Research Article

2-Hydroxymelatonin confers tolerance against combined cold and drought stress in tobacco, tomato, and cucumber as a potent anti-stress compound in the evolution of land plants

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ABSTRACT

Melatonin (M) is an endogenous molecule found ubiquitously in animals and plants that helps maintain various biological functions. Of note, plants also synthesize a melatonin metabolite, 2-hydroxymelatonin (2M) by the action of melatonin 2-hydroxylase, but the biological functions of 2M remain largely unknown. Here, we found that exogenous foliar application of 2M conferred tolerance against combined cold and drought stress in tobacco (*Nicotiana benthamiana*), tomato (*Solanum lycopersicum* L. cv. Micro-Tom), and cucumber (*Cucumis sativus* L. cv. Baecdadaki), whereas no such tolerance was observed against these stresses applied individually. Accordingly, endogenous 2M was induced in tobacco and tomato leaves in response to combined stress, whereas M levels remained unchanged in tobacco leaves and decreased in tomato leaves. After challenging tobacco and tomato leaves with prohexadione-calcium, an inhibitor of 2M synthesis, 2M levels decreased and led to hypersensitivity to combined stress. Because the gene encoding 2M is found only in land plants, and is absent in cyanobacteria and algae, we propose that 2M may have evolved as aquatic plants invaded land to overcome the stressors of virgin terrestrial environments, such as cold and drought.

Key words: cold stress, double stresses, drought stress, 2-hydroxymelatonin, land plants, melatonin.

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1. INTRODUCTION

Melatonin (M) is estimated to have emerged on Earth between 2,800 and 3,200 million years ago, in parallel with the appearance of non-purple sulfur bacteria and prokaryotic cyanobacteria (1, 2). M is believed to have initially functioned primarily as an antioxidant, and thereafter acquired many other functions, including circadian rhythm and sleep regulation in animals (1, 3). In light of the endosymbiotic theory, which states that cyanobacteria are the origin of chloroplasts, it is unsurprising that plants synthesize M. However, the discovery of M in plants, first identified in 1995 in many plant tissues, lagged 38 years behind that in animals, where M was first identified in bovine pineal gland in 1958 (4–6).

Despite the relatively short history of M studies in plants, many biological functions of M

have been uncovered in plants, i.e., as a signaling molecule for growth and development (7–9) as well as for a defense against pathogens (10, 11), and to confer tolerance against various abiotic stresses (e.g., cold, drought, heat, endoplasmic reticulum, and oxidative) (6, 12–15). All genes in the M biosynthetic pathway starting from tryptophan have been cloned in plants (6). Moreover, M is metabolized into the major metabolite 2-hydroxymelatonin (2M) by melatonin 2-hydroxylase (M2H) (16). Reports have suggested that 2M levels are much higher than those of M in various plant species (17), suggesting an important physiological role of 2M in plants. Although more than 100,000 plant metabolites have been identified, the presence of 2M in plants has not been reported to date except our previous reports (17, 18). Thus, whether 2M is a nonfunctional metabolite or possesses a novel function in plants should be clarified. Our previous study supported the former hypothesis because 2M treatment induced MAP kinase activation comparable to that of M in *Arabidopsis* (19). A similar study in rice seedlings showed enhanced resistance in response to combined cold and drought stress (20).

These findings inspired us to explore the effects of 2M in a number of other crop plants against combined abiotic stresses. In this study, we subjected tobacco (*Nicotiana benthamiana*), tomato (*Solanum lycopersicum* L.), and cucumber (*Cucumis sativus* L.) plants to combined cold and drought stress, to clarify whether 2M conferred resistance against these combined stressors. Our findings of an enhanced tolerance to cold and drought stress by exogenous 2M treatment, and reduced tolerance by application of a 2M synthesis inhibitor, revealed the involvement of 2M against combined cold and drought stressors in several land plant species.

2. MATERIALS AND METHODS

2.1. Plant materials and stress treatment.

The tobacco (*N. benthamiana*) and tomato (*S. lycopersicum* cv. Micro-Tom) seeds used in this study were provided by Dr. Y. S. Kim (Chonnam National University, Gwangju, Republic of Korea) and Dr. Y. H. Joung (Chonnam National University), respectively. Cucumber (*C. sativus* cv. Baecdadaki) seeds were purchased from Dongbu Farm Hannong Co., Ltd. (Seoul, Republic of Korea). The seeds were germinated and grown in plastic pots in a mixture comprising sterile bio soil and sterile perlite (3:1 ratio). The bio soil and perlite were purchased from Dongbu Farm Hannong Co., Ltd. and Ho Hyun Bio Co. (Hadong, Republic of Korea), respectively. In each experiment, we grew a maximum of five plants per plastic pot, which were watered with 50 mL of tap water every 2 days. Plants were grown in a controlled environment room under a constant temperature of 22°C and LED white fluorescent light (110 μmol m^{-2} s^{-1}), with a 15-h photoperiod for 3 weeks.

The stress treatments (cold, drought, and cold + drought) were applied to 4-week-old plants that had received either water (control) or pretreatment with M or 2M, as 20 mL of 10 μM of each solution sprayed on the tops of plants leaves. The pretreatment was repeated every 2 days for 1 week. To induce drought stress, watering was discontinued and plants were kept in the growth room. To induce cold stress, pots were transferred to a controlled cool chamber under a constant temperature of 4°C and LED white fluorescent light (96 μmol m^{-2} s^{-1}), with a 15-h photoperiod, and watering was continued. For the combined stress, pots were placed in the cool chamber and watering was discontinued.

2.2. Relative water content.

The relative water contents of leaves and soil were determined. The leaf relative water content (LRWC) was calculated using the following formula: LRWC (%) = [(fresh leaf weight

− dried leaf weight) / (turgid leaf weight − dried leaf weight)] × 100. The LRWC was estimated according to Turner (21). The soil relative water content (SRWC) was calculated using the following formula: SRWC (%) = [(actual pot weight − pot weight with dried soil) / (pot weight with saturated soil − pot weight with dried soil)] × 100. The SRWC was determined according to Wang *et al*. (22).

2.3. Lipid peroxidation.

The level of malondialdehyde (MDA) was evaluated as an end-product of lipid peroxidation using a slight modification of the thiobarbituric acid (TBA) assay described by Du and Bramlage (23). Briefly, 0.1 g of plant tissue was finely ground in liquid nitrogen with a TissueLyser II (Qiagen, Tokyo, Japan), and then homogenized in 4 mL of 0.5% (w/v) TBA (Sigma-Aldrich Inc., St. Louis, MO, USA) in 20% (v/v) trichloroacetic acid (Sigma-Aldrich Inc.). The homogenate was centrifuged at maximum speed for 20 min, and the clear supernatant was transferred into a new tube. The supernatant was heated for 25 min, and cooled on ice for 5 min. The cold supernatant underwent spectrophotometric analysis at absorbance (A) values of 532, 440, and 600 nm. The MDA equivalent (nmol mL^{-1}) was calculated as follows: {[(A532 − A600) − [(A440 − A600) × (molar absorbance of sucrose at 532 nm / molar absorbance of sucrose at 440 nm)]} / 157,000] × 10^6.

2.4. Melatonin and 2-hydroxymelatonin quantification.

For the determination of M and 2M contents in plants, 0.1 g of tissue material was finely ground using a TissueLyser II (Qiagen), and then extracted with 1 mL of chloroform (JENSEI, Tokyo, Japan) on a shaker with overnight incubation. The following day, 700 µL of clear supernatant was obtained via centrifugation at maximum speed for 15 min. The whole solution was vacuum-dried and resuspended in 100 µL of 40% (v/v) methanol. A 10-µL aliquot of methanol extract was used for the determination of M and 2M via high-performance liquid chromatography (HPLC) as described by Byeon *et al*. (24) and Byeon and Back (16), respectively. Briefly, M analysis was employed a fluorescence detector system (Waters, Milford, MA, USA) and detected at 280 nm (excitation) and 348 nm (emission). As for 2M, we used a UV detector system (Waters) at 254 nm. The 2M was separated on a Sunfire C18 column (Waters; 4.6 × 150 mm) with an isocratic elution with 35% MeOH in 0.3% trifluoroacetic acid at flow rate of 0.25 mL/min. The detection limit and recovery rate of both M and 2M were about 0.25 ng/g FW and 90% in these analytical methods, respectively. Under these conditions, M and 2M were eluted at retention time of 31 and 18.9 min, respectively.

2.5. Prohexadione-calcium treatment.

To inhibit 2M accumulation in tobacco and tomato plants (3-weeks old), prohexadione-calcium (Ph-Ca; Bibipul: Ph-Ca (0.1%) powder; Kyungnong Co., Ltd., Seoul, Republic of Korea) was applied as 50 mL of 10 µM, 50 µM, or 100 µM Ph-Ca solution. Of the 50 mL, 10 mL was sprayed on the tops of plant leaves and the rest was used to irrigate the soil. This treatment was repeated every 2 days for 1 week. One day after the final treatment, plants were transferred to the controlled cool chamber for the stress treatment.

2.6. Statistical analysis.

Data were analyzed using an ANOVA and followed by post hoc Tukey's HSD test with IBM SPSS Statistics 23 software (IBM Corp. Armonk, NY, USA). *P*-values < 0.05 denoted statistical significance. All data are presented as the means ± standard deviation.

3. RESULTS

3.1. 2M confers tolerance against combined cold and drought stress in tobacco.

To assess the involvement of 2M in protecting plants against stress, tobacco seedlings were exposed to cold, drought, or combined cold + drought. Pretreatment with 2M increased cold + drought stress tolerance in tobacco leaves compared to the control, but no increased tolerance was statistically observed against single cold or drought stress albeit both M and 2M application marginally enhanced cold tolerance (Fig. 1). After combined stress, 2M-pretreated tobacco leaves had a 35% higher LRWC and 2-fold higher biomass than control tobacco leaves. M-pretreated tobacco leaves also had slightly higher biomasses than control leaves after combined stress, possibly due to the conversion of M into 2M (16). The 2M level of 2M-treated tobacco was comparable to that of both control and M-treated tobacco after 25 days under the cold stress condition, suggestive of rapid degradation of 2M in plants.

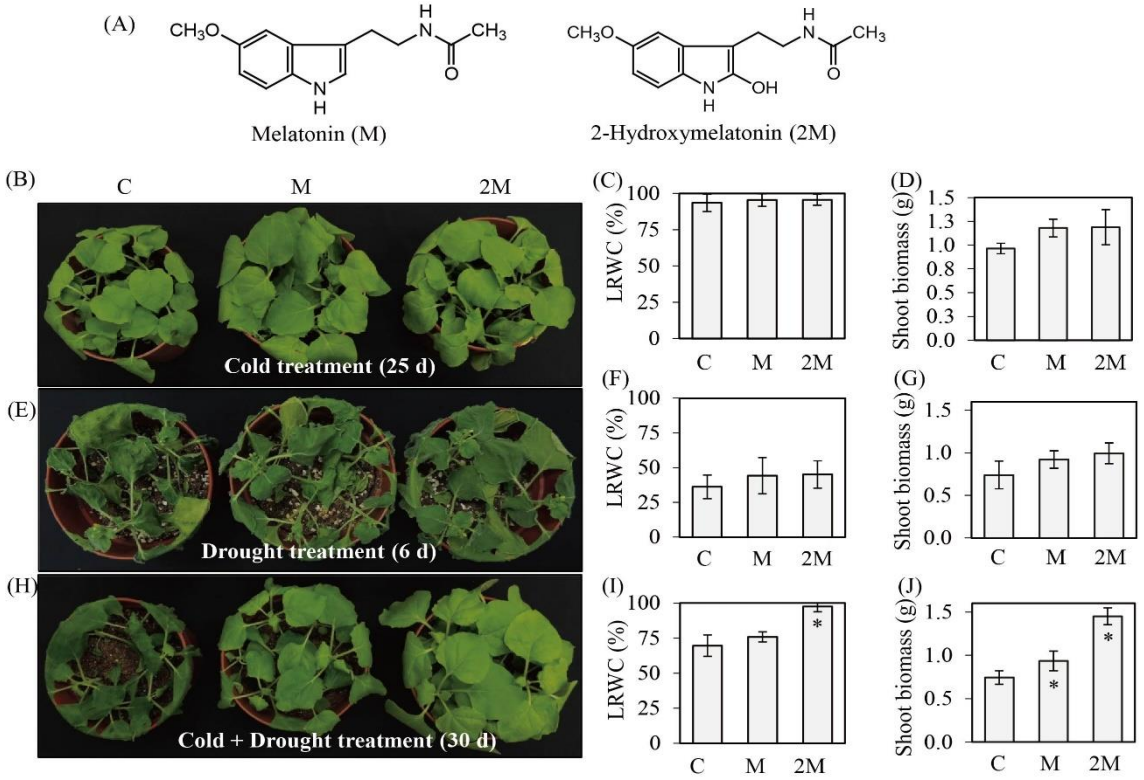

Fig. 1. Effects of 2-hydroxymelatonin on various abiotic stresses in tobacco.

*(**A**) Structures of melatonin (M) and 2-hydroxymelatonin (2M). (**B–D**) Effects of exogenous chemical treatments in tobacco leaves against cold stress. (**E–G**) Effects of exogenous chemical treatments in tobacco leaves against drought stress. (**H–J**) Effects of exogenous chemical treatments in tobacco leaves against cold + drought stress. For the treatment groups, 21-day-old tobacco plants of similar size were independently sprayed with water (control, C), 10 µM M, or 10 µM 2M for one week, and then exposed to cold (4°C) for 25 days, drought for 6 days at room temperature, or cold (4°C) plus drought for 30 days. After treatment, tobacco leaves were sampled for measurement of the leaf relative water content (LRWC) and shoot biomass per plant. Values are the means ± SD (n = 9). *: significant difference versus the control (post hoc Tukey's HSD test; P < 0.05). C: water; M: melatonin; 2M: 2-hydroxymelatonin.*

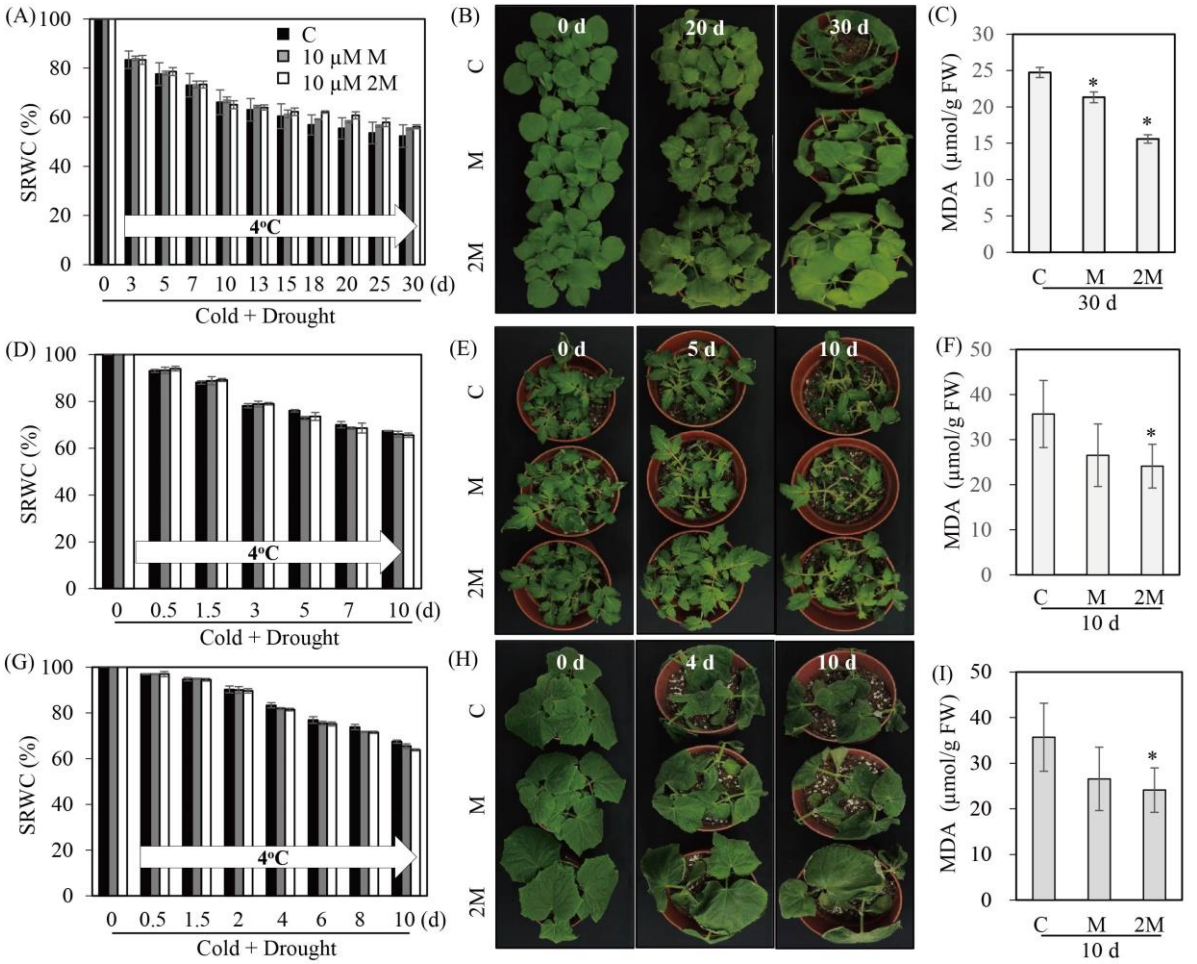

Fig. 2. Effects of 2-hydroxymelatonin against cold plus drought stress in tobacco, tomato, and cucumber.

*(**A–C**) Effects of 2M in tobacco against cold + drought stress. (**D–F**) Effects of 2M in tomato against cold + drought stress. (**G–I**) Effects of 2M in cucumber against cold + drought stress. For the treatment groups, 21-day-old seedlings were sprayed with water (control, C) or 10 µM M or 2M as described in Fig. 1. Soil relative water content (SRWC), phenotype, and malondialdehyde (MDA) content were determined at the indicated time points after treatment. Values are the means ± SD (n = 9). *: significant difference from the control (post hoc Tukey's HSD test; P < 0.05).C: water; M: melatonin; 2M: 2-hydroxymelatonin.*

3.2. 2M treatment lowers MDA production in tobacco, tomato, and cucumber exposed to combined cold and drought stress.

To assess whether the enhanced growth rate of 2M-pretreated tobacco exposed to cold + drought was coupled to biochemical stress tolerance, we measured the levels of MDA, a biomarker of lipid peroxidation induced by various stress conditions, including both cold and drought. After challenging tobacco plants with combined cold + drought stress (Fig. 2A–C), control tobacco showed severe wilting with higher MDA accumulation. In contrast, tobacco leaves pretreated with 10 µM M exhibited less wilting and lower MDA levels than the control tobacco. Moreover, 2M-pretreated tobacco showed a healthy phenotype and the lowest MDA content. During the cold + drought treatment, the SRWC did not differ significantly among the tobacco plants. Similarly, tomato (Fig. 2D–F) and cucumber (Fig. 2G–I) pretreated with 2M

exhibited healthier phenotypes with lower MDA contents than control and M-pretreated plants. Unlike tobacco, M-pretreated tomato and cucumber had non-significantly lower MDA levels than the control. This result further confirms that 2M treatment confers improved tolerance against combined cold and drought stress in several land plant species, including tobacco, tomato, and cucumber.

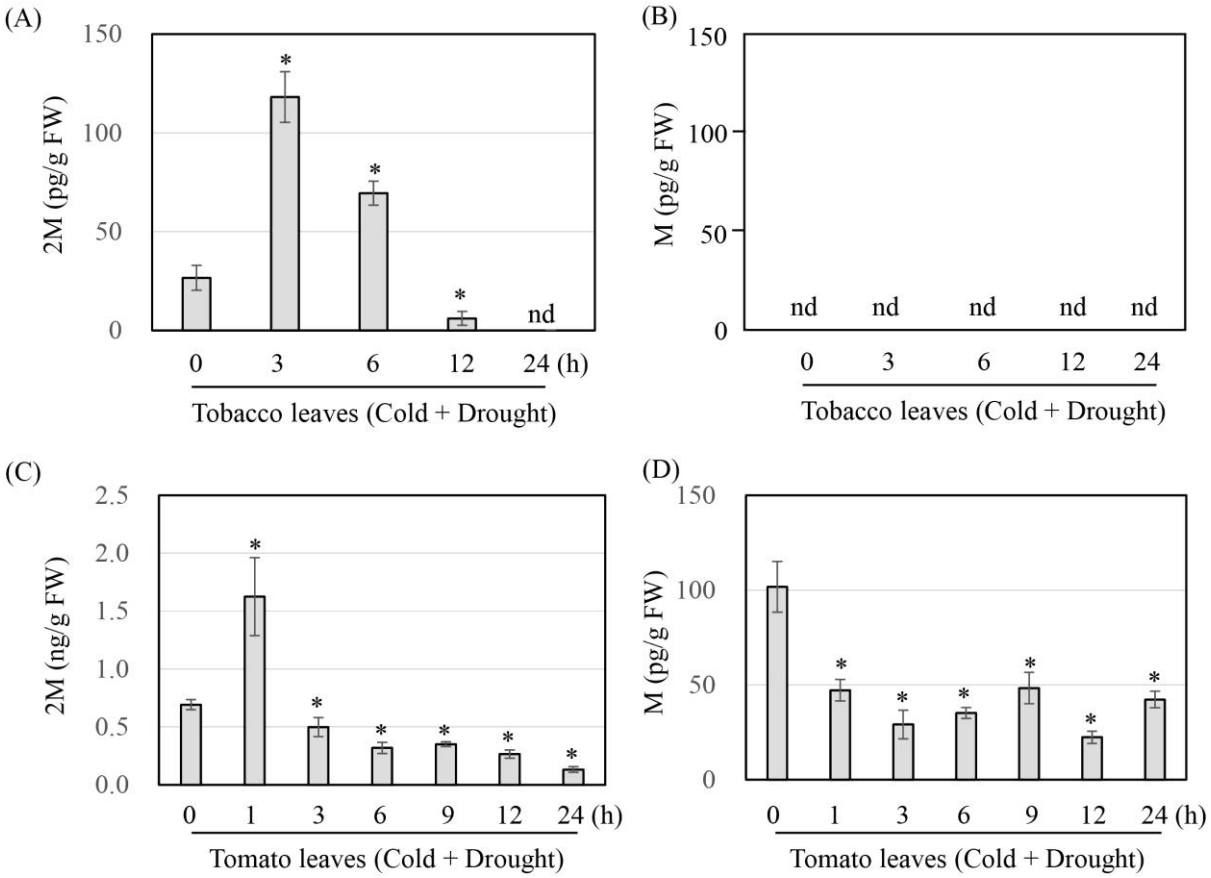

Fig. 3. Systemic induction of 2-hydroxymelatonin in response to combined cold and drought stress.

*(**A**) Endogenous 2M contents in tobacco plants after cold + drought stress. (**B**) M contents in tobacco plants after cold + drought stress. (**C**) 2M contents in tomato plants after cold + drought stress. (**D**) M contents in tomato plants after cold + drought stress. Samples were subjected to high-performance liquid chromatography analysis for M and 2M contents. Values are the means ± SD (n = 3). *: significant difference from the untreated control (post hoc Tukey's HSD test; P < 0.05). nd, not detected. M: melatonin; 2M: 2-hydroxymelatonin.*

3.3. 2M is induced in response to combined cold and drought stress.

To further clarify the role of 2M *in vivo*, we quantified 2M levels in tobacco and tomato in response to combined cold + drought stress. Tobacco leaves showed 2M induction, which peaked at 3 h and gradually declined at 6 h, reaching undetectable levels 24 h after the stress treatment (Fig. 3A). In marked contrast, M levels were undetectable in tobacco leaves before and after the stress treatment (Fig. 3B). Similarly, 2M was induced in tomato leaves 1 h after combined stress treatment, and returned to basal levels thereafter (Fig. 3C). By contrast, M was reduced 1 h after combined stress treatment, and the reduced levels were maintained until 24 h after treatment (Fig. 3D). The *in vivo* synthesis results suggest that 2M is transiently induced

upon combined cold and drought stress in both tobacco and tomato, whereas M fails to induce in both tobacco and tomato leaves, suggestive of distinct roles of 2M and M in terms of their biosynthesis in response to stress.

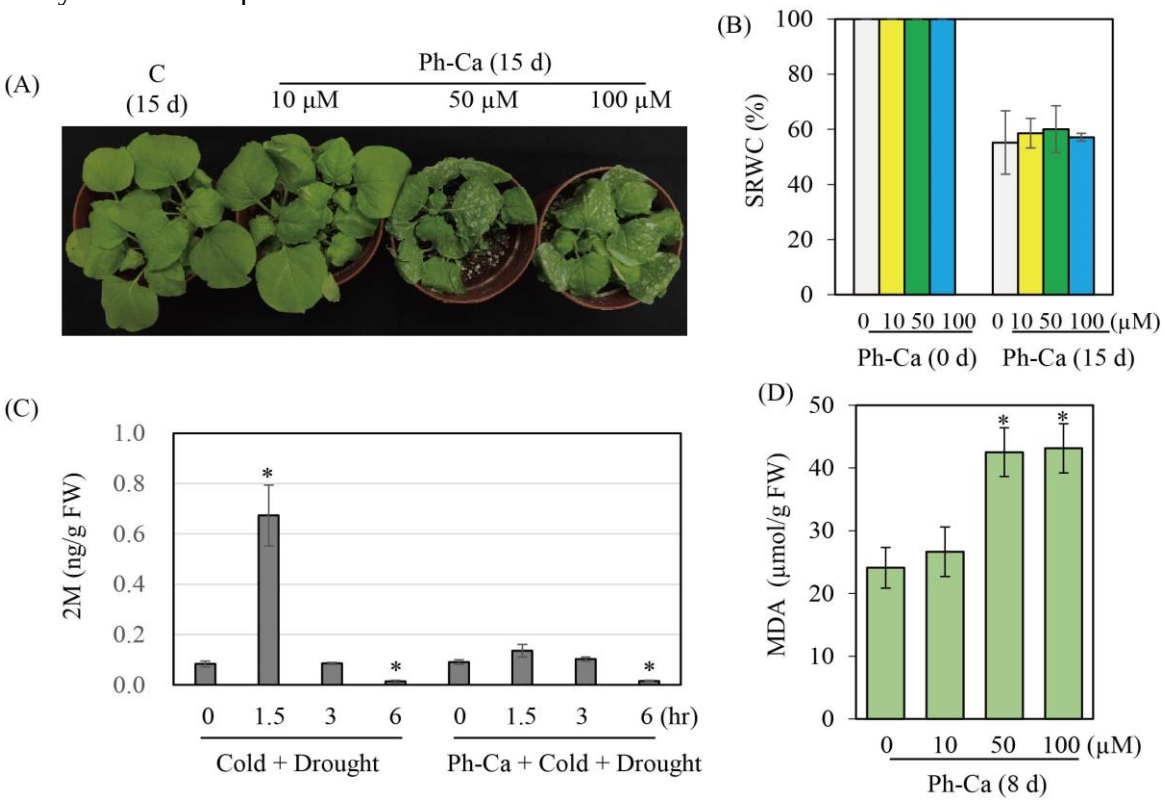

Fig. 4. Suppression of 2-hydroxymelatonin by prohexadione-calcium and increased susceptibility to combined cold and drought stress in tobacco plants.

*(**A**) Phenotypes of tobacco plants 15 days after prohexadione-calcium (Ph-Ca) treatment. (**B**) Soil relative water content (SRWC). (**C**) Leaf 2M levels. (**D**) Leaf MDA levels. For the treatment groups, 21-day-old tobacco plants were independently sprayed with water (control, C) or various concentrations of Ph-Ca and then exposed to combined cold (4°C) and drought conditions for 15 days. Values are the means ± SD (n = 3). *: significant difference from the control (post hoc Tukey's HSD test; P < 0.05. 2M: 2-hydroxymelatonin.*

3.4. Ph-Ca inhibits 2M synthesis and reduces tolerance against combined cold and drought stress.

To verify the in vivo role of 2M in conferring tolerance against combined stress in plants, we employed loss-of-function analysis via application of the 2M synthesis inhibitor Ph-Ca in tobacco and tomato plants. Ph-Ca is an inhibitor of 2-oxoglutarate-dependent dioxygenases (2-ODDs), such as gibberellin 2-oxidase (25) and M2H (16), because Ph-Ca has a similar structure to 2-oxoglutaric acid, a co-substrate of 2-ODDs. At 21 days old, tobacco leaves were treated with various concentrations of Ph-Ca or water (control) and then exposed to combined cold + drought stress for 15 days (Fig. 4A). The control and treated samples had identical SRWCs (Fig. 4B). Tobacco plants treated with 50 μM and 100 μM Ph-Ca had higher MDA levels than the control, suggesting that inhibition of 2M synthesis accelerated stress-induced membrane damage. However, tobacco treated with 10 μM Ph-Ca had MDA levels comparable to that of the control (Fig. 4D). Therefore, 50 μM Ph-Ca treatment successfully negate the transient induction of 2M in response to combined stress (Fig. 4C).

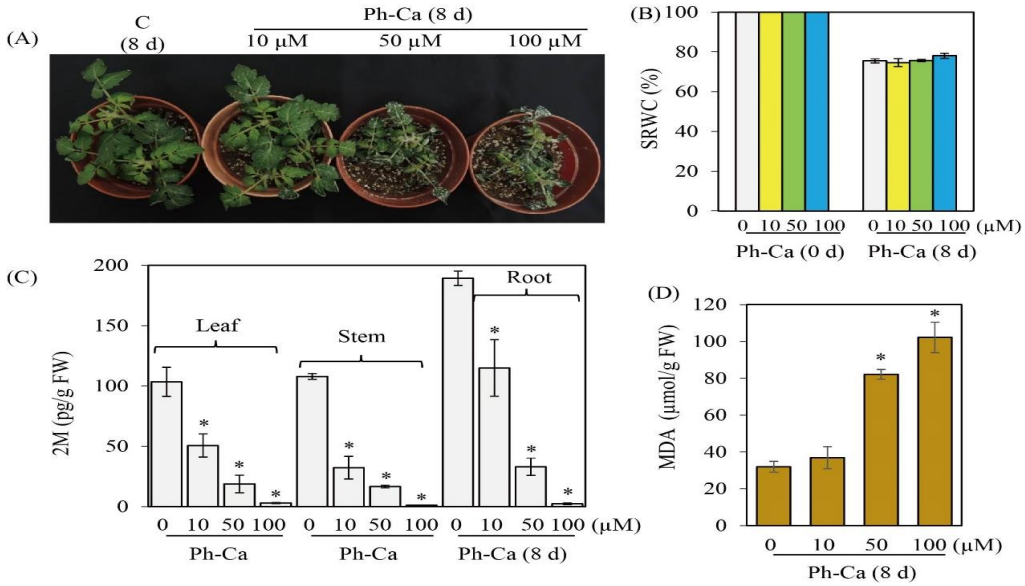

Fig. 5. Suppression of 2-hydroxymelatonin by prohexadione-calcium and increased susceptibility against combined cold and drought stress in tomato plants.

*(**A**) Phenotypes of tobacco plants in 8 days after Ph-Ca treatment. (**B**) Soil relative water content (SRWC). (**C**) 2M levels in various tissues. (**D**) Leaf MDA levels. For the treatment groups, 21-day-old tomato plants were independently sprayed with water or various concentrations of Ph-Ca and then exposed to the combined cold (4°C) and drought condition for 8 days. Samples were divided into leaf, stem, and root tissues and subjected to high-performance liquid chromatography analysis of 2M. Values are the means ± SD (n = 3). *: significant difference from the control (post hoc Tukey's HSD test; P < 0.05). C: water; 2M: 2-hydroxymelatonin.*

Similarly, 21-day-old tomato seedlings were treated with various concentrations of Ph-Ca and exposed to cold + drought stress for 8 days (Fig. 5A). Both control and 10-μM Ph-Ca-treated tomato plants appeared to be healthy, whereas tomato plants treated with 50 μM and 100 μM Ph-Ca were severely wilted and had high MDA levels (Fig. 5D). Moreover, Ph-Ca treatment resulted in significant dose-dependent decreases in 2M levels in all tissues (i.e., leaf, stem, and root; Fig. 5C). For example, treatment with 50 μM Ph-Ca led to the synthesis of 20 pg g^{-1} fresh weight 2M in both leaf and stem, which were 5-fold lower than control concentrations. Moreover, no 2M was detected in any tissues after treatment with 100 μM Ph-Ca. Interestingly, treatment with 10 μM Ph-Ca resulted in a roughly 50% decrease in 2M in tomato tissues compared to the control, although the phenotype and MDA production were comparable to those of the control. The SRWCs were identical in all treatments (Fig. 5B). These results clearly show that 2M has an important role in conferring tolerance against combined cold + drought stress in multiple land plant species.

4. CONCLUSION

The study of M in plants has a relatively short history compared to that in animals. In marked contrast with animals, plant M is enzymatically metabolized, via multiple M2H enzymes, into 2M as a predominant form of M derivatives. M2H enzymes are classified in the 2-ODD superfamily, which are reported to have been amplified in number during the transition of aquatic plants into land plants (16, 17, 27). Analogous to many other genes in the 2-ODD

superfamily, orthologs of *M2H* are absent in cyanobacteria and aquatic plants, but present in land plants. By contrast, apparent orthologs of serotonin *N*-acetyltransferase (*SNAT*), the penultimate enzyme for M synthesis, are present in plant species from cyanobacteria to land plants (Fig. 6A). Therefore, the emergence of *M2H* genes matches well with the diversification of the 2-ODD superfamily during land plant evolution (27). This implies that 2M had a role in the evolution of land plants from aquatic plants, which required adaptation to drastically different environments characterized by cold, drought, ultraviolet radiation, and gravity. To defend against such stressors, plants produce various metabolites, such as flavonoids and lignin against ultraviolet radiation and gravity, respectively (28). However, little is known of the mechanisms by which primitive terrestrial plants coped with cold, drought, and combined cold and drought. Although plant invasion from water to land progressed very slowly via unstable waterfronts, plants had to develop strategies against combined cold and drought stresses. To this end, it would be much easier for plants to evolve preexisting compounds found in aquatic plants, such as M. Consequently, exogenous M treatment improved cold resistance in some plants (6, 7), supporting possible functions of 2M against cold-related stresses.

Interestingly, many genes encoding 2-ODD members are involved in the synthesis of flavonoids, which protect plants from the ultraviolet radiation that was likely abundant in early terrestrial environments. However, the genes of 2-ODD members involved in flavonoid biosynthesis have evolved before the onset of land plant appearance, because flavonoids are also found in algal charophytes. Similarly, lignin, which confers resistance against gravity stress in land plants, is also found in aquatic algae (28). In contrast to flavonoids and lignin, the emergence of 2M was likely synchronized with the evolution of aquatic plants transition to land plants; this is supported by two lines of evidence (Fig. 6B). First, there is an absence of *M2H* genes responsible for 2M synthesis in aquatic plants, including red algae and green algae, which is in sharp contrast with the presence of M biosynthetic genes, such as *SNAT*, in both aquatic and land plants. Such diversification fits well with the common strategy employed by plants to cope with various adverse environments. This is similar to that animals acquire new functions by diversifying the regulatory modes of existing molecules such as M (1). Second, the novel function of 2M against combined stressors would not be developed in aquatic plants as in land plants since the former would not experience such harsh environments as in land plants.

(A)

	Aquatic plants			Land plants			
	Cyano-bacteria	Red algae	Green algae	Moss	Pine	Tobacco	Rice
M	+	+	+	+	+	+	+
2M	-	-	-	+	+	+	+

(B)

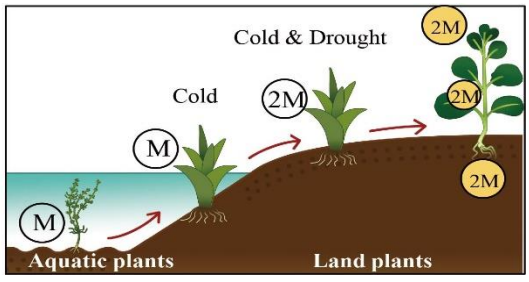

Fig. 6. Presence of homologous melatonin and 2-hydroxymelatonin biosynthetic genes and proposed function of 2-hydroxymelatonin.

*(**A**) Presence (+) or absence (-) of genes encoding M and 2M biosynthesis. (**B**) Proposed model for 2M as a key molecule supporting the land invasion of aquatic plants. Serotonin N-acetyltransferase (GenBank accession no.: AK059369), the penultimate gene for M synthesis, was used as the reference gene for M synthesis, while the melatonin 2-hydroxylase gene (GenBank accession no.: AK119413) encoding melatonin 2-hydroxylase in rice was used as the reference gene for 2M synthesis. M: melatonin; 2M: 2-hydroxymelatonin.*

Among the many strategies driving the land invasion of plants, no compounds have been shown to overcome the combination of cold and drought, which is considered to be a major limitation of plant survival on land. Moreover, many crops encounter a combination of various abiotic stressors, such as drought and heat, rather than one abiotic stress in isolation (29). Combined stressors can cause more damage than a single stressor, and elicit unique acclimation responses compared to single stressor (30). Among combined stressors, drought plus heat, salinity plus heavy metal, cold plus light, and abiotic plus biotic stresses have been investigated frequently; however, limited research has considered cold plus drought stress in plants. Although current climate predictions warn of global warming and increased drought, the combination of cold and drought also severely affects plant survival in northern regions; for example, plants such as grapevine suffer from dry winters in North China (31). Together with its important effects on optimal plant growth, combined cold and drought stress is a key combined abiotic stress encountered by land plants, necessitating strategies to ensure plant survival. Since 2M appears to have evolved concurrently with early land plant evolution, the diversification of land plants may have been achieved, at least in part, with the aid of 2M to protect plants from cold, dry terrestrial environments.

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AUTHORSHIP

Hye-Jung Lee carried out the experiments. Kyoungwhan Back designed, advised and wrote the article.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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