



# Melatonin Synthesis and Function: Evolutionary History in Animals and Plants

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Melatonin is an ancient molecule that can be traced back to the origin of life. Melatonin's initial function was likely that as a free radical scavenger. Melatonin presumably evolved in bacteria; it has been measured in both  $\alpha$ -proteobacteria and in photosynthetic cyanobacteria. In early evolution, bacteria were phagocytosed by primitive eukaryotes for their nutrient value. According to the endosymbiotic theory, the ingested bacteria eventually developed a symbiotic association with their host eukaryotes. The ingested  $\alpha$ -proteobacteria evolved into mitochondria while cyanobacteria became chloroplasts and both organelles retained their ability to produce melatonin. Since these organelles have persisted to the present day, all species that ever existed or currently exist may have or may continue to synthesize melatonin in their mitochondria (animals and plants) and chloroplasts (plants) where it functions as an antioxidant. Melatonin's other functions, including its multiple receptors, developed later in evolution. In present day animals, via receptor-mediated means, melatonin functions in the regulation of sleep, modulation of circadian rhythms, enhancement of immunity, as a multifunctional oncostatic agent, etc., while retaining its ability to reduce oxidative stress by processes that are, in part, receptor-independent. In plants, melatonin continues to function in reducing oxidative stress as well as in promoting seed germination and growth, improving stress resistance, stimulating the immune system and modulating circadian rhythms; a single melatonin receptor has been identified in land plants where it controls stomatal closure on leaves. The melatonin synthetic pathway varies somewhat between plants and animals. The amino acid, tryptophan, is the necessary precursor of melatonin in all taxa. In animals, tryptophan is initially hydroxylated to 5-hydroxytryptophan which is then decarboxylated with the formation of serotonin. Serotonin is either acetylated to *N*-acetylserotonin or it is methylated to form 5-methoxytryptamine; these products are either methylated or acetylated, respectively, to produce melatonin. In plants, tryptophan is first decarboxylated to tryptamine which is then hydroxylated to form serotonin.

**Keywords:** melatonin, evolution, antioxidant, biological rhythms, biosynthesis enzymes, endosymbiosis, regulation of melatonin

## INTRODUCTION

After its isolation and identification in the pineal gland of the cow, in subsequent years melatonin was identified in a wide variety of animals and plants (1–7). The extensive distribution of melatonin, especially in the primitive bacteria (cyanobacteria and  $\alpha$ -proteobacteria) indicates that the chemical is an ancient molecule that has been retained throughout the evolution of all organisms (8, 9) (Figure 1). It is speculated that melatonin evolved in bacteria prior to the process referred to as endosymbiosis. After cyanobacteria and  $\alpha$ -proteobacteria were engulfed by early prokaryotes, they eventually evolved into chloroplasts and mitochondria, respectively, such that all unicellular and multicellular organisms ultimately produce this critical indoleamine in these organelles (11–13). With organismal diversification, melatonin universally spread to all organisms and, accordingly, its functions, biosynthetic pathway, generation sites and biosynthetic regulation have also diverged.

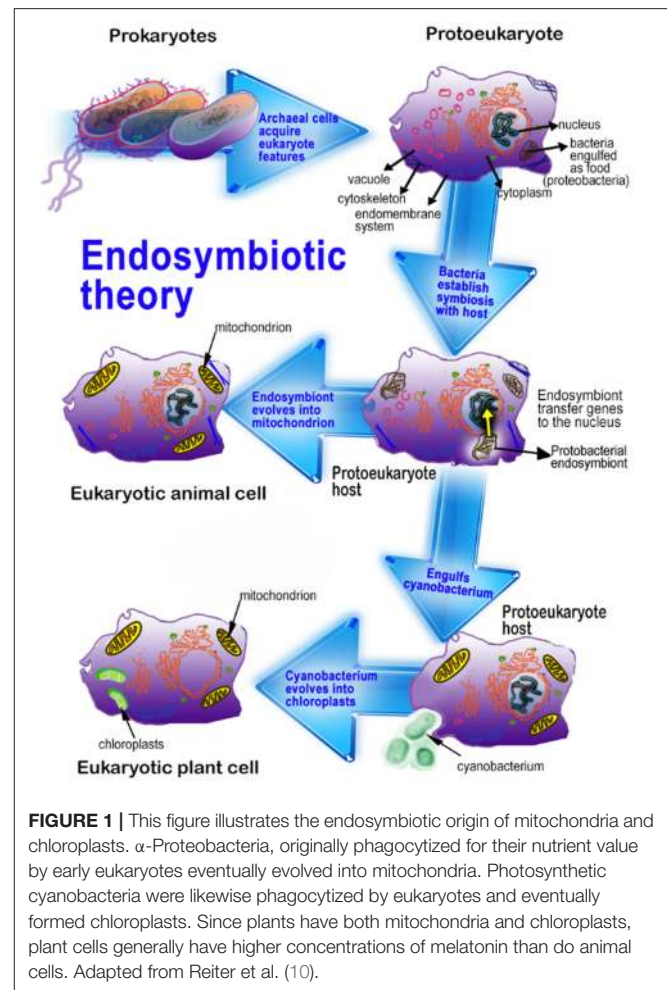
The proposed initial function of melatonin was to detoxify free radicals generated during the processes of photosynthesis and metabolism (8, 14–17). With the bio-divergence during organismal evolution, melatonin became a pleiotropic molecule that resists oxidation-related stress but also influences biological rhythms, suppresses inflammation, etc. (18–21).

The genes encoding the biosynthetic enzymes for melatonin have been identified in a number of species; these proteins potentially catalyze different substrates further determining the diverse biosynthetic routes of melatonin (22, 23). The multiple biosynthetic pathways provide direct evidence for melatonin's evolution. From unicellular to multicellular organisms, the subcellular localization of the enzymes related to melatonin biosynthesis may have changed somewhat (24, 25). The separation of the sites of subcellular localization may have been beneficial for the efficient control of melatonin synthesis (22, 26–28).

To exploit the multiple functions of melatonin, organisms developed various mechanisms to regulate its biosynthesis. For example, when faced with stress, activator protein-1 (AP-1), a transcription factor, promotes the synthesis of melatonin via up-regulating melatonin synthesis genes (29–34). Based on its evolutionary history, it seems clear that melatonin not only kept its primary function as an antioxidant but extended its functions to other important biological actions. Moreover, since it co-habitated with other key molecules such as sirtuins for eons, melatonin also learned to functionally cooperate with them (13).

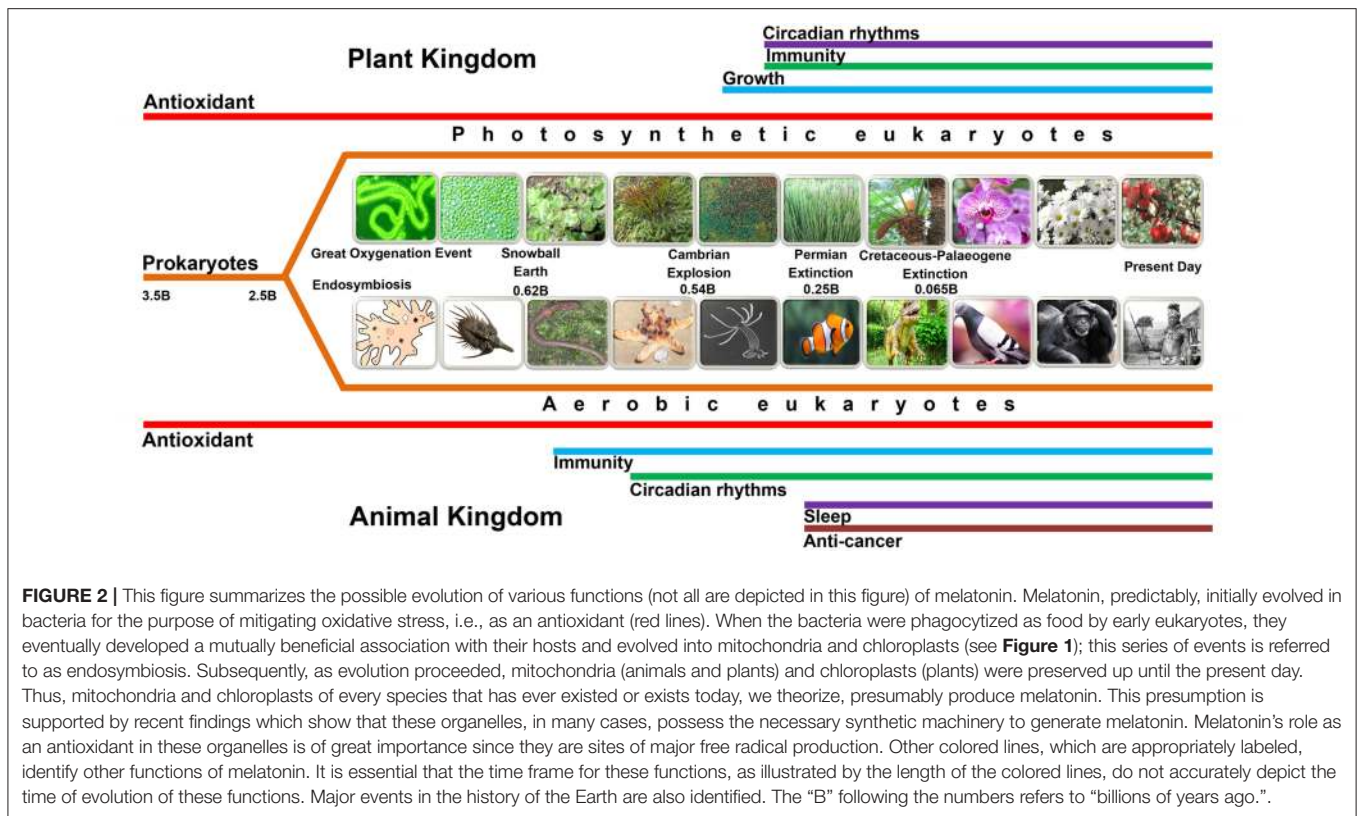
## FUNCTIONAL EVOLUTION OF MELATONIN

Molecular oxygen ( $O_2$ ) began to rise in the Earth's atmosphere (the Great Oxygenation Event) (Figure 2) around 2.5 billion years ago due to its persistent release from photosynthetic bacteria that had evolved an estimated billion years earlier (35–37). The rise of atmospheric  $O_2$  was a highly selective pressure for the evolution of organisms to use  $O_2$  as the basis of their metabolism (38, 39). During aerobic metabolism, reactive oxygen species (ROS) are invariably generated when  $O_2$  accepts leaked electrons from the electron transport chain (ETC) (24, 40, 41).



It is estimated that up to 4% of the  $O_2$  consumed by organisms during the aerobic metabolism eventually is reduced to ROS (42, 43). These large amounts of ROS are toxic to cells and organisms, inducing the development of complex and effective mechanisms to neutralize them; this initially occurred in early life forms such as bacteria and subsequent unicellular organisms (8, 44). To control oxidative stress, melatonin presumably emerged primarily as an antioxidant and free radical scavenger in early photosynthetic prokaryotic bacteria (12, 13, 45, 46). Melatonin has retained, until the present time and in all organisms, its ability to control oxidative stress that results from free radical production that occurs during photosynthesis and respiration (8, 47–49).

The special structure of melatonin determines its high efficiency in detoxifying free radicals based on its ability to donate an electron or a hydrogen atom, or depending on the radical type, potentially by other means as well (5, 15, 17). The superior antioxidant capacity of melatonin to limit oxidative stress is, at least partially, attributed to what is referred to as the cascade reaction which occurs when it generates derivatives that are likewise free radical scavengers (12, 15, 50–52). Melatonin interacts with a variety of ROS to produce cyclic 3-hydroxymelatonin and other melatonin metabolites,



e.g., *N1-acetyl-N2-formyl-5-methoxykynuramine* and *N-acetyl-5-methoxykynuramine* (53–56). These metabolites function as radical scavengers, sometimes even more aggressively than melatonin regarding their capacity to neutralize ROS (15, 57).

Despite its very long evolutionary history and its multiple functions, the chemical structure of melatonin has remained unchanged for billions of years (13). Moreover, melatonin may have been retained by all organisms even with their very wide biodiversification during evolution. This relates to the conservation of mitochondria and chloroplasts (or both) in most cells of all organisms. One exception is red blood cells which, during erythropoiesis, eject certain organelles including mitochondria.

As already noted, melatonin originally exclusively functioned as an antioxidant in primitive bacteria; however, over billions of years of evolution it became a pleiotropic molecule in multicellular organisms (**Figure 2**). The development of new functions of melatonin logically expanded the spectrum of its antioxidant activity (8). Regulation of biological rhythms is one of the key functional extensions. In early primitive unicellular plants and animals, more free radicals were produced during the photophase than during the scotophase; thus, larger amounts of melatonin were presumably consumed during the detoxification of excessively-produced free radicals during the day (58, 59) resulting in a diurnal rhythm of melatonin. In contrast to unicellular organisms, which directly perceive photoperiodic changes and synchronize their biological activities accordingly (60), complex multicellular organisms could no longer respond

directly to the photoperiodic changes (24). A signaling molecule, therefore, was required to ensure the photic information was transduced into a circadian signal for all cells (61). The alteration in melatonin levels in bacteria due to its differential utilization as a scavenger accurately reflected the photoperiodic changes of the light/dark cycle; theoretically, multiple organisms adopted the melatonin cycle as a signaling system for this purpose (24, 62, 63).

Multicellular organisms, therefore, co-opted a melatonin rhythm that already existed; but rather than depending on the metabolic utilization of melatonin to determine the cycle, they developed the subcellular framework to produce more melatonin during the scotophase than during the photophase, thereby ensuring a day:night melatonin cycle. In most vertebrates, but seemingly not all (64), this required the evolution of the pineal gland which is the location of the circadian production and, importantly, cyclic secretion of melatonin allowing all cells access to light:dark information. In present day animal species, the melatonin cycle, with highest levels at night, is the same regardless of the activity pattern of the species, i.e., nocturnal, diurnal or crepuscular. In addition to the neural connections between the eyes and the pineal gland, most clearly described in mammals, the pineal of some lower vertebrates responds directly to light stimuli (65). While the photic information has a direct impact on the electrophysiology of the organ, there is no proof that it alters melatonin production or secretion. In non-vertebrate animals and in plants, much less is known about the circadian production of melatonin (66, 67), although

these species do exhibit other circadian rhythms (68, 69) as well as possible 24 h fluctuations in melatonin, but sometimes the highest levels occur during the day (70).

Other actions of melatonin that have evolved and relate to the antioxidant activity of melatonin include retarding some age-related processes, anti-inflammatory activity, resisting neurodegenerative changes, the prevention of apoptosis in normal cells, and the preservation of mitochondrial and chloroplast physiology (8, 71–74) (**Figure 2**). These functions are associated, at least in part, with melatonin's ability to neutralize free radicals.

The actions of melatonin in different species have clearly diverged during the differentiation of major animal and plant taxa. These functions show a close relationship with the characteristics of the specific taxa. In mammals, melatonin is a molecule with hormonal properties (10, 75–77). The hormonal properties of melatonin are apparent in the regulation of seasonal reproductive activity, facilitation of sleep physiology, promotion of immunoresponsiveness, suppression of carcinogenesis, promotion of stem cell proliferation, anti-inflammation, and modulating aging (73, 78–83). Some of these actions are surely mandated by the interaction of melatonin with cell membrane receptors, i.e., MT1 and MT2, and/or perhaps with nuclear binding sites and are, therefore, considered hormonal (see below). For example, melatonin's ability to constrain cancer cell proliferation often involves membrane receptors (84, 85). There is also evidence, however, that receptor-independent actions such as its free radical generating capacity ("pro-oxidant"), an action possibly unique to cancer cells (86), also kills tumor cells (87, 88). Moreover, melatonin's multiple means by which it limits cancer metastases have not been unambiguously shown to be receptor-mediated (89). Thus, in mammals, melatonin is not a typical hormone and functions via receptor-dependent and receptor-independent means. On the evolutionary scale, the free radical scavenging properties, which continue to exist in mammalian cells, preceded the evolution of receptors for this indoleamine; thus, the initial actions of melatonin were receptor-independent.

In early non-mammalian vertebrates, the pineal organ directly responded to light that penetrated a cartilaginous plate overlying the epithalamus (90). This photic information was detected by photoreceptive elements similar to those in the retinas, with the electrical messages being sent to adjacent neural structures (91). In higher vertebrates, the pineal gland is no longer directly light-sensitive, although it does contain evolutionary morphological remnants of photoreceptive rods/cones (92), but it remains influenced by light and darkness via complex retina-suprachiasmatic nucleus-sympathetic neural connections (93).

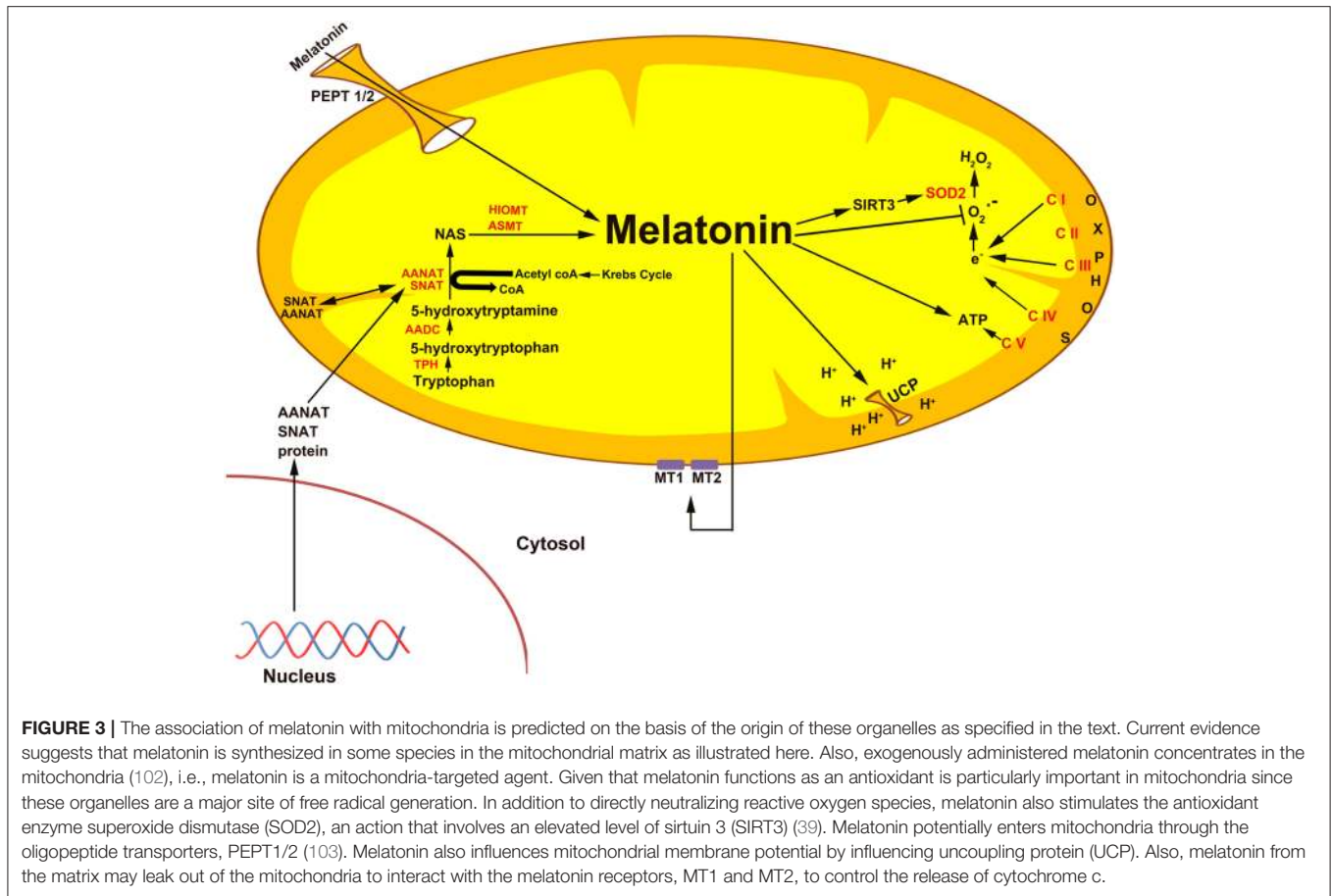
The mammalian pineal gland probably did not evolve as nor should it be strictly classified as an endocrine gland. Endocrine glands are typically regulated by the secretory products from other glands and exhibit either feedback or feedforward responses when contacted by these agents. Also, because of their primary regulation by hormones, hormone production and secretion typically are only modestly impacted by depriving endocrine glands of their sympathetic innervation. These features are in marked contrast with those of the pineal gland, where other

hormones have barely perceptible effects on pineal melatonin production (94, 95). The sympathetic denervation destroys the function of the pineal (96), while for other endocrine organs (e.g., anterior pituitary, thyroid gland) denervation is essentially inconsequential. Giving norepinephrine or isoproterenol to animals in which the pineal has been sympathetically denervated induces a rapid increase in pineal melatonin synthesis indicating that pineal synthetic processes are under the control of the nervous system rather than by hormones (97); there may, however, be some unique experimental conditions that perturb melatonin synthesis in the pineal gland independent of its innervation (98); how these actions are mediated remain unknown. For example, high melatonin production can be shifted to immune competent cells (99) under inflammatory conditions. This important observation was unanticipated and opens up the possibility that there may be other conditions under which melatonin synthesis is enhanced in other cell types.

Another argument against melatonin being exclusively a hormone comes from the observation that an estimated 99% of the melatonin in vertebrates is likely not produced in the pineal gland and is never released into the circulation. The discovery of melatonin in mitochondria, where it likely functions as a direct free radical scavenger and as an indirect antioxidant, means that the total quantity of melatonin synthesized in vertebrates is much greater than originally envisioned. In addition to functioning as a scavenger at the site at which it is produced, i.e., mitochondria, melatonin generated at the subcellular level may be locally released to function as a paracrine or autocrine agent (100). There is also preliminary evidence that mitochondria-produced melatonin is discharged from this organelle after which it interacts with receptors on the outer mitochondrial membrane where it may influence the release of cytochrome c (101) (**Figure 3**). In invertebrates (104) which lack a pineal gland, endogenous melatonin production may respond to external environmental alterations that do not involve the light:dark cycle.

In the plant kingdom, melatonin is demonstrated to be a multi-regulatory molecule with diverse functions in plant growth and development, such as seed protection and germination, root development, fruit ripening, and senescence (105–108). Compared to animals, plants face more environmental challenges because of their sessile nature. As a protection against these stresses, they rapidly unregulate melatonin synthesis which then functions in the protection against oxidative stress induced by these challenges (109). As mentioned, melatonin works independent of receptors when it clears ROS (110, 111). The major membrane melatonin receptors in animals, MT1 and MT2, activate different signaling cascades to improve or antagonize biological effects (112–115). To date, only one phytemelatonin receptor (CAND2/PMTR1) has been identified; it regulates stomatal closure via a  $H_2O_2$  and  $Ca^{2+}$  signaling transduction cascade (116). In addition, phytemelatonin can interact with unknown receptors with active  $H_2O_2/NO$  signaling pathways, and further improve plant stress tolerance by activating a variety of antioxidant enzymes, alleviating photosynthesis inhibition, and modulating transcription factors; these transcription factors are involved with stress resistance, chelating and promoting transport of heavy metals, or activating other stress-relevant





**FIGURE 3 |** The association of melatonin with mitochondria is predicted on the basis of the origin of these organelles as specified in the text. Current evidence suggests that melatonin is synthesized in some species in the mitochondrial matrix as illustrated here. Also, exogenously administered melatonin concentrates in the mitochondria (102), i.e., melatonin is a mitochondria-targeted agent. Given that melatonin functions as an antioxidant is particularly important in mitochondria since these organelles are a major site of free radical generation. In addition to directly neutralizing reactive oxygen species, melatonin also stimulates the antioxidant enzyme superoxide dismutase (SOD2), an action that involves an elevated level of sirtuin 3 (SIRT3) (39). Melatonin potentially enters mitochondria through the oligopeptide transporters, PEPT1/2 (103). Melatonin also influences mitochondrial membrane potential by influencing uncoupling protein (UCP). Also, melatonin from the matrix may leak out of the mitochondria to interact with the melatonin receptors, MT1 and MT2, to control the release of cytochrome c.

hormones such as salicylic acid, ethylene, and jasmonic acid (109, 117, 118). In animals, melatonin also binds directly to the catalytic site of quinone reductase 2 (QR2, E.C. 1.10.99.2), a cytosolic molecule, to modulate the activity of this enzyme; that modulation may be either up or down regulation (119, 120). Importantly, the change in QR2 activity may further play a key role in ROS generation or detoxification (121).

## ORIGIN OF MELATONIN RECEPTORS

The presumed original function of melatonin, i.e., as a direct free radical scavenger, required nothing of the cell except positioning melatonin in close proximity to where the bulk of the ROS are usually formed. Such positioning of an antioxidant is essential since free radicals have an extremely short half-life and instantaneously damage molecules in the immediate neighborhood of where they are formed. If a free radical scavenger is not properly situated, it cannot prevent the initial damage inflicted by a highly reactive radical. To accomplish this proper placement, evolution arranged for the uptake by and synthesis of melatonin in mitochondria (101, 102) and chloroplasts (108, 122), both major contributors to the total oxidative burden of cells.

In currently-surviving vertebrates, melatonin has a very extensive physiological toolkit. To broaden its functional repertoire, it was necessary for its binding sites/receptors and associated signaling transduction processes to also evolve. Many of the currently known activities of melatonin are mediated by G-protein coupled receptors in the membranes of animal cells (113, 123, 124). The best known receptors associated with cell membranes are members of the G-protein coupled receptor family (111, 125, 126); they are designated MT1 and MT2 (127–129).

Perhaps, of special relevance to the current discussion, is the finding that the MT1 receptor, generally considered to be confined to the limiting membrane of cells has also been recently associated with the outer membrane of mitochondria (101). According to the researchers who made this discovery, melatonin from the mitochondrial matrix diffuses out of these structures and interacts with MT1 receptors on the outer membrane of these organelles; they coined the term “automitocrine” to define this process. Via this receptor-mediated pathway, mitochondria-generated melatonin may control the release of cytochrome c from the matrix. This self-regulatory process has implications for apoptosis resulting from extensive free radical damage.

In addition to the well-characterized and highly relevant cell membrane receptors which are indispensable for a number of

melatonin's key functions, there are also binding sites in the cytosol (130) and in the nucleus (131–133). In the cytosol, the enzyme quinone reductase 2 (QR2) has been designated as receptor MT3 (134). The activity of this detoxifying enzyme may be related to some of the actions of melatonin in reducing oxidative damage. Melatonin also couples with calmodulin in the cytosol, an action that is reportedly linked to the cancer-inhibitory effect of the indoleamine (135, 136).

In an invertebrate, the crayfish (*Procambarus clarkii*), melatonin functions in the modulation of the reticular photoreceptor potential amplitude with the intensity change differing between the day and night (137). With the aid of the commonly used melatonin receptor blockers, the authors deduced that the actions of melatonin on visual photoreceptors are mediated by a site reminiscent of the mammalian MT2 receptor. In another crustacean, the crab (*Neohelice granulata*), some of the metabolic actions of melatonin are inhibited by luzindole, a classic MT1/MT2 receptor blocker (138).

In the honey bee, *Apis cerana*, a melatonin receptor with the typical seven transmembrane domains has been characterized (139); the authors named it AccMTNR1A. It mediates the response of this species to cold stress, a feature that is common with that of plants. Silencing of the receptor also interfered with the transcription of some antioxidant signaling pathways. This illustrates that antioxidant enzyme activity may be regulated in the honey bee as they are in plants (105). Collectively, the data from invertebrates show that not only do they produce melatonin but they probably have receptors that mediate some of its actions. Fossil records indicate that insects evolved more than 400 million years ago, so the melatonin receptor has likely existed for at least the same time duration.

*Tetrahymena* are nuclear dimorphic, unicellular, ciliated eukaryotes. Like many other eukaryotes, *Tetrahymena* feed on bacteria so this evolutionarily-early organism would be expected, due to endosymbiosis, to have retained the melatonin synthetic potential of the engulfed bacteria. There is evidence that *Tetrahymena* contain biogenic amines including possibly melatonin (140). Whether this species possesses melatonin binding sites/receptors has not been established. If they are found to contain melatonin-binding molecules, it would show that some type of melatonin receptor evolved about one million years after melatonin arose. As it currently stands, there is little information related to when melatonin receptors originated.

As noted above, melatonin was discovered in land plants about 25 years ago (2, 3) where it functions as an antioxidant in a receptor-independent manner (141) as in animals. The first phytomelatonin receptor, designated CAND2/PMTR1, was described in *Arabidopsis thaliana* (116). This receptor is located on epithelial cells which govern the closure of the leaf stomata. For this process, the signal transduction cascade involves G $\alpha$  subunit-activated H<sub>2</sub>O<sub>2</sub> production and Ca<sup>2+</sup> signaling. It is estimated that land plants came into existence on Earth about 200 million years ago. Whether the first land plant that appeared or any plants that preceded them possessed melatonin receptors is unknown.

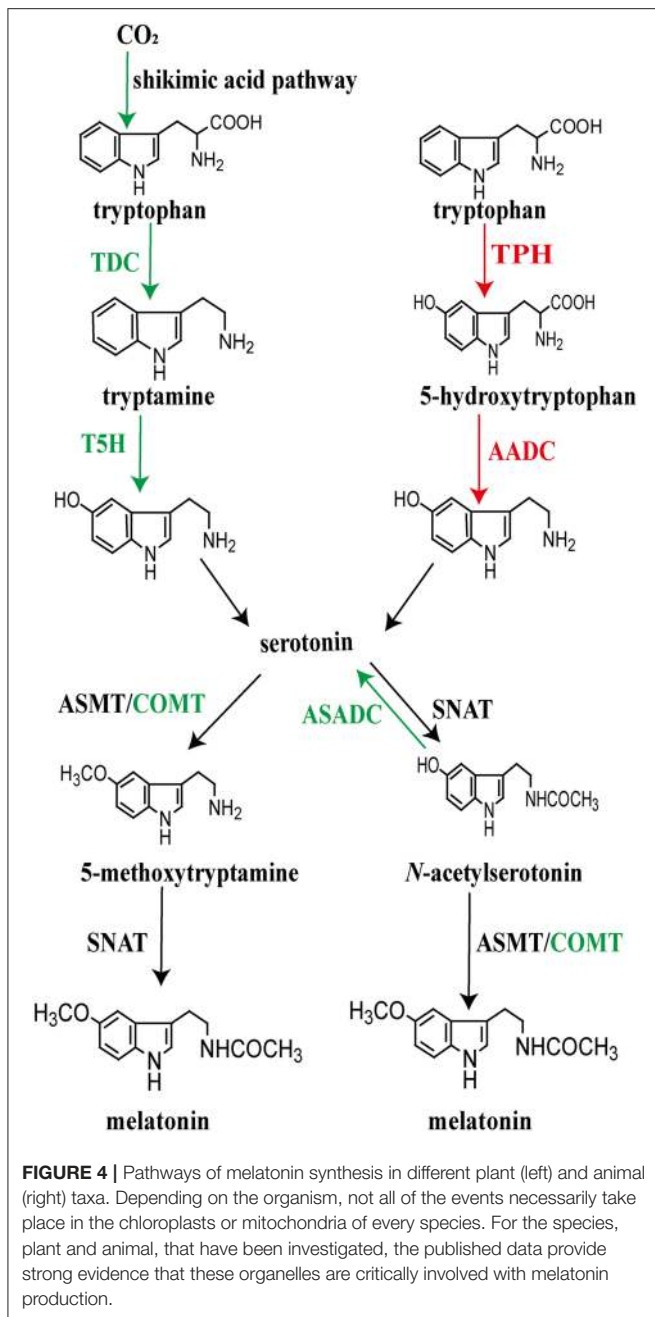
In addition to the direct scavenging of radicals and radical products by melatonin, land plants also have many of the antioxidant enzymes that exist in animals. In plants, the enzymes are melatonin-influenced and are quickly upregulated when the plant is exposed to an abiotic stress, e.g., draft, heat, cold, toxin, etc. (105, 142). It is presumed that this upregulation involves melatonin receptors as is likely the case for animals as well.

For additional details on the pharmacological characterization, cloning and signal transduction pathways of melatonin receptors, the reader is referred to comprehensive reviews of this subject by the groups of Jockers et al. (114, 115), Oishi et al. (130), Tosini et al. (143), Liu et al. (128), Dubocovich (114). Obviously, melatonin receptors developed subsequent to the evolution of melatonin. Based on what is currently known of their distribution, they probably originated with the origin of multicellular organisms, both plant and animal.

## DIVERGENCE OF MELATONIN BIOSYNTHESIS IN DIFFERENT TAXA

Melatonin is believed to exist in all living organisms including bacteria, yeasts, fungi, animals, and plants (144, 145). This molecule is formed exclusively from the amino acid tryptophan (146). While tryptophan is consumed in the diet, it can also be synthesized via the shikimic acid pathway starting with D-erythrose-4-phosphate, phosphoenolpyruvate, or carbon dioxide in some species (147). With the evolution of organisms (apart from animals) bacteria, fungi, and plants retained the ability to synthesize tryptophan (24). Conversely, mammals only attain tryptophan, an essential amino acid, during food intake. A reduction of tryptophan leads to the marked lowering of melatonin production in animals compared to that in plants (145, 148). Since plants cannot behaviorally avoid extremely stressful conditions, they require extra protection from stress; hence, the biosynthesis of tryptophan is presumably retained in plants to ensure that melatonin is available for relieving oxidative stress levels under environmentally-stressful conditions.

Beginning with tryptophan, melatonin biosynthesis includes four enzymatic steps in all organisms (22, 67). During its evolution lasting billions of years, the pattern of the melatonin synthesis became diversified (**Figure 4**). Tryptophan is first converted to serotonin which involves decarboxylation and hydroxylation. There are two strategies for the synthesis of serotonin that leads to melatonin production in different taxa. The biosynthetic pathway of serotonin in microorganisms and plants is different from that of vertebrates. Tryptophan is decarboxylated to tryptamine by tryptophan decarboxylase (TDC), followed by serotonin biosynthesis catalyzed by tryptamine 5-hydroxylase (T5H) in plants (149, 150). In contrast, rather than tryptophan decarboxylation being the initial step in serotonin production, animals first hydroxylate tryptophan using tryptophan hydroxylase (TPH) to form 5-hydroxytryptophan and then 5-hydroxytryptophan is decarboxylated by aromatic amino acid decarboxylase (AADC) to form serotonin (7).



Between tryptophan and melatonin, serotonin is a key intermediate after which the biosynthetic process utilizes two potential pathways, each of which includes two consecutive enzymatic steps to generate melatonin (15, 22). These steps catalyze serotonin to form the final product, melatonin; this involves serotonin *N*-acetyltransferase (NAT) and *N*-acetylserotonin *O*-methyltransferase (ASMT; formerly known as hydroxyindole-*O*-methyltransferase, HIOMT) (151–155). The penultima enzyme, NAT, plays a key role in the conversion of serotonin to *N*-acetylserotonin while the last enzyme, ASMT, catalyzes NAS to produce melatonin (153, 154, 156).

Alternatively, serotonin can be first *O*-methylated to 5-methoxytryptamine (5-MT) by ASMT; thereafter, 5-MT is *N*-acetylated by NAT to produce melatonin (7, 155). Differing from the formation of serotonin, the two alternative pathways for the conversion of serotonin to melatonin, likely occur in both plants and animals as well as in the microorganisms. However, different homologs of NAT have been detected between plants and animals, as well as ASMT in the two groups, revealing their different origins during evolution (122, 157–159). NAT seems to have originated independently as indicated by the few shared amino acid residues between animals and plants as well as between primitive cyanobacteria and archaea (24, 158). Thus, the enzymes controlling the biosynthetic steps of melatonin seem to have different origins at the emergence of melatonin; these enzymes further evolved divergently after endosymbiosis.

As already mentioned, the intermediate processes in melatonin production display a variety of differences among taxa. The present-day organisms possess diverse melatonin biosynthetic pathways. The enzymes that produce melatonin may play roles in catalyzing different substrates. Beyond the four key enzymes, other evolved enzymes are reported to directly participate in the biosynthesis of melatonin. This is supported by evidence that plants evolved caffeic acid *O*-methyltransferase (COMT), which is involved in the synthesis of 5-methoxytryptamine/melatonin by methylating serotonin/*N*-acetylserotonin (160). Apart from the classic enzymes strictly required for the melatonin biosynthetic pathways, Lee et al. (161) found that *N*-acetylserotonin can be converted to serotonin in rice seedlings by *N*-acetylserotonin deacetylase (ASDAC), which may result in a reduction in the content of melatonin (Figure 4).

## SUBCELLULAR LOCALIZATIONS OF ENZYMES ASSOCIATED WITH MELATONIN BIOSYNTHESIS FROM THE VIEW OF ENDSYMBIOSIS

Based on the endosymbiotic theory, when the early eukaryotic cells (having nuclei but no mitochondria) endocytosed  $\alpha$ -proteobacteria or cyanobacteria (Figure 1), rather than digesting these bacteria, the proto-eukaryotic cells developed a symbiotic association with them (162–164). The observations that the NAT protein, the rate-limiting enzyme of melatonin synthesis, is abundantly located in mitochondria of animals and chloroplasts of plants further support the different origins of the melatonin biosynthetic enzymes as a result of endosymbiosis (26, 165, 166). Furthermore, the DNA sequences and protein residues of cyanobacterium, a plant-type species, and rice are closely related, implying that the plant NAT gene was likely endosymbiotically-derived from cyanobacteria (167, 168). NAT genes of other eukaryotic organisms including fungi, invertebrates, and vertebrates seemingly evolved from *Rhodospirillum rubrum* (the presumed precursor of mitochondria) or closely related species since their NAT genes share similarity to some extent (24, 122, 169).

With endosymbiotic evolution, the function of melatonin synthesis was carried into multicellular organisms. Thus,

mitochondria and chloroplast, which resulted from the endosymbiosis of  $\alpha$ -proteobacteria or cyanobacteria, respectively, became the major melatonin generating subcellular organelle in both animals and plants (165, 166). In terms of function, melatonin produced in these two organelles most likely detoxifies excessive ROS and reactive nitrogen species (RNS) generated during oxidative phosphorylation and other metabolic actions (55, 73). The production of melatonin in mitochondria provides maximal on-site protection of these critical organelles (**Figure 4**). In plants, melatonin in chloroplasts provides a similar defense against oxidative stress with subsequent evolution after endosymbiosis. The melatonin-associated genes of the incorporated bacteria were gradually transferred from both mitochondria and chloroplasts to the nuclear genome of each host (24, 164–166, 170). While mitochondria and chloroplasts are considered major sites of melatonin synthesis, it does not preclude the possibility that some melatonin is not also formed in the cytosol ((171); also, see below).

With subsequent evolutionary processes, melatonin-related genes were modified by mutations and in response to natural selective pressures in different species (24, 155, 172). Specifically, the major structural differences of the melatonin synthases among phylogenetically distant species are the regulatory regions which could further influence the subcellular localization of these proteins (7, 24). At least in plants, the subcellular localization analysis documented that the rate-limiting enzyme for melatonin synthesis, NAT, is found in both chloroplasts and mitochondria (26). TPH, ASMT/COMT, and TDC/AADC locate in the cytoplasm while T5H is distributed in the endoplasmic reticulum (7, 22, 150, 154). This subcellular location of melatonin synthesis enzymes suggests that during evolution (**Figure 2**), the sites of melatonin synthesis became more diverse and extended to the cytoplasm and endoplasmic reticulum (22). This divergent distribution of melatonin production shows a good relationship with the transformation from prokaryotic cell to eukaryotic cell. Regarding the efficiency of melatonin biosynthesis, the present biosynthetic model is consistent with adequate substrate availability. For example, acetyl-CoA, a key substrate for melatonin production, is synthesized in the mitochondria through pyruvate dehydrogenase complex reaction (39, 173, 174). Furthermore, the different subcellular sites of melatonin synthetase avoid substrate competition by other enzymes preferring the same substrate (13, 22, 175). Thus, multiple subcellular sites of melatonin biosynthesis in both plants and animals could promote synthetic efficiency of this essential molecule (171).

## MULTIPLE MECHANISMS PRECISELY REGULATE MELATONIN BIOSYNTHESIS

The presence of melatonin at several sites correlates with its biological functions. As in animals, 24-h rhythms have been described in plants, e.g., in *Chenopodium*, which shows a nocturnal maximum growth rate around light/dark transition (176). Also, the dinoflagellate *Lingulodinium* and numerous other microalgae including chlorophytes (like plants, members of viridiplantae), exhibit robust circadian rhythms of melatonin

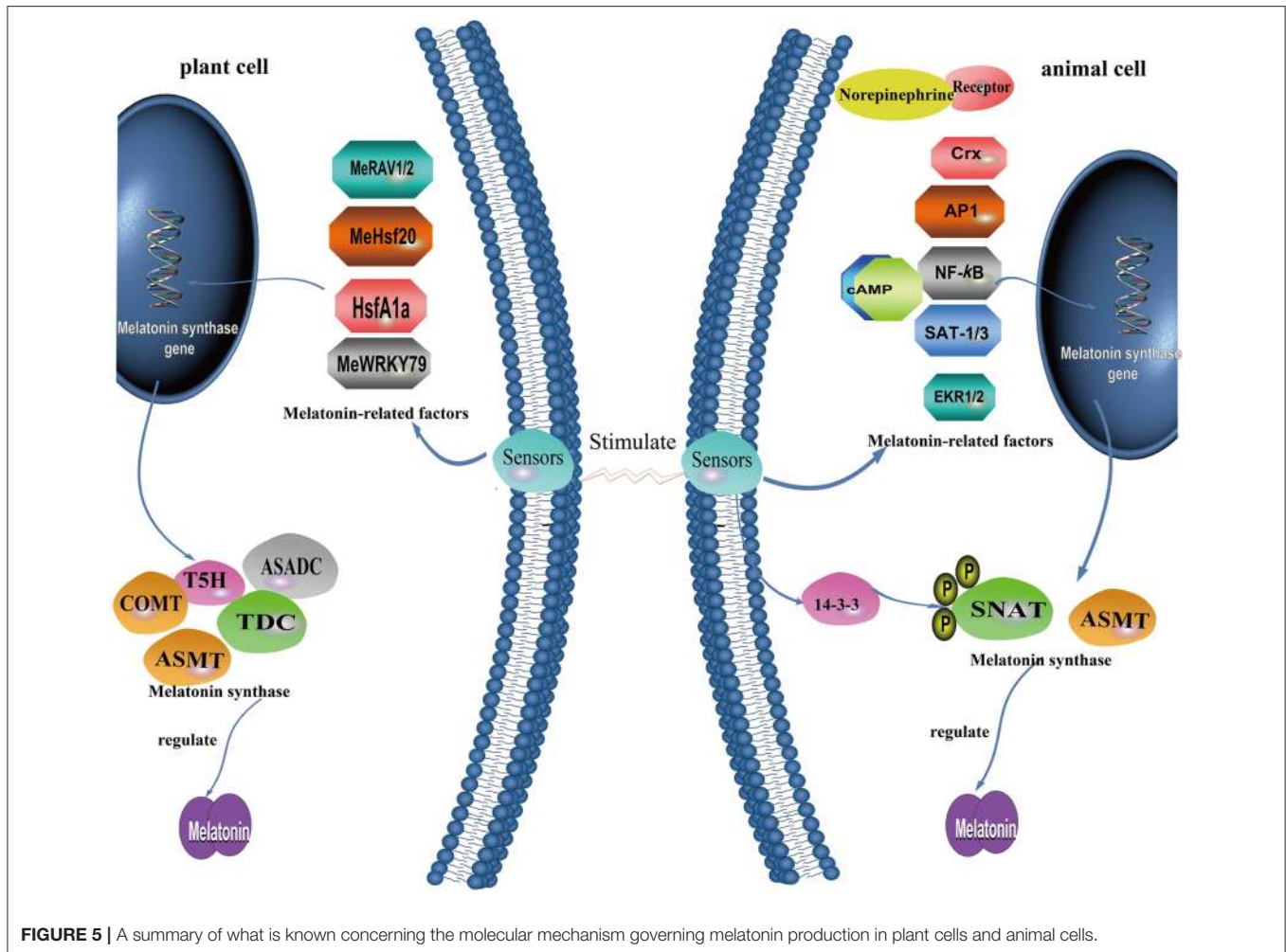
(177). In comparison to animal cells, plant cells contain much higher levels of melatonin, probably because they have two melatonin producing organelles, mitochondria and chloroplasts (77, 158, 178). In some species such as in *Glycyrrhiza uralensis*, cranberry and several medicinal herbs melatonin levels are reportedly several orders of magnitude higher than those in the serum of animals. Remarkably, the levels of melatonin in the pistachio nut may reach 230  $\mu\text{g/g}$  (15, 145, 179, 180). This may relate to the high environmental stress condition under which this plant normally grows. The immobility of plants results in them being subjected to more unavoidable environmental stressors, causing elevated ROS production and oxidative damage. Thus, they require additional protection from stressors by means of intrinsic mechanisms including high levels of endogenously-produced antioxidants, such as melatonin (158). This speculation is supported by the observation that a variety of environmental insults induce a dramatic increase in melatonin levels in plants (24, 141, 181). Under some conditions, stressful situations may also induce melatonin production in animals, e.g., physiological ischemia/reperfusion events (182). Also, plant cells generally have higher levels of melatonin than animal cells; this likely relates to the fact that plant cells have two sources of melatonin (mitochondria and chloroplasts) while animal cells have a single source.

The presence of melatonin in both plants and animals raises the question as to whether animals and plants have different mechanisms for modulating the biosynthesis of this indole-containing compound. Current data indicate that the regulatory mechanisms of melatonin synthesis are fundamentally different between animals and plants (**Figure 5**). In vertebrates, melatonin is referred to as the chemical expression of darkness (based only on pineal and blood levels); in general, in plants a day/night rhythm is less common, although not totally absent in some species, with the melatonin concentrations sometimes not varying much throughout the light:dark cycle (24, 183, 184). Hence, melatonin synthesis in some plants is non-rhythmic, as in the mitochondria of animals (101).

In vertebrates, light detection by the retinas suppresses the activity of NAT and melatonin production (185, 186). In plants, there is positive correlation between light intensity and melatonin levels with plants growing in habitats exposed to high light intensities, such as Mediterranean or alpine environments, usually having higher melatonin levels than the same or related species growing in other locations (187). In some species, melatonin was reported to be enhanced by darkness of even short duration, e.g., 1 h in rice seedlings (188), findings supported by an elevated ASMT expression in response to darkness (189). Moreover, heat-induced elevation of melatonin was antagonized by light in *Oryza* (188). In addition to these exceptions, the signal transduction pathways and regulatory mechanisms in vertebrates differ substantially from those in plants. The major regulatory components of melatonin synthesis in mammals, e.g., norepinephrine and its receptors, are not detected in plants and are unlikely to exist, indicating the loss of this pathway in the plants during evolution (24).

As noted above, the biosynthesis of melatonin is closely associated with four successive enzymes leading to the production of this compound. NAT usually is believed to





**FIGURE 5 |** A summary of what is known concerning the molecular mechanism governing melatonin production in plant cells and animal cells.

be the rate-limiting enzyme in pineal melatonin synthesis in vertebrates, although ASMT may limit this level around the nocturnal melatonin maximum (190). For plants, under most circumstances, NAT activity correlates well with the quantity of melatonin produced. In rice seedlings, however, melatonin was reported to be highest at the time of enhanced ASMT expression (191, 192). Typical for animals, the activities of NAT and melatonin production usually exhibit a good positive relationship (185, 193). The regulation of NAT in the pineal gland depends on the animal taxa examined. In many mammals, NAT gene expression in the pineal is either up- or down-regulated by the signals received from the suprachiasmatic nucleus (SCN) while in some species with a predominantly transcriptional control of NAT, its mRNA levels are stimulated up to two orders of magnitude (194). For primates and ungulates, NAT is mainly controlled post-translationally by phosphorylation and dephosphorylation, and stabilization of the phosphorylated form by a 14-3-3 protein (195). While the four successive enzymes positively regulate the pineal concentration of melatonin in all organisms, the overexpression of melatonin biosynthetic genes, such as *TDC*, *NAT*, and *ASMT* do not always lead to the accumulation of melatonin in plants (33, 122, 155, 159, 196, 197).

Recently, the *ASDAC* gene was found to catalyze the conversion of *N*-acetylserotonin to serotonin, a reverse reaction from the usual melatonin biosynthetic pathway in plants (161). Clearly, plants possess two genes encoding both NAT and ASDAC proteins, which impact the biosynthesis of melatonin; NAT favors melatonin synthesis, whereas ASDAC lowers melatonin levels.

Especially in plants, melatonin is maintained at a relatively constant level under normal conditions; however, it can be greatly and rapidly upregulated in response to unfavorable conditions such as cold, heat, salt, drought, oxidative and nutrient stress, and bacterial infection (142, 198–203). The underlying mechanisms for the rapid regulation of melatonin production have not been identified including the translation and post-translational regulation of melatonin synthesis enzymes, and the upstream transcription factors of these rate-limiting enzymes or isoenzymes (204, 205). Melatonin biosynthesis genes may have a role at the transcriptional level to control the content of melatonin (Figure 5). For this process, different taxa evolved divergently to work with other factors for self-development or coping with stressful conditions.

Activator protein-1 (AP-1) is a stress-responsive transcription factor that can be regulated by oxidative stress in many cell types (206). AP-1 seems to promote both NAT and ASMT activities to enhance melatonin synthesis. Structural analysis of the human *ASMT* gene promoter shows that it contains an AP-1 site at position−166 and similarly, there is an AP-1 transcription factor-binding site in the *AANAT* mouse gene (29, 30). Interestingly, stress uniformly stimulates glucocorticoid production in organisms (207). Glucocorticoids upregulate the transcriptional activity AP-1 and thereby promote gene expression for melatonin synthesis (171, 208). AP-1, in addition to acting as a signal transducer and activator of transcription-1 and 3 (STAT-1; STAT-3), competes with NF- $\kappa$ B for binding to nat- $\kappa$ B1 to regulate the transcription of NAT (32). Transcription of NAT driven by NF- $\kappa$ B dimers mediates pathogen-associated molecular patterns (PAMPs) or pro-inflammatory cytokine-induced melatonin synthesis in macrophages by binding to one or two upstream  $\kappa$ B binding sites (nat- $\kappa$ B1 and nat- $\kappa$ B2) of the NAT promoter in RAW 264.7 cells (209). LIM homeobox transcription factor *Isl1* positively modulates melatonin synthesis by targeting NAT at the (ATTA/TAAT) motif, via the ERK signaling pathway of norepinephrine (34). Regarding the binding site of TFs, the chicken NAT gene does not contain a canonical cAMP-response element (CRE) sequence TGACGTCA (210) but a TTATT8 repeat sequence and a CLS (6/8 identical to the canonical CRE) in basal and cAMP-driven promoters which bind c-Fos, JunD, and CREB to enhance basal and forskolin-stimulated NAT transcription (211). This motif was not found in NAT genes from other species, including mouse, rat, and zebrafish (211). For the rat, a cAMP- response element (CRE)-like sequence (CLS; TGCGCCA)-CCAAT complex in the flanking region and a canonical CRE in the first intron drives cAMP-dependent induction of the NAT gene as well (212, 213). In addition, the cone-rod homeobox (*Crx*) transcription factor was reported to regulate the expression of NAT in the mouse pineal gland.

For plants, limited information related to transcriptional regulation is available compared with that of animals. Evidence from recent studies show that a multifunctional enzyme, namely caffeic acid O-methyltransferase (COMT), can also catalyze the last step of melatonin biosynthesis (28). In rice, melatonin biosynthesis requires ASMT or COMT activity (154). Cai et al. (33) revealed that, in tomato, cadmium stress induces the expression of *HsfA1a*, which acts as a positive regulator of COMT1 transcript levels by binding to the COMT1 gene promoter heat-shock element (HSE) sequence (GAANN TTC), and induces melatonin accumulation. For cassava, three TFs were found to modulate melatonin biosynthesis. Cassava bacterial blight induces the expression of *MeWRKY79* and *MeHsf20*, which activate the expression of *MeASMT2* via binding to W-box (TTGACC/T) and HSEs (GAAnnTTC) in the *MeASMT2* promoter; this, in turn, increases melatonin accumulation and confers improved disease resistance (205). *MeRAV1* and *MeRAV2* may directly regulate three melatonin biosynthesis genes (*MeTDC2*, *MeT5H*, and *MeASMT*) by binding their promoter containing CAACA motif as transcriptional activators, and thus up-regulate melatonin biosynthesis

in response to disease resistance against cassava bacterial blight (214).

## CONCLUSION REMARKS

The acquisition of additional functions by melatonin, which is believed to have originally evolved to provide molecular protection from free radicals, occurred over a very long evolutionary period. It is theorized that melatonin first appeared in bacteria about 3.0–2.5 billion years ago. When these melatonin-synthesizing bacteria were phagocytized by early eukaryotes as food, over time they established a symbiotic association with their hosts and developed into mitochondria and chloroplasts. Since the bacteria that were ingested had the ability to synthesize melatonin, this important function was retained by the mitochondria and chloroplasts. As a consequence, we hypothesize that these organelles have produced melatonin in every plant and animal species that has ever existed and that this occurs in present day animal and plant cells as well. Thus, every cell that possesses mitochondria (animals and plants) or chloroplasts (plants), we feel, has the capacity to produce melatonin. Melatonin at these sites is important to provide protection against free radicals which are abundantly generated in these organelles. Over its very long evolutionary history, melatonin has acquired other essential functions that have been retained by this physiologically-diverse molecule.

Tryptophan is the starting molecule for melatonin production in cell species. The sequence of the enzymatic steps that convert tryptophan to melatonin, however, varies among species. These steps include hydroxylation, decarboxylation, acetylation, and methylation. In some plant species, melatonin may not be the end product; in at least one variety of rice, melatonin can be hydroxylated at either 2, 4, or 6 position with 2-hydroxymelatonin possessing significant antioxidant activity, like melatonin itself. While the synthetic pathway of melatonin has changed throughout evolution and differs among plant and animal species, the structure of melatonin persists as originally designed in bacteria billions of years ago. It is pointed out, however, that what is known about melatonin synthesis has come primarily from mammals and the pathway in other vertebrates has been sparingly investigated. Moreover, the pathway of melatonin production in invertebrates remains to be examined.

## AUTHOR CONTRIBUTIONS

RR initiated the review and checked all drafts of the report. DZ, YY, and YS worked together to write the initial drafts of the manuscript. QL and RS prepared figures for the article and read the final version. ZZ and RS participated in the discussion of the functional evolution of melatonin.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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