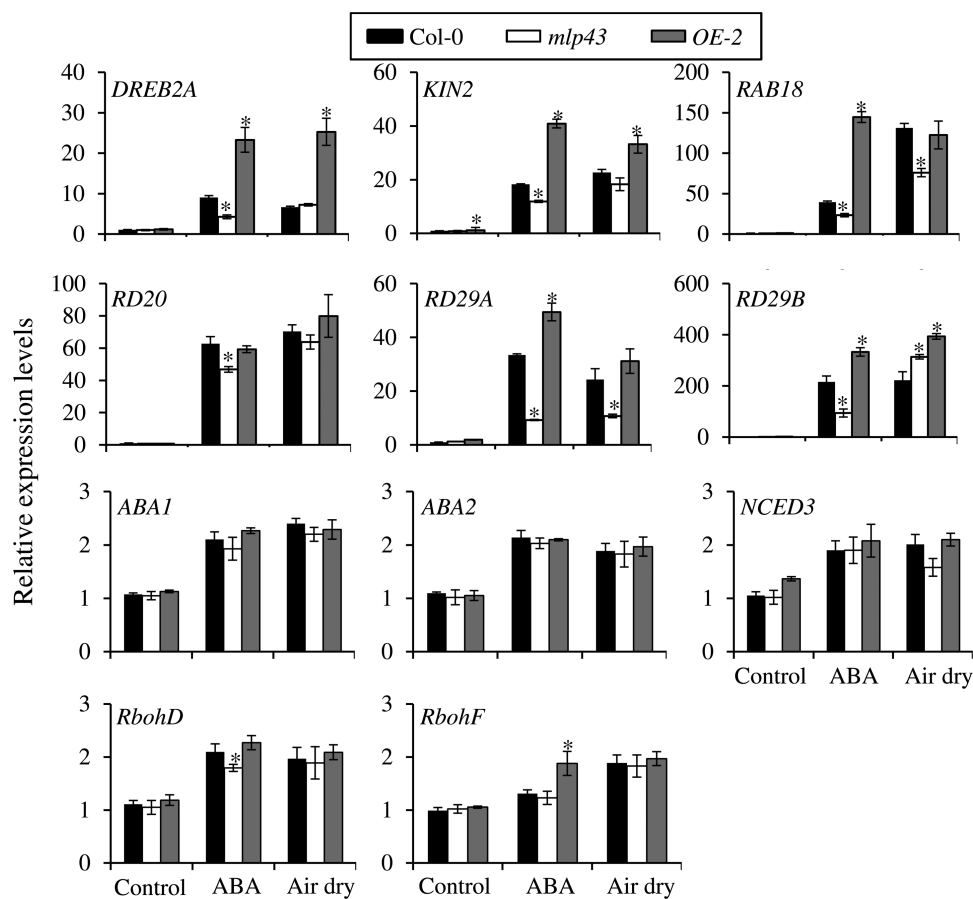
**Fig. 7.** Relative expression levels of drought- and ABA-responsive genes in *mlp43-1* mutant and *MLP43 OE* plants. The two-week-old plants were subjected to ABA treatment (50 μM) for 3 h and air dried for 6 h, respectively. The expression levels of the selected drought- and ABA-responsive genes were analysed quantitatively by qRT-PCR. Gene expression levels were analysed relative to the corresponding gene expression levels in Col-0 plants. The values of gene expression levels of Col-0 seedlings without ABA treatment were taken as '1'. Expression levels of β-*ACTIN8* represent the internal control. The data represent means ±SEs of three reproducible experiments (*P*<0.01).

detect any interactions between MLP34 or MLP168 and the key ABA signal components, except the interaction between MLP168 and wildtype ABI5 protein (Supplementary Fig. S5A). Interestingly, mutated proteins carrying single or triple phosphoamino acid mutations (S42A, S145A and T201A) in ABI5 could eliminate its interactions with MLP168 (Supplementary Fig. S5A). Taken together, the above results indicated that *MLP43* might function as a positive regulator through interacting with SnRK2.6 and ABFs in ABA signalling responses, and ABA could negatively regulate the expression of *MLP43* as a complementary mechanism to modulate the function of *MLP43 in vivo*.

## Discussion

In this study, the function of MLP43 was characterized during plant stress responses. Both MLP43 and ABA receptors PYL/PYR belong to the Bet v 1 family and share similar protein structure. We speculated that *MLP43* might be involved in the ABA signalling pathway. The efficiency of water loss is responsible for plant tolerance to drought stress, and rapid water loss leads to greater drought sensitivity. As indicated, the rosette leaf water loss of the *mlp43-1* mutant was significantly more rapid than wildtype (Col-0) under the same

drought stress conditions, resulting in higher electrolyte leakage and lower survival rates (Fig. 3). The above results were consistent with ABA insensitivity of *mlp43* in seed germination. As we know, ABA and GA$_3$ antagonistically regulate seed germination (Lee *et al.*, 2002; Piskurewicz *et al.*, 2008). Interestingly, *MLP43* expression was significantly inhibited by ABA, but promoted by GA$_3$ (Fig. 2A). Moreover, drought and salt treatments also negatively regulated *MLP43* expression. We also analysed the 1769 bp promoter sequence of *MLP43* and found two ACGT-containing ABRE-like elements in the promoter region, located at −1291 bp to −1294 bp and −1088 bp to −1092 bp, respectively. Interestingly, a functional G-box (CACGTG) was also identified in the *MLP43* promoter, located at −603 bp to −608 bp close to the ATG start codon of *MLP43*. The occurrence of both an ABRE and G-box motif in the *MLP43* promoter is consistent with previous studies that indicated that ABRE/G-box elements were usually co-recognized by ABA responsive transcription factors (Menkens *et al.*, 1995; Shen and Ho, 1995; Ho *et al.*, 1999).

To further elucidate the mechanism of *MLP43* in mediating ABA and drought responses, the comparisons of expression levels of ABA- and drought-responsive genes were performed. As expected, the expression levels of *DREB2A*,
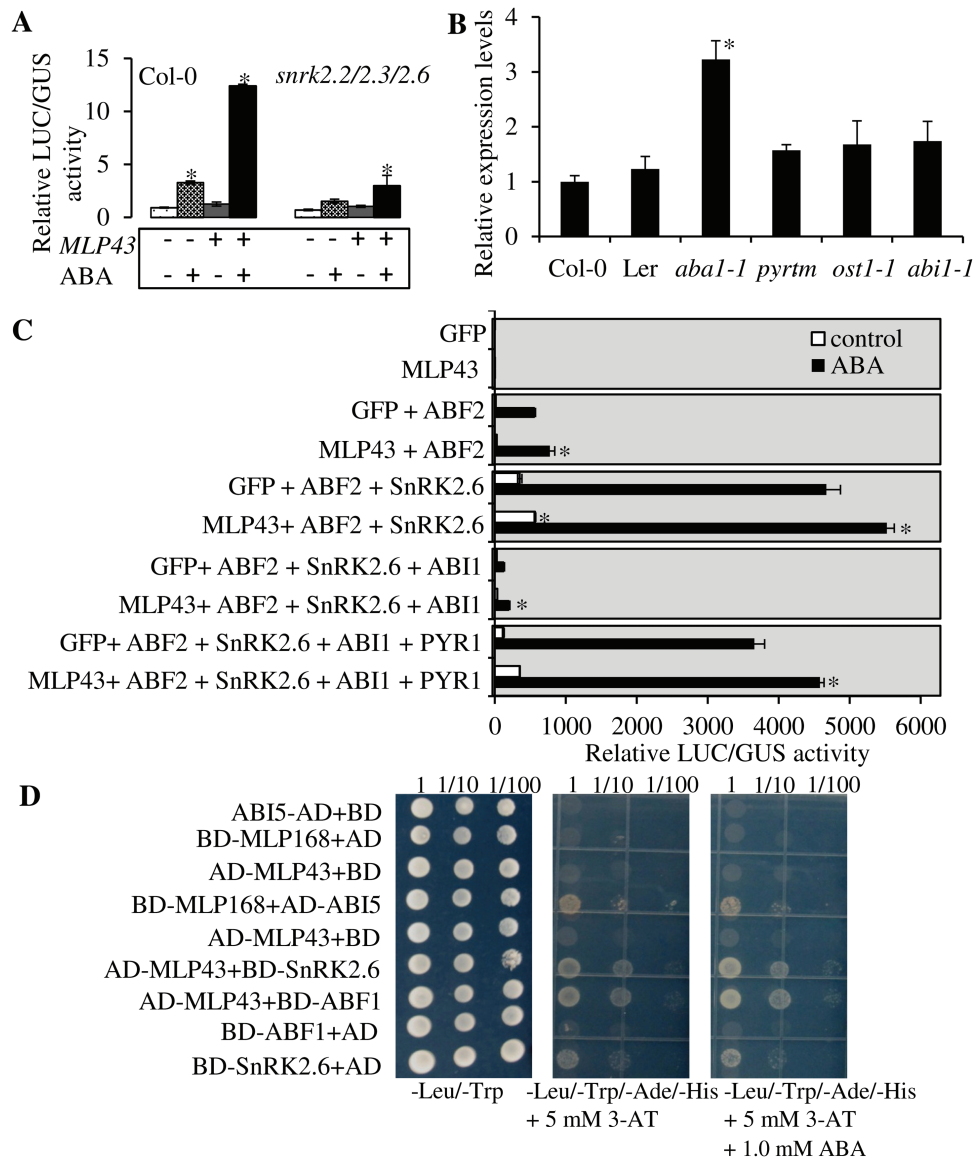
**Fig. 8.** *MLP43* functioned as a positive regulator in the reconstituted ABA signalling pathway. The data represent means ±SEs of five reproducible experiments (*P*<0.01). (A) Relative activities (LUC/GUS) in co-transformation with empty vector (Pro35S::GFP) or MLP43 (Pro35S::MLP43-GFP) in Col-0 and *snrk2.2/2.3/2.6* with or without ABA (50 μM) treatment for 12 h, respectively. (B) Relative expression levels of *MLP43* in the primary ABA signal components related gene mutants using qRT-PCR. The value of gene expression levels of Col-0 seedlings was taken as '1'. Expression levels of β-*ACTIN8* represent the internal control. (C) Relative activities (LUC/GUS) in the reconstituted ABA signal pathway. Co-transformation of *MLP43* enhanced the relative activities (LUC/GUS). ABF2, SnRK2.6, ABI1 and PYR1 were co-transformed as effectors, respectively. (D) Interactions between MLP43 and SnRK2.6 or ABF1 using the yeast two-hybrid assay. The homologous gene MLP168 could interact with wildtype ABI5 in yeast.

*KIN2*, *RAB18*, *RD29A* and *RD29B* were up-regulated by ABA treatment, but showed lower expression levels in *mlp43-1* and higher expression levels in *MLP43 OE* when compared with those in Col-0 after ABA treatment. These results indicated that *MLP43* enhanced ABA signal transduction and drought stress tolerance through modulating downstream targets of ABA and drought stress responses. In addition, expression levels of ABA biosynthesis-related genes, such as *ABA1*, *ABA2* and *NCED3* showed no significant changes in *mlp43* and *MLP43* OE lines indicating that *MLP43* modulated ABA responses independent of the ABA biosynthesis pathway. However, *MLP43* expression was up-regulated in the ABA receptor triple mutant *pyr1/ pyl1/pyl4*, as well

as in *ost1-1*, *abi1-1* (dominant negative mutant) and *aba1-1* mutants, when compared with those in wildtype Col-0 and Ler (Fig. 8B). In other words, disturbance of ABA signalling transduction or ABA biosynthesis blocked the inhibitory effect of ABA on *MLP43* transcription, indicating that the above key ABA signal components are important intermediators for the negative regulation of ABA on *MLP43* expression. Moreover, the re-constituted ABA signal pathway confirmed that transient over-expression of *MLP43* in protoplasts enhanced ABA responses by determining the reporter RD29A::LUC activity (Fig. 8C). However, mutation in *SnRK2.2/2.3/2.6* eliminated the positive effect of *MLP43* on ABA responses in the transient expression assay,

which provides evidence that *MLP43* functions up-stream of *SnRK2*s in the ABA signal pathway (Fig. 8A).

Furthermore, the interactions between MLP43 and SnRK2.6 or ABFs could be detected in the yeast two-hybrid assay (Fig. 8D; Supplementary Fig. S5), which provided direct evidence for the involvement of MLP43 in the ABA signalling pathway. In the core ABA signalling pathway, PYL/PYRs interact with PP2Cs in the present of ABA to form PYL/PYR-PP2C complexes, which in turn inhibits the activity of the PP2Cs in an ABA-dependent manner, allowing activation of SnRK2s (Ma *et al.*, 2009; Park *et al.*, 2009). To date, no direct interactions between ABA receptors PYL/PYRs and SnRK2s have been reported. Therefore, even though MLPs and the ABA receptor RCAR/PYR/PYL family of START proteins share amino acid and structural similarity at the protein level, MLP43 does not interact with ABI1, a key negative regulator of PP2Cs in the ABA signalling pathway. We speculated that MLPs might function as positive ABA regulators through direct regulation of downstream SnRK2s or ABF activities. Solid evidence through the yeast two-hybrid assay verified that MLP43 interacts with SnRK2.6 and ABF1 (Fig. 8D). However, the MLP homologous genes might be functionally diverse in their ABA responses as there were no interactions between MLP34/MLP168 and other ABA signalling components, except that MLP168 could interact with ABI5 in yeast (Supplementary Fig. 5). Presence or absence of ABA had no different effect on the interactions between MLPs and SnRK2/ABFs, indicating the interaction between MLP43 and SnRK2.6 or ABF1 is ABA independent. Therefore, our study provides experimental evidence to elucidate how *MLP43* functions as a positive regulator in ABA signalling and drought stress responses.

Accumulating evidence indicates that ABA-enhanced water stress tolerance is associated with induction of antioxidant defence systems, including ROS-scavenging enzymes such as SOD, CAT and APX (Mittler *et al.*, 2011). ROS are small molecules generated during development and in response to stress, and function as eukaryotic intracellular second messengers (Finkel, 1998; Mittler *et al.*, 2011). In *Arabidopsis*, *AtrbohD* and *AtrbohE* encode NADPH oxidases which catalyse NADPH into NADP and ROS especially after ABA and drought treatment (Torres *et al.*, 2002; Kwak *et al.*, 2003; Miller *et al.*, 2010). Our results showed that mutation of *MLP43* increased ROS accumulation under drought stress conditions (Fig. 5A), indicating the involvement of *MLP43* in ROS-mediated drought responses. However, *MLP43* did not modulate *RbohD* and *RbohF* transcript levels according to the qRT-PCR results, which indicated that *MLP43* preferably regulated ROS scavenging but not production (Fig. 7). The further accumulation of excessive ROS causes more severe oxidative damage to plant cells and stimulates antioxidase activities (Mittler, 2002). SOD and CAT are two important antioxidases that scavenge excessive ROS to prevent cell damage. We determined significant increases of both SOD and CAT activities in *MLP43*-overexpressed transgenic plants (Fig. 5C, D), which suggests that *MLP43* played a role in scavenging the excessive ROS production under dehydration to enhance drought tolerance.

During plant abiotic stress responses, compatible solutes are important in helping plants balance external osmotic pressure and maintain cellular function of macromolecules. In this study, we further examined primary metabolic profiles using GC-TOF MS. The results indicated several carbohydrates and amino acids accumulated in *MLP43* plants (Fig. 6). The increased amounts of alanine and glutamic acid in *MLP43* OE plants during drought stress treatment might regulate photosynthesis intensity (Bocian *et al.*, 2015). Moreover, overexpression of *MLP43* resulted in accumulation of free proline, maltose, trehalose and glucose, which in turn contributed to conferred drought tolerance (Garg *et al.*, 2002). Galactinol has a positive effect in protecting plants from drought stress and oxidative damage (Taji *et al.*, 2002; Nishizawa *et al.*, 2008). In our study, galactinol showed significant increases after drought treatment, especially in *MLP43* OE plants (Fig. 6). Moreover, overexpressed *GhMLP* led to a two-fold increase in flavonoid contents, which suggested that *MLP* might be involved in flavonoid metaolism (Chen and Dai, 2010). All these results indicated that *MLP43* modulated primary and secondary metabolic profiles, which might be contributed to increased abiotic stress tolerance.

In this study, we dissected the functions of *MLP43* in ABA signals and drought stress responses. First, ABA and drought stress treatments inhibited the expression of *MLP43*, but this inhibition was disturbed in the primary ABA signal components mutants. Second, *MLP43* overexpression enhanced ABA responses in seed germination and improved drought stress tolerance by modulating ABA- and/or drought-responsive gene expression, ROS homeostasis and primary metabolic profiling. Third, *MLP43* promoted RD29A::LUC activity in the reconstituted ABA signalling pathway, and that was dependent upon SnRK2.2/2.3/2.6. Finally, there were direct interactions between MLP43 and SnRK2.6 or ABF1 in the yeast two-hybrid assay. Collectively, we concluded that *MLP43* functioned as a positive regulator upstream of *SnRK2*s, and ABA down-regulated *MLP43* expression as a negative feedback loop, so regulating drought stress responses.

## Supplementary data

Supplementary data is available at *JXB* online.

Supplementary Fig. S1. Phenotypic analysis of another *MLP43* T-DNA insertion line *mlp43-2* (Salk_033347).

Supplementary Fig. S2. Comparison of primary root length and lateral roots number among Col-0, *mlp43-1*, *MLP OE* plants after ABA treatment.

Supplementary Fig. S3. Complementary over-expression of *MLP43* into the *mlp43-1* mutant (*Com-3*) showed drought-tolerance in soil.

Supplementary Fig. S4. Phylogenetic relationships of nine *AtMLP* genes.

Supplementary Fig. S5. Yeast two-hybrid assay to detect the interactions between MLP43/MLP34/MLP168 and the key components in the ABA signalling pathway.

Supplementary Table S1. The primers used for identification of the *mlp43* mutant (Salk_109337 and Salk_033347).

## Acknowledgments

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