

REVIEW PAPER

Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science

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Abstract

The presence of melatonin in plants is universal. Evidence has confirmed that a major portion of the melatonin is synthesized by plants themselves even though a homologue of the classic arylalkylamine *N*-acetyltransferase (AANAT) has not been identified as yet in plants. Thus, the serotonin *N*-acetylating enzyme in plants may differ greatly from the animal AANAT with regard to sequence and structure. This would imply multiple evolutionary origins of enzymes with these catalytic properties. A primary function of melatonin in plants is to serve as the first line of defence against internal and environmental oxidative stressors. The much higher melatonin levels in plants compared with those found in animals are thought to be a compensatory response by plants which lack means of mobility, unlike animals, as a means of coping with harsh environments. Importantly, remarkably high melatonin concentrations have been measured in popular beverages (coffee, tea, wine, and beer) and crops (corn, rice, wheat, barley, and oats). Billions of people worldwide consume these products daily. The beneficial effects of melatonin on human health derived from the consumption of these products must be considered. Evidence also indicates that melatonin has an ability to increase the production of crops. The mechanisms may involve the roles of melatonin in preservation of chlorophyll, promotion of photosynthesis, and stimulation of root development. Transgenic plants with enhanced melatonin content could probably lead to breakthroughs to increase crop production in agriculture and to improve the general health of humans.

Key words: Agriculture, antioxidant, free radical biology, melatonin, plant.

Introduction

Melatonin, a ubiquitously present, low molecular weight molecule in living organisms, has a simple structure; however, it exhibits pleiotropic biological activities in species from bacteria to mammals (Hardeland *et al.*, 2011). Some of these activities are receptor mediated, including via melatonin membrane receptors and nuclear receptors (Reppert, 1997; Dubocovich *et al.*, 1999; Dubocovich and Markowska, 2005; Imbesi *et al.*, 2009; Shiu *et al.*, 2010). Other functions are melatonin receptor independent (Reiter, 1996; Tan *et al.*, 2002) including the interactions of melatonin with reactive oxygen species (ROS) and those mediated by its bioactive metabolites (Silva *et al.*, 2004,

2005; Mayo *et al.*, 2005; Manda *et al.*, 2007, 2008; Hardeland *et al.*, 2009; Schaefer and Hardeland, 2009).

Previously, three melatonin membrane receptors were identified, originally denoted as Mel_{1a}, Mel_{1b}, and Mel_{1c}. The latter is only found in non-mammalian vertebrates. The first two, currently referred to as MT₁ (= MTNR1A, in gene sequence nomenclature) and MT₂ (= MTNR1B) are classified as high-affinity melatonin membrane receptors with *K_i* values in human tissue in the picomolar range (Hardeland, 2009). These membrane receptors are classic G protein-coupled proteins. The classic, first-discovered signalling pathway consists of an inhibition of adenylyl cyclase via the

G α_i subforms α_{i2} and α_{i3} that leads to a reduction of cAMP causing down-regulation of protein kinase A (PKA; Godson and Reppert, 1997; Dubocovich and Markowska, 2005). Meanwhile, various other pathways have been identified that involve different α subunits, $\beta\gamma$ heterodimers, and protein recruitment to receptor- β -arrestin complexes. These additional pathways include phospholipase C activation, changes in cGMP, stimulation of Ca²⁺-dependent, mitogen-activated protein (MAP) kinase or Akt/PKB routes as well as the control of Ca²⁺ and K⁺ channel subtypes (summarized in Hardeland (2009)). MT₁ and MT₂ are widely distributed in the central nervous system (Hunt *et al.*, 2001; Jin *et al.*, 2003; Unfried *et al.*, 2010) and also in peripheral tissues (Viswanathan *et al.*, 1990, 1997; Pang *et al.*, 1993a, b).

Identification of the central functions of these two receptors in circadian synchronization, reproductive regulation, and immuno-responsiveness are still huge challenges for scientists. In peripheral tissues, especially in small blood vessels, these two high-affinity membrane melatonin receptors seem to exhibit physiological antagonistic activities (Doolen *et al.*, 1998; Masana *et al.*, 2002). For example, activation of one of these receptors in small blood vessels of the skin or extremities results in dilation (Aoki *et al.*, 2006, 2008; Krauchi *et al.*, 2006); however, stimulation of the other receptor results in contraction of the blood vessel (Viswanathan *et al.*, 1997; Aarseth *et al.*, 2001; Regrigny *et al.*, 2001). Such seemingly contradictory findings are likely to be the expression of multiple signalling pathways of melatonin exceeding by far the original observations which were based only on decreases in cAMP. Thus, an exclusive cAMP-suppressing action could hardly explain the numerous activating actions of melatonin (see Hardeland *et al.* (2011)). The presence of different signalling pathways may be of importance for studies in organisms other than animals, if functionally related membrane receptors should be discovered there.

In mammals, a third binding site was originally believed to represent another membrane receptor and was introduced into the literature as MT₃. However, this protein turned out to be an enzyme, quinone reductase 2 (NQO₂) (Nosjean *et al.*, 2000; Mailliet *et al.*, 2004; Vella *et al.*, 2005; Boutin, 2007). Although specific ligands have been developed and several effects have been ascribed to this binding site, the importance of melatonin binding to this enzyme should be judged with caution since no signalling pathways are known to originate from this 'receptor' or enzyme (Hardeland *et al.*, 2011).

Nuclear melatonin receptors belong to the retinoid-related orphan receptor (ROR) family within the retinoic acid receptor superfamily. Melatonin binding has been described for three splice variants of ROR α (isoforms a, b, and d) and for ROR β (RZR β), but does not occur with ROR α isoform c (Carlberg, 1995 and Wiesenberg *et al.*, 1995; Wiesenberg *et al.*, 1995, 1998; Carlberg, 2000). The biological actions mediated by these receptors are still being debated. The accumulated evidence indicates that the nuclear melatonin receptors are genuine, and their activation as well as the subsequent biological effects may involve

circadian rhythm synchronization (Agez *et al.*, 2007) and immune enhancement in vertebrates (Guerrero *et al.*, 2000; Pozo *et al.*, 2004). Although retinoids are also produced by plants, though some effects of retinoic acid have been described in corn (Cho *et al.*, 1993), and despite their structural similarity to the phytohormone abscisic acid (Xie *et al.*, 2005), no direct evidence has been published for melatonin binding to this category of receptors, their orthologues or paralogues in plants. A suppression of flowering was obtained with both melatonin and a putative pharmacological ROR agonist, CGP 52608 (Kolar *et al.*, 1999b). However, far-reaching conclusions should not be based solely on an observation of inhibition.

In vertebrates, melatonin also binds to two ubiquitous proteins of utmost biological significance, calmodulin (CaM) (Benitez-King *et al.*, 1993), especially in the CaM-CaM kinase II complex (Landau and Zisapel, 2007), and calreticulin (CRT) (Macias *et al.*, 2003). The role of melatonin binding to the endoplasmic reticulum (ER) stress protein and chaperone CRT is not entirely clear. However, modulation of CaM and, in particular, CaM kinase II activity may have a plethora of effects in countless cell types (Colbran, 2004). In plants, both CRT (Jia *et al.*, 2009; Del Bem, 2011) and CaM (DeFalco *et al.*, 2010) are also present in several subforms, but interactions with melatonin have not been tested to date. Compared with mammals, sometimes much higher melatonin concentrations are present in plant organs (Chen *et al.*, 2003; Hardeland *et al.*, 2007; Murch *et al.*, 2009). This is of great interest. Moreover, binding to CaM might explain some cytoskeletal effects of melatonin in plant cells, as will be discussed below.

Melatonin is an amphiphilic molecule (Shida *et al.*, 1994; Ceraulo *et al.*, 1999), which can freely cross cell membranes and distribute to any aqueous compartment including the cytosol, nucleus, and mitochondria (Menendez-Pelaez *et al.*, 1993; Acuna-Castroviejo *et al.*, 2001, 2003). In plants, kelps, and other algae, the distribution of melatonin between cytoplasm/symplast, vacuoles, and the apoplast has been discussed (Hardeland, 1999; Hardeland and Poeggeler, 2003; Hardeland *et al.*, 2007), but specific details have not yet been elaborated. The amphiphilicity indicates that membrane receptors are not necessarily required for mediating all the functions of melatonin. Actually, non-receptor-mediated actions of melatonin have been identified. One of them is its free radical scavenging and antioxidant capacity (Tan *et al.*, 1993; Reiter *et al.*, 2008, 2010b; Romero *et al.*, 2010; Hibaoui *et al.*, 2009; Um and Kwon, 2010; SC Xu *et al.*, 2010). Melatonin effectively scavenges a variety of ROS and reactive nitrogen species (RNS), and protects cells, tissues, and organisms from oxidative stress (Tan *et al.*, 2002, 2003, 2007c; Jung *et al.*, 2010; Kaur *et al.*, 2010; Laothong *et al.*, 2010; Nopparat *et al.*, 2010). Since the formation of ROS/RNS and their metabolism are basic mechanisms related to cellular biology and physiopathology in all aerobic organisms, it is deduced that the initial function of melatonin in organisms was to serve as an antioxidant, with the other functions being acquired during evolution (Hardeland *et al.*, 1995; Tan *et al.*, 2010a). The

receptor-dependent and receptor-independent activities of melatonin are attributable to its highly pleiotropic nature.

Making the melatonin story even more complicated is the unconventional metabolism of melatonin. Melatonin can be enzymatically, pseudoenzymatically, or non-enzymatically transformed to several biologically active metabolites including 5-methoxytryptamine (5-MT), cyclic 3-hydroxymelatonin (c3OH M), *N*¹-acetyl-*N*²-formyl-5-methoxykynuramine (AFMK), and *N*¹-acetyl-5-methoxykynuramine (AMK) (Hardeland *et al.*, 2009; Tan *et al.*, 2010a). Several enzymes including indoleamine 2,3-dioxygenase (IDO), eosinophil peroxidase (EPO), horseradish peroxidase (HRP), myeloperoxidase (MPO), cytochrome P₄₅₀ (CYP) subforms, and NQR₂ can catalyze degradation of melatonin to AFMK. Further details and additional routes of AFMK and AMK formation are summarized in another report (Hardeland *et al.*, 2009). CYPs alternately metabolize melatonin to 6-hydroxymelatonin, *N*-acetylserotonin, or AFMK (Tan *et al.*, 2010a). Several biological activities of melatonin are believed to be mediated by its metabolites (Mayo *et al.*, 2005; Rosen *et al.*, 2006; Manda *et al.*, 2007; Tan *et al.*, 2007c; Hardeland *et al.*, 2009; Schaefer and Hardeland, 2009; Kuesel *et al.*, 2010). The multiple active metabolites of melatonin greatly expand the functional spectrum of this ubiquitously acting indoleamine.

The majority of observations summarized above are from animal studies or from cultured animal cells. In recent years, the number of reports of melatonin in plants has dramatically increased (Paredes *et al.*, 2009; Posmyk and Janas, 2009) and it is anticipated that additional mechanistic studies related to plant melatonin will appear in the near future. The major questions that remain unanswered are, what nutritional role does melatonin play in human health when plants containing substantial amounts of melatonin are consumed? Also, does melatonin share similar functions in plants and animals, particularly in relation to its antioxidant capacity? If so, would the beneficial effects of melatonin in plants promote their acclimation to harsh environments and would high plant melatonin levels increase production of crops in agriculture? These issues will be discussed relative to the currently available literature and based on emerging considerations.

Events leading to the discovery of melatonin in plants

Melatonin was originally identified in the pineal gland of cows and shown to cause melanosome concentration in amphibian and fish melanophores (Lerner *et al.*, 1958). Thereafter, melatonin was portrayed exclusively as an animal hormone, specifically a neurohormone (Reiter, 1991), for nearly four decades. During this period, with a very few exceptions, all research related to melatonin was performed either in animals or in tissues and cells of animals. However, some early observations were also made on cytoskeletal effects of melatonin in plants, for example in endosperm cells of the amaryllidacean *Scadoxus multiflorus* (syn. *Haemanthus katherinae*) (Jackson, 1969) and in epidermal cells of *Allium cepa* (Banerjee and Margulis, 1973). Changes were observed in mitotic spindles, which

could lead to mitotic arrest. Later findings on growth inhibition in the pheophycean kelp *Pterygophora californica* (Fuhrberg *et al.*, 1996) may be explained on this basis.

In the mid to late 1970s, melatonin was removed as a by-product of the decaffeination process of coffee beans. This was presumably the first indication that plants or plant products contain melatonin. At that time, it was not imagined that melatonin existed in plants. As a consequence of the decaffeination process, it was presumed that melatonin was a result of an unexpected chemical reaction. This episode, however, had little impact on the subsequent discovery of melatonin in plants.

The systematic search for melatonin in photoautotrophs was initiated in 1987, when members of Hardeland's group screened effects of biogenic amines in unicellular organisms, in particular, the bioluminescent dinoflagellates *Pyrocystis acuta* and *Lingulodinium polyedrum* (syn. *Gonyaulax polyedra*). Fischer and co-workers (Fischer, 1987; Poeggeler *et al.*, 1989; Fischer and Hardeland, 1994) observed phase shifts by melatonin in the overt circadian rhythm of bioluminescence in *P. acuta*. Balzer *et al.* found, in *L. polyedrum*, strong stimulations of bioluminescence by the melatonin metabolite 5-methoxytryptamine and the melatonin analogue *N,N*-dimethyl-5-methoxytryptamine (Balzer *et al.*, 1989, 1990; Poeggeler *et al.*, 1989; Balzer and Hardeland, 1991b) as well as effects of melatonin and other methoxylated indoleamines on the induction of a resting stage (Balzer and Hardeland, 1991a). She also measured, in this organism, serotonin *N*-acetylation and *N*-acetylserotonin *O*-methylation, namely the steps of melatonin biosynthesis (Balzer *et al.*, 1990). Behrmann and colleagues (Behrmann, 1989; Hardeland *et al.*, 1995) discovered the photocatalytic conversion of melatonin to AFMK by protein-free extracts from *L. polyedrum*. These findings and other details which would exceed the scope of this review gave rise to the detection of melatonin in photoautotroph dinoflagellates, mainly achieved by Poeggeler *et al.* (Balzer *et al.*, 1989; Poeggeler *et al.*, 1989).

The circadian rhythm of melatonin concentration, detected in *L. polyedrum*, represents the first demonstration outside the animals and in a photoautotroph (Poeggeler *et al.*, 1991). The rhythm was also shown to persist in constant darkness (Balzer *et al.*, 1993) and to be strongly enhanced with regard to average level and amplitude by a decrease in temperature from 20 °C to 15 °C (Fuhrberg *et al.*, 1997). In the following years, many more details on concentrations, actions, and metabolism were obtained in *L. polyedrum*, numerous other dinoflagellates, euglenoids, phaeophyceans, rhodophyceans, and chlorophyceans, to mention only the taxa containing photoautotrophs. Various actions of melatonin in these groups are summarized elsewhere (Hardeland, 1999).

These observations had not been expected by many melatonin scientists. Indeed, it seemed rather difficult to believe that a 'neurohormone', melatonin, would exist in neuron-free unicellular organisms and kelps. However, this observation stimulated experimentalists to consider the possibility of the presence of melatonin in non-vertebrates.

In the last half of 1992, another unusual observation was made when it was found that melatonin, mainly perceived at that time as a neurohormone, is a potent free radical scavenger and antioxidant; the resulting paper was published in 1993 (Tan *et al.*, 1993).

Antioxidative protection in cells and tissues represents a highly conserved function in all aerobic organisms including plants. Stimulated by this role, Manchester and co-workers tested a primitive photosynthetic bacteria (*Rhodospirillum rubrum*) for the presence of melatonin in 1994. They showed that not only was melatonin present in *R. rubrum* but, like some algae, this organism had the capacity to produce melatonin, with peak levels during darkness and a lower concentrations during the day. This observation was published in 1995 (Manchester *et al.*, 1995). Later, similar findings were obtained in another photosynthetic bacterium, *Erythrobacter longus* (Tilden *et al.*, 1997). Temporal fluctuations, but no robust rhythmicity of melatonin, were detected in *Escherichia coli* (i.e. a heterotroph bacterium) (Balzer *et al.*, 2000). Bacteria probably utilize melatonin to protect against oxidative stress, as far as they live under aerobic conditions.

Thus, it was natural to speculate that if melatonin is present in the photosynthetic unicellular organisms and also in animals as an antioxidant, there is, from an evolutionary point of view, no reason to doubt that melatonin would be present in green plants.

Two abstracts, one of which appeared in May 1993 (Van Tassel *et al.*, 1993) and the other in June 1995 (Van Tassel *et al.*, 1995), authored by Van Tassel and colleagues, claimed that melatonin was detected in a plant, *Pharbitis nil* (Japanese morning glory); the method of measurement was radioimmunoassay (RIA). However, these preliminary observations were not published as full reports before the end of 1995. In 1994, almost simultaneously, two research groups, Dubbels *et al.* in Germany and Hattori and colleagues in Japan submitted reports showing the presence of melatonin in plants. Dubbels and co-workers submitted their paper to the *Journal of Pineal Research* on 31 August 1994; this manuscript was accepted in October and published in January 1995 (Dubbels *et al.*, 1995); Hattori *et al.* submitted their observations to *Biochemistry and Molecular Biology International* on 20 December 1994; their manuscript was published in March of 1995 (Hattori *et al.*, 1995). These groups used different extraction and detection methodologies and independently reported that melatonin is present in many plants and plant products. The plants/plant products studied include tobacco plant, rice, corn, wheat, banana, etc. It seems that both the Dubbels and Hattori groups were not aware of the abstracts of Van Tassel *et al.* and did not mention their observations in their publications.

Later in 1995, a research group in the Czech Republic summarized their observations on melatonin in a higher plant, *Chenopodium rubrum*, at the World Conference on Chronobiology and Chronotherapeutics held in Ferrara, Italy, on September 6–10, 1995. Their observations were published later in 1995 as an abstract in *Biological Rhythm Research* (Kolar *et al.*, 1995).

In 1997, high levels of melatonin were reported to exist in European medicinal herbs, feverfew (*Tanacetum parthenium*) and St. John's wort (*Hypericum perforatum*), by Murch *et al.* (1997). Melatonin levels in these plant tissues are as high as several micrograms per gram of tissue, values that are orders of magnitude higher than those detected in animal blood. In the same year, the daily rhythm of melatonin in the plant *C. rubrum* was uncovered, with a rhythm similar to that seen in the majority of animals with a melatonin peak during darkness and baseline levels during the day (Kolar *et al.*, 1997). Shortly thereafter, this observation was questioned by Murch *et al.* (2000) who demonstrated that the daytime melatonin concentrations in St. John's wort are positively related to the light intensity to which these plants are exposed. The discrepancies between these observations may not be worth a dispute, since different plants can certainly behave differently. *Chenopodium* is a low melatonin organism (Kolar *et al.*, 1997), containing concentrations orders of magnitude lower than *Hypericum* (Murch *et al.*, 2000). Meanwhile, it was shown in various plants containing high amounts of melatonin that they respond to intense light, in particular UV, by an elevation of the methoxyindole (Conti *et al.*, 2002; Afreen *et al.*, 2006). This indicates a photoprotective role, which had already been assumed because of the numerous photo-reactions melatonin can undergo and its capability for neutralizing UV-induced free radicals and singlet oxygen (Balzer and Hardeland, 1996; Behrmann *et al.*, 1997; Hardeland, 2008b). For detailed discussion of this issue the readers should consult Hardeland *et al.* (2007).

To elucidate the role that melatonin plays in plants, Tan *et al.* (2000) reported that when plants were moved from room temperature to a cold (4 °C) environment, their melatonin content was significantly elevated. This finding is reminiscent of the temperature effects in *Lingulodinium* (Fuhrberg *et al.*, 1997), which are, however, orders of magnitude higher. Based on melatonin as a potent antioxidant, Tan *et al.* (2000) hypothesized that melatonin may be the first line of defence to protect plants against harsh environments such as cold, heat, drought, and air or soil pollution. This hypothesis has been subsequently supported by other published observations (Murch *et al.*, 2009; Tan *et al.*, 2010a). This is particularly in line with the observation of elevated melatonin in plants from alpine and Mediterranean environments exposed to intense UV radiation and, additionally, in the first case, large natural temperature variations (Conti *et al.*, 2002; Hardeland *et al.*, 2007; Murch *et al.*, 2009; Tan *et al.*, 2010a).

A large-scale melatonin screening study on plants was performed by Chen *et al.* (2003) using advanced technologies. In this study, 108 Chinese medicinal herbs were screened and all contained melatonin, with the levels ranging from a few nanograms per gram of tissue to several thousand nanograms per gram of tissue. These enormous differences between the species demonstrate that melatonin's roles in plants must be diverse. In addition to melatonin, one of its metabolites, AFMK, has also been identified in a plant (the water hyacinth, *Eichhornia*

crassipes) (Tan *et al.*, 2007a). This indicates that at least one metabolic pathway of melatonin in plants may be similar to that in animals. It remains to be determined to what extent AFMK formation in plants depends on photochemical processes, since extracts from several photoautotrophs catalysed AFMK formation from melatonin (Behrmann, 1989; Hardeland *et al.*, 1995; Behrmann *et al.*, 1997). Even chlorophyll has been shown to destroy melatonin photocatalytically *in vitro*, but, under the conditions used, AFMK was not recovered as the main product (Gawron and Hardeland, 1999).

To date, numerous publications have reported the presence of melatonin in plants and plant products with a wide range of concentrations from picograms to micrograms per gram of tissue [see the details in recent reviews (Tettamanti *et al.*, 2000; Paredes *et al.*, 2009; Posmyk and Janas, 2009)]. The extremely high levels and marked differences in melatonin concentrations in plants as reported in the literature have created concerns among scientists. Based on the data of animal studies during the past few decades, melatonin levels in the blood and tissues of animals from invertebrates to mammals are quite uniform and are in the range of low picograms per millilitre during the day to hundreds of picograms per millilitre at night (Reiter, 1986). There are, however, exceptions, with significantly higher levels of melatonin found in the third ventricle fluid of the brain of sheep (Tricoire *et al.*, 2002; Tan *et al.*, 2010b) and in the bile of several mammals (Tan *et al.*, 1999a). It was difficult for some investigators to understand why the levels of a 'neurohormone' were higher in plants than those measured in the blood of animals. Initially, differences were interpreted to be due to the different methodologies used by researchers in terms of plant melatonin extraction and measurement (Reiter *et al.*, 2007; Arnao and Hernandez-Ruiz, 2009a).

The initial concerns about high melatonin levels in some plants reflect an ignorance of two fundamental differences in limits for methoxyindole formation between vertebrates and plants. (i) The high concentrations of haemoglobin in vertebrate blood do not seem to be compatible with high melatonin, since combinations of NADH and melatonin, especially in the presence of H₂O₂, favour the oxidation of oxyhaemoglobin to methaemoglobin (Tan *et al.*, 2005). (ii) Tryptophan availability is much better in organisms producing aromatic amino acids via the shikimic acid pathway than in vertebrates which are devoid of this metabolic route. These arguments are likewise valid for other high-melatonin organisms, such as dinoflagellates (Fuhrberg *et al.*, 1997), euglenoids (Hardeland, 1999), and yeast (Sprenger *et al.*, 1999).

More recent studies have uncovered that melatonin concentrations differ not only among plant species, but also between varieties of the same species, and depend, to a considerable extent, on growth state, location, specific plant organ, as well as the timing and season of harvest (Burkhardt *et al.*, 2001; Hernandez-Ruiz and Arnao, 2008; Murch *et al.*, 2009; Okazaki and Ezura, 2009; Stege *et al.*, 2010). These findings are discussed in detail below. Several groups of plant biologists have now genetically modified the genes which are responsible for melatonin synthesis and

metabolism in plants including AANAT, ASMT (HIOMT), and IDO (Okazaki *et al.*, 2009, 2010; Kang *et al.*, 2010, 2011). These transgenic plants have shown both increases and decreases in melatonin production accordingly. At the current level of understanding, the presence of melatonin in plants is, for the most part, accepted, although there are still numerous unanswered questions. Moreover, a number of plant biologists have now become involved in studying the 'neurohormone' melatonin in plant materials.

Significance of melatonin in popular beverages and crops

Melatonin exists in almost all plants and plant products tested. Based on this, it is probably not surprising that melatonin has also been discovered in beverages which are derived from plant products. What is unexpected, however, is the uncommonly high levels of melatonin in some beverages.

Melatonin has been identified in the most popular beverages of the world. These include coffee, tea, wine, and beer (Chen *et al.*, 2003; Mercolini *et al.*, 2008; Iriti, 2009; Maldonado *et al.*, 2009; Iriti *et al.*, 2010; Stege *et al.*, 2010). Ramakrishna *et al.* (2011b) recently reported extremely high melatonin levels in harvested coffee seeds of *Coffea canephora*; the levels reported are $115 \pm 6 \mu\text{g g}^{-1}$ fresh weight. In coffee beans obtained from market, the melatonin levels in green beans are $5.8 \pm 0.8 \mu\text{g g}^{-1}$ and in the roasted beans are $8.0 \pm 0.9 \mu\text{g g}^{-1}$. Melatonin in coffee products reported in this study were measured using high-performance liquid chromatography (HPLC) and confirmed by mass spectrometry (MS) which are state-of-the-art methodologies used to identify melatonin. As mentioned previously, in the 1970s, large amounts of melatonin were found in the process of coffee decaffeination; these levels were presumed to be a result of an unexpected chemical reaction. Based on the current observations, however, the melatonin uncovered may not have been a by-product of an unknown chemical reaction, but rather melatonin produced by the coffee plant.

The melatonin concentrations in Chinese green tea and black tea have also been measured. Melatonin levels in Shiya tea (green tea) (Chen *et al.*, 2003) produced in Guangxi province of China are $\sim 2.12 \mu\text{g g}^{-1}$. Melatonin concentrations in Chinese Longjing tea (green) and Wulong tea (black) are several hundred nanograms per gram (DX Tan *et al.*, unpublished observations).

Several groups have reported widely diverse melatonin levels in wines. The ranges are from several picograms per millilitre to many nanograms per millilitre (Mercolini *et al.*, 2008; Stege *et al.*, 2010). Based on the published reports, melatonin levels are higher in red wine than in white wine (Stege *et al.*, 2010). Melatonin has been found in grapes, with especially high levels in skins and seeds of grapes (Iriti *et al.*, 2006; Stege *et al.*, 2010; Vitalini *et al.*, 2011b). Melatonin in wine, thus, is believed to be derived from the grapes. It should be noted, however, that yeast also produces (up to 100 ng mg^{-1} protein) and releases melatonin (Sprenger *et al.*, 1999; Hardeland and Poeggeler, 2003). It is our hypothesis that melatonin in wine is, at least partially, derived from the

yeast during the process of brewing. This may also apply to beer since melatonin has been reported in this beverage (Maldonado *et al.*, 2009) as well as in one of its raw materials, namely barley (Hernandez-Ruiz and Arnao, 2008).

The beneficial effects of melatonin as a potent antioxidant potentially to improve human health have been extensively reviewed (Reiter *et al.*, 2009, 2010a; Iriti *et al.*, 2010; Paradies *et al.*, 2010) and it has been suggested for clinical use in a variety of pathologies, including neurodegenerative diseases (Alzheimer's, Parkinson's disease), heart disease, metabolic disorders, tumours, and accidental nuclear radiation (Korkmaz *et al.*, 2009; Hong *et al.*, 2010; Rosenstein *et al.*, 2010; Sanchez-Barcelo *et al.*, 2010; Srinivasan *et al.*, 2010; Reiter *et al.*, 2011). The significance of high levels of melatonin in popular beverages could prove beneficial for some of these conditions. Generally, one serving of coffee is ~4–5 g of roasted coffee and contains ~40 µg of melatonin. Drinking one cup of coffee is theoretically sufficient to elevate blood melatonin levels. An increase in circulating melatonin in human subjects after consuming coffee has been reported (Ursing *et al.*, 2003). This was initially attributed to the inhibitory effect of coffee on the hepatic melatonin metabolizing enzyme CYP1A2 (Ursing *et al.*, 2003). At the time, the authors were not aware that coffee contains such a high level of melatonin.

Recent studies have shown the protective effects of coffee against liver fibrosis, steatohepatitis, and CCl₄-induced liver cirrhosis in experimental animal models (Shin *et al.*, 2010; Vitaglione *et al.*, 2010; Moreno *et al.*, 2011). The protective effects of coffee on these hepatopathologies are attributed to the antioxidant properties of the constituents in coffee. As recently discovered, the potent antioxidant melatonin is present in high concentrations in coffee. Numerous previously published articles have proven that melatonin effectively protects against the hepatopathologies mentioned above (Cruz *et al.*, 2005; Wang *et al.*, 2005; Tahan *et al.*, 2009, 2010; Solis-Munoz *et al.*, 2011). In addition to experimental animal studies, several epidemiological investigations provide solid evidence showing that regular coffee consumption significantly reduces the prevalence of human prostate cancer (Nilsson *et al.*, 2010; Park *et al.*, 2010; Wilson *et al.*, 2011; Yu *et al.*, 2011). It was estimated that the incidence of prostate cancer decreased by 30% in persons who drank three cups of coffee daily and was reduced by 60% in individuals who consumed six cups. The active ingredients accounting for this protection have not been identified; however, the active agent is not caffeine *per se*, since decaffeinated coffee did not jeopardize the prostate cancer-protective effects of coffee drinking. It is estimated that those individuals who benefited from drinking three or six cups of coffee daily also consumed at least 120 µg or 240 µg of melatonin, respectively, in the consumed beverage. It is well documented that melatonin strongly suppresses human prostate tumour cell proliferation in culture, as well as in animal models and in humans (Xi *et al.*, 2001; Shiu *et al.*, 2003; Joo and Yoo, 2009; Jung-Hynes *et al.*, 2011).

To explore the potential beneficial effects of melatonin in wine, Lamont *et al.* (2011) formulated the melatonin

concentration found in red wine (75 ng l⁻¹) and applied this to isolated rat hearts which were subjected to ischaemia/reperfusion injury. They observed that the melatonin concentration measured in red wine, when infused into the ischaemic/reperfused heart model, significantly reduced heart infarct size from 69% to 25%. The protective effect of melatonin on heart injury was equivalent to or better than that provided by resveratrol which is considered to be a major beneficial ingredient found in red wine; this was particularly remarkable since the resveratrol concentration tested in this study (2.3 mg l⁻¹) was almost 300 000-fold higher than that of melatonin.

As in the most common beverages, melatonin has been discovered in widely consumed crops including corn, rice, wheat, barley, and oats (Hattori *et al.*, 1995; Hernandez-Ruiz and Arnao, 2008; J Wang *et al.*, 2009). Melatonin concentration in these crops varies from several nanograms per gram to several thousand picograms per gram. Detailed studies have shown that even in the different varieties of the same species, substantial differences in melatonin concentrations exist, for example in different varieties of corn and rice (J Wang *et al.*, 2009). These variations can be greater than several hundred fold (Tables 1, 2). It appears that melatonin concentrations in plants are, to a great degree, determined by genetic traits. However, it is noted in some plants including tomato (Okazaki and Ezura, 2009), grapes (Murch *et al.*,

Table 1. Melatonin in corn

The numbers indicate the 58 different corn varieties that were tested; melatonin concentrations are listed from high to low values. All varieties were collected at the same site, the Institute of Rice, Fujian Academy of Agricultural Sciences, Fuzhou, China (26°3'41''N/119°18'22''E).

Variety	Mel (µg kg ⁻¹)	Variety	Mel (µg kg ⁻¹)	Variety	Mel (µg kg ⁻¹)
Corn 056	2034	Corn 031	41	Corn-N06	21
Corn 049	1981	Corn 129	41	Corn-N14	21
Corn 054	1110	Corn 143	39	Corn 159	20
Corn 115	983	Corn 057	37	Corn 042	19
Corn 046	803	Corn-N24	37	Corn-N13	19
Corn-N01	803	Corn-N04	35	Corn 150	18
Corn 047	614	Corn 155	35	Corn-N07	18
Corn-N02	614	Corn 038	33	Corn-N11	18
Corn 055	529	Corn 004	32	Corn-N18	18
Corn 163	446	Corn-N21	32	Corn 034	17
Corn 139	425	Corn 148	29	Corn-N19	17
Corn 032	325	Corn 007	28	Corn 140	16
Corn-N03	325	Corn-N23	27	Corn-N17	16
Corn 151	233	Corn-037	26	Corn-N22	16
Corn 033	172	Corn-N05	26	Corn-N16	15
Corn 142	142	Corn 040	25	Corn 009	14
Corn 149	81	Corn-N12	24	Corn-N10	14
Corn 144	78	Corn-N08	23	Corn-N09	11
Corn 035	62	Corn-N15	22		
Corn 004	60	Corn-N20	22		

Mel, melatonin. The table is modified from J Wang *et al.* (2009) with kind permission of the authors.

Table 2. Melatonin in rice

The numbers indicate the 25 different rice varieties that were tested; melatonin concentrations are listed from high to low. All varieties were collected at the same site, the Institute of Rice, Fujian Academy of Agricultural Sciences, Fuzhou, China (26°3'41''N/119°18'22''E).

Variety	Mel ($\mu\text{g kg}^{-1}$)	Variety	Mel ($\mu\text{g kg}^{-1}$)	Variety	Mel ($\mu\text{g kg}^{-1}$)
Rice 001	264	Rice 030	105	Rice 018	18
Rice 075	256	Rice 056	94	Rice 044	17
Rice 103	204	Rice 087	94	Rice 006	15
Rice 093	196	Rice 005	60	Rice 098	14
Rice 025	193	Rice 028	54	Rice 083	13
Rice 067	190	Rice 143	40	Rice 066	12
Rice 125	187	Rice 036	24	Rice 051	11
Rice 119	133	Rice 040	23		
Rice 141	115	Rice 029	21		

Mel, melatonin. The table is modified from J Wang *et al.* (2009) with kind permission of the authors.

2010), barley (Hernandez-Ruiz and Arnao, 2008), and water hyacinth (Tan *et al.*, 2007a) that the melatonin concentration is also impacted by the age, photoperiod, growing locations, environmental stresses, and harvest time. Melatonin concentrations in these popular crops, especially in rice and corn, are unexpectedly high. The consumption of corn or rice in some developing countries is ~500 g per adult per day; this intake would contain up to 1 mg of melatonin for corn and in excess of 100 μg of melatonin for rice. A human study has shown that individuals who consume vegetables rich in melatonin increase their urinary melatonin metabolite excretion, which is consistent with an elevated level of melatonin in the blood (Oba *et al.*, 2008). In these individuals their daily melatonin consumption derived from vegetables was calculated to be 1.288 μg . This amount is much less than what individuals would obtain from eating corn or rice. A majority of the population of the world depends on these crops as their main food. The impact of melatonin consumed from these popular crops currently remains unknown. There are two significant features related to the melatonin levels in these foodstuffs. First, melatonin, as an antioxidant, keeps these plant products from undergoing peroxidation, thereby improving their quality and prolonging their shelf life. Secondly, over the course of a lifetime, the regular consumption of melatonin-rich foods such as coffee, tea, corn, and rice may improve the general health of consumers.

Melatonin in plants: origins, metabolism, and genetic modification

The biosynthetic pathway of melatonin in vertebrates has been thoroughly studied and is well defined (Reiter, 1991). Beginning with the amino acid precursor tryptophan, four enzymes are sequentially involved in biosynthesis of melatonin. They are tryptophan hydroxylase (TPH) which converts tryptophan to 5-hydroxytryptophan; aromatic amino acid decarboxylase (AAAD) which converts

5-hydroxytryptophan to serotonin; arylalkylamine *N*-acetyltransferase (AANAT) which synthesizes *N*-acetylserotonin from serotonin; and *N*-acetylserotonin *O*-methyltransferase (ASMT), formerly called hydroxyindole *O*-methyltransferase (HIOMT), which forms melatonin. AANAT is believed to be the rate-limiting enzyme for melatonin biosynthesis in vertebrates; however, current studies point out that ASMT may also be involved in limiting melatonin production in some circumstances (Tan *et al.*, 2007c). The biosynthetic pathway of melatonin in higher plants appears similar to that in vertebrates (Arnao and Hernandez-Ruiz, 2006), but this is not without controversy. The first two enzymes in animals required for melatonin biosynthesis have been identified in plants (Facchini *et al.*, 2000; Fujiwara *et al.*, 2010). In accordance with these findings, serotonin has likewise been detected in numerous plants, often in concentrations much higher than those of melatonin (Feldman and Lee, 1985; Murch *et al.*, 2009). Park *et al.* (2012), however, recently reported that the first two steps of melatonin biosynthesis from tryptophan in a plant (rice) are reversed compared with that in animals. That is, the first enzymatic reaction product is not 5-hydroxytryptophan, but rather tryptamine, which is catalysed by tryptophan decarboxylase (TDC), and consequently the resulting tryptamine is catalysed to serotonin by tryptamine 5-hydroxylase, a cytochrome P450 enzyme. It seems that the biosynthetic pathways of melatonin in plants are more complex than that in animals.

Recently, the gene of the final enzyme (ASMT) in the synthetic pathway was also cloned and expressed in rice (Kang *et al.*, 2011). However, there are no published data available to show whether the activity or the AANAT is present in plants. The analyses of genomic sequences have revealed AANAT homologues in bacteria, unicellular chlorophytes, fungi, cephalochordates, and vertebrates, but not in trypanosomes, nematodes, arthropods, or in higher plants (Coon and Klein, 2006; Pavlicek *et al.*, 2010) (Fig. 1). Fungi, nematodes, and chordates share the closest common ancestor. In the tree of evolution, the nematodes evolved between fungi and chordates. It is, at least, surprising that only the nematodes are without an AANAT homologue, whereas it is present in fungi and vertebrates. However, that gene losses do occur in evolution is amply documented for numerous genes and cannot be ruled out, in this specific case without more detailed analysis. Recently, the claim that nematodes are without an AANAT homologue has been challenged by Migliori *et al.* (2012). This group not only detected the activity of AANAT but also found nine putative AANATs in *Caenorhabditis elegans*, the structures of which are similar to AANAT of vertebrates having both a GCN5-related *N*-acetyltransferase (GNAT) domain and a PKA phosphorylation site. Chlorophytes and plants form a clade that has evolved from the same ancestor. Yet, those green algae which have been analysed express AANAT variants, but plants obviously do not. One should, however, consider that the chlorophytes tested belong to rather atypical groups, *C. reinhardtii* being characterized, like other members of this family, by genetic

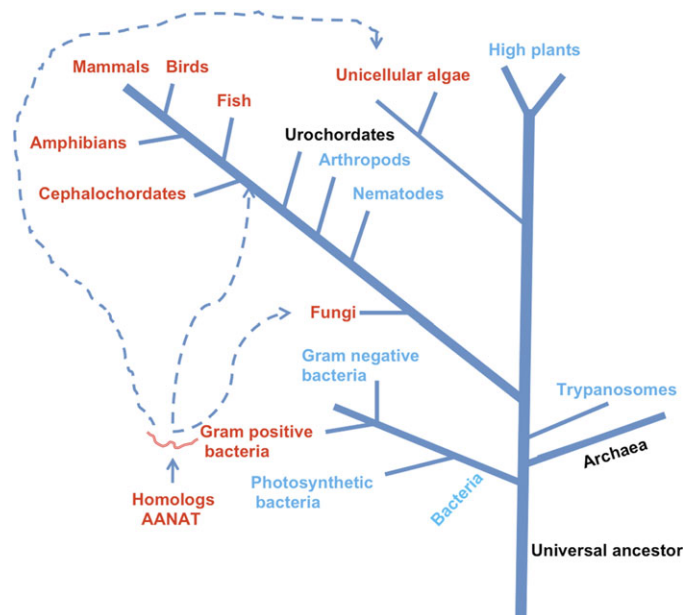


Fig. 1. A scheme of the speculated horizontal transfer of AANAT homologues from Gram-positive bacteria to other species. Species in red share the AANAT homologues. Species in green are void of AANAT homologues but they have the capacity to synthesize melatonin. There has been no attempt to examine whether melatonin is present in Archaea, so this information is lacking. The arrows indicate the direction of gene transfer. This scheme is modified from *Molecular and Cellular Endocrinology* 252, Coon SL, Klein DC. Evolution of arylalkylamine N-acetyltransferase: emergence and divergence, 2–10. Copyright (2006), with permission from Elsevier

peculiarities based on an unusual GC/AT ratio, and *Ostreococcus tauri* and *O. lucimarinus* representing nanoalgae with some of the smallest genomes known. To explain the phylogenetic patterns of distribution of AANAT among different taxa, it is speculated that an ancestor gene of AANAT may have been horizontally transferred from bacteria, particularly Gram-positive bacteria, to some species, such as photosynthetic green algae (Iyer *et al.*, 2004), but not to all, for example plants. This pattern is different from the conventional mechanism of gene transfer which is vertically passed to descendants.

As mentioned, melatonin has been identified in primitive photosynthetic bacteria (Manchester *et al.*, 1995; Tilden *et al.*, 1997) and also in Gram-negative bacteria (*E. coli*) (Hardeland and Poeggeler, 2003). Moreover, except for archaea, which have not yet been analysed in this regard, melatonin has been found in numerous species mentioned above, many of which lack an AANAT homologue (Hardeland and Poeggeler, 2003; Markowska *et al.*, 2009). The origin of AANAT within bacteria is unknown and difficult to identify, since horizontal gene transfer is a frequent phenomenon among bacteria, and also between bacteria and other organisms, including archaea and eucarya (Boto, 2010; Smillie *et al.*, 2010). It might be argued that melatonin in the nematode, *C. elegans*, originated from their food, *E. coli*, since this bacterium possesses melatonin.

However, the temporal patterns of melatonin in *C. elegans* and *E. coli* matched only partially (Balzer *et al.*, 2000).

If plants lack AANAT, it seems difficult to explain, at first glance, why melatonin is present in virtually all plants tested. The origin of melatonin in plants has been extensively discussed. Since melatonin is such a highly conserved molecule and since it is present in microorganisms including bacteria, fungi, and unicellular algae, it is likely that melatonin originally contained in microorganisms will inevitably be released into the soil during the process of decomposition of these organisms. This ‘soil melatonin’ could be absorbed by the rootlets of the plants and transported to all plant tissues. That plants take up exogenous melatonin via root absorption has been reported in several species (Arnao and Hernandez-Ruiz, 2007b; Tan *et al.*, 2007a, b). However, this is by no means the only possible interpretation. Paralogues of AANAT that could be genetically highly different may serve the same metabolic function. Even in mammals, other *N*-acetyltransferases that accept serotonin as a substrate and are involved in melatonin formation have been discussed for some tissues and specifically reported for the skin (Gaudet *et al.*, 1993; Semak *et al.*, 2004). With regard to the remarkable diversity of *N*-acetyltransferases in the living world and their frequently low substrate specificity, one could also imagine that functional changes have occurred during plant evolution, so that indoleamines were accepted as substrates by enzymes originally acting on other compounds. This possibility is not entirely unlikely. For instance, a parasitic nematode, the filaria *Brugia pahangi*, forms *N*-acetylserotonin using an enzyme that otherwise *N*-acetylates octopamine and dopamine (Isaac *et al.*, 1990). A polyamine *N*-acetyltransferase from the trematode, *Fasciola hepatica*, also converts tryptamine and serotonin to *N*-acetyltryptamine and *N*-acetylserotonin, respectively (Aisien and Walter, 1993). The relationship of polyamine and indoleamine acetylation may be of importance, since a parallel was observed in the cephalochordates, *Branchiostoma lanceolatum* and *B. floridae*, in which some of the seven AANAT isoforms detected were obviously capable of acetylating polyamines (Pavlicek *et al.*, 2010). Polyamines are ubiquitously present in plants (Kusano *et al.*, 2008; Takahashi and Kakehi, 2010), in which they are involved, among numerous other functions, in abiotic stress responses (Alcázar *et al.*, 2010; Gill and Tuteja, 2010), and in which a number of polyamine *N*-acetylating enzymes has been recently characterized (Fuell *et al.*, 2010).

In plants the enzymes required for melatonin biosynthesis from serotonin remain to be identified and genetically characterized. Whether or not AANAT has been lost in evolution from chlorophycean-like ancestors will have to be clarified, since the basis of only two, rather exceptional chlorophyceans is insufficient for a judgement. Moreover, the eventual contribution of soil organisms, bacteria, and fungi to plant melatonin in the natural environment should be determined.

Evidence for direct melatonin biosynthesis in plants has been presented by Murch *et al.* (2000). They observed that

when St. John's wort was treated with the radioactively labelled precursor tryptophan, radioactive melatonin was subsequently detected in plant tissue. This indicates that this plant at least is equipped with the necessary machinery for melatonin biosynthesis and sequentially converts tryptophan to melatonin. This conclusion is supported by findings in a melatonin-rich germplasm line of St John's wort (*H. perforatum*), which produces 12-fold more melatonin than the wild type, under identical environmental conditions (Murch and Saxena, 2006). Similarly, a spectrum of varieties of a Chinese medicinal herb (Huan qin) contain highly different amounts of melatonin varying by a factor of 2000-fold, under the same culture conditions (Murch *et al.*, 2004). Collectively, these observations are strongly in favour of an endogenous origin of melatonin in plants.

At the present state of knowledge, it seems likely that the *N*-acetylation of serotonin is catalysed in plants by AANAT paralogues rather than by homologues inherited from chlorophytes or obtained via horizontal gene transfer. To date, the possible existence of isoenzymes based on different genes, polymorphisms, and splice variants is not entirely clear, and should be investigated in the future. Gene structure and origin remain to be identified. For reasons of convenience, this/these 'protein(s)' are temporarily denoted as a plant AANAT/AANATs (pAANAT/pAANATs) in the text. It was hypothesized that AANAT isoenzymes may have multiple origins during evolution (Tan *et al.*, 2010a).

When the chlorophycean AANAT homologue from *C. reinhardtii* (crAANAT) is transfected into Micro-Tom tomato (Okazaki *et al.*, 2009) or when the human AANAT (hAANAT) is transfected into rice (Kang *et al.*, 2010), St John's wort (*H. perforatum*), or tobacco plant (*Nicotiana tabacum*) (Wang, 2008), the AANAT transgenes are fully expressed and the corresponding melatonin levels are highly elevated. The homology of crAANAT and vertebrate AANATs is rather limited, not only in terms of percentage, but more importantly with regard to functional domains. Like other non-vertebrate AANATs, the crAANAT shows differences in the N- and C-terminal regulatory regions, which are partially absent, and in particular the lack of a PKA phosphorylation site presumably prevents stabilization by a 14-3-3 protein, which would have consequences for circadian rhythmicity at the post-transcriptional level (reviewed in Hardeland, 2008a). It is believed that the efficiency of vertebrate AANAT is 1000-fold higher than that of non-vertebrate AANAT (Coon and Klein, 2006). It would be interesting to test the relative efficiencies of the algae AANAT homologue and the vertebrate AANAT homologue regarding their capacity to produce melatonin transfected into the same plant.

Transfection of various plants with genes for melatonin biosynthetic enzymes of different origin (crAANAT, hAANAT, plant ASMT, and combinations of vertebrate AANAT and ASMT) have shown that melatonin production is consistently promoted. These plants include tomato, rice, tobacco, and a medicinal herb (*H. perforatum*) as mentioned above. Considering the plasticity of melatonin production in plants, the ease of gene manipulation could

result in the development of larger quantities of edible foods including crops, vegetables, and fruits rich in melatonin.

The catabolic pathways of melatonin in plants are currently unknown, with the exception of its non-enzymatic degradation by free radicals and other reactive oxygens, such as singlet oxygen, and the direct photocatalytic effects of UV light, both of which are possible in all organisms. It seems likely that the non-enzymatic processes contribute significantly to melatonin degradation in plants, especially in locations exposed to high light and UV intensities. A large portion of melatonin consumed may be attributed to the protection of plant tissues against UV irradiation, detoxification of ROS generated during photosynthesis, and as a consequence of various stressors, such as harsh weather and air or soil pollutants.

Some evidence indicates, however, that enzymatic mechanisms may also be involved in melatonin degradation. Melatonin levels in IDO transgenic rice are significantly reduced (Okazaki *et al.*, 2010). IDO in vertebrates catalyses melatonin degradation to AFMK, although its main substrate is tryptophan. Therefore, it remains to be clarified whether IDO overexpression leads to a reduction in tryptophan availability. AFMK has been identified in a plant, the water hyacinth (Tan *et al.*, 2007a), and this melatonin metabolite exhibits a diurnal rhythm with its peak near to sunset. AFMK may be the major melatonin metabolite in plants since a product of melatonin's interaction with ROS is also AFMK (Tan *et al.*, 2007c).

Among the other possible routes of melatonin catabolism, enzymatic 6-hydroxylation, which represents the major pathway in vertebrates, seems to be rather unlikely, although the product, 6-hydroxymelatonin, may be formed non-enzymatically by interactions with ROS. In animals, 6-hydroxylation is required to form a conjugatable intermediate, for purposes of urinary excretion (i.e. processes not applicable to plants).

In the future, an alternative pathway should be considered which is a major means of degradation in photosynthetic dinoflagellates (summarized in Hardeland *et al.*, 2007). It has also been demonstrated in yeast (Sprenger *et al.*, 1999). In these organisms, melatonin is deacetylated to 5-methoxytryptamine (5-MT) by an aryl acylamidase (AAA). 5-MT is converted by a monoamine oxidase to 5-methoxyindole-3-acetaldehyde, which can be transformed by aldehyde dehydrogenase to 5-methoxyindole-3-acetic acid (5-MIAA) or, to a lesser extent, by alcohol dehydrogenase to 5-methoxytryptophol (5-ML). 5-MT and 5-ML have been detected in various dinoflagellates, rhodophytes, phaeophytes, and yeast (Hardeland, 1999), but have not been thoroughly investigated in plants. In *Lingulodinium*, the key enzyme AAA is regulated by melatonin and temperature, and it exhibits a pronounced circadian rhythm (summarized in Hardeland *et al.*, 2007). It should also be noted that the end-product 5-MIAA has a remarkable structural homology to the main physiological auxin, indole-3-acetic acid, and additionally displays antibacterial properties (Wang and Ng, 2002). Whether these findings are of physiological relevance in plants, remains to be studied.

Roles of melatonin in plants

The presence of melatonin in plants appears to be a universal phenomenon even though its biosynthetic pathway and metabolic mechanisms are yet to be revealed. Of course, the purpose of melatonin in plants is not for the benefit of consumers of plant products even though they obviously are the beneficiaries when these plants are consumed. Given that melatonin plant research is in its infancy, the functional significance of this indoleamine in diverse plant tissues remains unestablished. Several early studies have shown that melatonin exhibits effects similar to those of the plant growth hormone, auxin, which stimulates plant growth. Other studies have pointed out that melatonin may promote or inhibit plant flowering as well as the maturation of plants (Kolar *et al.*, 1999a; Hernandez-Ruiz *et al.*, 2005; Kolar and Machackova, 2005; Arnao and Hernandez-Ruiz, 2006, 2007a; Jones *et al.*, 2007). It is hypothesized that those functions were in fact acquired during evolution (Tan *et al.*, 2010a), but they were not the original purpose of melatonin in plants.

Rather, progressively more data support the idea that a primary function of melatonin in plants is to serve as an antioxidant and a plant protector against environmental insults. This suggestion is initiated by the observations that cold stress significantly elevates melatonin production in plants (Tan *et al.*, 1999b). This observation was followed by the identification of high levels of melatonin in a pollutant-resistant plant, the water hyacinth *E. crassipes* (Tan *et al.*, 2007a). This species tolerates extremely polluted environments and, therefore, it is used in phytoremediation (Agunbiade *et al.*, 2009). The high levels of melatonin in this plant are believed to play a pivotal role in preparing it against the environmental insults that generate ROS. Numerous studies have, in fact, mentioned the protective effects of exogenously applied melatonin to plants against a variety of oxidative stresses and harsh environments. These stressors include extremely cold or hot environments (Lei *et al.*, 2004; Posmyk *et al.*, 2009; Xu, 2010; XD Xu *et al.*, 2010b, c), UV irradiation (Afreen *et al.*, 2006; Zhang *et al.*, 2011), copper (Tan *et al.*, 2007b), cadmium, salted soil and hydrogen peroxide toxicity (Arnao and Hernandez-Ruiz, 2009b). The protective effects of melatonin against these stressors were attributed to its free radical scavenging and antioxidant capacity (Tan *et al.*, 2010a). Melatonin concentrations used in these studies ranged from several nanomolar to hundreds of micromolar.

Clearly, some of the melatonin concentrations used in these studies exceeded the physiological concentrations in the plants investigated; therefore, the physiological significance of melatonin in plants may be questioned. It should be noted, however, that the insults mentioned above also do not generate physiological levels of toxic radical species. The number of radicals produced under these dire conditions is pharmacological/pathological; to combat this excessively high production of reactive species, pharmacological concentrations of melatonin must be given. Secondly, some of the melatonin levels used in these studies are higher than the physiological levels detected in the blood of

vertebrates; however, they are physiological for some plants such as corn, coffee, tea, and medicinal herbs (*Glycyrrhiza*, Huan qin, or St John's wort) as noted above.

The much higher levels of melatonin detected in plants than in animals may be attributed to the differences in mobility of animals versus plants. Animals can actively avoid challenging environmental insults including cold, heat, UV irradiation, and heavily polluted environments, and, thus, they reduce oxidative stress simply via behavioural avoidance. Plants lack mobility like that of animals, except for leaf movements, and the high levels of antioxidants including melatonin in plants may be a compensatory mechanism for them to tolerate and survive in hostile environments. Oxidative stress such as UV irradiation probably consumes large quantities of melatonin in plants. This is supported by a recent field study which showed that when grapes are in the shade of leaves and branches (passive avoidance of UV irradiation compared with active avoidance by animals), melatonin concentrations in these grapes are 10-fold higher than those that are directly exposed to the sunlight (Boccalandro *et al.*, 2011). It is documented that melatonin directly scavenges ROS in plants and decreases the levels of malondialdehyde (MDA) in leaves of mung bean plant (*Vigna radiata*) exposed to UV-B (Y Wang *et al.*, 2009b) and dose-dependently protects against oxidative DNA damage as well as promoting DNA repair in plant protoplasts of *Gentiana macrophylla* after irradiation with different doses of UV-B (Zhang *et al.*, 2011). In seedlings of cucumbers subjected to heat stress, melatonin sprayed on the leaves significantly reduced the concentration of MDA, superoxide anion, and hydrogen peroxide in these tissues (XD Xu *et al.*, 2010b).

In addition to the direct interactions of melatonin with ROS, melatonin application also elevates the endogenous antioxidant enzymes in plants. These include peroxidase, superoxide anion dismutase (SOD), catalase (CAT), ascorbate peroxidase, monodehydroascorbate reductase (MDHAR), and glutathione reductase (Xu, 2010). As a result, the content of other antioxidants, namely ascorbic acid, vitamin E, and glutathione, are protected from oxidative damage.

Studies also have shown that not only exogenously applied but also endogenously produced melatonin in the plants possesses the same antioxidant capacity. Transgenic plants rich in endogenous melatonin have a higher total antioxidative capacity and lower oxidatively damaged or damaging products (reduced MDA, O₂⁻, and H₂O₂) compared with their non-transgenic wild-type counterparts which have lower endogenous melatonin levels (Y Wang *et al.*, 2009a; Xu, 2010). Outcomes of high melatonin levels in plants are elevated rates of germination of seeds under unfavourable conditions (Posmyk *et al.*, 2009), an enhanced survival rate (Zhao *et al.*, 2011), and improved quality of the plant products. The quality of the plant products can be judged from the contents of the nutritional ingredients they contain. Studies document that elevated levels of melatonin in plants essentially completely preserve the content of β-carotenoids, double the levels of vitamin E, and significantly increase the quantities of vitamin C and glutathione

(Wang, 2008; XD Xu *et al.*, 2010a; Wang *et al.*, 2012). The preservation of other antioxidants by melatonin strongly suggests that melatonin is the first line of defence against the oxidant stress occurring in plants. Other antioxidants preserved by melatonin may be used for other purposes, for example to intensify the colour of the flowers or function as back-up protection after melatonin is consumed (Tan *et al.*, 2010a).

Another important feature of melatonin as the first-line defence against oxidative stress is its stress-inducible characteristic in plants. A broad spectrum of stresses including cold temperature, UV exposure, H₂O₂, copper, and other environmental toxic chemicals significantly up-regulate endogenous melatonin production (Tan *et al.*, 1999b; Afreen *et al.*, 2006; Arnao and Hernandez-Ruiz, 2009b). An obvious advantage of a stress-inducible antioxidant such as melatonin is to upgrade the antioxidant capacity of the organisms when they experience low intensity insults and to pre-condition them to cope with upcoming stronger stressors. This increases their chance of survival; without pre-conditioning, a given species would be damaged or even destroyed by a rapid onset intensive stress. Whether the antioxidant capacity of melatonin in plants is exclusively dependent on its direct interaction with ROS or whether this is mediated by melatonin receptors is currently unknown. Melatonin receptors have not been reported in plants.

Chlorophyll preservation, photosynthetic stimulation, and the potential for improving crop production

In green plants or other organisms such as green algae, chlorophyll is the major molecule responsible for photosynthesis. Via this process, energy contained in photons is captured by chlorophyll and used for the synthesis of carbohydrates. Chlorophyll is fragile and easily injured by ROS which are generated either during the photosynthetic process or by the environmental variations including cold or hot temperatures and extensive visible or UV irradiation (Yamamoto *et al.*, 2008; Triantaphylides and Havaux, 2009). Preservation of the functional chlorophyll is of importance for the survival, growth, and finally the production of plants.

As an antioxidant, melatonin certainly has the capability of protecting against the degradation of chlorophyll. Arnao and Hernandez-Ruiz (2009c) incubated barley leaves with a solution containing 1 mM melatonin for 48 h and found that the chlorophyll content in the melatonin-treated leaves was 2-fold higher than that in untreated leaves. This was the first report to show that melatonin protected chlorophyll against oxidative stress. Thereafter, the chlorophyll-preserving effects of melatonin were confirmed in cucumber seedling subjected to heat stress (Xu, 2010) and also in a photosynthetic green macroalga (*Ulva* sp.) (Tal *et al.*, 2011). In *Ulva*, both endogenously produced and exogenously applied melatonin preserved the chlorophylls when the algae were subjected to hostile weather or metals which are toxic for this organism, namely zinc, lead, and cadmium. The chlorophyll protective effects of melatonin

in the green macroalga relate to the radical scavenging activity of this indoleamine. Accordingly, melatonin levels in the green macroalga under these environmental stresses were found to be significantly up-regulated, which indicates an inducible characteristic of melatonin responsive to stressors also in this organism.

In a transgenic rice plant, the ectopic overexpression of human AANAT with elevated endogenous melatonin levels causes 8-day-old etiolated plants to have much higher chlorophyll concentrations than their wild-type counterparts below a temperature of 12 °C (Kang *et al.*, 2010) (Fig. 2). The findings on chlorophyll preservation may be in accordance with earlier findings on melatonin's capacity to delay leaf senescence under conditions of abiotic stress, in both monocots (corn) and dicots (cucumber and tobacco) (Fletcher and Sopher, 1997). Unfortunately, these data presented at a conference are only available in abstract form.

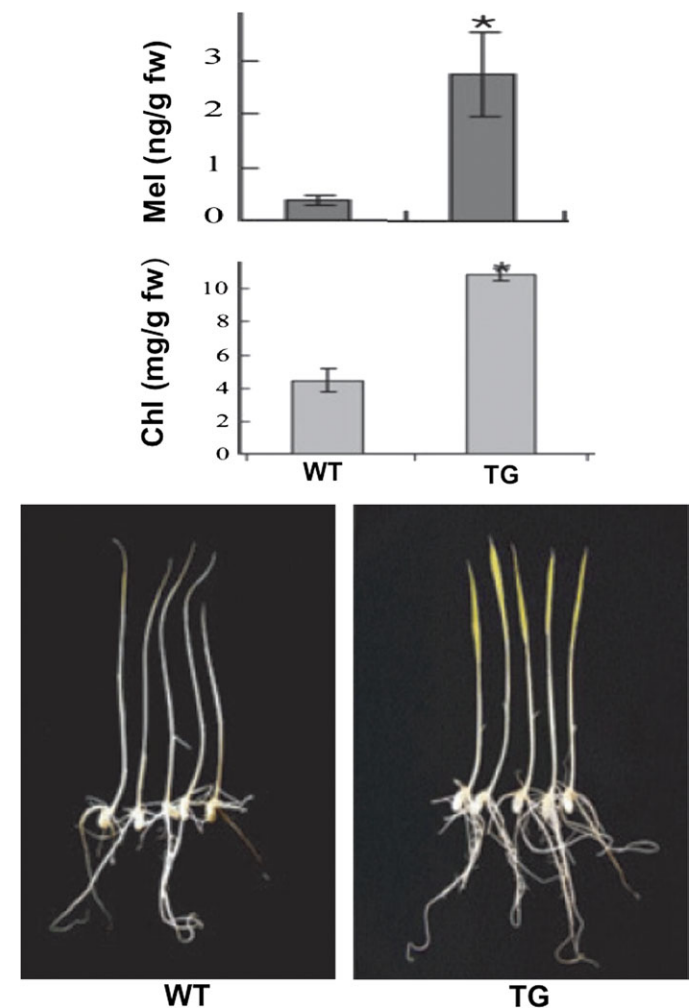


Fig. 2. The relationships among melatonin levels, chlorophyll concentrations, and the root system in 8-day-old etiolated rice seedlings which were grown at 12 °C under a light–dark cycle of 16 h/8 h. Data are expressed as means ± SE. **P* < 0.05. WT, wild type; TG, AANAT transgenic rice. The graphs are modified from Kang K, Lee K, Park S, Kim YS, Back K. *Journal of Pineal Research* 2010, with permission of John Wiley and Sons.

In addition to preserving the integrity of chlorophyll, melatonin also increases the photosynthetic efficiency of chlorophyll in plants. When melatonin was sprayed on cucumber seedlings at a concentration of 25–100 μM , the net photosynthetic rate was increased both under normal temperature conditions and when the seedlings were subjected to heat stress. Accordingly, the intercellular CO_2 concentrations were reduced (Xu, 2010). This indicates that more CO_2 contained in the leaves is being converted to carbohydrates with the accelerated photosynthesis as a consequence of melatonin treatment. Melatonin treatment also preserves the chlorophyll content of apple leaves which are subjected to oxidative stress following exposure to H_2O_2 ; the indoleamine also maximizes their photosystem II efficiency as well as elevates their ascorbic acid levels (Wang *et al.*, 2012).

A major question is that of whether elevated photosynthesis induced by melatonin can generate more biomass. Tomato plants carrying a rice IDO transgene not only had lowered endogenous melatonin levels, but also reduced their biomass (Okazaki *et al.*, 2010) (Fig. 3).

Another area in which melatonin apparently promotes plant growth seems to emerge in seeds, seedlings, and in the root system. Melatonin promotes seed germination as reported in cucumber and in red cabbage (Posmyk *et al.*, 2008, 2009). A currently ongoing study, conducted at cold temperatures, in which pea seeds were hydroprimed with or without melatonin (5 μM), showed that the germination rate of melatonin-treated seeds was 73.2%, whereas 53.7% of the non-melatonin-treated seeds germinated (RJ Reiter *et al.*, unpublished observations). In another preliminary study, Posmyk hydroprimed corn seeds with or without melatonin. After germination, the seedlings were planted in the field without further treatment and allowed to grow naturally. At harvesting, the melatonin-treated corn plants had a larger root system compared with non-melatonin-treated corn plants (Fig. 4B). This interesting observation implies that during the seed germination stage, melatonin initiated the irreversible gene expression for root development and this effect had consequences for, or even persisted throughout, the rest of the growing season. The one time melatonin-treated corn seeds also developed plants with notably larger corn cobs than those of non-melatonin-treated plants (Fig. 4B). It was estimated that the production of the corn from the melatonin-treated plants was $\sim 20\%$ greater than that from the non-melatonin-treated plants.

The findings on root development may be seen in the context of recent results showing that 10–160 μM serotonin, the precursor of melatonin, stimulated lateral root growth in *Arabidopsis thaliana*. At higher concentrations, serotonin inhibited primary and lateral root growth, but promoted formation of adventitious roots (Pelagio-Flores *et al.*, 2011). More details regarding the physiological roles of phyto-serotonin in plant development have been reviewed recently by Ramakrishna *et al.* (2011a). The bimodal concentration dependence is reminiscent of that of auxin. *Arabidopsis* lines expressing auxin-responsive marker constructs indicated anti-auxin effects in root primordia, and analyses of mutants defective in auxin-related genes showed that the

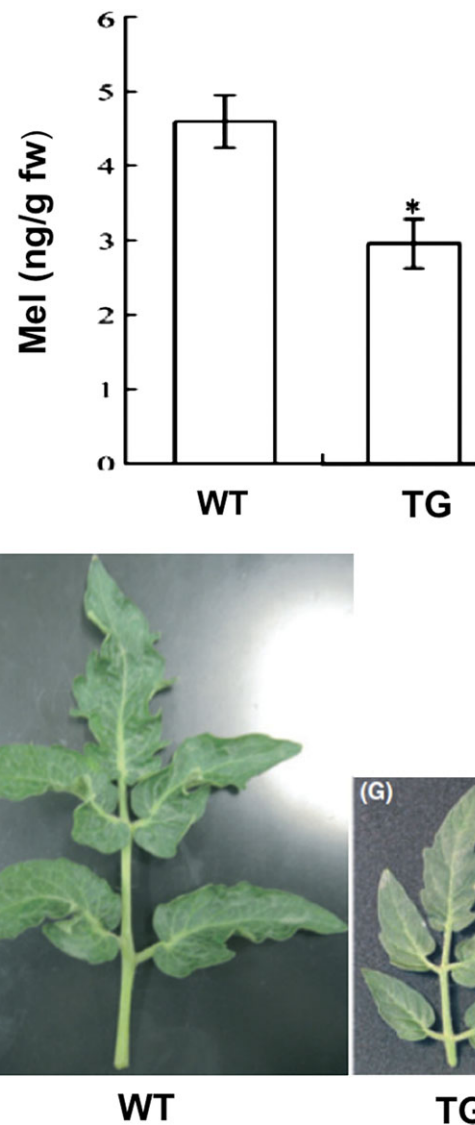


Fig. 3. The potential association of melatonin concentrations and biomass in tomato plants. WT, wild type; TG, IDO transgenic tomato plant. The white bars indicate 1 cm. Modified from Okazaki M, Higuchi K, Aouini A, Ezura H. *Journal of Pineal Research* 2010, with permission of John Wiley and Sons.

serotonin effect on lateral root formation required active AXR1 and AXR2 loci (Pelagio-Flores *et al.*, 2011). These findings may serve as hints for further studies, in which it should be also clarified to what extent exogenous serotonin is converted to melatonin and whether melatonin may antagonize the actions of high serotonin concentrations.

Currently, the mechanisms whereby melatonin promotes development remain unknown. They may be related to auxin-like effects or protection of root development. One well-designed study has reported that in plants (barley and lupinus) the highest melatonin levels are in the roots with an obvious gradient between different root parts. The order from high to low melatonin levels is apical, central, and basal portions of the root system (Hernandez-Ruiz and Arnao, 2008). The root-promoting action has also been observed in high level melatonin transgenic rice plants. In

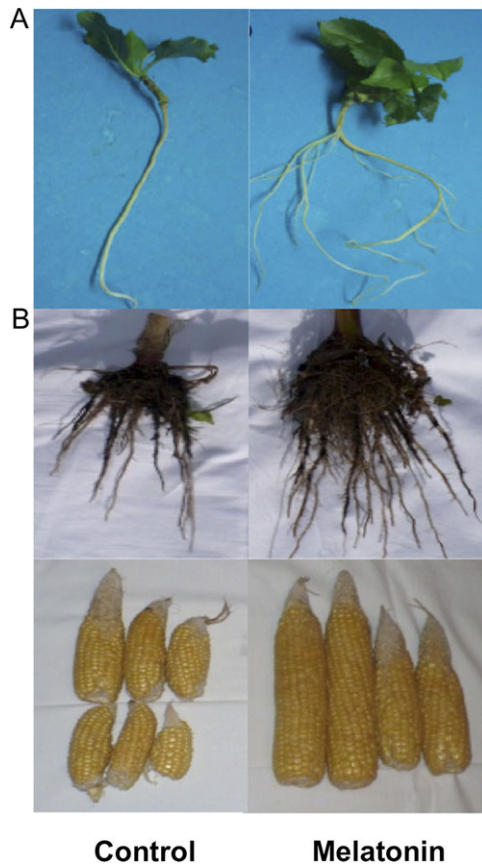


Fig. 4. Effects of melatonin application on root system development and crop production. (A) Effect of 1 μM melatonin on root growth in *in vitro* shoot tip explants of the commercial sweet cherry rootstocks CAB-6P (*Prunus cerasus* L.). The figure is modified from Sarropoulou VN, Therios IN, Dimassi KN. *Journal of Pineal Research* 2011, with permission of John Wiley and Sons. (B) Corn seeds were hydroprimed with 500 μM melatonin. After germination, the seedlings were planted in the field and grew under natural conditions without further treatment until harvest. The root system of melatonin-treated plants appears to be much better developed than that of the controls. It was estimated that the melatonin-treated corn plants produced up to 20% more corn than control plants. The data were kindly provided by Dr M.M. Posmyk

these plants, the chlorophyll content and root system are elevated above those in wild-type plants (Kang *et al.*, 2010) (Fig. 2). In addition to promoting root growth, melatonin stimulates root regeneration (Arnao and Hernandez-Ruiz, 2007b). Sarropoulou *et al.* (2011) recently observed that melatonin, at the concentration of 1 μM , dramatically enhanced adventitious root regeneration in *in vitro* shoot tip explants of commercial sweet cherry rootstocks CAB-6P (from *Prunus cerasus*) (Fig. 4A); however, at a concentration of 10 μM , melatonin had an inhibitory effect on root generation. This is the first report to show that melatonin influences root development in a tree.

Based on the preliminary observations that melatonin may increase corn production, stimulate root growth, preserve chlorophyll levels, and enhance photosynthesis,

the implications for increased food production are obvious. In the world, a major portion of the population depends on corn, rice, and wheat as their basic daily food source; this is also the case in many developing countries where food shortage is still a critical problem. Any method for enhancing the production of these basic foodstuffs would have massive ramifications. Melatonin, either by priming seeds or by transgenically enhancing plants with the indoleamine, enhances crop production, so that the nutritional status of billions of people could benefit.

Discoveries to date indicate that the beneficial effects of melatonin in plants may be multifaceted: (i) melatonin increases the plant germination rate; (ii) melatonin enhances the capacities of plants to resist a variety of environmental insults, especially cold and hot temperatures which are important factors in determining crop production; (iii) melatonin preserves the chlorophyll content and promotes its photosynthetic rate; as a result, plants synthesize more carbohydrates and increase their biomass; and (iv) melatonin promotes the development of the root system, which could lead to a greater absorptive capacity of nutrients from the soil.

Gene manipulations of melatonin's synthetic enzymes such as AANAT and/or ASMT have proven to be a feasible method to elevate endogenous melatonin production in plants. Moreover, it has been reported that application of agrochemicals including benzothiadiazole (BTH) and chitosan (CHT), which are used to activate the plant's own defence system, also increases the concentration of melatonin (Vitalini *et al.*, 2011a). A more direct way would be to provide exogenous melatonin for plants including priming seeds; this has been successfully employed in the case of cucumber seedlings, and corn and pea plants. It may also work on other crops such as rice, wheat, potato, etc. The utility of melatonin in agricultural production is a new frontier for melatonin research and deserves further investigation.

Concluding remarks

Melatonin is a pleiotropic molecule with multiple important physiological functions in many organisms. The multiplicity of melatonin's functions may be a consequence of its receptor-mediated and receptor-independent actions, as well as its biologically active metabolites. Melatonin was portrayed as being exclusively an animal hormone for nearly four decades; however, evidence shows that melatonin is a constitutive molecule present in plants as well. Currently, the biosynthetic and metabolic pathways of melatonin in plants have not been fully clarified. The homologue or a paralogue of AANAT has not been identified in plants. This implies that the enzyme which converts serotonin to *N*-acetylserotonin may have a different evolutionary origin.

Studies indicate that the primary function of melatonin in plants is to serve as the first line of defence against oxidative stresses which are a result of internal and environmental insults. Besides functioning as an antioxidant to protect against various forms of stresses, melatonin has acquired other functions in plants as well.

Compared with the melatonin levels in the body fluids of animals, the concentrations of melatonin in plants are significantly higher, often by several orders of magnitude. The much higher melatonin levels in plants than those found in animals may reflect the absence of limits, for example in tryptophan availability, but could also be interpreted in terms of greater needs for resisting damage from harsh environments which animals can actively avoid by behavioural changes. As an antioxidant, the high levels of melatonin in plants are also beneficial to animals including humans, who consume them. Melatonin levels in popular beverages including coffee, tea, red wine, and beer, and several major agriculture crops including corn, rice, and wheat are sufficiently high to raise blood melatonin levels after their consumption. Throughout the world, billions of people depend on these products as a major food source so the potentially beneficial effects of melatonin consumed in these products is obvious.

Based on the data available and preliminary observations, it is hypothesized that melatonin application in agriculture may significantly improve crop production. The reasons for this are: (i) as an antioxidant, melatonin enhances the tolerance of crops against the heat, cold, chemical pollutants, and other environmental insults; (ii) melatonin increases the germination rate of seeds under environmental stresses; (iii) melatonin preserves chlorophyll against oxidative stress and accelerates the rate of photosynthesis in plants; and (iv) melatonin stimulates the development of the root system and promotes root regeneration. Genetic manipulations to elevate melatonin production have proven feasible. Several transgenic plants have successfully expressed their transfected genes of enzymes for melatonin synthesis and have elevated the melatonin concentrations compared with their wild counterparts. The application of melatonin in agriculture is a new frontier to be explored.

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