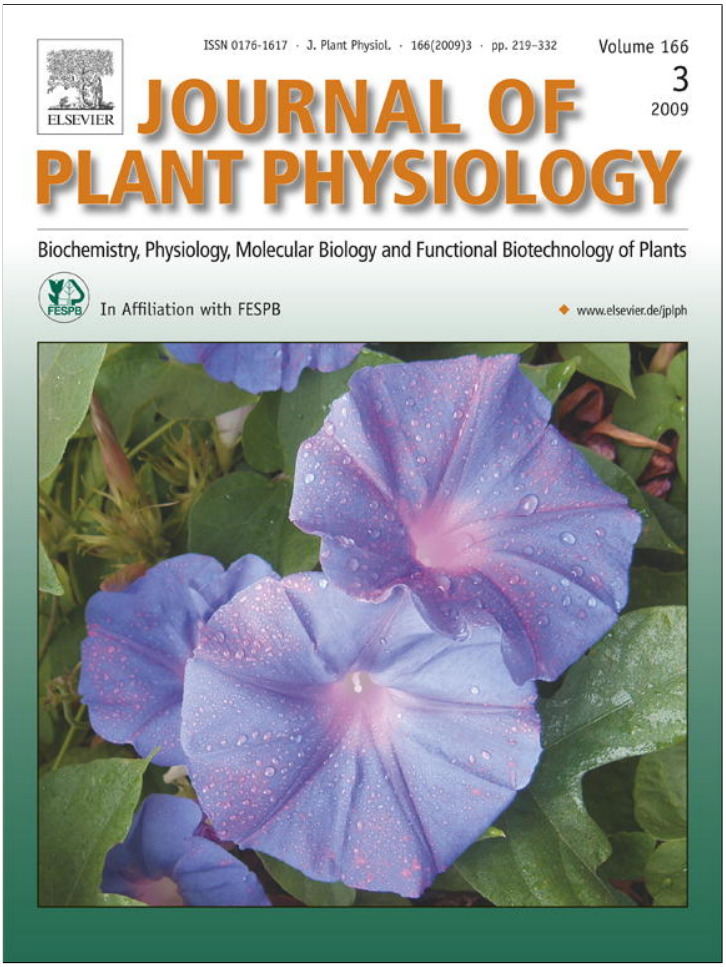

Journal of Plant Physiology **166** (2009) 324—328

Available online at www.sciencedirect.com

ScienceDirect

JOURNAL OF PLANT PHYSIOLOGY

www.elsevier.de/jplph

SHORT COMMUNICATION

Exogenously applied melatonin stimulates root growth and raises endogenous indoleacetic acid in roots of etiolated seedlings of *Brassica juncea*

Qian Chen[a,b], Wen-bo Qi[c], Russel J. Reiter[c], Wei Wei[a,*], Bao-min Wang[d]

[a]*State Key Laboratory of Vegetation and Environmental Changes, Institute of Botany, Chinese Academy of Sciences, 20 Nanxincun, Xiangshan, Beijing 100093, PR China*
[b]*Graduate University of Chinese Academy of Sciences, Beijing 100049, China*
[c]*Health Science Center, University of Texas, San Antonio, TX 78284, USA*
[d]*College of Agriculture and Biotechnology, China Agricultural University, Beijing 100094, China*

Received 13 February 2008; received in revised form 4 June 2008; accepted 4 June 2008

KEYWORDS
Indole-3-acetic acid;
Melatonin;
Root growth;
Wild leaf mustard

Summary

Exogenous melatonin was applied to etiolated seedlings of wild leaf mustard (*Brassica juncea*) and the effect on root growth and endogenous indole-3-acetic acid (IAA) levels determined. The results show that 0.1 μM melatonin has a stimulatory effect on root growth, while 100 μM is inhibitory. Furthermore, the stimulatory effect was only detectable in young seedlings (2-d old). Older seedlings (4-d old) appear to be less susceptible to both the stimulatory and the inhibitory effect of melatonin. Exogenous application of 0.1 μM melatonin also raised the endogenous levels of free IAA in roots, while higher concentrations had no significant effect. The specific mechanism that causes exogenous melatonin to increase the amount of free IAA in roots, paired with a stimulation of root growth, remains to be uncovered.

Introduction

Melatonin, *N*-acetyl-5-methoxytryptamine, is a naturally occurring indoleamine found throughout the animal kingdom. In 1995, the first report on melatonin in edible plant material initiated extensive research aimed at revealing the function of melatonin in plants (Hattori et al., 1995). Subsequently, melatonin has been found in essentially every plant that has been examined (Kolář and Macháčková, 2005; Reiter et al., 2007), currently more than 100 species (see reviews by Hardeland et al., 2007; Chen et al. 2008), including many medicinal herbs (Chen et al., 2003). The melatonin

Abbreviations: AFMK, *N*′-acetyl-*N*′-formyl-5-methoxykynuramine; ciELISA, competitive indirect enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; IAA, indole-3-acetic acid.

*Corresponding author. Tel.: +86 10 62836275; fax: +86 10 82596146.
E-mail address: weiwei@ibcas.ac.cn (W. Wei).

0176-1617/$ - see front matter © 2008 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2008.06.002

content seems to be especially high in plants exposed to high doses of UV irradiation (Hardeland et al., 2007).

Besides the known antioxidant function of melatonin in vertebrates, additional effects in plants have been described, for example on flowering in *Chenopodium rubrum*, the viability of carrot (*Daucus carota*) cells under cold stress (Kolář et al., 2003; Lei et al., 2004) or the tolerance of the water hyacinth (*Eichhornia crassipes*) to toxic organic chemicals and heavy metals (Tan et al., 2007a); see also reviews by Arnao and Hernández-Ruiz (2006) and Hardeland et al. (2007).

As in animals, the precursor of melatonin in plants is tryptophan, which also is the precursor of the auxin indole-3-acetic acid (IAA) (Murch et al., 2000; Arnao and Hernández-Ruiz, 2006). As an indoleamine, melatonin may be able to interfere with typical auxin functions such as the regulation of morphogenesis *in vitro*, promotion of coleoptile growth and inhibition of root elongation. In St. John's wort (*Hypericum perforatum*), a specific ratio of endogenous serotonin and melatonin regulates morphogenesis *in vitro* and melatonin increases *de novo* root formation (Murch et al., 2001). Similar to IAA, melatonin may stimulate growths in etiolated lupines (*Lupinus albus*) and coleoptiles of canary grass (*Phalaris canariensis*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and oats (*Avena sativa*), with a relative auxinic activity of 10–55% (Hernández-Ruiz et al., 2005).

Based on the functional relationship between melatonin and IAA as reported in previous studies (Hernández-Ruiz et al., 2004, 2005; Arnao and Hernández-Ruiz, 2006), we hypothesized that exogenous melatonin might cause changes in the concentration of endogenous free IAA. In this investigation, while monitoring the effect of exogenous melatonin on root growth of wild leaf mustard (*Brassica juncea*), IAA was measured by competitive indirect enzyme-linked immunosorbent assay (ciELISA) in roots after exposure to melatonin.

Materials and methods

Materials

Seeds of wild leaf mustard (*B. juncea* (L.) Czern.) were sterilized in 3% hypochlorous acid for 30 min, dipped in distilled water and grown on wet filter paper at 24 °C in darkness for 2 or 4 d. Melatonin (*N*-acetyl-5-methoxytryptamine) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA).

Root growth assay

Two or 4-d-old etiolated seedlings were incubated for 24 h at 24 °C in darkness in Petri dishes on filter paper with 3 mL melatonin solution of 100, 10, 1, 0.1 μM in 0.02% DMSO. Controls contained distilled water (C1) and 0.02% DMSO (C2). A line was drawn on each filter paper and the root tips of the etiolated seedling brought into line at the start of the experiment. The elongation of roots was observed using a binocular microscope (Olympus SZX12-DP70) with 10-fold magnification; photographs were taken after 24 h. The images were analyzed using National Institutes of Health Image J 1.37v software (Abramoff et al., 2004). Each of the 12 treatments consisted of three dishes with four seedlings per dish. Experiments were repeated three times. The root growth of all seedlings under all experimental conditions appeared normal. No morphological deviations could be observed.

Measurement of endogenous free IAA

Only roots of 2-d-old seedlings were analyzed for free IAA. In a first experiment, 100, 10, 1, 0.1 μM melatonin was applied (as described for the root growth assay) and the assay repeated twice. In a second experiment, the melatonin concentration was lowered to 0.01, 0.05, 0.1, 0.2 and 0.5 μM. This second assay was conducted only once. Each treatment had three replicates in both assays.

Approximately 0.15 g of roots, harvested from 35 seedlings per treatment, was cut and frozen in liquid nitrogen. At the time of assay, roots were crushed using mortar and pestle and extracted with 4 mL 80% methanol containing 1% 2, 6-di-tert-butyl-4-methylphenol. After 12 h at 4 °C, the extraction mixture was centrifuged at 4000 rpm for 10 min. The supernatant was isolated using solid phase extraction (SPE) with AccuBond C18SPE cartridge (Agilent technologies Inc., USA), and then dried using nitrogen gas. The residue was used for the subsequent ciELISA (Zhang et al., 1991). The reaction product was measured with a Multiskan Mk3 (Thermo, USA) at 490 nm, and the IAA concentration was estimated by the regressive equations between the optical density and the standard IAA concentration. The final concentration of IAA is given as mean of the three replicated samples per each treatment.

Statistical analysis

The SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. For the root growth assay, the four seedlings per Petri dish were considered a sample and ANOVA as well as post-hoc multiple comparison tests (LSD) were conducted on the data of root growth to examine the difference among the groups. ANOVA and multiple comparisons (LSD) were also performed on the IAA data. The data were transformed by logarithm where appropriate to meet the homogenous variance request.

Results

Effect of exogenous melatonin on root elongation

There was variation among the three replicates ($F_{2,10} = 34.330$, $p = 0.000$). A significant difference was found in the effect of different concentrations of melatonin ($F_{5,206} = 21.555$, $p = 0.000$) on the root growth of 2-d-old etiolated seedlings. In both experiments, different concentrations of melatonin had different effects on root growth ($F_{5,64} = 5.941$, $p = 0.000$ for the first assay; $F_{5,64} = 6.692$, $p = 0.000$ for the second assay; $F_{5,64} = 3.063$, $p = 0.015$ for the third assay). Treatment with 0.1 μM melatonin increased root elongation, while 100 μM melatonin had a significant inhibitory effect (Figure 1A).

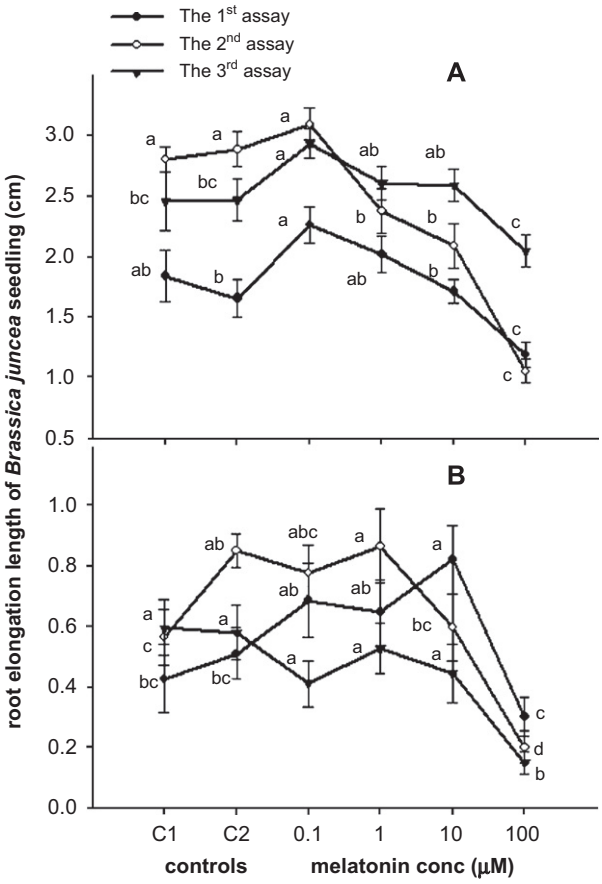

Figure 1. Effect of melatonin on root elongation in 2-d-old (A) and 4-d-old (B) seedlings of wild leaf mustard. The controls, C1 and C2, were grown in distilled water and distilled water with 0.02% DMSO, respectively. Bars represent standard errors of the mean. Different superscripts represent statistically significant differences at $P<0.05$ for each treatment.

For 4-d-old seedlings, a significant difference was found among different assays ($F_{2,10} = 6.099$, $p = 0.003$). While significantly different root growth was observed at various melatonin concentrations ($F_{5,203} = 9.912$, $p = 0.000$), treatment with melatonin had no positive effect on root growth. At 100 μM melatonin, root growth was strongly inhibited (Figure 1B). Significant differences in root growth were found among the various melatonin treatments in both experiments ($F_{5,63} = 3.380$, $p = 0.009$ for the first assay; $F_{5,63} = 7.515$, $p = 0.000$ for the second assay; $F_{5,63} = 4.119$, $p = 0.003$ for the third assay).

Effect of exogenous melatonin on endogenous IAA levels

The ciELISA data reveals that differences in IAA concentrations among the treatments were significant in the first test ($F_5 = 15.004$, $p = 0.000$) but not in the repeat. However, the overall difference in IAA levels after various melatonin treatments (0.1–100 μM) is significant ($F_{5,29} = 3.863$, $p = 0.008$) even considering the differences between the two tests ($F_{1,5} = 9.388$, $p = 0.005$). We established that 0.1 μM melatonin led to an increase in free IAA, while no change was observed at higher melatonin concentrations (Figure 2A).

In the second experiment, applying melatonin from 0.01 to 0.5 μM, IAA levels exhibited significant differences between treatments ($F_6 = 13.770$, $p = 0.000$). An obvious increase in IAA was observed at 0.01 and 0.05 μM melatonin, and the IAA levels were at the highest at 0.1 and 0.2 μM melatonin. The IAA content decreased when melatonin was increased to 0.5 μM but remained significantly higher than in controls (Figure 2B).

Discussion

Herein, we report for the first time that exogenously applied melatonin increases root growth in 2-d-old wild leaf mustard at low concentrations but inhibits root growth, as previously reported, at higher concentrations (see a review by Arnao and Hernández-Ruiz, 2006). In 4-d-old seedlings, root elongation was inhibited at 100 μM melatonin and no significant promotional effect on root growth at low melatonin concentrations could be observed. Thus, the sensitivity of the roots to melatonin treatments varied with growth stage. It has been shown that primary root length of many crucifer species reaches its maximum after 4 d of seed germination in Petri dishes (Huang

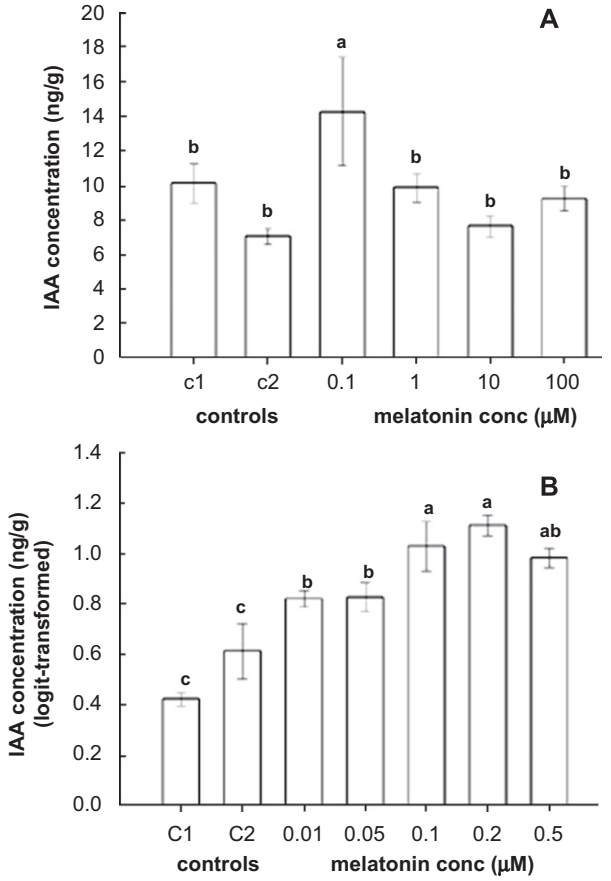

Figure 2. Free indoleacetic acid (IAA) in roots of 2-d-old seedlings of wild leaf mustard following treatment with different concentrations of melatonin. (A) IAA contents at different melatonin concentrations (0.1–100 μM). (B) Logit-transformed IAA contents at low melatonin concentrations (0.01–0.5 μM). Bars represent standard errors of the mean. The different superscripts represent statistically significant differences at $P<0.05$ for each treatment.

et al., 2002). In the case of wild leaf mustard, 2-d-old roots seem to possess maximum growth vigor. A promotional effect of melatonin on 4-d-old roots was clearly not obvious when compared to the response of 2-d-old roots to low melatonin concentrations.

A previous study showed that melatonin inhibited root elongation in some monocots even at very low concentrations. For canary grass and oats, 0.01 μM melatonin inhibited root growth (Hernández-Ruiz et al., 2005). In our study, the maximum inhibitory effect of melatonin on wild leaf mustard roots occurred at 100 μM, which is considerably higher than that for canary grass and oat. Although there are no accurate measurements of the melatonin concentrations in wild leaf mustard, the melatonin levels in two closely related *Brassica* species are much higher than the levels reported in canary grass and oats (Manchester et al., 2000; Hernández-Ruiz et al., 2005). These marked differences in endogenous melatonin concentrations may be related to the differential effect of exogenous melatonin on root growth. This possibility is supported by observations in wheat (Hernández-Ruiz et al., 2005), which contains similar melatonin concentration as found in several members of the genus *Brassica*.

Melatonin has the same precursor and similar physiological functions as IAA. The latter molecule also has bimodal effects on plant growth, i.e., it promotes growth at low concentrations and inhibits growth at high concentrations. In our experiments, 0.1 μM melatonin had the maximal positive effect on root elongation of 2-d-old seedlings. Endogenous free IAA levels also increased at low melatonin concentrations (0.1 and 0.2 μM). It is thus possible that stimulation of root growth by low concentrations of melatonin is actually triggered by melatonin-stimulated IAA synthesis. At high melatonin concentrations, however, IAA was not significantly increased, while root elongation was strongly inhibited. Thus, melatonin's suppressive effect on root growth seems to involve mechanisms not related to IAA, at least in the wild leaf mustard. The specific relationship between these two plant hormones remains unknown.

Melatonin has a high affinity to Ca^{2+}-activated calmodulin (CaM), which inhibits intercellular Ca^{2+}/CaM-dependent functions (Hardeland, 1997; Hardeland et al., 2007). It has been shown that CaM inhibitors reduce root growth and that this inhibitory action can be counteracted by the addition of exogenous CaM (Xing et al., 1998). Thus, the negative effect of high concentrations of melatonin on root elongation may be a result of melatonin-related changes in CaM antagonism.

Cell elongation is a key process in root growth. Reactive oxygen species (ROS), including the hydroxyl radical, reportedly activate Ca^{2+} channels allowing Ca^{2+} influx, which enhances cell elongation (Foreman et al., 2003). Another study indicated that the hydroxyl radical makes cell membranes more permeable, also leading to enhanced cell elongation (Schopfer, 2001). Moreover, one of the metabolites formed when melatonin scavenges radicals is N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) (Tan et al., 2007b), which likely functions as an antioxidant (Tan et al., 2007a). Thus, high concentrations of melatonin and AFMK might reduce ROS in root cells and thereby inhibit ROS-induced cell growth (Afreen et al., 2006). This might explain that melatonin at higher concentrations reduces root growth in wild leaf mustard without altering the

synthesis of IAA. The exact mechanism of the inhibitory effect of melatonin on root elongation requires further investigation.

Acknowledgments

This work was supported by a project of the Natural Science Foundation of China (Grant no. 30670316). We appreciate three anonymous reviewers and the editors whose comments help to improve the quality of this paper.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jplph.2008.06.002.

References

Abramoff MD, Magelhaes PJ, Ram SJ. Biophoton Int 2004; 11:36–42.
Afreen F, Zobayed SMA, Kozai T. J Pineal Res 2006;41: 108–15.
Arnao MB, Hernández-Ruiz J. Plant Signal Behav 2006; 1:89–95.
Chen G-F, Huo Y-S, Tan D-X, Liang Z, Zhang W-B, Zhang Y-K. Life Sci 2003;73:19–26.
Chen Q, Qi W-B, Li M, Wei W. Chin J Appl Environ Biol 2008;14:126–31.
Foreman J, Demidchik V, Bothwell JHF, Mylona P, Miedema H, Angel Torres MA, et al. Nature 2003; 422:442–6.
Hardeland R. Int J Biometeorol 1997;41:47–57.
Hardeland R, Pandi-Perumal SR, Poeggeler B. Funct Plant Sci Biotechnol 2007;1:32–45.
Hattori A, Migitaka H, Iigo M, Itoh M, Yamamoto K, Ohtani-Kaneko R, et al. Biochem Mol Biol Int 1995; 35:627–34.
Hernández-Ruiz J, Cano A, Arnao MB. Planta 2004;220: 140–4.
Hernández-Ruiz J, Cano A, Arnao MB. J Pineal Res 2005; 39:137–42.
Huang B, Liu X, Xue X, Chang L. Plant Breed 2002; 121:360–2.
Kolář J, Macháčková I. J Pineal Res 2005;39:333–41.
Kolář J, Johnson CH, Macháčková I. Physiol Plant 2003; 118:605–12.
Lei X-Y, Zhu R-Y, Zhang G-Y, Dai Y-R. J Pineal Res 2004;36: 126–31.
Manchester LC, Tan DX, Reiter RJ, Park W, Monis K, Qi W. Life Sci 2000;67:3023–9.
Murch SJ, KrishnaRaj S, Saxena PK. Plant Cell Rep 2000;19:698–704.
Murch SJ, Campbell SB, Saxena PK. In Vitro Cell Dev Biol Plant 2001;37:786–93.
Reiter RJ, Tan DX, Manchester LC, Simopoulos A, Maldonado MU, Flores LJ, et al. World Rev Nutr Diet 2007;97:211–30.
Schopfer P. Plant J 2001;28:679–88.
Tan D-X, Manchester LC, Mascio PD, Martinez GR, Prado FM, Reiter RJ. FASEB J 2007a;21:1724–9.
Tan D-X, Manchester LC, Terron MP, Flores LJ, Russel J, Reiter RJ. J Pineal Res 2007b;42:28–42.
Xing S-P, Li X-G, Zhang X-S, Yang Y-F. Chin Bull Bot 1998;2:41–5.
Zhang J, Han B-W, Wu Q, He Z-P. J Chin Agri Univ 1991; 17(S1):139–43.