

REVIEW PAPER

An evolutionary view of melatonin synthesis and metabolism related to its biological functions in plants

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Abstract

Plant melatonin research is a rapidly developing field. A variety of isoforms of melatonin's biosynthetic enzymes are present in different plants. Due to the different origins, they exhibit independent responses to the variable environmental stimuli. The locations for melatonin biosynthesis in plants are chloroplasts and mitochondria. These organelles have inherited their melatonin biosynthetic capacities from their bacterial ancestors. Under ideal conditions, chloroplasts are the main sites of melatonin biosynthesis. If the chloroplast pathway is blocked for any reason, the mitochondrial pathway will be activated for melatonin biosynthesis to maintain its production. Melatonin metabolism in plants is a less studied field; its metabolism is quite different from that of animals even though they share similar metabolites. Several new enzymes for melatonin metabolism in plants have been cloned and these enzymes are absent in animals. It seems that the 2-hydroxymelatonin is a major metabolite of melatonin in plants and its level is ~400-fold higher than that of melatonin. In the current article, from an evolutionary point of view, we update the information on plant melatonin biosynthesis and metabolism. This review will help the reader to understand the complexity of these processes and promote research enthusiasm in these fields.

Keywords: Abiotic stress, chloroplasts, melatonin, metabolism, mitochondria, plants.

Introduction

Melatonin, a derivative of the amino acid, tryptophan, is a phylogenetically ancient molecule which is found in primitive bacteria (Tan *et al.*, 2010). Historically, this molecule was classified as a neuroendocrine hormone and it was claimed to be present only in animals (Reiter, 1991a, b) due to the fact that it was first isolated from the pineal gland of cows (Lerner *et al.*, 1958). This notion has confined melatonin research exclusively to animals for several decades. In 1991, the concept that melatonin was an exclusive animal hormone was challenged by the discovery of Poeggeler *et al.* (1991) who identified melatonin in the unicellular dinoflagellate *Gonyaulax polyedra*. *Gonyaulax polyedra* (now known as *Lingulodinium polyedrum*, a

blooming dinoflagellate) is a photosynthetic alga. Since then, researchers have expanded melatonin research areas to bacteria (Manchester *et al.*, 1995; Tilden *et al.*, 1997) and fungi (Hardeland, 1999), as well as to plants. Melatonin research in plants had already been initiated by our group as early as in 1992. Due to the difficulties in the extraction of melatonin from plants and the unsuitable methods for a reliable plant melatonin assay (at the time all melatonin assays were designed for animal samples), the attempt to identify plant melatonin had failed initially. Finally, in 1995, two groups (both with connections to our laboratory) reported that melatonin is present in plants (Dubbels *et al.*, 1995; Hattori *et al.*, 1995).

The certainty of this new discovery remained questionable for the first 2 years since there were no confirmed reports from other laboratories to support it. In 1997, Murch *et al.* confirmed that melatonin was found in feverfew and other medicinal plants (Murch *et al.*, 1997). Thereafter, several papers consistently reported the presence of melatonin in plants (Kolár *et al.*, 1999; Manchester *et al.*, 2000; Murch *et al.*, 2000, 2004; Burkhardt *et al.*, 2001; Murch and Saxena, 2002; Chen *et al.*, 2003) and then the presence of melatonin in plants was thought to be reasonable and the idea was slowly accepted by the majority of melatonin scientists. However, during that time, the studies were often focused on the nutritional value of the plant melatonin for animals and humans (Reiter *et al.*, 2005; Simopoulos *et al.*, 2005). Readers are referred to a current review which has detailed the development of melatonin in plants chronologically (Arnao and Hernández-Ruiz, 2020a).

Logically, the purpose of melatonin production by plants would be for their own benefit rather than for the animals that consume them. Based on this principle as well as the fact that melatonin is a potent antioxidant, Tan *et al.* (1993, 2000) first hypothesized that the biological role of melatonin in plants is to protect them against environmental stressors including heat, cold, drought, and soil contamination. To test this hypothesis, Lei *et al.* (2004) examined the protective effects of melatonin on cold stress-induced injury in carrot suspension cells, and Afreen *et al.* (2006) found that *Glycyrrhiza uralensis* exhibited a strong response to UV-B radiation by increasing melatonin production. For further investigation, a pollutant-resistant plant, water hyacinth (*Eichhornia crassipes*), was selected to measure its melatonin level. As predicted, a high level of melatonin (two orders of magnitude higher than that in the serum of animals) was found in this plant and this high melatonin concentration presumably rendered its resistant to environmental pollution (Tan *et al.*, 2007a). Thereafter, an increasing number of plant biologists have engaged in melatonin research.

The turning point for plant melatonin research seemed to occur in 2009 (Arnao and Hernández-Ruiz, 2020a). Starting with this year, the number of reports of melatonin in plants sharply increased, and thereafter was maintained at a high level. Currently, the number of yearly publications related to plant melatonin are comparable with the number of reports associated with melatonin research in animals. Judging from this tendency, the number of plant melatonin papers published yearly is likely to surpass the number of animal-related melatonin research articles soon. Hundreds of publications have uncovered the biological activities of melatonin on plants. These include three major categories: (i) melatonin as a plant growth stimulator; (ii) melatonin as a stress protector of plants; and (iii) melatonin as a regulator of flowering and fruit ripening.

As a plant growth stimulator (Arnao and Hernández-Ruiz, 2019a, 2020a), melatonin promotes seed germination (Li *et al.*, 2018; Simlat *et al.*, 2018; Xiao *et al.*, 2019; Li *et al.*, 2019b) and lateral root generation (Liang *et al.*, 2017; Z. Chen *et al.*, 2018; Chen *et al.*, 2019; Ren *et al.*, 2019), enhances their photosynthetic efficiency (Debnath *et al.*, 2018; Y.-E. Chen *et al.*, 2018; Ahmad *et al.*, 2019), increases their biomass (Hernández-Ruiz *et al.*, 2004; Sarropoulou *et al.*,

2012; Fazal *et al.*, 2018), and elevates the yield in certain crops including soybean and wheat (Wei *et al.*, 2015; Qiao *et al.*, 2019). One potential mechanism is that melatonin *per se* mimics the activity of the plant hormone auxin, or acts on the upstream pathway of auxin to promote its action (Arnao and Hernández-Ruiz, 2018, 2019a). However, a recent study has reported that melatonin and auxin function in independent pathways and they share limited similarities in their biological activities (Zia *et al.*, 2019).

As a stress protector, the most studied effect is melatonin's action on environmental stressors. Melatonin elevates the resistance of plants against both abiotic and biotic stresses. For example, exogenously provided or endogenously produced melatonin allows plants to survive and thrive under conditions of hot, cold, drought, waterlogging, salinity, cadmium or other metals, and chemical pollutions (Zheng *et al.*, 2017b; Martinez *et al.*, 2018; Qi *et al.*, 2018; Li *et al.*, 2019a; D.-D. Liu *et al.*, 2019; Farouk and Al-Amri, 2019; Naghizadeh *et al.*, 2019; Wang *et al.*, 2019; Q. Zhang *et al.*, 2019); in the absence of melatonin, plants may possibly not survive these stresses. With respect to biotic stress, melatonin enhances plant resistance to virus, pathogenic bacteria, and fungal infections (Yin *et al.*, 2013; Qian *et al.*, 2015; X. Chen *et al.*, 2018; Zhang *et al.*, 2018; C. Liu *et al.*, 2019). The anti-stress effects of melatonin are mainly attributed to its capacity as a potent antioxidant (Arnao and Hernández-Ruiz, 2019b) and its ability to up-regulate a wide spectrum of stress response genes including *FaHSEF3*, *FaAWPM*, *FaCYTC2*, *SAD*, *CAT*, *APX*, *MAPK*, *bZIP60*, *BIP2*, *BIP3*, and *CNX1*, and to down-regulate the stress-related genes *CDPK1*, *MAPK1*, *TSPMS*, *ERF4*, *HSP80*, and *ERD15* (Gong *et al.*, 2017; Zhao *et al.*, 2017; Alam *et al.*, 2018; Lee and Back, 2018). It is unknown whether these responses are mediated by melatonin receptors or if they are receptor independent. The first melatonin receptor identified in plants is referred as phytomelatonin receptor 1 (PMTR1) (Wei *et al.*, 2018). This receptor is localized in the plasma membrane where it interacts with the G protein α subunit (GPA1). Activation of the receptor mediates the regulation of the status of the stomata of plants under physiological or pathological conditions, particularly during abiotic stress. Many extensive reviews have discussed the anti-stress effects of melatonin in plants (Hardeland, 2015, 2016; Sharif *et al.*, 2018; Arnao and Hernández-Ruiz, 2019a) and thus this issue is not discussed in detail in this review.

In reference to the third issue (iii, above), a couple of reports have claimed that melatonin delays flowering in rice and in apple (Byeon and Back, 2014b; H. Zhang *et al.*, 2019). In contrast, when a melatonin synthetic enzyme was knocked out in Arabidopsis to reduce melatonin levels, flowering was delayed (Lee *et al.*, 2019). Melatonin's effects on the rate of fruit ripening are complex, including delay or promotion depending on the species examined (Sun *et al.*, 2015, 2016; Xu *et al.*, 2018; Tijero *et al.*, 2019; Arnao and Hernández-Ruiz, 2020b). In addition to its biological roles in plants, the mechanisms of its biosynthesis and metabolism are also intriguing areas being explored. Herein, the focus is to update the developments in the areas of melatonin's biosynthesis and metabolism in plants.

The alternative biosynthetic pathways of melatonin in plants associated with biotic/abiotic stresses

The biosynthetic pathway of melatonin in animals has been extensively studied and well characterized (Axelrod and Weissbach, 1960; Champney *et al.*, 1984), but not without dispute (Tan *et al.*, 2016a). Generally, it starts from the amino acid, tryptophan, which is converted to melatonin in four consecutive steps. Initially, tryptophan is hydroxylated at the 5 position of the indole ring to form 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (TPH). Step 2 is the decarboxylation of 5-hydroxytryptophan to form 5-hydroxytryptamine (serotonin); this involves the enzyme aromatic amino acid decarboxylase (AADC). Step 3 is 5-hydroxytryptamine acetylation to form *N*-acetyl-5-hydroxytryptamine (*N*-acetylserotonin) by arylalkylamine *N*-acetyltransferase (AANAT, currently, SNAT). Step 4 (the final step) is *N*-acetyl-5-hydroxytryptamine being *O*-methylated to form melatonin, utilizing the enzyme hydroxyindole-*O*-methyltransferase (HIOMT, currently, ASMT). This is also referred to as the classic melatonin synthetic pathway in animals. These processes are illustrated in Fig. 1.

After the discovery of melatonin in plants (Dubbels *et al.*, 1995; Hattori *et al.*, 1995), to understand its synthetic pathway became a research topic for melatonin scientists. Murch *et al.* (2000) were the first to explore this issue. They treated the plant, St. John's wort (*Hypericum perforatum* cv. Anthos), with radiolabeled tryptophan ($[^{14}\text{C}]$ tryptophan). All of the radiolabeled precursors (Fig. 1) for melatonin biosynthesis and radiolabeled melatonin *per se* were identified in the plant extract by HPLC and LC-tandem MS. They deduced that the melatonin synthetic pathway in plants was the same as in animals. More detailed studies on the melatonin synthetic pathway in plants were performed by Park *et al.* (2012) and Byeon *et al.*

(2014). In a series of studies using the methodologies of molecular biology and enzymatic kinetics, these authors have shown that the melatonin synthetic pathway in plants is not as simple as in animals. It exhibits substantial differences from the melatonin synthetic pathway of animals. First, if the pathway begins with the tryptophan, the first two steps in plants are reversed compared those of animals. In plants, tryptophan is initially decarboxylated to form tryptamine by tryptophan decarboxylase (TDC) and then tryptamine is hydroxylated at the 5 position of the indole ring to form 5-hydroxytryptamine (serotonin) by tryptamine 5-hydroxylase (T5H). Secondly, in the final two steps, in addition to the process described above for animals, there is an alternative pathway which also has a reversed sequence to that in animals. Thirdly, the starting material is not necessarily tryptophan in plants as in animals since plants can *de novo* generate tryptophan via the shikimic acid pathway (Tan *et al.*, 2016a; Pérez-Llorca *et al.*, 2019). This may help to explain the much higher melatonin levels in plants than in animals since melatonin biosynthesis in plants would not be limited by the availability of tryptophan as it is in animals. The melatonin synthetic pathways in plants are illustrated in Fig. 2.

As illustrated in Fig. 2, melatonin biosynthetic pathways in plants and animals are substantially different; that is, they have different reaction sequences, synthetic enzymes, and intermediates, with their final product being melatonin. These differences imply the different evolutionary origins of the synthetic machinery for melatonin in plants and animals. There is no dispute regarding the process of conversion of tryptophan to 5-hydroxytryptamine (serotonin) in plants; however, the arguments have focused on the processes from serotonin to melatonin. Is serotonin initially acetylated to form *N*-acetyl-5-hydroxytryptamine and then *O*-methylated to form melatonin (we refer to this pathway as NM) as in animals, or is serotonin first *O*-methylated to form 5-methoxytryptamine and then acetylated to form melatonin (we refer this pathway as MN)?

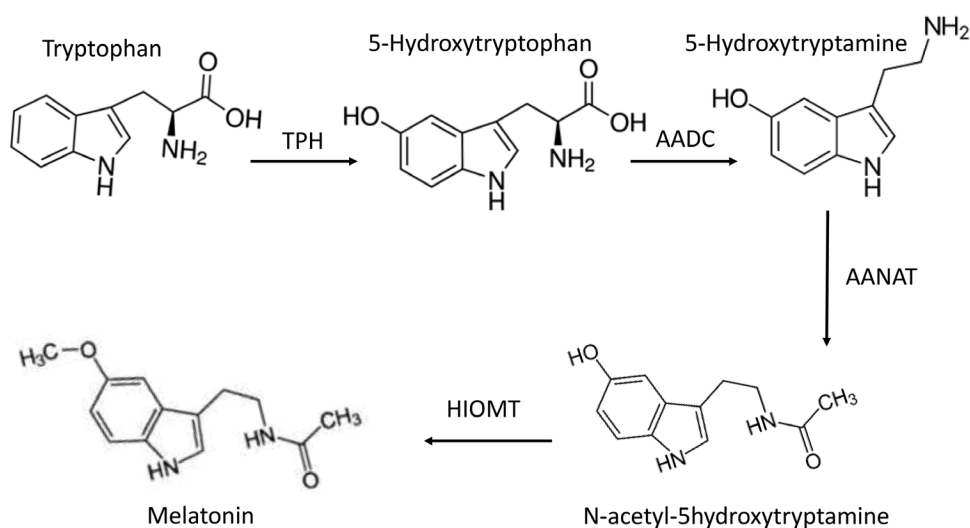


Fig. 1. The classic melatonin biosynthetic pathway in animals. This pathway was deduced by Axelrod and Weissbach in 1960. TPH, tryptophan hydroxylase; AADC, aromatic amino acid decarboxylase; AANAT, arylalkylamine *N*-acetyltransferase (also known as an arylamine *N*-acetyltransferase which is selective for indole-ethylamines as its substrate; it is different from the general arylamine *N*-acetyltransferase (NAT) which has a wide range of substrates; AANAT is currently also referred as serotonin *N*-acetyltransferase (SNAT)]; HIOMT, hydroxyindole-*O*-methyltransferase, also known as *N*-acetylserotonin *O*-methyltransferase (ASMT).

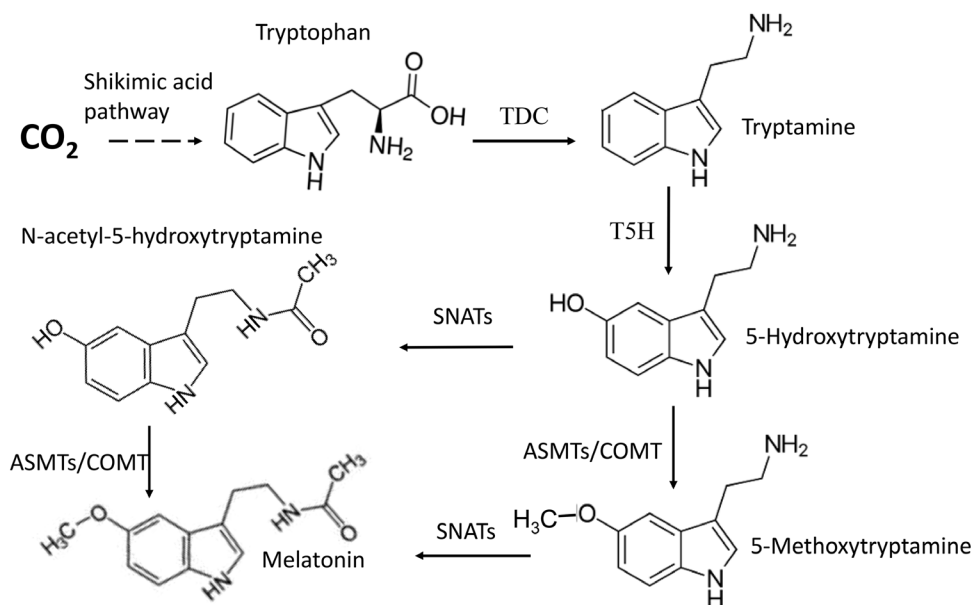


Fig. 2. Melatonin biosynthetic pathways in plants. TDC, tryptophan decarboxylase; T5H, tryptamine 5-hydroxylase; SNATs, several different serotonin *N*-acetyltransferases; ASMTs, several different *N*-acetylserotonin-*O*-methyltransferases (plant type SNATs and ASMTs seem to have different origins from those in animals); COMT, caffeic acid *O*-methyltransferase.

The different intermediates, *N*-acetyl-5-hydroxytryptamine for the NM route and 5-methoxytryptamine for the MN pathway, have both been found in plants. It seems that both NM and MN pathways exist in parallel in plants.

The remaining question is which pathway is the dominant one for melatonin biosynthesis in plants. A recent study has answered this question. It shows that under normal conditions, the NM pathway is dominant while, under abiotic stress, the MN pathway becomes dominant (Ye *et al.*, 2019). It seems that fungi share the same melatonin synthetic pathways as in plants, namely both the NM and MN routes. In fungi, the MN pathway is the dominant one in terms of melatonin biosynthesis (Muñiz-Calvo *et al.*, 2019). The origins of melatonin synthetic genes in fungi are probably different from those in plants, and are somewhat closer to those of animals. This indicates that the MN pathway may also be dominant in some animals.

The alternative melatonin synthetic pathways during stress in plants is not surprising considering the fact that several isoforms of SNAT and ASMT are present in plants (see below). Perhaps abiotic stress (maybe also biotic stress) up-regulates the expression of different isoforms of ASMT which exhibits a much higher affinity for serotonin than those isoforms expressed under normal conditions. The differential expression of melatonin synthetic isoforms under different conditions, for example under different light intensities or temperatures, has been reported in plants (H. Zhang *et al.*, 2019). The biological advantage of this is obvious. Under normal conditions, a relatively low level of melatonin may be required to promote plant growth, while extremely high levels of melatonin have some adverse effects on plant growth (Zhang *et al.*, 2014; Bychkov *et al.*, 2019; Yang *et al.*, 2019); however, the high levels of melatonin are required to protect plants against environmental hazards such as abiotic and biotic stresses. Usually, the highest levels of melatonin in plants occur under stressful conditions, and thus the differential expression of the enzymatic

isoforms in the melatonin synthetic pathways may serve this purpose.

Regulation of melatonin biosynthesis in plants and differences from animals

As mentioned, the regulation of melatonin biosynthesis in animals is well characterized. The greatest (essentially exclusive) regulatory factor in animals is normally light, particularly blue light (~420–480 nm) (Cahill *et al.*, 1998; Thapan *et al.*, 2001; Skene, 2003). Blue light irradiation at night suppresses melatonin biosynthesis by rapidly inhibiting the activity of AANAT via dephosphorylation of the enzyme (Ganguly *et al.*, 2005) to achieve an immediate effect, accompanied by down-regulation of gene expression (Velarde *et al.*, 2010) of this enzyme to achieve a relatively long-term effect. The normal light:dark cycle induces the classic melatonin circadian rhythm, with high levels during darkness and low levels during the day (Tan *et al.*, 2016b). It has also been hypothesized that near infrared (NIR) photons, which range from 650 nm to 1200 nm, penetrate cells and directly stimulate the mitochondria to synthesize melatonin (Zimmerman and Reiter, 2019). Other factors including food intake, temperature alterations, and disease state may impact animal melatonin biosynthesis (Tan *et al.*, 2011); generally, these impacts are overlooked by researchers.

Research on the regulation of melatonin biosynthesis in plants is still in its infancy and has not been fully characterized. Based on the available information, it seems that this process is more complicated than that in animals. There is an early study by Kolár *et al.* (1999) using a short-day flowering plant, *Chenopodium rubrum*, which suggest that melatonin levels exhibit a circadian rhythm similar to that in animals. This observation indicates that light also suppresses melatonin biosynthesis in plants. Surprisingly, an opposite observation was reported by Murch *et al.* (2000). They claim that light exposure does

not suppress melatonin biosynthesis but it significantly stimulates melatonin production with increased light intensities in plants. This observation has been strengthened by our own observations (Tan *et al.*, 2007a). By measuring the melatonin levels of an aquatic plant, water hyacinth, which grows under natural photoperiodic conditions, the highest melatonin level appeared during the day. Seemingly, sunlight promotes the melatonin production in some plants. Interestingly, even UV irradiation has also been found to significantly promote melatonin biosynthesis in a medicinal plant (*G. uralensis*) (Afreen *et al.*, 2006).

A recent study reported that blue light and far red light down-regulate gene expression of both isoforms of *SNAT* and *ASMT* in apple leaves, and thus suppress melatonin biosynthesis during certain seasons when the intensities of blue light and far red light are at their peaks (H. Zhang *et al.*, 2019), respectively. This observation does not necessarily contradict the reports that light intensity enhances melatonin biosynthesis in some plants as mentioned above. This inconsistency may be species specific or enzymatic isoform specific. More probably, this may relate to the light wavelengths, specifically during the different seasons. Plants are always exposed to a wide spectrum of wavelength irradiation from UV to NIR. The enzymes or their isoforms may exhibit different responses to the different wavelengths of light to transduce the information for seasonal alterations to plants. The photoperiodic changes are better indicators of seasons than other natural events including alteration of the temperature, humidity, etc., due to the fact that daily photoperiodic information has not changed during evolution. As noted above, since UV or green wavelengths promote, while blue or far red inhibit plant melatonin biosynthesis, the effects of other wavelengths on melatonin biosynthesis in plants should also be considered likely.

In addition to light wavelengths and intensities, abiotic stress is also a major regulator of melatonin biosynthesis in plants. High temperature, cold, drought, high salinity, and lead or cadmium pollution all positively regulate melatonin biosynthesis in a variety of plants (Arnao and Hernández-Ruiz, 2009; Tal *et al.*, 2011; Reiter *et al.*, 2015; K. Zhang *et al.*, 2019). These stressors are all likely to enhance reactive oxygen species (ROS) and reactive nitrogen species (NOS) production which results in oxidative stress. If this oxidative damage is not properly processed, it would result in even greater cell damage and death. To survive in hostile environments, plants have developed very strong antioxidative defense mechanisms. One of these mechanisms is to increase their antioxidant production. Melatonin is a highly effective antioxidant compared with some others (Tan *et al.*, 1993). Melatonin scavenges a spectrum of ROS and RNS with higher efficiency than other antioxidants (Tan *et al.*, 2007b). Under stressful conditions, plants will naturally enhance their melatonin production for protective purposes. This response is rooted in the evolutionary process. It has been shown that photosynthetic unicellular organisms (Hardeland, 1999), macroalgae (Tal *et al.*, 2011), fungi, and even bacteria (Jiao *et al.*, 2016; Fracassetti *et al.*, 2020) share a similar response with plants under oxidative stress conditions. Mechanistic studies have revealed that this response occurs at the level of gene expression. These stressors significantly up-regulate

mRNA and protein levels of both *SNAT* and *ASMT/COMT* in plants (Byeon and Back, 2014a; Liu *et al.*, 2017). The knockout or knockdown of these genes impedes the stress-induced positive melatonin response, while the overexpression of these genes enhances this response (Lee *et al.*, 2015; Byeon and Back, 2016; K. Lee and Back, 2017). There may be some exceptions. For example, in rice treated with cadmium, the expression of *SNAT* and *COMT* is down-regulated with the up-regulation of *TDC* and *T5H* and an increased melatonin level (Byeon *et al.*, 2015a). The upstream elements that regulate this stress-induced melatonin synthetic gene expression are currently unknown but at least involve the mitogen-activated protein kinase (MAPK) pathway (H.Y. Lee and Back, 2017; Gao *et al.*, 2019).

Location of melatonin biosynthesis in plants and its origin

The exact sites of melatonin biosynthesis in plant are an intriguing area to be further explored. There had been no convincing studies which attempted to identify the cellular location of melatonin biosynthesis until recently. The conventional notion was that melatonin is synthesized in the cytosol of all cells, no matter whether they were animals or plants. Based on the evolutionary evidence and the experimental observations that mitochondrial melatonin levels are very high (Venegas *et al.*, 2012; Tan *et al.*, 2013) have hypothesized that mitochondria and chloroplasts are the major sites of melatonin biosynthesis. Consistent with this hypothesis, one of the rate-limiting enzymes in melatonin biosynthesis, *SNAT*, has been found to localize in chloroplasts, whereas *ASMT* is present in the cytoplasm (Byeon *et al.*, 2014). The evidence indicates that at least the key step for melatonin biosynthesis occurs in the chloroplast. At that time, the authors deduced that *N*-acetylserotonin is synthesized in the chloroplasts and then transported into the cytoplasm for *O*-methylation to form melatonin. Conversely, it has been observed that the 5-hydroxytryptamine (serotonin) is methoxylated by either *ASMT* or *COMT* to form methoxytryptamine in the cytoplasm. This observation provides for the possibility that this methoxytryptamine may be transported into the chloroplast for acetylation by *SNAT* to form melatonin since *SNAT* is exclusively present in chloroplasts (Lee *et al.*, 2014). Following the introduction of the alternative melatonin pathway, namely to form methoxytryptamine from serotonin but not *N*-acetylserotonin as the dominant intermediate in plants, this pathway further supports the possibility of melatonin being finally produced in chloroplasts (Fig. 2).

Further study has found that *COMT* can also be overexpressed in the chloroplast. The overexpression of *COMT* in chloroplasts significantly increases melatonin production, while *COMT* overexpression in the cytoplasm failed to improve the quantity of melatonin formed (Choi *et al.*, 2017). This evidence proved the importance of the coordinated work of *COMT* and *SNAT* inside the chloroplast for melatonin biosynthesis. The most convincing results come from a study by Zheng *et al.* (2017a). They added serotonin to

purified apple chloroplasts and found that these chloroplasts generated melatonin in a dose–response manner. *In vivo* fluorescence and western blots both confirmed that the isoform of apple ASMT (MzASMT9) was localized in the chloroplasts. Actually, it was mainly present in the thylakoids of the chloroplasts. The overexpression of cloned *MzASMT9* in *Arabidopsis* results in enhanced melatonin production and elevated tolerance to salt stress. By using advanced technologies, additional data have confirmed that chloroplasts are the sites for melatonin biosynthesis since SNAT is exclusively present in these organelles (Yu *et al.*, 2019; Wang *et al.*, 2020).

In addition to chloroplasts, mitochondria also have the capacity to synthesize melatonin in plants. Wang *et al.* (2017) observed that isolated apple mitochondria generate melatonin, and an apple SNAT isoform, MzSNAT5, is present in the mitochondria of both *Arabidopsis* protoplasts and apple callus cells. Based on the theory of endosymbiosis (Sagan, 1967), the precursors of mitochondria are α -proteobacteria and the precursors of chloroplasts are cyanobacteria. We hypothesized that these organelles inherited the melatonin synthetic machinery from their prokaryotic ancestors (Tan *et al.*, 2013); thus, these two organelles should have different origins of their SNATs. Phylogenetic analysis showed that evolutionarily the *MzSNAT5* gene localized in apple mitochondria indeed has a closer relationship with animal SNAT than that of its chloroplast-localized MzSNAT9, or SNATs of rice, *Arabidopsis*, and cyanobacteria (Fig. 3); this suggests that the origin of MzSNAT5 might be similar to that of SNAT in animals. The results of multiple alignments also showed that MzSNAT5 had the *N*-acetyltransferase functional domain, indicating that it belongs to the *N*-acetyltransferase family of animals (Wang *et al.*, 2017).

It seems that these two melatonin-generating sites can crosstalk to maintain a stable supply of melatonin in plants. For example, Sekiguchi mutant rice completely lacks T5H activity (Park *et al.*, 2012). Theoretically, this plant cannot generate 5-hydroxytryptamine (serotonin) from tryptamine (Fig. 4). As a result, it should not produce melatonin.

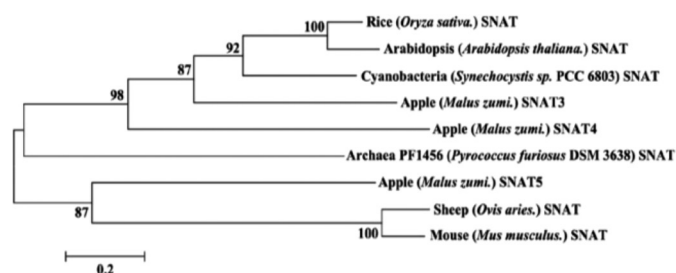


Fig. 3. The phylogenetic tree of MzSNAT5. The phylogenetic tree was constructed using the Neighbor–Joining method and a bootstrap test with 1000 iterations, using MEGA5.2 software. The GenBank accession numbers are NC_003413.1 (archaea PF1456, *Pyrococcus furiosus* DSM 3638, SNAT), NP_442603 (cyanobacteria, *Synechocystis* sp. PCC 6803, SNAT), AK059369 (rice, *Oryza sativa*, SNAT), ABD19662 (*Arabidopsis*, *Arabidopsis thaliana*, SNAT), NP_001009461 (sheep, *Ovis aries*, SNAT), NM_009591 (mouse, *Mus musculus*, SNAT), KJ156532 (apple, *M. zumi*, SNAT3), KJ156533 (apple, *M. zumi*, SNAT4), and KJ156534 (apple, *M. zumi*, SNAT5) (this figure was adapted from Wang *et al.*, 2017, with permission).

The fact is that the Sekiguchi rice still produces melatonin although the level is substantially lower than that of its wild type. Further analysis showed that Sekiguchi rice switched the melatonin synthetic pathway from the plant type to the animal type, which was indicated by the increased production of 5-hydroxytryptophan (Fig. 1). 5-Hydroxytryptophan is the product of tryptophan hydroxylase (dominant in animals, different from tryptamine 5-hydroxylase which is dominant in plants). 5-Hydroxytryptophan is decarboxylated to serotonin and avoids the T5H deficiency for melatonin biosynthesis in Sekiguchi rice. We speculate that this reaction probably occurs in the mitochondria. It seems that under normal conditions, plants synthesize melatonin in chloroplasts and, if this pathway is blocked, melatonin biosynthesis is switched to the mitochondria (Fig. 4).

The benefits of melatonin biosynthesis in chloroplasts and mitochondria are obvious. First, acetyl-CoA is synthesized and present at high levels in both organelles. Acetyl-CoA is the co-factor (substrate) of melatonin biosynthesis (Reiter *et al.*, 2019). From a substrate availability point of view, melatonin biosynthesis in chloroplasts and mitochondria is more efficient than its biosynthesis at other sites in the cells. Secondly, the major sources of ROS are from these two organelles and they face more oxidative stress than other cellular structures; thus, the

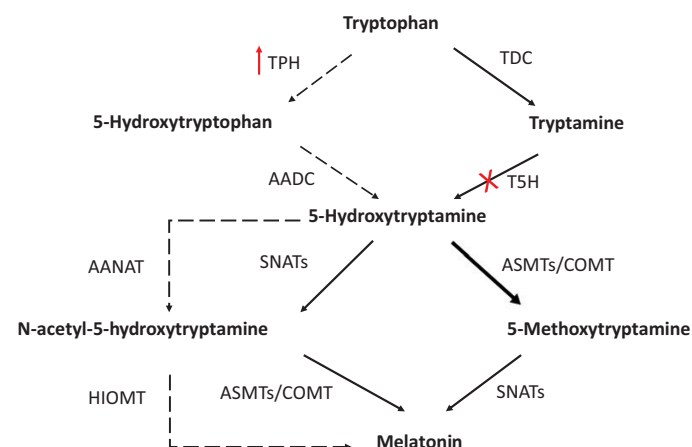


Fig. 4. The potentially switched pathways of melatonin biosynthesis from chloroplasts to mitochondria in plants. Solid arrows illustrate the melatonin biosynthetic pathway in chloroplasts. Broken arrows illustrate the melatonin biosynthetic pathway in mitochondria. Under normal conditions, chloroplasts are the major sites for melatonin biosynthesis. When this pathway is blocked, such as in the Sekiguchi rice, as indicated by the red cross, melatonin biosynthesis switches to the mitochondria, indicated by the red arrow. We speculate that these two pathways have independent enzyme systems which may have different origins. The enzymes in the mitochondrial pathway are similar to the enzymes found in animals, as shown in Fig. 1. TDC, tryptophan decarboxylase; TPH, tryptamine 5-hydroxylase; SNATs, several different serotonin *N*-acetyltransferases; ASMTs, several different *N*-acetylserotonin-*O*-methyltransferases (plant type SNATs and ASMTs seem to have different origins from those in animals); COMT, caffeic acid *O*-methyltransferase; T5H, tryptophan 5-hydroxylase; AADC, aromatic amino acid decarboxylase; AANAT, arylalkylamine *N*-acetyltransferase (also known as arylamine *N*-acetyltransferase which is selective for indole-ethylamines as its substrate); and HIOMT, hydroxyindole-*O*-methyltransferase/also known as *N*-acetylserotonin *O*-methyltransferase.

locally synthesized melatonin can produce on-site protective effects in these critical organelles.

The versatility of melatonin synthetic enzymes in plants and its biological significance

Tryptophan is considered as the starting material for melatonin biosynthesis (in animals this is the case since they cannot synthesize tryptophan, and tryptophan is acquired only via the diet). Plants can synthesize tryptophan *de novo*; thus, there are several precursors formed before tryptophan. It is accepted that SNAT or ASMT are the rate-limiting enzymes for melatonin biosynthesis in both animals and plants. In this section, the focus is directed on these two enzymes. In animals, no isoforms of either SNAT or ASMT have been found. It is reported that an arylamine *N*-acetyltransferase (NAT), particularly NAT1 or NAT2, may be present in mammals (Gaudet *et al.*, 1993a, c). NAT is different from AANAT/SANT. It does not specifically transfer the acetyl group to serotonin as does AANAT/SNAT but acetylates nitrogen or oxygen atoms of aromatic amines, hydrazines, and *N*-hydroxylamines. However, in hamster skin, NAT2 has the capacity to convert serotonin to *N*-acetylserotonin, as does AANAT (Gaudet *et al.*, 1993b), while under an AANAT/SNAT deficiency such as in melatonin-deficient mice (C57BL/6), NAT1 rather than NAT2 acetylates serotonin and participates in melatonin biosynthesis (Slominski *et al.*, 2003). This explains why the truncated AANAT C57BL/6 mice still produce a considerable amount of melatonin (Vivien-Roels *et al.*, 1998; Gómez-Corvera *et al.*, 2009).

In contrast to animals, many isoforms of SNAT and ASMT have been identified in different plants. These isoforms are listed in Table 1.

Interestingly, isoforms of SNAT or ASMT are not the homologs of those in animals. This indicates that they probably do not share the same origins. We have hypothesized that SNAT and ASMT of animals may be horizontally transferred from α -proteobacteria while SNAT and ASMT of plants were acquired from the cyanobacteria (Tan *et al.*, 2013). Thus, in some aspects including enzymatic kinetics, they exhibit major differences. For example, the suitable temperatures for the enzymatic activities of SNAT and ASMT in animals have relatively narrow margins from 25 °C to 45 °C (Ganguly *et al.*, 2001). However, these margins are greater, from 4 °C to 95 °C, for SNAT and ASMT of plants (Tan *et al.*, 2000; Byeon *et al.*, 2014; Wang *et al.*, 2017; Yu *et al.*, 2019). This temperature-tolerating feature in some plants also indicates that they face more hostile environments than those of animals due to their lack of mobility which prohibits avoidance of insults. Therefore, to survive and thrive requires that they be fit for the unpredictable environmental alterations (Reiter *et al.*, 2015). In plants, even isoforms of SNATs in the same plant show a great phylogenetic distance from their origins; for example, in rice, OsSNAT1 and OsSNAT2 have very little similarity in DNA sequence and also exhibit distinct enzymatic kinetics (Byeon *et al.*, 2016b). These differences seem to make each isoform of the

enzyme responsive to different environmental cues. For example, in apple plant, MdASMT9 mainly responds to far red light while MdASMT7 is exclusively responsive to blue light irradiation (H. Zhang *et al.*, 2019). Another example are the isoforms of ASMT in tomato. At least 14 isoforms of ASMTs (SIASMT1–SIASMT14) have been found in tomato plant (*Solanum lycopersicum*) (Table 1). They are distributed in different parts of the plant and exhibit different responses to a variety of pathogens (Liu *et al.*, 2017).

The versatility of melatonin synthetic enzymes in plants appears to be acquired from potentially multiple origins by horizontal gene transfer; in addition, evolution further enriches their variations. In terms of the versatility of plant SNATs, it seems that the catalytic core of this enzyme has been primarily conserved during the course of evolution and the alterations primarily occurred in its chloroplast transit peptides. It is estimated that the chloroplast transit peptides of plant SNATs were probably acquired 1500 million years ago at the appearance of unicellular green algae; thereafter, the length of these transit peptides progressively increased until vascular plants emerged ~450 million years ago (Byeon *et al.*, 2015c). The multiple origins and the evolutionary alterations as well as the splice variants contributed to the variabilities in melatonin synthetic enzymes in plants. In turn, these variations provide plants with a greater capacity to synthesize melatonin in a variety of environmental conditions including cold, heat, drought, waterlogging, and soil pollution. Melatonin, as the first-line defense antioxidant and plant stimulator, increases the tolerance of plants to these insults and promotes their ability to survive and thrive (Reiter *et al.*, 2015).

Melatonin metabolism in plants and the evolutionary consequences

Melatonin metabolism in animals has been extensively studied and is highly complicated, including enzymatic, pseudoenzymatic, and non-enzymatic processes. Thus, many metabolites can be generated via different processes. Making things more complicated is that the different processes can also generate the same product. For example, indolamine 2,3 dioxygenase (IOD) catalyzes melatonin to form N^1 -acetyl- N^2 -formyl-5-methoxykynuramine (AFMK). The pseudoenzymatic reactions of melatonin with cytochrome *c* or hemoglobin also generate AFMK, and melatonin's interaction with the superoxide anion ($O_2^{\cdot-}$) or singlet oxygen also produces AFMK. The complexity has been extensively reviewed by Tan *et al.* (2007b).

Studies on melatonin metabolism in plants were initiated only recently. The major obstacles for this research are the difficulties in extracting the specific melatonin metabolites from plant tissues and the questionable stabilities of these metabolites. As methodologies advance, these difficulties will be conquered and additional research in this field is expected.

The first melatonin metabolite identified in the plants was AFMK, which was found in the aquatic plant water hyacinth (Tan *et al.*, 2007a). In this plant, AFMK showed a similar circadian rhythm to melatonin but had a short phase delay after

Table 1. The list of isoforms of SNAT and ASMT in plants

Species	SNAT	ASMT/COMT	% homology to cynobacteria	Reference
Cyanobacterium (<i>Synechocystis</i> sp PCC 6803)	cSNAT		100	Byeon <i>et al.</i> (2013)
Green alga (<i>Chlamydomonas reinhardtii</i>)	CrAANAT		N/A	Okazaki <i>et al.</i> (2009)
Rice (<i>Oryza sativa</i>)	GNAT5		56	Kang <i>et al.</i> (2013)
Rice (<i>Oryza sativa</i>)	OsNAT1			Byeon <i>et al.</i> (2016a)
	OsNAT2			
Rice (<i>Oryza sativa</i>)		OsASMT15		Kang <i>et al.</i> (2011)
Rice (<i>Oryza sativa</i>)		OsASMT1 OsASMT2		Byeon and Back (2014a)
		OsASMT3		
<i>Arabidopsis thaliana</i>		AtASMT		Byeon and Back (2014b)
Alga (<i>Pyropia yezoensis</i>)	PySNAT		50	Byeon and Back (2014c)
Loblolly pine (<i>Pinus teada</i>)	PtSNAT		40	Park <i>et al.</i> (2014)
<i>Arabidopsis thaliana</i>	AtSNAT	AtCOMT	68% homology with rice SNAT	Lee <i>et al.</i> (2014)
Apple (<i>Malus domestica</i>)	MdAANAT1	MdASMT1 MdASMT3	% homology with rice SNAT	Lei <i>et al.</i> (2013)
Borkh. cv. Red)	MdAANAT2	MdASMT5 MdASMT7	MdAANAT1 (52.82%)	
	MdAANAT3 MdAANAT4		MdAANAT2 (41.54%)	
	MdAANAT5		MdAANAT3 (24.42%) MdAANAT4 (15.99%) MdAANAT5 (5.30%)	
Apple (<i>Malus zumi</i>)		MdASMT9	N/A	Zheng <i>et al.</i> , (2017a)
Tomato (<i>Solanum lycopersicum</i>)		SIASMT01	N/A	Liu <i>et al.</i> (2017)
		SIASMT02		
		SIASMT03		
		SIASMT04		
		SIASMT05		
		SIASMT06		
		SIASMT07		
		SIASMT08		
		SIASMT09		
		SIASMT10		
		SIASMT11		
		SIASMT12		
		SIASMT13		
		SIASMT14		
Grape (<i>Vitis vinifera</i> L.)	VvSNAT2		55% homology with rice SNAT2	Yu <i>et al.</i> 92019)
Tomato (<i>Solanum lycopersicum</i> L.)	SISNAT		71% homology with rice SNAT1	(Wang <i>et al.</i> (2020)

the melatonin peak. This indicated that a portion of melatonin was converted to AFMK. As we mentioned above, AFMK can be formed by different melatonin metabolic processes in animals and it has not been possible to deduce which process is related to plant AFMK formation. Until now, no homolog of the animal IDO has been found in plants. We speculate that plant AFMK is the product of melatonin interaction with $O_2^{\cdot-}$ and singlet oxygen since both are generated in large amounts during photosynthesis.

One function of melatonin is to detoxify these ROS to preserve an intact photosynthetic system. In addition, hydroxylated melatonin has been identified in plants. These include 2-, 4-, and 6-hydroxymelatonin. The levels of 4- and 6-hydroxymelatonin in plants are low. Unexpectedly, a large quantity of 2-hydroxymelatonin is found in rice. Its level is much higher than that of melatonin. The average ratio of 2-hydroxymelatonin to melatonin in rice is ~368:1 (Byeon *et al.*, 2015b). It seems that 2-hydroxymelatonin is the predominant indolamine rather than melatonin in this plant. Its synthetic enzyme has been identified and cloned: melatonin 2-hydroxylase (M2H) (Byeon and Back, 2015).

Similar to other melatonin synthetic enzymes, M2H also has several isoforms and these isoforms exhibit different responses to environmental challenges (Byeon *et al.*, 2015a). The protective effects of 2-hydroxymelatonin on abiotic stressors in plants seem stronger than those of melatonin. The results show that melatonin *per se* can only protect plants against a single abiotic stress, for example cold or drought; however, 2-hydroxymelatonin protects plant from joint or multiple abiotic stresses such as cold plus drought (Lee and Back, 2016, 2019). Phylogenetic analysis indicates that the M2H gene evolved during the transition of aquatic plants to land plants, since the water plants lack this enzyme (Lee and Back, 2019). This is understandable due to the fact that land plants face more overlapping environmental insults than do water plants, especially cold combined with drought or heat combined with drought.

2-Hydroxymelatonin is also present in animals (Tan *et al.*, 2007b). M2H has not been reported in animals. Its production in animals is the result of melatonin interaction with ROS. In contrast, 6-hydroxymelatonin is the major metabolite of melatonin in animals and its production is via an enzymatic process

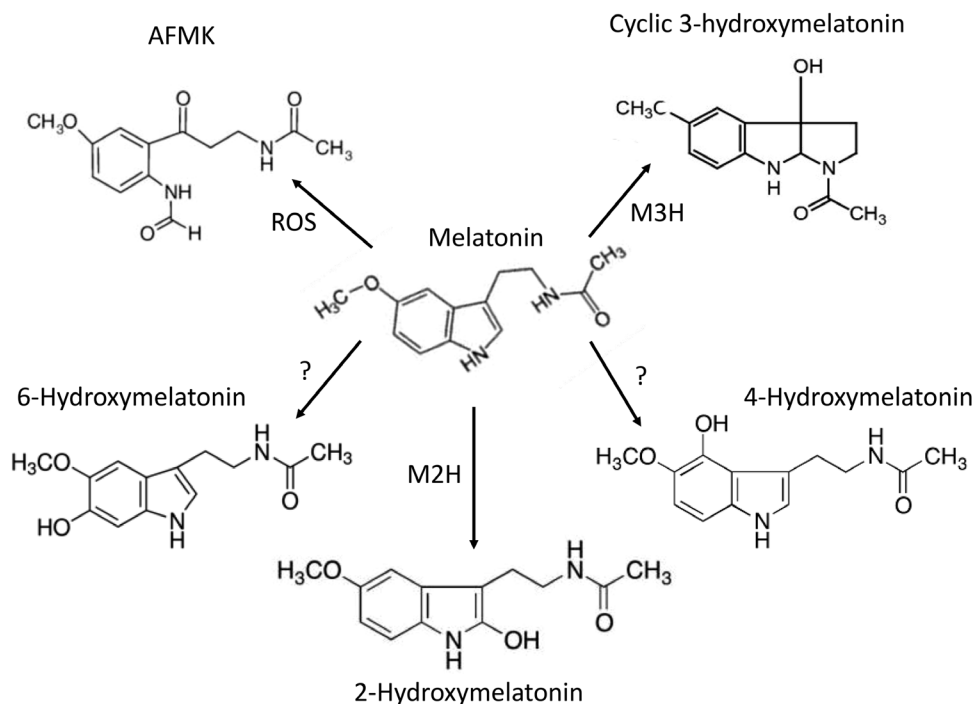


Fig. 5. Melatonin metabolic pathways in plants. AFMK, *N*¹-acetyl-*N*²-formyl-5-methoxykynurenamine; ROS, reactive oxygen species (in this case in particular they are superoxide anion and singlet oxygen); M3H, melatonin 3-hydroxylase; M2H, melatonin 2-hydroxylase; ?, unknown process.

involving CPY1A2 (Skene *et al.*, 2001). Recently, another melatonin metabolite, cyclic 3-hydroxymelatonin (c3OHM), has been identified in plants. This metabolite in animals is exclusively generated by the interaction of melatonin with the hydroxyl radical (Tan *et al.*, 1999). In contrast, c3OHM in plants is produced by the enzyme of melatonin 3-hydroxylase (M3H), which has been cloned from plants (Lee *et al.*, 2016). Its peak production occurs in darkness but without a regular circadian rhythm like melatonin. The function of c3OHM in plants is unclear. In rice, its production seems to be associated with tiller number (Choi and Back, 2019). Interestingly, if M2H gene expression is suppressed, the production of c3OHM is significantly increased. This suggests the potential association of c3OHM and 2-hydroxymelatonin for melatonin metabolism. The melatonin metabolic pathways in plants are illustrated in Fig. 5.

Concluding remarks

It has been more than two decades since the discovery of melatonin in plants and the existence of this molecule in plants is now common knowledge among scientists. Almost 800 articles have documented the biological activities of melatonin in plants, especially its protective effects against a variety of abiotic and biotic stressors which they encounter. Compared with that in animals, its biosynthesis and metabolism are not fully characterized due to the complexity of these processes. In contrast to animals, many isoforms of the melatonin synthetic enzymes, particularly the rate-limiting enzymes including SNATs and ASMTs/COMTs, are present in plants. Even in the same plant, these isoforms are quite different regarding their origins and enzymatic kinetics. The versatility of these isoforms

are thought to be responsible for the variety of environmental stimuli faced by the plants. For example, in the apple tree, one isoform of ASMT (MdASMT9) mainly responds to far red light irradiation while another isoform (MdASMT7) exclusively responds to blue light exposure. As a result, under many circumstances, the plants produce adequate levels of melatonin to fit the environmental changes by managing the expression of different melatonin synthetic isoforms.

The major melatonin synthetic sites in plants are the chloroplasts and mitochondria. These two organelles inherited the melatonin synthetic capacity from their ancestors (cyanobacteria and proteobacteria, respectively). They seem to have different sets of melatonin synthetic enzymes and also distinguishing synthetic processes; however, they crosstalk. For example, when the melatonin synthetic pathway is blocked in chloroplasts (enzyme mutation), melatonin biosynthesis shifts to the mitochondria to maintain melatonin production.

Melatonin metabolism in plants is also a new area under exploration. Several melatonin metabolites have been identified in plants, and the structures of these metabolites are the same as those found in animals. However, they do not share the same metabolic pathway as in animals. For example, c3OHM in animals is the product of melatonin interaction with the hydroxyl radical while in plants it is generated by a specific enzyme, M3H. The most interesting metabolite in plants is 2-hydroxymelatonin. In animals, the major melatonin metabolite is 6-hydroxymelatonin, and 2-hydroxymelatonin is a minor product. In plants, the level of 2-hydroxymelatonin is ~400-fold higher than that of melatonin. The protective effects of 2-hydroxymelatonin are also much stronger than that of melatonin. It is formed by the enzyme H2M which has not been found in animals. Phylogenetic analysis shows that this

enzyme probably evolved in the transition of aquatic plants to land plants since aquatic plants lack this enzyme. Since the research on melatonin metabolism in plants is in its early stage, additional melatonin metabolites and their pathways will probably be uncovered.

Author contributions

DXT initiated and drafted this article and RJR edited this article.

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