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Examination of the auxin hypothesis of phytomelatonin action in classical auxin assay systems in maize

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ABSTRACT

Melatonin has been found in a wide range of plant groups. Its physiological roles have been suggested to be diverse in stress protection and plant growth regulation. An attractive hypothesis is that phytomelatonin acts as an auxin to regulate plant development. However, the auxin hypothesis is controversial, since both supporting and contradictory evidence has been reported. We systematically investigated whether melatonin fulfilled the definition for auxin in maize (*Zea mays*). Melatonin did not affect coleoptile elongation, root growth or ACC synthase gene expression, contrary to 10^{-5} M IAA in our assay system. The auxin hypothesis of phytomelatonin action is not supported in maize, because melatonin appeared inactive in all of the auxin activity tests. On the other hand, melatonin was active in the protection of maize growth against salt stress, suggesting its importance in another context.

1. Introduction

Melatonin has been suggested as a versatile signal molecule found in diverse organisms including animals, fungi, mosses and plants (Reiter et al., 2013). Melatonin is found ubiquitously, both in monocot and dicot plants, from diverse phylogenetic groups, suggesting its physiological roles in plants (Paredes et al., 2009; Posmyk and Jana, 2009). The term 'phytomelatonin' was introduced to describe plant-derived melatonin (Blask et al., 2004). Melatonin is distributed in diverse plant organs, including leaves, fruits and seeds (van Tassel et al., 2001; Paredes et al., 2009). In some medicinal plants the level of melatonin is high enough to affect the physiology of herbivores (Chen et al., 2003; Murch and Saxena, 2006). Biosynthetic pathways of melatonin have been suggested to start with tryptophan (Murch et al., 2000). The identification of genes and activity of biosynthetic enzymes supports the concept of melatonin biosynthesis in plants (Facchini et al., 2000; Kang et al., 2007), although the entire pathway in a single plant species has not yet been reported.

The wide distribution of melatonin in the plant kingdom has called attention to its physiological roles. Daily rhythm-related function has been suggested based on the daily fluctuations in melatonin levels (Kolář et al., 1997; Tan et al., 2007). Melatonin is involved in plant responses to both abiotic and biotic stresses. Exogenously applied or endogenously induced melatonin modulates plant responses to cold (Bajwa et al., 2014), salt (Mukherjee et al., 2014) and oxidative (Tan et al., 2002) stresses. Changes in the level of melatonin during flower (Murch and Saxena, 2002) and fruit (Murch et al., 2010) development suggest its specific regulatory roles for plant development. An attractive hypothesis is that melatonin has auxin-like functions in plant growth regulation (Arnao and Hernández-Ruiz, 2006).

Auxin is a growth hormone and a representative developmental regulator in plants (Taiz and Zeiger, 2006). After Darwin and Darwin (1880) suggested the existence of a certain growth-promoting ingredient, its chemical nature was subsequently identified (Went, 1926). Kögl and Haagen Smit (1933) introduced the term "auxin," meaning "to grow" in Greek. Later "auxin" was used as the collective name for several natural and artificial chemical substances that primarily promote cell elongation, similar to indole-3-acetic acid (IAA) (Tukey et al., 1954). Any chemical with such activities can thus be regarded as an auxin.

Contrary to its growth-promoting action in the shoot system, auxin generally inhibits the growth of pre-existing roots (Thimann, 1936). Other hormones, such as gibberellins, stimulate the root growth as well as the shoot elongation. Thus, the concept of root growth inhibition was added to the auxin definition to distinguish it from other hormones (Thimann, 1969).

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Another typical auxin activity is to induce ethylene biosynthesis (Abeles, 1966). Ethylene is another plant hormone synthesized from AdoMet *via* ACC (1-aminocyclopropane-1-carboxylic acid). Auxin promotes ethylene biosynthesis by inducing ACC synthase gene expression, a key enzyme in ethylene production (Yi et al., 1999).

Auxin activities can be evaluated by measuring the stimulation of shoot elongation, inhibition of root growth and induction of ethylene biosynthesis. Therefore, it is possible to determine whether melatonin primarily acts as an auxin by evaluating the three activities described above.

There have been several reports leading to the auxin hypothesis to explain the melatonin function in plants. Hernández-Ruiz et al. (2004) showed dose-response curves of melatonin-induced lupin hypocotyl growth, supporting the auxin hypothesis of phytomelatonin action. Hernández-Ruiz et al. (2005) additionally observed similar growth-stimulating effects of melatonin in monocot plants. Furthermore, they reported the inhibitory effects of melatonin on root growth, fulfilling the second requirement for the definition of auxin. It was also observed that melatonin promoted root regeneration in de-rooted lupin hypocotyls (Arnao and Hernández-Ruiz, 2007). Because auxin stimulates the formation of new roots (Taiz and Zeiger, 2006), although it inhibits the growth of pre-existing roots, the finding appeared to support the auxin hypothesis of plant melatonin action (Arnao and Hernández-Ruiz, 2007). However, it was recently reported that melatonin stimulated lateral- and adventitious root formation independent of auxin signaling (Pelagio-Flores et al., 2012).

Some reports have suggested and supported the auxin hypothesis of melatonin action in plants after plant growth-promoting effects of melatonin were reported. However, several recent lines of evidence are not compatible with the auxin hypothesis. GUS gene under the regulation of auxin-responsive DR5 was not induced by melatonin and auxin-degradable AUX/IAA was preserved in the presence of melatonin (Pelagio-Flores et al., 2012). During careful reading of past work, we realized that preexisting documentation for the auxin hypothesis of phytomelatonin action contained only partial evidence for the definition of auxin. Therefore, it was necessary to determine whether melatonin meets all the requirements of the definition. We examined the auxin hypothesis of melatonin action in classical auxin assay systems using maize (*Zea mays*). We carried out a standard elongation assay with coleoptiles segments, root inhibition assay with young seedlings and ACC synthase induction test. To determine whether melatonin is really active in maize, we investigated the influence of melatonin on salt stress responses as well.

2. Materials and methods

2.1. Standard elongation assay

Kernels of maize (*Zea mays* L.) were soaked in distilled water on a shaker (30 cyles/min), spread on wet vermiculite prepared in a plastic box (410 mm × 297 mm × 182 mm), and kept for 3.5 days in a growth chamber at 26 °C in the dark. The coleoptiles were harvested and cut with 5-mm spaced twin razor blades at 5 mm from the tips. The isolated 5-mm coleoptile segments were floated on distilled water for 30 min to stabilize the spontaneous elongation and randomly mix the samples. Growth medium (10 ml, 5 mM potassium phosphate buffer, pH 6.8, supplemented when needed with melatonin and/or IAA) was prepared in 100-ml beakers. Methanol at a final concentration of 0.1% was used to dissolve IAA or melatonin. The same concentration (0.1%) of methanol was supplemented in the control medium. The coleoptile segments floated on distilled water were randomly selected and distributed to each beaker (10 segments). The length of 10 segments was measured to determine the initial value before distributing the segments. The prepared beakers were set in a covered plastic box that was wet with distilled water to saturate water vapor. The entire plastic box was placed on a shaker and incubated for 18 h. The total length was measured in 10 segments, after penetrating the hollow cylindrical segments with a 150 mm long injection needle. Statistical significance was examined using the Student's *t*-test.

2.2. Root growth test

Maize kernels were soaked in distilled water as described above, and grown in a paper roll system (Hetz et al., 1996). Paper towels were unrolled and prepared as a thin and long rectangle by half-folding (820 mm × 118 mm). Ten of the soaked maize kernels were then placed 1 cm below a long side of the paper towel rectangle, at an interval of 12 cm. The paper towels with maize kernels were rolled up and set vertically, orienting the maize-containing side upward in plastic culture vessels (65 mm × 65 mm × 100 mm, Sigma). Before the paper rolls were set up, 60 ml of potassium phosphate buffer-based growth medium (5 mM, pH 6.8) was prepared in the culture vessels. Melatonin and IAA was supplemented according to the experimental aim. The planted seedlings were grown at 26 °C for 3 d, and the primary root length was measured.

2.3. Examination of ACC synthase expression by RT-PCR and real-time PCR

Plant materials were prepared as described for the standard elongation assay. The incubated coleoptile segments were snap frozen with liquid nitrogen and pulverized with a mini-size homogenizer in Eppendorf tubes. Total RNA from the tissue powder was extracted with an RNA isolation kit (easy-BLUE™, iNtRON, Korea) following the supplier's directions. Reverse transcription of 1 μg RNA was performed with M-MLV (Promega, USA) using oligo-dT primers. The cDNA was used as a template in PCR reactions (35 cycles) to amplify the ACC Synthase 6 (ACS6) transcript in a thermocycler (Biometra, Germany) set to 60 °C for annealing. Actin transcripts were also amplified as internal controls and the ACS6 signals were normalized with the corresponding actin bands. PCR primers of the following nucleotide sequences were used for ACS6 (Geissler-Lee et al., 2009) and actin: ACS6 forward (AGCTGTGGAAGAAGGTGGTCTTCGAGGT), ACS6 reverse (AGTACGTGACCGTGGTTTCTATGA), Actin forward (ACCCAAAGGCTAACCGTGAG) and Actin reverse (TAGTCCAGGGCAATGTAGGC).

The level of transcripts was quantified by SYBR Green I-based real-time PCR with LghtCycler®Nano (Roche) using the same primer sets as used for the RT-PCR. Reaction mixture was prepared with 600 ng cDNA in a volume of 20 μl. For the quantitative PCR, 45 cycles (95 °C for 15 s, 66 °C for 15 s, 72 °C for 30 s) were run after holding at 95 °C for 600 s. Melting curve analysis was carried out in a temperature range between 60 °C and 95 °C with increase of 0.1 °C/s. Quantification data were obtained with LightCycler®Nano Software 1 (Roche).

2.4. Investigation of melatonin effects on the growth of salt-stressed maize

Plants were grown in paper rolls as for the root growth test. To induce salt stress 50, 100 or 150 mM NaCl was included in the paper rolls. The same NaCl concentration series were prepared in the presence or absence of 10^{-5} M melatonin to investigate the effect of melatonin on the salt stressed plants. After 3 days at 26 °C the length of coleoptiles and primary roots were measured. The statistical significance was examined using the Student's *t* -test.