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RESEARCH PAPER

Melatonin mediates the regulation of ABA metabolism, free-radical scavenging, and stomatal behaviour in two *Malus* species under drought stress

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Abstract

Melatonin pre-treatment significantly increases the tolerance of both drought-tolerant *Malus prunifolia* and drought-sensitive *M. hupehensis* plants. Its beneficial effects include better water conservation in leaves, less electrolyte leakage, steady chlorophyll contents, and greater photosynthetic performance under stress conditions. Melatonin selectively down-regulates *MdNCED3*, an abscisic acid (ABA) synthesis gene, and up-regulates its catabolic genes, *MdCYP707A1* and *MdCYP707A2*, thereby reducing ABA contents in drought-stressed plants. Melatonin also directly scavenges H_2O_2 and enhances the activities of antioxidant enzymes to detoxify H_2O_2 indirectly. These two mechanisms work synergistically to improve the functions of stomata, i.e. causing them to re-open. Plants can effectively regulate their water balance under drought conditions by up-regulating the expression of melatonin synthesis genes *MdTDC1*, *MdAANAT2*, *MdT5H4*, and *MdASMT1*. Therefore, inducing melatonin production is an important mechanism by which plants can counteract the influence of this abiotic stressor.

Key words: ABA, drought, abiotic stress, H_2O_2, *Malus*, melatonin, stomata, antioxidant.

Introduction

Drought stress presents one of the major limitations to crop productivity because it negatively alters plant physiology. The cellular changes that are associated with this stressor include turgor loss, changes in membrane fluidity and composition, and disturbances to protein–protein and protein–lipid interactions (Chaves *et al.*, 2003). Drought also increases the generation of reactive oxygen species (ROS) (Ippolito *et al.*, 2011), which induce membrane lipid peroxidation, ultimately leading to membrane dysfunction (Li *et al.*, 2011). Under a water deficit, plants activate a diverse set of physiological, metabolic, and defence systems to survive and sustain growth, such as reducing water losses by increasing diffusive resistance, improving water uptake through prolific and deep root systems, and developing smaller and more succulent leaves to minimize transpiration. Low-molecular-weight osmolytes, including glycine betaine, proline and other amino acids, organic acids, and polyols, also play vital roles in sustaining cellular functions under drought (Farooq *et al.*, 2012).

Abbreviations: AANAT, arylakylamine *N*-acetyltransferase; ABA, abscisic acid; APX, ascorbate peroxidase; ASMT, *N*-acetylserotonin methyltransferase; Car, carotenoid; CAT, catalase; Chl, chlorophyll; CYP707A, ABA 8′-hydroxylase; g_s, stomatal conductance; H_2O_2, hydrogen peroxide; NCED, 9-*cis*-epoxycarotenoid dioxigenase; PBS, phosphate-buffered saline; P_n, net photosynthesis rate; POD, peroxidase; REL, relative electrolyte leakage; ROS, reactive oxygen species; RWC, relative water content; SEM, scanning electron microscopy; TDC, tryptophan decarboxylase; T5H, tryptamine 5-hydroxylase.

A water deficit can also trigger production of the phytohormone abscisic acid (ABA), which in turn causes stomata to close and induces the expression of drought stress-related genes to modulate plant responses (Seki *et al.*, 2007).

The accumulation of endogenous ABA in plant tissues is regulated by a balance between biosynthesis and catabolism (Kushiro *et al.*, 2004). The 9-*cis*-epoxycarotenoid dioxigenase (NCED) is a key enzyme in ABA biosynthesis in multicellular plants, where it cleaves the 11, 12 double bonds of a C40 carotenoid (Car) and produces xanthoxin (Qin and Zeevaart, 1999). Of the five NCED family members in *Arabidopsis*, *AtNCED3* plays a crucial role in drought-induced ABA biosynthesis (Iuchi *et al.*, 2001). Although its transcripts are rapidly induced by water stress, T-DNA insertional *nced3* mutants have defects in ABA accumulation under drought conditions and, thus, they have impaired tolerance. Abscisic acid is primarily catabolized to form 8′-hydroxy ABA through hydroxylation with ABA 8′-hydroxylase (Nambara and Marion-Poll, 2005). This hydroxylation at the 8′-position seems to be the key step in ABA catabolism, and is catalysed by CYP707A, an ABA 8′-hydroxylase belonging to a class of cytochrome P450 monooxygenases (Kushiro *et al.*, 2004; Saito *et al.*, 2004). In apple seedlings, *MdCYP707A1* and *MdCYP707A2* regulate ABA metabolism under drought conditions (Kondo *et al.*, 2012).

The accumulation of ABA in plant cells is associated with the formation of ROS (Liu *et al.*, 2010; Zhu, 2002). For example, when less endogenous ABA is available, an accumulation of hydrogen peroxide (H_2O_2) is accordingly reduced (Ye *et al.*, 2011). As the most important ROS, H_2O_2 participates in a series of processes for plant development, stress responses, and programmed cell death (Apel and Hirt, 2004; Bethke and Jones, 2001; Foyer and Noctor, 2005; Pei *et al.*, 2000). Notably, H_2O_2 is directly involved in the regulation of stomatal movement. The opening and closing of stomata represent physiological responses by plants to drought stress (Dehesh *et al.*, 2014; Schroeder *et al.*, 2001; Xia *et al.*, 2014). Thus, the relationship between ABA and H_2O_2 plays a central role under drought stress. Although ABA activates the synthesis of H_2O_2 in guard cells, apparently via NADPH oxidase, H_2O_2 mediates ABA-induced stomatal closure. At micromolar concentrations, H_2O_2 stimulates the activation of important plasma membrane Ca^{2+} channels (Neill *et al.*, 2002). Induction of stomatal closure by H_2O_2 has been observed in *Vicia faba* and the epidermis of tobacco (*Nicotiana tabacum*) (Zhang *et al.*, 2001). In plants, H_2O_2 has two origins, the chloroplastic and cytoplasmic/plasma membranes (Zhang *et al.*, 2001).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a potent, naturally occurring antioxidant that effectively scavenges both ROS and reactive nitrogen species (RNS) in animals and plants (Tan *et al.*, 2012). It is synthesized from tryptophan sequentially by four enzymes (Arnao and Hernández-Ruiz, 2006; Kang *et al.*, 2011; Park *et al.*, 2012). Genes for the first two enzymes, tryptophan decarboxylase (TDC) and tryptamine 5-hydroxylase (T5H), have been cloned and characterized in rice (*Oryza sativa*; Kang *et al.*, 2007*a*, *b*). The third enzyme, serotonin *N*-acetyltransferase (SNAT) [also called arylakylamine *N*-acetyltransferase (AANAT)], was identified by Okazaki *et al.* (2009) in the unicellular green alga *Chlamydomonas reinhardtii*. Ectopic overexpression of SNAT in tomato (*Solanum lycopersicum*) results in enhanced melatonin production. *N*-acetylserotonin methyltransferase (ASMT), the last enzyme in the melatonin synthesis pathway, catalyses *N*-acetylserotonin into melatonin; it has been cloned and characterized in rice by Kang *et al.* (2011).

The benefits of melatonin have been documented in studies with various abiotic stressors, e.g. extreme temperatures (Kang *et al.*, 2010; Lei *et al.*, 2004; Shi and Chan, 2014; Xu, 2010), heavy metals (Posmyk *et al.*, 2008; Tan *et al.*, 2007*a*), UV radiation (Afreen *et al.*, 2006), and elevated salinity (Li *et al.*, 2012; Tal *et al.*, 2011; Zhang *et al.*, 2014). In addition to serving as an antioxidant, melatonin regulates root development (Park and Back, 2012; Pelagio-Flores *et al.*, 2012; Zhang *et al.*, 2013), seed germination (Tiryaki and Keles, 2012; Zhang *et al.*, 2013), leaf senescence (Wang *et al.*, 2013*a*, *b*; Wang *et al.*, 2014), and circadian rhythms (Arnao and Hernández-Ruiz, 2009; Kolář *et al.*, 1997). Even though this molecule positively influences plant responses to drought stress, such as by promoting seed germination and seedling growth in PEG-stressed cucumber (*Cucumis sativus* L.) (Zhang *et al.*, 2013) and retarding drought-induced leaf senescence in apple (Wang *et al.*, 2013*a*, *b*), the exact mechanisms by which these are accomplished are largely unknown.

To understand better how melatonin functions in plants exposed to drought conditions, two genotypes of apple species, the tolerant *Malus prunifolia* and the sensitive *Malus Hupehensis* were studied. Whereas melatonin-deficient plants are less well-protected against abiotic stress when compared with the wild type (Okazaki *et al.*, 2010), melatonin-rich plants are better able to withstand such challenges (Park *et al.*, 2013). Therefore, it is hypothesized that the amount of endogenously produced melatonin would be significantly higher in *M. prunifolia* than in *M. hupehensis*, such that the latter would be less tolerant of drought stress. If true, then pre-treatment with melatonin would be more beneficial if applied to the sensitive species because its melatonin content would rise more markedly than in the tolerant species. Two approaches were taken to test the hypothesis. First, a short-term water stress by withholding irrigation to selected plants of both species was induced. Second, the expression of genes involved in either the ABA metabolic pathway or the regulation of enzymes for melatonin production, as well as activities by antioxidant enzymes and stomatal behaviour were monitored. The objective was to increase our knowledge about the mechanisms by which melatonin can increase plant tolerance to ensuing drought conditions.

Materials and methods

Plant material

All experiments were performed in a greenhouse at the Northwest A and F University, Yangling (34°20′N, 108°24′E), China. Seeds of *M. prunifolia* and *M. hupehensis* were collected from Fuping, Shaanxi (34°75′N, 109°15′E) and Pingyi, Shandong (35°07′N, 117°25′E), respectively. Two-year-old plants were grown in plastic pots (30 cm×26 cm×22 cm) filled with a 5:1:1 (v:v:v) mixture of forest soil:sand:organic substrate. The plants were watered once a week

with half-strength Hoagland nutrient solution before the experiments. Standard horticultural practices were followed for disease and pest control.

Experimental designs

At the initiation of the study (July 2013), the stems, measured without branches, were approximately 1.0 m tall. Half of the plants for each species were treated with a half-strength nutrient solution containing 100 µM melatonin for 10 d. After this pre-cultivation period, the groups of control (CK) and melatonin-pre-treated plants (MT) were each randomly re-divided into two subgroups: normally watered controls and drought-stressed plants. For the latter, water was withheld for 6 d. This resulted in four study groups: (1) normally watered control (Control); (2) drought-stressed plants (Drought); (3) plants with melatonin (100 µM) pre-treated for 10 d, followed by normal irrigation (MC); and (4) plants receiving melatonin (100 µM) pre-treatment for 10 d but then exposed to drought stress (MD). Each group contained 40 plants. The fifth to eighth leaves from the top of a stem (fully mature leaves) were sampled from five trees per treatment group each day between 09.00 and 11.00 h. The collected leaves were rapidly frozen in liquid nitrogen and stored at –80 °C.

Relative water content and relative electrolyte leakage

During the drought period, the relative water content was computed according to the method described by Gaxiola *et al.* (2001). Leaves of each species were excised from each treatment group and their fresh weights were recorded immediately. After the leaves were floated in deionized water at 4 °C overnight, their rehydrated weights were determined. Finally, they were oven-dried at 70 °C for 48 h and weighed again. Relative water content was calculated as RWC=(fresh weight–dry weight) / (rehydrated weight–dry weight). Relative electrolyte leakage (REL) was determined from the leaves according to the method described by Dionisio-Sese and Tobita (1998).

RT-PCR analysis

Total RNA was extracted from leaves per the method described by Chang *et al.* (1993). Sequences for primers of *MdTDC1*, *MdT5H4*, *MdAANAT2*, and *MdASMT1* were obtained according to Lei *et al.* (2013). Sequences for primers of *MdCYP707A1* and *MdCYP707A2* were determined according to Kondo *et al.* (2012). Sequences for primers of *MdNCED3* and *EF-1a* were designed by Primer Premier 6 software (Biosoft International, Palo Alto, CA, USA). All primers are listed in Table 1. Poly(A)$^+$ RNA was purified with a poly(A)$^+$ Ttract® mRNA Isolation Systems III kit (Promega, USA) according to the manufacturer's instructions. Real-time PCR was performed on an iQ5.0 instrument (Bio-Rad, USA) using SYBR Green qPCR kits (TaKaRa) according to the manufacturer's instructions. To test the suitability of these primers, the specificity and identity of the reverse transcription (RT)-PCR products were monitored after each reaction by conducting melting-curve analysis of the products.

Transcripts of the *Malus* elongation factor 1 alpha gene (*EF-1a*; DQ341381) were used to standardize the cDNA samples for different genes. We had previously compared apple *EF-1α*, *actin*, and 18S rRNA as internal controls (Wang *et al.*, 2012) and found that *EF-1a* is more stable than the others as a reference gene under saline conditions. Three independent biological replications were performed for each experiment.

Quantification of net photosynthesis rate (P_n) and stomatal conductance (g_s)

The net photosynthesis rate (P_n) and stomatal conductance (g_s) were recorded between 09.00–11.00 h, with a portable system (Li-6400; LICOR, Lincoln, NE, USA). All measurements were performed at 1000 µmol photons m^{-2} s^{-1} and a constant airflow rate of 500 µmol s^{-1}. The cuvette CO_2 concentration was set at 400 µmol CO_2 mol^{-1} air, with a vapour pressure deficit of 2.0–3.4 kPa. The leaf temperature and ambient humidity were maintained at 30 °C and 65%, respectively. For each group, fully expanded and fully exposed leaves were sampled at the same position on each of five plants.

Table 1. *Primers used for quantitative real-time RT-PCR*

Gene	Primer sequence (5′–3′)
MdTDC1	F:TCACGCTGTGGTTGGAGGT
	R:CTGCATGCTCCTGAACCAAC
MdT5H4	F:TCGGTGACATGTTTGCTGC
	R:GGAAACCTTGGTCTGGCG
MdAANAT2	F:GAATCACCGTCCACGCTCC
	R:GAAATGCTTCCGATGTCCC
MdASMT1	F:AGAGGAGCGAGAAAGACTGGA
	R:CTAAAGAAAAACTTCAATGAGGGAT
MdNCED3	F:GCAGGAGATGATCGGCG
	R:CAGAAGCAGTCGGGGCAGT
MdCYP707A1	F:GAAGAGGTATGCTTTTGATGTGG
	R:TCAACAAGCCACCACTATCTTCT
MdCYP707A2	F:ACACTTTGCAAGAGATGAAGAGG
	R:TGTGAAAGGAAGTTCCAGGTAGA
EF-1α	F:ATTCAAGTATGCCTGGGTGC
	R:CAGTCAGCCTGTGATGTTCC

Determination of photosynthetic pigments

After the leaves were harvested on each sampling date, their photosynthetic pigments were extracted with 80% acetone. Contents were determined spectrophotometrically according to the method of Arnon (1949).

Observations of leaf stomata by scanning electron microscopy (SEM)

Four leaves were collected per treatment group for each species (fifth position from the top on each plant). The samples were immediately fixed with a 4% glutaraldehyde solution in 0.1 M phosphate-buffered saline (PBS; pH 6.8) to avoid any alterations during sample preparation. After being rinsed five times with PBS (for 5, 10, 15, 20, and 30 min), they were dehydrated in a graded ethanol series, vacuum-dried, and gold-coated. SEM was performed on a JSM-6360LV microscope (JEOL Ltd., Tokyo, Japan). Stomata were counted at random in 20 visual sections on the abaxial epidermis, and final tallies were used to compute their densities. Lengths, widths, and apertures were measured randomly from 20 stomata on the same specimens, using Image J software.

Determination of ABA content

Levels of ABA were measured as described by Zhang *et al.* (2008). Briefly, 30 µl of purified leaf extract was injected with an autosampler (Waters 2707; Milford, MA, USA) into a high-performance liquid chromatograph (HPLC). Analytic conditions were as follows: reverse phase column (Inertsil ODS-3, 250 × 4.6 mm, 5 µm); 30 °C; mobile phase, methanol with 0.6% acetic acid (dissolved in re-distilled water) (45:55, v:v); and flow rate, 0.8 ml min^{-1}. Phytohormone levels were determined by recording absorbance at 254 nm with a dual λ absorbance detector (Waters 2487). Data were acquired with an Empower chromatography workstation (Waters) and endogenous ABA was identified by comparing its retention time with that of ABA external standards (Sigma, St. Louis, MO, USA).

H_2O_2 content and activities of H_2O_2-scavenging enzymes

H_2O_2 was extracted with 5% (w/v) trichloroacetic acid and measured as described by Patterson *et al.* (1984).

For H_2O_2-scavenging enzymes, leaf samples (0.1 g) were ground in a chilled mortar with 1% (w/v) polyvinylpolypyrrolidone, then homogenized with 1.2 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA–Na_2 and 0.3% Triton X-100. For only the assay of ascorbate peroxidase (APX), 1 mM ascorbate was added to this mixture. Each homogenate was centrifuged at 13 000 g for 20 min at 4 °C. The supernatant was used for the following assays.

Catalase (CAT) activity was determined by monitoring the decrease in absorbance at 240 nm due to the decomposition of H_2O_2 (extinction coefficient of 39.4 mM^{-1} cm^{-1}) (Chance and Maehly, 1955). Peroxidase (POD) was assayed at 470 nm (extinction coefficient 25.2 mM^{-1} cm^{-1}) by using H_2O_2 and guaiacol as the reaction substrates (Chance and Maehly, 1955). APX activity was monitored as the decrease in absorbance at 290 nm when reduced ascorbate was oxidized (extinction coefficient of 2.8 mM^{-1} cm^{-1}) (Nakano and Asada, 1981).

Statistical analysis

Data were expressed as means±standard deviation (SD). The data were analysed via one-way ANOVA, followed by Tukey's tests. A p-value of <0.05 indicated a significant difference.

Results

Melatonin pre-treatment enables Malus plants to maintain water status and cell membrane stability under drought stress

The RWC values for each *Malus* species were significantly reduced under drought stress. However, prior application of 100 µM melatonin to the roots through the irrigation solution substantially alleviated this response over the stress period (Fig. 1). Similarly, REL was significantly increased in both species under drought conditions, with values being lower in the tolerant *M. prunifolia* than in the sensitive *M. hupehensis* (Fig. 2). The REL was also reduced significantly in melatonin-pre-treated plants compared with non-treated plants under the water deficit. It seemed that the beneficial effects of melatonin were more profound for the sensitive species than for the tolerant species, as indicated by the declines in REL, i.e. down by 21.47% (tolerant) vs 42.08% (sensitive) (Fig. 2).

Relative expression of melatonin synthesis genes

The melatonin synthesis genes *MdTDC1* (Fig. 3A, B), *MdT5H4* (Fig. 3C, D), *MdAANAT2* (Fig. 3E, F), and *MdASMT1* (Fig. 3G, H) were all significantly up-regulated by drought. Their relative expression levels were higher in sensitive *M. hupehensis* than in tolerant *M. prunifolia* when irrigation was withheld. Tan *et al.* (2012) have previously reported that melatonin synthesis is stress-inducible. Arnao and Herández-Ruiz (2013) considered that nearly all stressful factors caused an increase in melatonin in lupin plant tissues (*Lupinus albus* L.). The chemical stress provoked by $ZnSO_4$ or NaCl caused the most pronounced changes in the endogenous level of melatonin, followed by cold and drought stressors. It was concluded that imposition of drought induced

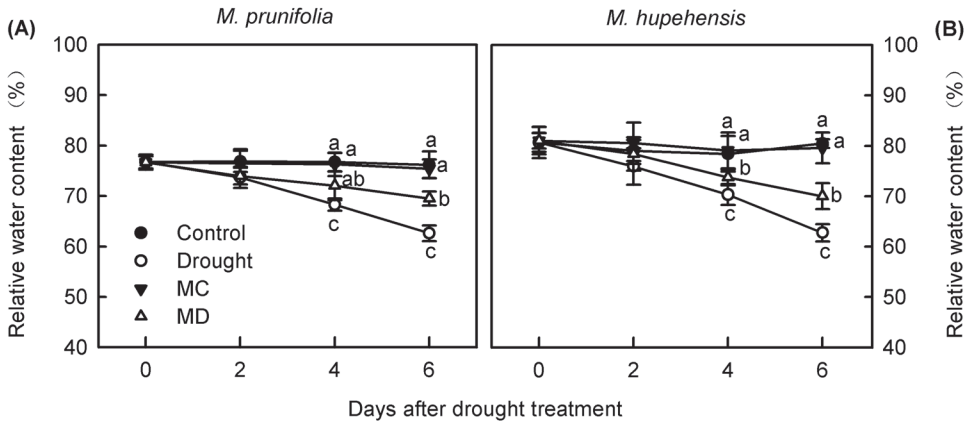

Fig. 1. Effects of melatonin on relative water content (RWC) for *M. prunifolia* (A) and *M. hupehensis* (B) under control and drought conditions. Closed and open circles indicate plants without melatonin pre-treatment under control and drought conditions, respectively. Closed and open triangles indicate plants with melatonin pre-treatment under control and drought conditions, respectively. Values are means of five replicates±SD. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different (P<0.05).

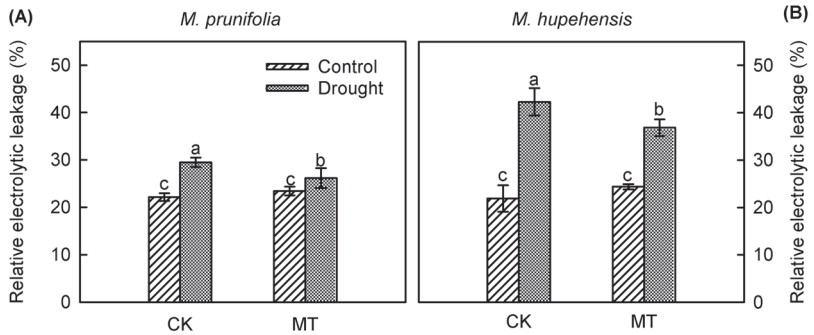

Fig. 2. Effects of melatonin on relative electrolyte leakage (REL) for *M. prunifolia* (A) and *M. hupehensis* (B) under control and drought conditions. CK, non-melatonin pre-treated control; MT, plants pre-treated with melatonin. Values are means of five replicates±SD. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different (P<0.05).

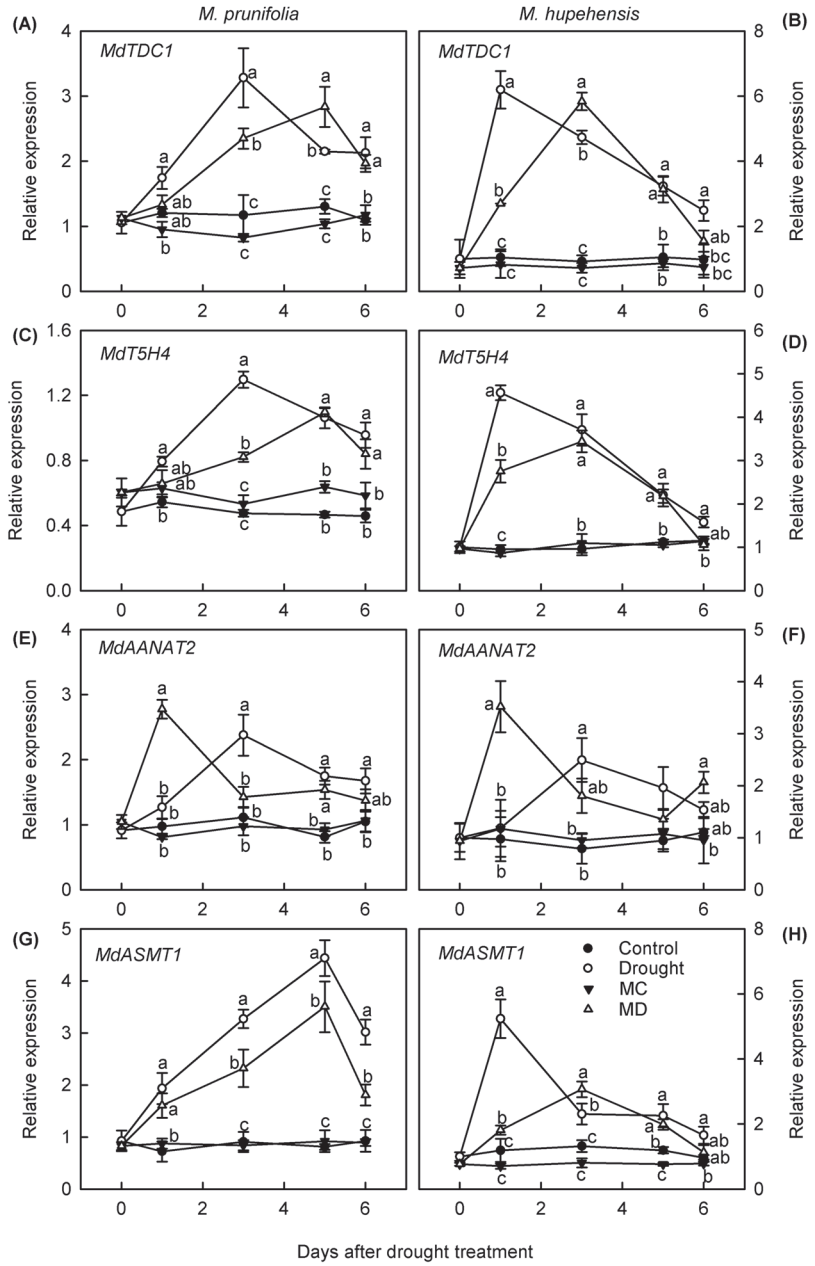

Fig. 3. Effects of melatonin on expression of melatonin synthesis genes: *MdTDC1* (A and B), *MdT5H4* (C and D), *MdAANAT2* (E and F), and *MdASMT1* (G and H) in leaves of *M. prunifolia* and *M. hupehensis*. Total RNA was isolated from samples at different time points, converted to cDNA, and subjected to real-time RT-PCR. Expression levels were calculated relative to expression of *Malus EF-1a* mRNA. Closed and open circles indicate plants without melatonin pre-treatment under control and drought conditions, respectively. Closed and open triangles indicate plants with melatonin pre-treatment under control and drought conditions, respectively. Values are means of five replicates±SD. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different (P<0.05).

up-regulation of those genes so that additional melatonin was produced to protect against that stressor. This was even more marked in the sensitive species, where expression was even further up-regulated, thus demonstrating that those plants could not produce melatonin as efficiently as plants from the more tolerant species could. Such a phenomenon may be related to possible genetic mutations for those associated enzymes. As expected, melatonin pre-treatment suppressed the expression of synthesis genes more intensely in the tolerant species, an outcome that provided additional evidence that *M. prunifolia* already had significantly higher levels of endogenously produced melatonin when compared with *M. hupehensis*.

Photosynthetic response and levels of photosynthetic pigments

In response to drought stress, P_n decreased in all groups throughout the experimental period, with rates being significantly lower for non-treated than for melatonin-pre-treated plants (Fig. 4A, B). Values for g_s displayed a similar trend (Fig. 4C, D).

Under drought conditions, contents of chlorophyll *a* (Chl *a*) (Fig. 5A, B), Chl *b* (Fig. 5C, D), carotenoid (Car) (Fig. 5E, F), and total chlorophyll (Chl t) (Fig. 5G, H) were reduced significantly in both species when compared with unstressed

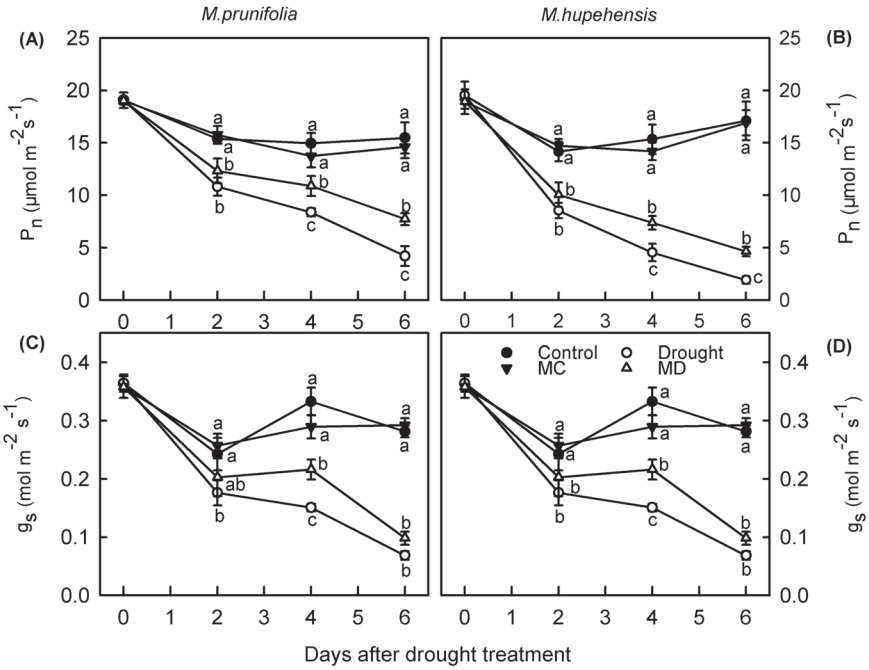

Fig. 4. Changes in net photosynthesis, P_n (A and B), and stomatal conductance, g_s (C and D) of *M. prunifolia* and *M. hupehensis* under control and drought conditions, with or without 100 μM melatonin pre-treatment. Closed and open circles indicate plants without pre-treatment under control and drought conditions, respectively. Closed and open triangles indicate pre-treated plants under control and drought conditions, respectively. Values are means of five replicates±SD. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different (P<0.05).

control plants. However, those pigments were largely preserved in the MD plants when compared with their melatonin-free counterparts (P<0.05). The protective effect of melatonin on Chl *b* and Car was greater in drought-sensitive *M. hupehensis* than in tolerant *M. prunifolia* (P<0.05).

Stomatal behaviour

The lower surfaces of the leaf samples were scanned at ×3000 magnification. The imposition of drought caused the stomata to close in both species whereas melatonin pre-treatment was associated with stomata that remained open under stress conditions (Fig. 6). Stress also resulted in stomata being significantly shorter (Fig. 7A, B) and narrower (Fig. 7C, D), as well as having smaller apertures (Fig. 7E, F) than in the control plants of either species. The MD plants also had significantly longer and wider stomata along with larger apertures (Fig. 7). In fact, when compared with those of the controls, stomatal apertures were 17.1% larger for pre-treated *M. prunifolia* and 50.6% for pre-treated *M. hupehensis* (Fig. 7E, F). Although stomatal density was not altered by drought in either species, exposure to melatonin was linked with a reduction in densities under both control and drought conditions (Fig. 7G, H).

ABA content and expression of genes in the ABA metabolic pathway

Melatonin did not alter ABA levels in control plants. Drought stress caused a significant elevation of ABA contents in both species whereas the melatonin pre-treatment resulted in 35.6% and 51.7% reductions in those contents in the drought-tolerant and -sensitive species, respectively (Fig. 8A, B).

Transcripts of the ABA biosynthesis gene *MdNCED3* were more abundant in leaves of both species under drought stress, but this up-regulation was significantly suppressed by melatonin pre-treatment (Fig. 9A, B). Two ABA catabolism genes, *MdCYP707A1* and *MdCYP707A2*, were also up-regulated. However, their levels of expression were significantly higher in melatonin-treated plants (Fig. 9C–F).

H_2O_2 content and activities of antioxidant enzymes

Drought stress caused a rapid increase in H_2O_2 levels in leaves from all sampled plants (Fig. 10A, B). By day 1, those had risen by 29.3% and 19.0% in *M. prunifolia* and *M. hupehensis*, respectively, and they remained elevated thereafter. Melatonin pre-treatment significantly suppressed this stress-related boost in H_2O_2 production. Both species showed significantly enhanced activities of CAT (Fig. 10C, D), POD (Fig. 10E, F), and APX (Fig. 10G, H) under stress, and those activities were further increased by exposure to melatonin. Levels of CAT and POD changed over time, being highest on days 1 and 3, respectively, after drought was initiated but then returning to normal levels by day 6. However, APX activity was significantly elevated throughout the 6-day experimental period. This suggested that APX was the key enzyme for detoxifying H_2O_2 in drought-stressed plants.

Discussion

Melatonin primarily functions in plants as the first line of defence against internal and environmental oxidative stressors (Tan *et al.*, 2012). This is accomplished when melatonin

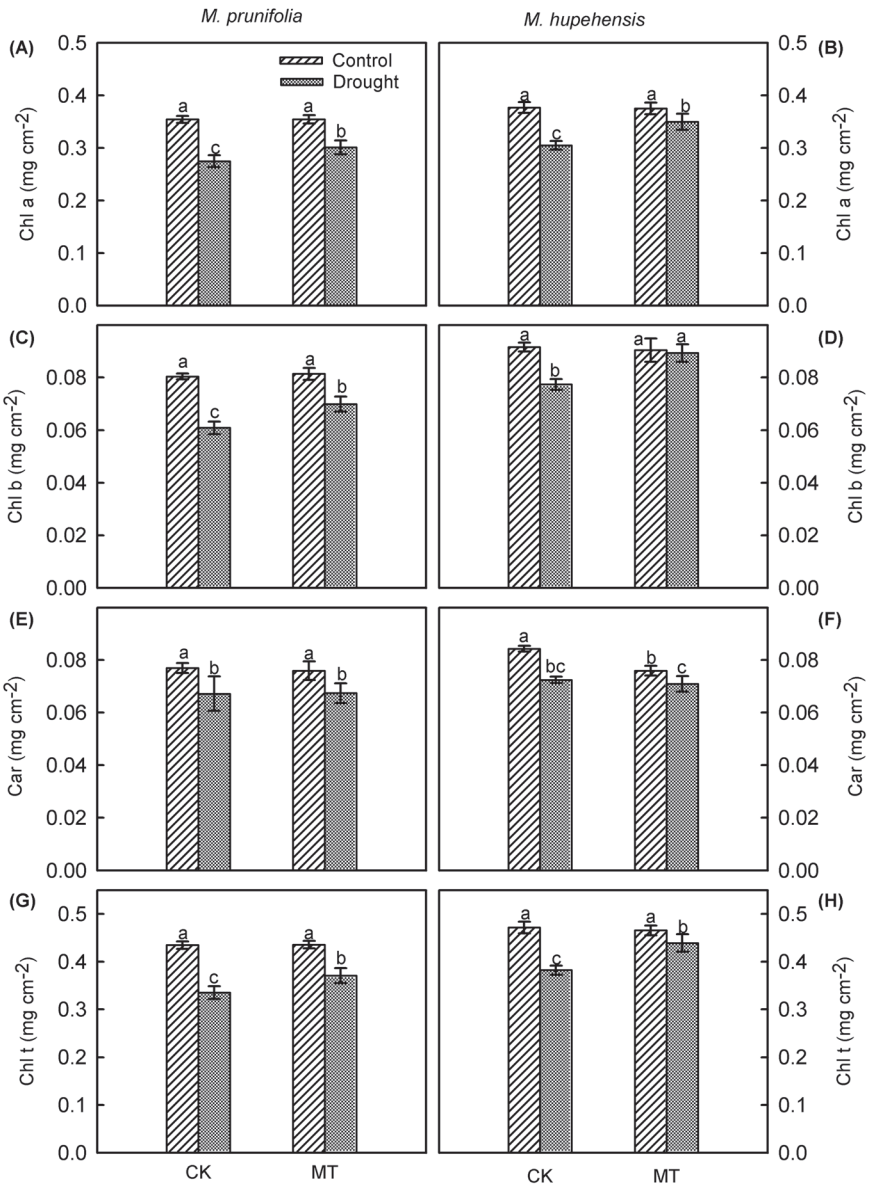

Fig. 5. Effects of melatonin on contents of photosynthetic pigments in *M. prunifolia* and *M. hupehensis* under control and drought conditions: Chl *a* (A and B), Chl *b* (C and D), carotenoid (Car; E and F), and total chlorophyll (Chl *t*; G and H). CK, non-melatonin pre-treated control; MT, plants pre-treated with melatonin. Data are means of five replicates±SD. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different ($P<0.05$).

directly scavenges free radicals and up-regulates the expression of genes for antioxidant enzymes. As a result, this molecule is applied to plants to protect them when exposed to various stress conditions (Arnao and Hernandez-Ruiz, 2014; Lei *et al.*, 2004; Posmyk *et al.*, 2008; Shi and Chan, 2014; Tiryaki and Keles, 2012; Xu, 2010; Yin *et al.*, 2013; Zhang *et al.*, 2014), including drought (Wang *et al.*, 2013*b*; Zhang *et al.*, 2013). However, the exact mechanisms by which melatonin improves drought tolerance have not been fully clarified. Here, we focused on the pathway for ABA metabolism.

Abscisic acid has a key role in conferring tolerance to environmental challenges (Fujita *et al.*, 2005; Iuchi *et al.*, 2001). Under a water deficit, plants accumulate ABA, which, at high levels, can promote ROS formation and eventually lead to oxidative damage, such as leaf peroxidation, electrolyte leakage, Chl degradation, and reduced photosynthetic performance (Jiang and Zhang, 2002; Zhu, 2002). These alterations were also observed in response to drought. Under stress conditions, expression of *MdNCED3* (for ABA biosynthesis), and *MdCYP707A1* and *MdCYP707A2* (both for ABA catabolism) was significantly up-regulated. The ABA content was also increased. Pre-treatment with melatonin selectively suppressed the up-regulation of *MdNCED3* and promoted the up-regulation of the other two, thereby reducing ABA levels when plants were later exposed to a water deficit. Therefore, this is the first report to show how melatonin buffers the effect of drought stress in plants.

Stomata have an essential role in determining drought tolerance. Their opening and closing is controlled by environmental and internal parameters, e.g. maintenance of the water balance and functioning of complex signal transduction pathways (Schroeder *et al.*, 2001). Abscisic acid mediates

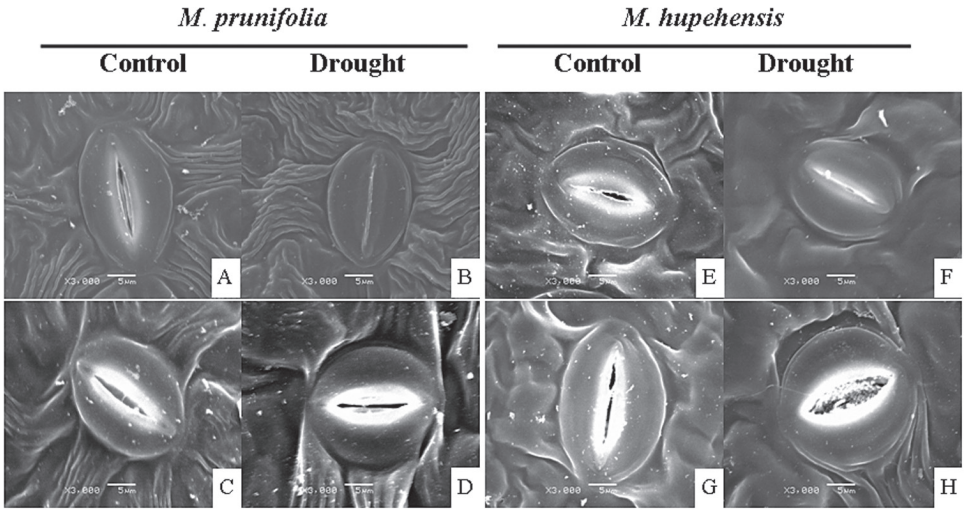

Fig. 6. SEM images of stomata from leaves of *M. prunifolia* and *M. hupehensis*: control leaves in which stomata are open (A and E); closed stomata in leaves exposed to drought stress for 5 d (B and F); leaves from control plants that were pre-treated with 100 μM melatonin (C and G); and leaves from drought-stressed, pre-treated plants for which all stomata are open (D and H). Magnification ×3000, scale bars=5 μm.

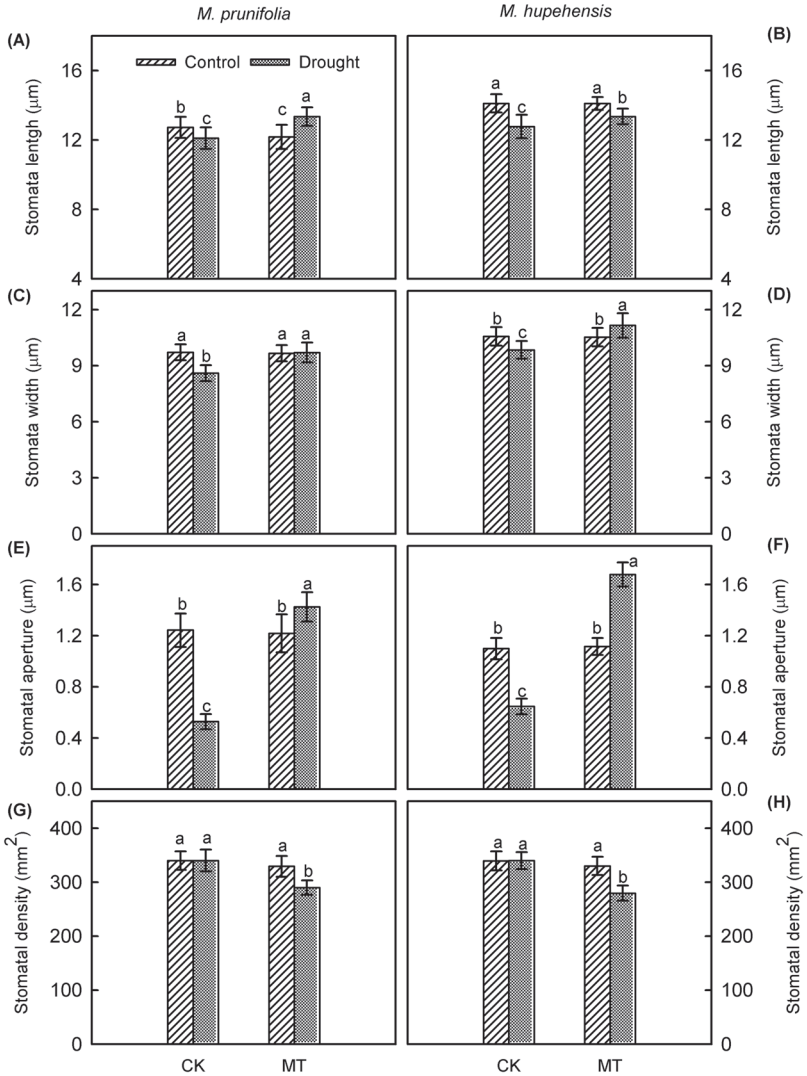

Fig. 7. Effects of melatonin on stomatal properties of leaves from *M. prunifolia* and *M. hupehensis* under control and drought conditions: length (A and B), width (C and D), aperture size (E and F), and density (G and H). CK, non-melatonin pre-treated control; MT, plants pre-treated with melatonin. Data are means of 30 images±SD. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different (P<0.05).

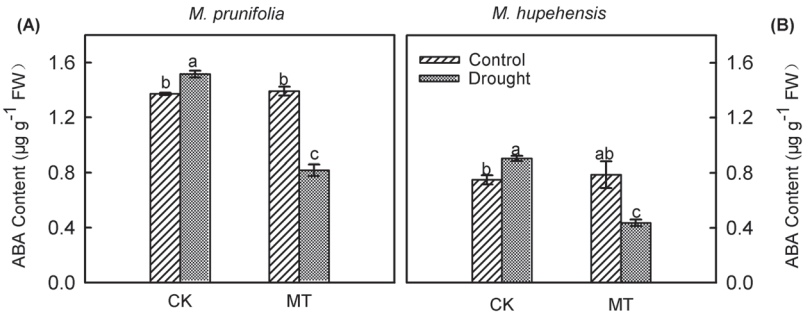

Fig. 8. Effects of melatonin on ABA contents in *M. prunifolia* (A) and *M. hupehensis* (B) under control and drought conditions. CK, non-melatonin pre-treated control plants; MT, plants pre-treated with melatonin. Data are means of five replicates±SD. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different (P<0.05).

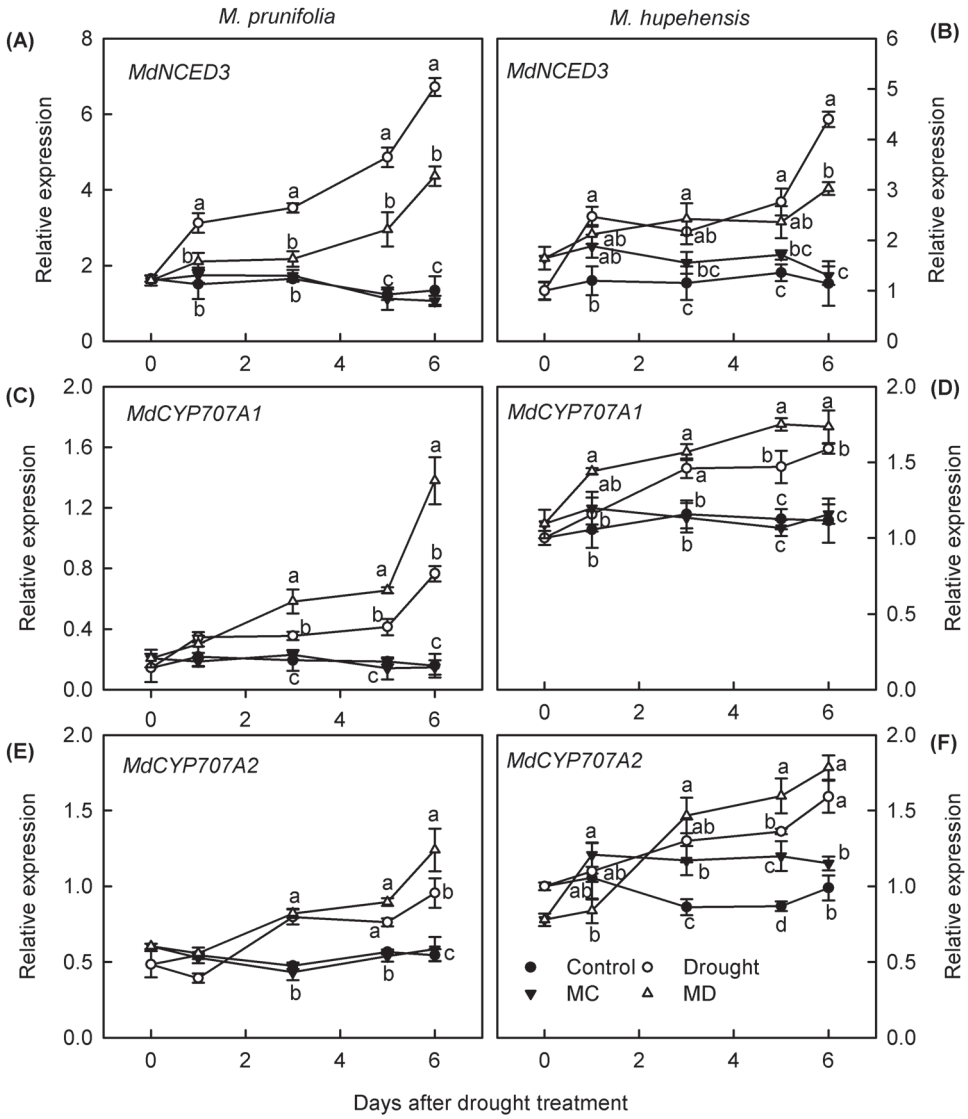

Fig. 9. Effects of melatonin on expression of genes in ABA synthesis and metabolic pathway: *MdNCED3* (A and B), *MdCYP707A1* (C and D), and *MdCYP707A2* (E and F) in leaves of *M. prunifolia* and *M. hupehensis*. Total RNA was isolated from samples at different time points, converted to cDNA, and subjected to real-time RT-PCR. Expression levels were calculated relative to expression of *Malus EF-1a* mRNA. Closed and open circles indicate plants without melatonin pre-treatment under control and drought conditions, respectively. Closed and open triangles indicate plants with melatonin pre-treatment under control and drought conditions, respectively. Data are means of five replicates±SD. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different (P<0.05).

the behaviour of stomatal apertures in response to environmental variables (Chaves *et al.*, 2009). It is considered fundamental in that process because it triggers activity in guard cell membrane channels and transporters, thereby decreasing guard cell turgor and ultimately closing the stomata (Brodribb and McAdam, 2013). For example, severely stressed *Callitris*

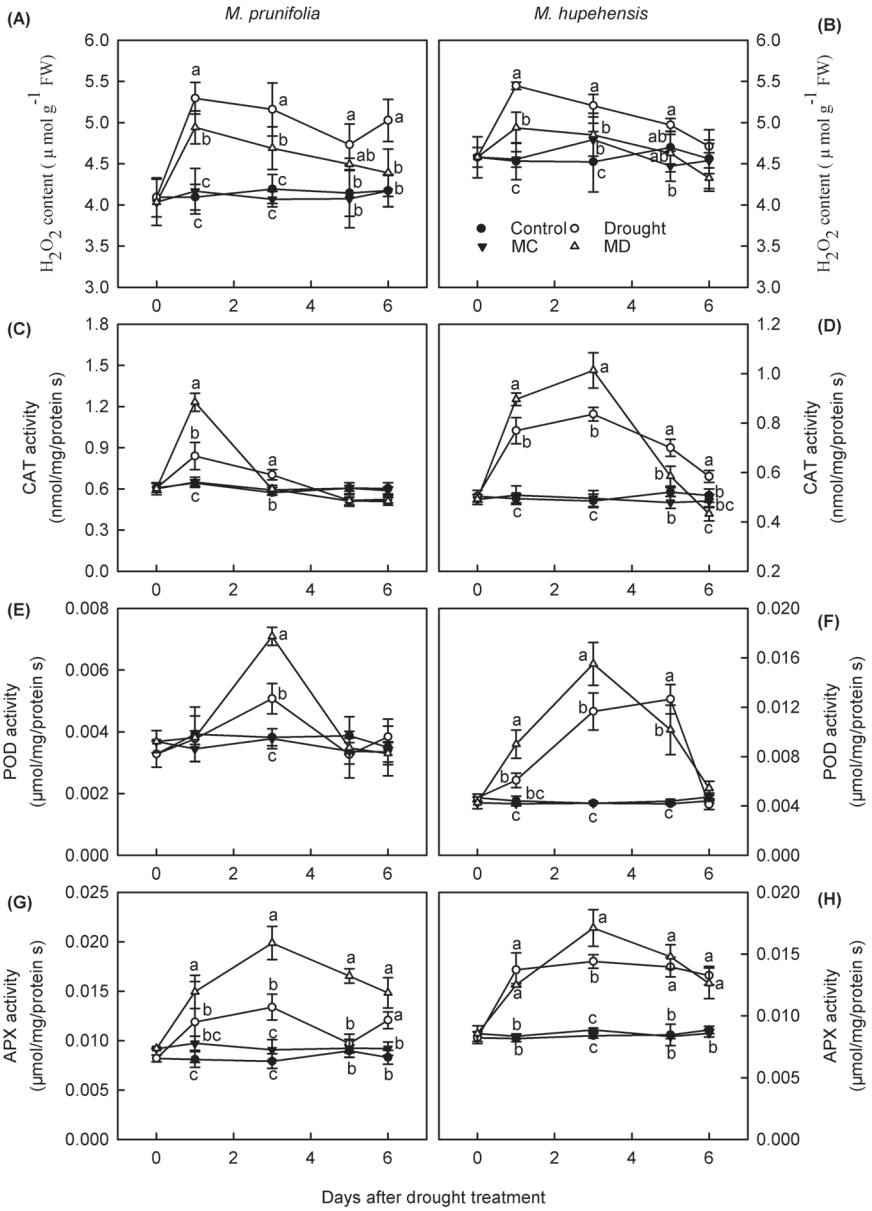

Fig. 10. Effects of melatonin on H_2O_2 accumulations and activities of antioxidant enzymes in leaves during stress period: H_2O_2 content (A and B), CAT activity (C and D), POD activity (E and F), and APX activity (G and H). Closed and open circles indicate plants without melatonin pre-treatment under control and drought conditions, respectively. Closed and open triangles indicate plants with melatonin pre-treatment under control and drought conditions, respectively. Data are means of five replicates±SD. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different (P<0.05).

rhomboidea plants that have depressed levels of ABA immediately re-open their stomata as soon as the leaves or soil are rehydrated (Brodribb and McAdam, 2013). The influence that ABA has on stomatal behaviour is related to its ability to increase the synthesis of H_2O_2 in guard cells via NADPH oxidase. Thus, elevated levels of H_2O_2 mediate ABA-induced stomatal closure.

Based on these observations, it is proposed that melatonin uses two means for increasing drought tolerance, first by enhancing ABA degradation and suppressing its synthesis. Consequently, less H_2O_2 is accumulated in the guard cells. Second, melatonin directly scavenges that H_2O_2 (Galano *et al.*, 2013; Reiter *et al.*, 2007; Tan *et al.*, 2000, 2007*b*). It also up-regulates the activities of antioxidant enzymes, particularly CAT, POD, and APX, which are very efficient in degrading H_2O_2 (Li *et al.*, 2012; Park *et al.*, 2013). Therefore, the reduced contents of both ABA and H_2O_2 work synergistically and lead to improved performance by stomata, so that they can re-open under drought conditions. This enhancement of stress tolerance is reflected in the diminished leakage of electrolytes, higher water contents in plant tissues, preservation of photosynthetic pigments, and improved functioning of the photosynthesis apparatus.

As expected, pre-treatment with melatonin had a more profound effect on the drought-sensitive species than on the tolerant species. When the expression of genes for melatonin synthesis enzymes was monitored, the resultant data also supported our speculation that melatonin is an important

molecule for regulating drought tolerance. Because this expression was stronger in the more sensitive species, it is believed that its machinery for melatonin synthesis cannot produce sufficient melatonin to guard against this stressor. Therefore, those genes must be further up-regulated so that adequate amounts of enzymes are produced. Future investigations should focus on identifying genetic mutations of that pathway in those two species.

In conclusion, when tolerant and sensitive apple plants are pre-treated with melatonin, they show greater tolerance and adaptability to drought stress. Dual protective mechanisms work synergistically to improve stomatal functioning, which regulates water status under either normal or drought conditions (Jones, 1998; Katul *et al.*, 2003; Sperry *et al.*, 2002). These findings provide evidence for the physiological role of melatonin and serve as a platform for possible applications in agricultural or related fields of research.

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